G Gyulai

Editor

PLANT GENETICS BIOTECHNOLOGY and FORESTRY

2nd Edition

Selected Chapters for University Courses of

Agricultural Biotechnology (BSc and MSc)
Forest Genetics and Biotechnology (MSc)
Environmental Genetics (MSc)
Ecological Genetics (MSc)
Plant Cell Genetics and *In Vitro* Breeding (PhD)

St. ISTVÁN UNIVERSITY
FACULTY of AGRICULTURAL and ENVIRONMENTAL SCIENCES
GÖDÖLLŐ
2017

Preface

The purpose of this University Textbook (‘*Egyetemi jegyzet’* in Hungarian) is to collect the most significant scientific information available in
Plant Genetics, Biotechnology and Forestry
edited for University Students (BSc, MSc, and PhD), Researchers, and the General Public.
Take and use this book with the best wishes and regards from the
Editor, Authors, and Publishers

2017 January 1

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Dedicated
to
Professor E Lehoczki (Szeged; 1979 - 1983)
and
Professor Gy Heltai (Gödöllő; 1983 - 1986)
and
Professor L Heszky (Gödöllő; 1986 - 1995)

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Nucleic acids (DNA and RNA), and the replication of DNA

(A) Introduction

Landmarks of Genetics

1819. FESTETICS, Imre (Keszthely, Hungary), Published the first formulation of *'genetic laws'*, '*mutation*', and '*F2 segregation*' *in sheep (Ovis aries)* breeding for wool fineness, 50 years before G Mendel.

1965. MENDEL G (Brno, Moravia) published the crossing experiments with green peas (*Phaseolus vulgaris*), and described the main principals of *heredity*.

1919. EREKY, KÁROLY (Budapest, Hungary), a Hungarian engineer, coins the term '*biotechnology'*, with meaning of the production of beer, cheese, bread *etc*., with the help of living organisms (*i.e.,* microbes) at industrial volumes.

1861. SEMMELWEISZ, Ignác MD (Budapest, Hungary) indroduced Ca- hypochlorite against *childbed fever* and *maternal mortality*.

1869. MIESCHER F (Switzerland, Europe) isolated a *phosphor-containing* material from the cells *nuclei* found in pus from discarded surgical bandages, and called it *nuclein*. It was also found later in salmon sperm.

1920. EREKY, Károly (Budapest, Hungary) coined the term '*biotechnology*' for high throughput agricultural technologies

1927. MULLER HJ (USA) demonstrated that X-rays are mutagenic in *Drosophila.*

1937. SZENT-GYÖRGYI, Albert (Hungary, UK, USA) Nobel Prize (1937) Nobel Lecture, December 11, 1937. 'Oxidation, Energy Transfer, and Vitamins'.

1941. BEADLE GW and EL TATUM (USA) propose the *one gene - one enzyme* (polypeptide) concept.

1944. AVERY OT, CM MACLEOD and M MCCARTY (Canada, USA) published the paper on 'Studies on the chemical nature of the substance inducing transformation of *Pneumococcal*' types (*J. Exp. Med*. 79, 137-158), which was the first report on the chemical identification of DNA. They proved that DNA was the '*hereditary material*' in the *F Griffith*'s (1928) *Pneuomococcus* experiments.

1946. LEDERBERG J and EL TATUM (USA) demonstrated *genetic recombination* (conjugation) of bacteria (Nobel Prize, 1958).

1950. CHARGAFF E (Hungary, Bukovina; Austro-Hungarian, USA) demonstrated that the numbers of adenine and thymine (A=T) are always equal similar to guanine and cytosine (G≡C).

1952. SANGER F *et al.,* (England; UK) have sequenced the amino acid sequences of insulin. (Nobel Prize, 1958).

1953. WATSON D, and FHC CRICK (Chicago, USA; UK). On the basis of Chargaff's chemical data (1950; numbers of A and T, and C and G are the same in DNA), and Wilkins and Franklin's available *X-ray diffraction* data, James D WATSON and Francis HC CRICK described the DNA's double helix structure (*Nature 171, 737-738)* (Nobel prize, 1962).

1956. TIJO JH and A LEVAN (Sweden, USA, China) showed that the *diploid chromosome number* of humans are 46 (which was not obvious that far).

1957. FRANKEL-CONRAT H, A GIERER and G SCHRAMM (Tübingen, Germany) independently demonstrated that the genetic information of *tobacco mosaic virus* is stored not in DNA rather in RNA (*i.e.,* discovery of RNA viruses).

1958. MESELSON MS and FW STAHL (USA) demonstrated that DNA replication is *semiconservative* (in *E. coli*), which means that DNA replication goes on both DNA strand.

1968. OKAZAKI RT *et al*., (Japan) reportd the discontinuous synthesis of the *lagging* DNA strand (*i.e.*. Okazaki fragments).

1968. KIMURA M (Japan) proposed the *Neutral* (and not the Darwinian *Natural*) selection and Gene Theory of Molecular Evolution.

1978. GILBERT W (USA) coined the terms *intron* (non coding DNA) and *exon* (coding DNA).

1984. JEFFREYS A (Oxford, UK) developed the term *genetic fingerprinting* (today *syn.*, *barcoding*).

1986. SAIKI RK and KB MULLIS *et al.* (USA) described the polymerase chain reaction (PCR) (Nobel prize, 1993).

2004. A HERSKO (born.:Karcag, Hungary - Izrael). Nobel Prize in Chemistry for his discovery with Aaron Ciechanover and Irwin Rose, of *ubiquitin*-mediated protein degradation.

2006. FIRE, AZ, CC MELLO (2006) Nobel Prize for their discovery of *RNA interference - gene silencing* by double-stranded RNA

2015. LINDAHL T, P MODRICH, A SANCER (USA) - finalized the molecular background of DNA repair (Nobel Prize, 2015).

2016. OHSUMI, Y. The Nobel Prize awarded to for his discoveries of mechanisms for *autophagy*.

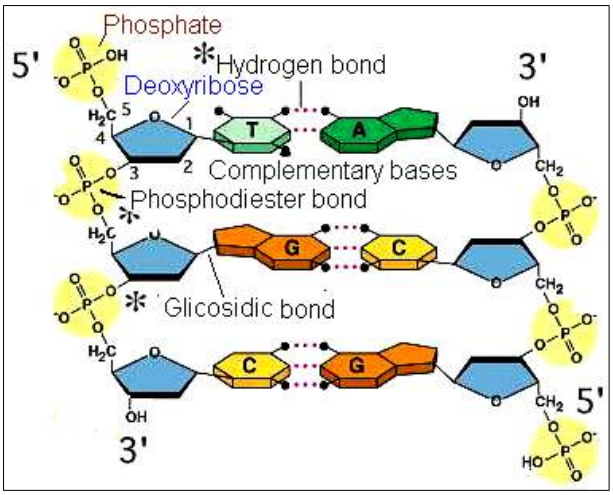


Figure 1a. Molecular structure of the double stranded DNA (*dsDNA*). The three differnt *chemical bonds* are indicated (*)

(B) Molecular structures of nucleic acids

DNA (*Deoxyribo Nucleic Acid*) (*Fig. 1a*) is made up of two *antipararell* strands forming a *twin molecule*. Biochemically, each strand is a very simple bio-*'polymer'* (hence the term *polymerization*, *syn*., DNA *replication*) as it is made up of molecular *units (i.e., nucleotides)*, which include the row of *deoxyribose* (a sugar) and the *covalently* bonded (*i.e., phospho-diester bond*) *phosphate* (PO_4^{3-}) between adjacent sugars (which forms the '*DNA backbone*').

The four *nitrogenous bases* (A, C, G, and T) (*Fig. 1b*) join to deoxyribose by *glycosidic bond** (*Fig. 1a*).

The nucleic acid backbone is unique as in it an inorganic molecule (the phosphor) joins an organic (the sugar) molecule. (To compare: in the biopolymers of *cellulose*, *starch*, and *glycogen* the sugar units -; and, in the *protein*s, the amino acid units join together without bridges of inorganic elements).

The two DNA strands are held together by *hydrogen bonds** stretching between the complementary bases (Adenine bonds to Thymine, and Guanine bonds to Cytosine; A═T and G≡C). In total, DNA is made up of only six kinds of molecules (*Fig. 1c*).

DNA strands are labeled by the numbers of carbon atoms of deoxyribose (1 to 5) and goes from 5'-end toward 3'-end (*Fig. 1a,c*).

The strands run in opposite directions (*i.e.,* antiparallel), one strand runs in a 5'-3' direction and the other runs in a 3'-5' direction.

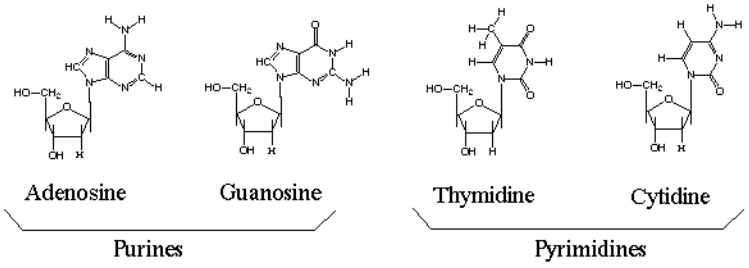


Figure 1b. Molecular structures of the four DNA nucleotides. See the contradiction in the terms of *Cytosine* (without sugar) *vs. Cytidine* (*with sugar*), and *Thymine* (without sugar) *vs. Thymidine* (with sugar) (which may follow the term of Pyrim*idine*s); and compared to *Adenine* / *Adenosine* – *Guanine* / *Guanosine* (which indicate the deoxirob*ose*).

The functional helical DNA (*i.e., double helix*) forms a minor and major *groove*s (*Fig. 1e*) (see later the intercalating fluorescent DNA dyes), and is wrapped around proteins (*histones*, which have 8-subunits), giving the structure of *nucleosome*. Nucleosomes help *DNA supercoiling* and *packaging to chromosomes* (prior to cell division); and they also play important role in *DNA replication, RNA transcription,* and ultimately the *gene expression.*

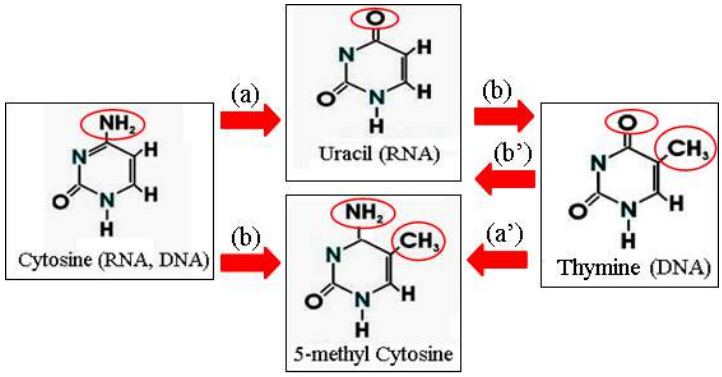


Figure 1d. Direct metabolic changes among nucleotides of DNA and RNA (including the 5mCitosine; see in gene silencing). Enzymatic reactions of *(a)* deaminations, *(a')* amination, *(b)* methylation and *(b')* demethylation are indicated by arrows (see *Chapter 4. Fig.1b, Chapter 12.Fig.3*)

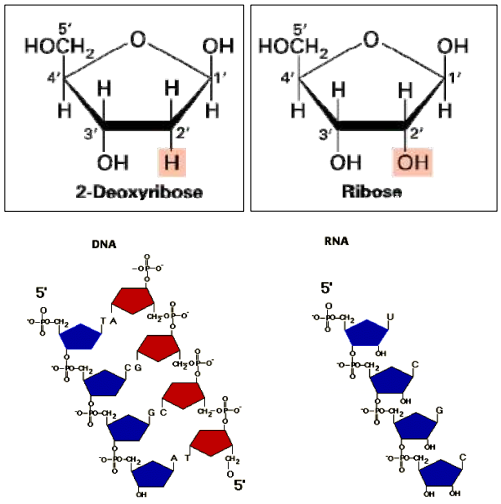


Figure 1c. The chemical difference between the sugar molecules of deoxyribose (DNA), and ribose (RNA).

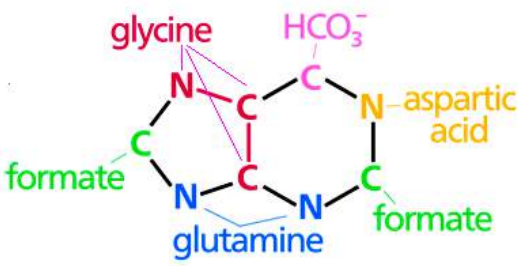


Figure 1f. The biosynthetic origins of the atoms of *purine* ring (*Adenine* and *Guanine*).

(C) Biosynthesis of nucleotides

De novo synthesis of *pyrimidines* and *purines* are carried out by several enzymatic steps in the cytoplasm of the cells. In *animals* the liver is the major organ of de novo synthesis of all four nucleotides (IUPAC - *International Union of Pure and Applied Chemistry*). The synthesis of the *pyrimidines* starts with the formation of *carbamoyl phosphate* from amino acid (aa) *glutamine* and CO_2.The atoms of *purine* nucleotides come from several sources from organic acids (*aspartic acid* and *formate*), amino acids (*glycine* and *glutamine*), and (HCO_3^-) (*Fig. 1f*).

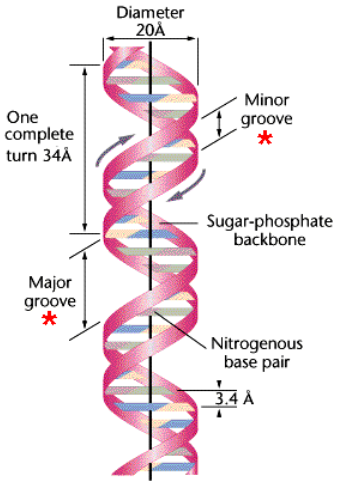


Figure 1e. The *helical* structure of DNA forms two grooves (indicated*)

(D) DNA Replication (syn.: DNA synthesis, DNA polymerization / amplification)

DNA POLYMERASES (DNA*pol; syn. DdDpol – DNA directed DNA polymerase*) are enzymes that catalyze DNA synthesis (*i.e., DNA replication, 'copy' of DNA*) on DNA template by assembling nucleotides - the building blocks of DNA. Before replication, another enzyme *HELICASE** and

The first DNA*pol*I of *E. coli* was discovered by *A Kornberg* and *S Ochoa* (Nobel Prize 1959); and DNA *pol*II, by *Kornberg T* and *ME Gefter* (1970) (*Fig. 2a*). Since *DNA polymerase* requires a free 3' OH group of deoxyribose, DNA synthesis can go on only one direction by extending the 3'-end of the DNA chain. Hence, DNA*pol* moves along the template strand (in 3'-5' direction), (*leading strand*). DNA replication proceeds *continuously* on the *leading strand*, but on the *lagging strand* the new DNA is made in fragments (*i.e., Okazaki fragments;* discovered *by R Okazaki* and *T Okazaki* in 1960s), which are later joined together by DNA *LIGASE ENZYME*. (*Fig. 2b*).

Extreme bacteria, *e.g., Thermus aquaticus* (discovered by *TD Brock* and *H Freeze* from Indiana University, USA, in 1969), living in the *geyser* proceeds DNA replication by extreme DNA*pol* (*i.e., TaqPol*) discovered and later used widely in PCR reactions by *A Chien, Edgar DB,* and *Trela JM* (*1976, J. Bact. 127: 1550–7*). By history, the first description of a method using an enzymatic assay to replicate a short DNA template was carried out by *K Kleppe* in 1971.

**HELICASE (Fig. 2a)*. The, human genome codes for 95 *helicases*, of them, 64 are *RNA helicases* (EC # 3.6.4.13), and 31 are *DNA helicases* (EC # 3.6.4.12). The *atrx* gene encoding the *ATP-dependent helicase* (also known as XH2 and XNP) of the SNF2 subgroup family, is thought to be responsible for functions of chromatin remodeling, gene regulation, and DNA methylation. *RecQ helicases* belong to the Superfamily II group of *HELICASES*, which help to maintain stability of the genome and suppress inappropriate recombination.

TOPOISOMERASE II opens up (*unwinds, 'unzips'*) the two DNA strands to facilitate replication (*i.e., replication fork*, *replication bubble*). The resulted two single stranded DNA (ssDNA) supply templates for replications (*i.e., semi conservative* replication, which literally means that both ssDNA strand are templates).

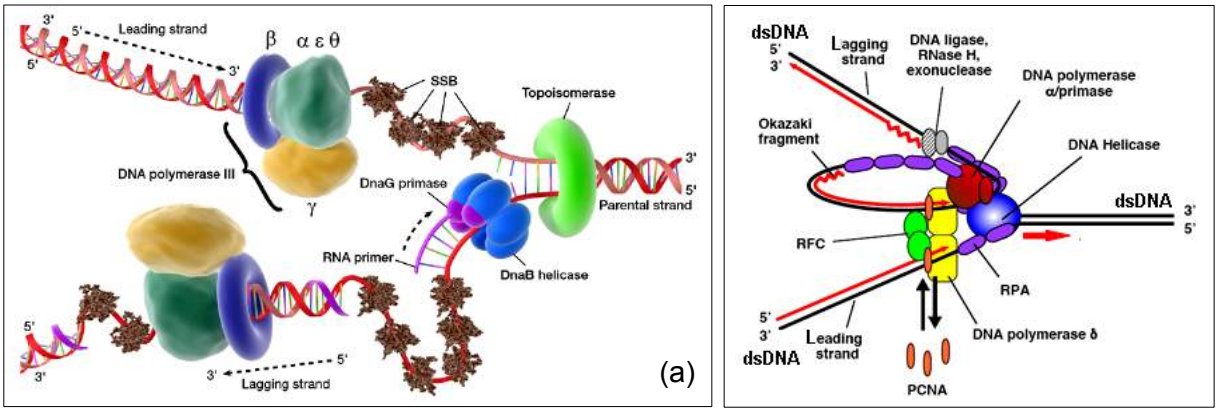


Figure 2a. Molecular components and comparison of the prokaryotic (*E. coli*) *(a)*, and eukaryotic (plants and animals) *(b)* DNA synthesis (*i.e.,* DNA replication). *SSB - Single-Stranded DNA Binding Protein. RFC - Replication FaCtors. RPA – Replication Proteins $A_{1\text{-}5}$. PCNA – Proliferating Cell Nuclear Antigen*

DNA*pol*, 'as everything', makes mistakes (*mismatch*) functionally for about 10^{6th} base pairs copied, which is corrected by the second function of *proof reading* activity of DNA*pol*, and when an incorrect base pair is recognized, DNA*pol* moves backwards to unpaired base pair of DNA *(i.e., 3'-5' exonuclease activity*), excises the wrong nucleotide and re-insert the correct nucleotide (*i.e.*, DNA repair). The speed/ rate of DNA synthesis (*i.e.,* processivity) in a living cell was first determined in phage T4 DNA*pol* of phage-infected *E. coli* (at 37 °C), which was 749 nucleotides per second.

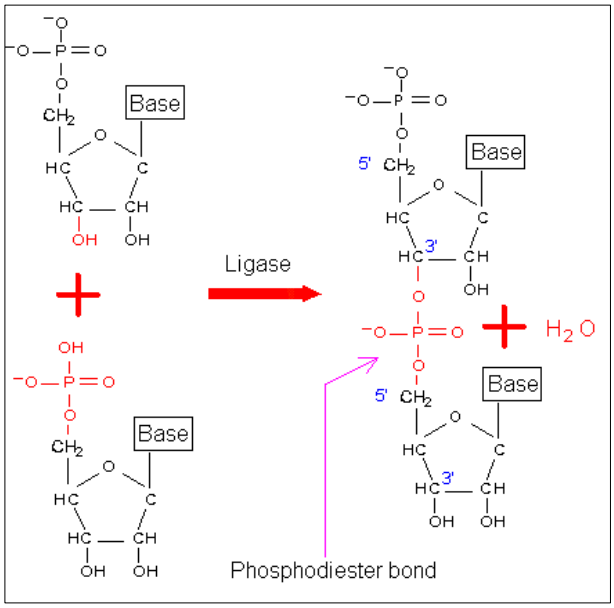


Figure 2b. The development of *phosphodiester bond* of DNA.

RNA HELICASES are essential for ribosome biogenesis, pre-mRNA splicing, and translation initiation. *RNA helicases* are involved in the mediation of antiviral immune response because they can identify foreign RNAs in *vertebrates* (about 80% of all viruses are RNA viruses, and they carries their own *RNA helicases*).

REVERSE TRANSCRIPTASE (RT) enzymes of *retroviruses* can transcribe (replicate) DNA on RNA template, hence the name *RdDpol – RNA directed DNA polymerase* (*e.g.,* EC# 2.7.7.49). *RdDpol* was discovered by *H Temin*, and independently, by *D Baltimore* in 1970 (isolated from RNA tumor viruses of R-MLV and RSV), and shared the Nobel Prize in 1975 (*Fig. 3*).

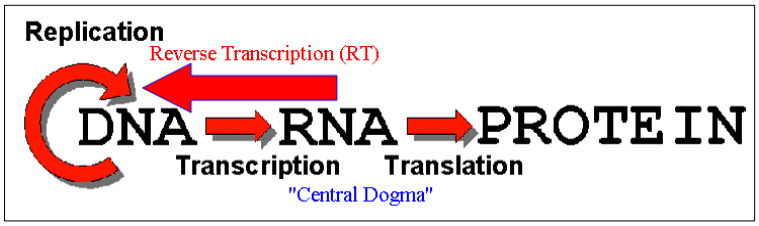


Figure 3. The function of Reverse Transcriptase, which turned back the "*Central Dogma*" (*H Temin* and *D Baltimore, 1970; Nobel Prize, 1975).*

(E) The RNA

RNA (*Ribo Nucleic Acid*) is a single stranded nucleic acid (ssRNA) containing *ribose* instead of deoxyribose (as in DNA) (*Fig. 1c*), and *uracil* instead of *thymidine* (*Fig. 1d*). The chemical structure of RNA is very similar to that of DNA, with only three differences, such as *(1)* RNA is a *single-stranded* molecule with much shorter chain than the DNA. However, RNA can also from parts of *double stranded helix* by complementary base pairing. *(2)* The sugar component of RNA is *ribose* (which has no *hydroxyl group* in the 2' position as in ribose), which structure makes RNA less stable than DNA for it is more prone to hydrolysis (see: self splicing of RNA). *(3)* In RNA the *thymine* is *demethylated* (*i.e.*, *uracil*) (*Fig. 1d*). Functionally and structurally RNAs are very divers molecules (*Table 1a,b,c,d*) *https://en.wikipedia.org/wiki/RNA*.

Table 1a. Types of RNAs involved in *protein synthesis*

Abbreviation	*RNA Types*	*Functions*	*Distribution*
mRNA	messenger RNA	Codes for protein	All organisms
rRNA	ribosomal RNA	Structural RNA of ribosomes	
tRNA	transfer RNA	Translation	
SRP RNA (7SL RNA)	Signal recognition particle RNA	Membrane integration	
tmRNA	Transfer-messenger RNA	Rescuing stalled ribosomes	Bacteria

Table 1b. Types of RNAs involved in *post-transcriptional modification*, and *DNA replication*

Abbreviation	*RNA Types*	*Functions*	*Distribution*
snRNA	Small nuclear RNA	Splicing and other series of functions	Eukaryotes and archaea
snoRNA	Small nucleolar RNA	Nucleotide modification of RNAs	Eukaryotes and archaea
SmY	SmY RNA	mRNA trans-splicing	Nematodes
scaRNA	Small Cajal body-specific RNA	Type of snoRNA; Nucleotide modification of RNAs	
gRNA	Guide RNA	mRNA nucleotide modification	Kinetoplastid mitochondria
RNase P	Ribonuclease P	tRNA maturation	All organisms
RNase MRP	Ribonuclease MRP	rRNA maturation, DNA replication	Eukaryotes
yRNA	Y RNA	RNA processing, DNA replication	Animals
TERC	Telomerase RNA Component	Telomere synthesis	Eukaryotes
SL RNA	Spliced Leader RNA	mRNA trans-splicing, RNA processing	

Table 1c. Types of *regulatory RNAs*

Abbr.	*RNA Types*	*Functions*	*Distribution*
asRNA	Antisense RNA	Transcriptional attenuation / mRNA degradation / mRNA stabilization / Translation block	All organisms
cis-NAT	Cis-natural antisense transcript	Gene regulation	

Table 1c. Types of *regulatory RNAs*

crRNA	CRISPR RNA	Resistance to parasites, probably by targeting their DNA	Bacteria and archaea
lncRNA	Long noncoding RNA	Regulation of gene transcription, epigenetic regulation	Eukaryotes
miRNA	MicroRNA	Gene regulation	Most eukaryotes
piRNA	Piwi-interacting RNA	Transposon defense	Animals
siRNA	Small interfering RNA	Gene regulation	Eukayotes
tasiRNA	Trans-acting siRNA	Gene regulation	Plants
rasiRNA	Repeat associated siRNA	Type of piRNA; transposon defense	*Drosophyla*
7SK	7SK RNA	negatively regulating CDK9/cyclin T complex	

Table 1d. Types of *parasitic RNAs*

Type	*Function*	*Distribution*
Retrotransposon	Self-propagation	Eukaryotes and bacteria
Viral genome	Information carrier	dsRNA viruses, (+)ssRNA viruses, (-)SSRNA viruses, and RT viruses
Viroid	Self-propagation	Infected plants
Satellite RNA	Self-propagation	Infected cells

(F) Artificial DNA; DNA analogs

Phosphorothioates. Changes of phosphodiester bond to *phosphorothioate* bond makes the DNA resistant to digestion by *nucleases*. The technology was already used for Human medicine (*VITRAVENE, 2007)* (*Fig 4a*).

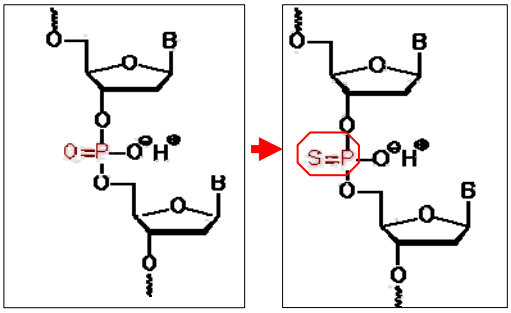


Figure 4a. Molecular structure of *phosphorothioates* (see the change of O to S)

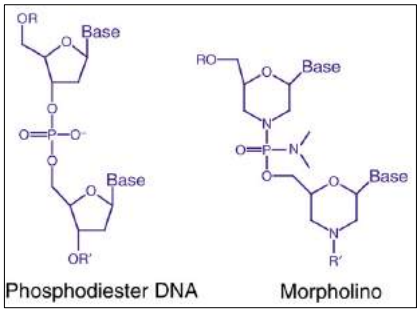


Figure 4b. Molecular structure of *morpholino DNA* (*oligonucleotides*) compared to natural DNA.

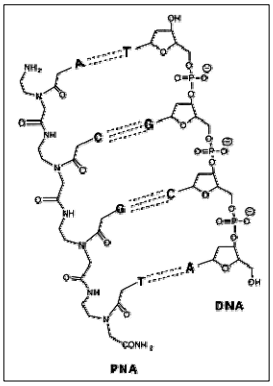


Figure 4c. The structure of *PNA:DNA.*

Morpholino. Morpholinos are *nonionic DNA analogs* (available from *Gene Tools LLC*), with *altered backbone* linkages. Morpholinos bind to complementary nucleic acid sequences by Watson-Crick base-pairing. The backbone makes morpholinos resistant to digestion by *nucleases*. Also, because the backbone lacks negative charge, it is thought that morpholinos are less likely to interact nonselectively with cellular proteins. Relatively long 25-base morpholinos are frequently used for antisense gene inhibition (*DR Corey* and *JM Abrams*, 2001) (*Fig. 4b*).

PNA (Protein Nucleic Acids). Backbone of proteins (N-C-C bonds) can also provide backbone for DNA (*i.e.,* PNA – *protein nucleic acid*), which also can hybridize to natural ssDNA strand (*Fig. 4c*).

(E) UV absorbance and fluorescence of nucleic acids

Due to the double bonds of DNA nucleotides (Adenine, Thymine, Guanine, Cytosine) DNA absorb UV (ultraviolet) light with a maximum peak at 260 nm (A_{260}). Due to aromatic amino acids (TYR, TRP, PHE) proteins also absorb UV light with a maximum peak at 280 nm (A_{280}) (*Fig. 5*). Therefore, UV absorbance is used for quantification of nucleic acids (and proteins). Absorbance A = 1.0 corresponds to a concentration of 50 µg/ml dsDNA. Surprisingly, ssDNA absorb more UV light than that of dsDNA (*i.e., hypochromic effect*). By the application of *fluorescence* dyes, which can be intercalated into DNA strands, the intensity of fluorescence after UV light excitation can also be used for quantification of DNA, *e.g.*, during *melting point* analysis. The melting temperature (*Tm*) is the temperature at which half of the DNA is in double stranded form and half is single stranded. The Tm depends greatly on base composition. Since G≡C bases pair with three Hydrogen bonds, DNA with high G≡C content have a higher *Tm* than that of DNA with higher A=T content.

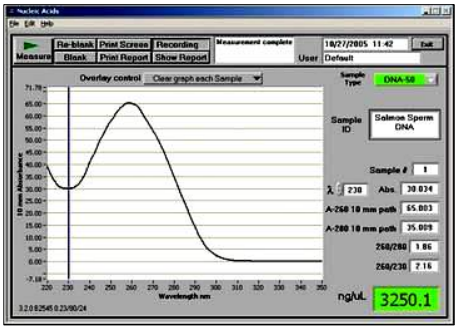


Figure 5. UV spectrogram of DNA samples measured by *NanoDrop* UV-vis spectrophotometer.

RNA transcription, and Protein translation

(A) RNA transcription

Eukaryotic transcription is the biochemical process of mRNA synthesis copied from DNA template catalyzed by enzyme RNA polymerase (*DdRpol* – DNA directed RNA polymerase). This is the first step of *gene expression* processed in the cell nucleus (*Fig. 1*).Transcription proceeds through main steps of (1) *Sigma factor proteins* binding to *DdRpol* to catalyze its binding to promoter site of the gene (*e.g.,* TATA box); (2) *DdRpol* creates the *transcription bubble*, which separates the two strands of the DNA helix, by breaking the *hydrogen bonds* between complementary DNA nucleotides, similar to the reaction during DNA replication; (3) *DdRpol* adds matching RNA nucleotides to the complementary nucleotides, and binds the *sugar-phosphate backbone* of RNA; (4) Hydrogen bonds of the untwisted RNA-DNA helix break, and the newly synthesized *pre-mRNA* strand releases; (5) The pre-mRNA is further processed in the cell nucleus through *polyadenylation*, *capping*, and *splicing*. After these steps, the edited (*mature*) mRNA exit to the cytoplasm through the *nuclear membrane* pore complexes. The transcribed gene may encode for *protein-coding-*, and *non-coding RNAs* (*e.g.,* microRNA), *ribosomal RNA* (rRNA), *transfer RNA* (tRNA), and other enzymatic RNA molecules called *ribozymes* (*Fig. 2a,b,c*).

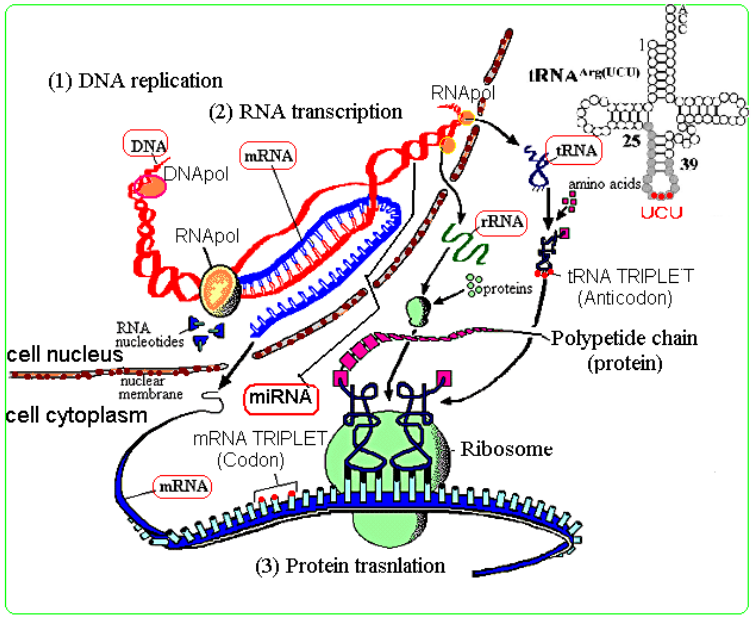


Figure 1. Compartmentalization of the three main genetic processes of *(1)* DNA replication, *(2)* RNA transcription, and *(3)* Protein translation.

The transcribed gene (*transcription unit*) contains not only the coding sequence, which will be translated into the protein, but the regulatory sequences, which direct and regulate the protein synthesis. This regulatory sequence before (*upstream*) the coding sequence is called the *five prime untranslated region* (5'UTR), and the sequence after (*downstream*) the coding sequence is called the *three prime untranslated region* (3'UTR).

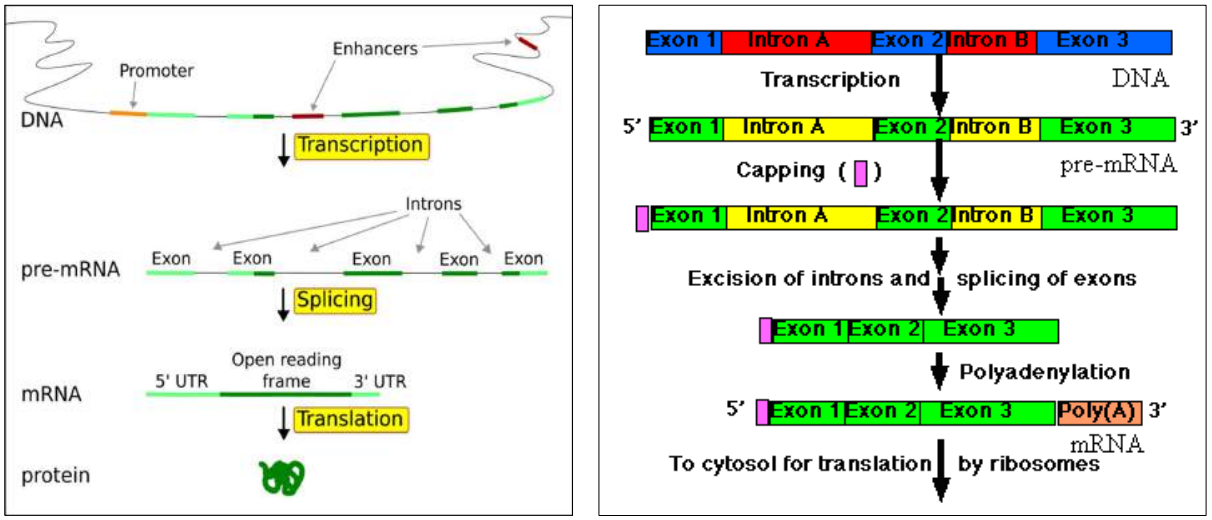


Figure 2a. Schematic *(left),* and detailed *(right)* molecular steps of '*mRNA processing*', which includes the *transcription* of RNA from DNA, *capping* of the pre-mRNA, *excision* of *introns* and *splicing* of *exons*, and *polyadenilation* of the mature mRNA.

The eukaryotic a gene has some functionally important gene sequences. The *initiation site* regulates the gene and helps *transcription factors (TF proteins)* to bind for starting the mRNA synthesis. *Exons* are the coding sequences, which are interrupted by *introns* that are removed during *mRNA*

processing (excision of introns, and spicing of exons). Before splicing, the pre-mRNA is capped with a *7-methylquanosine* (*i.e.*, cap) at the 5' end, and ffter splicing, *poly adenylation* takes place at the 3'end of mRNA (*Fig. 2abc, 3*).

The mature mRNA leaves cell nucleus and reaches ribosomes in the cell cytoplasm and the protein synthesis starts. The amino acid sequence of a protein is determined by the base sequence of the CDS of the mRNA. Starting with the *start codon* (AUG), the sequence of the mRNA is read three bases at a time (one codon). tRNA molecules bring (*transfer*) amino acids, and amino acids form a *peptide bound* with each other to form the protein (*Fig. 1*).

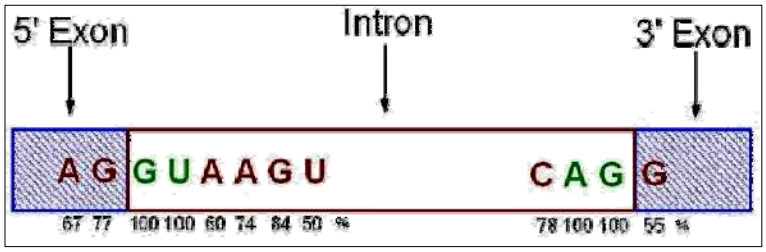


Figure 2b. The evolutionarily highly *conserved* DNA nucleotide sequences of the IEJ (*Intron Exon Junction*) region (see the % values).

By 1989, it turned out that RNA can be both a coding genetic material (like DNA), and a biological catalyst (like enzymes), and the term *ribozyme* (*ribo*nucleic acid en*zyme*) was coined (*S Altman and TR Cech, Nobel Prize, 1989*). These results also led to the *RNA world* hypothesis, which postulated that self-replicating and splicing (*Fig. 2c*) ribonucleic acid (RNA) may had been developed before DNA based life (*i.e.*, 'RNA World').

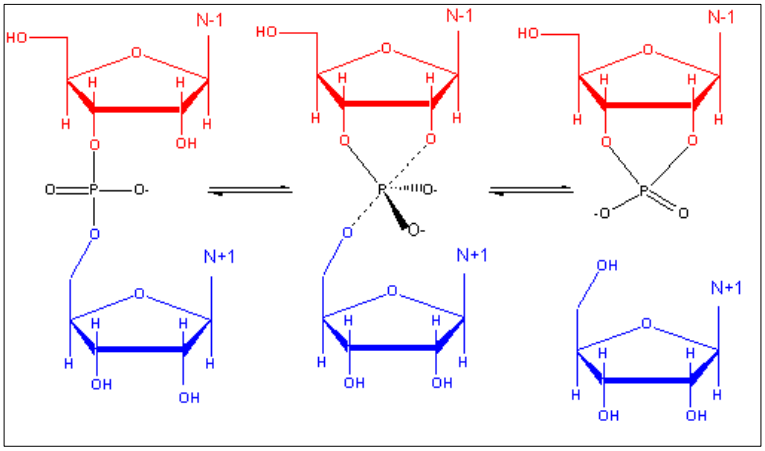


Figure 2c. The molecular mechanism of *ribosyme* (the self-cutting RNA)

In the function of ribosyme the point is that, as ribose also has an -OH group on the second carbon atom, it can react with phosphate group of the RNA backbone, and RNA breaks (i.e., splicing), without catalysis of any enzyme

Some amino acids (aa) may undergo *post-translational modification*. Glycosylation (addition of sugar molecules) and phosphorylation (the addition of phosphate molecules) are two common aa modifications. Only *methionine* and *tryptophan* have one codon. All the other aa have redundant codes. Some organisms may use some codons preferentially over others (*i.e.*, *code preference*) (*Fig. 4*). By history, *G Gamow* postulated that sets of three bases of DNA must be involved to encode the 21 amino acids (*Table 1*).

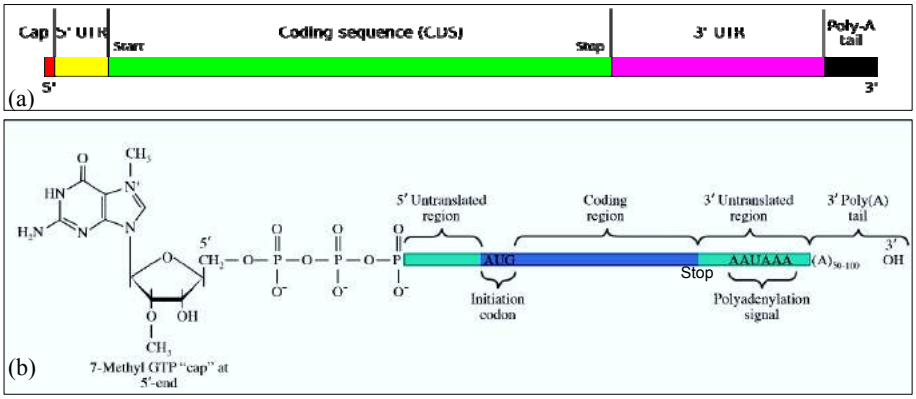


Figure 3. Schematic *(a)*, and molecular structure *(b)* of the mature eukaryotic mRNA

Quantitative PCR

The mRNA content can be easily determined by *quantitative PCR, which uses* poly-T primer that fish out the total mRNA molecules of tissue studied. The former technique of *Northern* blot is also useful for it.

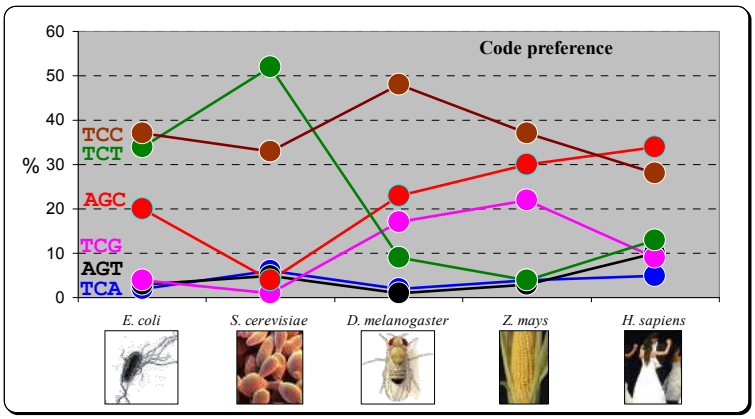


Figure 4. Sample of *code preference* of the six *synonym triplet* coding for *serine* amino acid compared in six different organisms

(B)Protein translation

During translation, the mRNA sequence, as a template, is "translated / read" to the amino acid sequence of proteins. Each group of three bases in mRNA constitutes a codon, and each codon specifies a particular amino acid (hence, it is a triplet code) (*Table 1*). Protein translation takes place in the cell cytoplasm on the surfaces of ribosomes (*Fig. 1*). When the mRNA binds to ribosomes the UTR sequences play crucial role, for 5'UTR, as the leader sequence, carries ribosome-binding site.

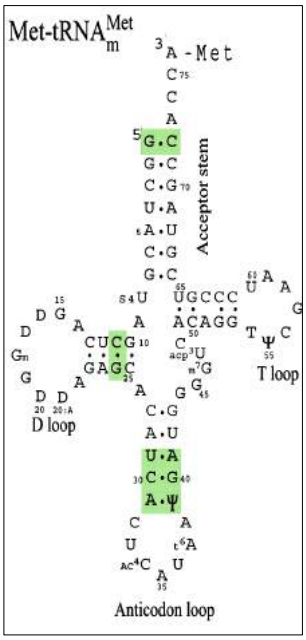


Figure 5. Sample for molecular structure of a tRNA (Met-tRNA; 76 nt)

Shortly, the translation includes four steps. (a) *Initiation*: The ribosome assembles around the target mRNA. The first tRNA (*Fig. 5*) is attached at the start codon. (b) *Elongation*: The tRNA transfers an amino acid to the tRNA corresponding to the next codon. *Aminoacyl tRNA synthetases* enzymes catalyze the bonding between specific tRNAs and the amino acids resulting in an *aminoacyl-tRNA*. This aminoacyl-tRNA is carried to the ribosome, where mRNA codons are matched through complementary base pairing to specific tRNA anticodons. (c) *Translocation*: The ribosome then moves (translocates) to the next codon along the mRNA to continue the process, and creating amino acid chain. (d) *Termination*: When a *stop codon* is reached along the mRNA, the ribosome releases the polypeptide.

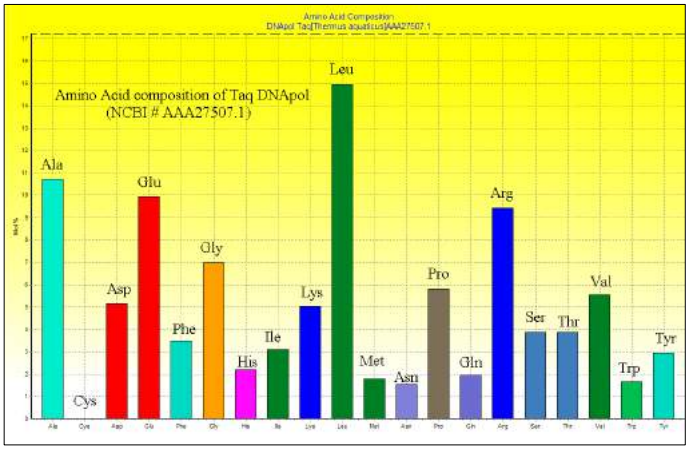


Figure 6a. Sample for amino acid composition of proteins (*e.g.*, *Taq*)

tRNAs are short (up to 93 nt) molecules. The most abundant is the methionine *tRNA*^{met} with partial double stranded forms (dsRNA) which makes with loops. The 3'-terminal group of tRNAs is the special sequence of the amino acid binding site (*Fig. 5*).

Table 1. The genetic codes (aa-s are indicated with three- and single letter codes) (see degenerated codes, *e.g.*, N for 'aNy')

Amino acid	Codons	Compressed	Amino acid	Codons	Compressed
Ala/A	GCU, GCC, GCA, GCG	GCN	Leu/L	UUA, UUG, CUU, CUC, CUA, CUG	YUR, CUN
Arg/R	CGU, CGC, CGA, CGG, AGA, AGG	CGN, MGR	Lys/K	AAA, AAG	AAR
Asn/N	AAU, AAC	AAY	Met/M	AUG	
Asp/D	GAU, GAC	GAY	Phe/F	UUU, UUC	UUY
Cys/C	UGU, UGC	UGY	Pro/P	CCU, CCC, CCA, CCG	CCN
Gln/Q	CAA, CAG	CAR	Ser/S	UCU, UCC, UCA, UCG, AGU, AGC	UCN, AGY
Glu/E	GAA, GAG	GAR	Thr/T	ACU, ACC, ACA, ACG	I
Gly/G	GGU, GGC, GGA, GGG	GGN	Trp/W	UGG	
His/H	CAU, CAC	CAY	Tyr/Y	UAU, UAC	UAY
Ile/I	AUU, AUC, AUA	AUH	Val/V	GUU, GUC, GUA, GUG	GUN
START	AUG		STOP	UAA, UGA, UAG	UAR, URA

The translated proteins, and they amino acid compositions are responsible for enzymatic function, even in extreme sample of the extremely heat tolerant protein of *Taq* DNApol (*Fig. 6a*).

The amino acids of human muscle also shows extreme compositions with high BCAAs (*Branched-Chain Amino Acids)* content of *leucine* (Leu), *isoleucine* (Ile) and *valine* (Val), which are among the nine essential amino acids for humans, and which account for 35% of the muscle proteins (*Fig. 6b*) (BCAAs are used frequently for body building) (*Appendix*).

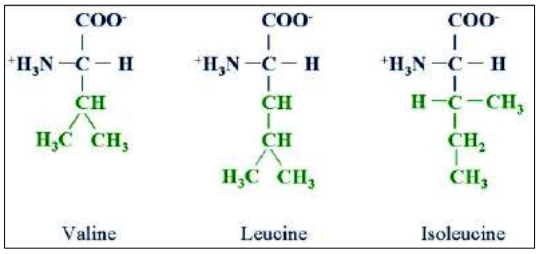
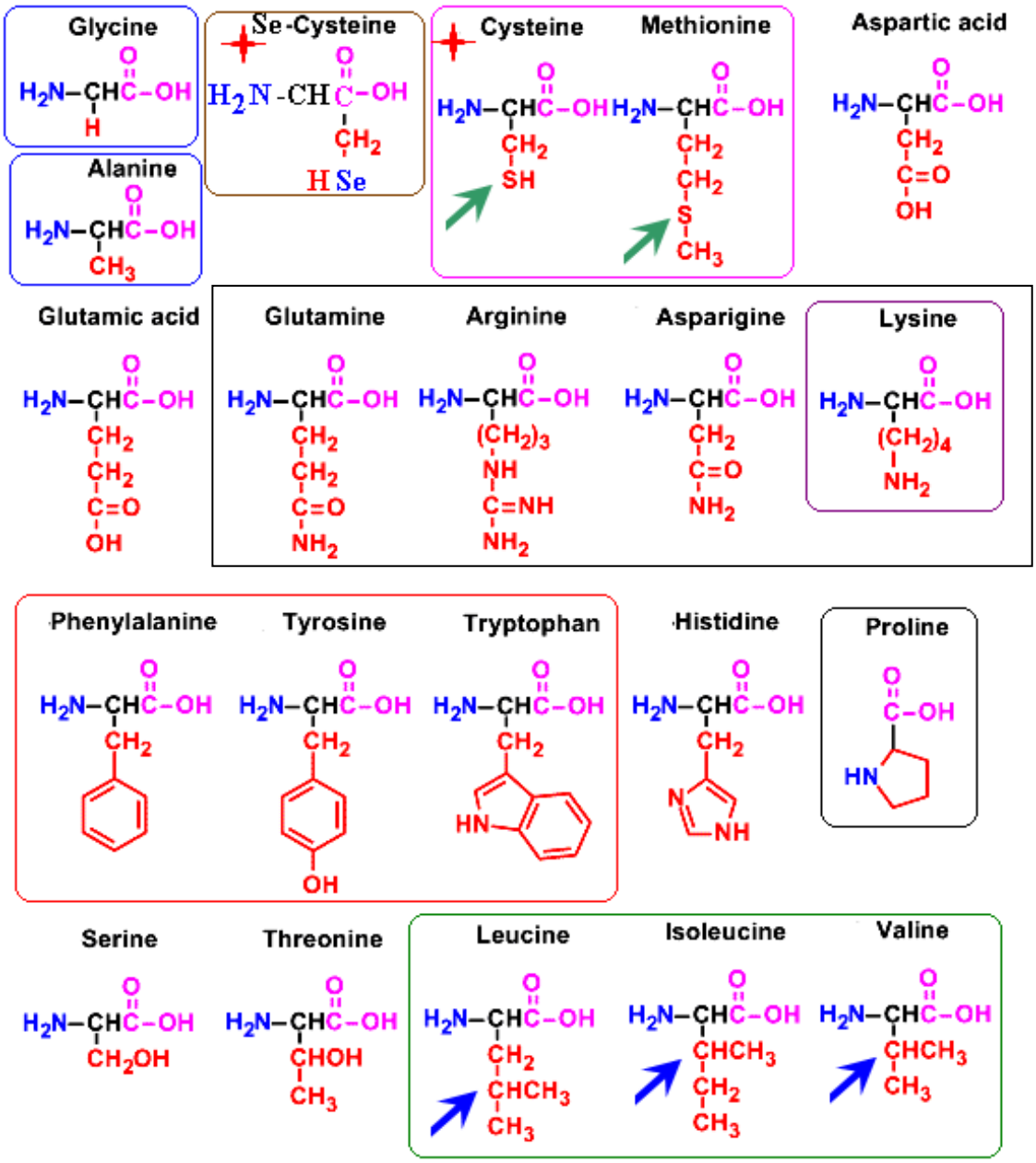


Figure 6b. Molecular structures of the three BCAAs (*Branched-Chain Amino Acids).*

Appendix



Chemical structures of the L-amino acids (AAs) (jn total 21). Significant, and unique (Se-Cys; and Pro) AAs are indicated, and also the two smallest AAs (Gly, Ala); the two S-containing AAs (Cys, Met); the AAs with amino (NH_2) group at the end of side chain, including Lys, used for Human virus infections; the tree aromatic AAs (Phe, Tyr, Trp) which provide precursors for plant hormone auxins; and the three BCAA (*Branched Chained Amino Acids*; Leu, Ile, Val), which are the main AAs of the Human muscles proteins (see *Mickey Hargitay*, 1955, and recently, *Arnold Swarzenegger*)

Mickey (Miklós) Hargitay (H-USA) (1926, Budapest – 2006, Los Angeles) the First Mr. Universe in the World, 1955. And recently, *A. Swarzenegger*

DNA methylation - Epigenetics of gene expression through DNA methylation in human and plant genomes

(A) Summary

Mechanisms of epigenetic control of DNA methylation, covalent histone modifications, and involvement of histone variants in the chromatin structure as a universal phenomenon in Human and plants are reviewed. These factors, together with chromatin remodeling, and non-coding RNAs, play key role in the determination of chromatin structure. These molecular processes interact with the chromatin in order to attain nuclear compartmentalization, as well as to establish euchromatic (gene-rich chromosome regions where nucleosomes are spaced apart and DNA is relatively accessible), and heterohromatic regions (gene-poor genomic regions where densely packed nucleosomes restrict DNA access). Crucial role of DNA methylation was observed in distinct human population phenotypes, physical appearance, social behavior, drug metabolism, sensory perception, response to environmental stimuli, and disease susceptibility. It was also shown that DNA methylation contributes to the biodiversity of natural populations. In plants, recent reports have demonstrated that different environmental stresses alter the DNA methylation pattern, and by this way, the stress response related gene expressions. Here we give a review of the molecular reasons and consequences of DNA methylation as a universal phenomenon in both human and plant genomes.

(B) Introduction

Although originally defined in a different context, the term *epigenetics* is currently used to describe the heritable changes in the DNA structure and its associated histone proteins, which is independent from the DNA sequence information (*Feinberg et al., 2010*).

Epigenetic control has been shown to be involved in all biological processes, which vary from embryogenesis and cellular differentiation to learning, memory and aging. *Epigenetics* also play crucial role in the development of various diseases, *e.g.,* cancer, neurodegenerative disorders, *ect*. Since *epigenetics* are more versatile in comparison to the genetic code, it is important to understand the mechanisms involved in the formation and upholding of these codes, in order to be able to reverse such errors by means of epigenetic therapy.

(C) Chemical background of DNA methylation

DNA methylation is a natural enzymatic process of TGS (Transcriptional Gene Silencing) catalyzed by DNA methyltransferases (MTases), which results in the meiotically heritable methylation pattern *i.e.*, 'genetic imprints' (*Park et al., 1996*).

Methylated DNA bases mainly include N6-methyladenine, C5-methylcytosine, and N4-methylcytosine. These methylated bases are natural components of DNA, which distinguish them from a large variety of chemically modified bases that can be formed by enzymatic alkylation or oxidative damage of the DNA. All these enzymes use *S-adenosyl-L-methionine (AdoMet)* as the donor of an activated methyl group, and modify the DNA in a sequence-specific manner, usually at palindromic sites, producing methylated DNA and *S-adenosyl-L-homocysteine (AdoHcy)* (*Jeltsch, 2002*) (*Appendix3*).

Biochemically, there are two types of DNA methylations: C-MTases, which form a C-C bond (cytosine-C5 MTases); and N-MTases, which form a C-N bond (adenine-N6 and cytosine-N4 MTases) (*Fig. 1a*). Different C5-cytosine MTases have been characterized in prokaryotes and eukaryotes. The methylation of C5-cytosine is the most prevalent DNA modification in eukaryotic genomes (*Fig. 1b*).

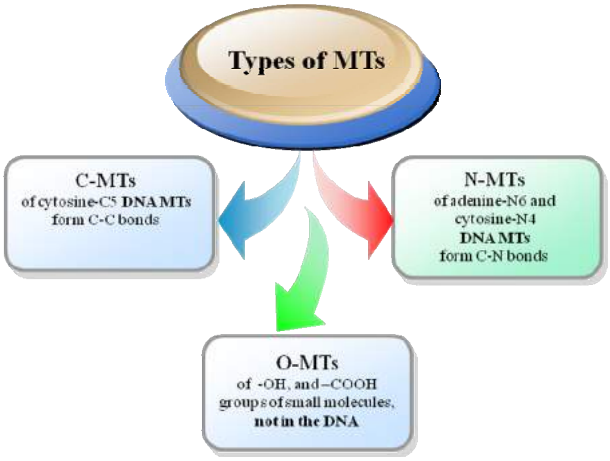


Figure 1a. Types of the three plant *AdoMet* directed (*syn., SAM*) MTs (C-, N-, and O-*MethylTransferases*) (*Alzohairy, Kovács L, Gyulai et al., 2016*)

In the human genome, most of the CpG (Cytosine-phosphodiesther-Guanine) dinucleotides (CpG islands of the genome) are methylated at the C5 position of cytosine base. About 50 % of the CpG dinucleotides are concentrated in gene gene promoter regions, as well as in the 5' untranslated regions of genes, and the remaining 50 % is located in the intragenic regions or in the genes (Delcuve et al., 2009).

DNA methylation, which occurs in the CpG islands of gene promoters results in gene silencing, in contrast to the DNA methylation in gene bodies, which might be involved in transcriptional activation (Hellman et al., 2007).

Additionally, methylated CpG islands were also observed in repetitive DNA sequences and transposable elements, which may play role in preventing translocations (Esteller, 2007).

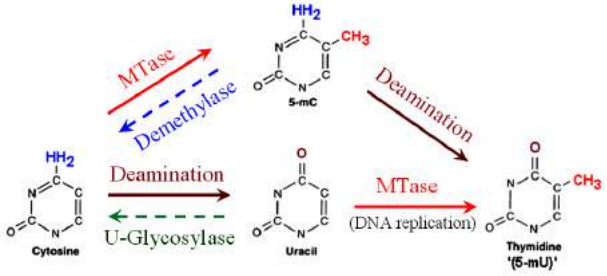


Figure 1b. Molecular pathways of enzymatic methylation, demethylation, and *spontaneous deamination* of DNA nucleotides (5-mC - C5-cytosine; MTase - DNA methyltransferase) *(see Ch.1.Fig.1d, Ch..12. Fig.3.)*

(D) Covalent histone modifications

DNA is wrapped around octameric protein structures, which consist of the four core histones H2A, H2B, H3 and H4. The histone octamer has a globular domain, which functions in packaging the DNA, as well as free tail structures, which protrude out from the N-terminal ends of histone proteins. These tail regions of histones are particularly of interest, due to the importance of diverse sets of covalent modifications that they can be subjected to. At least eight different types of such modifications have been described in no less than 60 different modification sites, which include: acetylation of lysines, methylation of lysine and arginines, phosphorylation of serine and threonines, ubiquitylation and sumoylation of lysines, deimination of arginines, ADP ribosylation of glutamic acids, and proline isomerization, which processes are all associated with certain biological roles (*Kouzarides, 2007; Wang et al., 2007*). Among these modifications, the best studied ones are the acetylations, methylations and phosphorylations.

Covalent histone modifications were shown to get involved in biological processes through two mechanisms. The first mechanism is interfering with nucleosome contacts, which results in disentanglement of the chromatin structure. The second, and better characterized mechanism, involves interacting with and recruiting certain proteins to the nucleosome, or oppositely, detaching certain proteins from the nucleosome. Hence, histone modifications are key players of the determination of the accessibility of the chromatin, and their involvement in cancer pathogenesis.

Methylation of *lysine* and/or *arginine* of histon H3 and H4 on specific sites, such as methylation of H3-K4, K36, K79, H3-R17, H3-R 26 and H4-R3, associates with the repression or activation of transcriptions (*Huang et al., 2005*). Methylation of *lysine* and *arginine* of H3 and H4 on other sites results in transcriptional deactivation, such as H3-K9, H3K27 H3K64 H4K20, H1.4K26, H3-R8, H3-R2, and H4K20 (*Appendix3*).

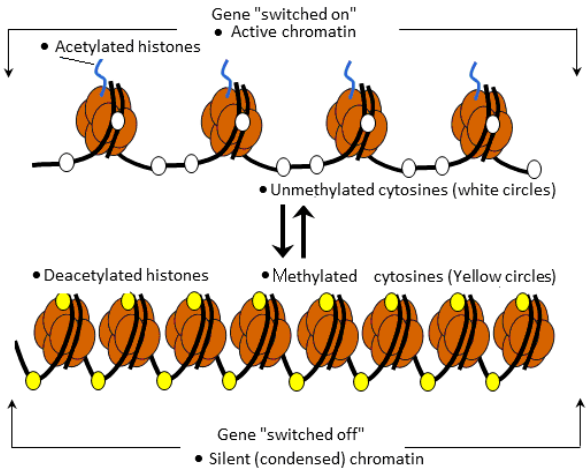


Figure 1c. Turning the gene transcription "on" and "off" by histone *de*/*acetylation*s and DNA *methylation*s

Although methylation of specific *lysine* and *arginine* has the same effect on transcriptional process, they vary in their functional responses for specific function. Methylation of H3-K9 and H4-K16, which are considered as an important modifications for gene silencing, has effect on the euchromatic and heterochromatic state of the gene and results in silencing of gene expression (*Stewart, et al., 2005*).

Methylation of H3K27 participates in the X chromosome inactivation by suppression of HOX genes (*Izzo and Schneider, 2011*).

H4K20me1 is involved in M phase of the cell cycle and in chromosomal condensation (*Huen et al., 2008*). These processes show that there is a strong relationship between histone modifications and DNA methylation. Despite the fact that each of these modifications is carried out by similar chemical reactions, each of them have its own molecular pathway in the regulation mechanism of transcription and gene expression (*Fig. 1a,c, d,e*).

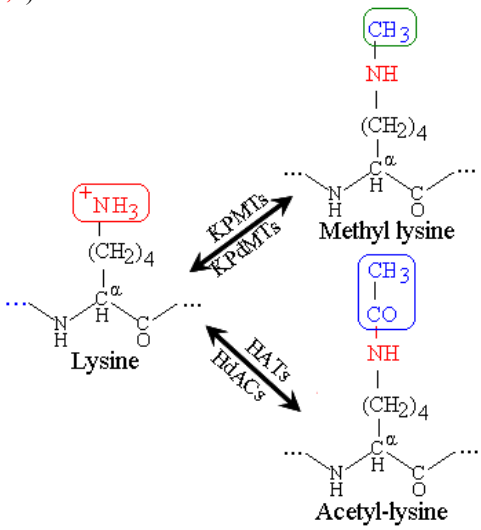
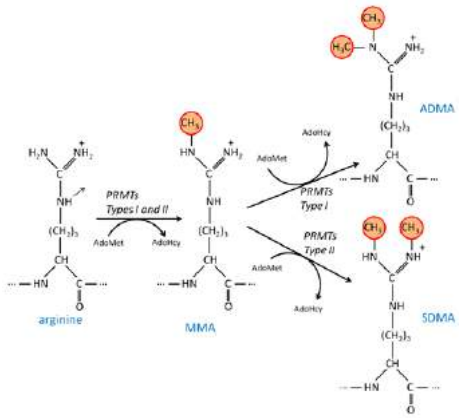


Figure 1d. Methylation (CH3-) of amino acid *Lysine* of histon proteins catalysed by **KPMTs** (K - stands for the single letter aa code of Lys) of *Lysine*-specific protein *MethylTrasferases;* and *Acetylation* (CH3-CO-) of *Lysine* catalyzed by **HATs** (*Histone Acetyl Transferases*). Both reactions increase the gene expression levels by neutralizing the strong positive charge of MH_3^+ group, which strongly binds to the negatively charged DNA (due to phospahete group of the DNA backbome). In the opposite reactions catalyzed by **KPdMTs** (Lysine Protein de-MethylTransferases), and **HdACs** (*Histone deACetylases*) genes are silenced as *RNApol* can not catalyze the transcription of mRNAs by opening the histon-DNA complexes (the symbols of the joined –N–C–C– backbones of AAs are indicated by three dots) (*Appendix3*)



Cont. Figure 1d. Methylation of *Agrinine* aa of the Histon proteins (MMA – *Mono Methyl Arginine*; ADMA – *Asymmetric DiMethyl Arg*., SDMA – *Symmetric DMA*) (*Esse er al., 2012*) (*Appendix 3*)

(E) DNA methylation

An emerging aspect of epigenetic play crucial role in the environment - genome interactions *(Jaenisch & Bird, 2003; Bonasio et al., 2010)*. Recently, many genome-wide association studies (GWAS) have established for genetic association with differences among human population phenotypes, physical appearance, social behavior, drug metabolism, sensory perception, response to environmental stimuli, and disease susceptibility *(Lachance et al., 2012)*. *Heyn et al. (2013)* identified DNA methylation differences that extricate three main human ethnic populations (Caucasian-American, African-American, and Han Chinese-American).

Two-thirds of population-specific CpG sites were associated directly with genetic background, showing the evolutionary genetic context of DNA methylation on the phenotype variation. Changes in CpG region density over the time were found to be responsible for DNA methylation levels observed among species (Feinberg & Irizarry, 2010). DNA methylation pattern showed differences between an African and an European population by studying 27.000 CpG sites *(Fraser et al., 2012)*, suggesting the linkage of population-specific methylation sites variance and the natural human variations (*Bell et al., 2012*). Thus, DNA methylation is a likely contributor to the different phenotypic characterisrics.

A recent genome-wide epigenetic analysis of honeybees also showed that, despite being genetically identical, the social behavior of the bees is strongly associated with their epigenetic profile, as DNA methylation significantly differ between nurse and forager bees (*Herb et al., 2012*).

(I.) Plant DNA methylation and its environmental stress response-specificity *(Appendix1,2)*

DNA methylation is not universal, as in the insect fruit fly *Drosophyla* has not been detected (*Hirochika et al., 2000*). In *Arabidopsis*, there are at least three classes of DNA methyltransferases (*METases*) catalyzing *asymmetric DNA methylation.* These are METs *(maintenance* methyltranferase), CMTs (chromomethylase3) and DRMs (*de novo* domains rearranged DNA methylases*)* (*Finnegan & Kovac, 2000)*.

In plants, levels of cytosine methylation was found to associate with elevated MET genes expression levels in both the symmetrical CG dinucleotide sites and the asymmetric CHH (H is A or T or C) sites. There are different enzymes, which are essential for the *de novo* and maintenance of DNA methylations. MET1 (DNA

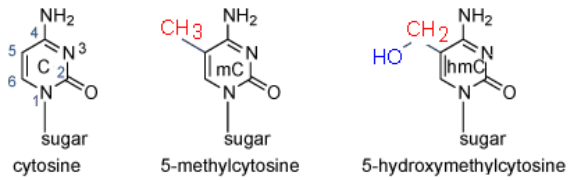


Figure 1e. Epigenetically important DNA bases of *cytosine* (C), *5-methylcytosine* (mC), and 5-*hydroxymethylcytosine* (hmC).

Methyltransferase1) and CMT1 (Chromomethylase1) are involved in the *de novo* cytosine methylation, and DRM2 (Domains Rearranged Methylase 2) maintains the symmetric DNA methylation *(Aufsatz et al., 2004, Gehring & Henikoff, 2007*). These enzymes accomplish a dynamic interchange between methylation and demethylation resulting in different gene expression levels (*Law & Jacobsen, 2010*).

Plant MET1 genes are similar in sequences and functions to mammalian *Dnmt*1, however, CMT3 is specific to the plant kingdom containing a chromo domain (*Henikoff & Comai, 1998*).

CMTs transfer a methyl group (CH_3) mainly from S-*ad*enosyl *met*hionine (AdoMet-dependent methyltransferases) mainly to the position of cytosine-C5 (EC 2.1.1.73), and also of cytosine-N4 (E.C. 2.1.1.13) (*Pósfai et al., 1989; Cheng & Roberts, 2001*), and adenine-N6 by adenine DNA methyltransferases (E.C. 2.1.2.72). The first eukaryotic adenine DNA methyltransferase was isolated from plants (wheat) and were found mainly responsible for the methylation of mitochondrial DNA (*Fedoreyeva & Vanyushin, 2002*).

The DRM class of *METases* includes DRM1 (624 aa) and DRM2 (626 aa) (syn. DNA-METase) (both EC 2.1.1.37) and contain catalytic domains which shows sequence similarity to mammalian *de novo Dnmt*3 (Cao & Jacobsen, 2002). In *Arabidopsis,* the same enzyme (DRM2) can methylate both cytosine and adenine nucleotides (*Vanyushin, 2006*).

A process of RdDM (*siRNA and micro-RNA directed DNA methylation*) also occurs in eukaryotes, which was also observed first in plants (*Wassenegger, 2000*).

The chromatin regulation through epigenetic modifications is important to the environmental responses. DNA methylation have a crucial impact on the plant response mechanism to biotic and abiotic stresses, which is crucial in the plant adaptation mechanisms.

(I.a) DNA methylation contributes to plant abiotic stress response

A number of recent reports have demonstrated that, different environmental stresses alter the DNA methylation status, and the levels of stress response related gene expressions. In pea and tobacco, the correlation between osmotic stress (water deficit) and the DNA methylation status was studied, and hypermethylation pattern was found due to osmotic stress *(Labra et al., 2002*). DNA methylation deficient mutant *MET1-3* was found hypersensitive to salt stress as a massive loss of cytosine methylation *(Baek et al., 2011*). Of the *Glyma11g02400* promoter of soybean (*Glycine max*), exposed to salt stress, most of the cytosines (from position 518 bp to -272 bp) were demethylated (*Song et al., 2012*).

Methylated DNA can be demethylated by exogenously applied DNA demethylating agent DHAC (5,6-dihydro-5'-azacytidine hydrochloride) as it was used in poplar (*Populus*) tree at the concentration of 10^{-4} M for 7 days, in aseptic tissue culture (*Gyulai et al., 2012*). In these study, DHAC treatments unregulated the endogenous poplar gene *gsh*1 by 19.7-fold, and

reactivated the 35S-*gsh*I transgene (cloned from E. coli) by 8.7-fold increment in the transgenic poplar.

Down regulation of *MET1* expression caused hypomethylation in maize roots exposed to cold stress, which affected Ac/Ds transposon region, coupled with demethylation level of *ZmMEI1* (*Zea mays* DNA methyltransferase1) *(Steward et al., 2000)* Consequently, demethylation of CG sites in the coding region caused an abiotic stress-induced gene activation. DNA methylation to demethylation shift was aslo found in rice grown under drought condition *(Wang et al., 2011)*. All these reports support the hypothesis that plant abiotic stress response mechanisms and adaptivity are crucially regulated by the genomic methylation status.

(I.b) DNA methylation contributes to plant biotic stress response

DNA methylation dynamics, as an 'immune' system to defense against biotic stresses have devolved in Plants. Many reports predicted a correlation between biotic stress induced defense-related genes and the levels of DNA methylation. *Dowen et al. (2012)* found a significant level of DNA hypomethylation of *Arabidopsis thaliana* exposed to bacterial pathogenes, which influenced elevated defense-related gene expressions. *Xa21G* R, the R-gene of rice, at demethylated level showed increased resistance to *Xanthomonas oryzae* pv. *Oryzae*. Both, hypomethylation level and resistance traits were stable inherited *(Akimoto et al., 2007)*. *NtAlix1*, a *tobacco mosaic virus* (TMV) responsive gene, was expressed at elevated level and associated with high levels of DNA methyltransferase transcripts *NtMET* activity (*Wada et al., 2004*).

However, *mungbean yellow mosaic India virus* (MYMIV) resistance of soybean was linked to a higher level of Intergenic Region (IR)-specific DNA methylation (Yadav & Chattopadhyay, 2011). Increased methylation level of IR cytosines coupled with *Tomato yellow leaf curl virus* (TYLCV) resistance was also reported in transgenic tobacco (*Bian et al., 2006*).

Useful plant DNA methylation data analysis was developed to identify the actual status of DNA methylation influenced by different experimental conditions (*Amin et al., 2014*).

All these results revealed that plant genome regulates gene-related pathogen defense mechanisms through the DNA methylation dynamics. Hence, epigenetic modifications through DNA methylation contribute critically to plant biotic stress defense mechanisms. The knowledge of this phenomenon will enhance the possibility of diminution of the diseases and pathogens stimuli on susceptible economic plants.

(II.) DNA methylation and human cancer development

Cancer development involves two type of DNA methylation processes of hypermethylation and hypomethylation. DNA hypermethylation was found in the promoter regions of several tumor suppressor genes, which silenced of those genes. This process is important for escaping apoptosis, loss of cell adhesion and angiogenesis, which are common features of tumors (*Herceg, 2007*).

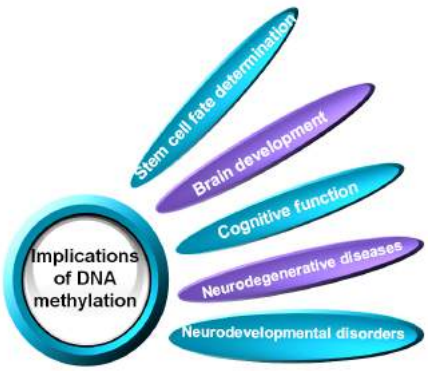


Figure 2. Implications of DNA methylation on *Neurodevelopmental and Neurodegenerative Disorders*

In contrast, hypomethylation was observed in almost all types of cancers, which may act through loss of imprinting, inappropriate cell type expression, genome vulnerability, and the activation of endoparasitic loci (*Esteller, 2007*). Studies have revealed that promoter hypermethylation of tumor suppressor genes is an early event in hepatocellular carcinogenesis of liver tissues including genes of p16INK4a, DLC1, E-Cadherin and PTEN (*Wong et al., 2007*)

Figure 3a. Intellectual disability, milder learning problems, autism, and anxiety are associated with CGG repeat expansion and methylation of the 5'UTR region of FMR1 gene resulting in Fragile X syndrome

(II) DNA Methylation in Neurodevelopmental and Neurodegenerative Disorders

In mammalian neuronal system, DNA methylation has been implicated in the regulation of neural stem cell fate determination, brain development, cognitive function, neurodevelopmental disorders, and neurodegenerative diseases (*Fig. 2*).

However, the number of neurodevelopmental disorders that have so far been associated with epigenetic aberrations is very limited (*Fuso, 2013*)

During the early embryo development, two major stages of epigenetic programming control the fate of embryonic cells. The first stage involves DNA demethylation/remethylation and reprogramming of histone PTMs (PostTranslational Modifications) in somatic cells.

The second stage involves the erasure and reestablishment of parental imprints by DNA methylation during embryo development. DNA methylation of neuron-restrictive silencer element also takes a part in preventing the non-neuronal cells from

differentiating into neurons by keeping proneural genes in an inactive state.

A part of chromosome n21 of people with Down syndrome associates with neurological changes and pathological aging due to DNA methylation.

(II.b) DNA methylation is associated with repeat instability

Expansion of trinucleotide repeats (TNRs) of a gene, either in the coding or non coding regions (*Fig. 3b*), during the formation of gametes can cause mutations and gene silencing.

Fragile X syndrome (FRAXA) is an X-chromosome-linked TNR disease (structurally a more then 200 CGG repeat expansion of the 5'UTR of FMR1 gene) (*Fig. 3a,b*), which affects the early neurodevelopment, and cause intellectual disability, milder learning problems, autism, and anxiety. Heterozygote females are not of high risk due to the presence of a non-expanded FMR1 allele. FMR1 (Fragile X Mental Retardation 1) is associated with aberrant CpG methylation pattern (*Fig. 4*). Unmethylated CGG repeats produce overexpression of the *FMR1* gene, which results in a toxic gain-of-function RNA that gives rise to the phenotypically distinct disorder called fragile X tremor/ataxia syndrome (FXTAS) (*Jacquemont et al., 2003*)

(II.c) DNA methylation and Human neurodegenerative disorders

Human neurodegenerative disorders are difficult to study. These diseases, such as *Parkinson's disease* (PD), *Alzheimer's disease* (AD), *Huntington's disease* (HD), Frontotemporal lobar degeneration (FTLD*), amyotrophic lateral sclerosis* (ALS), *Rett syndrome*, and *imprinting disorders*, are a group of disorders characterized by a progressive and specific loss of neurons. These diseases trigger neuronal cell death through endogenous suicide pathways. The neuronal death occurs relatively late in the degenerative process

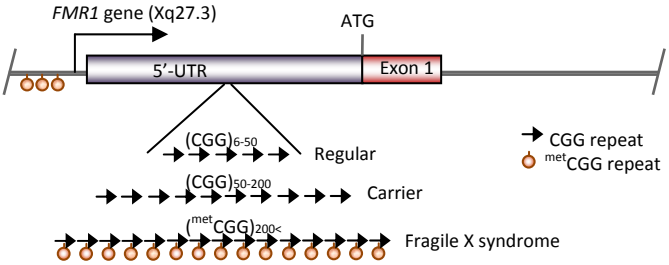


Figure 3b. Partial structure of the *FMR*1 (Fragile X mental retardation 1) gene. FMR1 is associated with the number and methylation level of trinucleotide repeat CGG (cytosine-guanine-guanine). *Non-FMR*1 (regular) people have unmethylated CGG within the 5'-untrunslated (5'-UTR) region of *exon* 1 with repeat number of 6 to 50. Fragile X carriers show repeat expansion with repeat number of 50 to 200. Fragile X syndrome patients show further repeat expansion over 200 CGG coupled with cytosine methylations of cgg repeats. Here, methylation also extends into the promoter region, which results in a silenced transcription of the *FMR*1 gene (after *Robertson and Wolffe, 2000*).

(d) Tissue specific DNA methylation patterns

DNA methylation patterns show high tissue specificity. In fact, different methylation levels were observed even across neuroanatomical regions within the brain. DNA methylation also plays a role in long-term memory formation and learning, although these mechanisms are not yet fully understood. Inactivation of DNA methylases was found to block contextual fear formation in rats, and genes such as PP1, RELN and the cell death itself may occur relatively late in the degenerative process of BDNF (brain-derived neurotrophic factor), which undergo changes in methylation during memory formation *(Liu et al., 2009*).

Alzheimer's disease (AD) is a neurological disorder and is the most prevalent form of age-related dementia in the modern societies. AD causes synaptic loss in specific brain regions, such as in the cortex and hippocampus *(Furuya et al., 2012*).

AD is characterized by neurofibrillary tangles, dystrophic neuritis, amyloid precursor protein, amyloid-β deposits coupled with increased activation of apoptosis pathways, impaired energy metabolism, mitochondrial dysfunction; oxidative stress, and DNA damage that lead to synaptic defects resulting in neuronal death (*Suzanne, 2008*). DNA hypomethylation and specific gene hypermethylation pattern were found among AD case studies. The methylation modification involved in a number of AD related genes such as *Tau* genes, which encodes proteins that stabilize microtubules. Aggregation of TAU protein in the brain is associated with a class of neurodegenerative diseases known as *tauopathies*. FK506- (an immunosuppressants) binding protein FKBP51, 51 kDa, forms a mature chaperone complex with Hsp90 (heat shock protein 90) that prevents TAU degradation. The decrease of DNA methylation in FKBP5 increased the expression of FKBP51, and high FKBP51 levels were associated with AD progression.

The conversion of 5-methylcytosine to 5-hydroxymethylcytosine (*Fig. 1e*) was discovered recently in mammalian DNA.

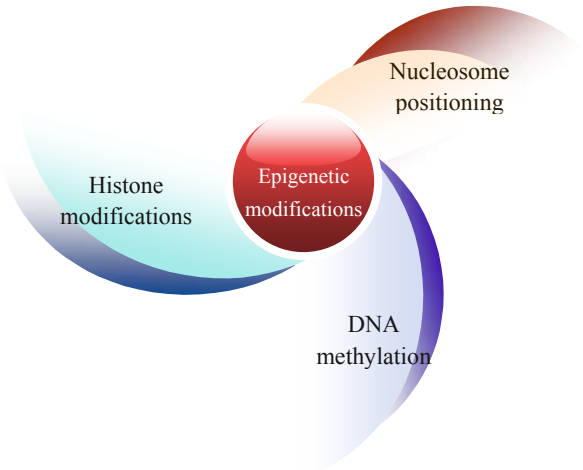


Figure 4. The *epigenetic* modifications of the DNA affect the gene functions through *histone modifications, nucelosome positioning* and DNA *methylation*

Huntington disease (HD) is a hereditary neurodegenerative disorder characterized by loss of

striatal neurons, and with motor, cognitive and psychiatric features that generally manifest in middle age. The *huntingtin* (*HTT*) gene (HTT) are large, spanning 180 kb and consisting of 67 exons (the large protein is 3144 aa, 13.7 Kb) (NCBI # NP_002102.4) (*Fig. 5*). Epigenetic mechanisms trigger apoptosis of motor neuron in HD through increasing DNA methylation, and upregulation of *DNA methyltransferases (DNMT)* (*Chestnut et al., 2011*). HD is caused by a CAG expansion in exon 1 of the huntingtin gene, which results in a poly-Q (polyglutamine) expansion in the *huntingtin* protein (HTT). There is an inverse correlation between CAG repeat length and age of onset with large expansions (*Evans-Galea et al., 2013*).

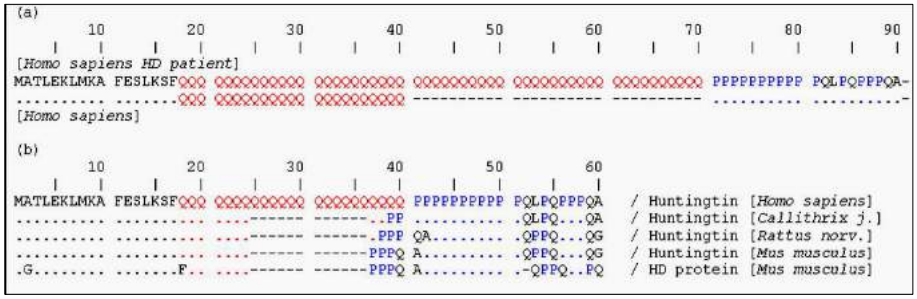


Figure 5. (a) Sample of amino acid (aa, indicated with single letter codes) sequence alignment of a domain (90 aa) (encoded by *exon*1 of *HTT* gene) of the HD patient (*Homo sapiens*) (*first row*), which shows #53 of the observed 51-57 *poly*Q; and the regular Huntingtin protein (HTT) with regular number (#23) of polyQ (poly-Glutamine; NCBI # NP_002102.4) (*second row*). *(b)* BLAST of the human HTT (NCBI #NP_002102.4) to other mammalian HTTs shows highly conserved regions of HTTs (*dot* indicates aa identical to the first row, and *dash* indicates aa absence). The *poly*P (P - *Proline*) stretches are also indicated (*in blue*)

Altered patterns of DNA methylation in cells expressing polyglutamine-expanded HTT (HunTingTin protein) was observed as compared with those of wild-type protein. The affected genes showed substantial overlap with genes that had been found to be deregulated in the presence of mutant HTT, which resulted in a changed gene expression in HD patients, which were partly attributable to DNA methylation (*Wood, 2013*). AP-1 and SOX2, the transcriptional regulators, are also associated with DNA methylation changes *(Ng et al., 2013*).

Parkinson disease (PD) is a multifactorial neurodegenerative and age related disorder, where environmental and genetic factors are involved in its etiology. Deregulation of DNA methylation was found to be associated with PD (*Masliah et al., 2013*). There is a significant decrease of DNA methylation levels in the frontal cortex of PD patients, and associated with the retention of DNMT1 (DNA cytosine-5-methyltransferase 1) in the cytoplasm (*Desplats et al., 2011*).

Detection of DNA methylation status of CpG islands of gene promoter regions of SNCA (*SyNuclein Alpha*) and LRRK2 (*Leucine-Rich Repeat Kinase* 2) showed that SNCA CpG-2 is hypomethylated in PD patients, and the methylation level is decreased in the early-onset PD patients. The methylation level of SNCA CpG-2 may use as a useful PD biomarker *(Tan al., 2014*).

The level of sulphur-containing amino acid homocysteine (Hcy), which is produced in S-adenosyl methionine cycle, showed increase in Parkinson's patients (*Doherty, 2013*).

Dr. Rett (the disorder was identified by *A Rett*) syndrome (originally termed as cerebroatrophic hyperammonemia) is caused by mutations in a methyl-CpG binding domain (MBD) protein (MeCP2) that causes loss of cognitive, motor and social skills, ending in severe mental retardation. MeCP2 participates in chromatin remodeling by recruiting key proteins to the methylated DNA sequences, which was characterized by mutations in genes coding for proteins involved in the regulation of DNA methylation *(Fuso, 2013*).

Ataxia-telangiectasia (A-T) is characterized as a neurodegeneration caused by protein deficiency of ATM (*Ataxia Telangiectasia Mutated*), and coupled with the accumulation of histone deacetylase-4 and the increase of trimethylation of histone H3 on Lsy27 *(Li et al., 2013*).

Frontotemporal lobar degeneration (FTLD) is a heterogeneous neurodegenerative disorder associated with personality changes and progressive dementia. It is due to methylation in the promoter region of *progranulin* gene, which may be a novel risk factor for the development of FTLD.

Mitochondrial DNA has an epigenetic regulation in normal and pathological conditions, particularly in neurodegeneration and aging. Changes in the level of 5mC and DNMTs are detected in neuronal mitochondria from patients with amyotrophic lateral sclerosis (ALS), which suggests that the motor neurons can engage epigenetic mechanisms involving DNMT upregulation and increased DNA methylation to drive apoptosis *(Iacobazzi et al., 2013*).

Since Recent studies have shed some light on the relationship between epigenetic alterations, neurodegenerative diseases (Portela and Esteller, 2010) and neurodevelopmental disorders *(Gapp et al., 2014*) new tools of genetics and methods of post-mortem brain tissue dissection would help in studying the relationship between epigenetics and cognitive decline *(Jones et al., 2013*).

Reference (for all paper cited):

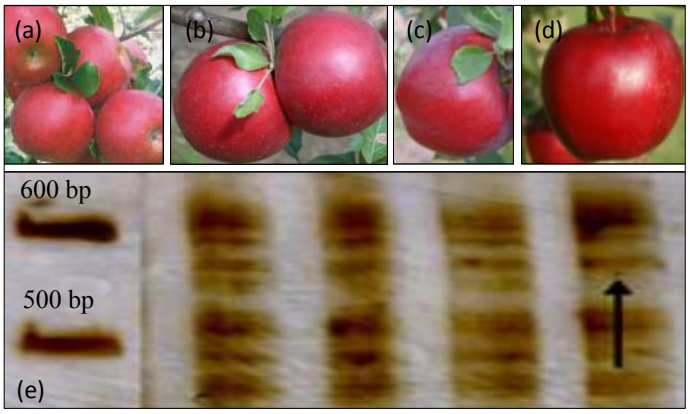
Alzohairy AM, II Amin, MH Elazma, H Elsawy, KY Kamal, AR Elhamamsy, G Gyulai, HMM Ibrahim, A Bahieldin, and MF Ramadan (2017) Universal Epigenetic Regulation of Gene expression through DNA methylation in Human and plant (*in press*)

Appendix1.

M-SAP (Methylation-Sensitive Amplification Polymorphism) (Reyna-L et al., 1997, MGG 253: 703-710) analysis of five apple varieties *(Malus domestica)* grown in Hungary of *cv. 'Jonathan' (a), 'Jonathan-M41' (b), 'Jonathan-Szatmarcseke' (c), and 'Jonathan'-Watson' (d).*

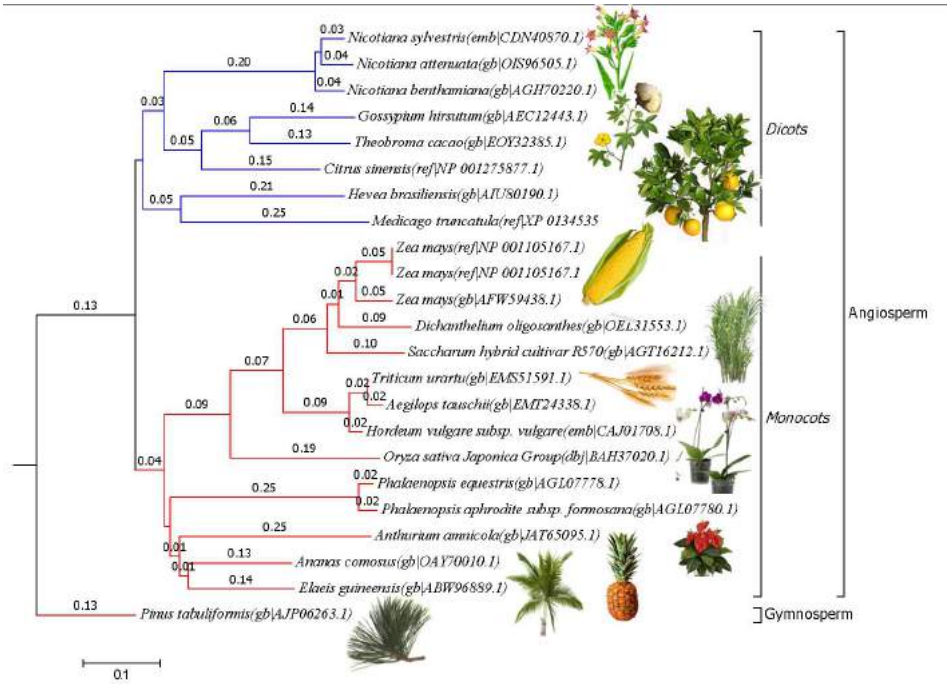
Total DNA were isolated in each case, DNAs were digested with *EcoRI* and methylation sensitive DNA restriction endonuclease *HpaII,* followed by selective PCR amplification with primer pairs of *Eco*RI-AGC (GACTGCGTACCAATTCAGC) and *Hpa*II/*Msp*I-TCAA (ATCATGAGTCCTGCTCGGTCAA).

Fragments were separated on silver stained PAGE gel (8%). *Mw*. sizes are indicated. The missing fragment is indicated with arrow *(from experiments of Szőke Antal et al., 2016).*



Appendix2.

Protein Phylogram (NJ – *Neigubor Joining*) of plant *DNA MTs* (*cytosine-5)-MethylTransferases*) aligned to *DNMT*3 of *Zea mays* (915 aa). The ID numbers (NCBI NP_001105167.1), branch length (MEGA7), relative genetic distance (scale 0.1), and the most significant plants are indicated (*Gyulai, 2016*)



Appendix3 (see Fig.1d).

(A) Methylation of aa *Arginine* of Histons. Molecular structure of *Arginine*, and the *mono- and di-methylarginines* are indicated. Type I and II *Protein Arginine MethylTransferases* catalyze the asymmetric and symmetric dimethylations, respectively.

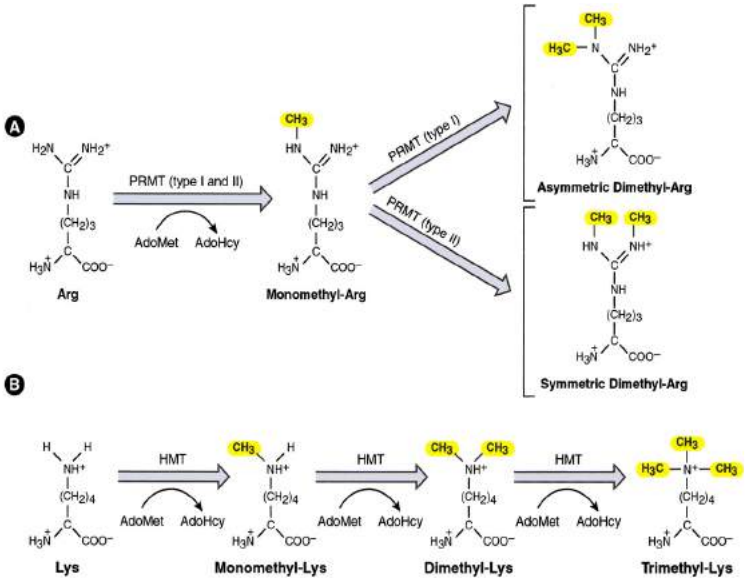
(B) Methylation of aa *Lysine* of Histons. Molecular structures of *Lysine* methylated to *mono-, di-,* and *tri-methyl-lysine* (*Zhang and Reinberg, 2001, Genes Dev.15:2343-2360*).

PRMTs (*Protein arginine methyltransferases*): The sequences used in the alignment include human *PRMT*1 (Q99873), human *PRMT*2 (P55345), human *PRMT*3 (O60678), mouse *PRMT*4 (AF117887), and human *PRMT*5 (AF015913).

HMTs (*Histone MethylTransferases*).

AdoMet (s-ADenOsyl-l-METhionine).

AdoHcy (s-ADenOsyl-l-HomoCYsteine).



Molecular markers, Genotyping, and ('Next generation') nucleic acids Sequencing

Introduction

The molecular markers based on PCR (*Polymerase Chain Reaction*) have been developed extensively for studies of molecular genetics, population genetics, genotype identifications and protection, monitoring of seed purity and hybrid quality, gene tagging, germplasm evaluation, phylogenetic studies, kinship studies, diagnostics, conservation genetics, forensic genetics, and *molecular barcoding*.

Molecular markers target different regions of the genomes (DNA) either at loci of *coding* vs. *non-coding*, *nuclear* vs. *organelle* (cp, and mt), *dominant* vs. *co-dominant*, or *linked* to genes or QTLs (*Quantitative Trait Loci*). Next to the biochemical (*i.e.* protein) markers such as IP (*Izoenzyme Polymorphism*) (*Hunter and Merkert, 1957*), there have been developed genetic (*i.e.* DNA) markers, such as RFLP (*Restriction Fragment Length Polymorphism*; *Grodzicker et al., 1974*), and the numerous PCR-based (*Saiki et al., 1985; Mullis and Faloona, 1987*) marker systems (*Table 1*). These markers are important and feasible tools to compare robust number of individuals, species and populations. Technically, these markers can be divided into five technical generations (*Table 1*).

Before application, all of these techniques need *primer design* (*e.g.* Primer3) at the first technical step including analyses for hairpin, self and heterodimer formations, and the suitable annealing temperatures (*Alzohairy et al., 2015*). The primer specificity should also be analyzed by BLAST (*Basic Local Alignment Search Tool*; *Altschul et al., 1997*) and MSA (*Multiple Sequence Alignments*) (*Fig. 1*) *in silico* with the software programs of either BioEdit (*Sequence Alignment Editor; North Carolina State University, USA*) (*Hall, 1999*), MULTALIN (*Combet et al., 2000*), CLUSTAL W (*Thompson et al., 1994*), MEGA4 (*Tamura et al., 2007*), or FastPCR (*Kalendar et al., 2009*). Useful servers are also available such as NCBI (*National Center* for *Biotechnology Information*), EMBL (*European Molecular Biology Laboratory*), IDT (*Integrated DNA Technologies*), GGB (*Grape Genome Browser*; *http://www.genoscope.cns.fr*), CGD (*Chloroplast Genome Database*; http://chloroplast.cbio.psu.edu/index.html), and *in-silico* PCR (http://insilico.ehu.es/PCR/).

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                                        2040       2050       2060       2070       2080       2090       2100
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
DQ886417 Vitis vinifera mybA1-RED 123 ACATGAAAGAGAAAGGGATCAGTATTTATTTGTGTTTTTTT-ACTTCTG---TTTTGCTTAAAGAGTTTC 188
DQ886419 V.vinifera mybA2-RED     123 .................G........AG.............-.......---..................... 188
DQ886420 V.vinifera mybA2-WHITE   123 ............................A..............T.......---..................... 189
DQ886421 V.vinifera mybA3-RED     123 ............................A..............-.......---..................... 188
DQ886422 V.vinifera mybA3-WHITE   123 ............................AG.............-.......---..................... 188

GU938680 Prunus avium mybA1       1072 --.---..A.C-------------------------------------------------------ATCACAT 1085
EU153581 P.avium myb10            1591 CT.GAG....T-------------------------------------------------------TTAC.. 1607
EU153578 P.armeniaca myb10        1610 CTGGAG....T-------------------------------------------------------TTAC.. 1626
EU153583 P.cerasifera myb10       1317 CTGGAG....T-------------------------------------------------------TTAC.. 1333
EU153582 P.cerasus myb10          1677 CT.AAG....T-------------------------------------------------------TTAC.. 1693
EU153580 P.domestica myb10        1394 CTGGAG....T-------------------------------------------------------TTAC.. 1410
EU155159 P.dulcis myb10           1259 --.CAG..A.T-------------------------------------------------------TTCACGA 1275
GU936492 P.persica myb10 RED      181  ...AAGC..G.-------------------------------------------------------TTGAA. 197
EU155160 P.persica myb10          1268 CTGGAG....T-------------------------------------------------------TTAC.T 1284
EU155161 P.salicina myb10         1335 CTGGAG....T-------------------------------------------------------TTAC.. 1351
GU938681 P.avium mybA2            1596 CT.GAG....T-------------------------------------------------------TTAC.. 1612
GU938682 P.avium mybF1            2012 CTGCTG.G...GCGATTGC.CTGGAGA.GAA.ACGCCC...CCTGGGCC---.AA..A.A.T.TT.A.A. 2078
```

Figure 1. Sequence alignments (70 nt) of the *MybR2R3* TF gene (*transcription factor* genes of nuDNA), which play crucial roles in the *fruit color development* including *Vitis* (Vitaceae) and *Prunus* (Rosaceae) species. Sequences were downloaded from the servers of NCBI, and GGB following the sequence alignments by *BioEdit*. Consensus nucleotides (.), deletions (-), SNPs (letters) and accession numbers (NCBI) are indicated.

(1) *Nested-PCR and Nested qRT-PCR*. Nested PCR involves two subsequent *uniplex* PCR reactions, in which the first PCR product (i.e. the nest) is used for the second set of primers, which amplifies a secondary target site within the first PCR product. One of the principal of this method is that if a wrong locus were amplified first by mistake the probability is very low to amplify it by the second time with the second primer pair. The other advantage is in the case of very low concentration of target sequence (*e.g.* viral infections). Based on qRT-PCR, nested qRT-PCR was found unique diagnostic tool to detect RNA viruses in the Human genome with the resolution rate of single tumor cell of 10^6 white blood cells.

(2) *Multiplex PCR***.** Compared to *uniplex* PCR, which amplifies single nucleic acid sequence of the genome, *multiplex* PCR (mPCR) is performed with more than one primer pairs in the same reaction mix, which amplify more than one target sequences. Several mPCR assays were suggested by research groups for microbiological quality control of food, water, clinical samples and pharmaceutical raw materials and products. An alternative PCR strategy can be applied by using *gradient thermocyclers*, which allow the use of primers of different annealing temperatures (T_{ann}) for simultaneous amplification of different targets in the same run.

Table 1. PCR-based DNA marker methods in sections and alphabetical orders (*Alzohairy, Gyulai et al., 2015*).

Acronym	Year	Methods	References
(1) *1st Generation Markers*			
ASO	1986	Allele Specific Oligonucleotides	Saiki *et al.* (1986)
AS-PCR	1988	Allele Specific Polymerase chain Reaction	Landegren *et al.* (1988)
OP	1988	Oligonucleotide Polymorphism	Beckmann (1988)
PCR	1985	Polymerase Chain Reaction	Saiki *et al.* (1985)
SSCP	1989	Single Stranded Conformational Polymorphism	Orita *et al.* (1989)
STS	1989	Sequence Tagged Site	Olsen *et al.* (1989)
VNTR	1985	Variable Number Tandem Repeats	Jeffreys *et al.* (1985)
(2) *2nd Generation Markers*			
AP-PCR	1990	Arbitrarily Primed Polymerase Chain Reaction	Welsh and McClelland (1990)
ARMS	1992	Amplification Refractory Mutation System PCR	Newton *et al.* (1989)
CAPS	1992	Cleaved Amplified Polymorphic Sequence	Akopyanz *et al.* (1992)
DOP-PCR	1992	Degenerate Oligonucleotides Primer - PCR	Telenius (1992)
ISJ-PCR	1991	Intron-Exon Splice Junction PCR	Weining and Langridge (1991)
ISSR	1994	Inter Simple Sequence Repeats	Zietkiewicz *et al.* (1994)
MAAP	1993	Multiple Arbitrary Amplicon Profiling	Caetano-Anolles *et al.* (1993)
RAPD	1990	Randomly Amplified Polymorphic DNA	Williams *et al.* (1990)
Double-RAPD	1991	RAPD by using two primers	Klein-Lankhorst *et al.* (1991)
RLGS	1991	Restriction Landmark Genome Scanning	Hatada *et al.* (1991)
SAMPL	1994	Selective Ampl. MicroSatellite Polymorph. Loci	Morgante and Vogel (1994)
SCAR	1993	Sequence Characterized Amplified Region	Paran and Michelmore (1993)
SSR	1992	Simple Sequence Repeats	Akkaya *et al.* (1992)
STMS	1990	Sequence Tagged Micro Satellite Sites	Beckmann and Soller (1990)
Tetra-PCR	1992	Allele specific amplification by tetra-primer PCR	Ye *et al.* (1992)
(3) *3rd Generation Markers*			
AFLP	1995	Amplified Fragment Length Polymorphism	Vos *et al.* (1995)
ASAP	1995	Allele Specific Associated Primers	Gu *et al.* (1995)
CFLB	1996	Cleavage Fragment Length Polymorphism	Brow (1996)
DAMD-PCR	1997	Directed Ampl. of Mini Satellite DNA-PCR	Bebeli *et al.* (1997)
M-SAP	1997	Methylation-Sensitive Amplified Polymorphism	Reyna-Lopez *et al.* (1997)
IMP	2001	Inter-MITE Polymorphism	Chang *et al.* (2001)
IRAP	1999	Inter- Retrotransposon Amplified Polymorphism	Kalendar *et al.* (1999)
ISTR	1996	Inverse Sequence-Tagged Repeats	Rohde (1996)
MITE	2000	Miniature Inverted-Repeat Transposable Element	Casa *et al.* (2000)
qRT-PCR	1996	quantitative Real Time PCR	Heid *et al.* (1996)
RBIP	1998	Retrotransposon Based Insertional Polymorphism	Flavell *et al.* (1998)
REMAP	1999	Retrotransposon-MicroSatellite Ampl. Polym.	Kalender *et al.* (1999)
R-ISSR	2005	Combinations of RAPD-ISSR and RAPD-SSR	Ye *et al.* (2005)
R-PCR	1995	Restricted-PCR	Puskás and Bottka (1995)
RT-PCR	1993	Real-Time PCR	Higuchi *et al.* (1993)
SNP	1994	Single Nucleotide Polymorphisms	Jordan and Humphries (1994)
SRAP	2001	Sequence Related Ampl. Polymorphism	Li and Quiros (2001)
SSAP	1997	Sequence Specific Ampl. Polymorphism	Waugh *et al.* (1997)
TE-AFLP	2000	Three Endonuclease AFLP	Van der Wurff *et al.* (2000)
Triple-RAPD	2008	Triple RAPD by using three primers, or more	Mansour *et al.* (2008)
(4) *New Generation Markers*			
DArT	2001	Diversity ARrays Technology	Jaccoud *et al.* (2001)
KASP	2013	Kompetitive Allele Specific PCR	Uitdewilligen *et al.* (2013)
MSAP	2003	Methylation Sensitive Ampl. Polymorphism	Baurens *et al.* (2003)
RGF	2008	Recursive Genome Function	Pellionisz (2008)
sRNA-qRT-PCR	2010	Small RNA qRT-PCR	Várkonyi-G and Hellens (2010)
(5) *Genome Sequencing (First- and Next Generation Sequencing)*			
AFFYMETRIX	1991	DNA and RNA Microarrays / Chip	Fodor *et al.* (1991, 2008)
ddNTPs	1980	Dideoxynucleotide Sequencing (ABI)	Sanger *et al.* (1980)
ILLUMINA / SOLEXA	2008/2006	The first Short Read Sequencer / bridgePCR	in: Bentley (2006)
Ion Torrent	2010	Proton sequencing (Portable sequencer) / emPCR	Pennisi (2010)
NanoPorSeq (?)	2015/1995	Nanopore genome sequencer	Hayden (2012)
ROCHE454	1996	Pyrosequencing (the 1st Next Gen. Seq.) / emPCR	Ronaghi *et al.* (1996, 1999)
RT-SEQ (SMRT)	2013/2010	Single Molecule Real Time seq./ PACBIOSCI	http://pacbiodevnet.com
SOLiD/ABI	2006	Seq. by Oligonucl. Ligation and Detection / emPCR	in: Tang *et al.* (2009)
StarLight (?)	2015	Single-molecule sequencing with quantum dots	in: Glenn *et al.* (2011)

(3) *ARMS-PCR* (*Amplification Refractory Mutation System* PCR). It is a multiplex type PCR by using tetra primers, which provides a fast SNP (*Single Nucleotide Polymorphic*) analysis coupled with sequencing or melting point analysis. Through the combinations of two *outer primers* and two allele-specific *inner primers* the genotyping requires regular PCR and fragment separations by electrophoresis.

(4) *SSR (Simple Sequence Repeats) PCR.*

In all prokaryotes and eukaryotes SSRs (*syn.*: microsatellites) are found universally with core sequence of SSR with 1 to 6 nucleotides (*Gupta et al., 1994*). The length polymorphism between individuals occurs due to the change in the number of this core repeats. *Dinucleotide core repeats* like (CA)n and (GA)n are the most abundant repeats. In humans, (CA)n repeat occurs once in every 30 kb. PCR primer pairs are designed to the sequences of flanking regions of the SSRs, and after the PCR amplification, fragment polymorphism is visualized on *agarose* or *polyacrylamide gels* (*Fig. 3a,b,c*). SSR PCR provides co-dominant and highly reproducible markers.

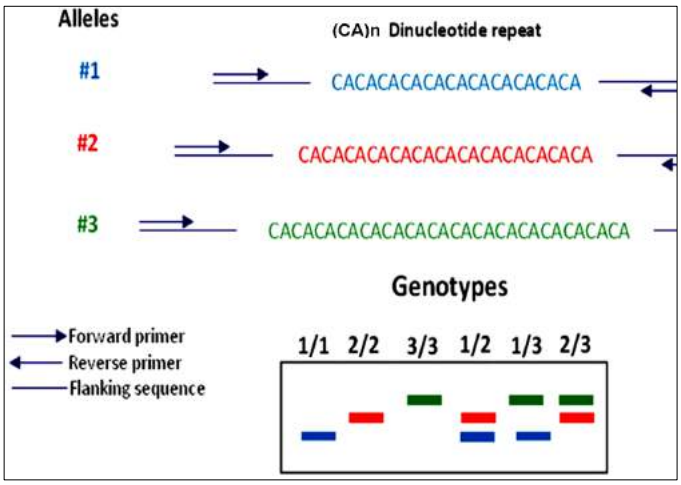


Figure 3a. Principle of the microsatellite (SSR) based barcoding in the case of a (CA)n repeat (*Alzohairy, Gyulai et al., 2015*).

Microsatellite markers were found very useful for population genetics, variety identification and protection, monitoring of seed purity and hybrid quality, gene tagging, germplasm evaluation, genome mapping and phylogenetic studies (*Lavin et al., 2003*) with or without bootstrap analysis (*Fig. 4*). In the case of hierarchical cluster analysis the *Maximum Likelihood* (ML) method was suggested to be the most comprehensive method (*Hillis et al., 1994*).

(5) *ALF-SSR*.

The SSR analysis can be automated and multiplexing by labeling the primers with fluorescent dye (*Fig. 3b*). A very sensitive and automated method is ALF-SSR PCR (*Automatic Laser Fluorometer*). In this method, one primer of each primer pair is labeled with Cy5 (*Phosphoramidite*), a cyanine type fluorescent dye, at the 5'-end (Sigma) according to *Röder et al. (1998)*.

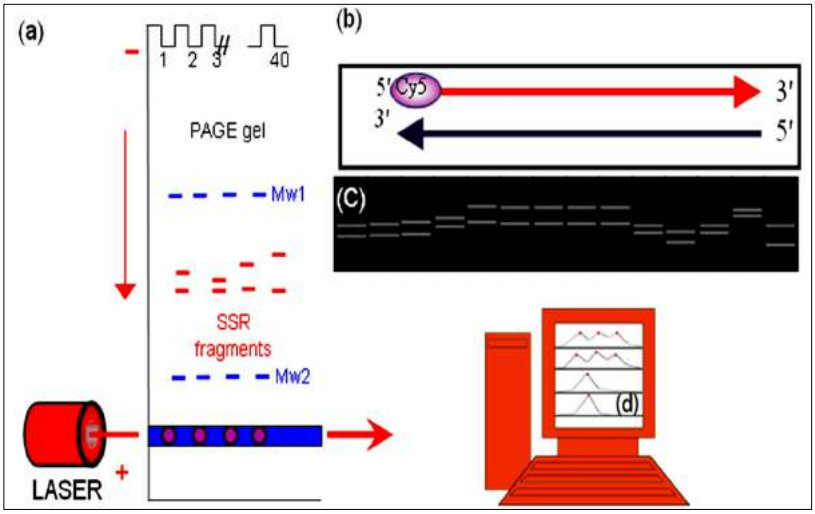


Figure 3b. Schematic flow chart of the ALF-SSR fragment separation by using ALF method (*Automatic Laser Fluorometer*; Amersham Bioscience) *(a)*. One strand of the PCR primer pair is labeled by Cy5 fluorescent die *(b)*. The outputs of gel image *(c)* and the real fluorograms *(d)* are shown. DNA loading lanes (1-40), electric current (poles + and -), Mw standards (Mw1 and Mw2), DNA fragments, and the data analyzing computer program (ALFwin Fragment Analyser) are indicated (*Alzohairy, Gyulai et al., 2015*).

The Cy5 labeled ALF-SSR fragments are excited by helium/neon *microlaser* at *643 nm*, and the emitted fluorescent signal of the Cy5 is detected at 667 nm by ALFexpress II DNA Analyser (Amersham Bioscience, Uppsala, Sweden - AP Budapest, Hungary). The ALF-SSR fluorograms are analyzed by computer program of ALFwin Fragment Analyser Version 1.00 (Pharmacia – Amersham Bioscience, Uppsala, Sweden. AP-Hungary, Budapest) according to *Röder et al. (1998), Huang et al. (2002)*. After sequencing, SSR fragments show a repetitions of the core nucleotide sequence (*Fig. 3c*).

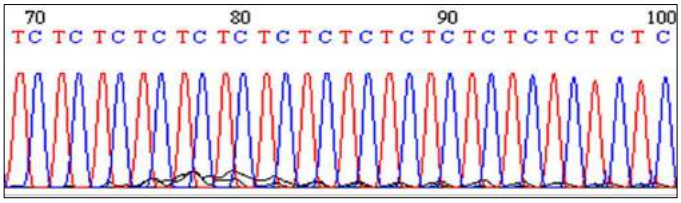


Figure 3c. Sample of the fluorogram of an SSR (TC)n repeat (68 to 100 nt) of watermelon (*Citrullus lanatus*) microsatellite amplified and sequenced by ABI PRISM 3100 *(Szabó and Gyulai, 2005)*

Figure 3d. Samples of melon (above) and watermelon (*below*) genotyping by microsatellite markers (*Szabó Z, G Gyulai, et al., 2005, Euphytica 146:87–94; Tóth Z, G Gyulai, et al., 2007, Agrártud Közl 27: 125–134*).

(6) *Inter-simple sequence repeats (ISSR).*

ISSR amplifies target DNA regions located between two identical microsatellite repeats (SSRs) (*Zietkiewicz et al., 1994*). ISSR was used extensively for producing molecular markers in several crops and horticultural plants (*Lágler et al., 2005*) (*Fig. 4*).

(7) *RAPD-ISSR.*

RAPD-PCR combines RAPD (discussed below) and ISSR primer pairs in PCR reactions, which are able to reveal new genomic loci that could not be detected with either of the primer alone. Further combinations of the different markers also suggest new possibilities in DNA barcoding.

(8) *R-PCR (Restricted PCR).*

For reduction of nonspecific PCR amplifications, which is caused by mispriming during PCR reactions, besides the standard pair of primers, 3'-dideoxy-terminated competitor oligonucleotides were applied in the R-PCR reactions (*Puskás and Bottka, 1995*). By this way an enhanced specificity of target site amplification was achieved. The competitor oligonucleotides act by masking the possible sites of nonspecific primer-template interaction, thus excluding undesired PCR extensions. This technique is generally applicable when highly degenerate primers are used.

(9) *DGGE-RAPD (Denaturing Gradient Gel Electrophoresis RAPD).*

The detection of DNA polymorphism in self-pollinating species was found to be difficult. To facilitate, DGGE (Denaturing Gradient Gel Electrophoresis) was used for RAPD analysis. In DGGE gel (12% acrylamide in TAE buffer with denaturant gradient, 10-50%, of 7 M urea and 40% formamide) the two alleles of a locus (if different) run separately. This method greatly improved the detection of reproducible DNA polymorphism among closely related plant species and lines. It was used first to estimate pedigree relationships among plant materials in wheat (*Triticum*), barley (*Hordeum*) and oat (*Avena*) (*Dweikat et al., 1993*). DDGE-RAPD was also found highly discriminative for the identification of barley (*Hordeum*) cultivars with different pedigree, and proved that DGGE-RAPD is a superior method for detecting DNA polymorphism when compared to agarose-RAPD, or polyacrylamide-RAPD methods (*Fig. 5*).

(10) *Single-, Double-, and Triple-Primed RAPD.*

RAPD (*Random Amplified Polymorphic DNA*) assay was one of the earliest and widely used PCR PCR by using single primer of arbitrary nucleotide sequence of 10 nt (*Williams et al., 1990*). The potential of the original RAPD assay was further increased by combining two and three primers (*Fig. 6*) in the same PCR reaction. This technique was extensively used for barcoding of economically important crops.

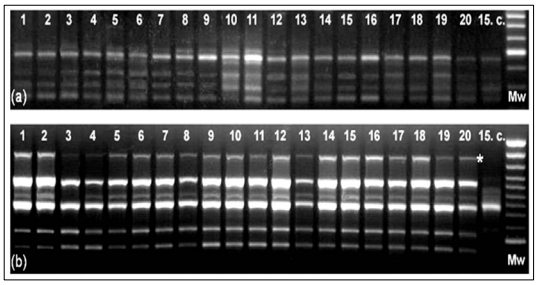


Figure 4. ISSR analysis. Samples of ISSR PCR with *monomorphic (a)* and *polymorphic (b)* band patterns on agarose gel (0.8 %), which were amplified by primers of FV835 [$(AG)_8YC$] *(a)*, and FV811 [$(GA)_8C$] *(b)*, in common millet (*P. miliaceum*) cultivars (1 to 20) compared to an ancient medieval (15^{th} CENT.) sample (indicated as 15.c). Mw – 100 bp DNA ladder. Asterisk indicates a missing ISSR fragment (*Lágler, Gyulai et al., 2005*).

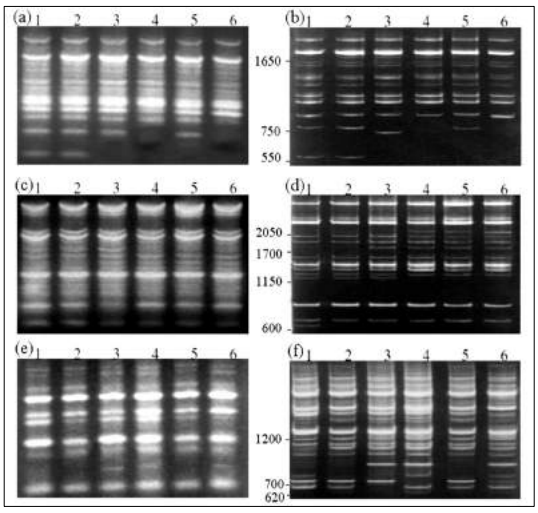


Figure 5. RAPD. Comparison of fragment patterns of agarose-RAPD *(a)*, *(c)* and *(e)*; and compared to DGGE-RAPD *(b)*, *(d)* and *(f)* generated by Operon RAPD primers of OP-A12 *(a)* and *(b)*; OP-B09 *(c)* and *(d)*; and OP-B15 *(e)* and *(f)* used for genotyping six barley (*A. s.*) cultivars (*Bahieldin et al., 2006*).

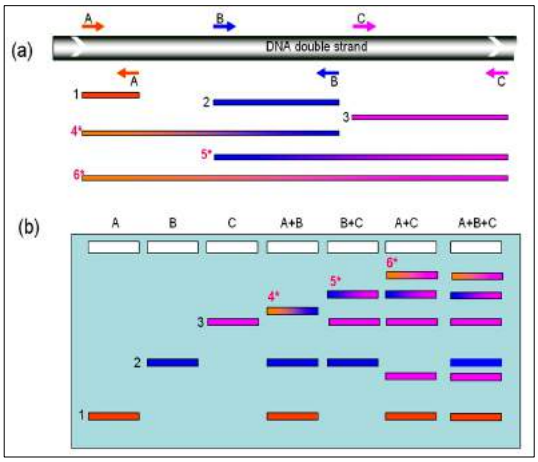


Figure 6. RAPD PCR. Principles of the Single- (A), Double- (A, B), and Triple- (A, B, and C) primed RAPD (*a*) with indications of hypothetical band patterns (*b*). Only a single locus (band) per primers is indicated. Extra bands amplified with double and triple primers are indicated with asterisk (*Alzohairy, Gyulai et al., 2008*).

(11) *Retrotransposon based PCR markers.*

Retrotransposons (RTs) are major constituents of most eukaryotic genomes; they are ubiquitous, dispersed throughout the genome, and their abundance correlates with the host genome sizes, which provides unique possibilities for molecular *barcoding* (*see Chapter 6*).

(12) *Organelle (Chloroplast and Mitochondria) specific PCR markers.*

DNAs of chloroplast (cpDNA) and mitochondria (mtDNA) have been used very frequently in plant systematic and phylogenetic studies (*Ali et al., 2014*). Both organelle DNAs are circular molecules ranging in size of 120 Kbp to 500 Kbp (*Fig. 7*), with unique exception of green alga *Floydiella terrestris* with huge cpDNA of 521.168 bp (NCBI# NC_014346); and *Cucumis melo* (*Alverson et al., 2010*) with giant mtDNA (2,900,000 bp).

Size correlations between cpDNA and mtDNA genomes of plants and algae show a shift (*Fig. 7*) from green algae (*Chara vulgaris*) with high cpDNA/mtDNA ratio (2.73) through mosses of *Physcomitrella patens* (1.16) and *Marchantia polymorpha* (0.65) towards flowering plants of dicots to monocots with exception of *Vitis*.

The decreasing ratio of cpDNA/mtDNA indicates an enlargening mtDNA during the evolution*: Spirodela polyrhiza* (0,74); *Brassica napus* (0,69); *Daucus carota* (0,55); *Helianthus annuus* (0,50); *Arabidopsis thaliana* (0,42); *Lotus japonicus* (0,40); *Vaccinium macrocarpon* (0,38); *Glycine max* (0,38); *Vigna radiata* (0,38); *Huperzia lucidula* (0,37); *Ricinus communis* (0,32); *Sorghum bicolor* (0,30); *Triticum aestivum* (0,29*); Liriodendron tulipifera* (0,29); *Oryza sativa Japonica* (0,274); *Oryza sativa Indica* (0,273); *Zea mays* (0,25); *Oryza rufipogon* (0,24); *Phoenix dactylifera* (0,22); and *Vitis vinifera* (0,21). NCBI data were plotted by XY plot of Microsoft Windows Xcel program (*Ali et al., 2015*).

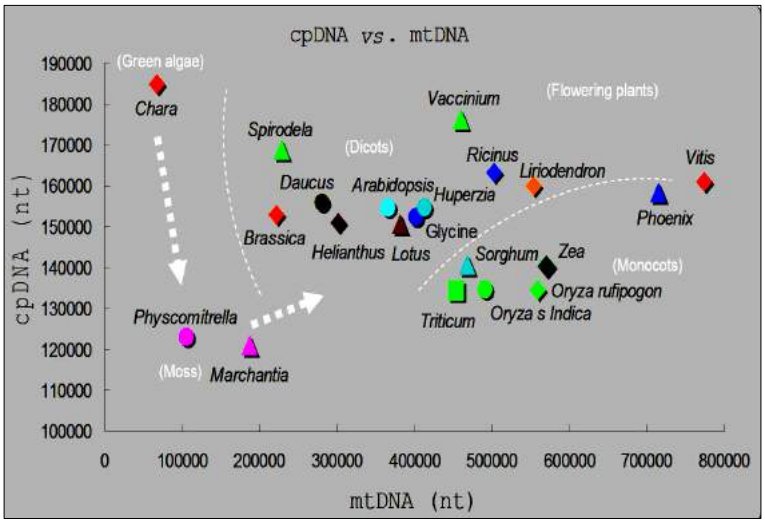


Figure 7. Size correlations between genomes of cpDNA (*i.e.,* plastom) and mtDNA (*i.e.,* mitom) of algae and higher plants *(Gyulai et al., 2015)*.

There are about 100 functional genes encoded in the chloroplast genomes, which contains, with few exceptions (IRL *vs.* IRless), two duplicate regions of *inverted repeats* (IR) in reverse orientation (from 10- to 76 kb). They divide the chloroplast genome into *large* (LSC) and *small single-copy* (SSC) regions.

The structural organization of chloroplast genome is highly conserved, *i.e.*, relatively free of large deletions, insertions, transpositions, inversions and SNPs (single nucleotide polymorphism), which make it advantageous for phylogenetic studies. Chloroplast DNA is abundant (generally, 50 chloroplasts are in a plant cells, and a single chloroplast have 50 cpDNA copy, which results 2.500 cpDNA copy per cell) compared nuclear DNA (which is generally 2n).

Organelle DNAs are usually uniparentally inherited (*paternally* in gymnosperms*, and maternally* in angiosperms, in general, with some exceptions), which facilitate to determine the maternal parent in hybrids and allopolyploids. Some chloroplast regions like *psb*A-*trn*H, and rps16 evolve relatively rapidly. There are a number of noncoding cpDNA regions which are also useful target of study such as the intergenic spacer of *atp*B-*rbc*L.

Due to the evolutionary high AT-content of cpDNA and mtDNA of higher plants compared to nuDNA (*Fig. 8*) lower temperatures are used for PCR reactions (*Demesure et al., 1995; Dane et al., 2004*).

(13) *SNP (Single Nucleotide Polymorphism) analysis*

Single nucleotides of DNA (A, T, C, or G) in the genome sequence is altered among individuals of the same species, which together give an individual-specific SNP pattern. SNPs can be determined either by *melting pint analysis* of qPCR, or by fragment or total genome sequencing (*Table 1*). SNPs occur approximately once every 100 to 300 bases of the genomes.

Besides that, *e.g.* the SNaPshot® Multiplex System (ThermoFisher Sci) was developed for the analysis of SNPs based on a primer extension method. Through its multiplexing capability, up to 10 SNPs can be analyzed in a single reaction, regardless of their positions on the chromosome or the amount of separation from neighboring SNP loci. The ability to use unlabeled user-defined primers allows researchers to incorporate SNPs of interest cost-

effectively. The Multiplex Ready Reaction Mix helps ensure robust, reproducible analyses of multiplexed samples. Researchers can analyze more than 23,000 SNP genotypes per day.

CAPS (*Table 1*), and ITS markers (*Chapter 7*) are also frequently used.

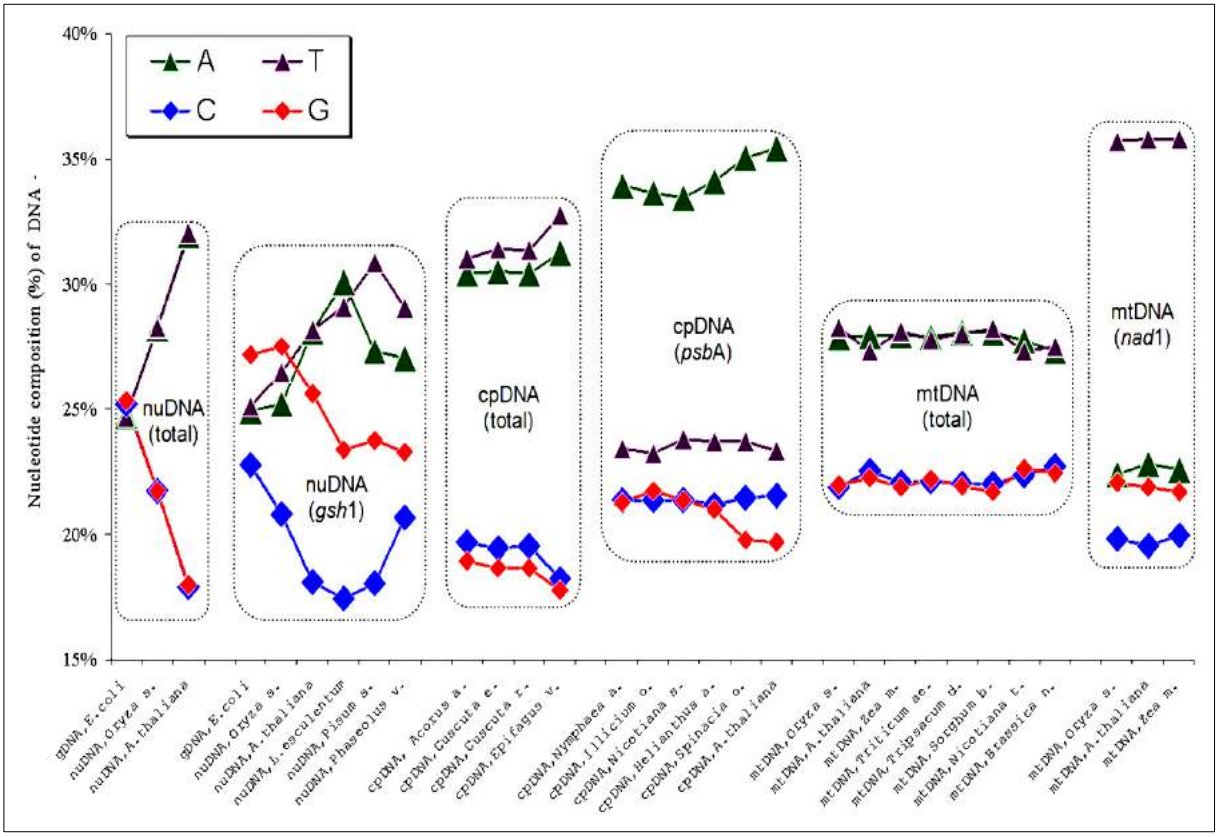


Figure 8. Nucleotide compositions of sequences of *coding* vs. *non coding*, and *nuclear* vs. *organelle* (cp and mt) DNAs. *Total genomes* (nuDNA, gDNA, cpDNA, and mtDNA) are compared to *coding gene* sequences (*gsh*1; *psb*A and *nad*1). Accession numbers of NCBI are NC_003070; NC_008402.1; NC_003076.5; X03954; AJ508916; Y09944; AF017983; AF128455; AF128454; NC_010093.1; NC_009963.1; NC_009766.1; NC_001568.1; NC_006050; EF380354; AB237912; DQ383815; AJ400848; AP000423; NC_007886.1; NC001284.2; NC_007982.1; NC_007579.1; NC_008362.1; NC_008360.1; NC_006581.1; NC_008285; NC_007886; NC_001284; and NC_007982 *(Gyulai et al., 2015)*.

(14) *Total Genome Sequencing*

The new generation markers, and the total genome sequencing (*Table 2*), such as the ddNTP technology (*Sanger et al., 1980*), the Chip technologies (*Fodor et al., 1991*), and the second- and new generation sequencing, and the newest *proton-* and *nanopore* sequencing have revolutionized genomics (*Table 2*). Total nuclear (nuDNA), mitochondrial (mtDNA) and chloroplast DNS (cpDNA) have been sequenced. The results provide basic knowledge for phylogenetisc (*Fig. 9, 10; and Appendix 1*).

The phylogram of 23S rRNA gene of cpDNA of grapevine (*Vitis vinifera*) was compared recently (*Fig. 9*) to the *evolutionarily first land plant* of ferns *Selaginella uncinata* (*Seun*); the ancient higher plant of gymnosperm *Pinus thunbergii (Pinth*) and *Pinus koraiensis* (*Pinko*); *evolutionarily the first angiosperms tree Amborella trichopoda* (*Amtr*) of the basal angyosperm ANITA (*Amborella, Nymphaeales, Illiciales, Trimeniaceae* and *Austrobaileya*) group,

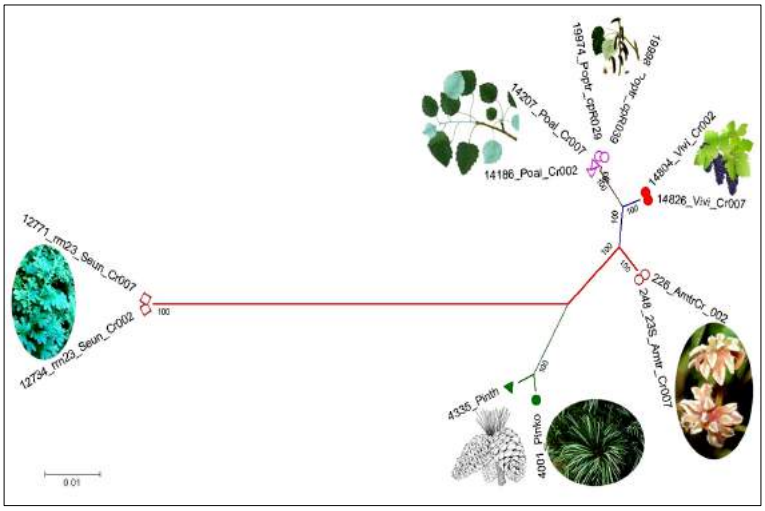


Figure 9. Bootstrap (1000 replicates) radial ML phylogram of plant 23S rRNA genes of cpDNA of significant plant species *(Gyulai et al., 2015)*.

which plant (a dwarf tree) by xylotomy still resembles to gymnosperms (with *homoxylon* trunk tissues), but by flowers do it to angiosperms. *Vitis vinifera* (*Vivi*) and two poplars (*Poal - Populus alba,* and *Poptr - P. trichocarpa*) were also included.

Total genome sequencing (*Table 2*).

(1) *Maxam-Gilbert DNA sequencing* (1977). *A Maxam* and *W Gilbert* DNA sequencing method (1977) is based on chemical synthesis of DNA (*i.e.* chemical sequencing) by using radioactive labeling at one 5' end of the DNA. Chemical treatment then generates breaks at a small proportion of one or two of the four nucleotide bases in each of four reactions (G, A+G, C, C+T). The fragments in the four reactions are electrophoresed side by side in denaturing acrylamide gels for size separation. To visualize the fragments, the gel is exposed to X-ray film for autoradiography.

(2) *Sanger sequencing (Chain-termination DNA sequencing)* (1977). The method was developed by *F. Sanger* and coworkers and soon became the most frequently used method, owing to its reliability. When invented, the chain-termination method used fewer toxic chemicals and lower amounts of radioactivity than the *Maxam and Gilbert* method. Sanger method was soon automated (by ABI) and was the method used in the *first generation of DNA sequencers*. Over the period, great advances were made in the technique, such as the usage of *fluorescent labeling*, *capillary electrophoresis*, and general automation. F Sanger is a double Nobel prized scientist (1958, for sequencing the insulin), and (1980) for DNA sequencing.

(3) *Shotgun sequencing* (*Staden, 1979*). It is a sequencing method designed for analysis of DNA sequences longer than 1000 base pairs, up to entire chromosomes. This method requires the target DNA to be broken into random fragments. After sequencing individual fragments, the sequences can be reassembled on the basis of their overlapping regions.

(4) *Bridge PCR* (Illumina). The method is used for *in vitro* clonal amplification by *bridge PCR*. Fragments are amplified by primers attached to a solid surface. This method is used in the *Illumina Genome Analyzer* sequencers. The Helicos *Bridge PCR* uses a single-molecule methods developed by *Stephen Quake's* laboratory (and later commercialized by Helicos), by using bright *fluorophores* and *laser excitation* to detect base addition events from individual DNA molecules fixed to a surface.

First generation sequencing.

These methods are applied to genome sequencing, genome resequencing, transcriptome profiling (RNA-Seq), DNA-protein interactions (ChIP-sequencing), and epigenome characterization. Resequencing is necessary, because the genome of a single individual of a species will not indicate all of the genome variations among other individuals of the same species.

(5) *Massively parallel signature sequencing (MPSS)*. It was the first of the *next-generation sequencing* technologies, developed in the 1990s at *Lynx Therapeutics*, a company founded in 1992 by *Sydney Brenner* and *Sam Eletr*. MPSS is *a bead-based* method that uses an *adapter ligation* followed by adapter decoding, and reading the sequence in increments of the four nucleotides. Because the technology was so complex, MPSS was only performed in-house by *Lynx Therapeutics* and *no DNA sequencing machines were sold* to independent laboratories. Lynx Therapeutics merged with *Solexa* (later acquired by *Illumina*) in 2004, leading to the development of *sequencing-by-synthesis*, a simpler approach acquired from *Manteia Predictive Medicine*, which rendered MPSS obsolete. However, the essential properties of the MPSS output were typical of later next-generation data types, including hundreds of thousands of short DNA sequences. In the case of MPSS, these were typically used for sequencing cDNA for measurements of gene expression levels.

(6) *Colony sequencing (SOLiD)*. The method was developed in the laboratory of *George M Church* at Harvard Univ., and used to sequence a full *E. coli* genome in 2005. The method combines an *in vitro* paired-tag library with *emulsion PCR*, an automated microscope, and ligation-based sequencing chemistry at an accuracy of >99.9999% and a cost approximately $1/9^{th}$ that of Sanger sequencing. The technology was licensed to *Agencourt Biosciences*, subsequently spun out into *Agencourt Personal Genomics*, and eventually incorporated into the *Applied Biosystems SOLiD* platform. *Applied Biosystems* was later acquired by *Life Technologies*, now part of *Thermo Fisher Scientific*.

Next generation sequencing.

(7) *Pyrosequencing (Roche 454)*. The methodwas developed by *454 Life Sciences*, which has since been acquired by *Roche Diagnostics*. The method amplifies DNA inside water droplets in an oil solution (*emulsion PCR*), with each droplet containing a single DNA template attached to a *single primer-coated bead* that then forms a clonal colony. The sequencing machine contains picoliter-volume wells, each containing a single bead and sequencing enzymes. Pyrosequencing uses *luciferase* enzyme to generate light for detection of the individual nucleotides added to the nascent DNA. This technology provides intermediate read length and price per base compared to Sanger sequencing.

(8) *Illumina HiSeq 2500 (Solexa Inc.) sequencing* (1988). *Solexa*, now part of *Illumina*, was founded by

S Balasubramanian and *D Klenerman*, and developed a sequencing method based on *reversible dye-terminators* technology, and engineered polymerases. The technology was invented in 1997 by *P Mayer* and *L Farinelli*, which is based on DNA clusters or DNA colonies, which involves the clonal amplification of DNA on a surface. The cluster technology was co-acquired with *Lynx Therapeutics* of California. *Solexa Ltd*. later merged with *Lynx* to form *Solexa Inc*. In the cluster technology, DNA molecules and primers are first attached on a slide or flow cell and amplified with polymerase so that local clonal DNA colonies (later coined DNA clusters) are formed. To determine the sequence, four types of *reversible terminator bases* (RT-bases) are added and non-incorporated nucleotides are washed away. A *camera* takes images of the *fluorescently labeled nucleotides*. Then the dye, along with the terminal 3' blocker, is chemically removed from the DNA, allowing for the next cycle to begin. There is an *Illumina MiSeq sequencer* also.

(9) *SOLid Sequencing*. *Applied Biosystems*' SOLiD technology (now a *Life Technologies* brand) employs *sequencing by ligation*. A pool of all possible oligonucleotides of a fixed length is labeled according to the sequenced position. *Oligonucleotides are annealed and ligated*; the preferential ligation by *DNA ligase* for matching sequences results in a signal, which is informative of the nucleotide at that position. Before sequencing, the DNA is amplified by *emulsion PCR*. The resulting beads, each containing single copies of the same DNA molecule, are deposited on a *glass slide*.

(10) *Proton sequencing (Ion Torrent semiconductor sequencing)*. Ion Torrent Systems Inc. (now owned by *Life Technologies*) developed a system based on using standard sequencing chemistry, but with a novel, *semiconductor based detection* system. This method of sequencing is based on the detection of *hydrogen ions* that are released during the polymerisation of DNA. A microwell containing a template DNA strand to be sequenced is flooded with a single type of nucleotide. If the introduced nucleotide is complementary to the leading template nucleotide it is incorporated into the growing complementary strand. This causes the release of a hydrogen ion that triggers a *hypersensitive ion sensor*, which indicates that a reaction has occurred.

As the results of the function *ligase* enzyme during both the natural process of DNA replication and the DNA synthesis during sequencing, pyrophosphate molecule releases which is detected by *Pyrosequencing*. A H ion (proton) also releases every step of DNA elongation, which proton is detected by *Proton sequencing* (*Fig. 10*).

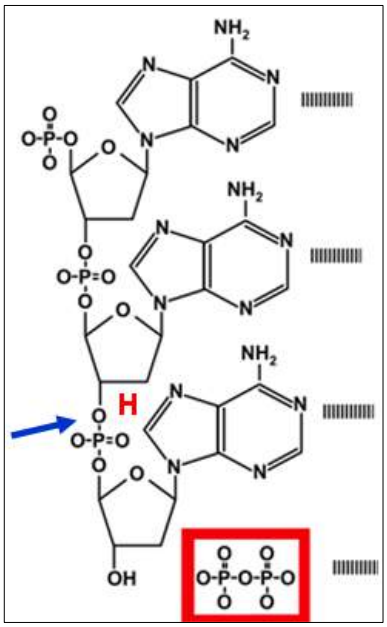


Figure 10. The release of Hydrogen ion (*i.e.,* proton) and pyrophosphate molecule during DNA synthesis.

New generation sequencing under development (for information only).

(11) *DNA nanoball sequencing.* The method is a type of high throughput sequencing technology used to determine the entire genomic sequence of an organism. The company *Complete Genomics* uses this technology to sequence samples submitted by researchers. The method uses *rolling circle replication* to amplify small fragments of genomic DNA into DNA nanoballs. Unchained sequencing by ligation is then used to determine the nucleotide sequence. The method allows large numbers of DNA nanoballs to be sequenced per run and at low reagent costs compared to other next generation sequencing platforms.

(12) *Heliscope single molecule fluorescent sequencing*. It is a method of single-molecule sequencing developed by Helicos Biosciences. It uses DNA fragments with added *poly-A tail adapters* which are attached to the flow cell surface. The next steps involve *extension-based sequencing* with cyclic washes of the flow cell with *fluorescently labeled nucleotides* (one nucleotide type at a time, as with the Sanger method). The reads are performed by the Heliscope sequencer. The reads are short, averaging 35 bp. *In 2009 a human genome was sequenced using the Heliscope*, however in 2012 the company went bankrupt.

(13) *SMRT (Single Molecule Real Time) sequencing (PacBio)*. SMRT sequencing is based on the *sequencing by synthesis* approach. The DNA is synthesized in zero-mode wave-guides (ZMWs) – small well-like containers with the capturing tools located at the bottom of the well. The sequencing is performed with use of unmodified polymerase (attached to the ZMW bottom) and *fluorescently labelled nucleotides* flowing freely in the solution. The wells are constructed in a way that only the fluorescence occurring by the bottom of the well is detected. According *to Pacific Biosciences (PacBio)*, the SMRT technology developer, this methodology allows detection of nucleotide modifications (such as *cytosine methylation*). This happens through the observation of polymerase kinetics. This approach allows reads of 20,000 nucleotides or more, with average read lengths of 5 kilobases. In 2015, Pacific Biosciences announced the launch of a new sequencing instrument called the *Sequel System*, with 1 million ZMWs compared to 150,000 ZMWs in the PacBio RS II instrument.

(14) *Nanopore DNA sequencing (Oxford Nanopore Technologies, UK).*
This method is based on the readout of *electrical signals* occurring at nucleotides passing by *alpha-hemolysin pores* covalently bound with *cyclodextrin*. *The DNA passing through the nanopore changes its ion current*, which depends on the shape, size and length of the DNA molecule. *Each type of the nucleotide blocks the ion flow through the pore for a different period of time*. Two main areas of nanopore sequencing are in development, the *solid state-*, and *protein based nanopore sequencing*. Protein nanopore sequencing utilizes membrane protein complexes Hemolysin and MspA (Mycobacterium Smegmatis Porin A), which show great promise given their ability to distinguish between individual and groups of nucleotides. In contrast, *solid-state nanopore* sequencing utilizes synthetic materials such as *silicon nitride* and *aluminum*

oxide and it is preferred for its superior mechanical ability and thermal and chemical stability. The concept originated from the idea that single stranded DNA or RNA molecules can be *electrophoretically driven* in a strict linear sequence through a *biological pore* that can be less than *eight nanometers*, and can be detected given that the *molecules release an ionic current* while moving through the pore. The *MinION* equipment (not yet available, need further development) has a handheld sequencer capable of generating more than 150 megabases of sequencing data in one run.

(15) *Tunnelling currents DNA sequencing.* Another approach uses measurements of the *electrical tunnelling* currents across single-stranded DNA as it moves through a channel. Depending on its electronic structure, each base affects the tunnelling current differently, allowing differentiation between different bases. The use of tunnelling currents has the potential to sequence orders of magnitude faster than ionic current methods. (IV.16) *Sequencing by hybridization (under development).* It would be a *non-enzymatic method* that uses a DNA *microarray*. A single pool of DNA whose sequence is to be determined is *fluorescently labeled* and hybridized to an array containing known sequences. Strong hybridization signals from a given spot on the array identifies its sequence in the DNA being sequenced. This method utilizes the binding characteristics of a library of short single stranded DNA molecules (*oligonucleotides*, also called DNA probes), to reconstruct a target DNA sequence. Non-specific hybrids are removed by washing and the target DNA is eluted. The benefit of this sequencing type is its ability to capture a large number of targets. (IV.17) *MALDI-TOF MS Sequencing.* Mass spectrometry, *i.e. Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry* (MALDI-TOF MS), has specifically been investigated as an *alternative method to gel electrophoresis* for visualizing DNA fragments. With this method, DNA fragments generated by *chain-termination sequencing* reactions are compared by mass rather than by size. The mass of each nucleotide is different from the others and this difference is detectable by mass spectrometry. Single-nucleotide mutations in a fragment can be more easily detected with MS than by gel electrophoresis alone. MALDI-TOF MS can more easily detect differences between RNA fragments, so researchers may indirectly sequence DNA with MS-based methods by converting it to RNA first. The higher resolution of DNA fragments permitted by MS-based methods is of special interest to researchers in *forensic science*, as they may wish to find single-nucleotide polymorphisms in human DNA samples to identify individuals. These samples may be highly degraded so forensic researchers often prefer *mitochondrial DNA for its higher stability* and applications for lineage studies. MS-based sequencing methods have been used to compare the sequences of human mitochondrial DNA from samples in a Federal Bureau of Investigation database and from bones found in mass graves of World War I soldiers. (IV.18) *Microfluidic Sanger sequencing*. The entire thermocycling amplification of DNA fragments as well as their separation by electrophoresis is done on a single *glass wafer* (approximately 10 cm in diameter), or use of *microchips*. Research will still need to be done in order to make this use of technology effective. (IV.19) *Transmission electron microscopy DNA sequencing.* This approach directly visualizes the sequence of DNA molecules using *electron microscopy*. (IV.20) *RNAP (RNA polymerase) sequencing*. In this method *RNA polymerase* (RNAP) is attached to a polystyrene bead, and one end of DNA to be sequenced is attached to another bead, with both beads being placed in optical traps. RNAP motion during transcription brings the beads in closer and their relative distance changes, which can then be recorded at a *single nucleotide resolution*. (IV.21) *In vitro virus high-throughput sequencing.* A method has been developed to analyze full sets of protein interactions using a combination of *454 pyrosequencing* and an *in vitro* virus mRNA display method. Specifically, this method covalently links proteins of interest to the mRNAs encoding them, and then detects the mRNA pieces using reverse transcription PCRs. The mRNA may then be amplified and sequenced. The combined method was titled IVV-HiTSeq and can be performed under cell-free conditions.

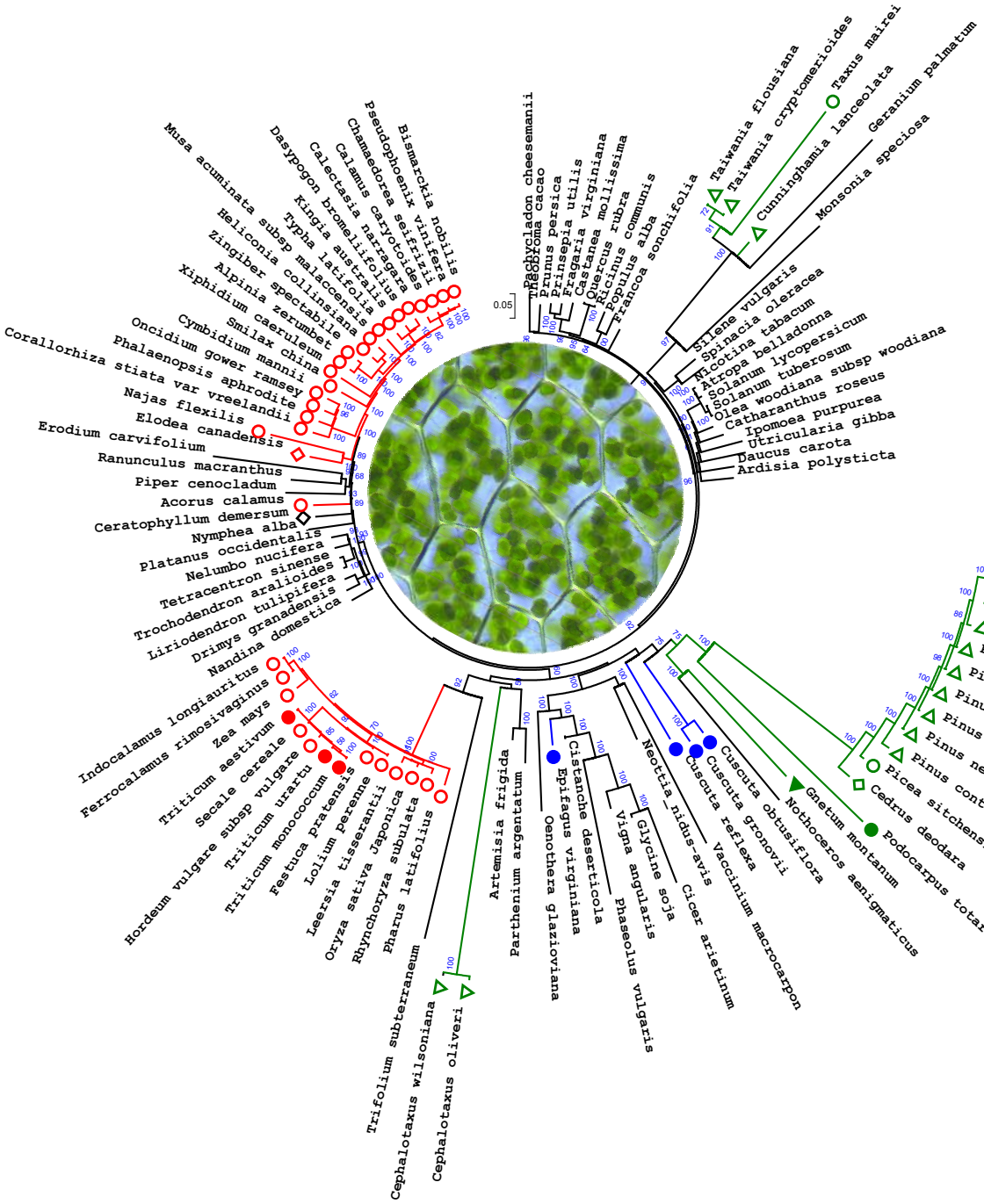
Table 2. Comparison of automated sequencing methods.

Method	Read length (nt)	Accuracy	Reads per run	Time per run	Cost per 1 million nt	Advantages	Disadvantages
1. Chain termination (Sanger Seq) (**ABI**)	400 - 900	99.9%	N/A	20 min. – 3 hours	$ 2400	Long individual reads. Useful.	Expensive and impractical for larger sequencing projects.
2. Pyrosequencing (**Roche 454**)	700	99.9%	1 million	24 hours	$ 10	Long read size. Fast.	Expensive. Homopolymer errors.
3. Sequencing by synthesis (**Illumina**)	50 - 300	99.9% (Phred30)	up to 6 billion	1 - 11 days,	$ 0.15	Potential for high sequence yield	Equipment can be very expensive. Requires high conc. of DNA.
4. Single-molecule real-time sequencing (**PacBio**)	10,000-15,000 > 40,000	87% single-read accuracy	50,000 per SMRT cell, or 500–1000 megabases	30 minutes-4 hours	$ 0.40	Longest read length. Fast. Detects methylations: 4mC, 5mC, 6mA.	Moderate throughput. Equipment can be very expensive.
5. Sequencing by ligation (**SOLiD**)	50+35 50+50	99.9%	1.2 to 1.4 billion	1 - 2 weeks	$ 0.13	Low cost per base.	Slower than other methods
6. Proton Seq (**Ion Torrent**)	up to 400 bp	98%	up to 80 million	2 hours	$ 1	Less expensive equipment. Fast.	Homopolymer errors.

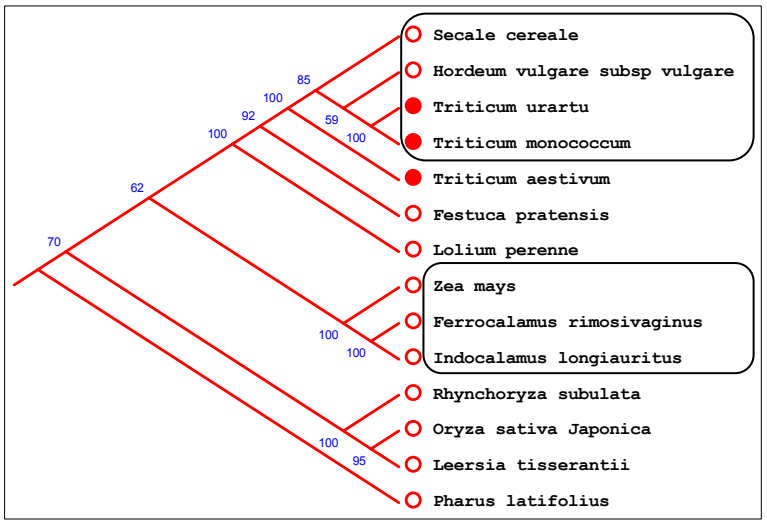
References (for all paper cited)

Alzohairy AM, G Gyulai, H Ohm, Z Szabó, SM Ragheb, M Ajmal Ali, H Elsawy, A Bahieldin (2015) Nuclear and Organelle Specific PCR Markers. *In*. Ali MA, G Gyulai, F Al-Hemaid (*Eds*) *Plant DNA barcoding and phylogenetics*. LAP Lambert Academic Publ. Germany. Chapter 4. pp. 53-78. ISBN-13: 978-3-659-28095-5.

Appendix 1



Total cpDNA genomes comparisons. ML (*Maximum Likelihood*) cladogaram of the total cpDNA genomes with 1000x bootstrap values (MEGA4). Monocots (○) show three cpDNA lineages including the individual *Acorus*. Of them, *Triticum* species are labeled (●). ***Gymnosperm trees*** (green) which show three lineages (also indicated), the (1) Gnetales (▼) – *Pinaceae* of Coniferales-I. It does indicate the most ancient family of *Podocarpaceae* (*Podocarpus,* ●), the *Pinaceae* species of *Pinus, Picea, Abies, Cathaya* (▲), and *Cedrus. Cathaya (NC_014589*) has the smallest cpDNA genome (107.122 bp) of Coniferales. This clad shows the closest similarity to the bryophyte *Nothoceros*. (2) *Taxodiaceae* of Coniferales-II: *Taiwania*, *Taxus*, and *Cunninghamia*. (3) The evolutionarily youngest *Cepahlotaxaceae* (*i.e.*, *Cephalotaxus*) of Coniferales-III. Water submerged ***Angiosperm*** plants (*Elodea* ◊, and *Ceratophyllum,* ◊); and parasite plants with reduced cpDNA genomes (*Cuscuta* and *Epifagus,* ●) are also labeled. Scale (0.05) shows the relative genetic distance. Accession #s are available at NCBI and CGP. For computing the about 21 million nucleotides, an eight core computer with 24 GB RAM was used (*Gyulai, Kerti, Katona M, and Hidvégi, 2015; in: Ali MA, G Gyulai, N Hidvégi, B Kerti, FMA Al Hemaid, AK Pandey, J Lee (2014) Saudi J Biol Sci 21: 204–231*)



Sub-clade of total cpDNA analysis, with indications of the closest relatives of *Secale*, *Hordeum* and *Triticum*, except *T. aestivum*. See also the *Zea* clade.

LTR-Retrotransposon based markers

(A) Introduction

Retrotransposons (RTs) are major components of most eukaryotic genomes; they are ubiquitous, dispersed throughout the genome, and their abundance correlates with the host genome sizes (*Fig. 1a*). *Copy-and-paste* life style of the RTs consists of three molecular steps, which involve *transcription* of an RNA copy from the genomic RT, followed by *reverse transcription* to cDNA, and finally a *reintegration* event into a new locus of the genome. This process leads to new genomic insertions without excision of the original RT (*Kalendar and Schulman, 2006*).

The target sites of insertions are relatively random and independent for different plant taxa; however, some elements cluster together in '*repeat seas*' or have a tendency to cluster around the *chromosome centromers* and *telomeres*. The structure and copy number of *retrotransposon types (i.e. families)* are strongly influenced by the evolutionary history of the host genome (*Fig. 1b*).

Molecular barcoding of RTs play an essential role in all fields of genetics and genomics, and represent a powerful tool for molecular barcoding. To detect RT polymorphisms, marker systems generally based on the amplification of sequences between the ends of the retrotransposon sequences, such as *long terminal repeats* (LTR) of LTR-retrotransposons (LTR-RTs) and the *flanking genomic* DNA.

Interspersed repetitive DNA sequences comprise a large fraction of the eukaryotic genomes. They predominantly consist of transposable elements (TEs) with two main families, Retrotransposons (Class I) and DNA transposons (Class II) (*McClintock, 1984*). Retrotransposons (RTs) are the most abundant class of TEs.

There are two major groups of RTs depending on the presence *vs*. absence of long terminal repeats (LTRs) the LTR-retrotransposons (LTR-RTs) and non-LTR-retrotransposons. LTR-RTs comprise two main subgroups, *copia* (with high copy number) and *gypsy* (with high transposing activity) (*Fig. 1c*).

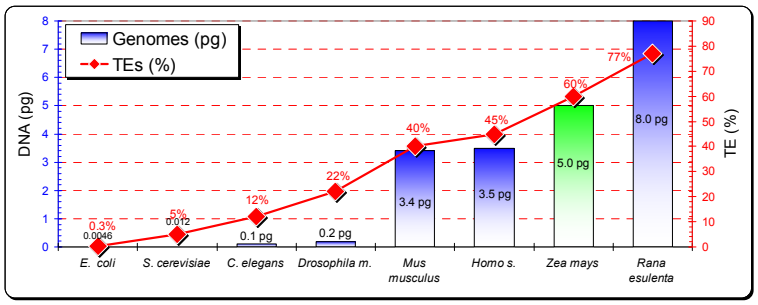


Figure 1a. Correlation between levels of transposable elements (TE, %) dispersed in the genome, and the sizes (DNA pg) of host genomes of the species at different stages of evolution *(Alzohairy,Gyulai et al., 2013, Plasmid 69:1–15)*.

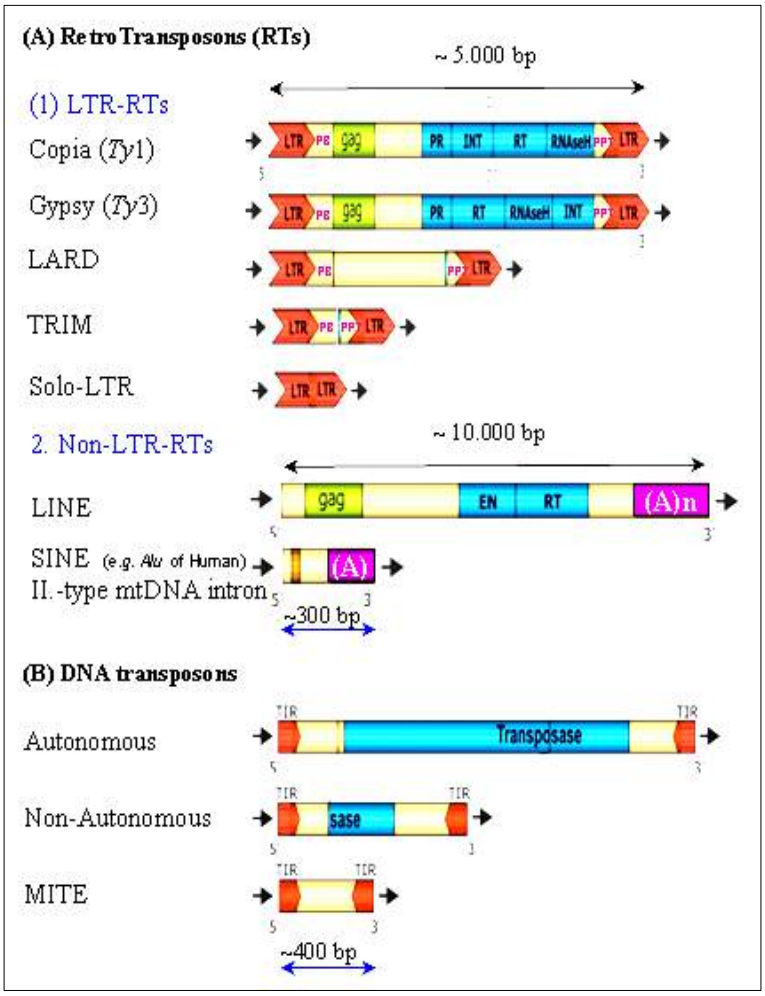


Figure 1b. Types, families, molecular structures, and estimated length of transposons (*i.e.,* jumping genes) *(Casacuberta and Santiago, 2003)*. **GAG** ***(gag)*** **-** *Group-specifikus AntiGene.* **CP -** *Coat Protein* of a type of *transit peptide* (**TP**). Ihe three domains of GAG are the *matrix* (**MA**), *capsid* (**CA**), and the *nucleocapsid* (**NC**) proteins, which form the whole *capsid protein* (**CP**). **EN/ENV -** *ENVelop* protein with two domains of **SU** (*surface*) and **TM** (*TransMembrane*) proteins (*syn.* **MP** - *Movement Protein*). **INT** ***(int)*** - *INTegráz*. **LARD -** *Large Retrotraspozon Derivatives*. **LINE -** *Long Interspersed Nuclear Elements*. **LTR -** *Long Terminal Repeat,* which ends up in short (1-5 nt) **IR** (*inverted repeat*), *i.e.,* **TIR**, *Terminal Inverted Repeat*. **MITE -** *Miniature Inverted-repeat Transposable Elements*. ***pol***: *mRNA-polymerase* comprising domains of ***ap-pr*** (*aspartic proteáz*), ***rt*** (*reverz transzkriptáz*), ***RH-H*** *Ribonukleáz-H*, and rarely ***INT***. ***PBS*** **-** *Primer Binding Site* (18 bp) for tRNAs. ***PPT*** **-** *PoliPurin Tract* (with high adenine and guanine content of 7-18 bp), which supply the primer site for the viral +cDNA synthesis. **PR -** *PRoteáz*, which is rich in Aspartic Acid aa (**AP** – *aspartic protease*). **RH -** *RNAase-H*. **RT -** *Reverse Transcriptase* (*RdDpol*). **SINE -** *Short Interspersed Nuclear Elements*. **solo-LTR -** *solo-LTR*, composed of the two LTRs 'only'. **TRIM -** *Terminal repeat Retrotransposons In Miniature*. **TIR -** *Terminal Inverted Repeat*. **Transposase** – DNA endonuclease with *Ligase* activity.

Both, *copia* and *gypsy* LTR-RTs, carry regulatory sequences of *gene promoters* such as CAAT box (*e.g.,* CCATT), TATA box (*e.g.,* TGGCTATAAATAG), *transcription start* (*e.g.,* CCCATGG), *polyadenylation signal* (*e.g.,* AATAAG), and *polyadenylation start* (*e.g.,* TAGT) (*Ramallo et al., 2008*). All these domains are required for replication and integration of RTs. The long *internal domain* (*Gag-Pol* Region) of the LTR-RTs encodes the structural proteins of the *virus-like particle* (which encapsulate the RNA copy of the RT), and the enzymes *Reverse Transcriptase* and *Integrase* (*Fig. 1b*).

The size of LTR-RTs varies from long (*e.g., Bare*1 *copia* LTR-RT; 13,271 bp; #NCBI Z17327) to short (*e.g., Bare1 copia solo*-LTR-RT; 3,130 bp; #NCBI AB014756; and the truncated RLC_Lara *copia* RT; 735 bp; NCBI #EF067844, # TREP2298). In plants, LTR-RTs are more plentiful and active than non-LTR-RTs (*AGI, Arabidopsis Genome Initiative, 2000*).

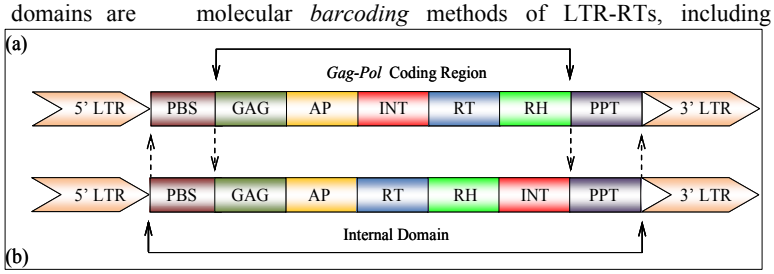


Figure 1c. Structural differences between *Copia (a)* and *Gypsy (b)* type LTR-Retrotransposon families. 5`LTR - 5`-end of long terminal repeat; PBS - *primer binding site*; GAG - *group-specific antigen* (*syn.*: *capsid* protein gene); AP - *aspartic protease* gene; INT - *integrase* gene; RT - *reverse transcriptase* gene; RH - *ribonuclease*-H gene; 3`LTR - 3`-end of the long *terminal repeat*; PPT - *polypurine tract* *(Alzohairy,Gyulaiet al., 2014).*

Due to the recombinational processes of chromosome during the meiotic prophase, active retrotransposons tend to lose their activity due to sequence breakage, and recombination resulting in recombinant LTR-RTs, which lost the transposition activity (*i.e.* non-autonomous), such as derivatives of LARD (*Large Retrotransposon Derivatives*), TRIM (*Terminal Repeat Retrotransposon in Miniature*) and *solo*-LTR (sequence carrying 5' and 3' LTRs only).

Here we review the utility of some main PCR-based molecular *barcoding* methods of LTR-RTs, including RBIP (*Retrotransposon-Based Insertion Polymorphism*), IRAP (*Inter Retrotransposon Amplified Polymorphism*), IPBS (*Inter Primer Binding Sequence*), REMAP (*Retrotransposon-Microsatellite Amplified Polymorphism*), and SSAP (*Sequence-Specific Amplified Polymorphism*) (*Schulman, 2007*).

(B) Utilization of retrotransposons as molecular markers

Molecular barcoding methods (*Schulman, 2007*) based on RTs are based on PCR technology, and detect large portions of the genome (*Poczai et al., 2013*). Retrotransposon-based markers follow *Mendelian inheritance* (*i.e.* dominant *vs.* recessive) with high levels of genetic variability (*Manninen et al., 2000*). In the genome, three different orientations of RTs are possible (*i.e. head-to-head*, *tail-to-tail*, and *head-to-tail*), either at a single locus, or inserted next to or within each other (nested RTs). This feature increases the variation available for revealing polymorphism within and among species. If the RT sequence and the adjacent genomic sequences are known, then all types of PCR-based molecular techniques can detect RT polymorphisms.

As the new cDNA copy of RT integrate into a new locus of the genome the old copy persist in the genome across generations, new sequence variations between ancestral and derived RT loci are generated. The presence of a given retrotransposon suggests its *orthologue* integration, while the absence indicates the *plesiomorphic* condition prior to integration. The presence *vs*. absence of RTs can be utilized to construct phylogenetic trees of species due to the distribution of retrotransposons across the organism. This is the reason why RTs have been suggested to provide powerful phylogenetic markers with little if any *homoplasy* (*Schulman, 2007*).

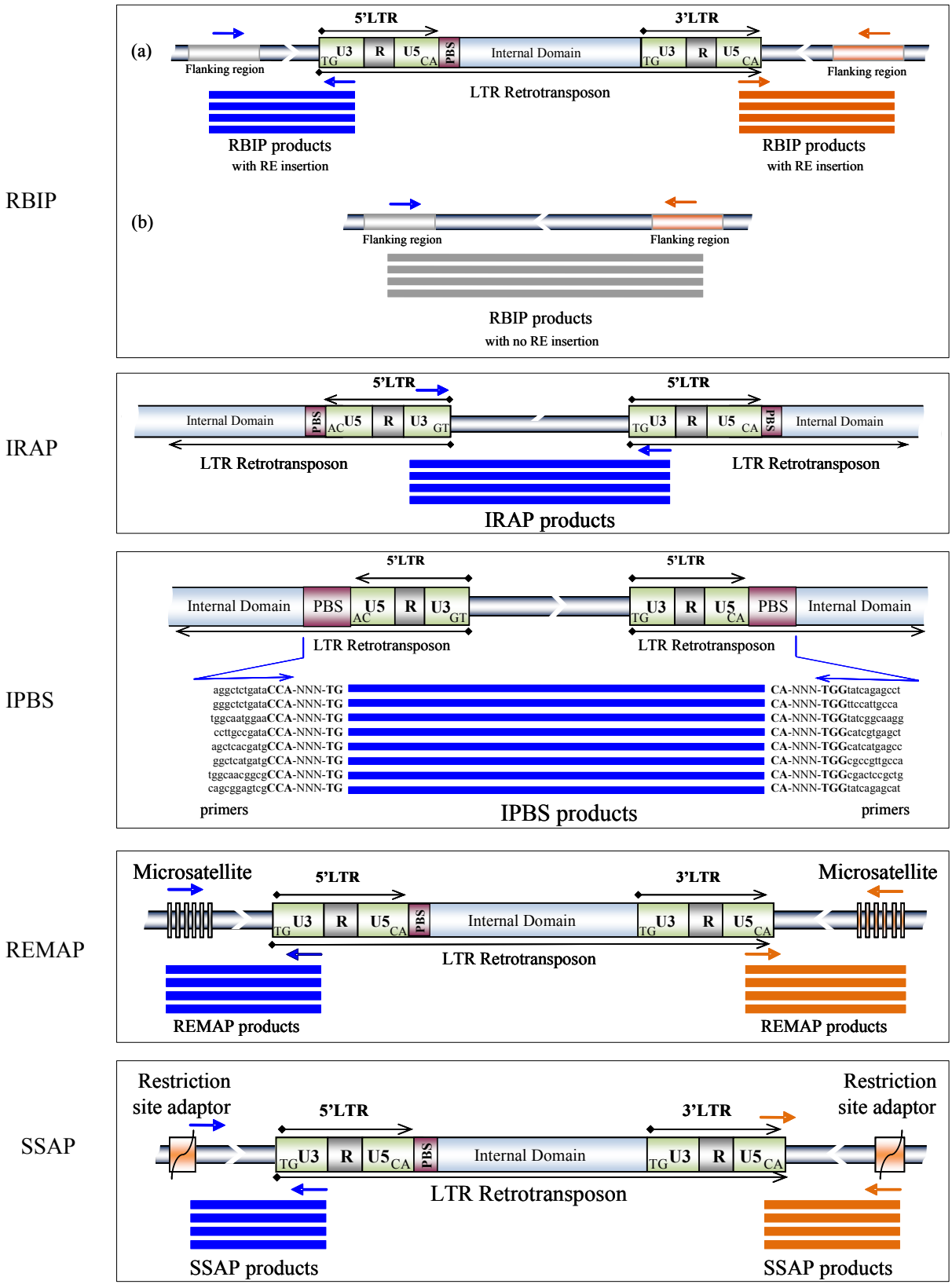
(C) Primer design for detection LTR-RTs

For PCR amplification, the LTR regions of the LTR-RTs are chosen to minimize the size of the target DNA to be amplified. A primer facing outward from the 5'-en LTR will necessarily face inward to a 3' LTR of a neighboring LTR-RT, in the case of direct repeats (*head-to-tail* position). The long sequences of LTRs may also interfere with the production of amplicons within the size range of standard PCR. The conservative regions of LTR sequences are also used for designing inverted primers for *Long-PCR*, which can be used for cloning entire RTs. Primers, in REMAP, one primer is designed from the LTR and another from a nearby simple sequence repeats (microsatellites, *syn.*: SSRs). RBIP detects both the presence and absence of the LTR-RT insertion using three primers to generate single-locus codominant markers. In SSAP, two primers are designed to produce amplification between RTs and adaptors ligated to a restriction site (usually *Mse*I or *Pst*I). In IPBS, primers are designed to match and amplify the conserved regions of the primer binding sequences (PBS).

LTR-RT genotyping

(1) RBIP *(Retrotransposon-Based Insertion Polymorphism)* (*Flavell et al., 1998*).

This method detects retrotransposons in the genome using one primer binding to the flanking genomic site at the LTR-RT insertion, and ananother primer binds to the LTR-RT at either sites of 3`- or 5`-end of LTRs (*Fig. 1a*). Unfortunately, an alternative reaction can also take place between the left and right flanks in the case of empty (RT-unoccupied) site (*Fig. 1b*). The basic RBIP was developed for studying the *PDR*1 retrotransposon in *Pisum sativum* (*Flavell et al., 1998*). One of the disadvantages of this method is its high costs compared to other methods. RBIP also allows the *dot blot* approach used in microarrays (*Flavell et al., 1998*). RBIP requires information on the sequences of the 5' and 3' flanking regions of the LTR-RT insertions. A further limitation of RBIP is due to its large size range compared to standard PCR (about 3-5 Kbp). By using three primers, RBIP can detect both the presence and absence of the TE insertions.



Figures 2. **RBIP** (*Retrotransposon-Based Insertion Polymorphism*) detects the presence (*a*) or absence (*b*) of LTR-RTs in the host genome *(Flavell et al., 1998)*. **IRAP** (*Inter Retrotransposons Amplified Polymorphisms*) amplifies DNA stretches between two adjacent LTR-RTs located in tail-to-tail outward orientation *(Kalendar et al., 1999)*. **IPBS** (*Inter Primer Binding Sequence*) utilizes the sequence of PBSs (Primer Binding Sites) of two adjacent LTR-RTs located in tail-to-tail position *(Kalendar et al., 2010)*. **REMAP** (*Retrotransposon-Microsatellite Amplified Polymorphism*) amplifies DNA stretches between LTRs of the LTR-RT and a neighboring genomic microsatellite (indicated by vertical bars) *(Kalendar et al., 1999)*. **SSAP** (*Sequence-Specific Amplified Polymorphism*) amplifies sequence between the LTR-RT and a restriction site anchored by an adaptor *(Waugh et al., 1997)*. (Primers are indicated by arrows). *(In: Alzohairy, Gyulai et al., 2014)*

(2) IRAP (*Inter Retrotransposons Amplified Polymorphisms*) amplifies DNA stretches between two adjacent LTR-RTs located in tail-to-tail outward orientation *(Kalendar et al., 1999)*.

IRAP does not require restriction enzyme digestion or ligation. Different retrotransposon insertions increase the number of sites amplified and sizes of inter-RT fragments, which can be used as marker to detect genotype polymorphism. The primers should be pointing outwards from the LTRs of LTR-RTs to amplify the region between two RTs (*Kalendar et al., 2011*). The two primers could be designed to either the same or different RT families. IRAP can be also carried out with a single primer, which matches either the 5' or 3' end of the LTRs but pointing outwards from the LTR itself. IRAP may produce too many fragments to give good resolution on gels, or no products because target amplification sites are too far apart to generate amplicons. Yet, IRAP overcomes some of the drawbacks of other techniques, such in the case of SSAP, where either radioactive or fluorescent labeled primers are needed. The method was used widely for *BARE*-1 LTR-RT (*Hordeum vulgare*), and for genotyping *Oryza sativa*, *Musa*, *Brassica*, *Spartina*, *Triticum* and *Solanum* species.

(3) IPBS (*Inter Primer Binding Sequence*) (*Kalendar et al., 2010*)

The method is frequently used for displaying retrotransposon polymorphisms (*Fig. 2*). The need for sequence information is a prerequisite to design LTR-RT-based molecular barcodes. IPBS tends to overcome this problem as the primer binding sequence (PBS) of the LTR region is part of the *internal domain* of retrotransposons (*Fig. 1*), which is highly conserved region for duplexing with tRNAs during the reverse transcription of the life cycle of retrotransposons. PCR amplification occurs between two nested PBSs of two neighboring LTR-RTs (*Fig. 2*). While the process of reverse transcription is conserved among all retroviruses, the specific tRNA capture varies for different retroviruses and retrotransposons. Thus, the IPBS amplification method can be useful for all retroviruses that contain conservative PBS sites for $tRNAi^{Met}$, $tRNA^{Lys}$, $tRNA^{Pro}$, $tRNA^{Trp}$, $tRNA^{Asn}$, $tRNA^{Ser}$, $tRNA^{Arg}$, $tRNA^{Phe}$, $tRNA^{Leu}$ or $tRNA^{Gln}$ (*Kalendar et al., 2010*). As plant LTR-RTs are frequently nested, mixed, inverted or truncated in the geneome, LTR-RTs can be easily amplified using conservative IPBS primers. IPBS sequences can also be used for detecting other retrotransposons when the retrotransposon density is high within the genome. Retrotransposon movements and recombinations can also be monitored because new inserts or recombinations will be polymorphic, which will appear only in plant lines in which the insertions or recombinations took place.

(4) REMAP (*Retrotransposon-Microsatellite Amplified Polymorphism*) (*Kalendar et al., 1999*)

REMAP combines one primer binding to LTRs of LTR-RTs, and the other primer binding to neighbouring (if so) locus-specific simple sequence repeats (SSRs) of the genome. This technique is applicable only when SSR locates near the retrotransposons (*Fig. 2*). Amplification between retrotransposon and a nearby SSR requires neither digestion with restriction enzymes, nor adaptor ligation to generate the marker bands. This protocol can be completed in 1-2 days, and has been used to scale diversity, similarity and cladistic relationships in many genotypes of *Oryza sativa*, rice pathogens (*Magnaporthe grisea*), *Spartina* sp. and *Avena sativa*.

(5) SSAP (Sequence-Specific Amplified Polymorphism) (*Waugh et al., 1997*)

The method was one of the first retrotransposon-based barcoding technology based on the technical steps used first in AFLP (*Amplified Fragment Length Polymorphism*) (*Vos et al., 1995*) (*Fig. 2*). SSAP uses one primer matching the end of an LTR (*e.g.,* 3' end) and the other primer matches to a restriction site (usually *Mse*I or *Pst*I) adaptor as in AFLP. Primer pairs (labeled either ^{32}P- or fluorescently) contains two or three selective nucleotides. Non-selective primer pair can also be useful when restriction enzymes have a long recognition site sequence, or when the copy number of the LTR-RTs is low.

SSAP primers are often designed to the LTR region, but could also match to an internal sequence of the RTs, like the polypurine tract (PPT), which is located to the internal part the 3'-LTR (*Ellis et al., 1998*). The number of selected bases may be increased in the case of high-copy-number RT families. The use of single or double enzyme digestions (or infrequently cutting enzymes) allow the survey of all insertion sites for a given RT, which method can be considered as a variant of *anchored PCR*. Primers that give highly polymorphic, clear, and reproducible SSAP banding patterns are candidate primers for subsequent work.

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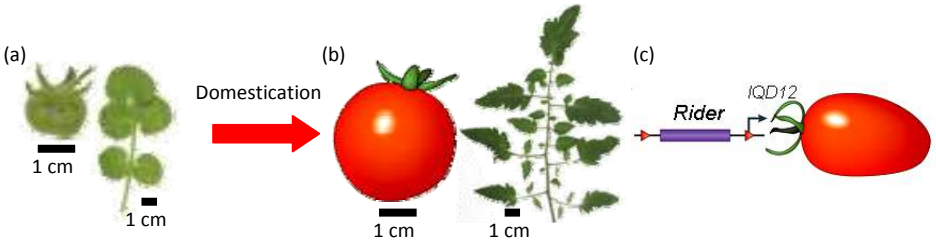
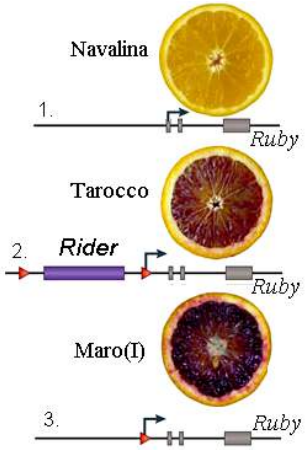
Kalendar R, AH Schulman (2006) IRAP and REMAP for retrotransposon-based genotyping and fingerprinting. *Nature Protocols* 1: 2478-2484.

Schulman AH (2007) Molecular markers to assess genetic diversity. *Euphytica* 158: 313-321.

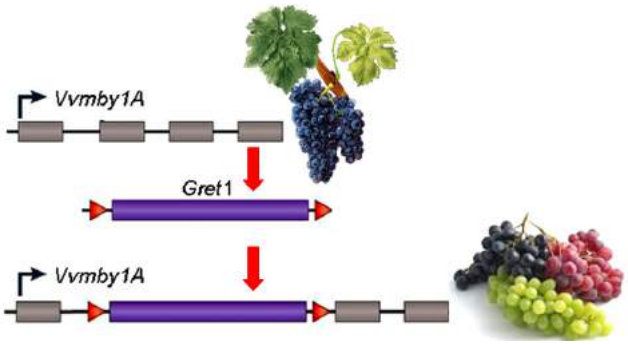
Appendix – Samples for the transpositions (‘*jumping*’) of RetroTransposons, which resulted in several very important achievements for the Agriculture.



(A) The wild tomato (*Solanum pennellii*) *(a)*, growing in South America (with small leaves and small green fruits), is the ancestor of the cultivated tomato *(S. lycopersicum) (b),* in which an LTR-RetroTransposon ‘*Rider*’ got activated, and ‘*jumped further in the genome*’ into ‘*IQD*12’ locus, and, as it’s resulted, a new from of oval tomato fruits developed *(Lisch, 2013; Lookhart, 2013; Gyulai et al., 2017)*



(B) The activity of *(1)* LTR-RetroTransposon ‘*Rider*’ and a further partial excision (‘jumping further’ in the DNA out from gene ‘*Ruby*’) resulted in a new orange (*Citrus x sinensis*) flesh colors of ‘red/bood oranges’ of cv. ‘*Navalia*’, ‘*Tarocco*’ and ‘*Maro*(I)’ *(Lisch, 2013; Lookhart, 2013; Gyulai et al., 2017)*



(C) The activity of of the ’*Gret*1’ LTR-RT has developed several new grape colors of *Vitis vinifera* by ‘just *jumping*’ (about 6.000 years ago) into the *anthocyaine*-related gene *Vvmby*1A (which is related with the black grape color - *notice*; all wild, and ancient grapes have black color only), and ‘*had braked*’ it, so new grape colors had developed (*Kobayashi et al., 2004, Science 304:98; Lisch, 2013, Nature Reviews Genetics 14: 49–61; Gyulai et al., 2017*).

(D) DNA sequence identificication of a transposition (‘jumping’) of ‘*Cila-1’* (298 nt) TRIM type Retrotranszpozon (*underlined*) into a *Seed Nucellus-Specific Protein* (WM403) gene (AF008925) in watermelon (*Citrullus_lanatus*) (2.726 nt). *NCBI# EU009625, Kalendar R, Gyulai G, Tóth Z, Schulman AH (2007) Citrullus lanatus* var. *lanatus* transposon ’*Cila*-1’, partial sequence. gi|152926315|gb|EU009625.1|[152926315]

[1]GGATCTTTTTAAGTGTTGGGGCTGGTTTTTATTTTGTTTACGAGTTTCAAAATGTTACCTAATTTAGTATTTCAGTTTCAAAATATTACAA
TTTTACTATTTAAATTTGAATTTTGTTTTAATATGGTCACTAGGTTTCAACGTTTTATATTTTTAACCTTAATTTTTCATTAAATACTCATC
TTCAATCGTTGACGTTAATGTCTATTAATTAATTAAAATTAATTATAAAGTGAGATTTCAAAAGTGATGAAAATCTGTGAAACTTAATTAAT
TATAGTTTTTTTAATTAATTTAAATTTATTAACATACATTAACATTGAAAATGAAAAAATGAATATTTAGTAAAAAATCGAGGTTAAATGTG
TAAATATTGAAACTTGGGGACCAAATTAAAATAAAGCTCAAATGTCAATTCATAACAAAGTTCAAATGAATTTCGTTGTTTGTTATGGTAGG
AAAGAGAATAACTTTAAAATACGAAATGATTTCAAATCCTGTTTTTACTTTAAAAAAACCTTCCCAACCAAAATGCTCGGATTTTGCAGAAT
TTCATAGGATTTAAAAATAAATTACCTAGTCGACCTTGTCATATTCTAATTTTCAATTAACAGTAAATTTTTAACTTTTTTTTTTCTCTTTT
TCTGTTAACATCCAAACACAATTTTAAATACATTCATTCAAATCTTTCTCGTTCCAAATAAAATAATTAATTTTAAATCATTTTTTAAATTT
CAAATTACAAAATTTAAAATCATTTGTTCAAATTTGTTAAATCTATATTTAGGGTAAAAATTCTAAAAGTTACCGAAAGTTGAGCAAACCAT
TTTGTTGTCTCCTTTCTTATATTAGTCCACCTTTTTGTTTGAAAGACATTTATGCAATTCACGAAATAATTAAATTAGAAATCCAACTAAGA
TCTACGTTGATGCAATTTTGAACTTACCTTGTTCGGGTCCACCACATTGTATTATTAAAAAACAAATTAAGAACATCTTGGTATATGAACAA
TTCGAGTACTCACTTCAAGATGAGATACAACAATTACCTATAAGTTATACTAATTTAAAAAGTGGTCCCAATTCATAACTTTGTTTGTTGTT
TAATTTCCGTAATCATAATGAATTGTAGGACCCGCTTGTAAATCTGTAATCAAAACGCGTAATTTGATTGAAGAACGAAGGATGTTCAATCT
AATTGTAGTTGAAAATTGTGTGAGATCCACTACATTTACCGTTAGTGTTATAGATATTGTTGCTATAACGGTACGAATGTTATGAATCTGTC
ACATTTATTTTTTACGTTTATTATTACCATTCGGGTTAAACAGTAACGGTGACGATAACGATAATAATAAAATGTGACAAATTCATAATTTT
AAGTAAACGTGATCAAAAGTAATCCAA**TTACA<***TGTTGGAATGCCTTGAATCATAACCTAATTAAAATTGGTATCTTATATATTTAATTATT*
TAATTTTTTTCCTTCTCCACTTAGGGTTTAAAAAGTGCACTATATAAACTCTTTAATTCTATAGACTTTATTCATTTTGTGGTATTATATAA
TTTTATAACATTCTCTCTAATAAAATTGTTATCCCTCTATCTCGTGGACGTAACTAACACATTGTTAGTGCACAACGTAAATTTGTGTGTCG
*ATCTTTATTTCTATTTTTTACGTTATTTTGTATTATTTAATTGTCGATTTCATAAC***>A****TTATA***TTG...CTCTCG[2726]

Plant molecular systematics

(A) Introduction – Plant Systematics

Systemetics/Taxonomy of plant/animal morphology (*i.e.* Linnaean systematics) (*Table 1, 2*) are based on morphological characterization of the specimen, and uses *binomial names*, which was introduced first by *Gaspar Bauhin* in his book of *Pinax Theatri Botanici (1623)*. *Linnaeus (1753)* also based his classification on morphological characters, *e.g.,* for birds, the shape of the bill; and for plants, the structure of flowers, which are the evolutionarily most conserved plant organ including the stamens, pistils, sepals and petals (*Linnaeus, C*: *Sytema nature*; and *Systema plantarum;* describing about 8000 species). *JB de Lamarck (1744–1829)* (*e.g.,* heritability of acquired characteristic); and later *A Wallace* and *Ch. Darwin (1858, 1859)* gave a theory of evolution (*e.g., in: On the origin of species by means of natural selection*), which was also based on morphological traits which are changing by selection and adaptation. *Darwin* also introduced the principle of *common descent* into the classification.

Recently, molecular systematics provided several genetic tools for the identification, classification and description of (new) species. The rapid development in molecular technologies has resulted in three main fields of techniques of the isoenzyme (IP, Izoenzyme Polymorphism; *Hunter and Merkert, 1957*), RFLP (*Botstein et al., 1980*), and PCR-based *(Mullis and Faloona, 1987*) methods. For systematics, PCR based methods and whole genome sequencings were found to have distinctive resolutions. Some of the first PCR-based technologies of RAPD (*Williams et al., 1990*), SSR (*Gupta et al., 1994*), ISSR (*Zietkiewicz et al., 1994*), and ISJ-PCR (*Weining and Langridge, 1991*) were followed by numerous new approaches (*see Chapter 5*).

Table 1. Plant systematics based on morphology – From the Antiquity to the modern periods

- *Before Linnaeus*

THEOPHRASTUS (BC 350 – BC 287): *Historia Plantarum*.

PLINIUS, Gaius Secundus (AD 23 – AD 79): *Historia Naturalis*.

DIOSCORIDES, Pedanius (about AD 70): *De Materia Medica*.

AL-AWWAM Ibn (1158) Libro de agricultura. Madrid

FUCHS, L (1542): *De historia stirpium*. Basel.

CESALPINO, Andrea (1583). *De plantis libri XVI.*

BAUHIN, Caspar (1596): *Phytopinaxí.* and *Pinax theatri botanici* (1623).

CLUSIUS, Carolus (1526–1609): *Histoire des plante* (1557). *Fungorum in Pannonis observatorium brevis historia* (1601).

BENTHAM G and JD HOOKER (1863) *Genera Plantarum.*

RAY, JOHN (1686): *Historia Plantarum*.

- *After Linnaeus*

LINNAEUS, Carolus (1707–1778): *Species Plantarum* (1753).

ADANSON, Michel (1763): *Familles des plantes*.

DE JUSSIEU, ANTOINE LAURENT (1789): *Genera Plantarum, secundum ordines naturales disposita juxta methodum in Horto Regio Parisiensi exaratam*.

DE CANDOLLE, AP, *et al.* (1824–1873): *Prodromus systemati naturalis regni vegetabilis sive enumeratio contracta ordinum, generum specierumque plantarum huc usque cognitarum, juxta methodi naturalis normas digesta*.

LINDLEY, John (1846): *The Vegetable Kingdom*.

DARWIN Ch. and A. WALLACE (1858). Ch. DARWIN (1859): *Origin of Species*.

ENGLER A. (1844–1930) and K.A.E. PRANTL (1849–1893): *Die Naturlichen Pflanzenfamilien* (1887–1915).

BESSEY, E. Charles (1845–1915): *Synopsis of Plant Phyla* (1907).

SOÓ, Rezső: *Fejlődéstörténeti növényrendszertan – Evolutionary plant systematic*. Budapest (1947, 1953).

CRONQUIST, A (1919–1992) *The Evolution and Classification of Flowering Plants* (1957, 1968) (2nd edition, 1988). and *An Integrated System of Classification of Flowering Plants* (1981).

TAKHTAJAN, A. (1910–2009) *Flowering plants: origin and dispersal* (1954, 1969).

THORNE, R.F. (1920–1915) *A phylogenetic classification of the Angiospermae* (1976).

BORHIDI, Attila (1995) *A zárvatermők fejlődéstörténeti rendszertana – Evolutionary systematic of Angiosperms*. Budapest (1932–).

PODANI, János (2003, 2007) *A szárazföldi növények evolúciója és rendszertana – Evolution and systematic dry land plants*. Budapest (1952–).

APG III system (*Angiosperm Phylogeny Group III system*) (2009).

Some important further references: Halley (1912), Bessey (1915), Troll (1944), Busch (1944), Grossheim (1946), Gundersen (1950), Pulle (1952), Hutchinson (1959), Meeuse (1961) and Melville (1962).

In Hungary, there is a long list of famous botanists working in plant systematics, floristics, lichenology, mycology, palinology, pomology, physiology, and dendrology of Hungary from the Middle Ages (*Table 2*).

Table 2. Plant systematics in Hungary

16th CENT.
MÉLIUSZ JUHÁSZ, Péter (1532–1572)
BEYTHE, István (1532–1612)

17th CENT.
LIPPAI, JÁNOS (1606–1666)

18th CENT.
BRASSAI, Sámuel (1797–1897)
CSAPÓ, József (1734–1799)
DIÓSZEGI, Sámuel (1760–1813)
HABERLE, Károly (1764–1832)
KITAIBEL, Pál (1757–1817)
SADLER, József (1791–1849)
SCOPOLI, J Antal (1723–1788)
WALDSTEIN-W., F. Ádám (1759–1823)

19th CENT.
AMBRÓZY, István (1869–1933)
ANDREÁNSZKY, Gábor (1895–1967)
BAROSS, László (1865–1938)
BERNÁTSKY, Jenő (1873–1944)
BORBÁS, Vincze von (1844–1905)
BUDAI, József (1851–1939)
CHOLNOKY, László (1899–1967)
CSAPODY, Vera (1890–1985)
CSEREY, Adolf (1851–1918)
CSERHÁTI, Sándor (1852–1909)
DEGEN, Árpád (1866–1934)
DEININGER, Imre (1844–1918)
DOBY, Géza (1877–1968)
ENDLICHER, L István (1804–1849)
ENTZ, Ferenc (1805–1877)
FEHÉR, Dániel (1890–1955)
FILARSZKY, Nándor (1858–1941)
FLEISCHMANN, Rudolf (1879–1950)
GOMBOCZ, Endre (1882–1945)
GRABNER, Emil (1878–1955)
GREGUSS, Pál (1889–1984)
GYÖRFFY, István (1880–1958)
HAYNALD, Lajos (1816–1891)
HAZSLINSZKY, Á Frigyes (1818–1896)
HERMAN, Ottó (1835 – 1914)
HOLLÓS, László (1859–1940)
ISTVÁNFFI, Gyula (1860–1930)
JANKA, Viktor (1837–1890)
JÁVORKA, Sándor (1883–1961)
JURÁNYI, Lajos (1837–1897)
KABAY, János (1896–1936)
KUBACSKA, András (1871–1942)
LEGÁNY, Ödön (1876–1944)
MÁGÓCSY-DIETZ, Sándor (1855–1945)
MAGYAR, Gyula (1884–1945)
MOESZ, Gusztáv (1873–1946)
MOKRY, Sámuel (1832–1909)
NATTER-NÁD, Miksa (1893–1982)
PAÁL, Árpád (1889–1943)
PÉNZES, Antal (1895–1984)
RAPAICS, Rajmund (1885–1954)
SIMONKAI, Lajos (1851–1910)
SZABÓ, Zoltán (1882–1944)
SZATALA, Ödön (1889–1958)
THAISZ, Lajos (1867–1937)
TUZSON, János (1870–1943)
WÁGNER, János (1870–1955)

20th - 21st CENT.
BARABÁS, Zoltán (1926–1993)
BARTHA, Dénes (1956–)
BODROGKÖZY, György (1924–)
BORHIDI, Attila (1932–)
BOROS, Ádám (1900–1973)
DEBRECZY, Zsolt (1941–)
FACSAR, Géza (1941–)
FALUDI, Béla (1909–1984)
FARKAS, Gábor (1925–1986)
FEKETE, Gábor (1930–)
FELFÖLDY, Lajos (1920–)
FRIDVALDSZKY, Loránd (1923–1994)
GULYÁS, Sándor (1933–1996)
GYŐRFFY, Barna (1911–1970)
GYULAI, Gábor (1953–)
HARASZTY, Árpád (1907–1987)
HORÁNSZKY, András (1928–2015)
HORTOBÁGYI, Tibor (1912–1990)
HORVÁTH, Imre (1926–1979)
ISÉPY, István (1942–)
JAKUCS, Pál (1928–2000)
JÁNOSSY, Andor (1908–1975)
JEANPLONG, József (1919–2006)
JUHÁSZ, Miklós (1938 –)
JUHÁSZ-NAGY, Pál (1935–1993)
KÁRPÁTI, Zoltán (1909–1972)
KEDVES, Miklós (1933–2003)
KOMLÓDI, Magda (1931–)
LEHOCZKY, Endre (1941–)
MAJER, Antal (1910–1996)
MÁNDY, György (1913–1976)
MÁTHÉ, Imre (1911–1993)
MILKOVITS, István (1937–)
ORBÁN, Sándor (1947–)
ORSÓS, Ottó (1911–1939)
PENKSZA, Károly (1963–)
PÓCS, Tamás (1933–)
PODANI, János (1952–)
PRÉCSÉNYI, István (1926–2007)
PRISZTER, SZANISZLÓ (1917–2011)
RÁSKY, M Klára (1908–1971)
SEREGÉLYES, Tibor (1949–2005)
SIMON, Tibor (1926–)
SIMONCICS, Pál (1918-2002)
SOÓ, Rezső (1903–1980)
SUBA, János (1929–)
SZELÉNYI, Gusztáv (1904–1982)
SZERDAHELYI, Tibor (1953–)
TERPÓ, András (1925–2015)
TUBA, Zoltán (1951–2009)
UBRIZSY, Gábor (1919–1973)
UHERKOVICH, Gábor (1912–2002)
UJHELYI, József (1910–1979)
UJVÁROSI, Miklós (1913–1981)
VIDA, Gábor (1935–)
ZÓLYOMI, Bálint (1908–1997)

(B) Molecular systematics and phylogeny based on ITS analysis

Plant molecular systematics relies primarily on the nuclear genes of ribosomal rDNA coding for ribosomal RNAs (*Appendix 1*), which is arranged in thousands of tandem repeats, and locates in one or a few chromosomal loci. In total, they can comprise 10% of the total plant genomes. Each repeat consists of a transcribed region that comprises an

The coding regions of the genomes show little sequence diversity among the closely related species; however the ITS1-5.8S-ITS2 regions show detectable rates of variability. Therefore, nuclear ribosomal ITS sequence data have a great potential to resolve plant phylogeny. ITS technology is also useful for technical purposes (*Fig. 1a*).

External Transcribed Spacer (ETS) followed by the 18S gene, an *Internal Transcribed Spacer* (ITS1), the 5.8S gene, a second *Internal Transcribed Spacer* (ITS2), and finally the 26S gene. Each repeat is separated from the next repeat by an intergenic spacer (IGS).

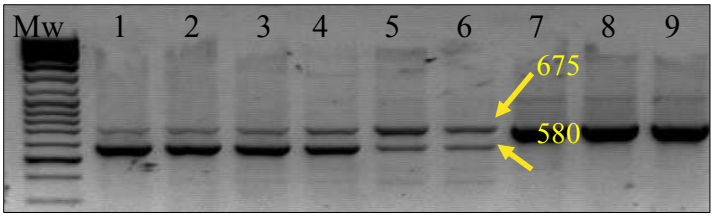


Figure 1a. Samples of ITS analysis for separation of soil bacterial contaminated seeds (1 - 9) from aseptic seeds (7 - 9) of melon (*Cucumis melo*). The fragment sizes of melon ITS (675 bp) and the contaminating bacterial ITS (580 bp) are indicated *(Szabó Z, Tóth Z, and Gyulai G, 2005).*

The approximate lengths of the three ITS genes are very similar throughout plants, the 18S gene is about 1,800 bp, the 26S gene is 3,300 bp, and the 5.8S gene is 160 bp. In contrast, the length of the IGS varies more considerably (from 1 to 8 kb).

This variation in IGS length is the major concern to the large range of variation with a total length of the repeat unit in plants ranging from approximately 8-15 kb. The variation of the length of the ITS1 and ITS2 regions are also large. The sequence diversity of the external transcribed spacer (ETS) region (especially the 3'end of the 5'-ETS adjacent to 18S) was found to be low.

Sequences of the single or low-copy nuclear genes have also great potential to elucidate phylogenetic relationships. The advantages of nuclear genes include the availability of many genes, their overall faster rate of evolution, and their *biparental* inheritance.

Although reliability of the use of ITS sequence of rDNA, as the sole source of phylogenetic evidence has come under serious criticism, it was found to be useful to reveal species-specific phylogenetic linkages in plants, fungi and animals, until the use of total genome analyses.

(C) Case Studies 1. Phylogenetic study of Euphorbia scordifolia vs. Euphorbia supina (Euphorbiaceae) *growing in Saudi Arabia by ITS1-5.8S-ITS2 sequence analysis.*

The genus *Euphorbia* (*Euphorbiaceae*) comprises 2000 species, and is one of the largest genus of the flowering plants. It has four subgeneric clades of *Rhizanthium*, *Esula*, *Euphorbia*, and *Chamaesyce*. In Hungary, 24 *Euphorbia* species grow. In Saudi Arabia, the genus is represented by 38 species. Of them, *Euphorbia scordifolia* distributes in western region of Saudi Arabia, and in Cape Verde Island, Somalia, Ethiopia, Sudan, and Yemen.

For PCR amplification of ITS1-5.8S-ITS2 analysis, ITS1 (5'-gtccactgaaccttatcatttag-3') and ITS4 (5'-tcctccgcttattgatatgc-3' primer pair was used.

For comparative analyses, ITS sequences of 28 species of *Euphorbia* were downloaded from GenBank database NCBI).

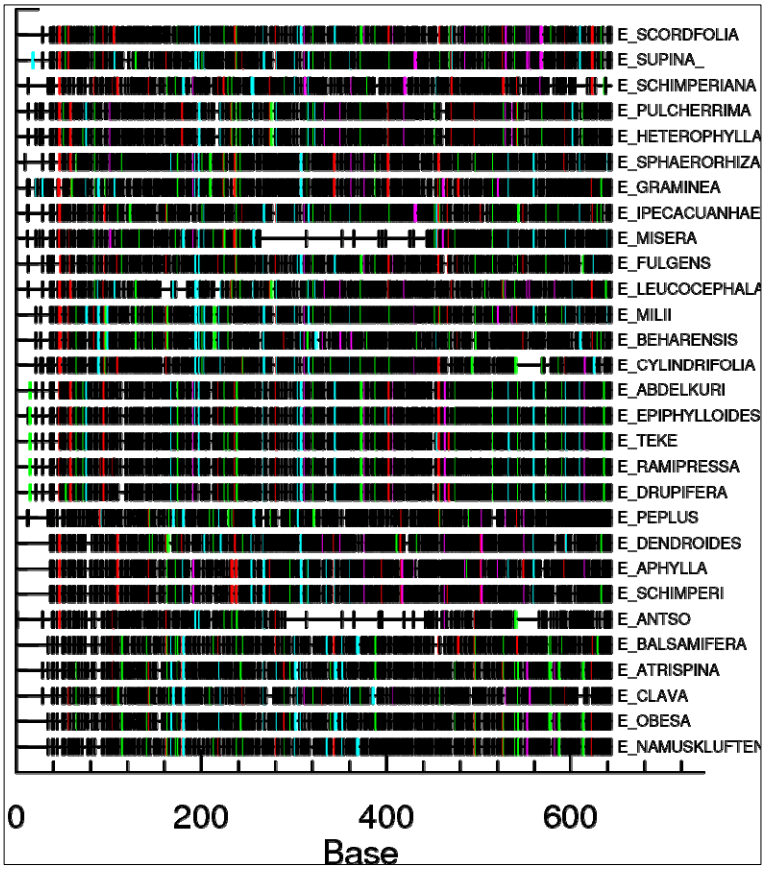


Figure 1b. HYPERMUT (*Rose and Korber, 2000*) illustration of the distribution of substitution sites along the ITS region obtained from 29 species of *Euphorbia*, using *Dichostemma glaucescens* as reference (red = GG > AG, cyan = GA > AA, green = GC > AC, magenta = GT > AT, black = not G > A transition, yellow = gap) *(Ali et al., 2015).*

After cloning, and sequencing, the combined length of ITS1-5.8S-ITS2 region in *Euphorbia scordifolia* was found to be 642 bp, including the ITS1 region (266 bp, 53% GC content), the 5.8S gene (162 bp, 56% GC content), and the ITS2 region (213 bp, 58% GC content). Molecular taxonomy of ITS sequences revealed that *E. scordifolia* belongs to the subgenus *Chamaesyce* with the closest genetic distance to *E. supine*. The morphological characters of *E. scordifolia* are very similar with *E. supina*. The distribution and pattern of nucleotide substitution was investigated by using HYPERMUT program (*Rose and Korber, 2000*) (*Fig. 1b*).

(D) Case study 2. Molecular taxonomy of Astragalus collenettae (Fabaceae) *an endemic legume species to Saudi Arabia by ITS1-5.8S-ITS2 sequence analysis.*

Term endemism refers to the unique geographic area/habitat (*e.g.* island, mount, cave, or country, *etc.*) of a species which is indigenous to only that geographical region.

Literally, the most unique endemism is the *relictum endemism*, which refers to those endemic species which survived the last Ice Age at the same geographical location. To our knowledge, there are only three plant *relictum endemisms* in the World, the gymnosperm *Welwitschia mirabilis* growing in the Kalahari Desert, the Hawaii *Argyroxiphium* (*Asteraceae*); and the *Linum dolomiticum*, (*Linaceae*) growing in Hungary (personal communication of *I. Milkovits and J Suba, 1978*).

Astragalus (family *Fabaceae*, subfamily *Faboideaea*) is the largest genus of the higher plants by comprising the largest numbers of species (about 2,500 - 3,000) of a genus (with 703 entries of NCBI), which are herbs and small shrubs.

This genus was found to be a remarkable example of studying *speciation* through *adaptive radiation* (*i.e.* increase in genetic diversity and consequently in species numbers) on a global scale. *Astragalus* species distributed primarily around the northern hemisphere, and also around South America, and spread mainly in the cool temperate semiarid and arid continental regions of southwest and central Asia (with about 1500 species) and the Sino-Himalayan region (500 species), to western North America (400–450 species) and along the Andes in South America (with about 100 species).

A recent phylogenetic analyses of the ITS cladogram (*Fig. 2*) revealed that two species of *Astragalus filicaulis* and *A. arpilobus* show the closest sequence similarities to *Astragalus collenettae*. This result may indicate the putative ancestors of *A. collenettae,* and also shows the genetic background of plant speciation through *irradiation* and environmental/geographical *isolation*.

In Saudi Arabia, due to the extreme climatic and soil conditions, there are a high numbers of plant endemism with a total of 50 species. *Astragalus collenettae* is one of the three legume endemism of Saudi Arabia along with *Astracantha echinus* ssp. *arabica*, and *Indigofera rubromarginata* var. *longipedunculata*. A detailed literature survey revealed that the taxonomic status of *Astragalus collenettae* is unknown.

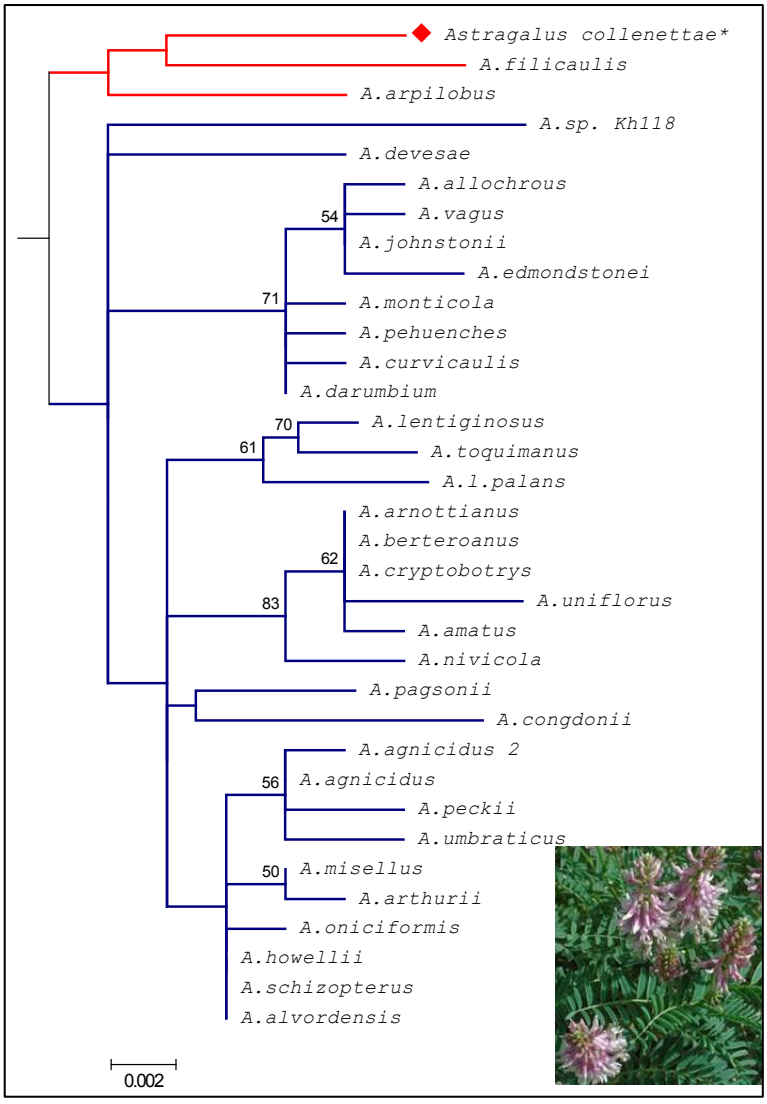


Figure 2. Consensus ITS ML-phylogram of *Astragalus* species. Bootsrap values above 50% (1000 replication) are indicated *(Ali et al., 2016).* For orientation, photo of *A. bisulcatus* is included

(E) Case Study 3. Phylogenetic study of Euryops jaberiana vs. Europs arabicus (Asteraceae), *growing in Saudi Arabia, by ITS1-5.8S-ITS2 sequence analysis.*

Euryops jaberiana (#KU577443) (tribe *Senecioneae*, family *Asteraceae*) is an endemic plant to northern Saudi Arabia. Its taxonomic status was revealed recently by using internal transcribed spacer (ITS) sequence of nuclear ribosomal DNA (nrDNA).

The genus *Euryops* comprises approximately 100 species and displays a restricted distribution in Africa to Arabia and Socotra. Species of *Euryops* are perennial shrubs (except *E. annuus*), with *coriaceous leaves* and yellow or orange-flowered *capitula* on simple peduncles, and usually devoid of leaves or bracts. Despite the genus was divided into six sections of *Euryops*, *Angustifoliae*, *Brachypus*, *Chrysops*, *Euryops*, *Leptorrhiza* and *Psilosteum*, based on morphology, its *phylogeny* and *phytogeography*, based on molecular data, remained poorly understood.

In Saudi Arabia, the genus *Euryops* is represented by only two species of *E. arabicus* and *E. jaberiana*. *E. arabicus* is the only species found outside of Africa and is endemic to Arabian Peninsula, while *E. jaberiana* is endemic to northern Saudi Arabia.

Morphologically *E. jaberiana* is very closely resembles with *E. arabiucs* and difficult to distinguish the two species. The key morphological features which differentiate *E. jaberiana* from *E. arabicus* are the 3-lobed leaves at the tips, pappus hairs transparent or rarely dull while, and achenes glabrescent, while the leaves of *E. arabicus* are unlobed, pappus hairs are dull white, and achene densely lanate hairy.

For PCR amplification ITS1 and ITS4 primers were used. For phylogenetic analysis the sequences (645 bp) ITS of *E. jaberiana* (#KU577443) was aligned with a total number of 17 representative sequences belonging to sections of the genus *Euryops* with an outgroup of *Gymnodiscus capillaries* (#EU667515).

For sequence alignment CLUSTAL X version 1.81 program was used, followed by manual adjustment of the aligned sequence using *BioEdit*. The Neighbor Joining (NJ), Maximum Parsimony (MP), and Maximum Likelihood (ML) dendrogram analyses were carried out using PAUP and MEGA5 programs.

Of the length of ITS region (ITS1-5.8S-ITS2) of *E. jaberiana* was found to comprise the ITS1 region (260 bp, with 43% GC content), the 5.8S gene (154 bp, 54% GC content), and the ITS2 region (231 bp, 50% GC content).

A total of eight specific nucleotide differences *i.e.* at the alignment position 93 (A → T), 116 (G → C), 201 (T → C), 443 (C → G), 461 (T → G), 531 (T → C), 573 (C→T) and 611 (T→C) were detected between *E. jaberiana* and *E. arabicus*.

The phylogenetic tree constructed by the NJ (and also by *Maximum Parsimony* and *Maximum Likelihood*) analyses showed a clear resolution, which nested within the clade of the section *Angustifoliae*, and showing the closest genetic relationship to *E. arabicus* (# EU667464) (*Fig. 3*).

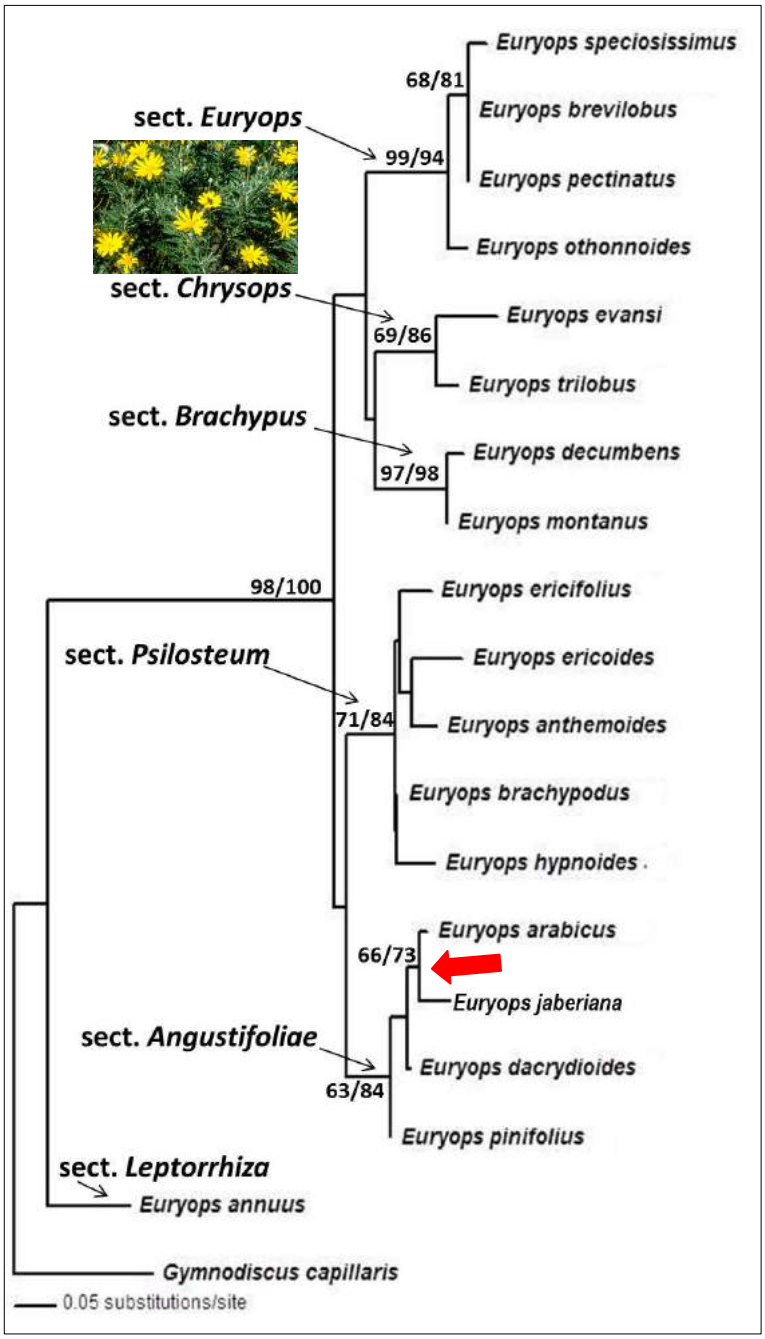


Figure 3. The NJ tree of ITS sequence of nuclear ribosomal DNA of 18 species of *Euryops* including *E. jaberiana* The bootstrap (MP/ML) support greater than 50% in 1000 replicates is indicated (*Ali et al., 2016*). For orientation, photo of *E. pectinatus* is included.

References

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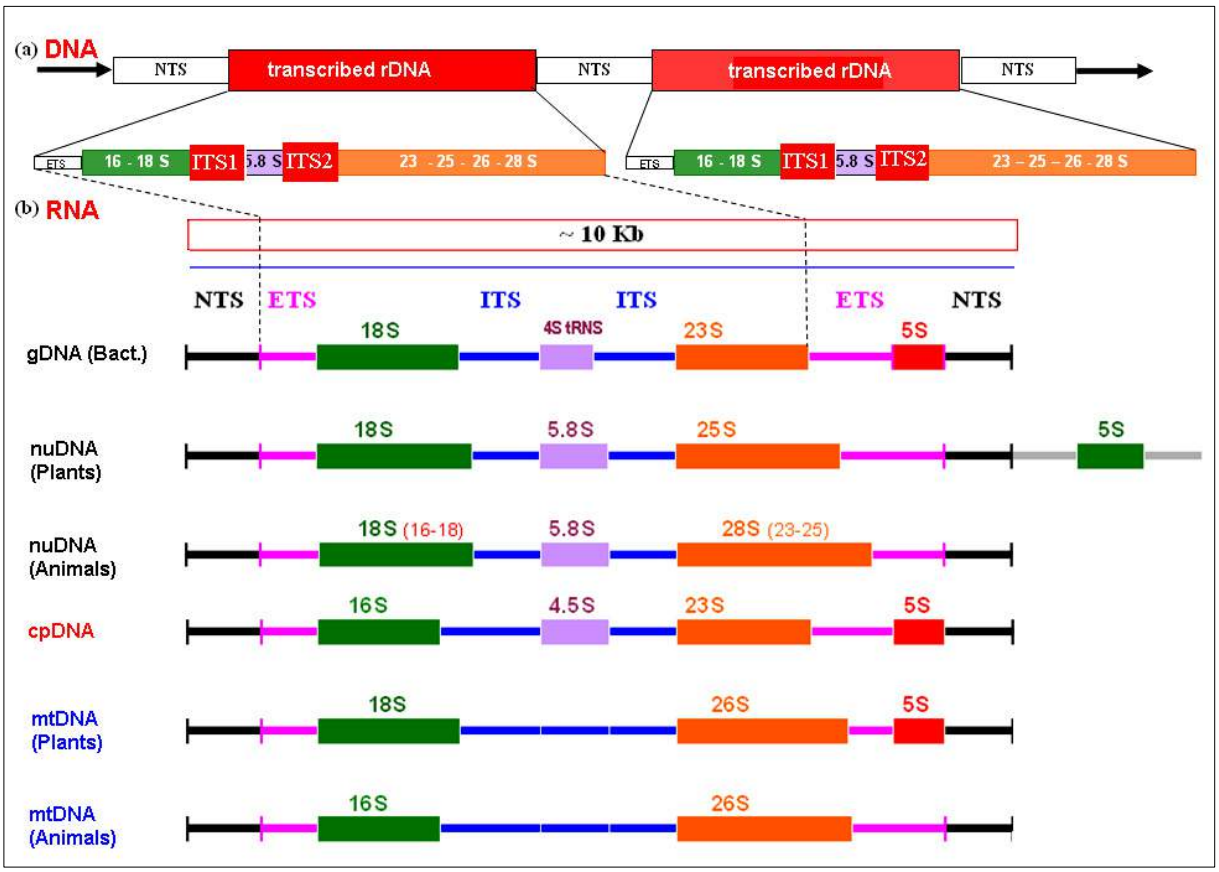
Appendix 1. Organization of the rDNA genes and subunits including ITS-1 and 2, by organisms and organs (*Gyulai, 2015*)



Molecular metabolomics – Tomato carotenoids (*Solanum lycopersicum*)

Introduction

Cultivated tomato (*Solanum lycopersicum;* chromosome number 2n = 24; genome size $0.9x10^9$ bp) (*TGC, 2012; SGN, 2014*) has twelve wild relative tomato species, which are divided into two complexes, the *Peruvianum* complex with two self-incompatible species (*S. chilense* and *S. peruvianum*), and the *Esculentum* complex, which includes the intercrossable species of *S. arcanum, S. chmielewskii, S. corneliomuelleri, S. habrochaites, S. huaylasense; S. neorickii, S. pennellii, S. pimpinellifolium,* and the two endemic species of *S. cheesmaniae* and *S. galapagense* (*Peralta et al., 2005*).

Tomato occupies the top position of the total World production of the ten major *fleshy fruits* with 28% (about 10^8 MT), followed by *banana* (20%, 7.6×10^7 MT); *apple* and *grapes* (about 15% and 5.6%, respectively, a total of 5.9×10^7 MT); *pear* and *pineapple* (about 5%, 1.9%, respectively, a total of 1.8×10^7 MT), and *papaya* and *strawberry* (2% and 1%; 7×10^6MT and 3.4×10^6MT, respectively).

Carotenoids and Chlorophylls

More than 700 carotenoids (including the annotated 281 molecules) have been identified from different plant sources (*Appendix 1*). Carotenoids are divided into non-oxygenated carotenoids, the *Carotenes*; and oxygenated carotenoids, the *Xanthophylls*. Carotenes are divided by *Acyclic-carotenes* (*e.g.,* phytoene, phytofluene, ζ-carotene, neurosporene, and lycopene), and *Cyclic-carotenes* (*e.g.,* δ- and γ carotene, α- and β-zeacarotene with one ring; and α- and β carotene with double rings).

Xanthophylls (*syn*. carotenols or hydroxycarotenoids) are also divided as *acyclic* (*e.g.,* lycoxanthin and lycophyll) and *cyclic* groups (*e.g.,* rubixanthin with one ring; and α- and β-cryptoxanthin, zeinoxanthin, zeaxanthis and lutein). A third group of xanthophylls includes *Epoxy-carotenoids* (*e.g.,* antheraxanthin, auroxanthin, neoxanthin, luteoxanthin, violaxanthin, lutein-5-6-epoxyde, and β-carotene-5,6-epoxide). The fourth group of xanthophylls comprises some *Unique carotenoids* (*e.g., capsanthin, capsorubin, crocetin* and *bixin*). In the whole carotenoid biosynthesis the phytoene ($C_{40}H_{64}$) synthase (PSY) is the rate-limiting enzyme.

Colour of tomato fruit skin and flesh is one of the most important quality components of the tomato. The colors slightly depend on the elution solvents used for the analyses. The main acyclic carotenoids of ζ-carotene (its colour is *light-yellow*), neurosporine (*yellow-orange*) and lycopene (*red-orange*); and cyclic-carotenoids of γ-carotenes (*pink-orange*), and β-carotene (*orange*) are the main tomato fruit colorant. The main carotenoids of β-carotene and lycopene cause *red-to-orange* coloration, which are the predominant tomato pigments.

Chlorophylls, the green pigments of unripe fruits, breaks down during ripening, except in cultivars with 'black' coloured fruits, where persistent chlorophyll content gives a *purplish-brown* colour. This colour is regulated by *gf* (green flesh) gene that encodes a *Stay-Green* senescence-related regulator, and finally results in the mix of red and green colour, which seems *black-brown-chocolate* colour.

Functionally, carotenoids, especially β-carotene, primarily act as accessory and photo protective pigments for *chlorophyll a* and *b* of LHCs (*Light Harvesting Complex*) of *Photosystem I, and II* (PSI and PSII) during photosynthesis in leaves, fruits and flowers. Carotenoids absorb the sunlight in a broader range of the blue spectrum (400-500 nm) than chlorophylls, and they transfer this absorbed energy to chlorophyll *a* of the photosynthetic reaction center. Carotenoids also supply substrates for the biosynthesis of the plant growth regulator abscisic acid (ABA). All carotenoids can be crystallized in *chromoplasts* of the flowers during the transition of chloroplast to chromoplast, or transported and accumulated in lipid bodies.

In animals, *ceto-carotenoid* type *astaxanthin* (*Appendix 1*) is responsible for the *orange* colour of *salmon meat* and *lobster shell*. *Feather colours* of the bird's also came from carotenoids. Chicken *egg yolks* are rich in lutein and zeaxanthin. In human nutrition and health, carotenoids act as anti-aging and anti-cancer substances, and provide provitamin-A (*e.g.,* β-carotene, β-cryptoxanthin and α-carotene). Carotenoids of algae, fungi and bacteria have also been identified and characterized.

Genes (*Table 1*) of enzymes involved in carotenoids synthesis are encoded in plant nuclear genomes, and gene products are transported either to the cytoplasm (including mitochondria) (*i.e.,,* mevalonate pathway) or to the plastids (*i.e.,,* non-mevalonate pathway) where they are post-translationally modified and activated.

Tomato fruits are also rich in *phenols* and *polyphenols* (like gallic acid, catechin, rutin, ferulic acid, *etc*.) and *vitamin E* (α-, and γ-tocopherol), which are also responsible for the antioxidant capacity of the soluble phase of fruit sap.

As the monoculture of tomatoes (*i.e.,* less then ten cultivars are cultivated in the World), the unique heirloom tomato genotypes may provide basal material for new feeding experiments and medical studies. *E.g.*, eggs of carotenoid-fed female birds (*Larus fuscus*) contained high carotenoid but low Ig contents (*i.e.,,* passive immunity), whereas control females produced eggs contained low carotenoid but high Ig content, which results indicated a carotenoid-mediated effects on phenotype for ecological fitness in mother birds and their offspring.

Lutein- and zeaxanthin supplemented male zebra finch birds (*Taeniopygia guttata*) an elevated blood carotenoid levels were found with increased cell mediated and humoral immune responses than control birds, and with brighter beak coloration,

which results suggested that carotenoids-based colour signals in birds may directly signal male health via the *immunostimulatory* action of ingested and circulated carotenoids.

In an experiments with depletion of carotenoids of nineteen healthy adult human that were fed at low carotenoids diets for 10 weeks, of the six major *human serum carotenoids* of lycopene, β-carotene, α-carotene, lutein, zeaxanthin and β-cryptoxanthin, the lycopene concentration showed sharp decrease compared to other carotenoids. This result indicated that *lycopene* appears to be the physiologically most important antioxidant of human body (*Appendix 1*).

Plant materials. A greenhouse study was conducted in the summer (June 1 to Sept 31) of 2014 at the spring at Experiment Station, Szent István University, Hungary, Europe. Tomatoes were seeded into Canadian Growing Mix 2 (*Conrad Fafard Inc*., Agawan, MA) in 72-cell flats and transplanted into trade gallon pots after five weeks in accordance with standard transplant production (Peat Brown OPM Multipack 025W; Kekkila) (*Fig. 1*).

Transplants were fertilized once a week with a 20N-4.4P-16.6K water soluble fertilizer (Peter's Water Soluble Plant Food 20-10-20) (Scotts Co, Marysville, OH) at a rate of 265 mg/L of N. Plants were allowed to grow for three weeks and then potted into three-gallon (24.13 cm tall, 27.94 cm diameter, 11.36 L) containers.

Plants were watered twice daily and fertigated weekly with 20N-4.4P-16.6K water soluble fertilizer (Peter's Water Soluble Plant Food 20-10-20) (Scotts Co, Marysville, OH) at a rate of 265 mg/L of N. Tomato plants were staked with three foot bamboo stakes attached with twist ties. All suckers below the first flower cluster were removed.

Table 1. Tomato genes encoding for fruit colors (*TFM, 2014; TGC, 2012*).

Gene Symbols	Gene Names	Description
Abg	Aubergine	*Purple* fruit epidermis particularly on shoulder
Af	Anthocyanin fruit	*Anthocyanin* in green and ripe fruit (absent when shaded)
ant	aurantia (*ant*1)	Short thick stems, light green pinnae; *light orange fruit* with colourless pericarp
at	apricot (yellow)	*Yellow-pink colour* of fruit flesh
aur	aurantiaca (*aur*1)	Small, pointed, yellowish light-green pinnae, and *orange fruit*
B	β-carotene	High β-carotene, low lycopene in ripe fruit
B^c	crimson (og^c)	Increased fruit lycopene content, phenotype similar to B^{og}
B^m	minutum	High β-carotene, low lycopene in ripe fruit
B^{og}	old gold (*og*)	Corolla tawny orange; increased fruit lycopene
del	Delta	Reddish-orange fruit, due to inhibition of lycopene, and increase of δ-carotene
dg	dark green	Darker green colour appears as fruit develops, then persists until onset of ripening, high chlorophyll compared to normal or *hp*
dps	diospyros	Fruit tissue is dusky orange
gdf	Gold Fleck	Small dark green spots on immature fruit, which turn yellow on ripe fruit
gf	green flesh	Persistent chlorophyll giving ripe fruit *purplish-brown colour*
glu	glutinosa (*glu*1)	*Dark green*, shiny fruit with sticky epidermis; poor germination
gr	green ripe (*gr*)	Resembles *gf*, except that center of *fruit turns red*
gs	green stripe	Radical green stripes in epidermis of unripe fruit; *golden in ripe fruit*
hp-1	high pigment (*hp*2)	Chlorophyll, carotenoids, ascorbic acid content of fruit intensified
$r^{\cdot}$	yellow flesh	*Yellow* colour of ripe fruit flesh
$r^{(1s)}$	yellow flesh (1s)	*Yellow* colour of ripe fruit flesh.
$r^{(2s)}$	yellow flesh (2s)	*Yellow* fruit flesh; lighter yellow flowers
r^{prov4}	yellow flesh	Yellow colour of ripe fruit flesh
r^{prov5}	yellow flesh	*Yellow* colour of ripe fruit flesh
r^{y}	yellow flesh	*Reddish yellow*: Likely allele of r with reddish flesh tones in ripe fruit
sh	sherry	Fruit flesh *yellow* with reddish tinge
t	tangerine	Fruit flesh and stamens *orange* coloured
vo	virescent orange	Fruit flesh orange, redder in outer walls
y	colourless fruit epidermis	Fruit epidermis lacks pigmentation (*clear skin*)

Treatments consisted of the following selected American heirloom tomatoes: '*Aunt Ruby's German Green*'; '*Black from Tula*', '*Cherokee Purple*' and *German Johnson Regular Leaf*'; and '*Kellogg's Breakfast*', and '*Yellow Brandywine Platfoot Strain*' (*Fig. 2*).

The germination vigor was found the strongest in *'Kellogg's Breakfast'* followed by *'Yellow Brandywine Platfoot Strain', 'Cherokee Purple', 'German Johnson Regular Leaf', 'Aunt Ruby's German Green'* and *'Black from Tula'* heirloom. The '*Cherokee Purple'* (3rd heirloom) was found to be a 'potato leaved' type (*Fig. 1*).

All heirlooms showed '*indeterminate*' growing habit. Nearly a century ago, a spontaneous mutation in *sp* (*self pruning*) gene family spawned the *'determinate' tomato* development, which now dominate the tomato market being beneficial for mechanical harvesting (*TGC, 2012*)

By fruit shape, all heirlooms showed extremely *puffy fruits* with hollow locules (*i.e.,* fruit cavity with seeds), which suggest the presence of *spf*2 genes (superpuff) with bell pepper shaped fruit. Fruits of

'Aunt Ruby's German Green' showed serious radial crakes, which phenomenon is regulated by two dominant genes of *CE* and *RA*. All the other tomatoes showed some radial crack resistance, which is controlled by recessive alleles of *cr* and *ra*; however *'Black from Tula'* was found susceptible to fruit bursting, which is regulated by dominant gene *BT* (burst types). The first three American heirlooms (1-3) may also carry *Abg* (*Aubergine*) gene, which cause purple fruit epidermis particularly on shoulder, and the *'Yellow Brandywine Platfoot Strain'* (6th heirloom) probably carries *fs* (fruit stripe) gene, which causes dark green radial stripes at the opposite locules (*Fig. 2, Table 1*).

Treatments were randomly assigned to six individual plants in a completely randomized design with three replications. Data gathered included germination vigor (*Fig. 1*) and fruit quality characteristics. The first six ripened fruit (*Fig. 2*) grown on the same vine nodes were collected and processed for HPLC analysis.

Figure 1. Germination vigor of the six American heirloom tomatoes (*Solanum lycopersicum*) (three pots each). (1) *'Aunt Ruby's German Green';* (2) *'Black from Tula';* (3) *'Cherokee Purple';* (4) *'German Johnson Regular Leaf';* (5) *'Kellogg's Breakfast';* and (6) *'Yellow Brandywine Platfoot Strain'.*

Figure 2. Fruit samples of the six American heirloom tomatoes (*Solanum lycopersicum*). (1) *'Aunt Ruby's German Green';* (2) *'Black from Tula';* (3) *'Cherokee Purple';* (4) *'German Johnson Regular Leaf';* (5) *'Kellogg's Breakfast';* and (6) *'Yellow Brandywine Platfoot Strain'.*

Extraction of carotenoids. Lipids and fat soluble pigments collected from the raw tomatoes were extracted according to a previously described procedure with slight modification. Five-gram samples from tomato fruits were taken from each variety in four replicates (6 varieties x 4 replicates = 24 samples), and disintegrated in a crucible mortar with quartz sand. The water was removed by adding 20 mL methanol. The mixture was then transferred quantitatively to a 100 mL conical flask, and 70 mL of a 6:1 dichloroethanemethanol solution was added.

The mixture was shaken for 15 min by a mechanical shaker. Distilled water was with hand shaking till the dichloroethane phase was clearly separated from the polar phase (water + methanol). The two phases were separated into different funnels, and the lower layer containing lipids in dichloroethane was dried over anhydrous sodium sulphate. Finally, the organic solvent was evaporated under vacuum by rotary evaporator at not higher than 40°C. The residues were re-dissolved in HPLC acetone, as the best organic solvent that ensures high solubility of most of carotenoids before injection onto HPLC column (*Fig. 3*).

HPLC analysis. For Reverse Phase (RP) High-Performance Liquid Chromatography (HPLC) a Chromaster Hitachi HPLC instrument was used, couple with diode-array detector (Model 5430), an autosampler (Model 5210) and a gradient pump (Model 5110). The instrument and analyses was operated by EZchrom Elite software (version 3.3.2.SP2) and the separation of carotenoids was performed on Accucore (Thermo Scientific) C-30, 2.7 µ, 150 x 4.0 mm column with gradient elution of (a) tetra-butyl-methyl-ether (TBME) and (b) methanol (MetOH).

The gradient elution started with 100% TBME, and changed to 30% TBME in MetOH for 25 min, stayed isocratic for 5 min, and finally turned to 100% TBME for 5 min. Peak identification was based on the comparison of spectral properties and retention time of carotenoids with those of available standard materials like lycopene, β-carotene and zeaxanthin, which were purchased from (Sigma-Aldrich, Budapest, Hungary).

In case of compounds with no available standards, the peaks were tentatively identified according to their spectral characteristics and chromatographic retention as compared to references. The cis isomers of lycopene were identified on the basis of appearance of an extra absorption wavelength at 340 nm and 361 nm. The 9-Z and 13-Z cis lycopene isomers were identified according to II-value, which equals to absorbance at 361 nm over absorbance at the maximum wavelength of cis lycopeneThe column effluents were detected and integrated at their maximum absorption wavelength for quantitative determinations. They were quantified as either lycopene- or β-carotene-equivalent (µg g^{-1}, equal to g kg^{-1}) according to their spectral characteristics (*Fig. 3, Table 1*).

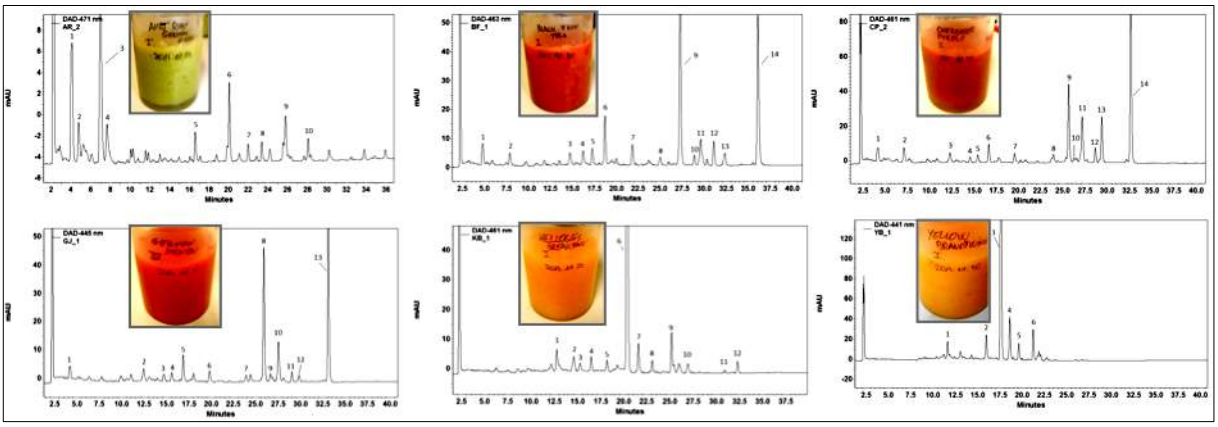


Figure 3. HPLC profiles of carotenoids of the six American heirlooms tomatoes (*Solanum lycopersicum*) studied **AR** - *'Aunt Ruby's German Green'*.: 1 - Lutein, 2 - Chlorophyllid B, 3 - Chlorophyll B, 4 - Chlorophyll B, 5 - Neoxanthin, 6 - β-carotene, 7- 13-cis-lycopene, 8 - Rubixanthin, 9 - Lycopene, 10 - γ-carotene. **BF** - *'Black from Tula'*: 1 - Lutein, 2 - Chlorophyll B, 3 - Lycopene diepoxide, 4 - Mutatoxanthin, 5 - Neoxanthin, 6 - cis-neoxanthin, 7 - Lycopene-epoxide 1, 8 - cis-β-carotene, 9 - β-carotene, 10 - Lycoxanthin, 11 - 13-cis-lycopene, 12 - Rubixanthin, 13 - 9-cis-lycopene, 14 - Lycopene. **CP** - *'Cherokee Purple'*: 1 - Lutein, 2 - Chlorophyll B, 3 - Lycopene diepoxide, 4 - Mutatoxanthin, 5 - Neoxanthin, 6 - Cis-neoxanthin, 7 - Lycopene-epoxide 1, 8 - Lycoxanthin, 9 - β-carotene, 10 - 15-Cis-lycopene, 11 - 13-Cis-lycopene, 12 - Rubixanthin, 13 - 9-Cis-lycopene, 14 - Lycopene. **GJ** - *'German Johnson Regular Leaf'*: 1 - Lutein, 2 - Lycopene diepoxide, 3 - Mutatoxanthin, 4 - Neoxanthin, 5 - Cis-neoxanthin, 6 - Lycopene-epoxide 1, 7 - Lycoxanthin, 8 - β-carotene, 9 - 15-Cis-lycopene, 10 - 13-Cis-lycopene, 11 - Rubixanthin, 12 - 9-Cis-lycopene, 13 - Lycopene. **KB** - *'Kellogg's Breakfast'*: 1 - Prolycopene epoxide, 2 - Mutatoxanthin, 3 - Neoxanthin, 4 - Cis-neoxanthin, 5 - Neochrome, 6 - Prolycopene, 7 - Proneurosporene, 8 - Violaxanthin, 9 - β-carotene, 10 - 13-Cis-lycopene, 11 - 9-Cis-lycopene, 12 - Lycopene; **YB** - *'Yellow Brandywine Platfoot Strain'*: 1 - Prolycopene epoxide, 2 - Neochrome, 3 - Prolycopene, 4- Proneurosporene, 5 - ζ -carotene, 6 - α-cryptoxanthin; Photos of the meshed fruit saps prepared for HPLC analysis are indicated.

Table. 2. Carotenoids components of the fruits of six American heirloom tomatoes (*Solanum lycopersicum*)

Carotenoids contents (μg g^{-1} FW)	Mol. Formulas	MW	Aunt Ruby's German Green (1)	Black from Tula (2)	Cherokee Purple (3)	German Johnson (4)	Kellog's Breakfast (5)	Yellow Brandywine (6)
ζ-carotene	$C_{40}H_{60}$	540.904	0	0	0	0	19.14±15.39	32.65±22.21
ζ-carotene like	$C_{40}H_{60}$	540.904	0	0	0	0	1.68±1.58	3.33±3.21
Neurosporene	$C_{40}H_{58}$	538.890	0	0	0	0	1.55±0.59	1.42±0.38
Neurosporene-epoxide	$C_{40}H_{58}O$	549.449	0	0	0	0	2.49±1.76	3.68±2.33
Prolycopene	$C_{40}H_{56}$	536.889	0	0	0	0	100.87±51.4	70.99±15.27
Neochrome	$C_{41}H_{58}O_3$	598.8974	0	0	0	0	3.1±1.54	2.26±0.41
α-cryptoxanthin	$C_{40}H_{56}O$	552.872	0	0	0	0	5.33±3.12	5.96±1.29
Violaxanthin	$C_{40}H_{56}O_4$	600.870	0	0	0	0	2.92±1.17	1.89±0.65
Lycopene	$C_{40}H_{56}$	536.873	0.23±0.26	13.33±7.48	19.25±14.25	17.93±6.29	0	0
β-carotene	$C_{40}H_{56}$	536.873	1.15±1.98	23.56±9.17	11.51±3.32	12.2±3.34	0	0
9-Cis-lycopene	$C_{40}H_{56}$	536.873	1.35±2.31	2.2±3.82	4.5±5.39	7.84±2.24	0	0
13-Cis-lycopene	$C_{40}H_{56}$	536.873	0.08±0.02	5.04±13.06	3.68±3.15	5.28±2.77	0	0
Lycopene-epoxide 1	$C_{40}H_{56}O$	552.433	0	1.45±0.74	2.55±2.63	0.81±0.36	0	0
Lycopene-epoxide 2	$C_{40}H_{56}O$	552.433	0	0.81±0.74	2.83±5.61	0.76±0.18	0	0
β-cryptoxanthin	$C_{40}H_{56}O$	552.872	0	0.35±0.22	0.96±1	0.67±0.19	0	0
Lycoxanthin	$C_{40}H_{56}O$	552.872	0	7.13±13.3	0	0.77±0.29	0	0
Lycopene-diepoxide_1	$C_{40}H_{56}O_2$	568.428	0.2±0.28	0.77±0.7	1.1±0.07	1.04±0.35	0	0
Lycopene-diepoxide_2	$C_{40}H_{56}O_2$	568.428	0.2±0.28	1.06±1.27	1.03±0.14	1.09±0.13	0	0
Lutein	$C_{40}H_{56}O_2$	568.871	1.77±1.99	2.93±2.87	0.99±1.2	0.19±0.04	0	0
Mutatoxanthin	$C_{40}H_{56}O_3$	584.871	0	1.62±0.74	0.75±0.28	1.25±0.27	0	0
Neoxanthin	$C_{40}H_{56}O_4$	600.870	0	2.11±2.41	1.52±1.05	2.69±0.5	0	0
TOTAL			4.98	62.36	50.67	52.52	137.08	122.08

Statistical analysis. Mean values of four (n=4) HPLC measurements and 95 % Confidence Interval for Mean ($CI_{95\%}$ = x ± d) confidence intervals were calculated at P 95% confidence level by SPSS program package. For Dendrogram (using *Average Linkage Within Group*) - and Canonical Discriminant Functions Analyses also the SPSS program package was used.

Tomato fruit carotenoids

The synthesis of plant carotenoids links genetically with the processes of fruit ripening (*i.e.,* cell wall softening), which is regulated by about 50 genes in tomato. Regulation/modulation of fruit ripening of all fleshy fruit plant species has profound agronomic importance

Recently, the fresh market tomatoes include only '*long shelf-life*' varieties, which are natural mutants, and carry ripening inhibitor (*RIN*) genes. Another unique natural tomato mutants is a dwarf type '*Micro-Tom*'. The main *RIN* genes are the *rin* (*ripening-inhibitor* MADS-box gene), *nor* (*non-ripening transcription factor gene*), *nr* (*never-ripe ethylene signaling*), *nr*-2 (*never-ripe 2*) / *gr* (*green-ripe*), and *cnr* (*coulorless non-ripening*). Of them, one of the earliest tomato fruit ripening mutants was the dominant *Nr* (*Never-ripe)* mutation. This mutation was shown to be the consequence of a single amino acid change in one of the seven ethylene receptors (*Le*TR1-7).

The first registered *RIN* tomato, the '*Daniela*' (FA144) an indeterminate long-self life hybrid, was released in about 1992 by the *BonTom Tomato Breeding Group* (Faculty of Agriculture, Hebrew University of Jerusalem, Israel). *Green-Ripe* (*Gr*) and its allele, *Nr-2*, were also found to be dominant nonripening mutant.

During the transgenic GM programs, the first FDA-approved transgenic food of *Flavr-Savr* tomato was released in 1994, followed by another delayed ripening tomatoes (DNAP) by *Zeneca/Peto and Monsanto*.

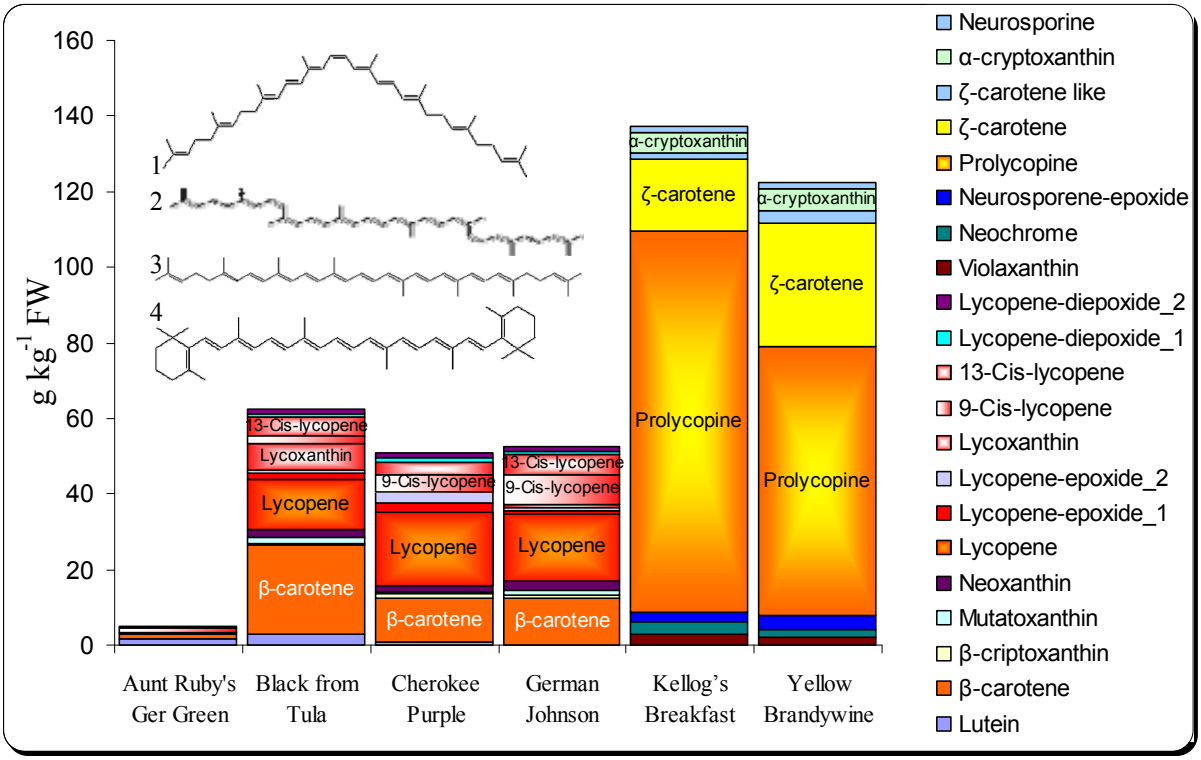


Figure 4. Cumulative carotenoids components of the six American heirloom tomatoes (*Solanum lycopersicum*). The main metabolic steps (numbers 1 to 4), and molecular formulas from the *non-cyclic carotenoids* ζ-carotene ($C_{40}H_{60}$) *(1),* → prolycopene ($C_{40}H_{56}$) *(2),* → lycopene ($C_{40}H_{56}$) *(3),* to → *cyclic carotenoid* β-carotene ($C_{40}H_{56}$) *(4),* are indicated (*syn.* of prolycopine are: *cis-lycopene*; *all-trans-lycopine*) (*Pék, Helyes, Gyulai et al., 2016*)

By visual observation of fruits (*Fig. 2*), none of the heirloom tomatoes showed the expression of *RIN* genes, as the fruits were ripened and softened very quickly in some days after total fruit size development. These fruit characters obviously suggested these heirlooms for fresh consumption or for tinned juice.

The fruit pigment composition of the heirlooms showed three main groups (*Table 2, Fig. 4*). In the first group, on the contrary of the extremely low carotenoids compositions (*Fig. 4*), *'Aunt Ruby's German Green'* (1st heirloom) was found with delicious taste due probably to its tasty compositions

of other organic fruit components such as *carbohydrates* and *organic acids*.

In the second group, *'Black from Tula'* (2nd heirloom) showed the highest content of β-carotene (23.56 ug g^{-1} FW). This β-carotene content showed higher level compared tomato cv. *'Strombolino'* (3.27±0.17 ug g^{-1} FW) measured in our lab using the same technology. The highest lycopene content (19.25 ug g^{-1} FW) was found in the '*Cherokee Purple*'(3) and '*German Johnson'*(4) (17.93±6.29 ug g^{-1} FW) tomatoes. These lycopene contents showed slower levels then in tomato cv. *'Strombolino'* (62.6±2.77 ug g^{-1} FW), however it was higher levels compared to a *vine-ripened* tomato *'Lemance* F1*'*, which yielded 4.5±1.40 ug g^{-1} FW (*Pék et al., 2010*).

The third group comprised the two yellow fruited tomatoes of *'Kellogg's Breakfast'*(5) and *'Yellow Brandywine Platfoot Strain'*(6) with extremely high level of prolycopene content (100.87 and 70. 99 ug g^{-1} FW, respectively). As the later stages of main metabolic *desaturation* steps of the synthesis of plant acyclic carotenoids ($C_{40}H_{60}$), which goes through *ζ-carotene* → neurosporene → *prolycopene* (and → *lycopene*), we may suggest that these two heirloom tomatoes may be metabolically 'economic' by blocking the further enzymatic reactions (and saving *metabolic energy* by this way), which would have been included the cyclizations of lycopene either to *α-zeacarotene* → *δ-carotene* → *ε-carotene*, or to *β-zeacarotene* → *γ-carotene* → *α-* and β-carotene (*Fig. 4, Appendix 1*)

Dendrogram- (*Fig. 5a*) and Discriminant Analysis (*Fig. 5b*) of carotenoids contents (*Table 2*) revealed that *'Aunt Ruby's German Green'* shows the most homogenous clade (Clade 1 of *Fig. 5a*) and is close to *'Kellogg's Breakfast'* and *'Yellow Brandywine Platfoot Strain'*(Clade 2 of *Fig. 5b*). Heirlooms of *'Black from Tula'*, *'Cherokee Purple'*, and *'German Johnson Regular Leaf'* showed separate carotenoids compositions (*Fig. 5b*).

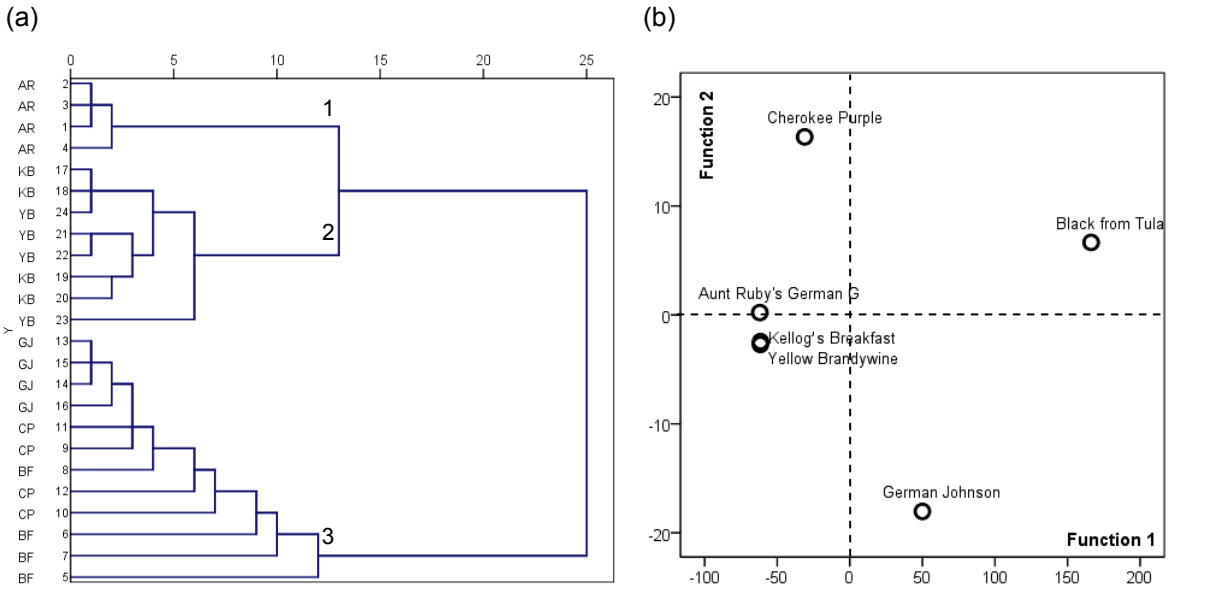


Figure 5. Dendrogram *(a)* and Canonical Discriminant Functions *(b)* analysis of the carotenoids contents of six American heirloom tomatoes (*Solanum lycopersicum*) studied. AR – (1) *'Aunt Ruby's German Green'*. BF – (2) *'Black from Tula'*. CP – (3) *'Cherokee Purple'*. GJ – (4) *'German Johnson Regular Leaf'*. KB – (5) *'Kellogg's Breakfast'*. YB – (6)*'Yellow Brandywine Platfoot Strain'*. The level of dissimilarity (*i.e.* Squared Euclidean Distances) (0 to 25), the three main clades, and the number of four measurements of each heirloom (1 to 24) are indicated.

In conclusion, as a result of high prolycopene and ζ-carotene contents, the two yellow fruited heirloom tomatoes *'Kellogg's Breakfast'*(5) and *'Yellow Brandywine Platfoot Strain'*(6) are suggested to be involved in a breeding program to identify further gene markers for yellow fruit coloration. Due to good fruit taste of *'Aunt Ruby's German Green'*(1); and for high lycopene and β-carotene contents the red-fruited '*Black from Tula'*(2), '*Cherokee Purple'*(3), and '*German Johnson'*(4) tomatoes have high potential for recultivation.

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SGN (2014) Sol Genomics Network website: http://solgenomics.net

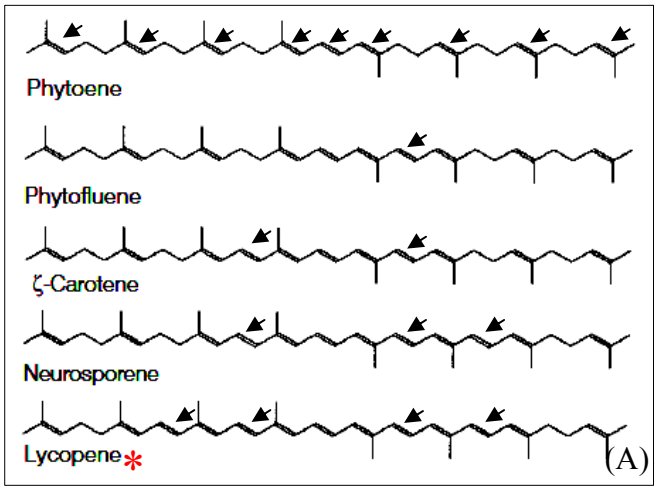
TFM (2014) Tomato Fruit Mutation website: http://kdcomm.net/~tomato/Tomato/mutant.html

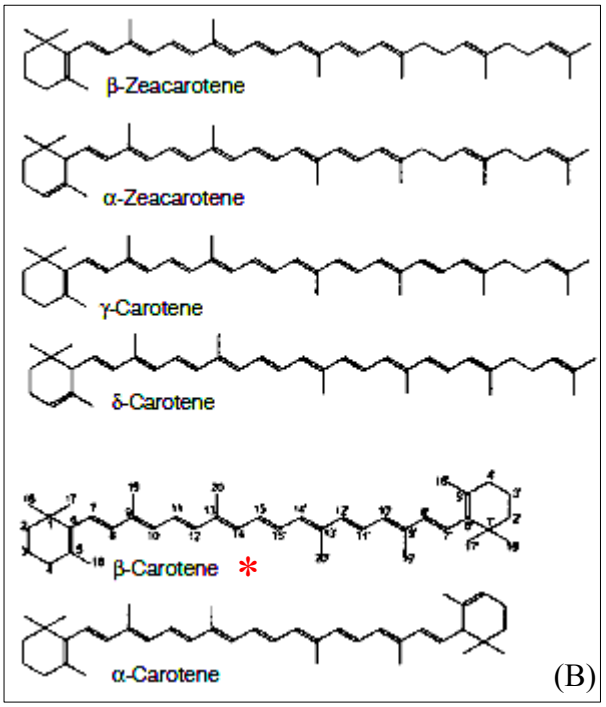
TGC (2012) Tomato Genome Consortium. *Nature* 485, 635–641.

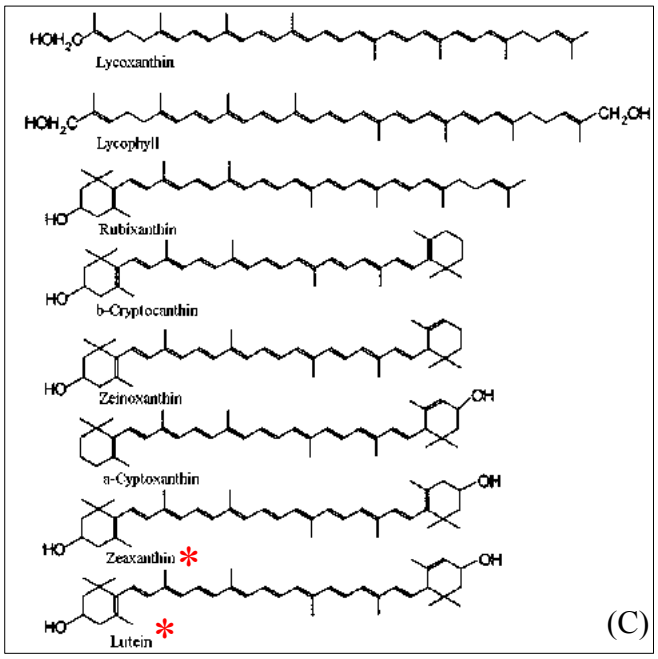
Appendix 1. Molecular structures of:

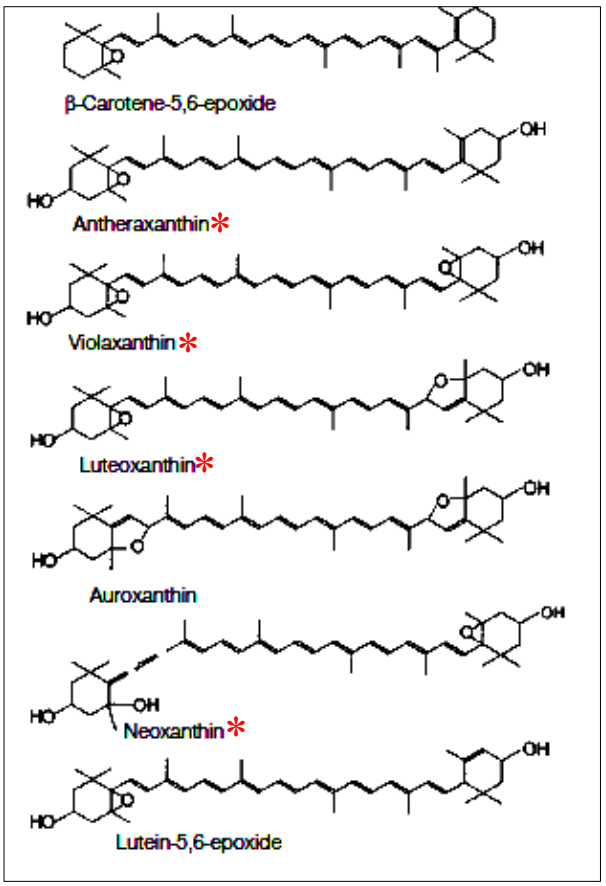
(A) Acyclic Carotenes (*the increasing number of double bounds are indicated by arrows*)
(B) Cyclic Carotenes
(C) Xanthophylls (Hydroxycarotenoids, Carotenols,)
(D) Epoxy-Carotenoids
(E) Unique plant carotenoids
(F) Unique animal carotenoid *Astaxanthin*

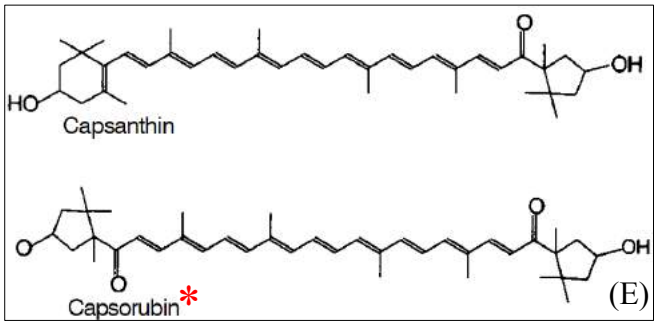
The most important carotenoids are indicated with asterisk (*).

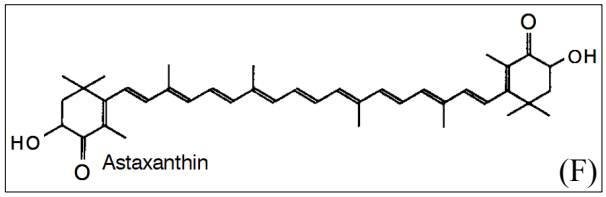












Plant Cell, Tissue, and Organ Cultures – Basics of plant biotechnology

Introduction

Basics of Plant Tissue Cultures of Plant Biotechnology includes several laboratory techniques and processes at cell and organ levels, and apply *in vitro* cultures under *aseptic* conditions with using artificial nutritive media (*Table 1*) supplemented with plant hormones. Biotechnology is a very broad term and is not new. The term biotechnology, first was coined in 1919 by a Hungarian engineer *EREKY K* (*1878-1952*) (*Appendix 1*).

Regular plant tissue culture lab is equipped with *Laminar air flow cabinets*, *Autoclaves*, *Water purification systems*, and several types of *Glasswares* (*Fig. 1, 6-8, 11a, 14ab; Appendix*). The most important advantage of these techniques is the capability of the production of 10 million plants per year developed from a single cell (or *explant*), and to keep and treat one million plants in a flask at 'cell level'.

(A) Main fields of Plant tissue cultures

1. Seed culture. Similar to agriculture, seeds, after *surface sterilization* (after using *sodium-* or *calcium hypochlorite*; which was used first in human medication by the Hungarian medical doctor *Ignác Semmelweis*, Budapest, 1818-1865) (*Fig. 2b,c*) can be used *in vitro* for whole plant development (*Fig. 3*) for further mass propagation (*Fig. 8*).

2a. Explants. Micropropagation (micro – propagation). It is a laboratory processes of whole plant developments, which applies small (*i.e., micro*) parts (*i.e., explants*) of a plant, and carried out under aseptic conditions, *in vitro* (*i.e.,* in '*bottle'*). The most frequently used explants are the shoots (*i.e., Shoot culture* - *Fig. 1, 6*); roots (*i.e., Root culture* - *Fig. 1*); buds (*i.e.,* both *apical* and *axillary Bud cultures*) (*Fig. 4*); leaves (*i.e., Leaf-*, and *Leaf disc* cultures - *Fig. 1*); and *Petioles* (*e.g.,* of poplar leaves) (*Fig. 6*). These techniques also called as 'basic plant organ cultures', or simply 'cloning'. Cloning means *vegetative* (*syn*., *axexual*) *propagation*.

2b. Meristem culture. Mericlones. Virus elimination. Meri(stem)-*cloning* uses not the whole buds (*Fig. 4*) for plant development, rather the excised *meri*stems (*Fig. 10*) of both roots or buds (*Fig. 9*). As meristems are free of *pathogenes*, mericloning is a unique technique to produce *virus-free* lines for crop production.

3. Embryo, Embryo rescue, Ovary and Ovulum cultures. Seed organs having the whole *embryo* (*Fig. 1*), and *female* sexual organs of *ovary* and *ovulum*, or the single *egg cell* also can be excised from both mature or immature *vs.* fertilized or unfertilized seeds for further manipulation. *Sexually incompatible* parents also can be hybridized by embryo saving (*i.e.*, *embryo rescue*), and develop the hybrid plants *in vitro*.

3b. Anther and Pollen cultures. Male sexual organs of the whole *anther* (full with undeveloped pollens – *i.e., anther culture*) (*Fig. 11a,b*), or the excised *pollens* (*i.e., pollen culture*) (*Fig. 11c, 12*) can also be used for whole plant development (*Fig. 13*) with 'half genome' (n) *in vitro* either in suspension or agar culture (*Fig. 11a*).

3c. Haploid cultures, DH breeding. Twin embryos. Sex cells of both *egg-*, and *pollen* cells are haploids (carrying only half of the genome) (n), so when plants develop from them, the whole plant will be also *haploid* (n). Through *colchicines* treatment, the genome can be *diploidized* and the developed *diploid* (2n) plants will be completely *homozygous* (*i.e.*, *DiHaploid*, DH). Besides *fixed meiotic recombinants* (*syn., sexual 'clones'*), DH-breeding also uses DH-plants for further hybridization to use up the phenomenon of *hybrid vigor* (*i.e.,* hybrid produce more yield then either parent lines alone). Conventional *Twin embryo* technology (*Fig. 11d*), and *haploid marker systems*, also can produce haploid plants.

3d. Pollination and fertilization in vitro. Direct pollination and fertilization can be carried out *in vitro* for breaking down the *sexual incompatibility* prior to *embryo rescue* technology.

4. Callus cultures. Totipotency. Cell suspensions. Organogenesis. Embryogenesis. Somatic embryos. Totipotency. Somaclones. Somaclonal variation. Artificial seeds.

All plant organs cultured *in vitro* (summarized above) can be turned to an *undifferentiated* developmental way by stimulating them with *plant hormones* (*Fig. 17*), which produce clumps of undifferentiated cells (*i.e., callus*), either on the surface of agar-solidified media (*Fig. 2a*), or in liquid (*Fig. 3*), media (*i.e., cell suspension*).

Whole plants can develope from these *single callus cells* through either *organogenesis* (root develops first and later the shoot does), or through *embryogenesis* (not organs rather full embryo develops directly; *i.e., somatic embryos*) (*i.e., somaclones*).

Plants, developed from *somatic embryos* (*Fig. 2a*) are *identical* to the original plants (*i.e.,* all cells are *totipotent* for they carry the same DNA), however, with slight genetic differences *(i.e., somaclonal variation*) due to the effects of *in vitro* conditions and mainly to the plant hormones (*Fig. 18*) applied. *Somatic embryos* can be coated with special compounds (*e.g.,* graphite, and calcium alginate), and can sow (!) directly in the field (*i.e., artificial seeds*).

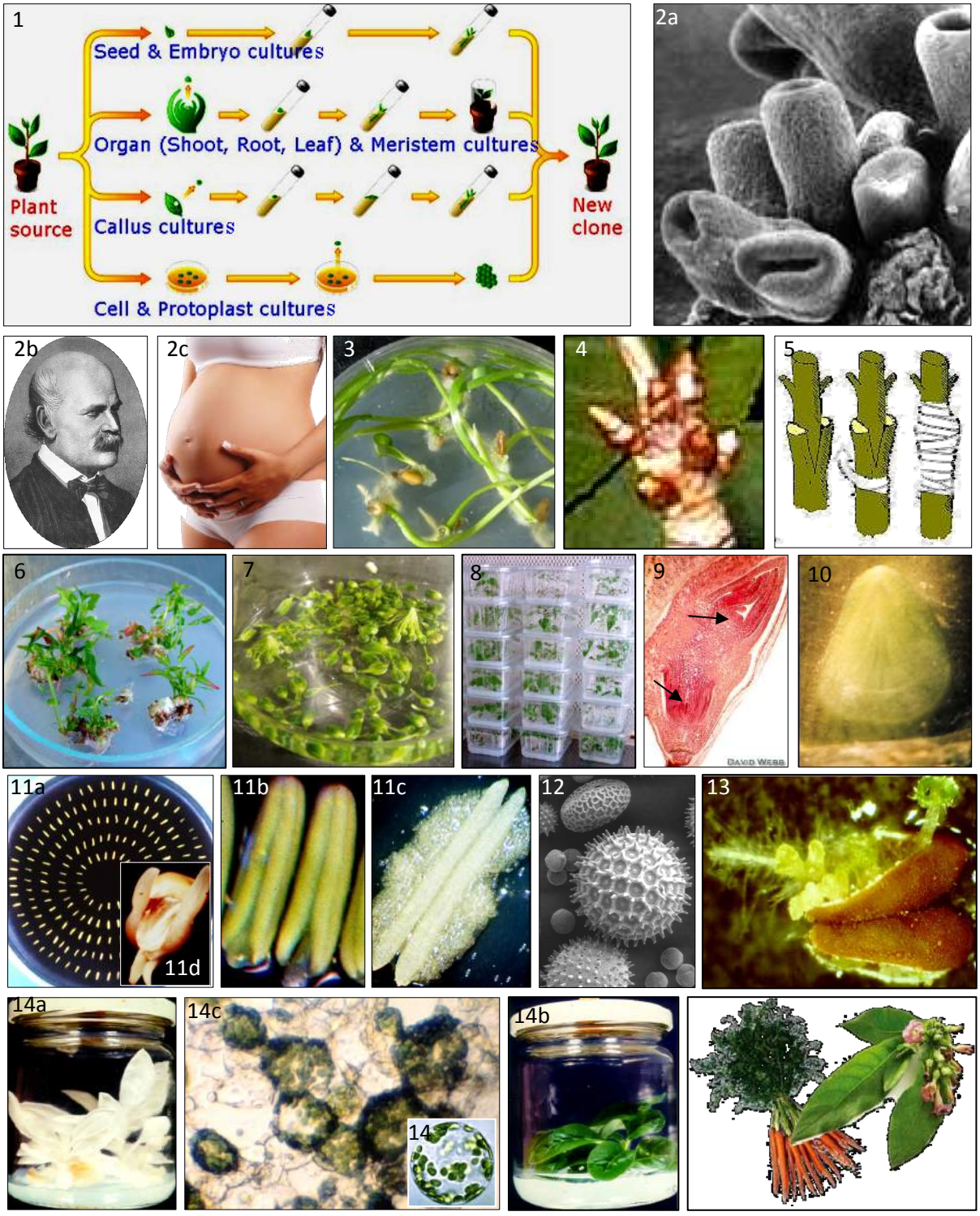


Figure 1-15. Basic technologies of Plant tissue and organ cultures **(1)**. *VEGETATIVE ORGANS.* Somatic embryos of dicot *Aesculus* trees (**2a**) (*Gyulai, Jekkel, and J. Kiss, 1998*), developed *in vitro*, after surface sterilization of explants by Ca/Na-hypochlorite, which was used first in human medical sciences (against *childbed fever* and *maternal mortality*) (**2c**), by the Hungarian MD *Semmelweis, Ignác* (1818-1865) (**2b**). Seed germination of wheat (*Triticum*) under aseptic conditions (**3**). Bud explants for *bud cultures* (**4**). Samples of cloning (*i.e., grafting*) used in horticulture (**5**). Cloning *in vitro*: *shoot culture* of poplar tree in agar-solidified media (**6**), or in aseptic *suspension culture* incubated in liquid medium (**7**). Mass propagation of poplar in *shoot cultures* (**8**) (*Bittsánszky and Gyulai, 2005*). Seed meristems (of shoot and root) (**9**), excised for *meristem cultures*. Shoot *apical meristem* (**10**) excised for *mericloning*. *GENERATIVE ORGANS*. Anthers excised for *anther culture* (**11a**), close up of anthers (**11b**), which release pollens (**12**) onto the surface of agar medium (**11c**) (*Gyulai et al., 1986*). Haploid plantlet can develop from twin embryos (*e.g.,* germinating maize) (**11d**), or developed from *pollen* (**13**) in anther culture of paprika (*Capsicum*) (*Gyulai and Gémesné, 2000*). Protoplasts (**14**) isolation from parental tobacco (*Maliga P, L Márton, P Medgyesy, M Czakó, et. al., 1973*) plants (**14a**, and **14b**), and *cell fusion* (**14c**) (*Gyulai and Malone, 1986*) of tobacco; and carrot (NICA) (*NIcotiana* x *Daucus/CArrot*) (*Dudits D, M Deák, A Páy, et al., 1987*).

5. Protoplast. Cell immobilization. Protoclone. Cybrid.

Callus cells, after treating with *cell wall digesting enzymes* of *cellulase*, *hemicellulase*, and *pectinase* (*etc.*), develop to *protoplast* (*i.e.,* plant cell without cell wall, only with cell membrane) (*Fig. 14*), which can either directly grow to *protoplast originated plant* (*i.e.*, *protoclone*), or can be *fused* (*i.e.*, cell fusion) in the case of two parental populations of protoplasts (*Fig. 14c*), which produce *cybrids* (*i.e.,* CYtoplamic hyBRIDS). Immobilization of protoplasts with embedding them in *low melting agar*, or *calcium alginate* (similar to artificial seeds, or enzyme immobilization for 'champagne production') can protect cells from bursting.

(B) Plant tissue culture media

Plant tissue culture applies 'artificial soils' (*i.e.,* nutritive media), which is composed of *mineral elements* (*Table 1; Fig. 16*), *chelated iron* for Fe-uptake (EDTA-Fe), *vitamins* for helping sugar digestion, *plant hormones* (*Fig. 17, 18*) to help organs development, and the solidifying agents of *agar, agarose, gerlite*, *etc*.

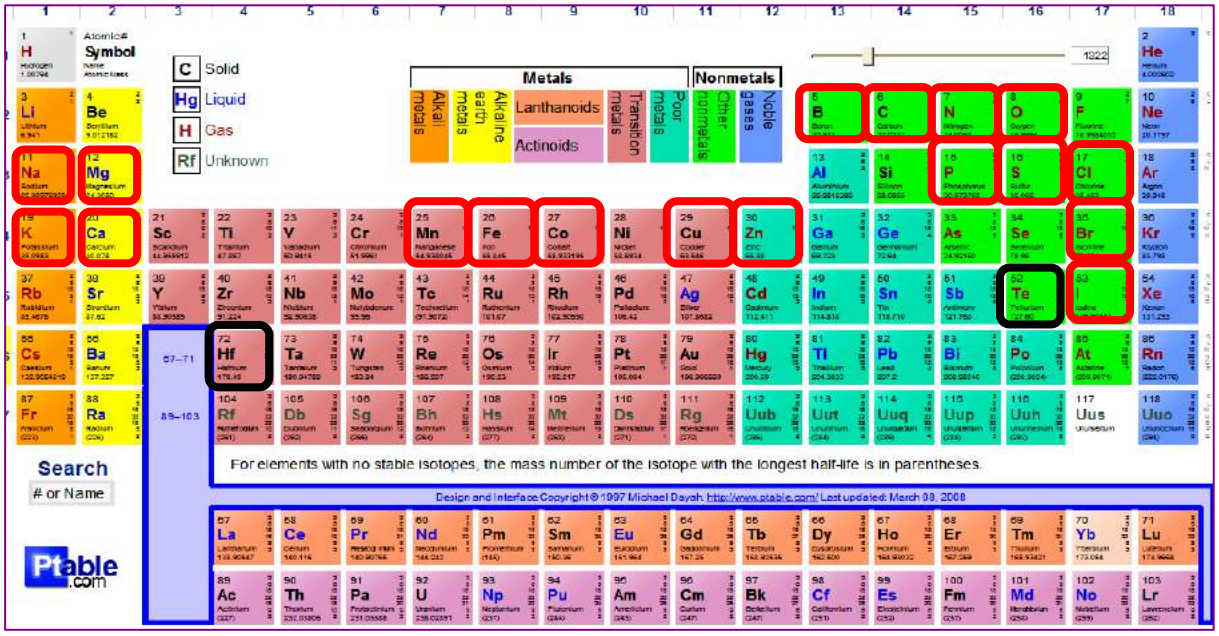
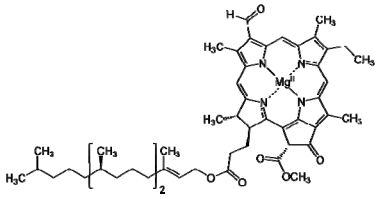


Figure 16. Periodic table of elements (pre-edited first by *DI Mendelejev*, 1837-1907; in 1875). The most important plant nutritive elements, and the two elements of Hf and Te, which were discovered by Hungarian chemists (*Tellurium* - by *Ferenc Müller*, 1782; and *Hafnium* by *György Hevesi*, 1911, Nobel Prize, 1943) are indicated.

(a) The main physiological functions of the Major plant nutritive elements

(Ca^{2+}): Calcium is a co-factor of several enzymes and is particularly important in plant cell wall development. *(PO_4^{3-})*: Phosphorus is an integral part of nucleic acids (DNA and RNA) and other structural compounds (*e.g.*, ATP). It is added to culture medium as phosphate (PO_4^{3-}) salt. *(K^+)*: Potassium is the major cation in plants, its positive charge balances negative ions. *(Mg^{2+}):* Magnesium is critical (*i.e.,* cofactor) for the functioning of several enzymes, it is an integral component of the *chlorophyll* molecule, and is a cation that balances negative ions. Mg^{2+} is the central atoms of chlorophyll molecules, which molecule is the basis of Life in the earth by sunlight harvesting!



(SO_4^{2-}): Sulfur is a part of two S-containing amino acids (*cysteine* and *methionine*) and has an important function in protein structure by *sulphur bridge bounds.* It is applied as SO_4^{2-} salts.

(b) Chelating agents

EDTA (*Ethylene Diamine Tetraacetic Acid*) is a complexing (chelating) compounds, which chelate *metal ions*. It is an *polyprotonic acid* containing four *carboxylic acid* groups and two *amine* groups, with *lone pair* electrons (EDTA is synthesized on an industrial scale from *ethylenediamine*, *formaldehide*, and a source of cyanide either HCN or NaCN).

EDDHA (*Ethylene Diamine DihHdroxy phenylacetic Acid*) is also a powerful complexing (chelating) molecule with an additional rooting effect due to the *phenylacetic acid* (*i.e.,* it is a natural auxin) component.

In molecular biology, ion depletion is commonly used to deactivate metal-dependent enzymes *e.g.,* to suppress the damage to DNA or proteins. EDTA forms especially strong complexes with Mn(II), Cu(II), Fe(III), Pb(II), and Co(III).

As Human medicine, EDTA is used to bind metal ions in the practice of *chelation therapy*, *e.g.,* for treating mercury and lead poisoning. It is used in a similar manner to remove excess iron from the body.

Table 1. Mineral compositions of the main tissue culture media

		White (1943)	MS (1962)	B5 (1968)	SH (1972)	N6 (1975)	WPM (1980)	AA (1985)	Gyulai (1992)
(I) MAJOR ELEMENTS	*Mw*								
KNO_3	*101.1*	0.08	1.90	2.5	2.5	2.83	-	-	-
NH_4NO_3	*80.4*	-	1.65	-	-	-	0.4	-	0.956
KCl	*74.6*	0.065	-	-	-	-	-	2.95	-
$CaCl_2 \times 2H_2O$	*147.0*	-	0.44	0.15	0.2	0.166	0.096	0.15	0.096
$MgSO_4 \times 7H_2O$	*246.5*	0.737	0.37	0.25	0.4	0.185	0.37	0.25	0.37
KH_2PO_4	*136.1*	-	0.17	-	-	0.4	0.17	-	1.16
$NaH_2PO_4 \times H_2O$	*138.0*	0.0165	-	0.15	-	-	-	0.15	-
$(NH_4)_2SO_4$	*132.1*	-	-	0.134	-	0.463	-	-	-
Na_2SO_4	*142.0*	0.2	-	-	-	-	-	-	-
$Ca(NO_3)_2 \times 4H_2O$	*236.2*	0.288	-	-	-	-	0.556	-	-
$(NH_4)H_2PO_4$	*115.3*	-	-	-	0.3	-	-	-	-
K_2SO_4	*174.3*	-	-	-	-	-	0.99	-	-
TOTAL (**g L^{-1}**)		1.4	4.5	3.2	3.4	4.0	2.6	3.5	2.6
(II) MINOR ELEMENTS	*Mw*								
$MnSO_4 \times 4H_2O$	*223.1*	6.65	22.3	13.5	13.5	4.4	22.3	13.5	22.3
$ZnSO_4 \times 7H_2O$	*287.4*	2.67	8.6	2.0	1.0	1.5	8.6	2.0	8.6
H_3BO_3	*61.8*	1.50	6.2	3.0	5.0	1.6	6.2	3.0	6.2
KI	*166.0*	0.75	0.83	0.75	1.0	0.8	-	0.75	0.83
$Na_2MoO_4 \times 2H_2O$	*242.0*	0.1	0.25	0.25	0.1	-	0.25	0.25	0.25
$CuSO_4$ Xx5H_2O	*249.7*	0.1	0.025	0.025	0.2	-	0.25	0.025	0.025
$CoCl_2 \times 6H_2O$	*237.9*	-	0.025	0.025	0.025	-	-	0.025	0.025
TOTAL (**mg L^{-1}**)		**11.8**	**38.2**	**57.8**	**20.8**	**8.3**	**37.6**	**19.3**	**38.2**
(III) VITAMINS (mg L^{-1})	*Mw*								
Myo-Inositol	*180.2*	-	100.0	100.0	1000.0	-	100.0	20.0	100.0
Tiamin (B_1)	*337.3*	0.1	0.1	10.0	5.0	1.0	1.0	0.4	2.0
Nicotinic acid (B_3)	*123.1*	0.5	0.5	1.0	5.0	0.5	0.5	0.5	1.0
Pyridoxine (B_6)	*169.2*	0.1	0.1	1.0	0.5	0.5	0.5	0.1	0.5
Glycin	*77.1*	-	2.0	-	-	2.0	2.0	7.5	2.0
Glutamine	*146.2*	-	-	-	-	-	-	876.0	-
Asparagine	*132.1*	-	-	-	-	-	-	266.0	-
Arginin	*174.2*	-	-	-	-	-	-	174.0	-
(IV) IRON (mg L^{-1})	*Mw*								
$Fe_2(SO_4)_3$	*400.1*	2.5	-	-	-	-	-	-	-
$FeSO_4 \times 7H_2O$	*278.0*	**27.80**							
EDTA (-Na2)	*373.2*	**37.30**							

References

AA (1985) - Amino Acid containing medium – Müller and Gráfe, 1978, *Mol Gen Genet 161:67-76; and* Toriyama and Hinata *1985, Plant Sci* 41:179-183. *B5 (1968)* – Gamborg *et al*., *Exp Cell Res 50:151-158. Gyulai* G *et al*., *F-medium – Plant Cell Rep 1992 11:266-169. MS (1962)* – Murashige and Skoog, *Physiol Plant 15:473-497. N6 (1975)* – Chu *et al*., *Scientica Sinic. 18:659-668. SH (1972)* – Schenk and Hildebrandt, *Can J Bot. 50:199-204. White (1933)* – White medium; in.: *Protoplasma 19:97-116*; and Hoagland medium – *Proc. Am. Soc. Hort. Sci. 30:288-296. WPM (1980)* – Woody plant medium, Lloyd and McCown, *Com.Proc.Int.Plant Prop. Soc*.,30:421-427. *Semmelweis 1 (1861) Die Ätiologie, der Begriff und die Prophylaxis des Kindbettfiebers. And. Semmelweis's 1862 Open Letter to all Professors of Obstetrics*

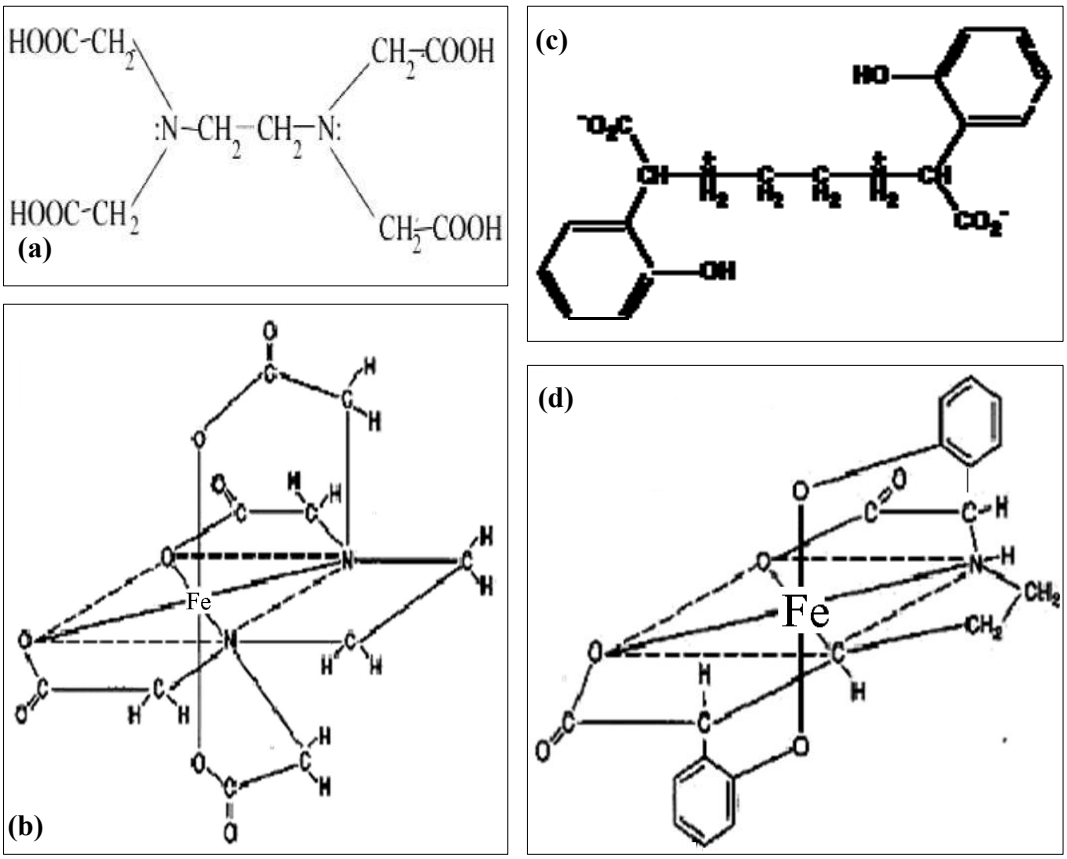


Figure 17. Molecular structures of chelating agents of EDTA *(a)* with Fe^{III}-EDTA *(b);* and EDDHA *(c)* with Fe^{III}-EDDHA *(d).*

(c) Plant hormones

Major plant hormones (and their functions) are the (1) *Abscisic acid* (closes of stomata; and initiate seed dormancy); (2) *Auxins* (elongate roots and shoots; and regulate *phototropism* – plant turn to light, and *gravitropism* – roots grow downward); (3) *Cytokinins* (promote sprouting of lateral buds, and delay plant aging/senescence); (4) *Ethylene* (ripens of fruits; and accelerates blooming and flowering, and seed germination); (5) *Gibberellins* (initiate seed germination, and sprouting of buds; elongate of shoots, and enlarge fruits) (*Fig.18*).

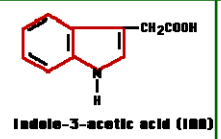
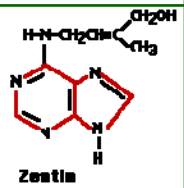
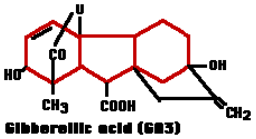
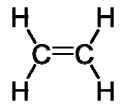
Main groups of *Plant Hormones* and they main physiological functions				
Auxins	Cytokinins	Gibberellins	Abscisic Acids	Ethylene
Rooting	*Shooting*	*Elongating*	*Dorming*	*Ripening*
 Indole-3-acetic acid (IBA)	 Zeatin	 Gibberellic acid (GA3)	 Abscisic acid (ABA)	
			 Leaf/Fruit falling	

Figure 18. The main groups of plant hormones with indications of the main physiological functions, and molecular structures.

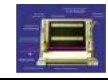
Appendix 1.
Biographic data of Károly EREKY

Reference: M HOFMAN (Belgium), 2002: "Károly EREKY, a famous Hungarian economist, engineer, biotechnologist,and politician":

- Born: 1878.Oct.20, Esztergom, Hungary (Europe)
- Graduated: Technical University of Budapest
- Machinery engineer worked as a mechanical engineer in Vienna
- Research travels (1907-1910) through Europe and USA to study Agriculture: He was extremely satisfied about Danish Agricultural Cooperatives, next to the Belgian and French organizations.
- 1905: Senior lecturer at the Technical University of Budapest
- 1911: Founded of the "Animal Trade Society" (`*Állatértékesítő Egyesület*`)
- 1912: Developed and launched a huge pig farm at Nagytétény, suburb of Budapest (about 15 km South of Budapest), (Dr. G Gyulai was grown up in that region, and the people there were very proud of this pig farm that was the` biggest of Central Europe`, even if they were 'less pleased by its flies and smell'
- Served 22 months in World War I.
- 1919: Minister of Social Affairs (`Népélelmezési Miniszter`) of the Friedrich's Government
- 1920-22: Senator in the Hungarian Parliament
- Later: retired from politics and was consulting expert of the Hungarian National Bank.
- Later his interest turned to *sugar industry*, and the reconstruction of the Hungarian Agriculture.
- 1952: Killed in kommunist's prison: 1952. June. 17., Budapest, Hungary.

Appendix 2.

The main labwares (glasswares, plasticwares, and equipments) used in labs of biotechnology and genetics

									
Beaker	Mortar With Pestle	Funnel	Plastic Bottle	Glass bottle	Petri Dishes	Micro tubes	PCR tubes	Filter unit	pH Testing Strip
									
Cilinders	Conical flask	Volumetric flask	Syringe	Syringe filter	Magnetc needle	Magnetic stirrer	Measuring pipetts	Rack for PCR tubes	Tray
									
Centrifuge	Microfuge	cf. rotor	Refrigirated centrifuge	Minifuge	Scale	Balance	Microscope	Binocular rmicroscope	Stereo microspcope
									
Pressure cooker	Autoclave (steam sterilizer)	Portable autoclave	Heat sterilizes	Laminar flow hood	Alcohol burner	Gas burner	NanoDrop	ALF Express II	PCR
Scissors	Pruner	Forceps	Scalpel (handle)	Scalpel blade	Blade	Lancet needle	Bead sterilizer	Vacuum desiccator	Pipetts

Plant ammonium (NH_4^+) metabolism

Summary

Most of the plant nutritive elements (molecules and ions) are taken up from the soil as anions and cations of inorganic salts. However, oxygen (O_2) is taken up directly from the atmosphere to run respiration and synthesis of metabolites. The major carbon (C) source has also been fixing as atmospheric CO_2 during the last three billion years through Calvin-cycle (CO_2); nevertheless, vast non-photosynthetic carbon fixation (CO_2/HCO_3^-) also accrues from the soil solution. Nitrogen (N_2) also has two main uptake sources, one is from the N-containing soil inorganic salts as nitrate (NO_3^-) and ammonium (NH_4^+); and the other is from the atmosphere. Plants can not metabolite inert nitrogen gas (N_2), only it's physically generated (*e.g.* by lightings) gaseous NOx forms of NO and NO_2; and the microbiologically fixed ammonium (NH_4^+) converted from N_2. Whatever N-sources enter plants only NH_4^+ can incorporate metabolically, first into amino acids. Next to exogenous ammonium, endogenous ammonium also releases metabolically in the living plant cells, which may lead to tissue damage, if there were no several routes of detoxification / reassimilation reactions catalyzed by enzymes and transporters. This chapter provides an overview of these enzymes based on data collected from Plant Metabolic Network (PMN), Genevestigator and The Arabidopsis Information Resource (TAIR).

1. Introduction

Availability of nitrogen as a plant mineral nutrient is the strongest limiting factor (up to 50%) to plant growth and yield. Ammonia/ammonium (NH_3/NH_4^+), nitrate (NO_3^-), and NOx (NO and NO_2) are the N-sources of plants. Nitrogen fertilizers contain ammonium and nitrate and applied primarily as KNO_3, NH_4NO_3, $(NH_4)_2SO_4$, $(NH_4)_3PO_4$ in solid form or in solution.

Ammonia gas can also be injected into the soil, which rapidly diffuses from the injection point and dissolves in the soil solution as ammonium ion. Urea, the worldwide leading N-fertilizer, also releases ammonia.

Whatever N-sources enter plants only NH_4^+ (*Fig. 1*) can incorporate into amino acids and amides. NO_3^- ion also turns to ammonia through reductions catalyzed by nitrate- and nitrite reductases. Forms of atmospheric nitrogen (N_2) fixed and turned to NH_4^+ by N-fixing bacteria (*e.g. Rhizobium, Frankia, etc.*, *see Chapter 15*), and NOx gases (NO and NO_2) are also taken up by plants (*see Chapter 17*).

As a result of global warming, ammonium will become increasingly important nutrient in the future, because under the globally elevated levels of CO_2 the nitrate assimilation was found to be inhibited in sea algae.

In the opposite, both ammonium and nitrate, are strongly phytotoxic at high concentrations, especially when the rate of the conversion of ammonium into amino acids and amides becomes lower than the rate of its uptake and assimilation. Surprisingly, plant ammonium toxicity was first demonstrated by *Ch. Darwin in 1882*, when described the inhibition of growth of *Euphorbia peplus* by ammonium carbonate.

The question addressed is: is it possible to reduce or eliminate ammonium phytotoxicity? In theory, toxic action of a chemical can be counterbalanced at different levels. First, at the stage of uptake and translocation. At later stages, toxicant can be converted into less toxic or nontoxic derivatives (*i.e.,* detoxification), and/or excreted out of the plant or by compartmentalization (deposition into an organelle where it harmless). There are well-demonstrated examples for all these processes, most of them discovered by studying the selective phytotoxicity of *herbicides* (Fig. 2). In cases of ammonium, several detoxifying pathways were observed including amino acid catabolism, nitrate reduction, phenylpropanoid metabolism, proteolysis, and *photorespiration, etc*. (Fig. 3).

For studying all these ammonium related plant metabolic reactions (Fig. 1) data were collected from Plant Metabolic Network (PMN), Genevestigator and The Arabidopsis Information Resource (TAIR). First, terms of *"ammonia"* and *"ammonium"* were searched in the Compound Search of PMN that listed the reactions and pathways. Then, using the tabulated reactions, the catalyzing enzymes, and the protein-coding genes in *Arabidopsis thaliana* were analyzed. Later, changes of the expressions of the *Arabidopsis* genes under different N-supply and stress were collected from Genevestigator. Finally,

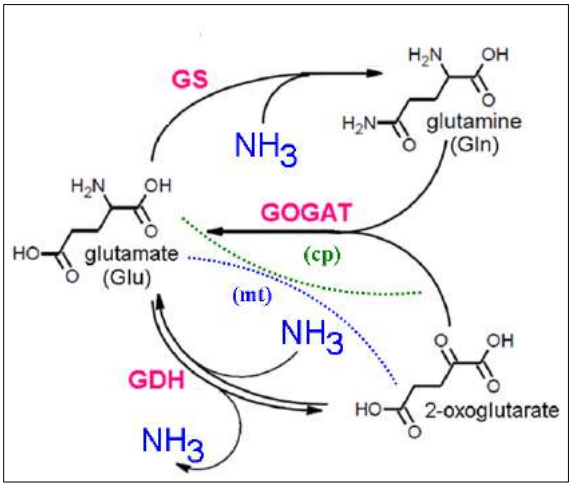


Figure 1. Key pathways of ammonium metabolism in plants. *Transaminase enzymes* of GS (*glutamine synthase*, EC 6.3.1.2; *syn.*: GLUL - *glutamate-ammonia ligase*) and GOGAT (*glutamine oxoglutarate aminotransferase*; EC 1.4.1.13; *syn.*: *glutamate synthase*) localized in the chloroplasts (*cp*); and reversible enzyme GDH (glutamate *dehydrogenase*, EC 1.4.1.3) in the mitochondria (*mt*) are indicated (Kömíves *et al,. 2015*; *Bittsánszky et al., 2015*).

TAIR (The Arabidopsis Information Resource) searched to identify enzymes and transporters in mutants. In total, 92 enzymes were found being coupled to plant ammonia metabolism.

2. Uptake and translocation of exogenous ammonium in plants

Uptake of ammonium from the soil solution and its translocation within the plant tissues are carried out by highly selective plasma membrane-located transporters of the AMT (*AMmonium Transporter*) enzyme family (*Fig. 3*). AMTs are characterized by the presence of a conserved hydrophobic molecular pore through which ammonium moves. Cellular ammonium concentrations depend on the *phosphorylation* levels of AMTs. This reaction protects plants against ammonium toxicity. External, and not internal, ammonium levels as low as 50 µM were found to trigger the *phosphorylation* of *At*AMT1.1 at the conserved *threonine* amino acid (T_{460}) in the C-terminus. As a result, ammonium uptake stops as soon as its cellular level reaches the optimum

The expression of AMT gene (*amt*1) of *Arabidopsis* was shown to be down-regulated by glutamine, rather than ammonium itself, which result indicates a feedback regulation, for glutamine is the first metabolite of *ammonium assimilation* (Fig. 1). Further regulation of ammonium uptake may involve the proteolysis of the transporters.

The discovery that bacterial and fungal transporters act as receptors as well, the term was coined *transceptors*. *At*AMT1;3, which carries a phosphomimic residue in its C terminus, is a candidate of plant ammonium *transceptors* for it has the ability to regulate lateral root branching in response to localized ammonium supply. Ammonium-induced plastid-derived retrograde signaling (chloroplast to nucleus) was also detected.

3. Biochemical processes leading to the production vs. consumption of ammonium

Ammonium production and consumption are catalyzed by *oxidoreductases, transferases, hydrolases, C-N bond lyases* and *ligases*, and *proteases* identified here (Plant Metabolic Network).

Photorespiration, coupled to *Calvin cycle*, is considered the major route of metabolic ammonium production in C3 plants (see *Chapter 14*.) when the oxidative decarboxilation of glycine to serine releases NH_3. The production of the photorespiratory

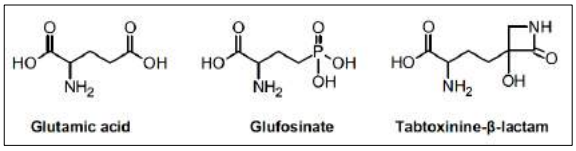


Figure 2a. Chemical structures of amino acid *glutamic acid*, herbicide *glufosinate*, and the bacterial toxin *tabtoxinine-β-lactam* (*Komíves et al., 2016*).

ammonium exceeded by about 10-fold of the primary nitrate reduction in the vegetative leaves of nonleguminous C3 plants.

Eexcess of ammonia generated by *photophosphorylation* was found excreted through the leaves as a volatile NH_3 gas in rice (*Oryza*).

Large amount of ammonium is also producing during protein hydrolysis especially in the senescing leaves. All endogenous ammonium should be *reassimilated* immediately to escape toxicity.

Detoxification of ammonium in plants involves the formation of C-N bonds. There are several *anaplerotic* reactions in response to ammonium stress as they can provide the necessary C for the assimilation of N.

Ammonium conjugation to amino acid *glutamate* (Glu) to form *glutamine* (Gln) and the synthesis of glutamate from 2-oxoglutaric acid (2oG) are considered, not only the main way of ammonia assimilation, but the critical pathways to protect cells from ammonium toxicity (*Fig. 1*). These processes are catalyzed by enzymes of GS (*glutamine synthase*, EC 6.3.1.2; *syn.*: GLUL - *glutamate-ammonia ligase*), and GOGAT (*glutamine oxoglutarate aminotransferase*; EC 1.4.1.13; *syn.*: *glutamate synthase*) located in chloroplast, and GDH (*glutamate dehydrogenase*, EC 1.4.1.3) in the mitochondria (*Fig. 1, 3*).

The importance of these enzymes in the ammonium detoxification is clearly indicated by the powerful *phytotoxicity* of the herbicide *glufosinate* (2-amino-4-phosphonobutanoic acid) (*Fig. 2a*), the ingredients of *Finale, Liberty Basta, Rely, Ignite*, and *Challenge*. *Glufosinate* (as structural analog of glutamate, is a cosubstrate in the GS-catalyzed reactions) kills plants by inhibiting GS, which results in no ammonia incorporation to glutamate, consequently no glutamine synthesis. Furthermore, less glutamine content will not facilitate *glutamate synthase* catalyzed by GOGAT as well, with the final consequences of a rapid accumulation of ammonium in the cells, which led total plant death. For a protective clue for crops in agriculture, an increased levels of external potassium cation (K^+) was found to decrease ammonium toxicity by stimulating the activities of the GS and also PEPC (*phosphoenolpyruvate carboxylase*; EC 4.1.1.31).

An alternative pathway in the mitochondria is the reversible reaction of ammonia with 2-oG, which also results glutamate synthesis, catalyzed by GDH (*glutamate dehydrogenase*, EC 1.4.1.3) (*Fig. 1*).

Although the most important N-containing endogenous compounds usually are generated from amino acids, there are some notable exceptions requiring ammonium as an intermediate.

There were found several enzymes, which release ammonia during amino acid synthesis: *e.g.,* in the cysteine synthesis (*cystathionine γ-lyase*, 4.4.1.1); in methionine synthesis (*cystathionine γ-synthase*, 2.5.1.48) and methionine metabolism (*cystathionine β-lyase*, 4.4.1.8); in

glutamine synthesis (*glutamate-ammonia ligase*, 6.3.1.2) and *glutamate dehydrogenase* (1.4.1.2, 1.4.1.3, 1.4.1.4, 1.4.1.13, 1.4.1.14); in proline synthesis (*citrullinase*, 3.5.1.20); and in the seleno-amino acid degradation. Further enzymes were also identified in the general amino acid catabolism, like *L-amino acid oxidase* (1.4.3.2); *D-amino-acid oxidase* (1.4.3.3); *monoamine oxidase* (1.4.3.4); *putrescine oxidase* (1.4.3.10); *asparaginase* (3.5.1.1); *L-glutaminase* (3.5.1.2); *amidase* (3.5.1.4); *arginine deiminase* (3.5.3.6); *aspartate ammonia lyase* (4.3.1.1); *D-serine ammonia lyase* (4.3.1.18); *threonine ammonia lyase* (4.3.1.19); *methionine γ-lyase* (4.4.1.11); *D-cysteine desulfhydrase* (4.4.1.15); *Proteases*; enzymes of polyamine synthesis of *N-carbamoylputrescine amidase* (3.5.1.53); and *ornithine cyclodeaminase* (4.3.1.12).

Ammonia monooxygenase (AMO; EC 1.14.99.39) was identified in the prokaryotic ammonia oxidation to nitrite in single enzymatic step of nitrifying bacteria (*e.g.*, *Nitrosomonas*) and *Archaea* (*Fig. 2b*).

Arginase (EC 3.5.3.1, *syn.: arginine amidinase*, *canavanase, L-arginase, arginine transamidinase*) is a manganese-containing enzyme, and catalyzes the reaction of arginine + H_2O → ornithine + urea, which reaction is the final step in the *urea cycle* (*Fig. 4a,b*). Similar to fishes, plants lack the excretion system of *urea cycle.*

Xanthine oxidoreductases (XOR; EC 1.17.3.2), a type of enzyme that generates reactive oxygen species when produce urate. Most of the enzymatic reactions of ammonia metabolism are irreversible; however transaminases (*aminotransferases*) catalyze reversible reactions, including GS, GDH, and probably Fd-GOGAT (*Fd-glutamate synthase*, EC 1.4.1.7) and NADH-GOGAT (*NADH-glutamate synthase*, EC 1.4.1.14). *Histidine ammonia-lyase* (EC 4.3.1.3), which catalyze the elimination of ammonia from L-histidine, was also reported as a reversible reaction.

The multi-ion model of potassium channel showed that the single channel current carried by potassium or ammonium ions is a nonmonotonic function of the mole fraction of these two ions in the same solution. Tests also showed that low-affinity NH_4^+ influx may be mediated by both K^+ and non-selective *cation channels*.

As compared to roots, shoot tissues of *Arabidopsis thaliana* are hypersensitive to exposure to ammonium. Therefore, compartmentalization of ammonium in the vacuoles of root cells could serve as an effective strategy to avoid ammonium toxicity in the more sensitive shoot, when in direct contact with ammonia, however, in *vtc*1-1 and *hsn*1-1 mutants shoots, unlike roots, did not show enhanced sensitivity to NH_4^+. Both observations may connect with the *auxin* transport and gradient from apical buds through shoot to the roots, and the ethylene production in shoots and not in roots

5. Detoxification of ammonium

Although several direct effects of ammonium on cell biochemistry were described, the exact mechanisms responsible for its toxic action have not been fully clarified. Ammonium toxicity typically occurs at high concentrations (mM), or alternatively, when plant cells themselves overproduce ammonium

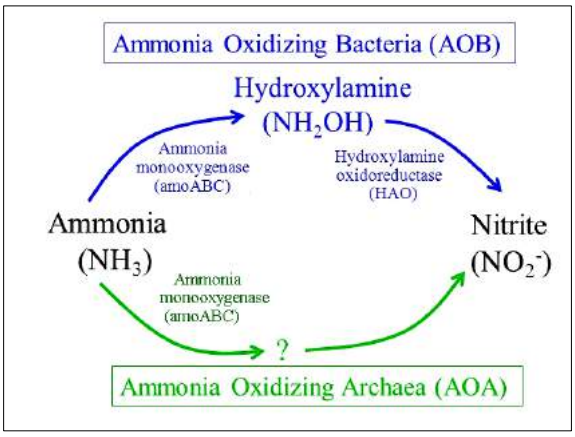


Figure 2b. Metabolic reactions of ommonia-oxydizing Bacteria and *Archaea* with indications of the catalyzing enzymes.

by increased *proteolytic* activity as a result of environmental stresses. Plants can consume ammonia at low (μM) concentrations. To compare, in *in vitro* aseptic conditions, ammonia is applied at 10 – 20 mM concentrations as NH_4NO_3 to the nutritive agar media, however prior to 20 min. autoclaving (*see Chapter 9*).

Mechanisms proposed to explain the toxic action of ammonium to plants are the depletion of carbon supply, damaged chloroplast ultrastructure, deficiency of mineral cations, disruptions in hormonal homeostasis and in photosynthesis, energy-demanding, futile transmembrane ammonium cycling, increased proton efflux, oxidative stress, shifting the cellular pH to intolerable levels, and uncoupling of photo- and oxidative *phosphorylation*.

Exogenously applied NH_4Cl is able to 'uncouple' electron transport chains from the ATP synthesis (no oxidative- and *photophosphorylation*) by abolishing the proton gradient in the chloroplast and mitochondria.

4. Compartmentalization

In plant cells, several toxic metabolites and pollutants are converted to non-toxic derivatives (*e.g.,* phytochelatin complexes and conjugates with glutathione or sugar), which are *sequestered* into the vacuoles catalyzed by membrane bound ATP-driven pumps. Since the pH in the plant cell vacuoles are more acidic than in the cytosol, the vacuole may contain about 100-fold higher ammonium concentrations without the necessity of an active transport. A similar mechanism was detected in barley roots which are capable to actively extrude ammonium in order to reduce its cellular concentration. A respiratory increase is associated with the extrusion process results in intensive cycling of ammonium across the plasma membrane.

An ammonium-dependent increase of O_2 uptake was found to linked to the up-regulation of ATP-coupling *cytochrome* pathway, which may be related

to the ATP consumption by plasma-membrane H^+-*ATPase*. The importance of this mechanism is supported by the observation that increased external potassium cation (K^+) concentrations can protect ammonia sensitivity in rice (*Oryza*) by diminishing the excessive rates of ammonia flux.

increased the level of cellular ammonium concentrations by inducing protease activity, which is putatively responsible for the degradation of oxidatively damaged proteins. Long-term ammonium nutrition of *Arabidopsis* increased the extrachloroplastic NADPH / $NADP^+$ ratio and the

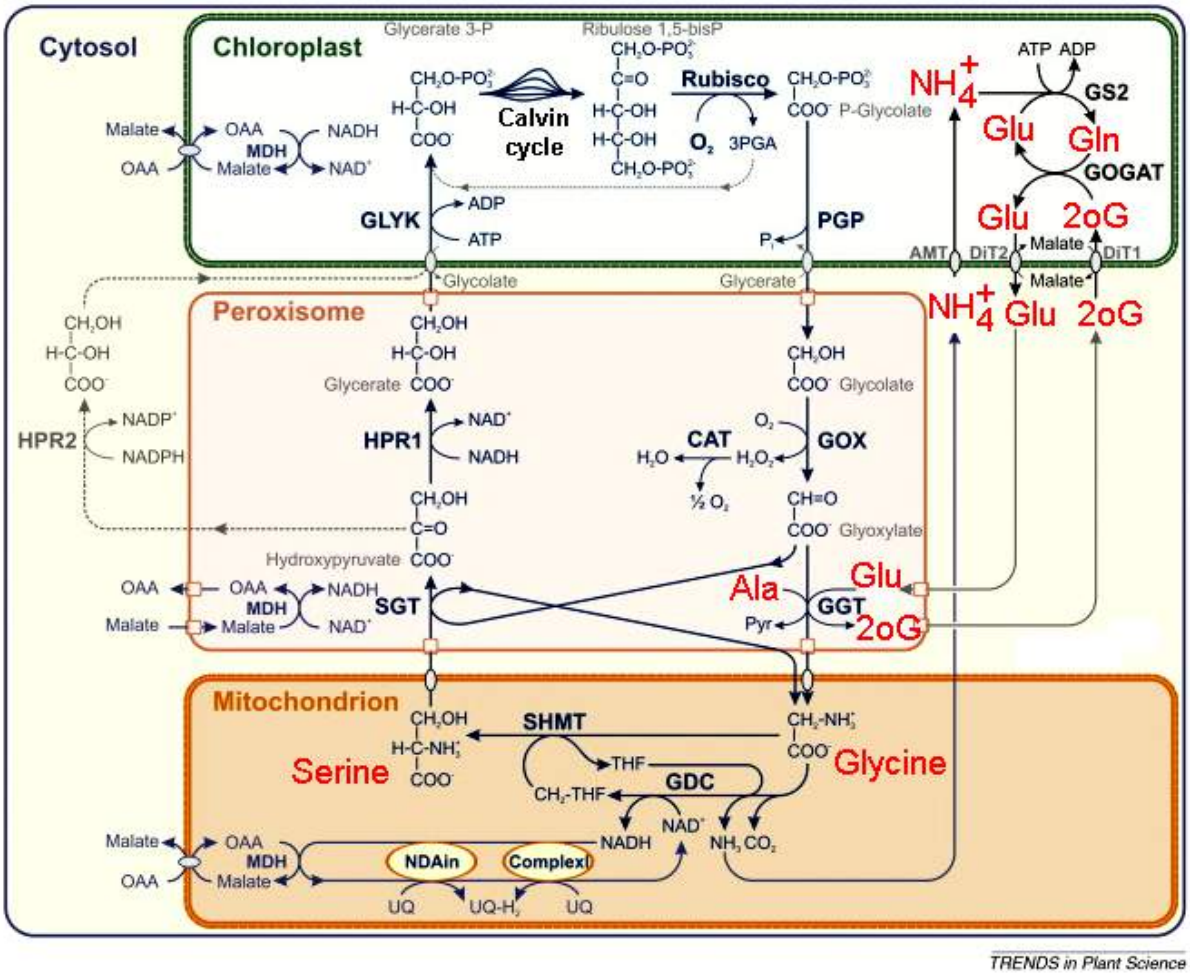


Figure 3. Metabolic pathways of plant photorespiration. Flow-chart of reactions constituting the core reaction of the *C_2 cycle* (*blue*), the photorespiratory nitrogen cycle (*black*), and several associated reactions (*grey* and *light blue*). The enzymes involved are CAT - *catalase*; ComplexI - *NADH:ubiquinone reductase* of the mitochondrial electron transport chain; GDC - *glycine decarboxylase*; GLYK - *glycerate 3-kinase*; GOGAT - *ferredoxin-dependent glutamate synthase*; GOX - *glycolate oxidase*; GGT - *glutamate:glyoxylate aminotransferase*; GS - *glutamine synthetase*; HPR1 - *peroxisomal hydroxypyruvate reductase*; HPR2 - *cytosolic hydroxypyruvate reductase*; MDH - *malate dehydrogenase*; NDAin - *internal NADH:ubiquinone reductase*; PGP - *2PG phosphatase*; SGT - *serine:glyoxylate aminotransferase*; and SHMT - *serine hydroxymethyltransferase*. N-containing substances, *Calvin-cycle* reactions (with multiple arrows from *3PGA* to *RubP*) are indicated. (*Bauwe et al., 2010. Photorespiration: players, partners and origin. Trends in Plant Sci 5:330–336).*

6. Detoxification of reactive oxygen species induced by ammonium

Stunted root growth, and a not iron-deplated leaf chlorosis are the main phenotypic markers of NH_4^+ toxicity in plants.

Millimolar concentrations of ammonium were found to induce oxidative stress response reactions in leaf tissues of the aquatic plant *Myriophyllum mattogrossense*. A 24-hour treatment of tobacco (*Nicotiana*) with ammonium elicited the generation of reactive oxygen species. Interestingly, oxidative stress itself generated by hydrogen peroxide level of the mitochondrial reactive oxygen species in leaves but did not impaired with photosynthetic capacity (*Podgorska et al., 2013*).

7. Ammonium production under environmental stress

Ammonium accumulates in foliar tissues exposed to biotic (*e.g.,* nematode infection) and abiotic (*e.g.,* water and salinity) stresses, which observations indicate the possibility of an ammonium bioassay for monitoring environmentally induced stresses in plants. High level of irradiation

increased ammonia tolerance in *Pisum sativum*. Inner content of plant ammonium showed increment when ammonium concentration increased in the outside, which result indicates an ammonium efflux at high external concentration, which wastes metabolic energy of the plant (*Britto and Kronzucker, 2001*).

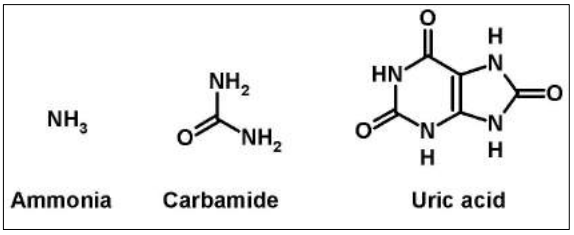


Figure 4a. Animals excrete *excess nitrogen* arising from metabolic breakdown of amino acids in one of three ways: (a) fishes and all aquatic animals excrete ammonia; (b) most terrestrial vertebrates excrete urea, syn.: Carbamide (a bound from of ammonia); and (c) birds and terrestrial reptiles excrete uric acid (*Komives, Gyulai et al., 2016).*

The stress-induced production of ammonium is controversial for ammonium which may be the stressor and may be the part of defense mechanism, which induces enzyme activities of protease, *phenylalanine ammonia lyase* (PAL, EC 4.3.1.24) (*Tanaka et al., 1989*); and *asparagine synthetase* isozymes.

PAL catalyzes the non-oxidative *deamination* of L-phenylalanine yielding ammonia and producing *trans-cinnamic acid* (*Fig. 5*), which is the precursor of N-containing secondary metabolites (*lignin, alkaloids, amides, etc.*) that were found to accumulated in the stressed plants. In cases of biotic

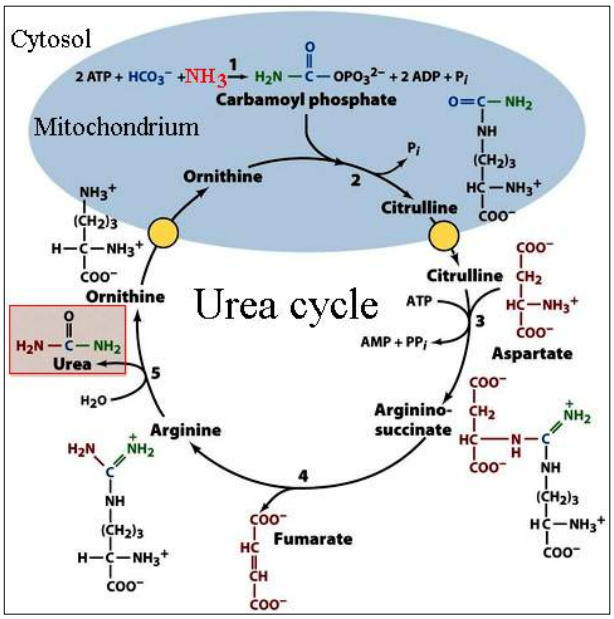


Figure 4b. The synthesis of urea in the Urea cycle of terrestrial animals (the enzymatic reactions are numbered).

stress, *At*AMT1.1 was found as a negative regulator

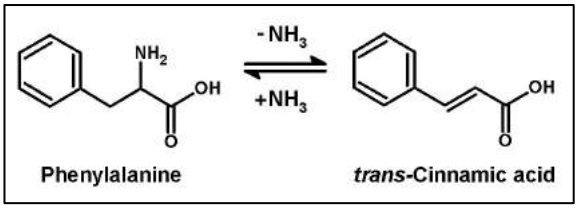


Figure 5. The catalytic activity of the enzyme PAL (*Phenylalanine Ammonia Lyase*) that converts phenylalanine to *trans-Cinnamic acid* (which is the precursor of *lignin*, and *alkaloids*), and release ammonia *(Komives et al., 2016).*

in plant defenses against pathogens.

Plant pathogens also generate ammonium stress. The necrotrophic tomato pathogen *Colletotrichum coccodes* secrete ammonium into the fruit tissues during ripening, and induces host programmed cell death. Ammonium initiated programmed cell death was also observed, yeast, and mammals.

Plant-microbe interactions can also interact with ammonium toxicity. Numerous plant species live in symbiosis with bacterial and fungal microorganism. Roles of *Rhizobium* bacteria and *mycorrhiza fungi* in plant nitrogen uptake have been extensively studied, however very little information is available how these microorganisms influence plant ammonium toxicity. Symbiotic plant hemoglobines (*symHbs*) found in the root nodules carry oxygen to the N-fixing bacteria ('*legHbs*'), and may also contribute to the ammonia transportation (Seregélyes *et al.,* 2000).

Plants infected by *Agrobacterium tumefaciens* and *A. rhizogenes* highly express the ammonium-producing enzyme ornithine cyclodeaminase (EC 4.3.1.12). *Pseudomonas syringae* pv. *tabaci* produces *tabtoxinine-ß-lactam*, a potent inhibitor of the enzyme GS (*glutamine synthase*, EC 6.3.1.2; *syn.*: GLUL - *glutamate-ammonia ligase*) (*Fig. 1*), which leads to the accumulation of ammonium at phytotoxic concentrations.

8. Selection and Breeding for ammonium tolerance

A combination of physiological and genomic analysis (including structural and functional genomics, proteomics and metabolomics) leads to the optimization of the traits in new crops. Marker assisted breeding and selection for ammonium tolerance is viable option for selection enzyme mutants with ammonium tolerance. Enzymes linked to glutamate synthesis (GOGAT) were found to be upregulated by the effect of ammonia either to short or long exposure.

Rice cultivar-dependent volatile gas ammonia excretion generated by photophosphorylation, showed correlation with GS activities of the cultivars, which indicates the possibility to select new crop cultivars with high GS activity. Some mutant plants were found slightly more resistant to *methylammonium* than the wild type. Methylammonium, a non metabolizing structural analogue of ammonium used for studying endogenous ammonia transport, was used to select resistant plants (*mea*R).

NRT1.1 mutants showed enhanced tolerance to concentrated ammonium as sole N-source in *Arabidopsis*.

As the opposite, *Arabidopsis* with reduced ammonium transporter activity usually show normal growth (TAIR germplasm names: AMT1;1-1, CS6168, AMT1;2-1, AMT1;2-2, AMT1;3-1, AMT2;1-1), and the ascorbic acid-deficient *Arabidopsis thaliana* mutant (*vtc*1-1), which is defective for *GDP-mannose pyrophosphorylase* (GMPase), showed conditional hypersensitivity to ammonium.

Morphological responses of *Arabidopsis* mutants lacking the gene of the ammonium producing enzyme *S-(hydroxymethyl)glutathione dehydrogenase* (EC: 1.1.1.284; TAIR germplasm names: FUG1-2. CS66011, CS66011, CS66012, CS67832, CS67833, FLAG_298F11, GABI_315D11) were found sensitive to heat stress, and showed reduced fertility, short stamens and shriveled small petals.

Arabidopsis plants deficient in *glutamate dehydrogenase* (GLDH; EC: 1.4.1.4; TAIR germplasms: CS8606, CS8611, CS8612, CS6376) show *chlorosis* and reduced growth (carbon dioxide restores normal growth and appearance), and *glutamate synthase* (GOGAT) activity is also reduced in these mutants.

Plants with genetically reduced *glutamate synthase* (GOGAT) activity (EC 1.4.7.1) show similar phenotype (TAIR germplasms: CS6376, CS8606, CS8611, CS8612), which underline the close functional relations between these enzymes.

Arabidopsis mutants for *formyltetrahydrofolate deformylase* (EC: 3.5.1.10) (TAIR germplasm: CS860079) are small, pale, delayed in flowering, infertile, the mature seeds are shriveled and carrying colorless embryos with low amounts of accumulated lipids, and fail to germinate.

Plants lacking serine *endopeptidases* activity (EC 3.4.21) (TAIR germplasms: SALK_004770C, SALK_099162) are susceptible to high light intensity.

Ammonium sensitivity of plant species living in sea water, fresh water and terrestrial ecosystems are different, and can indicate evolutionary lineages by sequence analysis of ammonium related proteins and genes. AMTs of a gymnosperm *Pinus pinaster,* two monocots (*Os – Oryza sativa*, *Zm – Zea mays*); and three dicots of *Cruciferae* (*At – Arabidopsis thaliana*), *Solanaceae* (*Le – Solanum lycopersicum*) and *Rosids* (*Malus hupehensis*) revealed two main clades (Fig. 6). AMTs of tomato (*Le*), and maize (*Zm*) clustered in a single clade, and AMTs of all the other species were found in both. *At*AMT1.3, -1.5, and -1.1 showed the closest sequence similarity, which may predict shared functional similarity in transceptor functions (Fig. 6).

The approach of using genetically modified (GM) plants is more promising. Transgenic plants expressing the bacterial GDH (*glutamate dehydrogenase*) enzyme showed increased tolerance to drought. GM plants with either overexpressed enzymes responsible for ammonium assimilation and detoxification, or with silenced enzyme gene technology may result in immediate ammonium tolerance.

9. Conclusions

Plant ammonium metabolism including sensing, uptake, short and long-distance transport, assimilation and detoxification, and exudation by the roots and leaves, are equally important enzymatic reactions. Detailed research is necessary to clarify genetic regulations of all these steps to improve ammonium-uptake to increasing yield and to escape metabolic ammonium loss and toxicity.

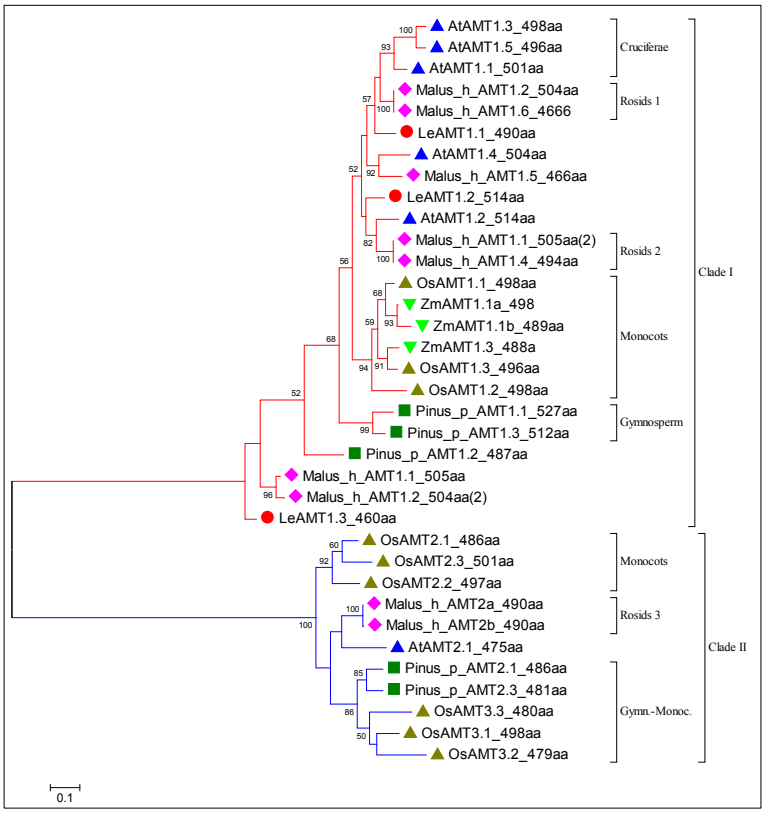


Figure 6. In silico protein phylogram (ML – maximum likelihood; *Hillis et al., 1994*) of plant AMTs (*ammonium transporters* of membrane channel proteins) edited by MEGA5 program (*Tamura et al., 2004*). Plant species are indicated from gymnosperm *Pinus pinaster,* through monocots (*Os – Oryza sativa*, *Zm – Zea mays*), to dicots of *Cruciferae* (*At – Arabidopsis thaliana*), *Solanaceae* (*Le – Solanum lycopersicum*) and Rosids (*Malus hupehensis*). Substitution rate (aa per site, scale 0.1), and bootsrap values (% of the 1000 times computer running replicates) are indicated at the branch nodes (sequences were downloaded from websites of *http://aramemnon.botanik.uni-koeln.de/, and http://www.uniprot.org*) (*Gyulai et al., 2016*).

Reference (for all paper cited).

Bittsánszky A, K Pilinszky, G Gyulai, T Komives (2015) Overcoming ammonium toxicity. *Plant Sci* 231:184-190.

Chemical plant protection

Moore's Law: the number of transistors in a dense integrated circuit doubles approximately every two years (Moore, 1965).

Eroom's Law (spelled as Moore's Law backwards): drug discovery is slowing down and the cost of developing a new drug doubles every nine years (Scanwell *et al.*, 2012).

(1) Insecticide DDT

After the discovery (1938) and huge commercial and popular success of the *insecticide* DDT (*DichloroDiphenylTrichloroethane*) (*Fig. A*), (Nobel Prize for its discoverer *Paul Herman Müller* in 1948) a number of chemical companies started to develop new pesticide active ingredients. The demand for such chemicals was high: they were inexpensive and highly efficient replacements for earlier labor-intensive crop protection practices. As a result, the number of newly developed pesticides increased continuously and their use spread out to almost all areas of human activities from household crop production to households, and forestry, *etc*.

This upward curve was broken suddenly by an alarming number of reports published on the negative effects of pesticides on human health and the environment (summarized by *Rachel Carson* in her exceptionally influential book "*Silent Spring*") (*Carson, 1962*). As a consequence, DDT was banned (*first in Hungary, 1968*) and increasingly higher standards of pest control ability and environmental toxicity were demanded by the regulatory agencies for the registration and use of pesticides.

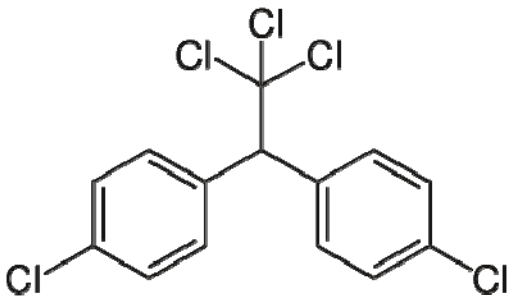


Figure A. The molecular structure of DDT

Pesticide research and development (R&D) has been at the crossroads ever since then. Pesticide producers had to realize that commercial success is more and more difficult to achieve and the R&D methods used earlier (based on trial and error: testing random chemicals for phenotypic pesticidal effects) are not satisfactory any longer. In a bold move, the agrochemical giant *Monsanto* shifted away from the chemical approach in 1981 and set *biotechnology* as the company's strategic research focus. At the same time, for the companies that remained in the agrochemical arena, R&D costs of new pesticide active ingredients increased dramatically (similarly to pharmaceuticals: *cf. Eroom's Law*), and the success rate to find a marketable one dropped sharply. Companies responded by increasing their R&D expenditures, introduced *combinatorial chemistry* to generate a larger number of test compounds and applied high-throughput screening to detect *phenotypic responses*.

In the practice of plant protection, pesticides remained indispensable but more and more efforts were taken to apply them in the framework of integrated pest management (IPM). IPM was formulated in 1972 on the prevention, monitoring and rapid identification of the pest(s) and disease(s) and their effective control by a coordinated use of all accessible technologies. IPM requires a systematic knowledge on crop biology and the biology of their pests and diseases, as well the existing plant protection procedures (*Owen et al., 2015*).

In pesticide R&D the phenotype approach changed with the accumulation of knowledge in *biochemistry* and *molecular biology*, and research shifted toward a target-based one in order to take advantage of various new technologies, such as *next-generation sequencing*, *transcriptomics*, *metabolomics*, and *proteomics*. These methods are known to generate a huge number of data ("big data"), the interpretation of which is a new challenge for the science.

Up until very recently, mining of the "big data" generated by the so-called *"-omics"* methods did not lead to breathtaking results: the number of new pesticide active ingredients has been declining continuously. A recent announcement by *Syngenta,* the largest pesticide manufacturer in the world, indicates that this evident trend may change in the near future: the company has *ca.* 50 new crop protection innovations in the U.S. pipeline, including 19 new active ingredients (*Syngenta, News Release on March 30, 2016*). These numbers are remarkably high, well in the range in the "golden era" of the industry - even if only a fraction of the new active ingredients in the pipeline will actually reach commercialization. Still, it is very worrisome that discovery of compounds characterized with a new *biochemical mode of action* has been extremely rare during the last decades.

Nevertheless, the current R&D approach served well the largest chemical companies: they managed to increase their market share by mergers and acquisitions (*cf.* the merger of *Dow Chemical* and *DuPont* as well as *Monsanto*'s and *ChemChina*'s recent multibillion dollar bids to take over *Syngenta*) (*Kaskey, 2015*).

Other negative consequence of the current prevalence of *Eroom's Law* in pesticide R&D is that today generic products account for 70% of the pesticide market share. Needless to say, this situation is detrimental in many ways: it hurts not only the producers, but also the users who have to deal with the reoccurrence of *pest resistance*, and even the environment suffers from the accumulation of overused old compounds.

Now, is there a "chemical" way out? There are two interesting paths to be explored.

(1) Designing pesticides that fit in the concept of "*Ecological plant protection*" (*EPP, coined in 1957*:

Nagy, 2008) – an almost 60 years old idea, preceding IPM with more than a decade. EPP is a close analogy to the recently introduced "*Systems biology*" approach in the R&D and practical use of pharmaceuticals. Unfortunately, agro-ecosystems are so complex, that there are no "big data" systems developed yet for their description. Still, the combination of the continuously increasing capacity of data collection (and evaluation, *cf.* Moore's Law) with target site ("*-omics*") pesticide R&D may bring us pleasant surprises in the near future.

(2) We should be aware of the "-omics" technologies opening new insights into the chemical kingdom of nature: plants and microorganisms produce hundreds of thousands of metabolites, many of them "designed" to be *bioactive* (*e.g.*, for signal and defense). These may be major sources for new pesticides.

In addition, it is interesting to speculate on how the newly developed gene editing technologies, *e.g.,* the CRISPR (*Clustered Regularly Interspaced Short Palindromic Repeats*) – Cas (*CRISPR-Associated Protein*; *i.e.*, enzyme, which is an *RNA-guided DNA endonuclease*) systems (*Fig. B*) can serve the chemical plant protection by (1) reversing pesticide resistance in insects and weeds (*n.b.*, this may change agro-ecosystems), and (2) increasing the production of natural pesticides in plants (in addition to their known possible roles in breeding stress-tolerant and disease-resistant crops) (*Wright et al., 2016*).

In summary, in the light of the most recent developments, 2017 could be a most interesting year for chemical plant protection research and for the pesticide industry.

Appendix (Fig. B)

The CRISP Repeats (CRISPR) are prokaryotic DNAs, and provide an *immune system* that confers resistance to foreign invading genetic elements such as plasmids and phages. Each repeat is followed by short sequence of *spacer* DNA. Spacer sequences *from invading viruses* are incorporated at CRISPR loci within the bacterial genome and serve as a memory of previous infection. Re-infection triggers the complementary mature CRISPR RNA (crRNA) to find a matching sequence – which provides the CRISPR-associated (Cas) nuclease the specificity to form a double-strand break at specific foreign DNA sequences. CRISPR spacers recognize and cut the *exogenous genetic elements* in a manner analogous to *RNA interference* in eukaryotic organisms. CRISPRs are found in approximately 40% of sequenced bacterial genomes and 90% of sequenced archaea.

The recent discovery of the CRISPR/Cas9 complex has provided useful tool to target and modify any genomic sequence with high levels of specificity. The system, consisting of an RNA-guided nuclease (Cas9) and guide RNA (gRNA) complementary to a target sequence, allows for sequence-specific cleavage of target loci across the genome.

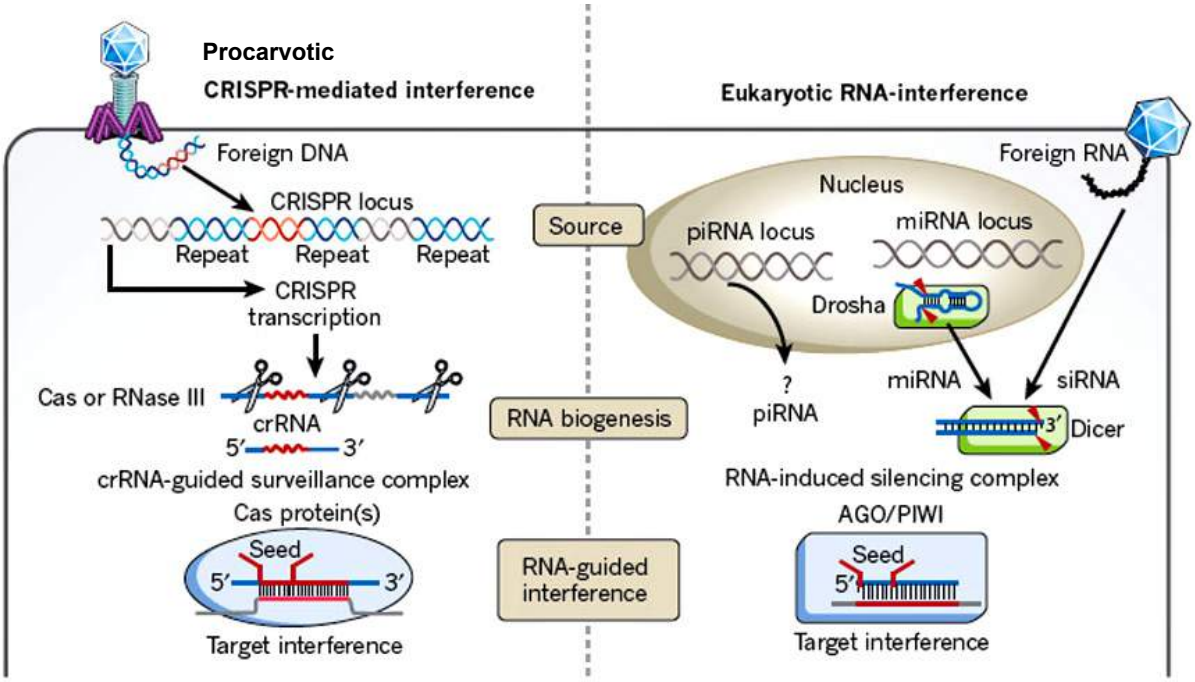


Figure B. Camparison of CRISPR and RNAi. CRISPR systems and RNAi recognize long RNA precursors that are processed into small RNAs, which act as sequence-specific guides for targeting complementary nucleic acids. In CRISPR systems, foreign DNA is integrated into the CRISPR locus, and long transcripts from these loci are processed by a CRISPR-associated (*Cas*) or RNase III family nuclease. The short CRISPR-derived RNAs (*crRNAs*) assemble with Cas proteins into large surveillance complexes that target destruction of invading genetic material. In some eukaryotes, long double-stranded RNAs are recognized as foreign, and a specialized RNase III family endoribonuclease (*Dicer*) cleaves these RNAs into short-interfering RNAs (*siRNAs*) that guide the immune system to *invading RNA viruses*. PIWI-interacting RNAs (*piRNAs*) are transcribed from repetitive clusters in the genome that often contain many copies of *retrotransposons* and primarily act by restricting transposon mobility. The biogenesis of *piRNAs* is not yet fully understood. MicroRNAs (*miRNAs*) are also encoded on the chromosome, and primary miRNA transcripts form stable hairpin structures that are sequentially processed (shown by red triangles) by two RNase III family endoribonucleases (*Drosha* and *Dicer*). miRNAs do not participate in genome defense but are major regulators of endogenous gene expression. Like *crRNAs*, eukaryotic piRNAs, siRNAs and miRNAs associate with proteins that facilitate complementary interactions with invading nucleic acid targets. In eukaryotes, the *Argonaute* proteins pre-order the 5′ region of the guide RNA into a helical configuration, reducing the entropy penalty of interactions with target RNAs. This high-affinity binding site, called the 'seed' sequence, is essential for target sequence interactions. Recent studies indicate that the CRISPR system may use a similar seed-binding mechanism for enhancing target sequence interactions. (*Wiedenheft et al., 2012. Nature 482: 331–338*).

(2) Herbicide Glyphosate, Gluphosinate (syn., Phosphinothricin)

- "In nature nothing exists alone." (*Rachel Carson in "Silent Spring")* (*Carson, 1962*)

- „Alle Dinge sind Gift, und nichts ist ohne Gift; allein die Dosis machts, daß ein Ding kein Gift sei. (All things are poison, and nothing is without poison: the dose alone makes a thing not poison)". Paracelsus in *‚Sieben Verteidigungsreden'* (*von Hohenheim, 1538*)

(a) Brief history

Glyphosate (N-[PhosphonoMethyl]Glycine), as a *glycine analogue,* is a widely applied *synthetic, non-selective, systemic herbicide* (*JE Franz, 1970*) has became the most controversial *herbicide* ever used.

Glyphosate is an *aminophosphonic analogue* of the *natural amino* acid *glycine*, and like all amino acids, exists in different *ionic states depending on pH*, both the *phosphonic* acid and carboxylic acid moieties can be *ionised* and the *amine* group can be *protonated* and the substance exists as a series of *zwitterions* (*i.e.,* dipolar ion) (*viz.*, *gly-phos-ate* - ***gly***cine and a ***phos***phon***ate***). The molecule is very simple, and shows rather unique *chemical structure* (*Fig. 1a*). Unlike most of other *herbicides*, it is highly *water soluble* and, and depending on the soil properties, it may be very strongly bound to *soil matrices*.

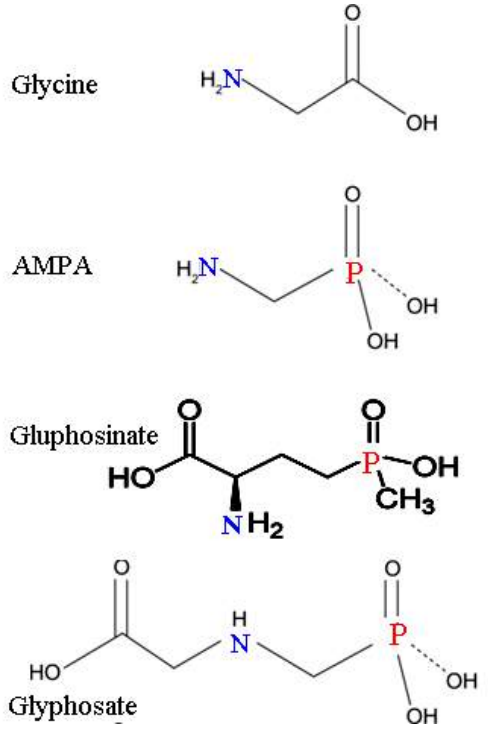


Figure 1a. Chemical structure of *glyphosat*e compared to amino acid *glycine*, AMPA (*AminoMethylPhosphonic Acid*), and *gluphosinate* (see *Fig. 5*)

The chemical structure (that was named as *glyphosate* decades later) was first synthesized in 1950 at the *Swiss company CILAG* (*Franz et al., 1997*). About ten years later the herbicide manufacturer *Stauffer Chemical Company* patented it as a *chelator* (*complexing agent* for metals such as magnesium, copper, and zinc) (*Fon and Uhing, 1964*). To their later deep regret, researchers of the company missed to identify the molecule as a potential *herbicide* because of the short duration of the company's standardized biological assays (only five days, while the first, glyphosate-induced phytotoxic symptoms usually appear after about one week) (*FM Pallos*, personal communication in 1982).

It took another ten years until the molecule's herbicidal properties were discovered at *Monsanto Chemical Company* (*Irani, 1969*). The molecule was patented, obtained "*glyphosate*" as the common name, and the production of the first glyphosate-based commercial herbicide ("*RoundUp*") began in 1974 (*Székács and Darvas, 2012*). In 2002, *glyphosate* was additionally patented by *Monsanto* as an antibiotic (*Abraham, 2010*).

At first, as a non-selective, so-called *total herbicide*, *glyphosate* had a narrow market, although its unique properties (low price, low mammalian toxicity, high efficacy due to a unique systemic action *via* basipetal and acropetal translocation) were rapidly acknowledged. *Glyphosate* linked to the *phytotoxic activity* when contacted with the soil attributed to rapid degradation of the active ingredient to *non-phytotoxic metabolites*, but later explained by the binding of *glyphosate* molecules to soil particles (*Duke et al., 2012*).

Unlike *glyphosate, glufosinate* (*Fig. 1a*) (*syn.*, *phosphinothricin*) is a naturally-occurring broad-spectrum systemic *herbicide* produced by *Streptomyces* soil bacteria (and later synthesized first *by scientists at Hoechst in the 1970*s).

For many years, *glyphosate* has been considered as a 'virtually ideal' herbicide (*Duke and Powles, 2008*). *Nota bene*, as it turned out later, the mechanism of binding of *glyphosate* to soil particles is very similar to that of *phosphate*. Thus, as a result of a *displacement reaction*, the leaching of soil-bound *glyphosate* to the groundwater is increased by phosphate fertilizers (*Munira et al., 2016*).

(b) GM crops

The real breakthrough for *glyphosate* came only with the nenefits of the *genetically modified (GM)* crops: a field in which *Monsanto* pioneered (*in: Kőmies, 2016*). *Glyphosate resistance* of *transgenic crops* (GM) was achieved by introducing non-sensitive *EPSPS* enzyme gene (from *Agrobacterium*) (*Pollegioni et al., 2011*), which was resistant to *glyphosate* inhibition. (*Agrobacterium* strain called *CP4* was found surviving in a waste-fed column at a *glyphosate* production facility. This *CP4 EPSPS* gene was cloned and *transfected* into soybeans. In 1996, genetically modified soybeans were made commercially available. Current glyphosate-resistant crops include *soy* ("*RoundUp Ready*"), *maize,*

canola, alfalfa, sugar beets, and *cotton*, with *wheat* still under development.

Glyphosate-resistant GM crops have been in production for twenty years by now, and their planting area is still increasing – making *glyphosate* the most-used pesticide in the world with a predicted annual sale of 1.35 million metric tons in 2017 (*Newman et al., 2016*).

However, the use of an glyphosate-insensitive *EPSPS* implies that *glyphosate* itself remains unmetabolized in the tissues of resistant plants – in contrast to *glufosinate-tolerant GM plants*, in which the resistance is due to their ability to chemically modify (by the introduced bacterial enzyme gene GAT (*Fig. 1b*), which made it much less toxic to plants and to the environment (*Muller et al., 2001*).

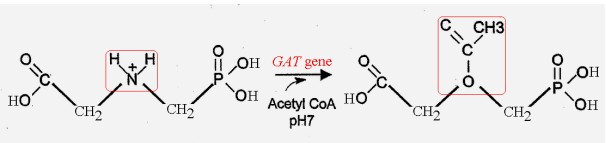


Figure 1b. The function of the prokaryotic GAT enzyme gene (*Glyphosate-N-AcetylTransferase*) in the *deactivation* of herbicide *glyphosate* by acetilation

(c) Biological 'mode of action' of glyphosate

Jaworski (1972) suggested that the *phytotoxic action of glyphosate* is due to an interference with the biosynthesis of *L-phenylalanine* (*Fig, 2*) and, in particular, with the metabolism of *chorismic acid* (*Fig. 2*), the key metabolite in the biosynthesis of the so-called *aromatic amino* acids (*L-phenylalanine, L-tyrosine*, and *L-tryptophan*; *Fig. 2*). An inhibition of the formation of *aromatic amino acids* certainly leads to toxic symptoms, since it leads to a halt of protein *de-novo* synthesis of the production of the natural plant growth hormone *indole-3-acetic acid* (a derivative of amino acid *L-tryptophan*).

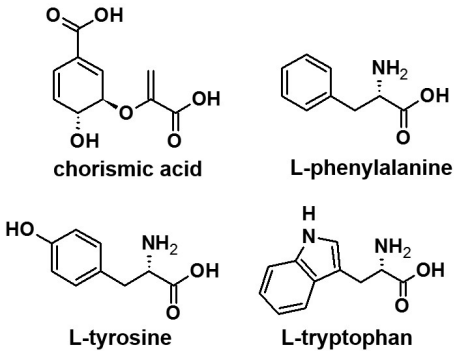


Figure 2. Chemical structures of *chorismic acid* and the aromatic amino acids *L-phenylalanine*, *L-tyrosine*, *and L-tryptophan*

An alternative mode of action was suggested by *Duke and Hoagland (1978)*, as they found an elevated levels of *phenolic* secondary metabolites and also of the key enzyme of phenolic biosynthesis *Phenyllanine Ammonia-Lyase, PAL* (# EC 4.3.1.5) in the roots of dark-grown, glyphosate-treated maize seedlings. They concluded that the *phytotoxic action* of *glyphosate* might be due to a stimulation of the biosynthesis of growth-inhibiting *phenolic*s (*Duke and Hoagland, 1978*).

The subsequent lack of *aromatic amino acids* in *glyphosate*-treated plants with time (but not immediately, the results in *pleiotropic effects* on the physiology of plants were observed in reduced photosynthesis, growth retardation, oxidative stress, *etc.* (*Gomes et al., 2014*).

The proper biochemical mechanism of action of *glyphosate* was identified by *Steinrücken and Amrhein (1980)* by identifying the specific inhibition of *5-enolPyruvylShikimate-3-phosphate synthasee (EPSPS)* (#EC 2.5.1.19; *Fig. 3a,b*), by confirming the first findings of *Jaworski (1972)*.

It was also suggested that part of the *phytotoxic effects of glyphosate* may be due to the accumulation of *shikimic acid* and *shikimic acid-3-phosphate* (*Fig. 3a,b*) as a result of the inhibition of *EPSP synthase* (*de Maria et al., 2006*).

(d) Effects of the secondary metabolism on plants

Aromatic amino acids are precursors of a large number of chemicals of plants synthesized. These chemicals are called *secondary metabolites* and they have important functions in plant life affecting responses to *biotic and abiotic environmental stresses*, interactions with other (beneficial or harmful) organisms, and normal growth and development (*Vogt, 2010*). However, deriving from *chorismate*, a key metabolite originated from the *shikimic acid cycle* (*Fig. 4*), it multiples other metabolic pathways can also be influenced by *glyphosate*.

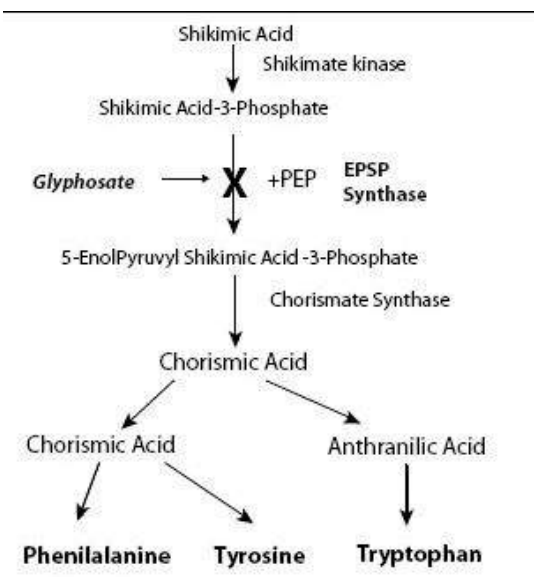


Figure 3a. Phytotoxic mechanism of action of *glyphosate* by inhibiting the enzyme *EPSP synthase* in the synthesis of the *aromatic amino acids* (*Phe*, *Tyr*, *Try*) (*see Fig.3b*)

Early mode of action studies suggested an induction of the *phenolic* biosynthesis. In contrast,

later investing the anions confirmed that synthesis of phenolic compounds originated from *phenylalanine* was blocked by *glyphosate* in plants (*Carbonari et al., 2014*).

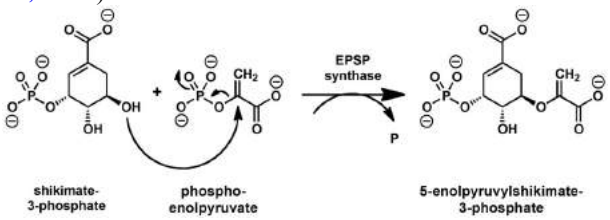


Figure 3b. *Glyphosate* kills plants by inhibiting the enzyme *5-EnolPyruvylShikimate-3-Phosphate Synthase (EPSPS)*, which catalyzes the reaction of *shikimate-3-phosphate (S3P)* and *phosphoenolpyruvate* to form *5-enolpyruvyl-shikimate-3-phosphate (EPSP)* (*see Fig.3a*)

It was also proved that the levels of some phenolic compounds (*e.g., hydroxybenzoic acids*) not directly synthesized by the *shikimic acid pathway* were induced by *glyphosate* in several plant species. The authors concluded that *hydroxybenzoic acids* can overflows metabolites of the *shikimic acid pathway.* (*Lydon and Duke, 1988*).

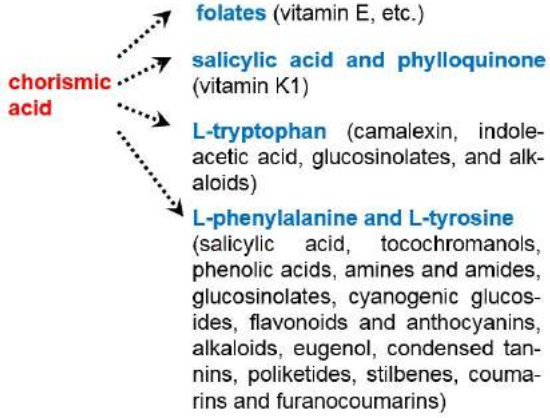


Figure 4. Synthetic routes of the major classes of plant metabolites derived from *chorismic acid*

A recent study reported a response of *adventitious root* cultures of *Echinacea purpurea* to *glyphosate*-induced starvation as g*lyphosate* reduced the tissue growth and the levels of *phenolic* secondary metabolites.

Feeding of *L-tryptophan* and, to a less extent *L-phenylalanine* counteracted the phytotoxic effects of the herbicide and led to the accumulation of higher levels of secondary metabolites (*Mobin et al., 2015*). The authors concluded that in *Echinacea purpurea* plants the *phytotoxicity of glyphosate* affects the *indolic pathway* significantly stronger than the *phenolic pathway*.

The main building blocks of *lignins* are produced from *L-phenylalanine* via the *phenolic biosynthesis* pathway. Lignins are cross-linked structural polymers in plant tissues. They are important components of *cell walls*, and protect the cells against physical injury and also against *pathogen attack*. *Glyphosate* strongly inhibits *lignification*, making the plants physically weaker and also prone to infections by pathogens (*Zobiole et al., 2010*).

Further studies indicated *a glyphosate*-induced disturbances in the biosynthesis of *glucosinolate phytoalexins (Pedras and Yaya, 2014),* flavonoids (*Margna et al., 1988*), cocaine (*Casale and Lydon, 2007*), and other secondary metabolites derived from *aromatic amino acids* (*Tzin and Galili, 2010*).

(e) Fate in the environment; and side effects

Sprays the leaves with *glyphosate* are rapidly taken up and translocated into the newly developing plant parts (*Klier et al., 2008*), and appreciable amounts are exuded from the roots into the soil solution (*Coupland and Caseley, 1979*). In many soils, *glyphosate* is not readily decomposed and may remain there for a long period of time unchanged, or *chelated* with soil cations.

Saska et al. (2016) found that population parameters of the aphid *Metopolophium dirhodum* were significantly negatively affected by applications of sub-lethal doses of *glyphosate*.

Not surprisingly, the continuous use of very large amounts of *glyphosate* led to environmental problems: first, *glyphosate-resistant weeds* started to appear in the fields (*Nandula, 2010*). At the same time, residues of *glyphosate* and its primary metabolite *aminomethyl-phosphonic acid (AMPA*, *Fig. 5*) were detected in the soil, in freshwater, and in the organisms that live there (*Perez et al., 2011*), in the crop products, and in the animals that were fed with them, and in humans, too. Therefore, studies were initiated to determine whether continuous exposure to *glyphosate* leads to unwanted effects to the environment, such as soil microorganisms (*Kremer and Means, 2009*), insects (*Saska et al., 2016*), fish (*Giannini, 2013*), and to humans. Results of these studies were presented in a number of papers (*Székács and Darvas, 2012; Myers et al., 2016*) and discussed in books (*e.g., Nandula, 2010*).

Most pesticides contain substances in addition to the active ingredient(s) that are referred to as *inerts*, *adjuvants* or *co-formulants*, *e.g.*, increasing the penetration of the active molecule. *Nota bene*, the science of *toxicology* does not know about "*inert*" chemicals: this would be against the law of *Paracelsus* (in: *von Hohenheim, 1538*). Consequently, those *adjuvants* and their *glyphosate* formulations have gone under secrutiny (*Mesnage et al. 2013*), also in conjunction with parallel exposure to *Bacillus thuringiensis* toxins (*Mesnage et al. 2013*). As regards to *glyphosate-based herbicides*, it is still not clear how many or what percentage of its *side effects* described in the scientific literature are due to those "*inert*" components (*Defarge et al., 2016*). However, a certain group of *adjuvants*, the frequently used tallow amines, have been banned recently (*AgriLand, 2016*).

Unfortunately, the results of studies on the side effects of *glyphosate* led to a number of conflicting conclusions. For example, *nephropathy*, *cancer*, *etc.,* was attributed to *glyphosate.*

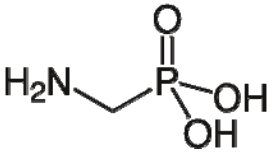


Figure 5. Chemical structure of *AminoMethylPhosphonic Acid (AMPA)*, the breakdown of *glyphosate* which has so far only been detected in *soil bacteria*

Recently, *glyphosate* was shown to induce birth defects in rats (*Schimpf et al., 2016*) and was suggested (without experimental evidence) to produce DNA and epigenetic effects (*Nardemir et al., 2015*), and was suggested to act as the *analogue* of the *amino acid glycine* to replace it in diverse proteins (*Samsel and Seneff, 2016*).

In contrast, *glyphosate* and its *AMPA metabolite* were found to inhibit the proliferation, and promote the *apoptosis* of cancer cells (but not normal ones), leading to a suggestion that they may be lead molecules to new anticancer therapies (*Li et al., 2013*).

(f) Effects on plant-pathogen interactions

Another intriguing subject is the very complex influence of *glyphosate* may exert on plant-pathogen interactions.

First, *glyphosate* has antimicrobial effect *per se* (*Samac and Foster-Hartnett, 2012*).

Second: *glyphosate* may alter the chemical, physical, and biochemical mechanisms plants use to fend off to pathogens. Some of the protection barriers are constitutive, such as the cell walls and wax epidermal cuticles of the leaves. Furthermore, plant cells are capable of detecting attacking pathogens and react with inducible responses, *e.g.*, the production of toxic chemicals (*hydrogen peroxide* and other reactive oxygen species; *e.g., phytoalexins*, *etc.*), pathogen-degrading enzymes, and deliberate cell suicide (*hypersensitive reaction*, HR) (*Barna et al., 2012*).

Glyphosate, by blocking the synthesis of *chorismic acid* (and though the *aromatic amino acids; Fig. 3*) may negatively influence virtually all of the induced defense plant responses. To mention a few: 1) Higher levels of the natural *antimicrobial* products (called *phytoalexins*) are synthesized at infection sites (*Mhlongo et al., 2016*). 2) New *lignin* (built from hydroxylated *benzoic acids*) in cell walls and localized at the point of infection keeps the pathogen isolated and blocked its spread in the plant tissues. 3) *Salicylic acid* (a signal compound in plant response to pathogen infection) is produced by two parallel routes, both of which need intermediates (*i.e., chorismic acid* and *L-phenylalanine*) from the *shikimic acid pathway*. It is interesting to note that *salicylic acid antagonizes* the biological activity of *glyphosate* (*Akbulut, 2014*). 4) Naturally, the synthesis of pathogenesis-related proteins is important for plant resistance depending on the availability of the *aromatic amino acid* building blocks (*Johal and Huber, 2009*).

Since most *phytoalexins* are synthesized from *L-phenylalanine*, their levels are strongly reduced in *glyphosate*-treated plants (*Keen et al., 1982*) leading to a synergistic toxic action between the herbicide and the pathogen (*Descalzo et al., 1996*). In fact, herbicidal activity of *glyphosate* on bean seedlings was partly attributed to parasitization of plants by *soilborne fungi* (*Johal and Huber, 2009*). This suggestion was confirmed in a later investigation by *Schafer et al. (2013)*, by concluding that *rhizosphere i*nteractions play a major role in the phytotoxic action of *glyphosate* and might be involved in the evolution of *weed tolerance* and resistance to *glyphosate* in the field.

A practical application of the phenomenon of synergistic phytotoxic action between pathogens and glyphosate was invented by *Sharon et al. (1992)*, and they increased the weed controlling ability of the *Alternaria crassicae*-based mycoherbicide by adding very low (non-phytotoxic) concentrations of *glyphosate*. Unexpectedly, *glyphosate* did not influence the pathogenicity of *Fusarium solani* f. sp. *pisi* to pea seedlings (*Kawate et al., 1992*), although *pisatin* (the key p*hytoalexin* of pea) is also a product of the phenolic biosynthesis pathway (*Kőmíves and Casida, 1983*).

Hypersensitive response (HR) was considered for a long time as a key process of *plant disease resistance* until *Király et al. (1972)* who showed that HR is a consequence, and not the cause of plant resistance to infections. Interestingly, *glyphosate* had no effect on the *hypersensitive responses* of the host cells in leaves infected with incompatible bacteria (*Holliday and Keen, 1982*). Bacterial counts showed that inhibition of the biosynthesis of the phytoalexin *glyceollin* by *glyphosate* only partially prevented the expression of resistance to bacteria. In contrast, *glyphosate*-treated plants, fed with amino acids *phenylalanine* and *tyrosine*, were capable to synthesize and accumulate *glyceollin*, and were also resistant to incompatible bacteria. These results were interpreted on the basis that *glyceollin* accumulation is only a component of the complex mechanism of resistance to bacteria (*Holliday and Keen, 1982*).

In addition to their roles as *phytoalexins*, phenolic *stress metabolites* may contribute to plant resistance to pathogens via taking part in complex redox reactions that lead to the formation of *antimicrobial reactive oxygen species and quinines* (*Boeck et al., 2015*). Since *glyphosate* inhibits only the formation of *phenols* originated from intermediates after *chorismic acid*, *phenols* derived from earlier steps (such as *hydroxybenzoic acids*) may be important for the production of reactive oxygen species.

Salicylic acid plays a central role in plant defense signaling by regulating multiple biochemical pathways (*Wang, 1014*). Since *glyphosate* blocks both synthetic routes leading to *salicylic acid*, some of its effects on plant pathogen interactions may be due to the absence of this key molecule. Unfortunately, no study was published yet on the *salicylic acid* levels in *glyphosate*-treated plants.

(g) Future prospects

Although, there are significant arguments to ban *glyphosate*, most probably it will remain in use in the foreseeable future. It seems that further, thorough and rather expensive investigations are necessary to determine the environmental and human health risks of the use of *glyphosate*, its by-products and formulations. In this respect "*omics*" studies, such as those of *Martyniuk and Simmons (2016)*, could be the most promising. Additional research is also needed on the biological functions and the regulation of the individual steps in the biosynthesis of secondary plant metabolites in order to elucidate the *antagonistic* (and possibly *synergistic*) interactions of different metabolites derived from the *shikimic acid pathway*.

A further but not less important challenge for future research is the identification of key mechanisms by which residues of *glyphosate* may affect *ecosystems* and *ecological cycles*.

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Genetically modified (GM) plants I. qRT-PCR, and gene upregulation

(A) Introduction

The Biological Research Centre (Szeged, Hungary) is considered as one of the *Genius Loci* (*Smil, 2001*) of the current plant biotechnology since methodologies of plant cell line selections for chloroplast mutants (*Maliga et al., 1973; Garab et al., 1974*), cell fusion (*Dudits et al., 1976*), genetic transformation (*Márton et al., 1979; Koncz et al., 1984*), bacterial nitrogen fixation (*Kiss et al., 1980*), and artificial chromosomes (*Hadlaczky, 2001*) were either fundamentally developed or highly improved there.

The first stable higher plant mutant, the antibiotic (*i.e.* streptomycin, SR) resistant (*i.e.* mutant) tobacco (SR1) was selected (*Maliga et al., 1973*) *in vitro*, followed by the selection (*Maliga et al., 1975*) and identification of SR1A15 (*Sváb and Maliga, 1986*) the first double mutant of higher plants, the albino (chloroplast) tobacco (*Maliga et al., 1975; Páy and Smith, 1988*).

Later, as the early forms of gene transfer, plants developed from protoplast cell fusions (*i.e.* cybrids) were developed in several laboratories (*Kao and Michayluk, 1974; Melchers and Labib, 1974; Power et al., 1976; Dudits et al., 1977; Medgyesi et al., 1985*).

Alternatives to the conventional haploid genome transfer (*i.e.* pollination), the technologies of single and pyramided gene transfers resulted in stable transgenic crops (*i.e.* GM - Genetically Modified, or GMO - Genetically Modified Organism), were developed in four labs at the same time in 1983: GM *Nicotiana plumbaginifolia* (resistant to the antibiotic *kanamycin*) (*Bevan et al., 1983*), other tobacco lines resistant to kanamycin and methotrexate (a drug used to treat cancer and rheumatoid arthritis) (*Herrera-Estrella et al., 1983*), GM petunia resistant to kanamycin (*Fraley et al., 1983*), and GM sunflower transformed by phaseolin gene isolated from bean (*Murai et al., 1983*).

The first field trial of GM plants, a GM cotton was carried out in 1990, followed by the first FDA-approved (Food and Drug Administration of the Unites States) transgenic food of *Flavr-Savr tomato* in 1994 (*in: Bruening and Lyons, 2000*). A series of further GM crops were released in 1995, such as the *canola* oil seed rape (*Brassica napus*) with modified oil composition (Calgene), Bt (*Bacillus thuringiensis*) corn (Ciba-Geigy) resistant to the herbicide *bromoxynil* (Calgene), Bt cotton (Monsanto), GM soybeans resistant to herbicide *glyphosate* (Monsanto); virus-resistant squash (Asgrow), and a delayed ripening tomatoes (DNAP, Zeneca/Peto and Monsanto) (*in: Conner et al., 2003*). Later, a series of woody plants were also bred by genetic transformation (*Arisi et al., 1997; Noctor et al., 1998; Bittsánszky et al., 2005; Gyulai et al., 2012, 2014*) (*Table 1*).

Transgenes incorporated in the host genome represent genetic markers of GMOs (*Genetically Modified Organisms*) and also in its vegetative and sexual progenies. Here we show a case study of detection and monitoring of CaMV-35S-*gsh*I transgene of poplar (*Populus x canescens*), with a study which may be useful for both developing GM plants and for anti-GM controls.

Table 1. Samples of insect resistant transgenic GM poplars (*Populus* ssp.)

Poplar Species and Hybrids	Transgenes	Target Insects	References
P. alba × *P. grandidentata*	*cryIA(a)*	○ *Malacosoma disstria* ○ *Lymantria dispar*	M^{C}Cown *et al.*, 1991.
P. alba × *P. grandidentata*	*pinII*	○ *Lymantria dispar*	Heuchelin *et al.*, 1997.
[P. alba × (*P. davidiana* + *P. simonii*)] × *P. tomentosa*	*cryIA(cI)*	○ *Lymantria dispar* ○ *Clostera anachoreta*	Yang *et al.*, 2003.
P. nigra	*cryIA(c)*	○ *Lymantria dispar* ○ *Apochemia cinerarius*	Wang *et al.*, 1996.
(P. tomentosa × *P. bolleana)* × *P. tomentosa*	*CpTI*	○ *Lymantria dispar* ○ *Malacosoma disstria* ○ *Stilpnotia candida*	Zhang *et al.*, 2005.
P. tremula × *P. tremuloides*	*cryIIIA*	○ *Chrysomela tremulae*	Cornu *et al.*, 1996.
P. tremula × *P. tremuloides*	*ocI*	○ *Chrysomela tremulae*	Leple *et al.*, 1995.
P. tremula × *P. grandidentata*	*pinII*	○ *Plagiodera versicolora*	Klopfenstein *et al.*, 1997.

(B) PCR amplification of CaMV-35S-gshI transgene expressed in GM poplar (P. x canescens).

Double strand breaks (DSBs) of DNA as the initial events of recombination can occur not only in the meiotic but also in the somatic cells, which can cause transgene (*Fig. 1b*) elimination from the host genome. In this study, the *gsh*I transgene was found to be stably incorporated in the tested poplar lines (*ggs*11 and *lgl*6), and no transgene elimination or segregation was detected, which can occur during cycles of micropropagation *in vitro* (*Fig. 1c*).

The phytoextraction and remediative capacity of poplars was improved significantly by genetic transformation of *Populus* x *canescens* (*P. tremula* x *P. alba*) to overexpress the bacterial gene coding for *γ-glutamylcysteine synthetase* (# EC 3.2.3.3), which is the rate-limiting regulatory enzyme in the biosynthesis of the ubiquitous tripeptide thiol compound *glutathione* (*GSH*, γ-L-glutamyl-L-cysteinyl-glycine). Here we show how *gsh*I transgene is detected by using *gsh*I-specific PCR primers (*Koprivova et al., 2002*). The sequence differences between the eukaryotic plant *gsh*1 gene and the prokaryotic *gsh*I transgene of *E. coli* (*Fig. 1a*) made it feasible to design transgene specific PCR primers.

MSA (Multiple Sequence Alignments) for primer design. Nucleotide sequences of genes *gsh*1 were downloaded from the NCBI (*National Center for Biotechnology Information*) databases (*Altschul et al., 1997*). MSA were applied *in silico* with the software programs of BioEdit Sequence Alignment Editor (North Carolina State University, USA) (*Hall, 1999*), MULTALIN (*Combet et al., 2000*), CLUSTAL W (*Thompson et al.,*

1994), FastPCR (*Kalendar et al., 2009*), and computer program MEGA4 (*Tamura et al., 2007*).

DNA extraction. Total DNA samples of 0.1 g leaf tissue in each case are extracted in CTAB buffer (*cethyltrimethylammonium bromide*) followed by RNase-A treatment (extracted from bovine pancreas, Sigma, R-4875) for 30 min at 37 °C. DNA samples of ten individuals of each line were pooled in one bulk and subjected to PCR analysis.

Barcoding of transgene. The *gsh*I-transgene (*E. coli*, NCBI #X03954) in the transformed poplar clones is amplified by the *gsh*I specific primer pair of 5'-ATCCCGGACGTATCACAGG-3' (position from 341 to 359 bp of *gsh*I) and its reverse 3'-GATGCACCAAACAGATAAGG-5' (position from 939 to 920 of *gsh*I) (*Fig. 1b,c*).

Hot Start PCR is combined with *Touchdown PCR* by using AmpliTaq Gold™ DNA Polymerase (0.5 U). Reactions are carried out in a total volume of 25 µl (transgene detection) containing 50 ng of genomic DNA diluted in 1 x PCR buffer (with 2.5 mM $MgCl_2$), dNTPs (200 µM each) and 20 pmol of each primer.

Touchdown PCR is performed by decreasing steps of the annealing temperature from 66 °C to 56 °C by 0.7 °C / 30 s increments per cycle in each of the initial 12 cycles (PE 9700, Applied Biosystems), followed by a 'touchdown' annealing temperature for the remaining 25 cycles at 56 °C for 30 s with a final cycle of 60 °C for 45 min with a final hold at 4 °C. A minimum of three independent DNA preparations of each sample are used. PCR amplifications are assayed by *agarose* (1.8 %, SeaKem LE, FMC) gel electrophoresis (Owl system), stained with *ethidium bromide* (0.5 ng/µl) after running at 80 V in 1 X TAE buffer. Each successful reaction with scorable bands is repeated at least twice. Transilluminated gels are also analyzed by the ChemiIlmager v 5.5 computer program (Alpha Innotech). A negative control which contained all the necessary PCR components except template DNA is included in the PCR runs as negative control.

(C) Gene activation through DNA demethylation

DNA methylation of genes is a general process of gene silencing catalyzed by a nuclear enzyme family MTases (DNA *m*ethyl*t*ransfer*ases*). These enzymes transfer a methyl group (CH_3) mainly from S-*ad*enosyl *met*hionine (*AdoMet-dependent methyltransferases*) either to the position of cytosine-C_5 (EC 2.1.1.73), cytosine-N_4 (E.C. 2.1.113) or adenine-N_6 of DNA (E.C. 2.1.2.72). The cytosine methylation to 5-methylcytosine (5mC) is the most frequent, and it results in *down regulation* of genes (*Fig. 3*). Different methylation patterns are associated with different types of silencing. For example, methylation of symmetrical sequences of cytosines (*e.g.* CpG and CpNpG) in the promoter region is often associated with transcriptional gene silencing (TGS), while symmetrical methylation in the coding region is often associated with post-transcriptional silencing (PTGS).

The function of DNA methylation can be blocked by *MTase-inhibitors*, which act via covalent complex formation when present either in the *cytosol* or when incorporated into genomic DNA results in gene reactivation. (A demethylation independent gene reactivation through the application of *thymidine* analogues can also occur).

		490				500				510
HIGHER PLANTS		...	.|.	...	|..	..|	...	.|.	...	|..	..|
Populus trichocarpa (*gsh*1)	426	GGC	ATC	AAT	TTG	TTG	TCG	CCG	CAA	GCC	CTC
		Gly	His	Gln	Phe	Val	Val	Ala	Ala	Ser	Pro
Brassica juncea (*gsh*1)	261	.T.	...	.G.	.GA	...	.T.	.G.	...	.T.	...
		Gly	His	Gln	Leu	Ile	Val	Ala	Ala	Ser	Pro
Solanum lycopersicum (*gsh*1)	444	.AG	.C.	TGA	CAA	...	.A.	.T.	...	.T.	...
		Gly	Asp	Leu	Thr	Ile	Val	Ala	Ala	Ser	Pro
Phaseolus vulgaris (*gsh*1)	348	.CG	G.A	G.G	.GA	...	.T.	.T.	.G.	...	...
PROKARYOTES		Gly	Gly	Arg	Val	Ile	Val	Ala	Ala	Ser	Pro
E. coli (*gsh*I)	429	T..	G.G	TTA	A..	C..	AT.	G.A	..C	TGG	.AA
		Cys	Val	Leu	Met	Leu	Met	Ala	His	Trp	Gln

Figure 1a. Samples for sequence diversity of four *orthologous* plant *gsh*1 genes (stretches of 30 nt) and their amino acid translates (10 aa) of GSH proteins (*Glutathione synthase*), compared to the *non-orthologous* prokaryotic *gsh*I and aa of *GSH*I of *E. coli*. Synonymous and non-synonymous nucleotide substitutions, and the translated aa (by BioEdit; *Hall, 1999*) changes are indicated in boxes in different colors. (*gsh*1; NCBI # EF148665) (*Gyulai et al., 2005*)

(D) Functional analyses of the genes (i.e. gene expression analyses; RT-PCR; qRT-PCR)

Methods of RT-PCR (*Reverse Transcriptase PCR*), and qRT-PCR (*Quantitative Real-Time PCR*) combine RTase (*Reverse Transcriptase)* enzymes (*Baltimore, 1970; Temin and Mizutani, 1970*), which transcribe cDNA copy from mRNA template, and the real-time PCR (RT-PCR) (*Holland et al., 1991; Higuchi et al., 1993; Heid et al., 1996*). It detects the actual level of mRNA of the target gene studied, and detects the amplifying PCR fragment cycle by cycles. The qRT-PCR replaced the *Northern blot* analysis (*Alwine et al., 1977*) which basically follows the DNA blot of *Southern blot* analysis *(Southern, 1975*) leading to the *Western blot* analysis of proteins.

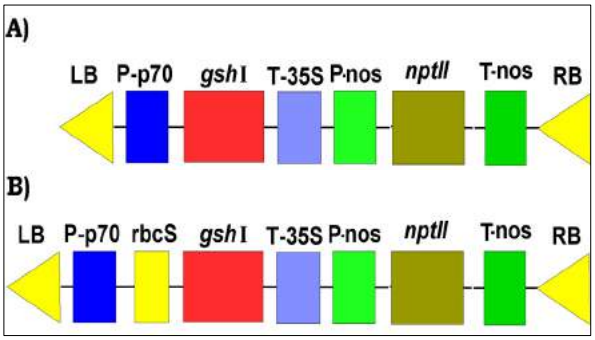


Figure 1b. Binary vector construct used for *Agrobacterium tumefaciens* mediated transformation of poplar (*Populus x canescens*) tree to overexpress ***gsh*I** transgene (γ-glutamylcysteine synthetase; syn.: γ-ECS, EC 3.2.3.3) either in the cytosol (*ggs*11) ***(A)*** or in the chloroplast (*lgl*6) ***(B)***. **LB/RB** – left/right border of *Agrobacterium* T-DNA; **P-p70** - 35S promoter of CaMV (Cauliflower mosaic virus) with double-enhancers; ***gsh*I** - γ-glutamylcysteine synthetase gene; **T-35S** - terminator sequence; ***npt*II** - neomycin phosphotransferase gene; **P-nos** - 5' promoter of nopaline synthase gene, **T-nos** - 3' terminal signal of nopaline synthase gene; ***rbc*S** – RUBISCO transit peptide gene of pea *(Koprivova et al., 2002; Bittsánszky, Gyulai et al., 2005).*

In qRT-PCR, the isolated RNA templates of either mRNA or small RNA are first converted to complementary DNA (cDNA) using a RTase enzymes, and then the transcribed cDNA is used as a template for *regular-*, *semi quatitative* (*Fig. 2a*) or *quantitative* PCR (*Fig. 2b*). It depends on the ways of fragment analysis, which may conducted by end-point detection on agarose gel (*i.e.* semi quantitative PCR), or by qPCR equipments (qPCR). In qPCR, to follow the amplifying qRT-PCR products cycles by cycles, DNA staying fluorescent dyes (EtBr, EvaGeen, SybrGreed, *etc.*) are applied either in single or combined (*e.g.* TaqMan; LifeTechnologies) forms.

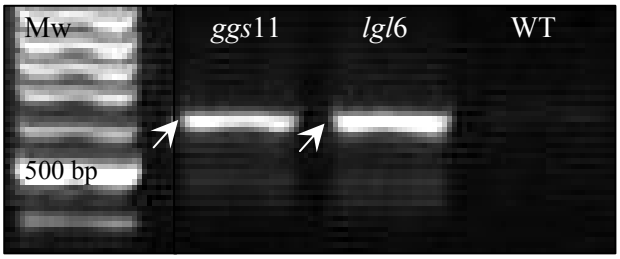


Figure 1c. PCR amplification of partial sequence (598 bp) (arrows) of the CaMV-35S-*gsh*I transgene cloned from *E. coli* (NCBI # X03954) in poplar (*Populus* x *canescens*) clones of *ggs*11 (cyt-ECS) and *lgl*6 (chl-ECS), and in the non-transformed (WT) clone (1.8% agarose gel).
Primer pair used was:
5'-ATCCCGGACGTATCACAG G-3' (341-359 nt of *gsh*I) and
3'-GATGCACCAAACAGATAA GG-5' (939-920 nt of *gsh*I) *(Bittsánszky, Gyulai et al., 2006).*

*(E) Gene expression analysis of gsh*I-transgene *by RT-qPCR – Determination of gsh*1-mRNA.

The relative expression of genes (and transgenes) can be easily triggered *in vitro* by both *down-regulation* (*syn.*: *gene silencing*, and *hypomethylation*), and *up-regulation* (*syn.*: *reactivation, hypermethylation,* and *demethylation*). The DNA *hypermethylating* drug *3-aminobenzamide* has been used for *down-regulation* a series of genes. For gene *up-regulation* MTase-inhibitors, such as the structurally modified *cytosine* analogues *zebularine, 5-azacytidine (5-azaC)*, *5-aza-2'-deoxycytidine* and the heat-stable form of *DHAC* (*5,6-dihydro-5'-azacytidine hydrochloride*) (10^{-4} M for 7 days) are used. In the samples here, gene expression levels of *gsh*I-transgene (*E. coli*) and endogenous poplar *gsh*1 gene were analyzed. For control, constitutively expressed housekeeping poplar gene *a-tubulin* is used. The effects of DNA demethylating agent *DHAC* and herbicide *paraquat* on the expression of *gsh*1 and *gsh*I were also studied.

Reverse transcription (RT) of *gsh*1-mRNA followed by quantitative real time polymerase chain reaction (qPCR) analysis (RT-qPCR) has proven to be an exceptionally sensitive method for both absolute and relative quantification of gene expression levels. Relative quantification is more relevant to compare expression levels of different treatments rather then absolute quantification.

Total RNA is extracted from 0.05 g leaf disc tissues by using the Absolutely RNA Miniprep Kit (# 400800, Stratagene, USA - Biomedica, Hungary) following the manufacture's protocol. The quality and quantity of extracted total RNA samples (2 µl) are measured by NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA – BioScience, Budapest, Hungary).

cDNA synthesis. Reverse transcription of first strand cDNA was synthesized on the templates of total mRNA by RT (reverse transcriptase of *Moloney Murine Leukemia Virus*: M-MuLV) with oligo(dT)$_{18}$ (0.5 µg) primer following the manufacturer's protocol (# K1622; Fermentas – Biocenter, Szeged, Hungary).

Gene expression analysis. First strand cDNA samples (2.5 µl) were directly applied in qRT-PCR (in 25 µl vol.) and probed by *gsh*I gene specific primer pair (400 nM) designed by *'Primer3'* computer program (*Rozen* and *Skaletsky, 1997*), with the application of DyNAmo HS SybrGreen I qPCR kit. Reactions were performed in forty cycles (95 °C/20sec, 60 °C/20sec, 72 °C/20sec) prior to a hold at 95 °C for 10 min, and closed by a final hold at 4°C. Reactions were run either by Rotor Gene 6000 (Corbett Res., Australia – Izinta, Hung.) (*Fig. 2a,b*).

Data analysis. For both calibration and quantification of reactions, ten-fold serial dilutions (1x, 10x, 10^2x, 10^3x cDNAs) were applied including controls of NTC (*N*on DNA-*T*emplate *C*ontrol) and ddH_2O (*Fig. 2a,b*). Data were analyzed by relative quantification of the $2^{-\Delta\Delta Ct}$ method (*Livak* and *Schmittigen, 2001*).

Ct values (*t*hreshold *C*ycles). The threshold of fluorescence value (dR) of the amplified PCR products was determined manually above the background of fluorescence signals. Standard curve correlating Ct values to log amount of DNA were plotted at high R^2-ratio (0.987) (*Fig. 2a,b*).

ΔCt. Δ*Ct* valaues were calculated as $Ct_{gsh1\text{-}E.coli} - Ct_{\alpha\text{-tubulin}}$ and $Ct_{gsh1\text{-poplar}} - Ct_{\alpha\text{-tubulin}}$. *ΔΔCt values.* ΔΔ*Ct* values were determined as mean of $Ct_{untreated}$ – mean $Ct_{DHAC\text{-treated}}$.

Table 2. Calculation processes of relative gene expression levels (values of $2^{-\Delta\Delta Ct}$) of endogenous poplar gene *gsh*1 *(A)*, and transgene *gsh*I *(B)* in the untreated, DHAC-treated (10^{-4} M, 7 days) and paraquat treated (PQ, $4x10^{-7}$ M, 7 days) leaf discs samples of *gsh*I transgenic (11*ggs*) and wild type (WT) poplars (*P. x canescens*).

(A)

*gsh*1 *(Populus)*	$Ct_{gsh1.\ Populus}$		$Ct_{\alpha-\ tubulin}$		ΔCt		ΔΔCt	Gene expression ($2^{-\Delta\Delta Ct}$)		
	mean	S.D.	mean	S.D.	mean	S.D.	mean	-	+	value
Untreated										
11*ggs*	23.3	0.14	20.2	0.16	3.1	0.11	-1.7	2.9	3.4	**3.1**
WT	23.6	0.21	18.9	0.16	4.7	0.12	0.0	0.9	1.1	**1.0**
DHAC-treated										
11*ggs*	23.3	0.20	19.6	0.21	3.7	0.40	-1.0	2.9	3.4	**2.0**
WT	20.4	0.20	19.9	0.10	0.4	0.11	-4.3	0.9	1.1	**19.8**
DHAC + PQ-treated										
11*ggs*	19.7	0.02	19.8	0.07	-0.1	0.09	-4.8	26.4	29.8	**28.1**
WT	18.5	0.26	17.8	0.06	0.6	0.23	-4.1	14.8	20.3	**17.3**

(B)

*gsh*I *(E.coli)*	$Ct_{gsh1.\ E.coli}$		$Ct_{\alpha-\ tubulin}$		ΔCt		ΔΔCt	Gene expression ($2^{-\Delta\Delta Ct}$)		
	mean	S.D.	mean	S.D.	mean	S.D.	mean	-	+	value
Untreated										
11*ggs*	34.4	0.48	21.7	0.03	12.8	0.46	0.0	0.7	1.4	**1.0**
DHAC-treated										
11*ggs*	35.0	0.60	21.2	0.01	13.9	0.61	1.1	0.3	0.7	**0.5**
DHAC + PQ -treated										
11*ggs*	32.4	0.60	21.6	0.04	10.7	0.64	-2.0	2.6	6.3	**4.0**

(F) Gene reactivation and upregulation by DNA de-methylation

GM poplar clones were maintained in aseptic shoot culture for about a decade, with no *gsh*I transgene elimination (see above). However, transgene has been exposed to *gene silencing* processes especially by the methylation of the constitutive CaMV 35S promoter region, which aimed to be reactivated by DHAC-induced transgene demethylation (*Table 2*).

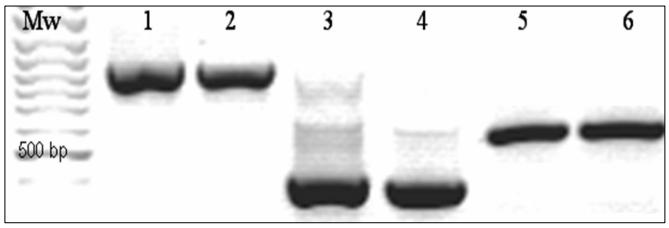


Figure 2a. Samples of *semi quantitative RT-PCR* (*Real Time Polymerase Chain Reaction*) (Corbett Research, Co) with end-point detection on agarose gel (1.8 %). Gene expression levels of the constitutively expressed gene 26SrRNA (**1, 2**) for standard, and the nuclear encoded gene rbcS (**3, 4**), and the stress responsive gene gst (**5, 6**) were determined in the cDNA samples of two poplar (*Populus* x *canescens*) clones *Bittsánszky, Gyulai et al., 2005*).

The expression level of endogenous *gsh*1 gene of transformed 11*ggs* clone did not increase by DHAC-treatment, but under *paraquat* stress it showed 9-fold increase (from 3.1 to 28.1 rel. unit) along with elevated co-expression of the transgene *gsh*I with a 4-fold increment (from 1.0 to 4.0).

In contrast, the expression level of endogenous poplar gene *gsh*1 of WT clone showed an extreme response to DHAC-induced demethylation (20-fold increment) without further increment to *paraquat* (17-fold). These results indicate a more extensive stress activation capacity of endogenous poplar *gsh*1 over the transgenic *gsh*I, which may imply an alternative gene technology for DHAC induced up-regulation of endogenous genes rather than transgenic technology.

Paraquat (*N,N'-Dimethyl-4,4'-bipyridinium dichloride*; syn.: *methylviologen*) primarily affects as an electron acceptor in the electron transport chains located in chloroplasts (*Gyulai, 1984; Lehoczki et al., 1992*). At bleaching concentrations of *paraquat* it totally impairs chloroplast function by reducing superoxide radicals that react with unsaturated lipids in membranes (*Bittsánszky et al., 2006*). This deleterious effect was efficiently eliminated by the DHAC-induced upregulation of both *gsh* genes (*Table 2*).

Increased levels of *gsh*1-mRNA (syn.: γ-ECS-mRNA) has also been reported in *Brassica juncea* and *B. napus*, and *Arabidopsis thaliana*. The moss *Physcomitrella patens* also showed a high level of *gsh*1 overexpression in response to 10 μM Cd^{2+} (treated for 3 days). These results indicate a wide oxidative stress-response capacity of *gsh*1 not only to herbicides but also to heavy metals.

The lower responsiveness of both the *gsh*I transgene (from 1.0 to 0.5 rel. unit) and the endogenous *gsh*1 gene (from 3.1 to 2.0 rel. unit) of the poplar clone to DHAC-induced demethylation, compared to that of the WT clone (from 1.0 to 19.8 rel. unit of *gsh*1) might indicate a *co-repression* between the two *gsh*1 and *gsh*I genes. It is either the results of *multicopy integration* of transgene at the same locus in the host genome, or the *position effect* of transgene (surrounded by host genes) due to random integration, or, the AT/CG composition of the transgene, or, the presence of inverted repeats in the integration site, or, the overexpression of the transgene, or, even the

environmental conditions played role. Nevertheless, under PQ stress the high expression level of poplar *gsh*1 (9-fold increment from 3.1 to 28.1 rel. unit) and transgene *gsh*I (4-fold from 1.0 to 4.0 rel. unit) of 11*ggs* clone exposed to the combined treatment of DHAC and PQ may indicate an elevated *co-expressions* of the two genes (*Table 2*).

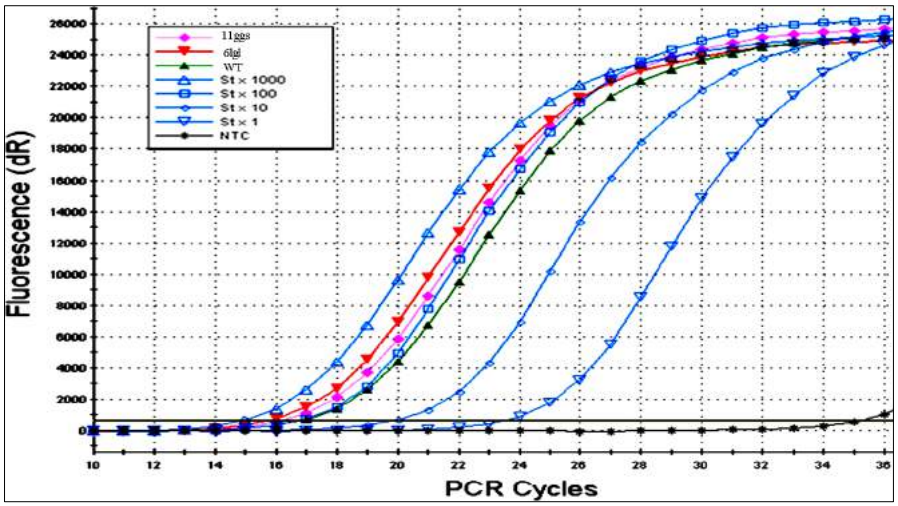


Figure 2b.
Samples for quantitative RT-PCR (*quantitative Real Time Polymerase Chain Reaction*) measurements (Corbett Res., Co) of the expression of plant *gsh*1 genes in three poplar (*Populus* x *canescens*) clones (11*ggs*, 6*Lgl*, and Wild Type - WT), and compared to the concentration series of control DNAs (1 to 1000 times dilution series) and NTC (*Non Template Control*). Relative fluorescence (dR) and PCR cycle numbers are indicated *(Bittsánszky, Gyulai et al., 2006; Gyulai et al., 2012).*

To conclude, by means of molecular barcoding the transgenes, either coding or reporter genes, can be detected in the genetically modified GMO plants for both GM and anti-GM purposes. The sequence differences between the foreign gene and the resident genes make it feasible to design GMO-specific barcodes. These results also imply an alternative gene technology of DHAC induced up-regulation in poplars (*Appendix 1*) rather than any transgenic technology (*Appendix 2*).

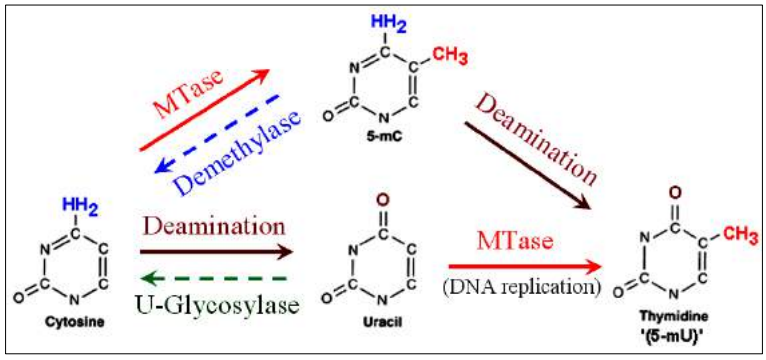


Figure 3. Molecular pathways of enzymatic *methylation*, *demethylation*, and *spontaneous deamination* of DNA nucleotides (5-mC = C5-methyl cytosine, MTase = DNA methyltransferase) *(Alzohairy, Gyulai et al., 2016)* (*see Chapter 1. Fig.1d., Chapter 4. Fig.1b*).

References (for all papers cited:

Bittsánszky A, G Gyulai, I Bock, LY Murenets, M Czakó, T Kömíves, H Rennenberg (2015) Barcoding of transgenes in GM plants. *In*. Ali MA, G Gyulai, F Al-Hemaid (Eds) *Plant DNA barcoding and phylogenetics*. LAP Lambert Academic Publ. Germany. Chapter 17. pp. 269-278. ISBN-13: 978-3-659-28095-5

Appendix 1.

Lists of sections (I - VI), and distribution of *Populus* species (1 - 24).

I. section ***Populus*** - aspens and white poplar. Circumpolar subarctic and cool temperate)

1. *Populus tremula* (Common Aspen, Trembling Aspen or Eurasian Aspen) (Europe, northern Asia)
2. *Populus tremuloides* (Quaking Aspen or Trembling Aspen) (North America)
3. *Populus grandidentata* (Bigtooth Aspen) (Eastern North America)
4. *Populus adenopoda* (Chinese Aspen) (Eastern Asia)
5. *Populus sieboldii* (Japanese Aspen) (Eastern Asia)
6. *Populus alba* (White Poplar) (Southern Europe to central Asia) (warm temperate)
7. *Populus* × *canescens* (*P. alba* × *P. tremula*) (Grey Poplar)

II. section ***Aegiros*** - black poplars or cottonwoods. North America, Europe, western Asia; temperate

8. *Populus nigra* (Black Poplar) (Europe
9. *Populus* × *canadensis* (*P. nigra* × *P. deltoides*) (Hybrid Black Poplar)
10. *Populus deltoides* (Eastern Cottonwood) (Eastern North America)
11. *Populus fremontii* (Fremont Cottonwood) (Western North America)
12. *Populus petrowskyana* (Russian Poplar) (Europe/Asia)

III. section ***Tacamahaca*** balsam poplars. North America, Asia; cool temperate

13. *Populus angustifolia* (Willow-leaved Poplar or Narrowleaf Cottonwood) (Central North America)
14. *Populus balsamifera* (Ontario Balsam Poplar) (Northern North America)
15. *Populus trichocarpa* (Western Balsam Poplar or Black Cottonwood) (Western North America)
16. *Populus laurifolia* (Laurel-leaf Poplar) (Central Asia)
17. *Populus simonii* (Simon's Poplar) (Northeast Asia)
18. *Populus maximowiczii* (Maximowicz' Poplar) (Northeast Asia)

IV. section ***Leucoides*** necklace poplars or bigleaf poplars. E-N America, eastern Asia; (warm temperate)

19. *Populus heterophylla* (Swamp Cottonwood) (Southeastern North America)
20. *Populus lasiocarpa* (Chinese Necklace Poplar) (Eastern Asia)
21. *Populus wilsonii* (Wilson's Poplar) (Eastern Asia)

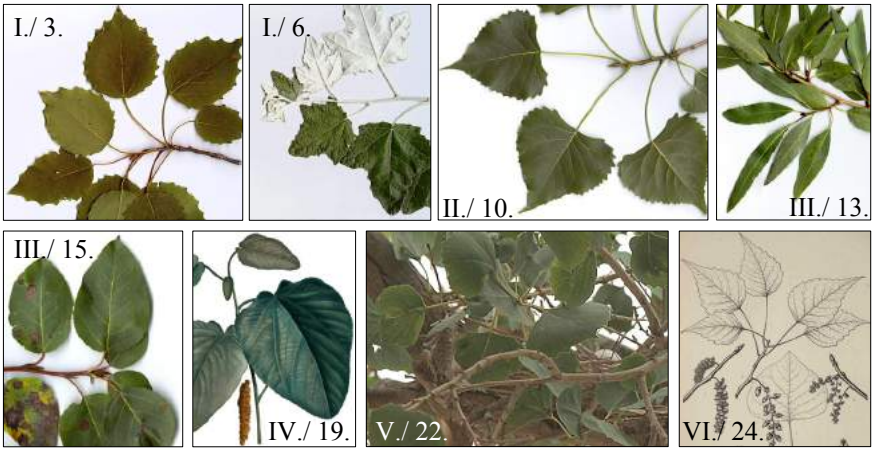
V. section ***Turanga*** subtropical poplars. Southwest Asia, east Africa; subtropical to tropical

22. *Populus euphratica* (Euphrates Poplar) (Southwest Asia) (*highly tolerant to soil salinity*!)
23. *Populus ilicifolia* (Tana River Poplar) (East Africa)

VI. section ***Abaso*** Mexican poplars. Mexico; subtropical to tropical

24. *Populus mexicana* (Mexico Poplar) (Mexico)

Illustrations



Appendix 2.

Owen Paterson: 'Anti-GMO stance of Green Blob, Greenpeace condemn poor to starvation, death

Owen Patterson | February 24, 2015

http://geneticliteracyproject.org/2015/02/24/owen-paterson-anti-gmo-stance-of-green-blob-greenpeace-condemn-poor-to-starvation-death/

Speaking Tuesday in Pretoria, South Africa, former UK environment secretary Owen Paterson accused the European Union and Greenpeace of condemning millions of people in developing countries to economic dependency on aid, starvation and death by their refusal to accept the science behind genetically modified crops and other life saving advances in plant sciences. Owen denounced what he called the "green blob" of officials and gullible media that misrepresent the consensus science, likening Greenpeace to the Luddites who smashed textile machinery in the nineteenth century. He accused the EU of "neo-colonialism at its worst" by restricting food production within its own borders. Paterson said the world is on the cusp of a green revolution of the kind that fed a billion people in the 1960s and 1970s as the world's population soared–but that revolution is threatened by well-meaning Westerners who he maintains are imposing their food "fetishes" on the developing world. Here is the full text of Paterson's speech to the International Service for the Acquisition of Agri-biotech Applications (ISAAA):

Thank you all very much.

Thank you, Doctor Obokoh for that kind introduction. It's a great pleasure to be able to speak at the Annual South African agricultural biotechnology industry/ISAAA media conference. You are all doing so much important – indeed life-saving – work to bring the benefits of modern agricultural technology to this continent.

This is a time of extraordinary opportunity for Africa. Progress in the plant sciences is opening up the promise of a second Green Revolution, one that can not only feed the 9 to 10 billion people that will inhabit our planet in 2050, but feed them well – one that can finally end the shame of the nearly one billion who still go to bed every night hungry and malnourished.

It is a revolution, powered by cutting-edge science, that can drive economic development from the bottom up. I'm talking about authentic, indigenous growth – the only kind that really takes root that empowers individuals that breaks the cycle of aid and dependency and can make every nation on this Earth a strong, competitive player in global economic growth.

No place on Earth holds more promise in this respect than Africa.

With its vast, and as yet underutilized resources of land, soil, water and sun, Africa is wonderfully situated to match or exceed the success of Brazil – a nation that agricultural development helped catapult into the front ranks of world trade – but it will only happen if African countries embrace farming systems based on modern technologies.

The Green Blob

This is also a time, however, of great mischief, in which many individuals and even governments are turning their backs on progress. It's a strange time, really, in which the privileged classes increasingly fetishize their food and seek to turn their personal preferences into policy proscriptions for the rest of us.

Not since the original Luddites smashed cotton mill machinery in early 19th century England, have we seen such an organized, fanatical antagonism to progress and science. These enemies of the Green Revolution call themselves "progressive," but their agenda could hardly be more backward-looking and regressive.

They call themselves humanitarians and environmentalists. But their policies would condemn billions to hunger, poverty and underdevelopment. And their insistence on mandating primitive, inefficient farming techniques would decimate the Earth's remaining wild spaces, devastate species and biodiversity, and leave our natural ecology poorer as a result.

I call them the "Green Blob" – a reference to a 1950s Sci-Fi movie starring Steve McQueen in which a blob-like alien attacks Earth and swallows everything in its path: the environmental pressure groups, renewable energy companies and some public officials who keep each other well supplied with lavish funds, scare stories and green tape. This tangled triangle of unelected busybodies claims to have the interest of the planet and the countryside at heart, but it is increasingly clear that it is focusing on the wrong issues and doing real harm while profiting handsomely.

The Blob operates on two levels. First, by a pernicious grab for funding dollars with literally hundreds of them orbiting the honey pot of Europe. Secondly, with an ideological belief that Europe should abdicate its fundamental responsibility to feed its own people.

Neo-colonialism at its worst, Europe sits on some of the most fertile land on the planet, and yet imports food from the rest of the world which requires the equivalent of 35m hectares of farmland to produce.

There are many impediments standing between the vision of agricultural progress and Africa, of course, but none is more pernicious than the Blob. It is supported by massive funding provided by the EU itself, as well as numerous church and humanitarian groups, and the well-meaning but misguided generosity of the privileged classes in Europe and elsewhere. It has undue influence in the media, government and international institutions. Unfortunately, few question either its credentials or motives. I will be speaking more about the Green Blob and how we need to push back and reassert the fundamental primacy of science. But first I want to talk about the good news – and there is a lot of good news to talk about.

The Good News About Biotech

As you know, the International Service for the Acquisition of Agri-biotech Applications, ISAAA, has just come out with its latest report on the worldwide adoption of genetically modified crops. As before, it records a remarkable success story. 2014 was the 19th year of successful commercialization of biotech crops, 18 million farmers, of which 90 per cent were small and resource-poor, planted a record 181 million hectares of biotech crops in 28 countries. GMO-versions of food staples like potato in the United States and eggplant in Bangladesh have been approved for planting. The United States continues to lead the way and saw a 5.5-fold increase in hectares of drought tolerant maize planted.

Biotech continues to be the most rapidly adopted agricultural technology in history. During the 19 years GMO crops have been commercialized, we have seen a more than 100-fold increase in the area planted. The facts also completely belie the propaganda that GMOs are only for the wealthy nations. In fact, more than 90 per cent of the farmers planting biotech are smallholder farmers in nations in the developing world. For the third year in a row, less developed countries planted more biotech hectares than the entire developed world.

Farmers are famously risk-averse. They know that the misapplication of resources can spell the difference between a bumper harvest and total crop failure. That's why it's even more remarkable that nearly 100 per cent of all those farmers who plant biotech

crops have yet to go back to the old ways. They continue to choose to plant biotech year after year because biotech plants work. It's really that simple.

ISAAA report a number of very heartening breakthroughs. The drought-tolerant maize technology donated to Africa by Monsanto is expected to begin commercial planting in 2017.

Field trials have been given the go-ahead in Cameroon, Egypt, Ghana, Kenya, Malawi, Nigeria and Uganda while trials have been conducted on a broad range of new crops, from biotech bananas to maize to cotton and cowpeas. One can certainly hope that as the trials reveal the dramatic benefits of GMO, these countries too will make them available to all their famers.

They can certainly look to the success in South Africa, which is still the leader on this continent, with 2.7 million biotech hectares planted. But they might also take in the dramatic example of insect resistant Bt cotton in Burkina Faso, where farmers are rapidly and overwhelmingly embracing the efficiencies and improved yields represented by the GMO variety.

By 2013, in fact, almost 70 per cent of all cotton grown in Burkina Faso was Bt, which increased farmers' yields on average 20 per cent over non-GMO cotton. It has also dramatically decreased pesticide applications – which in Africa are often done by hand, a 40 to 80 pound backpack filled with older pesticides strapped to one's back. Bt-cotton has cut those applications from 6 to 2 or fewer and delivers a solution that is eminently more effective.

Within one season, Bt can transform the life of smallholder farmers, turning their farms into profit-making enterprises that allow them to send their children to school rather than out into the fields, and to buy their families enough to eat – and of course with better nutrition comes better health.

Even where farmers have voted overwhelmingly for a choice of GM technology and the benefits have been tested and demonstrated in numerous studies, the Green Blob has been tireless in myth making and misinformation. Take the allegations of Indian farmer suicides. Anti-GM green groups stated that the introduction of GM crops had brought about an increase of suicides among India's farmers. It is imperative in the case of such tragedy to be accurate about causes if you are to help people driven to suicide.

Professor *Ian Plewis* from the University of Manchester clarifies that farmer suicide rates in India are similar to the best estimates of the rates in Scotland and France, around 30 per 100,000 farmers. While these rates are still tragic, they existed at the same level prior to the introduction of GM cotton to India. He states, "In fact, the available data does not support the view that farmer suicides have increased following the introduction of Bt cotton. Taking all states together, there is evidence to support the hypothesis that the reverse is true. And in the global context, over 80 per cent of the world's cotton crop has been GM for several years. The success of insect protected GM cotton has given Burkina Faso, one of the poorest countries in the world, a new tool to boost their main economic activity: cotton production. Farmers have seen at least 66 per cent less pesticide applied, 20 per cent increase in yield, and at least $87 per hectare increase in their profit.

Green Blob myths like "GM Indian Farmer Suicide" are retarding the adoption of new science in the developing world. Yet Africa is showing Europe the way. In 2014 the 28 member states of the EU recorded 12 field trials of GM crops. This compares with 13 projects in Uganda, Kenya and Nigeria. These three countries are doing more active biotech field research than the entire rich continent of Europe.

Four Anti-GMO Myths and the Truth About Biotech

Around the globe, in fact, the increasingly widespread adoption of biotech is exploding the myths of the anti-GMO campaigners. It is worth taking a moment to examine four of these myths, taking them one at a time:

Myth #1 is their recurrent implication that farmers are stupid, fooled by biotech companies into paying more for GMO seeds when they would be far better off without them. Well, I tend to think farmers have a pretty good understanding of their bottom line, and I can't imagine any farmer – in my own country or in the developing world – spending one extra dollar, euro, pound, or rand that he absolutely didn't have to spend.

As it happens, the most current and extensive research on the subject bears that out. A recent analysis of previous major studies – conducted by researchers at Germany's Göttingen University, found that globally since their introduction almost two decades ago, biotech crops have increased crop yield overall by 22 per cent, increased farmer profits by 68 per cent, and reduced chemical pesticide use by 37 per cent. They also found that these yield and profit gains are the highest in less developed countries, not the industrialised countries. In other words, the supposedly dumb farmer of Blob mythology is actually a lot smarter than the Green Blob itself.

Myth #2 is that forsaking modern agricultural technology – going organic – will benefit the environment. The opposite is actually the truth.

A few years ago, another group of researchers at Stanford University in the United States found that without the advances in agricultural technology since 1960, we would need more than twice as much land to grow all the food we produce today. That's almost two billion more hectares of ploughed land than today, more than the entire landmass of Russia, the largest nation on the globe spreading over nine time zones. Two billion hectares is more than twice the entire area of the United States. The equivalent of three Amazon rain forests.

I can remember as a child seeing traumatic news bulletins with images of starving people on the Indian subcontinent. The father of the Green Revolution, Norman Borlaug – "The man who fed the world" and Nobel Peace Prize Laureate in 1970 – changed that by transferring wheat with new genetics from the Americas to the Indian sub-continent in the 1960s. India is now a major food exporter.

Borlaug and others harnessed innovation to completely change the way we farm. For example, it has been estimated that the production of a given quantity of a crop now requires 65 per cent less land than it did in 1961. Between 1967 and 2007 world food production increased by 115 per cent but land use only increased by eight per cent. Indur Goklany has calculated that if we tried to support today's population using the production methods of the 1950s, instead of farming 38 per cent of all land, we would need to use 82 per cent.

As Borlaug said, "There are 6.6 billion people on the planet today, with organic farming we could only feed 4 billion of them. Which two billion would volunteer to die?" Agriculture always needs to balance our demands for more food whilst improving the environment and biodiversity. It is clear that sustainable intensive agriculture produces more food on less land, and therefore protects wild lands for wild life, for recreation, for urban development.

There is less pressure on land that is being used for wildlife and recreation. And the whole huge area generates tourism cash and employment for local economies. In other

broad acre crops that contain oil in their leaves and stems. So GM developments in oil producing plants could help save vulnerable orangutan habitats encroached by palm oil plantations.

Few people know that the first biotechnology product approved for food was rennet, an enzyme used to make cheese. Today, 90 per cent of the cheeses we eat use GMO rennet because it's safer and more effective. GMO-enzymes are routinely used in the production of bread, wine and beer.

All the insulin routinely used to keep diabetics alive is from GMO-bacteria. Previously insulin was produced from the pancreases of cattle and pigs. A single diabetic

words, modern agriculture – with its GMOs, nitrogen fertilizer and modern pesticides – has probably done more to save natural habitat, support biodiversity, and save endangered species than all the other environmental, NGO and UN conservation activities put together. The Keystone Alliance, a collaborative effort of industry and conservation groups in the United States, has demonstrated the environmental benefits of modern agriculture on the micro-level as well. In each of the major crops studied, inputs of water, fertilizer and energy have been slashed and the environmental impact dramatically diminished at the same time that yields have skyrocketed. Maize yields, for instance, increased by 64 per cent in the 31 years between 1980 and 2011. Land use, however, decreased by 30 per cent, soil erosion by 67 per cent, irrigation water by 53 per cent, and energy use by 44 per cent.

The revolution in no-till farming, was invented in the later 1960s a long time before GMOs. Modern herbicides and GMO crops have significantly extended its scope because farmers in many locations no longer have to plough the land to manage weeds. Tractor fuel is saved and topsoil is increasingly returning to its original structure and beneficial microorganism content and, in the United States, rivers and streams are spared the soil run-off that the EPA used to define as one of the top environmental problems in that country.

And the most widely used herbicide in no-till – the glyphosate that NGOs so love to criticize – is enormously healthier for the environment and the humans and animals that live there than the chemistries it replaced. While glyphosate is indeed bad for weeds, its toxicity to animals is less than, not equivalent, but significantly less than, vinegar. Something to think about next time you dresses your salad.

Which brings me to *Myth #3*: the insistence by anti-GMO campaigners that biotech crops are somehow unsafe to eat. It's a claim they continue to hang onto in the face of many hundreds of studies testifying to GMO safety – the overwhelming majority of all the studies that have been conducted, a large number of them sponsored by governments and completely independent of industry.

It's a claim they persist in despite the universal opinion of every independent scientific institution globally, (including the European Commission!) that GMOs are as safe as any other food, and the fact that people in the United States have been consuming diets replete with GMOs for over 15 years now without one documented adverse health effect – not so much as a sniffle or a tummy ache. Even in Europe, overwhelmingly all the animal products produced – meat, milk, cheese, eggs – come from animals fed on imported GM maize and GM soya meal. Most European farmers have, for the last almost two decades, not been permitted to grow these crops – with the one exception of a single strain of maize. But European livestock farmers import millions of tons annually – without these imports currently the European livestock market would have collapsed.

In a rational world, a recent study out of the University of California Davis (one of the leading agricultural universities in the United States) would end the call for 'animal studies' of GMOs once and for all. The study compared health outcomes in over 100 billion cattle and other livestock before GMOs were introduced in 1996 and after – when quite quickly GMOs accounted for approximately 90 per cent of all animal feed. In effect, US livestock production has amounted to the largest animal feeding study ever conducted. And what was the difference in health outcomes found by the researchers? Zero. None. The animals were just as healthy after GMOs were introduced as before. Of course, as with the other myths, the myth of GMOs' adverse health effects isn't just wrong – it's the inverse of the truth.

Generally ignored, for instance, is the widespread problem of mycotoxin contamination, often the result of insect chewing and especially boring into the growing crop, which allows the entry into the plant of fungal pathogens. The problem afflicts a wide range of foods and feed, such as maize, sorghum and peanuts. Without doubt, many mycotoxins are most effectively controlled by planting GMO crops engineered with Bt insect resistance.

The FAO estimates that up to half of some food crops are affected. Globally, it is estimated that more than five billion people in the developing world are exposed to these naturally occurring toxins, which can suppress the immune system, retard growth and cause cancer and liver disease in both livestock and humans. In Africa, the rural poor are chronically exposed to unsafe levels of these poisons. In 2003, 120 people died in Kenya after eating maize with very high aflatoxin levels.

In the industrialized nations, organic growers have long sprayed with spores of the whole Bt bacterium to control for insects. This organism occurs naturally in the soil, after all, and has proved safe for mammals and humans. But when scientists engineered a plant that produced one protein found in the Bt cells as a part of its built-in defenses, the Blob fought tooth and nail to deprive the developed world of its benefits.

In a nationally funded trial conducted at the respected University of Milan, two varieties of maize were involved. Compared with conventional maize, Bt-maize not only increased yield by 28-34 per cent, but reduced the fungal toxin fumonisin from 6,000 parts per billion in the non-GMO maize to 60 parts per billion or less in the Bt-maize. The conventional maize containing over 6,000 parts per billion was unfit for human consumption under both Italian and European law. Despite the health implications, these results were shamelessly suppressed by the activist influenced Italian government which organized it. Once again, the myth is turned on its head: it's not GMOs, but the anti-GMO Green Blob that is the real danger to human health.

Myth #4 is that biotech is only good for farmers and has no consumer benefits. Once again an inverse of the truth. I count increased protection of wild lands by focusing production sustainably, and cheaper food, as being fundamentally important "consumer benefits". There are more specific examples too.

Biotechnology has already given us soybeans with higher oleic acid that don't produce cholesterol-elevating trans fats when heated. Currently, a new biotech tomato is being tested that mimics good cholesterol. Tomatoes are coming with high concentrations of cancer-fighting anthocyanins. Non-browning apples have recently been approved by US regulators, which should potentially greatly reduce waste through less spoilage. Healthier GMO potatoes have also been approved and peanuts are currently under development that lack two of the most intense allergens that pose such a danger to so many of our children.

Only ten days ago, I was in Canberra and saw real progress on oilseed crops that will provide a sustainable source of long chain omega-3 fatty acids providing better nutrition to humans and farmed fish. This could stop the obscenity of feeding huge numbers of farmed fish with wild fish.

In addition, in future, it might be possible to have the oil yields of oil palm replicated and even exceeded from GM

would require the pancreases of 50 pigs for a year's supply. Before GMO-bacteria produced insulin, one major industrial insulin producer processed eleven tons of pig's pancreases every day – from a daily slaughter of 100,000 animals. Unfortunately, hugely promising GMO techniques that could protect yoghurt starter cultures from infection have been kept off the market due to fear of consumer backlash. Perhaps the most promising development, however, is biofortification, especially for the developing world, where so many lack the nutrients essential for health and well-being.

Golden Rice, Greenpeace and the Anti-Humanitarians

The flagship biofortified technology was developed 15 years ago by two German Professors Ingo Potrykus and Peter Beyer. Called Golden Rice, it is a miracle grain enhanced with vitamin-A-producing beta-carotene. In 2001 the inventors donated the technology as a potential additional intervention for vitamin A deficiency, for development and deployment by the public sector in developing countries so that it could benefit the poor of the world.

Absence of a source of vitamin A in the diet, vitamin A deficiency, is the principal cause of childhood blindness globally, affecting 500,000 children annually of which half die within a year or two. Vitamin A deficiency is also a nutritionally acquired immune deficiency syndrome, so common diseases which should be survivable are lethal. Two million young children die as a result every year.

So let's be clear. Although these deaths are preventable, 6,000 children alive today will be dead tomorrow. (By comparison Ebola has tragically killed about 9,000 in the last year: about 25 a day). Many of those millions of lives could have been saved if Golden Rice had been available in their diet, and it could have been already for several years, but for the on-going opposition of well-financed anti-GMO activist groups and their ceaseless campaign to frighten people and pressure governments to keep Golden Rice off the market.

The leader of that opposition, with a combined global war chest estimated to exceed US $500 million, has been Greenpeace, with its combination of highly sophisticated PR and un-scientific scaremongering. Greenpeace originally claimed Golden Rice wouldn't work, but once its efficacy had been proven beyond a shadow of a doubt, they switched to saying that the poor should simply buy vitamin supplements and eat fresh vegetables instead – as if families living on less than $2 a day can afford such luxuries.

But Greenpeace doesn't content itself with mere PR. In 2013, an organization in the Philippines who lists Greenpeace amongst its partners, used a tactic that has been used all over the world by Greenpeace – violently attacking and destroying agricultural research they oppose. The group, known as MASIPAG, claims to be a "farmer-led network," destroyed a field trial of Golden Rice. But local officials reported that the thugs who attacked the fields had been bused in from the city.

Shamefully, Greenpeace isn't alone in its support for the MASIPAG anti-GMO eco-terrorists. MASIPAG's list of supporters reads like a directory of misguided European church and government sponsored social justice and development groups. Perhaps one should put the words "so-called" before social justice. A short list of MASIPAG's funding sources include:
(1) The Swiss Catholic pastoral development group known as The Fastenopfer Catholic Lenten Fund; (2) Misereor, the German Catholic Bishops' Organization for Development Cooperation, which receives financial support from the German government; (3) The Swedish Society for Nature Conservation, which is funded by the Swedish Ministry for Foreign Affairs; (5) Trocaire, the official development agency of the Catholic Church in Ireland, which receives funding from the Irish, UK and EU governments; It should be stressed that MASIPAG is just one of a proliferating network of anti- GMO groups and assorted activists that are operating in the developing world, often with NGO and EU support.

In 2011 Greenpeace attacked GM wheat in Australia which was part of exciting research to enhance the health benefits of this staple food crop. It was heartening to observe the very widespread backlash and condemnation by the Australian community against this criminal vandalism of trusted research. On 14 July 2011, the CSIRO Experimental Station at Ginninderra in Canberra was broken into and research plants were cut down. Some of the GM crop trial plots were partially destroyed. Greenpeace admitted liability. This incident was investigated by the Australian Federal Police and two Sydney women were charged in relation to the incident, and both women later pleaded guilty to charges of damaging Commonwealth property. On 1 August 2012, CSIRO received a reparation payment of $282,560 from Greenpeace. In November 2012 the two women received a 9 month suspended sentence, to be of good behaviour for 12 months with $1,000 security, for each defendant. The question must be asked, when did so many of our "humanitarian" organizations become so disdainful about the lives of the desperately poor, whom they are supposed to be helping? How long have they been putting ideology over humanity? Do Greenpeace supporters understand that the conduct of the organization that they give to has been truly wicked? Patrick Moore, one of the early leaders of Greenpeace in the 1970's when it took account of science and respected human life, has broken with his old organization for just this reason. He now works to expose Greenpeace's actions in the developing world and has joined with Golden Rice inventor Ingo Potrykus in calling for the organization to be tried for crimes against humanity. So I say to my friends in Europe and in the United States: next time some young volunteer stops you on the street to ask for money for Greenpeace, ask them about Golden Rice. They'll want to talk about all the polar bears and whales they claim to have saved, but ask them instead about the millions of children that their organization is helping condemn to blindness and early death. It should also be recognized, however, that there are some humanitarian and environmental groups that are coming to recognize the important role that biotech can play in alleviating human suffering and spurring development. I'm thinking particularly of organizations such as Oxfam and the Nature Conservancy, whose initial opposition to GMOs has softened in the light of the overwhelming scientific evidence of their efficacy and safety. It's time for these organizations to step up and show leadership on this urgent humanitarian issue.

Where also are the UN organisations WHO, FAO, UNICEF – all with nutritional improvement and development mandates? They have recognized the scourge of vitamin A deficiency as a very major – and cheap to control – problem for the last 25 years. Undoubtedly current interventions have saved millions of lives. But VAD (Vitamin A Deficiency) induced preventable deaths continue, and now, as a result of Golden Rice, the half of the world where rice is the staple could benefit from a free nutritional trait. But, cowed by activist polemic, these huge and capable institutions have chosen not to believe in science.Here is my plea to them: You have rejected the world of activist myth for scientific fact. Now use your moral authority to appeal to your colleagues in the NGO community. Convince them to do the right thing and support giving the developing world the GM tools it needs to feed its growing, and too often malnourished, population.

The EU's Retreat from Science

Of course, the greatest offender of all is the European Union itself, which in a twisted version of neo-colonialism has imposed its affluent organic affectations and anti-scientific policies on Africa. The Kenyan-born Calestous Juma, professor of the practice of international development at Harvard Kennedy School, is a former executive secretary of the UN Convention on Biological Diversity, who has repeatedly slammed the EU for strong-arming African nations not to grow GM crops and threatening to cut off imports if they dare to assert their independence. I am proud that when I served as the Secretary of State for Environment, Food and Rural Affairs, we were able to convince the European Council of Ministers to change European policy so that now individual countries can decide for themselves whether to plant GMOs or not. This is a significant reform. I hope that when we look back over these years it will prove to have been a watershed moment, as nation after nation in Europe seizes the enormous opportunity offered by this exciting technology. But we shouldn't fool ourselves into thinking that one reform will solve the problem. The EU's retreat from science has become more like a rout. Just last November, the incoming president, Jean-Claude Juncker, refused to renew the contract for the professor of cell biology, Anne Glover, who had been so ably filling the role of the Commission President's scientific advisor. For months, Greenpeace and other NGOs had been conducting a concerted campaign against Professor Glover, largely because of her outspoken, science- based support of GMOs. In response, some forty leading scientific organizations and over 770 individuals sent a letter in support of Professor Glover and her position. It is emblematic of Europe today – once the birthplace of modern science and home to many of its greatest achievements – that the anti-science know-nothings won the day. And to think that the Lisbon treaty was meant to ensure that Europe developed a knowledge-based economy! No chance when scientific evidence is only merited with the same influence as public opinion, which as we have seen, is so easily influenced by political activists.

The European retreat from science is often dressed up as the precautionary principle, which has the advantage of sounding "sciency," but is in fact neither science-based nor by any legitimate definition a "principle." It's more like an impulse, or reflex, as clichéd and inept when it comes to the serious work of regulation as saying "better safe than sorry." No one can really define it adequately. The best its advocates can do is say that if something could possibly cause harm, ban it. Former Greenpeace UK director Stephen Tindale now urges Europe to "move on from the theological dispute with respect to GMO crops". Everyday, activists warn us of "risks". But everything, everywhere and always, could cause harm. You can drown in an inch of water. Coffee in large enough doses causes cancer. And let's not get started on cell phones. The EU finds itself in the ridiculous position that, according to its own pesticide regulations, it would have to ban coffee – and beer, and a thousand other consumer items – if they were sprayed on fields rather than sold in grocery stores.

The precautionary principle is so broad it effectively gives regulators the cover to ban or restrict anything at whim or according to whomever is exerting the most pressure. In other words, it replaces science-based regulation with politics. This drives the limits on the amount of pesticides in tap water, set at 0.1 parts per billion – the equivalent of one *paracetamol* tablet in an Olympic-sized swimming pool. We also saw the precautionary principle in action with startling clarity in the battle over *neonicotinoids*, or *neonics*, which activists accused of causing a "bee- *pocalyps*e," an imminent extinction of bees. Large-scale field studies and massive real-world science do not back up this claim. The EU's own science didn't back it up. Even at its most basic level, it was faulty: bee populations aren't falling at a rapid rate. They're not falling at all. For the last two decades that neonics have been on the market, bee populations have been rising, both in the EU and around the world. But the Green Blob was fierce on this one. I myself personally received 85,000 emails; very few of them were complimentary. Insisting, as we did at DEFRA, that policies be based on science – and the science did not support a ban – wasn't a popular position. Nor was it, as we know, the winning position. The EU Commission, as is its habit, caved in to the activists. Working with allies, I ensure that they couldn't muster a qualified majority for a ban among the member states, despite holding two votes on the issue. But democracy only goes so far in Brussels. So EU officials simply took it on themselves to make the final decision. They overrode their own scientists and banned neonics starting at the end of 2013. The result was predictable. I say that because it was indeed predicted by farmers and others who said the ban would remove their best defence against insect pests and force them to use older, less effective pesticides that are worse for bees. Which is precisely what has happened: despite multiple sprayings with pyrethroid, England's oil seed rape crop has been devastated, with losses over 40 per cent in some counties. Europe as a whole is looking at a 15 per cent reduction in rapeseed this year as a result of the ban. Another example of the precautionary principle creating realised risks.

Just recently this scandal developed further – some have called it Beegate – when a blogger in Brussels by the name of David Zaruck uncovered a memo that had mistakenly been left on the web by one of the scientists who was most vocal in pushing for a ban. That memo – the minutes of a meeting held back in 2010 – detailed in their own words how he and other leading scientists working for the EU-funded "Task Force" on neonics conspired to manufacture studies to support a ban. Perhaps one should put the word "scientists" here in quotes. Deciding on the outcome of your research before you even conduct it is not how science is supposed to be done. But we have apparently entered a brave new world in the EU where everything is backward.

For instance, there's the startling fact that the "technologically advanced" EU, which boasts incredibly fertile soils and extremely friendly climates for agriculture, cannot even feed itself and has been reduced to becoming a net importer of food. What that means, of course, is that we simply export our environmental footprint elsewhere. It also means Europe is not only not contributing, we are a net drag on humanity's foremost imperative in the 21st century – growing enough food to feed everyone on this planet, and to feed them well.

Today, nearly 805 million people do not have enough to eat, about one out of every nine people in the world. The majority of them are children, who will never have the same chance at a good life as others because hunger will stunt their development, shrink their innocent capacity for hope, and leave their small bodies prey to any number of terrible illnesses.One in three children in many developing nations today has been stunted by hunger. Sixty-six million attend school classes hungry every day. Poor nutrition causes nearly half of all the deaths of children less than five years of age – over three million children every year. This is the state of affairs that the EU's precautionary principle would effectively preserve. The world population is seven billion today and will burst through the ten billion mark by 2050 or sometime soon after. Not long ago, one of our English celebrities was delivering a petition to our Prime Minister protesting against genetically modified foods. Being a famous and wealthy fashion designer, apparently, has given her particular insight into this field. In the process she was asked by a BBC interviewer what she would say to those who can't afford to eat the high-priced organic food she recommends. Her answer was simple. They should simply "eat less."

Too many of our fellow human beings are already eating less. I don't believe that we, as Europeans, can really say that we belong to a humane society, a decent society, until every single one of them has access to good, nutritious food. Until every human being on this planet has enough to eat. That means that somehow we have to get right again with science. We need every possible tool available to meet this challenge. We simply cannot afford to take the most promising plant technologies off the table and keep them locked away in the regulatory drawer while children continue to go hungry.

Many centuries ago, science pulled the European continent out of the Dark Ages and established a whole new concept – one of on-going human progress. In our own time, the extraordinary science promoted by one-man, Norman Borlaug, accelerated that progress in the plant sciences just in time to save over a billion people from mass starvation in the 1960s and 70s. That was called the Green Revolution. We stand today at the beginning of a second Green Revolution – a period of extraordinary breakthroughs that can do the job if we let them.

We must let them. Despite the flack. Despite the propaganda. Despite the political setbacks we sometimes encounter. We must all push forward.

Myth versus fact; Green Blob versus Green Revolution. There is literally no challenge today that is more important. I commend African Nations for showing Europe the way. I am sure that science will overcome superstition. I am confident that together we will all succeed. Thank you very much!

Genetically modified (GM) plants II. Environmental risk assessment

(A) Introduction

Genetically modified organisms (GMO) interact with the environment through abiotic and biotic factors via trophic interactions. *Herbivores* are feeding on GM plants (direct effect), and are preyed by different predators (indirect effect) such as *spiders* (*e.g., Theridion impressum*), *ladybirds* (*e.g., Coccinella septempunctata*), and *rove beetles* (*e.g*., *Tachyporus hypnorum*).

The European Union (EU) and its member States are facing new challenges concerning the GM crop. According to the EU regulations, GM plants should be *environmentally risk assessed* before use (*EFSA, 2010a,b*). One of the key areas of *risk assessment* of GM plants is to assess its adverse effects on the *non-target organisms* (primarily the *arthropods*) (*Wolt et al., 2010*), in which processes the principal step is the so-called '*tiered approach'* (*USEPA, 1998, Romeis et al., 2006, 2008*). It is based on the consideration of *insecticidal protein* producing plants, and their tests in laboratory, glass-houses, and semi-field and field conditions (*Poppy, 2000; Wilkinson et al., 2003; Garcia-Alonso et al., 2006; Rose, 2007*). Ecotoxical *tiered approach* comprises the assessment of intended effects of *GM plants* based on three steps: *Tier1*: Laboratory studies (*in vitro* and *in planta*) for the worst case scenario; *Tier 2*: Semi-field studies in glass houses an cages which are close to real conditions; and *Tier 3*: Field studies under natural conditions. GM plants carry several points of economic, coexistence, environmental, animal feeding and food security, ethical, personal, and emotional concerns. This *Chapter* reflects a general scientific approach and is independent from concrete growing, economic, political and sociological relations and is exclusively to contribute to the scientific *risk assessment*.

(B) The environmental risk assessment (step-by-step approach) of GM plants

GM plants are capable of causing potential adverse effects to the environment. An important step in the *problem formulation* is to identify the aspects of the environment that need to be protected from *hazardous factors*. Nature needs of environmental protection concerning conservation of *natural habitats* of wild *fauna* and *flora*, and natural resources of *biodiversity* (*EFSA, 2010ab*). In the case of GM plants, the environment is exposed to transgene(s), which may spread and initiate *seven main sources of risks* such as Plant-to-plant *gene flow (vertical gene transfer)*; Plant-to-microorganisms gene flow (*horizontal gene transfer*); Interactions of the GM plant with target organisms; Interactions of the GM plant with non-target organisms; Impacts of the specific management techniques; Effects on biogeochemical processes; and Effects on human and animal health.

1. Plant-to-plant gene flow (vertical gene transfer)

In vertical gene transfers (VGT) the DNA flows from parent to offspring. In the case of a GM organism, potential adverse effects of *vertical gene flow are*: (a) Increased *fitness* of GM plant or of the transgenic (introgressed) *wild relatives* may make them more persistent, exacerbating weed problems which may need to be controlled by more complex *weed control* strategies causing environmental harm. (b) The increased fitness of *transgenic feral plants* or of transgenic (introgressed) wild relatives in semi-natural or natural habitats may reduce the diversity/abundance of valued flora and fauna. Native plant species may be displaced, which in turn, might affect species that use those plants as food, shelter, etc. (*EFSA 2010b*).

2. Plant- to-microorganisms gene flow (horizontal gene transfer)

During the *horizontal gene transfer* (HGT) an organism incorporates its genetic material from another organism without being the ancestors/offspring of that organism. The evaluation of the effect of this HGT contains analysis of the transfer of recombinant plant DNA to initially receiving micro-organisms and potential transfer to other organisms (microorganisms, or plants) and the potential consequences of such a gene transfer for the environment and human and animal health (*EFSA, 2010b*).

3. Interactions of the GM plant with target organisms

Target organisms (TO) are those on which a GM plant are intended to act and are usually plant pests or pathogens. All other organisms are considered as non-target organisms. The investigation/analysis of resistance developed by the target organisms is very important (*EFSA, 2010b*).

4. Interactions of the GM plant with non-target organisms

ERA considers the possible immediate and/or delayed environmental impact resulting from direct and indirect interactions of GM plants with non-target organisms (NTOs) (EFSA 2010b). Examples of non-target invertebrate organisms occurring in agro-ecosystems detailed by their ecological role are listed in *Table 1*.

5. Impacts of the specific management techniques The introduction of GM plants for cultivation may require specific management and cultivation

6. Effects on biogeochemical processes

Biogeochemical processes are connected with the movement, transformation and storage of energy, water, carbon, nitrogen and other elements in the ecosystems. Problem formulation should cover principally two scales, the production site, for example a field, in which the GM plant is grown; and the wider environment with which the field interacts through exchanges of energy, elements and materials. With respect to biogeochemical processes in the production site, the evaluation should address the potential impact of GM plants through factors such as:

(a) Release of *recombinant gene* products or GM specific metabolites into the *plant-soil system*, which may directly influence the soil fertility, nutrient transformations and *food webs*; *(b)* Altered movement of other compounds from roots to soil, which may directly influence soil fertility, nutrient transformations and soil food webs; *(c)* Altered plant litter that decomposes differently from that of non-GM plants due to either the presence of specific compounds (*e.g.,* toxic metabolites), or altered concentration of substances resistant to decomposition; *(d)* Altered uptake and cycling of plant nutrients within the *plant-soil system*, including the *fixation of atmospheric nitrogen* (*see Chapters 10 and 17*) (*EFSA, 2010b*).

Table 1. Examples of functional groups of different organisms (*EFSA, 2010a*) (*see Appendix) 1, 2*)

Functional group		Examples of taxonomic groups
Herbivores		Phloem-feeders: aphids (*Hemiptera: Aphididae*), leafhoppers (*e.g., Hemiptera: Cicadellidae*)
		Cell-content feeders: thrips (*Thysanoptera: Thripidae*), spider mites (*Acarina)*
		Chewing: leaf beetles (*Coleoptera: Chrysomelidae*), *Lepidoptera* larvae, *Diptera* larvae
Natural enemies	Predators	Beetles: *Coleoptera* (*e.g., Coccinellidae, Carabidae, Staphilinidae*)
		Predatory bugs: *Heteroptera* (*e.g., Nabidae, Anthocoridae*)
		Predatory flies: *Diptera* (*e.g., Syrphidae*)
		Lacewings: *Neuroptera* (*e.g., Chrysopidae, Hemerobidae*)
		Spiders: *Araneae*
		Mites: Acarina (*e.g., Phytoseiidae*)
	Parasitoids	Hymenoptera (*e.g., Ichneumonidae, Braconidae, Aphelinidae*)
	Parasites and Pathogens	Bacteria, fungi, viruses
	Entomopathogenics	*Nematoda* (*e.g. Heterorhabditidae, Steinernematidae*), pathogenic microorganisms
Pollinators		Solitary and social bees (*Hymenoptera: Apidae*), hover flies (*Diptera: Syrphidae*); Coleoptera (*e.g., Curculionidae, Scarabaeidae*)
Decomposers		Diptera larvae (*e.g,. Phoridae, Sciaridae*), Nematoda (*e.g., Rhabditidae, Dorylaimidae*), springtails (*Collembola*), mites (*Acarina*), earthworms (*Haplotaxida: Lumbricidae), Isopoda*, microorganisms
Plant symbionts		*Rhizobacteria*, mycorrhiza

7. Effects on human and animal health

In the case of GMO risk assessment, it is required to assess whether the GM plant and its products might be harmful for human and animal health. If a potential hazard has been detected, the risk to persons working with the GM plant, coming into contact with it or exposed to products from processed plants should be assessed (*EFSA, 2010b*).

(C) Post-Market Environmental Monitoring Plan

The monitoring plan should be targeted rather than considering every possible environmental aspect. The aims of environmental monitoring of the GM plant are: (1) to study any possible adverse effects of the GM plant identified in the formal risk assessment procedure, and (2) to identify the occurrence of adverse unforeseen effects of the GM plant or its use which were not anticipated in the ERA (*EFSA, 2010b*).

In the field (Tier 3), for instance in a maize stand (*Fig. 1a*) target organisms (TO) and non-target organisms (NTO) can be monitored by different sampling methods. *Arthropods* occurring on plant surface can be observed visually (*Fig. 1b*). Flying insects (aphids, thrips, leafhoppers, flea beetles, predatory flies etc.), can be collected by yellow sticky card (*Fig. 1d*). Soil dwelling arthropods (ground beetles, rove beetles, mites, spiders) can be sampled by pitfall trap (*Fig. 1c*), or by litterbag method (*Fig. 1e*). The arthropods collected by litter bag can be retrieved using Berlese-funnel (*Fig. 1f*).

Figure 1. Sampling methods of target and non-target arthropods occurring on maize (*Zea mays*) *(a)*: Visual observations *(b)*, pitfall trap *(c)*, yellow sticky card *(d)*, litterbag method *(e)*, and Berlese-funnels *(f)*.

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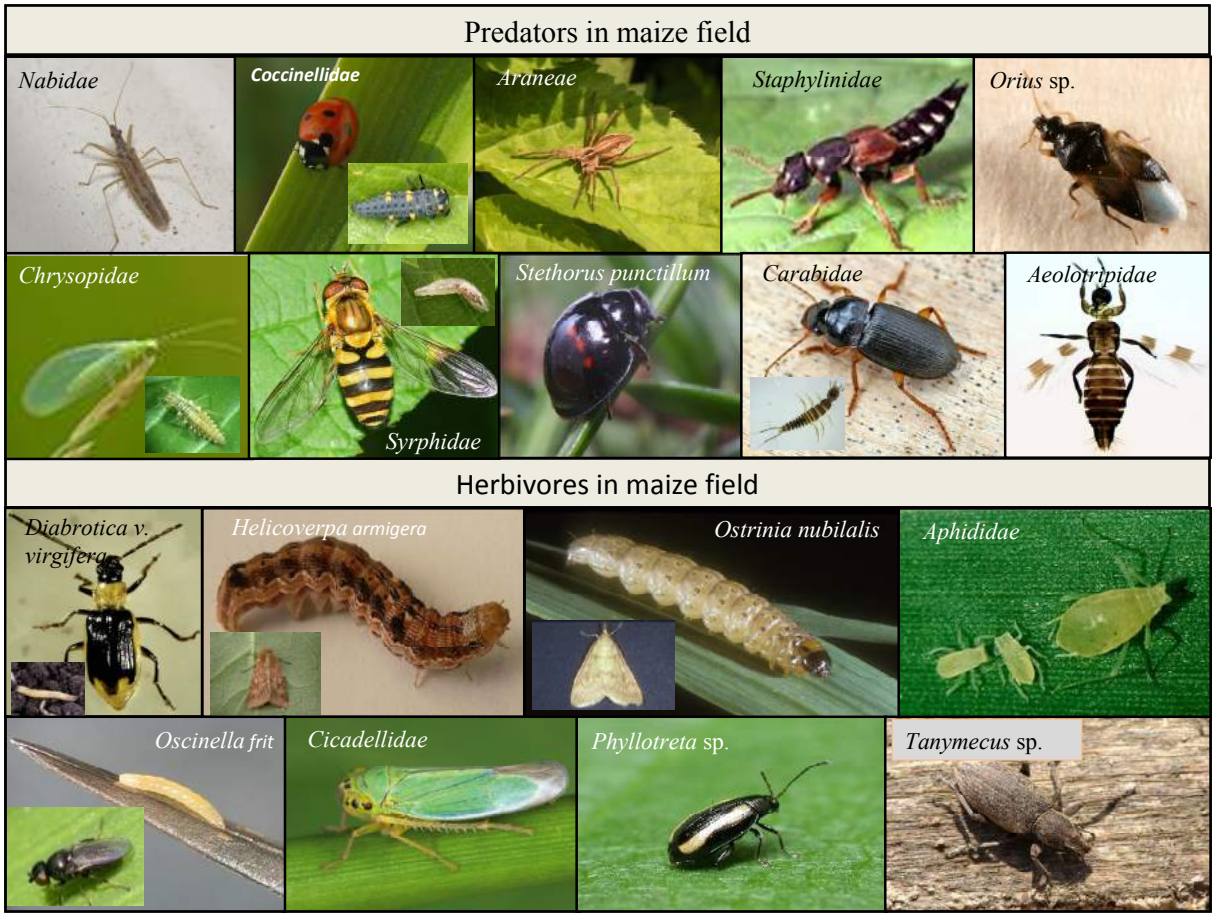
Appendix 1. Samples of insect *Predators* and *Herbivores* in maize field:

Predators: Damsel bugs (*Nabidae*). Ladybirds/Ladybeetles/Ladybugs/Coccinellid beetles (*Coccinellidae*). Spiders (*Araneae*). Rove beetles/Staphilinid beetles (*Staphilinidae*). Pirate bugs/Anthocorid bugs: *Orius* spp). Green lacewings (*Chrysopidae*). Predatory flies/Syrphids (*Syrphidae*). Spider mite destroyer coccinellid (*Stethorus punctillum).* Ground beetles/Carabid beetles (*Carabidae).* Predatory thrips (*Aelothripidae*).

Herbivores: Western corn rootworm (*Diabrotica virgifera virgifera*). Cotton bollworm (*Helicoverpa armigera*). European corn borer (*Ostrinia nubilalis*). Aphids (*Aphididae*). Frit fly (*Oscinella frit*). Leafhoppers (*Cicadellidae*). Flea beetles (*Phyllotreta* spp). Corn weevils (*Tanymecus* spp.).

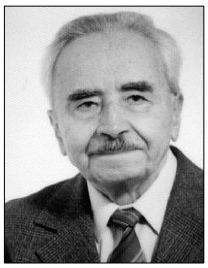


Appendix 2. Putative roles of GM plants in the agro-ecosystems - *Coevolution* vs. *Sequential evolution*

The *reciprocal evolutionary relationships* of *insects* (*e.g.,* butterflies) and their *food plants* have been examined on the basis of an extensive survey of *food plant choice*. The evolution of *secondary plant metabolites* and the subsequent *evolutionary responses* of *phytophagous* organisms have clearly been the dominant factors in the evolution of *phytophagous* insects. The secondary plant metabolites have probably been also critical in the evolution and phylogenesis of *angiosperm plant species*.

The examination of broad patterns of *coevolution* (or *sequential evolution*) permits several levels of predictions and shows promise as a route to the understanding of *community evolution*. The importance of *plant-herbivore interactions* in generating *terrestrial diversity* is a possible consequence. For instance, the *rich diversity of tropical communities* may be traced to the *warm climates* toward *poikilothermal phytophagous insects* (*Paul R. Ehrlich PR, PH Raven (1964) Butterflies and Plants: A Study in Coevolution. Evolution 18: 586-608*). Very significant Hungarian entomologists (*G Szelényi, T Jermy, and G Jenser*) have investigated *coevolution* vs. *sequential evolution* in agro-ecosystems.

Szelényi, Gusztáv (1904-2014)



Jermy, Tibor (1917-1982)

Jenser, Gábor (1931-2015)

C_4 photosynthetic trees

Introduction

C4 type of plant carbon uptake ('fixation' from the air) (*e.g.* sugarcane) is one of the three sub-types of photosynthesis including **C3** (*Calvin cycle*) and *CAM* (*Crassulacean Acid Metabolism*) (*e.g.,* pineapple). It is named for the 4-carbon molecules (*e.g. malate*) present in the first molecular product of the biochemical process, in contrast to the 3-carbon molecule products (*glycerate-3-P*) in *C3* plants. Both, C4 and CAM types of photosynthesis also ends in the *C3* photosynthesis (*Calvin cycle*), so we may refer them as 'C4-C3' and 'CAM-C3' photosynthesis, and just use the short forms (C4 and CAM) (*Table 1*).

Table 1. The main characteristics of the three types of photosynthesis (C3; C4, and CAM)

Characteristics	C3 plants	C4-C3 plants	CAM-C3 plants
The % of plant species	85 %	3 %	8 %
Types of Photosynthesis	C3	C4-C3	CAM-C3
CO_2 fixation pathway	via C3 cycle only	C4 in the mesophyll cells then C3 in the bundle sheath cells. Mainly C4 grasses and sedges, about 79% of all C4 plants.	Temporally C4 at night, C3 at day time. Mostly succulents.
Leaf anatomy	Large air spaces bordered by loosely arranged spongy mesophyll cells.	Leaf veins are surrounded by thick-walled BSC (*Bundle Sheet Cells*) and surrounded by thin-walled *Mesophyll Cells* (*kranz/wreath/koszorú leaf anatomy*).	Mesophyll cells have large, water-filled vacuoles.
Stomatal movement	Stomata open at daytime, close at night.		Inverted stomatal cycle (open at night, close in the day).
Environmental effect	Temperate Zone.	Tropical or semi-tropical zones, high light intensity, high temperature, drought conditions.	Desert or arid (xeric) habitats.

About *7,600* plant species (only angiosperm) use C4 carbon fixation, which represents about **3%** of all terrestrial plant species (C4 photosynthesis has not been developed 'yet' in Gymnosperms) (*Table 1*).

C4 carbon fixation dominates in monocots (40% of monocot species follows C4), compared to only 4.5% of dicots. However, species of only 3 monocot families utilize C4 carbon fixation compared to 15 of dicots (*Table 2*). About 46% percent of grasses are *C4*. These include the food crops of *maize, sugar cane, millet*, and *sorghum*.

Of the dicots, the order *Caryophyllales* contains the most species; of them *Chenopodiaceae* use *C4* carbon fixation the most, with 550 out of 1,400 species of the family. About 250 of the 1000 species of the related *Amaranthaceae* also use C4 (*Table 2*).

Table 2. List of the angiosperm plant families / orders having C4-species (*Sage, 2001*).

Plant Family	Orders
Monocots (# of C4 species / genera), (% of C4 species / % of the Family)	
1 *Cyperaceae* (1300/289), (18/27)	Poales
2 *Poaceae* (4600/372), (61/46)	
3 *Hydrocharitaceae* (1/1), (0.1/1)	Alismatales
Dicots (# of C4 species / genera), (% of C4 species / % of the Family)	
1 *Acanthaceae* (80/1)	Lamiales
2 *Scrophulariaceae* (14/1)	
3 *Chenopodiaceae* (550/45), (7/39)	Caryophyllales
4 *Amaranthaceae* (250/11), (3/25)	
5 *Aizoaceae* (30/5)	
6 *Caryophyllaceae* (50/1)	
7 *Molluginaceae* (3/1)	
8 *Nyctaginaceae* (25/3)	
9 *Polygonaceae* (80/1)	
10 *Portulacaceae* (70/2), (1/16)	
11 *Asteraceae* (150/8) (2/1.3)	Asterales
12 *Boraginaceae* (40/1)	Boraginales
13 *Capparidaceae* (2/1)	Brassicales
14 *Euphorbiaceae* (250/1), (3/5)	Malpighiales
15 *Zygophyllaceae* (50/4)	Zygophyllales

Biochemistry of C_4 photosynthesis

C4 photosynthesis is the most efficient form of plant photosynthesis on the terrestrial Earth, due to its ability to concentrate CO_2, delivered to the enzyme *ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo)*, which is responsible for "fixing" CO_2.

During this biochemical process,

the oxygenation of *ribulose 1,5-bisphosphate* (RuBP) is suppressed (this is the photorespiration, catalyzed by enzyme *RuBisCo*, which can effectively fix O_2 also, instead of CO_2).

Technically, the first experiments (in 1950s) indicated that some plants do not use only the C_3 carbon fixation, instead, they produce *malate* and *aspartate* in the first step of carbon fixation (*Karpilov, 1960*) and (*Kortschak et al., 1965)*. The C_4 pathway was finally elucidated by *MD Hatch* and *CR Slack* in Australia in 1966 ('*Hatch-Slack* pathway') (*Fig. 1a*). As C_4 photosynthesis is an *adaptation* to warm temperature and low atmospheric CO_2 concentration, C_4 evolution has been seen as a consequence of past atmospheric CO_2 decline, as it happened about 32–25 million years ago (Mya) in the Oligocene period. The first transition from C_3 to C_4 photosynthesis may have occurred in the *Chloridoideae* subfamily of *Poaceae*. C4 plants also have higher water use efficiency (WUE) and nitrogen use efficiency (NUE) that are typically 1.3–4 times greater than in C_3 plants.

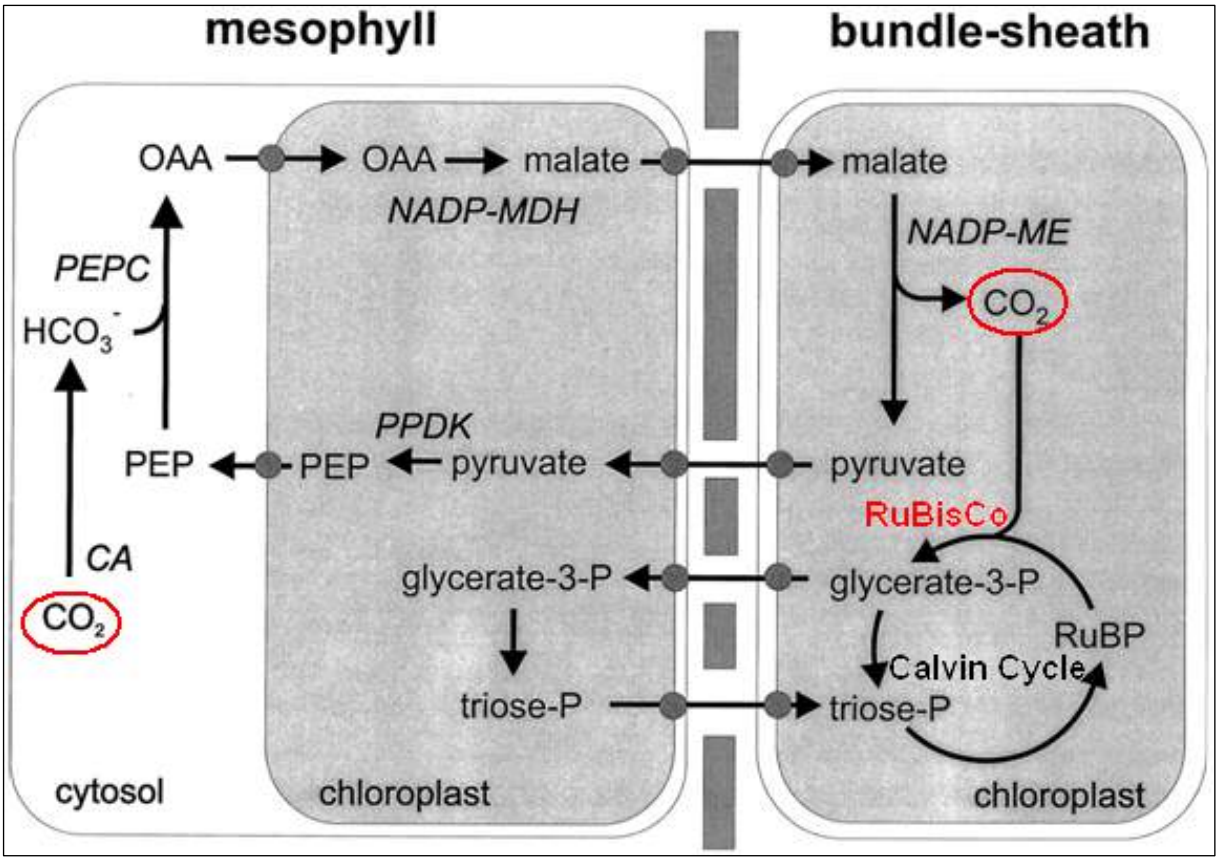
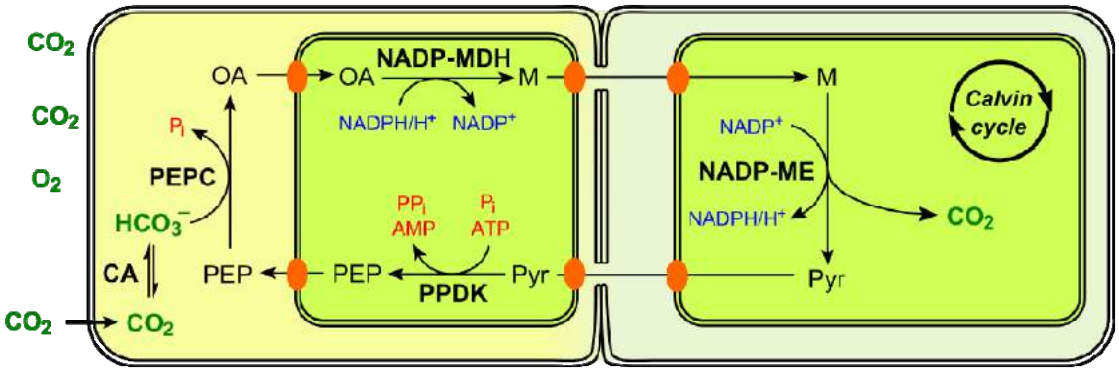


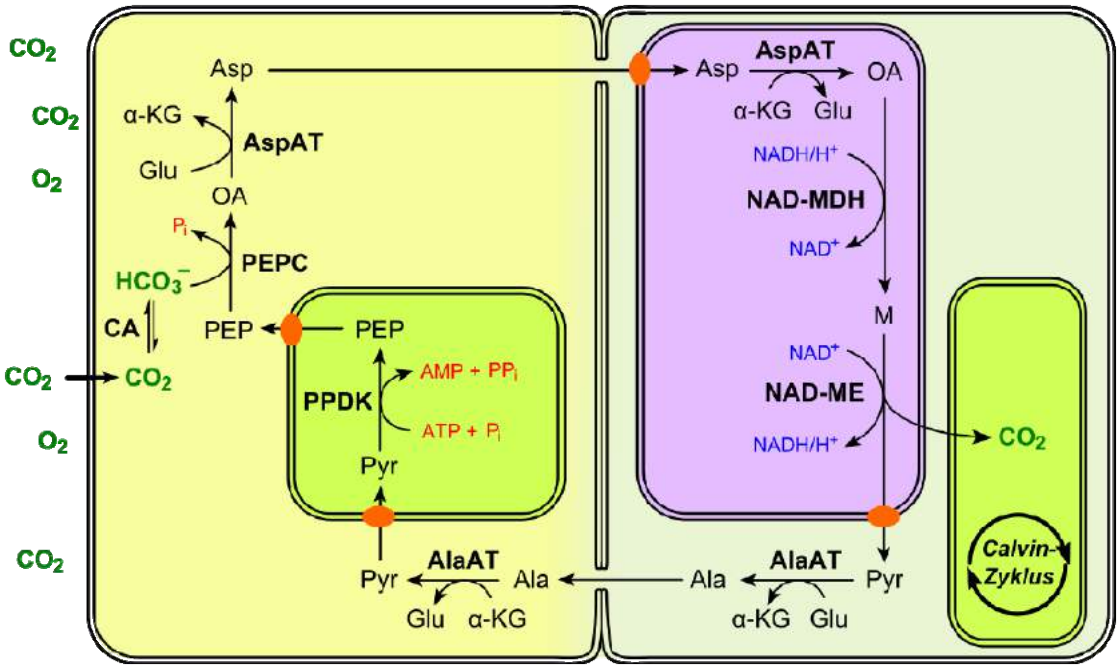
Figure 1a. C4-photosynthesis in the *mesophyll* and *bundle-sheath* leaf cells. The CO_2-concentrating mechanism in an *NADP-ME* type C_4 (*NADP-Malic Enzyme*) plant such as sugar cane (*Saccharum ssp.*): *PEPC* (*PhosphoEolPyruvate carboxylase*) in the *cytosol* of *mesophyll celles* fixes bicarbonate from CO_2 by *CA* (*Carbonic Anhydrase*) and produces OAA (*OxaloAcetAte*). OAA *is reduced to malate* in the mesophyll chloroplasts by *NADP-MDH* (*NADP-Malate DeHydrogenase*). Malate is transported to the bundle-sheath via the plasmodesmata and is decarboxylated in the bundle-sheath chloroplasts by *NADP-ME*. Finally, the released CO_2 is fixed by enzyme *RuBisCo* (*Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase*) in the Benson–Calvin cycle. PEP is regenerated from pyruvate by *PPDK* (*Pyruvate Pi DiKinase*) in the mesophyll chloroplast (combined actions of *PPDK* and adenylate kinase). *Glycerate-3-P* from the *Calvin Cycle* is shuttled to the mesophyll cells for reduction to *triose-P* (membrane transporters are indicated by filled circles). (*Leegood, 2002, J Exp Bot 53:581-590*).

Leaf anatomy generally is associated with the C_4 photosynthesis, called *kranz leaf anatomy*, which is characterized by rings of *Bundle Sheath Cells* (BSC) cells round the vascular cells, and surrounded by *Mesophyll Cells* (MC) (*Fig. 2*). Some C_4 species have no distinct bundle sheath tissue (*e.g.*, *Suaeda aralocaspica*, *Bienertia cycloptera*, *B. sinuspersici* and *B. kavirense*, all chenopods).

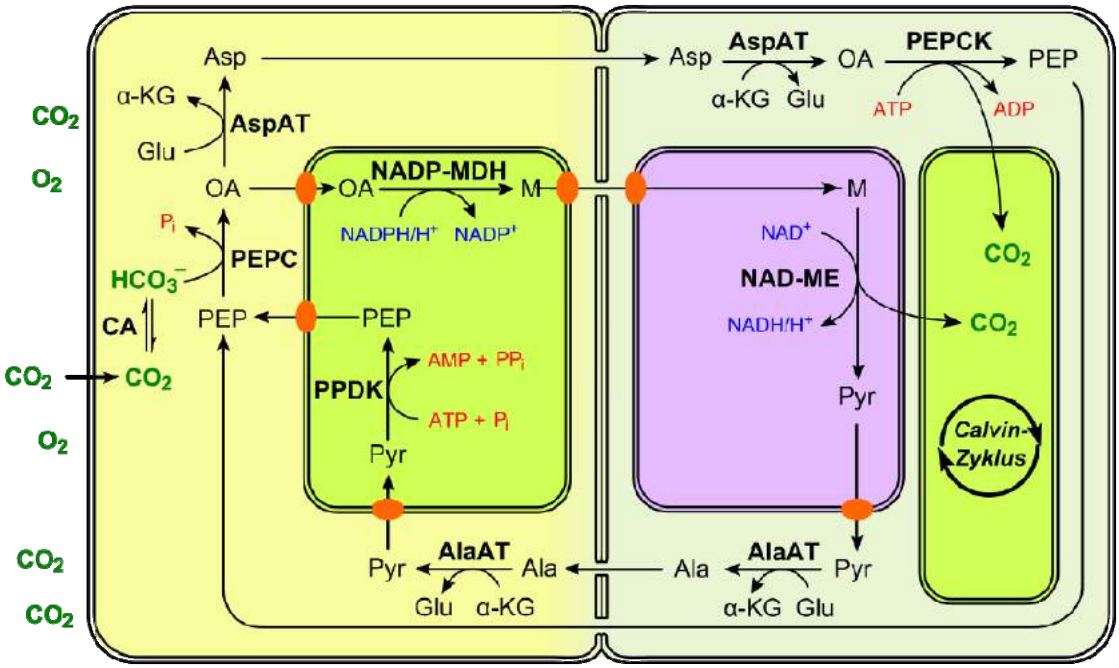
During *C4*-photosynthesis, atmospheric CO_2 reaches MC cells via the plant stomata and is converted into HCO_3^-, which molecule is then fixed on *phosphoenolpyruvate* (*PEP*) by *PEPC* (*PhosphoEnolPyruvate Carboxylase*) into oxaloacetate (OAA). This four-carbon organic acid is then transported to the BS cells via a biochemical path that varies among C_4 plants. Therein, decarboxylating enzymes *NADP-ME* release CO_2 from the four-carbon compound, which can then enter the *Calvin Cycle* as in all photosynthetic plants through its fixation by *RuBisCo* (Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase). *RuBisCo* is the main enzyme that catalyzes the reaction that incorporates ('*fixes*') CO_2 (carbon dioxide) into the *Calvin Cycle*. The 560 kilo Dalton (kDa) enzyme consists of two protein subunits, *large chain* (55 kDa) and *small chain* (13 kDa).



(a) C4 pathway: NADP-ME type



(b) C4 pathway: NAD-ME type



(c) C4 pathway: PEPCK type

Figure 1b. The three types of C_4 photosynthesis (for details see *Fig.1a*) *https://en.wikipedia.org/wiki/C4_carbon_fixation.*

The large chain is coded by the chloroplast *rbcL* gene, and the small chain is encoded in the cell nucleus. The *RuBisCo* enzyme can form large-small-, and also large-large subunit dimers.

Three types of biochemical pathways of the C4 photosynthesis were identified based on the decarboxylating enzymes: *NADP–ME* (Nicotinamide Adenine Dinucleotide Phosphate–Malic Enzyme), *NAD–ME* (Nicotinamide Adenine Dinucleotide–Malic Enzyme), and *PCK* (Phosphonolpyruvate CarboxyKinase) (*Fig. 1b*) (*Mol Biol Evol, 2009, 26: 1909-1919*).

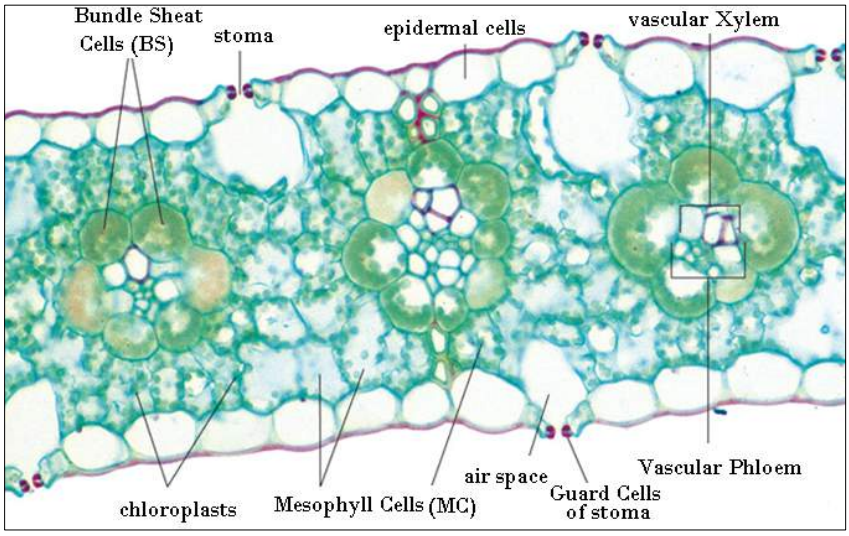


Figure 2. Cross section of a leaf of C_4 photosynthetic plant *ttp://www.majordifferences.com.*

C_4 genes

In the C_4 photosynthesis, PEPC is responsible for the initial fixation of atmospheric CO_2 into organic compounds. However, PEPC-encoding genes (*ppc* genes) form a ubiquitous multigene family not restricted to C_4 plants. These genes encode different enzymes responsible for various functions, mainly anaplerotic (*Latzko and Kelly, 1983*).

One of the first *ppc* gene sequences of *Cyperaceae* (EMBL # AB085948) was available in public databases. Besides grasses, *ppc* genes of eudicot C_4 systems have also been investigated, *e.g.*, in the genus *Flaveria* (*Asteraceae*) and *Alternanthera* (*Amaranthaceae*). The sedge family (*Cyperaceae*) is the second most important C_4 family with approximately 1,500 C_4 species (more than 20% of all C_4 plants).

Primers were developed recently amplify a long *ppc*-1 cDNA segment in monocots (2000 bp) *ppc*-850F (CAG TTC TCY TCY TGG ATG GG) and *ppc*-2872R (GCR GCR ATR CCC TTC ATG GT). Based on *ppc* sequences isolated from *Cy. eragrostis* (EMBL # FM208065 and FM208066) and *E. vivipara* (AB085948), primer pair was designed: *ppc*-1336F (TTT GGT CTC TCT YTT GTG CGT C) and *ppc*-2727R (GTT SGG GTC CCT GAT YCT TTT G).

Table 3. List of CAM plant families/orders

Monocots		*Dicocots*	
1. *Alismataceae*	Alismatales	1. *Apiaceae*	Apiales
2. *Hydrocharitaceae*		2. *Asteraceae*	Asterales
3. *Araceae*		3. *Moringaceae*	Brassicales
4. *Agavaceae*	Asparagales	4. *Aizoaceae*	Caryophyllales
5. *Asphodelaceae*		5. *Cactaceae*	
6. *Orchidaceae*		6. *Didiereaceae*	
7. *Ruscaceae*		7. *Portulacaceae*	
8. *Bromeliaceae*	Poales	8. *Cucurbitaceae*	Cucurbitales
9. *Cyperaceae*		9. *Apocynaceae*	Gentianales
10. *Commelinaceae*	Commelinales	10. *Rubiaceae*	
		11. *Ebenaceae*	Ericales
		12. *Geraniaceae*	Geraniales
		13. *Clusiaceae*	Malpighiales
		14. *Euphorbiacea*	
		15. *Passifloraceae*	
		16. *Gesneriaceae*	Lamiales
		17. *Lamiaceae*	
		18. *Oxalidaceae*	Oxalidales
		19. *Piperaceae*	Piperales
		20. *Celastraceae*	Rosids
		21. *Crassulaceae*	Saxifragales
		22. *Sapindaceae*	Sapindales
		23. *Convolvulaceae*	Solanales
		24. *Vitaceae*	Vitales

C_4 trees

C4 photosynthesis has developed in the woody species, *e.g., Chamissoa* (an *Amaranthaceae* genus of woody shrubs and lianas of wet forests in the neotropics). However, there is only one 'robust tree' with *C_4* photosynthesis, the only *C_4* species that forms a tree in the sense of high apical dominance from an early age is *Chamaesyce forbesii* (*Euphorbiaceae*) a rare understory tree from Hawaii that is less than 5 m tall. The unusual nature of *C. forbesii* is attributed to its evolution on a novel *island habitat* with low competitive pressure. Because it has evolved a tree-like growth habit, it demonstrates that *C_4* species are capable of evolving true arborescence.

In total, there are 5 to 10 species of sub-trees and arborescent shrubs of genera *Haloxylon* and *Calligonum*; and there are further 500 species of shrubs and sub-shrubs, all xerophytic, and many halophytic. The woody species of *Haloxylon aphyllum* grows in Central Asia. Woody *Atriplex canescens* grows in Nevada (USA).

The capacity for secondary growth of woody species demonstrates the potential to evolve the arborescent life form, since the key difference between shrubs and trees is the degree of apical dominance. However, only limited numbers C4 species approach arborescence, most are desert shrubs that only become short stature trees with advanced age.

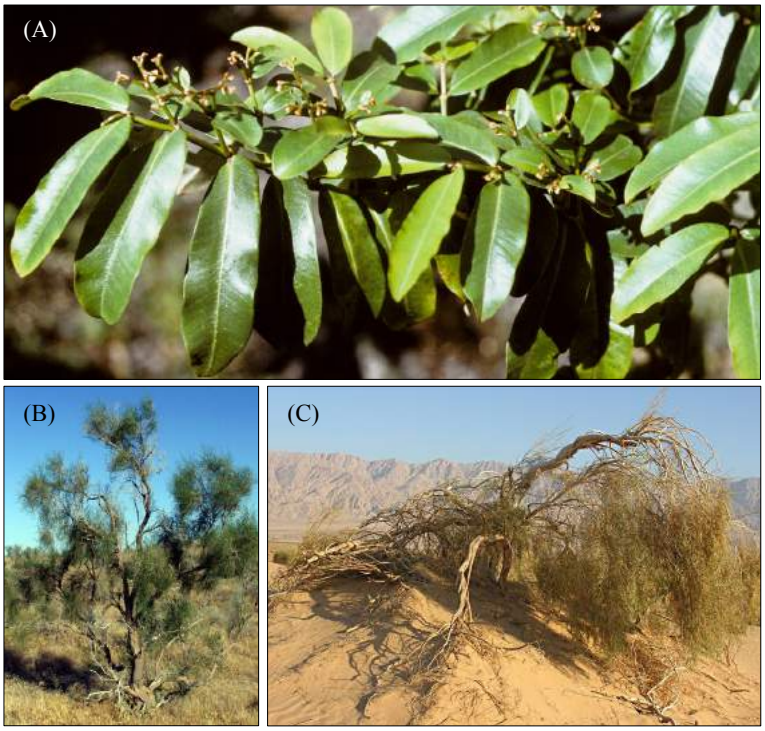


Figure 3. Woody species with *C_4* photosynthesis. (A) *Euphorbia (Chamaesyce) forbesii* (*Euphorbiaceae*) (Hawai). (B) black saxaul (*Haloxylon aphyllum*) and (C) white saxaul (*Haloxylon persicum*) (Central Asia).

The best examples are black saxaul (*Haloxylon aphyllum*) and white saxaul (*Haloxylon persicum*), which are halophytic xerophytes from the hot, sandy deserts of Central Asia (*Fig. 3*). No of *C_4* species evolved also among vines (*e.g.*, *Vitis*).

CAM photosynthesis

Plants using full *CAM* the stomata in the leaves remain shut during the day to reduce evapotranspiration, but open at night to collect carbon dioxide (CO_2). The CO_2 is stored in the four-carbon acid *malate* in vacuoles at night, and then in the daytime, the *malate* is transported to chloroplasts where it releases CO_2 for photosynthesis. This metabolism was first studied in plants of the *Crassulaceae* family of succulents (*Table 3*). In *CAM*, the carbon dioxide is fixed in the cytoplasm of mesophyll cells by a PEP reaction similar to that of *C4* pathway. But, unlike the *C4* mechanism, the resulting organic acids are stored in vacuoles for later use; that is, they are not immediately passed on to the Calvin cycle.

C_4 crops

Below 30° latitude, annual biomass production lists only one *C_3* species producing over 44 t ha^{-1} $year^{-1}$. In contrast, 12 *C_4* species with annual biomass yields over 50 t ha^{-1}; seven of these *C_4* species produce over 60 t ha^{-1} of biomass.

Only *C_4* species (like maize) have been recorded as producing over 80 t ha^{-1}, and as the top, the record yield for plant production per year is near 100 t ha^{-1} by tropical *C_4* grasses such as *Echinochloa polystachya* and *Pennisetum purpureum* (Napier grass) (*Table 4*).

Table 4. The photosynthetic pathway of the most valuable crops in the world with product values (US$ billion) (*FAOstat, 2011*).

C_3 crop		*C_4 crops*	
○ Rice, paddy	(136)	• Maize	• (40)
○ Wheat	(83)	○ Sugarcane	○ (35)
○ Soybeans	(47)	• Sorghum	
○ Potatoes	(38)	• Pearl millet (*Pennisetum glaucum*)	(100)
○ Cotton, lint	(34)	• Foxtail millet (*Setara italica*)	
○ Tomatoes	(32)	• Proso millet (*Panicum miliaceum*)	
○ Grapes	(31)	• Finger millet (*Eluasine coracana*)	
○ Apples	(20)	• Tef (*Eragrostis tef*)	
○ Peanuts	(18)	• Fonio (*Digitaria exilis*)	
○ Cassava	(16)	○ Amaranth (*Amaranthus* spp.)	

J Sheehy at IRRI predicted that "converting" *rice* into a C_4 plant could produce sufficient yield increases to meet the needs of Asia by 2050. *Global warming* also generates new considerations for C_3 *vs.* C_4 plants (*Fig. 4, 5*).

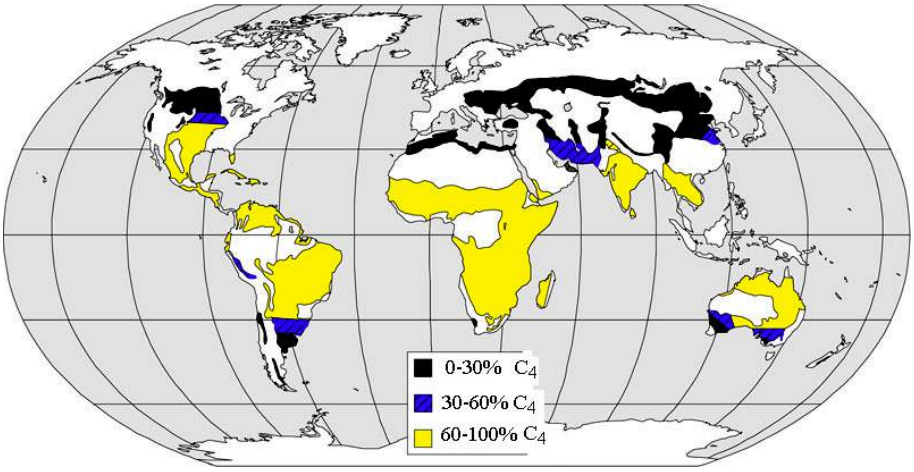


Figure 4. Distribution of C_3 and C_4 plant of the World. (*Ehleringer and Forseth, 1980). Solar tracking by plants*. *Science* 210: 1094–1098. *http://6e.plantphys.net/topic09.03.html).*

In Antarctica, near the South Pole, scientists are drilling into the ice to recover the atmospheric compositions of the past 600,000 years (*Fig. 5*). These ice core data show that levels of greenhouse gases is low in *glacial periods* and increase in *interglacial periods* as Earth moved in and out of ice ages. These repeated *glacial cycles* reflect the absorption of carbon dioxide into the oceans during *glacial* periods, and its release to the atmosphere during *interglacial* periods.

As a result of burning fossil fuels, atmospheric carbon dioxide levels have increased. Fossil-fuel consumption rates have accelerated since the 1950s, resulting in atmospheric carbon dioxide levels today that greatly exceed the levels recorded in the ice core records, which also show that temperature and atmospheric carbon dioxide levels are tightly correlated with each other.

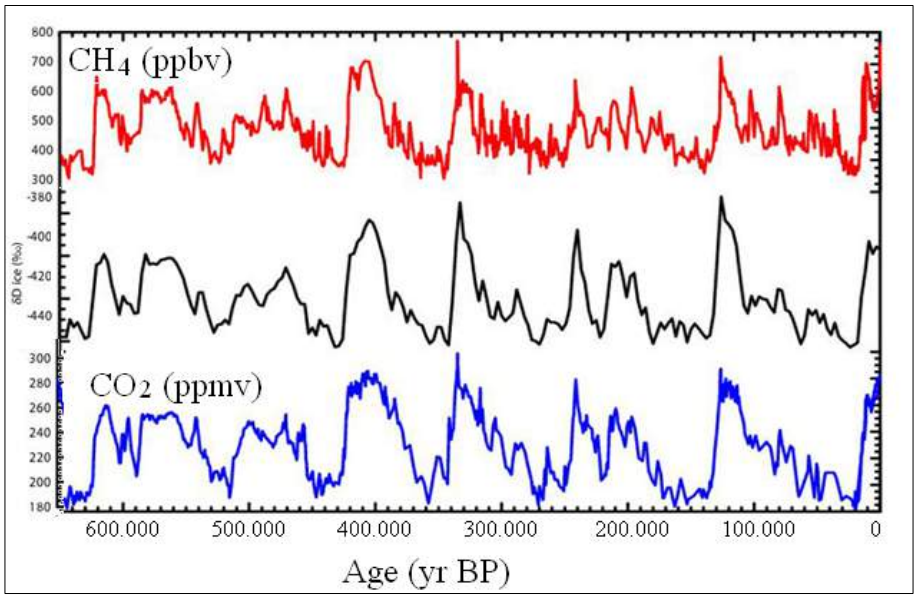


Figure 5. Changes of the concentration of greenhouse gases CH_4 (*up*) and CO_2 (*down*) of the ancient air trapped as *ice bubbles* for the past 600,000 years. The *middle line* represents the hydrogen isotope ratio of the ice which is a good proxy for the *air temperature* record

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Monoecious *vs.* Dioecious trees - Basics of Digital Seed Morphometry

(A) Introduction

None of the *gymnosperm* species (all the extant of about 860 'coniferous-like' species) has bisexual (*syn.*: hermaphrodite) flower, they have only unisexual - either *staminate* (male: ♂) or *pistillate* (female: ♀) flower that develop either on the same plant, *i.e., monoecious* species (*e.g., Pinus*), or on separate plants, *i.e., dioecious* species (*e.g., Taxus, Ginkgo*). This indicates the ancestral property of the unisexual flowers over bisexual flower, however, *angiosperms* with bisexual flowers tend to '*mutate back*' during the evolution towards *monoecy* and *dioecy* coupled with sex chromosome development to promote outcrossing and for escaping inbreeding. In the opposite, of the total *Salix* (300 species) and *Populus* (30 species) of the Dioecious *Salicaceae*, two species (*S. maritima* and *P. lasiocarpa*) 'tend' to change to Monoecious (*Field et al., 2012. Evolution 67-3:661-672)*.

All gymnosperm species are trees (there is no 'herbaceous gymnosperm'), wind pollinated, and all of them are either *Dioecious* or *Monoecious* (the bisexual flower has not been evolved yet in Gymnosperms). Angiosperms mostly have bisexual flowers, but there are also *Dioecious* and *Monoecious* species among them (*Fig. 1*).

Dioecious plant species account for 6% (14,620) of the total 240,000 (< 300,000) flowering plant species in 7.1% (959 of 13,500) of genera, and 43% (157 of 365) of families (*Table 1*).

Several Dioecious crops gained huge agronomical importance; *e.g.,* all-male *Asparagus* with higher yield then female; female *Phoenix* produced date; female *Taxus* bearing red 'berries' (*arils*) for ornamental use; and female *Cannabis* which gives flower used for brewing beer, *etc.*

Table 1. List of some Dioecious & Monoceous Trees

1. Dioecious Trees

1a. Gymnosperms

1 *Araucaria* (Norfolk Island Pine)
2 *Cephalotaxus* (Plum Yew)
3 *Ginkgo biloba* (Ginkgo)
4 *Juniperus* (Juniper)
5 *Podocarpus* (Podocarp)
6 *Taxus* (Yew)
7 *Torreya* (Nutmeg Yew) (Taxaceae)

1b. Angiosperm

8 *Acer negundo* (Box Elder)
9 *Actinidia* (Kiwi Fruit Vine)
10 *Ailanthus* (Tree of Heaven)
11 *Aucuba* (Non Hardy Dogwood)
12 *Broussonetia* (Paper Mulberry)
13 *Celastrus* (Bittersweet Vine)
14 *Cercidiphyllum* (Kadsura Tree)
15 *Chionanthus* (Fringe Tree)
16 *Cotinus* (Smoke Tree)
17 *Comptonia* (in Bayberry Family)
18 *Diospyros* (Persimmon)
19 *Eucommia* (Hardy Rubber Tree)
20 *Fraxinus* (Ash Trees)
21 *Gleditsia* (Honey Locust)
22 *Hippophae* (Sea Buckthorn)
23 *Ilex* (Holly)
24 *Lindera* (Spicebush)
25 *Maclura* (Osage Orange)
26 *Morus* (Mulberry)
27 *Myrica* (Bayberry)
28 *Nemopanthus* (Mountain Holly)
29 Palm trees
30 *Phellodendron* (Amur Cork Tree)
31 *Populus* (Poplar or Aspen)
32 *Salix* (Willow)
33 *Shepherdia* (Buffalo Berry)
34 *Zanthoxylum* (Prickly Ash)

2. Monoecious Trees

2a. Gymnosperm

35 *Abies* (Fir)
36 *Cedrus* (Cedar)
37 *Chamaecyparis* (False Cypress)
38 Cryptomeria
39 Cunninghamia
40 *Cupressus* (Cypress)
41 Keteleeria
42 *Larix* (Larch)
43 *Picea* (Spruce)
44 *Pinus* (Pine)
45 *Pseudolarix* (Golden Larch)
46 *Pseudotsuga* (Douglas Fir)
47 *Sciadopitys* (Umbrella Pine)
48 *Sequoia* (Coastal Redwood)
49 *Sequoiadendrom* (Giant Redwood)
50 *Taxodium* (Bald Cypress)
51 *Thuja* (Arborvitae)
52 *Tsuga* (Hemlock)

2b. Angiosperm

53 *Alnus* (Alder)
54 *Betula* (Birch)
55 *Carpinus* (Hornbeam, Ironwood)
56 *Castanea* (Chestnut)
57 *Carya* (Hickory)
58 *Corylus* (Hazelnut)
59 *Fagus* (Beech)
60 *Ficus* (Fig)
61 *Juglans* (Walnut)
62 *Ostrya* (Hop Hornbeam)
63 *Platanus* (Sycamore, Plane Tree)
64 *Quercus* (Oak)

(B) Terminology

The complexity of the morphology of flowers and its variation within populations has led to a rich terminology:

- *Androdioecious* have male flowers on some plants, and bisexual ones on the others.
- *Androecious* have only male flowers producing pollen but no seed.
- *Androgynous* is *syn.* of bisexual.
- *Andromonoecious* have both bisexual and male flowers on the same plant.
- *Bisexual* (*syn.*: androgynous, perfect, or hermaphroditic)**.** Each flower of each individual has both male (stamen) and female (pistil) organs.
- *Dichogamous* have sexes developing at different times; producing pollen when the stigmas are not receptive, either *protandrous* or *protogynous*. This promotes outcrossing by limiting self-pollination. Some dichogamous plants have bisexual flowers, others have unisexual flowers.
- *Dioecious* have either only male or only female flowers on the individuals. No individual plant of the population produces both pollen and ovules.
- *Gynodioecious* have hermaphrodite flowers and female flowers on separate plants.
- *Gynoecious* having only female flowers (the female of a *dioecious* population); producing seed but not pollen.
- *Gynomonoecious* have both bisexual and female flowers on the same plant.
- *Hermaphroditic* is *syn.* of *bisexual*.
- *Imperfect* is *syn.* of *unisexual.*
- *Polygamous* have male, female, and bisexual flowers on the same plant. Also called *polygamomonoecious* or *trimonoecious.*
- *Protandrous* (of dichogamous plants) have male parts of flowers developed before female parts, *e.g.*, having flowers that function first as male and then change to female or producing pollen before the stigmas of the same plant are receptive.
- *Protogynous* (of *dichogamous* plants) have female parts of flowers developed before male parts, *e.g.,* having flowers that function first as female and then change to male or producing pollen after the stigmas of the same plant are receptive.
- *Subandroecious* have mostly male flowers, with a few female or bisexual flowers.
- *Subdioecious* have some individuals in otherwise dioecious populations with flowers that are not clearly male or female. The population produces normally male or female plants with unisexual flowers, but some plants may have bisexual flowers, some both male and female flowers, and others some combination thereof, such as female and bisexual flowers. This is thought to represent a transition between bisexuality and dioecy.
- *Subgynoecious* have mostly female flowers, with a few male or bisexual flowers.
- *Unisexual* have either functionally male or functionally female flowers. This is also called diclinous, incomplete or imperfect.

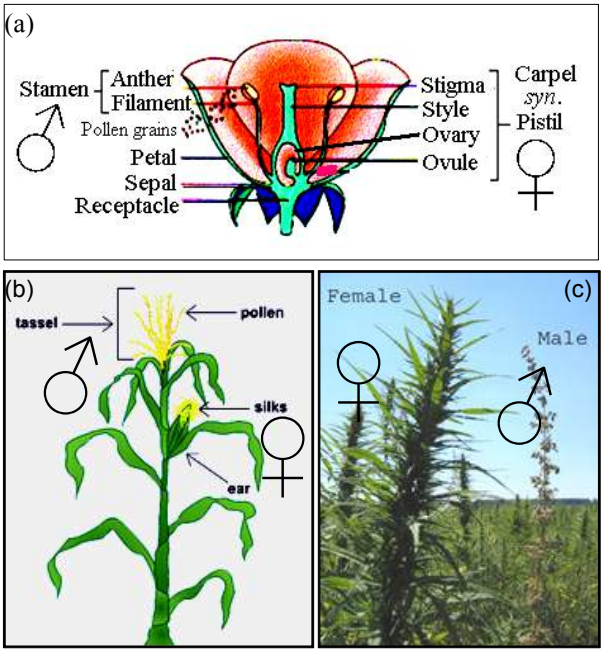

Figure 1. Parts and structures of bisexual flower *(a),* and compared to unisexual flowers of *Monoecious* (*e.g.*, maize) *(b),* and *Dioecious* (*e.g.*, hemp) *(c)* species

(C) Physiology, Ecology and Genetics

In a population, the ratio between pistillate (♀) and staminate (♂) plants is not necessarily 50% : 50%, as male and female individuals are differentially affected by biotic and abiotic environmental stresses, which deviates equilibrium expectation of 1:1 sex ratio.

Gender-specific physiological responses of Dioecious plants to environmental stresses, like rising air temperature and CO_2 concentration of global climate change, are different. It may lead to dramatic changes in sex ratio and consequently the ranges of distribution and growth pattern. In general, male plants show higher level of photosynthetic carbon fixation, and consequently higher fresh mass production. Male *Salix arctica* had a significantly higher photosynthetic rate than females at elevated CO_2 temperature. Male *Populus tremuloides* trees also showed higher photosynthesis activity than females throughout the growing season, regardless of CO_2 concentrations. Moreover, male *Populus cathayana* trees showed higher drought and chilling tolerance then females, which results in the change of the ratio of female to male trees in a population. It was also observed in *Populus tremuloides* populations on the Front Range in Colorado, where the ratio of female to male was 1.27 below 2450 m of elevation, but it was only 0.56 above 2900 m.

The growth form, clonality, fleshy fruits, pollen and seed dispersal vector, and the possession of sex chromosomes also affect sex-ratio bias, and results in male-biased sex ratios, which were found twice as much as female-biased ratios. Female-bias was found associated with herbaceous species, and abiotic pollen dispersal. There are species with extreme female-biased sex ratios; *e.g.*, seagrasses (*Phyllospadix* sp.) were the even sex ratio among

seedlings became female-biased to 90% at later life stages, which was driven by male-biased mortality.

The other reason of sex ratio bias is that there are plant species with more complex sex pattern including *gynodioecious vs. gynomonoecious*, *androdioecious vs. andromonoecious*, and *tridioecious vs. trimonoecious* plants (see point B. Terminology). In a populations of *Asparagus* (2n = 20) there may be four different individuals (*i.e.,* genotypes): the homogametic females (XX), the heterogametic males (XY), the andromonoecious males (also XY), and the supermales (YY). The crossing experiments revealed that when an andromonoecious (XY) plant is selved (XY x XY), it gives a segregation of 1 XX (with berries) : 2 XY : 1 YY progeny. When a *supermale* (YY) plant was crossed with a female (XX) plant (YY x XX) only male (XY) plants werew found in the progeny. Both observations indicate *Mendelian single dominant gene segregation*. For cultivation, male and supermale plants are desired because of higher yield and longevity. Breeders use difficult *testcrosses* to identify these genotypes, however molecular barcoding by PCR technology and, probably, the new *digital seed morphometry* may provide powerful tools to identify these genotypes at seedling stage.

When *Asparagus* seed populations of our study were re-analyzed by K-Means Cluster Analysis (IBM SPSS) setting not two, rather four putative clusters for the four possible *Asparagus* genotypes, the results was difficult to interpret with seed numbers of 1:48:56:44 in the four clusters (*Gyulai et al., 2015a*).

(D) Digital seed morphometry (by Fovea Pro 4.0 program)

Seed parameters (in total 20 of the 33 measured) can be analyzed by digital morphometry, and used to decide whether seeds carrying *pistillate* (♀) plant can be discriminated from seeds carrying *staminate* (♂) plants, which question has not been raised. In a study (*Gyulai et al., 2015a*), presented in this Chapter, seeds of four dioecious plants of the gymnosperm tree *Taxus baccata* (153 seeds) grown in Gödöllő, Hungary; the angiosperm dicot shrub *Hippophae rhamnoides* (268 seeds) (grown in Veresegyháza, Hungary) and tree *Diospyros virginiana* (103 seeds) (grown in Gödöllő, Hungary, after transplantation from Purdue University Camp, Indiana, USA) were compared to the angiosperm herbaceous monocot *Asparagus officinalis* (149 seeds). In total, 673 seeds were analyzed (*Fig. 2*).

Based on the analyses, new index of *Seed Diversity Index (SDI)*, based on the mean values of the *Relative Standard Deviation* (%RSD) of measured values of seed parameters, and *Seed Morphological Distances (SMD)*, based on Group Centroids of DA (*Discriminant Analysis*) were formulated. CRT (*Classification and Regression Trees*) diagram was found to reveal the most discriminative seed parameters. *Histogram analysis* was used to determine the 'levels of domestication' of the species by estimating the variations (*'Force of Mutation'*; X-axis) *vs.* selection (*'Force of Selection'*; Y-axis).

Previous studies, contrary to the expectations, revealed that Nature is not always 'natural' ('normal') statistically as histograms of seed parameters in wild taxa consistently show a pattern of multimodal (*syn.*: non-Gaussian, non-bell-shaped) and unpredictable distributions. On the contrary, domestic crop seeds, as the result of continuous human selection, show 'normal' (*syn.*: bell-shaped, unimodal) distributions, which are distinct and more predictable, and coupled with comparatively narrow range of variation.

The PCA (Principal Component Analysis) revealed 20 of the measured 33 seed parameters, which were grouped in the first three Principal Components, and used for the further analyses

List of the main digital seed characteristics (see Chapter 18)

Area(cm^2): the area of a seeds determined by counting the connected thresholded pixels.
Aspect_Ratio: *Length / Breadth.*
Breadth(cm): the minimum caliper dimension.
Circum.Rad.(cm): radius of the circle around the seed (*Circumscribed Radius*).
Convex_Area(cm^2): a shape is convex if there are no 'dents' or indentations in it.
Convex_Perim(cm): perimeter of the convex bounding polygon of the seed.
Convexity: Perimeter / Convex_Perimeter(cm).
Elongation: Area / ConvexHull Area.
Equiv.Diam(cm): the diameter of a circle with the same area as the seed.
External_Perimeter(cm): distances between the centres of pixels on the outer side of the boundary.
Filled_Area(cm^2): measures the presence of internal gaps or voids (seeds that contain internal holes have area fractions less than unity).
FormFactor: $4\pi \bullet$ Area / *Perimeter*^{2}.
Inscrib.Rad.(cm): radius of the inscribed circle of seed.
Length(cm): maximum caliper dimension.
Perimeter(cm): distances between the centres of pixels on the seed side of the boundary.
Radius_Ratio: Inscribed Radius / Circum.Rad.(cm).
Roundness: $4 \bullet$ *Area* / $\pi \bullet$ *Max Dimension.*
Skeleton_Length(cm): total lineal distance down the centre line of seed.
Solidity: Area(cm2) / Convex_Area(cm2).
Symmetry: the symmetry of a shape encoded in the Fourier transform of its boundary.

Relative Standard Deviations (%RSD) of seed parameters were calculated and plotted. Of them, the first three main %RSD parameters of seed *Area(cm²)*, *Filled_Area(cm²)* and *Convex_Area(cm²)* showed the highest levels in the *Hippophae* seed population (14.6 %, 14.6 % and 14.5 %, respectively). %RSD of seed parameter *Elongation* was the highest in *Taxus* seeds (19.4 %).

To scale homo/heterogeneity of seed populations, the mean values of the Relative Standard Deviations (%RSDs) of all the twenty seed parameters was calculated species by species, which results provided a general index coined SDI (*Seed Diversity Index*).

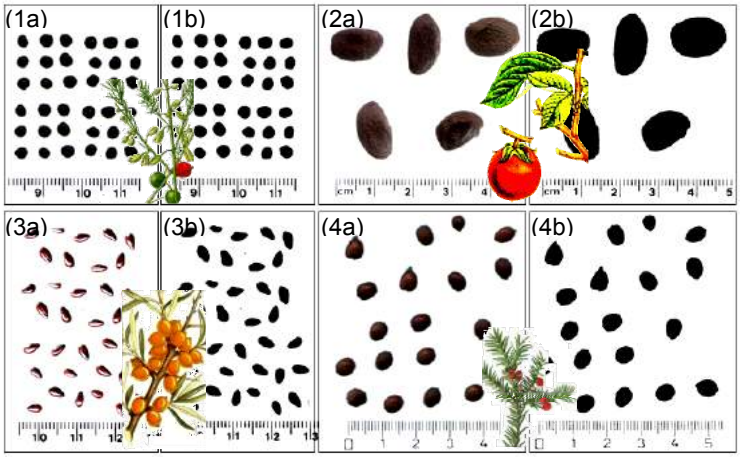

Figure 2. Digital *(a)* (*pdf*) and BitMap photos *(b)* of seed samples of the four *dioecious* plant species prepared for *digital seed morphometry* analysis with scales (mm) at the same magnification. Leaf and fruit samples of *(1) Asparagus officinalis*, *(2) Diospyros virginiana*, *(3) Hippophae rhamnoides*, and *(4) Taxus baccata* are indicated (*Gyulai et al., 2015a).*

Statistically, the level of %RSD below 10% indicates homogenous (seed) populations with low morphological diversity, and it is taken as heterogeneous with higher morphological diversity above 30 %. The %RSD of seed *Elongation* was the highest in *Taxus* (19.4 %) and *Diospyros* (18.6 %) above 10 %, however below 30 %. Seed *Area(cm²)*, *Filled_Area(cm²)* and *Convex_Area(cm²)* showed levels also above 10% in *Hippophae* (14.6 %, 14.6 % and 14.5 %, respectively).

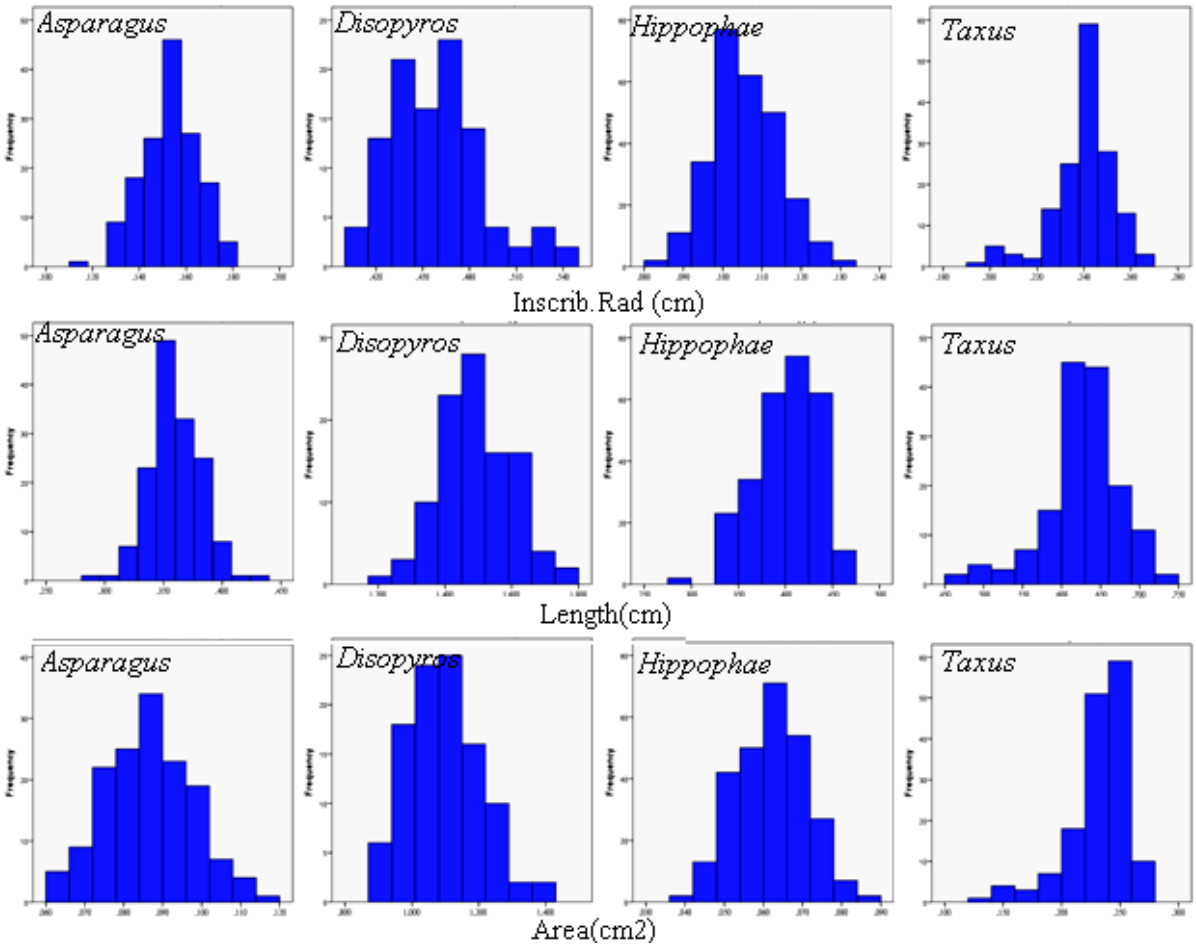

Figure 3a. Histogram analysis (with *ten ranks* on the X-axis, and the same scale on the Y-axis) (MS Xcel) of the three main discriminative seed parameters identified by CRT analysis of *Inscrib.Rad (cm)* (up); *Length (cm)* (middle); and *Area (cm2)* (down) of the four Dioecious plants (*Asparagus, Disopyros*, *Hippophae* and *Taxus*) (*Gyulai et al., 2015a).*

Histograms of the three most discriminative seed parameters determined by CRT analysis - *Inscrib.Rad(cm)*, *Length(cm)*, and *Area(cm2)* - revealed that *Asparagus* shows the closest to natural unimodal distribution in all of the three parameters by using ten histogram ranks (along axis-X). All parameters of *Diospyros*, *Hippophae* and *Taxus* show histogram bias especially in the parameter of *Inscrib.Rad(cm)* of *Diospyros*, which is exactly multimodal. The Y-values of histograms showed the lowest levels in *Diospyros* and the highest levels in Taxus with *Inscrib.Rad(cm)*, and *Are(cm^2)* (*Fig. 3a,b*).

Values of X- and Y-axis of histograms of seed parameters were also postulated to reflect ranks of X-axis (*i.e.,* width of diversity) as measuring the '*Force of Mutation*'; and the Y-axis (with high/low protrusions) as the '*Force of Selection*'. In the study of Dioecious plants, when automatically adjusted ten ranks were set, *Taxus* showed the highest Y-values (*i.e.,* '*Force of Selection*') in two - *Inscrib.Rad(cm)* and *Area(cm2)* - of the three most discriminative CRT parameters (*Fig. 3a*). This results may indicate a tendency of narrowing the variations of *Taxus* (as a type of overspecialization of a preferred type of seed), which, consequently, increases the vulnerability of this species to environmental changes.

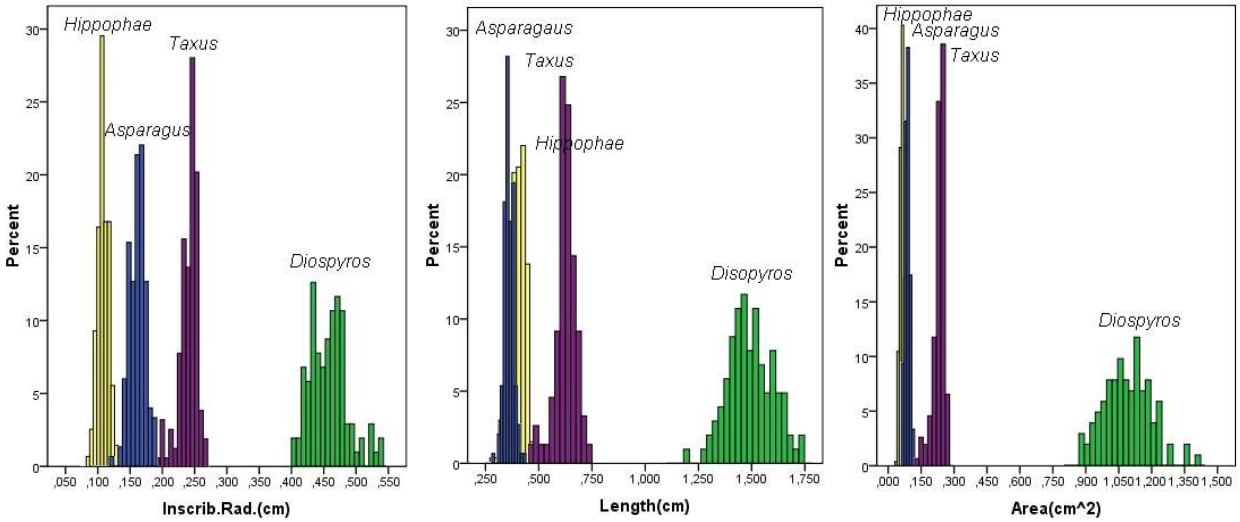

Figure 3b. Histogram analysis (with *twenty ranks* and setting the same scale on axis-X and -Y) (IBM SPSS) of the three main discriminative seed parameters of *Inscrib.Rad(cm)*, *Length(cm)*, and *Area(cm^2)* of the four *dioecious* plants studied (*Gyulai et al., 2015a).*

Recent ecological reports have indicated that the areas of *Taxus,* as a species of cold preferred gymnosperms, dramatically narrowing and tend to move north around the Mediterranean Sea. Small and isolated populations of *Taxus* were also found to be exposed to genetic drift (with a potential extinction) by the loss of genetic variation (*i.e.,* diversity) as it was observed by DNA markers. Moreover, isolated populations of *dioecious* plants tends to show a female bias of sex ratio (*i.e.,* higher fitness of female plants) due probably to the seed sets as it was also observed in *Taxus*. However, male plants show higher levels of fresh mass production (discussed above).

When the histogram analysis was repeated with higher resolution by using twenty ranks, with setting the same size ranges along the X-axis and percentages on Y-axis, the results revealed that the close to normal distribution of *Asparagus* seeds (*Fig. 3a*) show more detailed pattern (*Fig. 3b*). The multimodality of *Disopyros* showed the highest ranges of diversity along the X-axis ('*Force of Mutation*') and the lowest protrusions (selections) along Y-axis ('*Force of Selection*'), which result consequently indicates the most adaptability (*i.e.,* ecological fitness) of this species to the environmental changes.

This result seems to contradict the SDI-indexes, which indicated *Hippophae* to having the most divers seed populations with DSI 8.10, compared to *Dyospiros* (7.16). However, SDI-indexes show an average of all twenty parameters, and histograms reflect single seed parameters. Methodologically it can conclude that, of the two types of histogram analyses, histograms with higher (*i.e.,* twenty) ranks reveals more reliable seed diversity pattern then the regular, automatically ranked type of histograms with ten ranks.

K-means Cluster analysis used to separate the two (putative male and female) seed populations revealed that none of the K-means Cluster analysis of the twenty parameters of seed populations of the four plant species separated two seed subpopulations with ratio of 50 % (♀) : 50% (♂) for the putative male and female plant carrying seed populations. However, K-Means Cluster Analysis, by setting for two clusters, revealed the possibility to determine whether seeds carrying *pistillate* (♀) plant can be separated from seeds carrying *staminate* (♂) plant. When *Asparagus* seed populations were re-analyzed by K-Means

Cluster Analysis (IBM SPSS) setting not two, but rather four putative clusters for the four possible *Asparagus* genotypes (see point C. above), the results was difficult to interpret with the obtained seed numbers of 1:48:56:44 in the four clusters.

(E) Molecular markers used for male** vs. **female plants

Some dioecious plants, similar to animals, show differences not only in DNA fragments (*i.e.,* markers) but also in sex chromosomes (*Tale 2; Fig. 4a,b*). For molecular determination, numerous sex-linked DNA have been developed (*in Gyulai et al., 2015b*).

Plant sex chromosomes are not as rare as previously assumed. By families of angiosperms, *Amaranthaceae, Aracaceae, Asparagaceae, Cannabaceae, Caricaceae, Caryophyllaceae, Cucurbitaceae, Polygonaceae, Rosaceae* and *Santalaceae* comprise species with sex chromosomes.

Evolutionarily, *plant sex chromosomes* may evolved some million years ago, *e.g.,* in *Silene,* it might have developed about 5–10 Mya ago, however, in papaya (*Carica papaya*) it may have evolved more recently. To compare, *sex chromosomes in mammals* may developed 300 Mya ago, and were discovered only about a century ago in human (XX diploid

Heteromorphic sex chromosomes for males (XY) and homomorphic for females (XX) were described, for *e.g., Cannabis, Humulus, Silene,* and *Coccinia*. Some of them have a dosage compensation system, *e.g.,* in *Humulus*. Homomorphic sex chromosomes were characterized cytologically in *Actinidia, Asparagus, Antennaria, Carica, Vasconcellea, Silene, Spinacia, Bryonia, Ecballium, Dioscorea, Thalictrum,* and in *Vitis*. Female-bias mainly occurs in species with sex chromosomes and there is some evidence for a greater degree of bias in those with heteromorphic sex chromosomes (*in Gyulai et al., 2015b*).

Table 2. Samples of sex-linked (male/staminate/♂ *vs.* female/pistillate/♀) PCR based DNA markers of Dioecious trees (*Gyulai et al., 2015b*

Primer sequences (5'–3')	Fragm. Size (bp)	Gender (♂ or ♀)	Referenc es
Actinidia deliciosa *RAPD* CAGGCCCTTC (OPA-01)	1031	♀	Shirkot *et al.* (2002)
AATCGGGCTG (OPA-02)	2000		
GTGACGTAGG (OPA-08)	700		
CAATCGCCGT (OPA-11)	2800		
AGCCAGCGAA (OPA-16)	3000		
GTTTCGCTCC (OPB-01)	2000		
GATGACCGCC (OPC-05)	350	♂	
CTCACGTTGG (OPN-01)	600		
RAPD-SCAR TCTCGCCTACCTTTTACCA TCTCGCCTACAGGAACAACA	500		Li *et al.* (2010)
Carica papaya *RAPD* ACGGCGTATG (OPE-19)	2180	♀	Niroshini *et al.* (2000)
GAGGATCCCT (OPF-02)	800	♂	Parasnis *et al.* (2000)
ISSR (GATA)4	5kb		Parasnis *et al.* (1999)

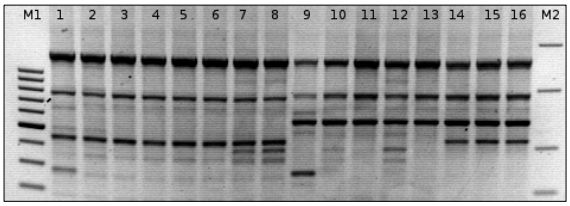

Figure 4a. Male-specific RAPD *OPC-20* DNA marker (500 bp) (*Sharma et al., 2010*) identified in common sea-buckthorn (*Hippophae rhamnoides ssp. Carpathica*) population. Females ♀ (L 1-8) and males ♂ (L 9-16) are indicated. M1 - Mw 100 bp DNA size marker. M2 - FastRuler Low Range DNA ruler. *See the male specific OPC / 20 / 911 bp DNA band in each male plant (L 9-16)* (*Kerti, Gyulai et al., 2015*).

homomorphic sex-chromosomes for females, and XY diploid heteromorphic sex chromosome for males). To compare, flowering plants may began appearing in fossil records at about of 124.6 million years ago (*Mya*), however *angiosperms* show about 158-179 *Mya* evolution based on DNA data.

Figure 4b. Molecular sex-linked marker identification at seedling *stage* in common sea-buckthorn (*Hippophae rhamnoides ssp. Carpathica*) population (*Kerti, Gyulai et al., 2015*).

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Pecan (*Carya illinoinensis* *Wang.; K. Koch*) - Breeding in the southern United States

(A) Introduction

Pecan (*Fig. 1*) is the most economically important nut species native to North America (*Molnar et al., 2013*). The native range of pecan extends from the southeastern part of the United States (U.S.), Southwest as far as Oaxaca, Mexico, with native stands surviving winters into the central Midwestern states like Illinois, Iowa, and Kansas (*Molnar et al., 2013; Radford et al., 1981; Smith, 1929*). While the native range covers parts of 13 states, pecan production in the U.S. is primarily located in the Deep South, with the state of Georgia being the largest producer (*USDA, 2009*). Other states like Alabama, Mississippi, Louisiana, and Texas also have thousands of acres of viable commercial pecan orchards.

Family Juglandaceae includes nine main genera with several species of *Alfaroa* (7 species), *Annamocarya* (1), ***Carya*** (19), *Cyclocarya* (1), *Engelhardia* (8), *Juglans* (20), *Oreomunnea* (2), *Platycarya* (1), and *Pterocarya* (6).

The most limiting factor for obtaining marketable yields from pecan production in the humid southeastern U.S. from a pest management standpoint is pecan scab (*Cladosporium caryigenum*) (*Demaree, 1924*). Crop losses in unsprayed orchards can be as high as 50 to 100 percent (*Goff, 1996*). A pecan scab epidemic in the early 1900s was attributed to the widespread introduction of susceptible cultivars '*San Saba*' and '*Georgia Giant*' (*Goff, 1996*). By 1956 all common cultivars (13 total) were susceptible to pecan scab.

Pecan scab will attack leaves, leaf petioles, and nut shuck tissue, reducing photosynthesis and defoliating trees very prematurely. Yields can be reduced by 100% in many orchards. Pecan tissues are most susceptible to scab when young and actively growing (*Goff, 1996*).

Pecan scab is a fungus that overwinters on shucks, leaf petioles, and stems infected the previous season (*Goff, 1996*). Spores will be released in early February and March, but most spores are released in mid-April, at pecan bud-break. Pecan scab spores are spread by rain, wind, and leaf wetness over long distances. Relative humidity plays a major role in development of this disease. Spores are released as humidity decreases during the morning as dew evaporates (*Goff, 1996*). Air temperature that favors pecan scab sporulation is from 19 to 29 C. During hot dry weather very few spores are released.

Pecans are *monoecious* with a long juvenility period ranging from 5 to 10 years (*Beedanagri, et al., 2005*). The *staminate flowers* are produced on three-stalked catkins that emerge in the spring (*Goff, 1996*). The *pistillate flowers* appear toward the end of new shoots with three to seven clusters (*Goff, 1996*). Pecans are *dichogamous*, meaning that pollen shed and stigma receptivity are not synchronized.

There are two types of flowering in pecans: *protandrous* and *protogynous*. The *protandrous* cultivars, whereby pollen is released ahead of stigma receptivity are called *Type I* flowering (*Goff, 1996*). Conversely the *protogynous* or *Type II* trees have stigma receptivity before pollen shed. Therefore, pecans need to be planted in with a mixed stand of *Type I* and *Type II* to ensure pollination (*Goff, 1996*). This phenomenon of *dichogamy* also decreases *selfing* of pecans since very few cultivars have periods of adequate overlap of *Type I* and *Type II*.

Pecans grown in the southeastern U.S. for commercial production have a tendency to alternate bear. This means that a heavy yield one year is most often followed by a much lower yield the following year (*Goff, 1996*). Much research has been conducted on reducing alternate bearing. Alternate bearing is attributed to a lack of stored carbohydrates in the off-year (*Goff, 1996*). In other words, the trees assimilated food reserves are used up to produce the heavy on-crop year yields, thus depleting carbohydrate reserves that are stored in the roots for the following year. When the pecans come out of dormancy the following spring, this lack of carbohydrate reserves reduces flower production and thus the lower yields in the *off-crop year*.

As trees age and yields increase, alternate bearing becomes more pronounced (*Goff, 1996*). Prevention comes in many forms, but *Goff (1996)* recommends maintaining healthy foliage until November (when most trees lose leaves) to store as much carbohydrate assimilates as possible. Recent research has evaluated mechanically thinning the crop in the heavy load year, which has been shown to reduce alternate bearing (*Smith and Gallott, 1990 and Reid et al., 1993*).

Figure. 1. Pecan nut (*Carya illinoinensis*) is a new tree crop in Hungary (Gödöllő) transplanted from Auburn University, AL, USA, in 2005, by *WG Foshee, WD Goff, and G Gyulai*. (*see the tree, leaves – up; and the young and old husks, and nuts - down*)

(B) Accessions to cultivars

Since the 1920s top-working trees with resistant cultivars was recommended as the best control for scab. Unfortunately, resistance was quickly overcome by scab leading growers to depend on preventative fungicide sprays starting at budbreak and spraying on 2-week intervals throughout the growing season (*Goff, 1996*).

Demand for resistant cultivars to pecan scab has been a priority for commercial growers and homeowners in the southeastern U.S. *Dr. Bill Goff* set out in the 1990s to identify superior seedling trees that displayed excellent scab resistance. In search for scab resistance, he traveled the swampy bottom lands of Louisiana and Mississippi, the center of origin of pecans where the most genetic variation can be found. During this discovery phase, trees would be marked and evaluated for pecan scab resistance and nut quality in the following spring and fall. This approach along with collaboration from producers allowed *Dr. Goff* and his research team to make several selections for grafting and further evaluations.

Of these wild selections over 25 were grafted onto seedling rootstocks at the *EV Smith* Research Center in central Alabama (*latitude* 32°30'N, *longitude* 85°40'W). The orchard selected for evaluations (referred to as the Headquarters orchard) was established for pecan scab screening. This orchard had never received any fungicide sprays and was planted with '*Cheyenne*', which is very susceptible to pecan scab, creating an environment with high scab pressure.

Goff (2015) updated his recommendations based on two consecutive rainy years (2014 and 2015) which increased pecan scab pressure. The recommendations include three cultivars that were rated excellent for pecan scab resistance, '*Gafford*' located in southwest Alabama (Butler County, Alabama) and '*Headquarters*' a cultivar that was discovered in the Headquarters orchard in Macon County, Alabama and '*Miss L*' a seedling selected by Dr. Goff from Pointe Coupee Parish, Louisiana (*Goff, 2015*). Other cultivars recommended for the southeastern U.S. were '*McMillan*' (Baldwin County, Alabama native) with a good rating on pecan scab resistance and '*Zinner*' (Baldwin County, Alabama native) with a mediocre pecan scab resistance rating (*Goff, 2015*).

The selection of native trees with inherent pecan scab resistance has been a successful approach for evaluating cultivars suited for the harsh conditions of the southeastern U.S. To date, this method of discovering scab resistance has been quite successful, however *introgressing* these new found traits into commercial cultivars must be done via conventional breeding to create pecan trees with good yield, nut quality, and disease resistance.

Pecans are clonally propagated asexually by taking a scion from a source tree during dormancy for grafting onto seedling rootstocks that are about 1 to 2 inches in diameter when the cambium layer is in a stage of rapid growth and the bark is slipping. Pecan nuts are borne in the fall in temperate zones in the U.S. and must be stratified (placed into a cooler at 35 to 45 °F for two months) to germinate (*Goff, 1996*). Fallen nuts generally have a high germination rate following natural stratification. Following stratification, nuts are then planted in early March into seedbeds or containers. Grown properly, seedlings can be whip grafted when they are 9 to 11 months old (*Goff, 1996*).

(C) Conventional Breeding Programs in the U.S.

Pecans are diploid *2n=2x=32*. *Sexual crosses* made via conventional breeding involve first bagging *pistillate flowers* to avoid cross pollination from unwanted pollen sources (*Conner, 2012*).

Catkins from the male parent are collected and dried releasing the pollen which is then collected to be applied via bulbs and syringes into the bagged *pistillate flowers*. Breeding is very labor intensive and pecans require several years to reach a reproductive phase. The breeding cycle from cross to releasing of cultivar is on average 25 years (*Thompson and Grauke, 1994*).

Currently all pecan ('*Carya illinoinensis*') cultivars are sold on *seedling rootstocks* which can have high variability (*Grauke and Gel, 1983; Smith, 1929*). Several techniques have been used to try to produce *clonal rootstocks* for pecans but most have failed. Rooting of pecan trees has shown marginal success for juvenile cuttings but has been very limited for adult-phase wood (*Smith, 1980; Sparks and Porkorney, 1966*). *Grauke and Gel (1983)* tried to propagate adult-phase pecans by *air-layering* three cultivars (Candy, Desirable, and Moreland).

All three cultivars formed roots but none survived after being transplanted. *Brutsch et al. (1966)* were successful in getting juvenile pecan cuttings to live, but non-juvenile cuttings did not survive. As a result of mediocre success with rooted cuttings, all pecan cultivars in the U.S. in commercial use continue to be *budded* or *grafted* on seedlings grown from open-pollinated flowers (*Goff, 1996*). Because open-pollinated seedlings are so variable there is a need for breeding cultivars for rootstocks via *recurrent selection* methods due to *inbreeding depression* (*Thompson and Conner, 2012*). Currently, there is no known breeding program developing rootstocks.

Most of the World's production of pecans comes from the U.S. (*Pena, 2007*). Most pecans were spread across the southern U.S. by seedlings from mother trees. Since pecans are highly heterozygous and exhibit inbreeding depression, clonal propagation was started as a way to obtain cultivars (*Thompson and Conner, 2012*). Scab resistance was serendipitously discovered by homeowners in this manner. '*Stuart*', a seedling from Alabama, was the first cultivar propagated back in the late 1800's (*Thompson, 1990; Thompson and Conner, 2012*).

Louis Romberg, a pecan breeder, began a pecan and *hickory* collection in Brownwood, Texas in the

1930s (*Thompson and Conner, 2012*). In 1984, this orchard was designated as the *National Clonal Germplasm Repository for Pecans and Hickories*.

Another orchard similar to this was later established at *College Station, Texas*. Currently, the National Repository has over 300 pecan cultivars. Georgia established a Pecan Breeding Program in 1989 (*Conner, 2016*).

Pecans, like many other domesticated crops, are bred conventionally using a pedigree method, *introgressing* useful traits found in the wild, such as resistance to diseases, into commercial cultivars. However, because of the long juvenility phase of pecans delaying reproduction (5 to 10 years), the release of a pecan cultivar can be upwards of 25 years from the initial cross, where other crops such as peaches only take 10 years (*Thompson and Grauke, 1994*). With the recent reduction in price of high-throughput genotyping, marker assisted selection is being utilized by breeders to help make selections in some species earlier through the use of molecular markers that have been identified for traits such as resistance to certain pathogens.

Markers developed for traits such as nut quality or precocity can significantly reduce the time it takes to select for these phenotypic traits observed in the reproductive stage while the plant is still a young seedling in a juvenile state (*Grauke et al., 2015*).

The United States Department of Agriculture, Agricultural Research Service (USDA-ARS) is in charge of a national pecan breeding program in College Station and Brownwood, Texas (*Thompson and Conner, 2012*). Two selection cycles are used: the first one being the *Basic Breeding Program* (BBP) where up to *4000 seedlings per year* are evaluated for primarily scab resistance along with yield, precocity, and other insect and disease resistance. Subsequently, these seedlings are clonally propagated via grafting to evaluate the same genotype in both College Station and Brownwood.

The second selection cycle is the *National Pecan Advanced Clone Testing System* (NPACTS) where only one or two per thousand clones from the Basic Breeding Program make it to national testing of the clones across the southeastern *United States pecan belt* to look at the same genotype in differing environments. It is estimated that the USDA releases a new cultivar every 2-5 years using this system. The University of Georgia has the only other active breeding program for pecans and is very similar to the USDA program.

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Legume trees (order Fabales) – Conservation genetics

(A) Introduction

Order *Fabales* comprises huge numbers of 20.055 species of 754 genera of 4 families (*Fabaceae, Polygalaceae, Quillajaceae*, and *Surianaceae*). Of them, family *Fabaceae* comprises the most numerous woody species (240 – 253) of a plant family! Genus *Astragalus* of family *Fabaceae*, is also unique being the largest plant genus, which comprises the largest numbers of species (about 2,500 species) of a genus (with 703 entries in the *NCBI*).

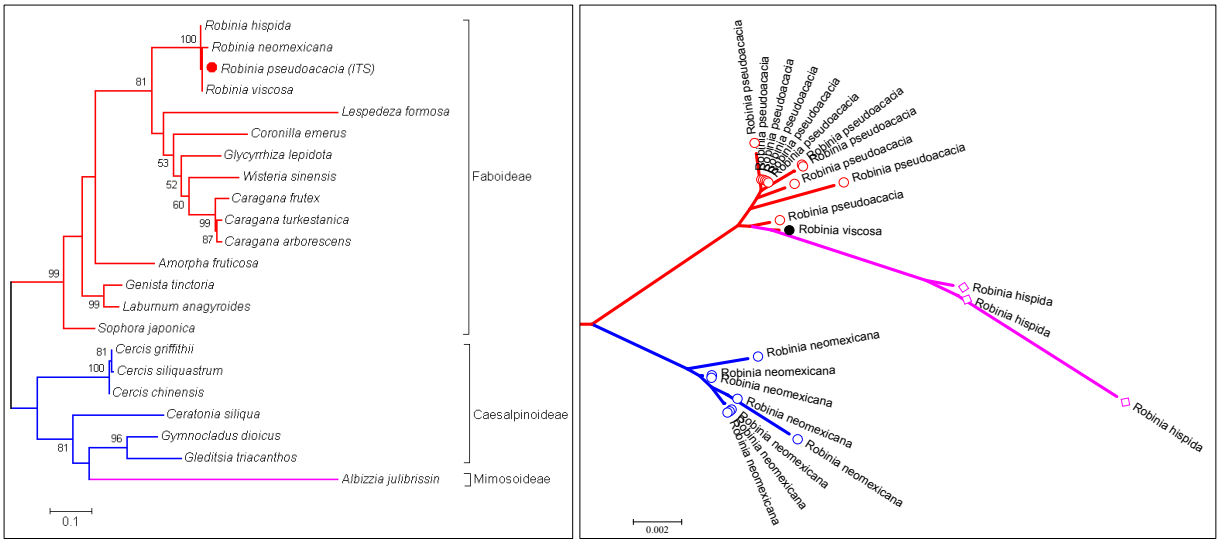

Figure 1. In silico ITS1-5.8S-ITS2 (683 bp) ML phylogram (Maximum Likelihood; *Hillis et al., 1994*) (MEGA4; *Tamura et al., 2007*) of legume trees of the three *Fabaceae* subfamilies (*Faboideae, Caeasalpinoideae*, and *Mimosoideae*) growing in Hungary *(a)* and including the main four *Robinia* species *(b)*. NCBI accession numbers are: *Albizia julibrissin* (FJ572041), *Amorpha fruticosa* (AFU59890), *Caragana arborescens* (FJ537262), *Caragana frutex* (FJ537285), *Caragana turkestanica* (FJ537256), *Ceratonia siliqua* (AJ245576, AJ245575), *Cercis chinensis* (FJ432284), *Cercis griffithii* (FJ432280), *Cercis siliquastrum* (FJ432281), *Colutea arborescens* (CAU56010, CAU56009), *Hyppocrepis* (*Coronilla) emerus* (AF450240), *Genista tinctoria* (AF330664), *Gleditsia triacanthos* (AF509969), *Glycyrrhiza lepidota* (U50759, U50758), *Gymnocladus dioica* (AF510032), *Laburnum anagyroides* (AY263679), *Lespedeza* (GU572199), *Robinia hispida* (AF398819), *Robinia neomexicana* (AF537351), *Robinia pseudoacacia* (AF450153), *Robinia viscosa* (AF398821), *Sophora japonica* (FJ528289), *Wisteria sinensis* (EU424072) (*Gyulai and Láposi,, 2010*).

Fabaceae is divided into three subfamilies (*Faboideae, Caeasalpinoideae*, and *Mimosoideae*) (*Fig. 1a*), and comprises 11 genera (*Hebestigma*, *Lennea*), (*Gliricidia*, *Poitea*) and (*Olneya*, *Robinia*, *Poissonia*, *Coursetia*, *Peteria*, *Genistidium*, and *Sphinctospermum*). The ITS DNA region used for phylogey (*Lavin et al., 2003*) is estimated at 3.1–3.5 x 10^{-9} substitutions/site/year.

The small genus *Robinia* comprises a limited numbers (about 30) of species and hybrids, such as *R. boyntonii, R. elliottii, R. hartwigii, R. hispida* (bristly locust; 2n = 30), *R. kelseyi, R. luxurians, R. nana, R. neomexicana* (New Mexican locust), *R. pseudoacacia* (black locust, false acacia), *R. viscosa* (clammy locust; 2n = 20)*;* and the hybrids: *Robinia* × *ambigua* (*R. pseudoacacia* × *R. viscosa*) (Idaho locust), *Robinia* × *holdtii* (*R. neomexicana* × *R. pseudoacacia*), *Robinia* × *longiloba* (*R. hispida* × *R. viscosa*) and *Robinia* × *margarettiae* (*R. hispida* × *R. pseudoacacia*) (*Table 1*).

Legume trees *Fabaceae* have several endemic genera with unique genome constitutions such as the Caribbean endemic *Hebestigma* and *Poitea,* and the Australian *Castaneospermum australe. Castaneospermum* (*Moreton Bay Chestnut* or *Blackbean*) is already on the market as an ornamental indoor plant.

Figure 2. Samples of the oldest European black locust (*Robinia*) trees. *(a)* the Royal Herbal Garden Paris, France, planted in 1602 by Jean Robin (1550-1629). *(b)* Doorwerth, Hollandia, planted in 1678. *(c)* Bábolna tree, Hungary, planted in 1710 by *Count Szapáry* at city Bábolna (*Fig. 6.b,c*). *(d)* Kew's '*Old Lions*', London, UK, planted in 1762 (*Gyulai et al., 2015*).

(B) Black locust (Robinia pseudoacacia)

The most important legume trees in Hungary is the black locust (*R. pseudoacacia*) (2n = 20 and 22; 2,4 pg DNA) (http://ccdb.tau.ac.il/countsByMatchedName/Robinia%20pseudoacacia/), which is native to the southeastern United States, but has been widely planted and naturalized elsewhere in the temperate North America, Europe, Southern Africa and Asia. It was introduced into Europe in 1602 (France) (*Fig. 2.*), however it was native to European flora as *Robinia* fossils were found in Germany from Miocene (14.3 to 3.8 million years BP) but later became extinct.

Fossilized *Robinia*-like trees (*Robinia zirkelii*) (*syn.*: *Cercidoxylon/Robinoxylon z.*; *Robinia/Robinoxylon brewerii*; and *Paleo-Robinoxylon z.*) were also found in several sites in North America from *Late Eocene* (Chadronian; 38 – 33.9 million years BP) Nebraska, USA. Petrified woods of *Robinia alexanderi*, and *R. breweri* were also identified, but all of these samples are considered today to be *R. pseudoacacia*, which indicate the genome stability of *Robinia* without morphological changes during the last ten millions years of evolution.

Further *Legume trees* were also introduced to Europe such as *Gleditsia triacanthos* (honey locust) (in 1600s), *Robinia hispida* (in 1743), and *Robinia viscosa* (in 1797). The ITS analysis of the four main *Robinia* species introduced and grown in Hungary indicated that *R. viscosa* is genetically the closest relative to *R. pseudoacacia* (*Fig. 1b*).

Table 1. Species and hybrids of genus *Robinia*

Species

1. *R. albicans*
2. *R. boyntonii*
3. *R. breviloba*.
4. *R. cubense*
5. *R. elliottii*
6. *R. fertilis*
7. *R. grandiflora*
8. *R. hartwegii*
9. *R. hispida* L. (2n = 30)
   a. *R. hispida* var. *hispida*
   b. *R. hispida* var. *rosea*
   c. *R. hispida* var. *fertilis*
   d. *R. hispida* var. *nana*
   e. *R. hispida* var. *kelseyi*
10. *R. kelseyi*
11. *R. leucantha*
12. *R. luxurians*
13. *R. nana*
14. *R. neomexicana*
    a. *R. neomexicana* var. *neomexicana*
    b. *R. neomexicana* var. *luxurians*
    c. *R. neomexicana* var. *subvelutina*
    d. *R. neomexicana* var. *rusbyi*
15. *R. pallida*
16. *R. pedunculata*
17. *R. pseudoacacia* L. (2n = 20 and 22)
    a. *R. p.* f. *inermis*
    b. *R. p.* var. *pyramidalis*
    c. *R. p.* var. *rectissima*
    d. *R. p.* var. *globosa*
18. *R. rusbyi*
19. *R. speciosa*
20. *R. viscosa* (2n = 20)
    a. *R. viscosa*. var. *hartwegii*
    b. *R. viscosa* var. *viscose*

Hybrids

21. *R.* × *ambigua* [*pseudoacacia* × *viscosa*]
22. *R.* × *ambigua* var. *bella-rosea*
23. *R.* × *dubia*
24. *R.* × *holdtii* [*neomexicana* × *pseudoacacia*]
25. *R.* × *coloradensis*
26. *R.* × *longiloba* [*hispida* × *viscosa*]
27. *R.* × *margarettiae* [*hispida* × *pseudoacacia*]
28. *R.* × *slavinii*

Black locust is the major *honey plant* in Europe**,** its spreading area exceeded 464.000 ha in Hungary in 2012, which is 24,1% of the total forests of Hungary. It is the source of the unique Hungarian acacia honey. Its wood is extremely hard, resistant to rot, and durable, making it prized for furniture, flooring and paneling, and also for firewood. On its root system it has nitrogen-fixing bacteria, which are able to fix the aerial nitrogen (N_2) from the atmosphere, so it can grow on poor soils.

By *leaf type,* black locust has at least four pinnate type, the *monophylla* (*syn.*: *unifolia*), *triphylla*, *oligophylla* (regular) and the *polyphylla* (*microphylla* up to 25 leaflets) types. These leaf type varieties showed different hybrid patterns in crossings. *Seed pods* have 4 to 8 seeds. By *tree shape*, new forms of *pyramidalis, rectissima* and *globosa* were selected (*Table 1*).

Figure 3. Thorny (*up*), and thornless (*down*) plant types. Thornless black locust (*Robinia pseudoacacia* f. *inermis*) (*left*); thornless gleditsia (*Gleditsia triacanthos* f. *inermis*) (*middle*); and thornless black berry (*Rubus fruticosus* f. *inermis*) (Rosales!) (*right*).

(C) Thornlessness

Several *thornless* (‘*inermis*’) *Robinia* clones were developed and selected such as *Robinia pseudoacacia* f. *inermis,* also in Hungary (*Fig. 3*). Thornlessness is caused mainly epigenetically by epidermis mutation in shoot meristems, and considered as chimera. Certain thornless varieties from other thorny species such as blackberry (*Rubus laciniatus/fruticosus* cv. ‘*thornless evergreen’*) were identified (in 1939), and also developed from tissue cultures after gamma irradiation (*Fig. 3*).

Thornlessness can be linked to morphological characters like in thornless *Rubus fruticosus*, where cotyledon marginal hairs of the seedlings are absent. Thornless honey locust (*Gleditsia triacanthos*) trees were also selected and grafted (*Fig. 3*).

Genetic markers linked to thornlessness can help the selection during breeding at seedling stage. In thornless *Robinia* a 262 bp long DNA fragment was found at the *scu*10 locus compared to 252 bb of the regular thorny trees (*Fig. 4*).

Thornless lemons (*Citrus x limon*) *e.g.,* cv. ‘*Eureka*’, and limes (*Citrus aurantifolia*) *e.g.*, cv. ‘*Mexican*’ were identified. “*Every rose has a thorn*”, however thornless and near-thornless Roses (*1960*) were also selected and cultivated.

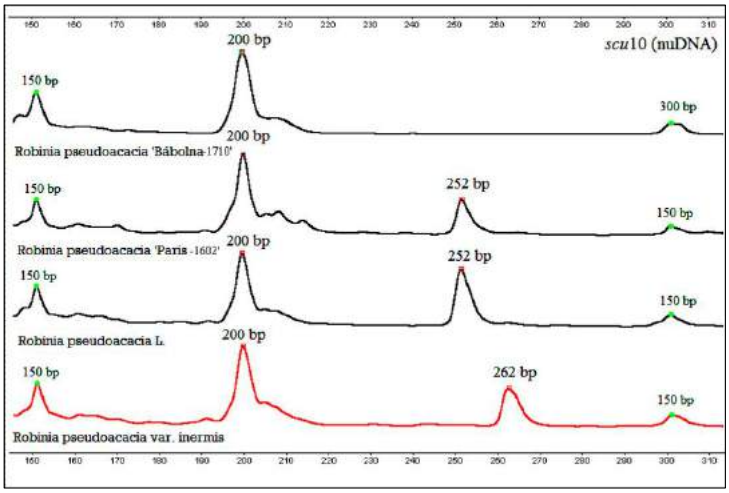

Figure 4. Molecular marker for thornlessness (*Robinia p. inermis*) at the nuDNA *scu*10 (262 bp) locus compared to ‘*Bábolna-1710*’ clone. PCR amplified *ALF fluorograms* (15-300 nt) of four *Robinia* clones were compared. Mw standards (150- and 300 bp), and the amplified DNA fragments (200-, 252-, and 262 bp) are indicated (*Gyulai et al., 2010*)

(D) Clonal propagation

Black locust trees mainly spread clonally through root sprouts. A recent analysis revealed that the genetic diversity of 31 black locust biotypes grown in Hungary, based on 170 DNA fragments of 28 alleles at three (*scu7, scu*10 and *ITS*1-5.8S-*ITS*2) loci of the genomes, follows a strict clonal propagation strategy (*Fig. 5*).

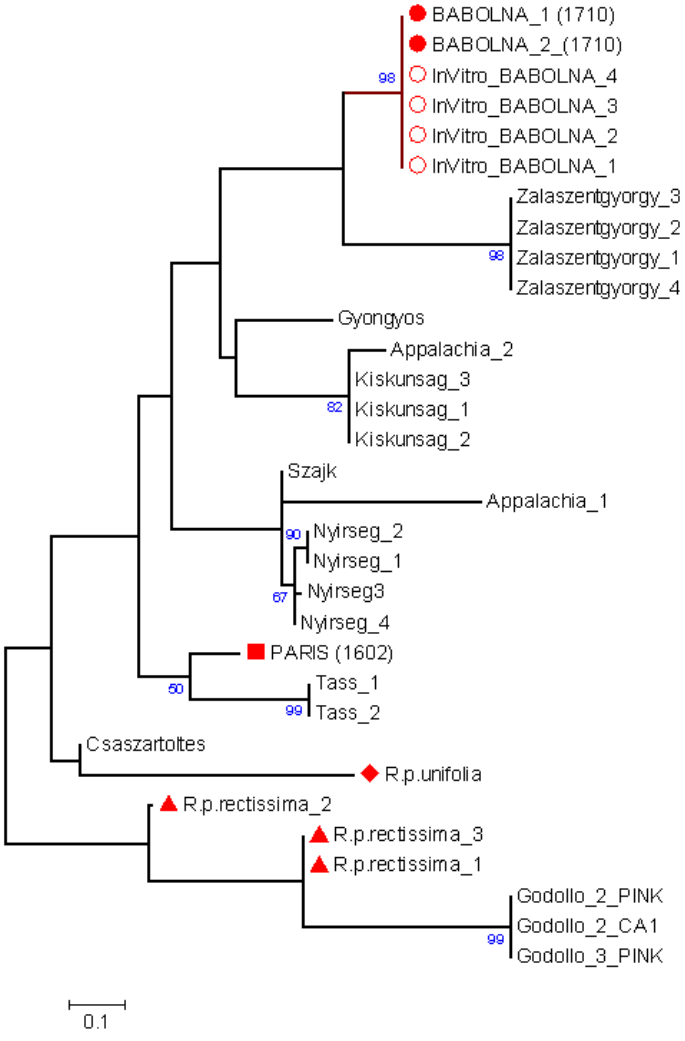

Figure 5. ML (Maximum Likelihood; *Hillis et al., 1994*) phylogram (MEGA4; *Tamura et al., 2009*) of *Robinia pseudoacacia* clones growing at different habitats of Hungary. The oldest Hungarian black locust (*Robinia pseudoacacia cv. ‘Bábolna-1710’*; (●) and its *in vitro* clones (○), and further 31 samples from natural black locust populations sampled in Hungary (names of cities are indicated) are compared to the oldest European clone ‘Paris-1602’ (France) (■), and two subspecies of *R. p. rectissima* (▲) and *R. p. unifolia* (♦). The phylogram was based on the patterns of 170 PCR fragments of 28 alleles at six SSR loci. Relative genetic distance (scale 0.1) and bootstrap values over 50% are indicated at the nodes (*Gyulai et al., 2010*).

(E) Conservation Genetics - Cloning of 'Living Fossils'

Delicate plant species, old varieties and heirlooms are threatened by extinction either due to changes in environmental conditions, overcultivation, or falling below the minimum viable population. Conservation genetics provides effective tools of biotechnology (*e.g.* micropropagation) to produce a large number of clones of old varieties. As the clones develop from somatic tissues or organs, the genome (*i.e.* total DNA) remains identical in each clone. In the case of the old *Robinia pseudoacaia* cv. '*Bábolna*', planted in 1710, micropropagation was successfully used for clonal propagation resulting in identical clones (*Fig. 6*). After rooting, clones will be planted in an ArchaeoBotanical garden in Gödöllő (Hungary).

By technique, shoot apical buds were dissected and processed for *aseptic shoot culture* following the general tissue culture protocols. Buds were cleaned, washed with detergent (3 min) and rinsed three times with distilled water (3 min), followed by surface sterilization with ethanol (70% v/v) for 1 min and a commercial bleaching agent (8% NaOCl w/v) for 1 min; followed by three rinses with sterile distilled water (ddH_2O), and incubated in aseptic tissue culture medium F6 (*Gyulai et al. 1992*) supplemented with 0.1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin, respectively. In a comparative experiment IBA (indole-3-butyric acid) was also used (1 mg/L) suggested. Clones, sprouted from aseptic buds in three weeks were further *micropropagated* with nodal segments (*Fig. 6*).

After the subsequent incubation time, clones were rooted on hormone-free medium, transplanted to pots and grown in green houses.

Figure 6. The oldest Hungarian black locust tree (*Robinia pseudoacacia cv. 'Bábolna'*) planted in 1710. *(a)* The memorial plate translation is: "*The black locust seeds were taken from North America in 1710.*" *(b)* The tree in the winter and *(c)* in the spring (also see in *Fig*. 2c) *(d)* blooming branches, *(e)* thorn, *(f)* the *polyphylla* type leaf, *(g)* the small, two seeded pod, and *(h)* the *micropropagated* clones *in vitro (Gyulai et al., 2011).*

Natural clones of the world's oldest trees indicate the importance of clonal propagation such as in the cases of an 11,700-year-old creosote bush (*Larrea tridentata*), the 'King Clone' (Mojave Desert, Lucerne Valley, California, USA), and the 9500-year-old Norway spruce (*Picea abies*) the 'Old Tjikko' (Sweden). Giant natural clone (8 km long) of marine *Posidonia oceanica* (Monocots, Alismatales) were identified recently and determined to be 100,000 years old. *Posidonia* species grow only in the Mediterranean and Caribbean seas and covers the seabed all round the islands. *Cymodocea* (also, Monocots, Alismatales) species also grow clonally in the seas and oceans, and also considered as land plant species, which 'went back' to the salty see water from where land plants evolved.

Giant natural clones among land plants also occur. The largest known clonal flowering plant, and indeed the 'largest plant and organism', is a grove of male (♂) 'trembling giant' poplar tree (*Populus tremuloides*) in Utah (USA), and estimated as 80,000 years old. Its nickname is 'Pando' (pando is from Latin for '*I spread*'). Its' been determined to be a single living organism by identical genetic markers and assumed to have one massive underground root system. The plant is estimated to weigh collectively 6,000,000 kg (6 tons), making it the heaviest known organism.

(F) Nitrogen fixation

Not only *Legumes* live in symbiosis with N_2-fixing soil bacteria. *Elaeagnus* (*Elaeagnaceae* of *Rosales*) and several other plant species are also able to grow in symbiosis with the N_2-fixing bacteria *Frankia*. This symbiosis results in nitrogen fixing nodules developing on the roots. Due to this symbiotic association, the growth of plants is independent from the soluble nitrogen nutrient content of the soil (*see Chapter 10*). The detailed N_2-fixation is summarized below:

(a) INF - Inorganic Nitrogen Fixation. Inorganic nitrogen fixation occurs at lightning in the atmosphere at terrestrial combustions, and during the industrial ammonia production (*i.e.* Haber - Bosch process) for manufacturing artificial fertilizers (*e.g.* NH_4NO_3).

(b) BNF - Biological (Organic/Prokaryotic) Nitrogen Fixation. BNF was discovered in 1901 (*Beijerinck, 1901*), which is the biological conversion of atmospheric nitrogen gas (N_2, dinitrogen) to ammonia (NH_3) catalyzed by the prokaryotic enzymes NITOGENAZE (EC 1.18.6.1, EC 1.19.6.1) of diazotroph organisms ($\mathbf{N_2 \rightarrow NH_3}$). As the result, the fixed aerial nitrogen is reduced to ammonia (NH_3) and became available not only to other prokaryotes in the soil or water but to the plants and finally to the animals.

BNF has been detected in various microorganisms *e.g.* in aerobic, phototrophic *Cyanobacteria,* anaerobic purple-sulfur bacteria (*e.g. Chromatium*), anaerobic green-sulfur bacteria (e.g., *Chlorobium*), chemolithotroph bacteria (*e.g., Alcaligenes, Thiobacillus, Methanosarcina,* and *Azospirillum*) and in the heterotroph bacteria (*e.g.* the anaerobe *Clostridium,* and microaerophile *Herbaspirillum*), and aerobe *Azotobacter*. In total, there are about 38 genera of Bacteria, 20 genera of Cyanobacteria and 87 species of Archaea, which are able to fix atmospheric nitrogen, and classified as diazotrophs.

N_2-fixation is a high energy consuming process, which needs at least 8 mol ATP per mol NH_4^+ produced. This high energy consumption is due to the very unique, triple chemical bond of dinitrogen molecules (N≡N), with high bond energy of 945 kJ/mol.

Both processes are catalyzed by enzyme nitrogenases, which are encoded by *nif* (nitrogen fixation) genes. The highly conserved sequences of the *nif* make it an ideal molecular tool to determine the BNF potential of agro/ecosystems and soils.

Structurally, the enzyme nitrogenase is a complex of two protein complexes of nitrogenase Mo-Fe (N*ase*; a tetramer, α2β2, Mo-Fe protein, encoded by ***Nif*DK**), and nitrogenase reductase **Fe-S** (NR*ase*; a homodimer, α2, Fe-S protein, encoded by ***Nif*H**) (*Fig. 7*). Basically, Nase, which is a very large enzyme with Mw about 240 kD, catalyzes the reduction of N_2 to NH_3, and NRase donates two high potential electrons at a time to N*ase*.

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                                      150       160       170       180       190       200       210
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|
P08925 Frankia alni           97  AGRGVITSITYLEEAGAYEN-LDFVTYDVLGDVVCGGFAMPIRQGKAQEIYIVTSGEMMAMYAANNIARG 165
Q8KQ04 Frankia sp             97  R.................D-.................................................. 165
F8B5Q3 Frankia sp             97  .......A..F...N....D-.................................................. 165
P0A3S2 Anabaena azollae       102 ....I..A.NF...N...QD-....S.................E.......................... 170
A8CIG7 Anabaena siamensis     102 ....I..A.NF...N...TD-....S.................E.......................... 170
Q3M5S8 Anabaena variabilis    102 ....I..A.NF...N...QD-V...S.................E.......................... 170
D4H6V6 Denitrovibrio acetiphil. 99  .......A.NF...E....ED....S.................E.......................SK. 168
G6FYF2 Fischerella sp         101 ....I..A.NF...N...QD-....S.................E.......................... 169
D7E3U2 Nostoc azollae         103 ....I..A.NF...N...TD-....S.................E.......................... 171
Q47917 Mastigocladus laminosus 101 ....I..A.NF...N...QD-....S.................E.......................... 169
D8G542 Oscillatoria sp        102 ....I..A.NF...N....D-....S.................E.......................... 170
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Figure 7. Consensus sequence alignments (*BioEdit; Hall, 1999*) of the highly conserved prokaryotic *Nif*H (nitrogenase reductase Fe-S) proteins (stretches of 140 aa to 210 aa) of *Frankia* ssp., and related N-fixing bacteria. Amino acids (single capital letters of the possible 21 aa), consensus aa (dots), and accession numbers (*UniProt*) are indicated.

Molybdenum in the Mo-Fe protein, (nitrogenise-1) may be substituted by vanadium resulting in *vnf*DK and *vnf*H (nitrogenise-2), or by iron in *anf*DK and *anf*H (nitrogenise-3). Genes for nitrogenase Mo-Fe (*nif*DK) and nitrogenase reductase Fe-S (*nif*H) contain highly conserved regions with strait phylogenetic resolutions especially in the case of *nif*H.

- *N-fixation by free-living soil bacteria.* These species are *Azotobacter*, *Bacillus*, *Clostridium*, and *Klebsiella.* Because nitrogenase can be inhibited by oxygen, free-living organisms are anaerobes.

- *Associative N-fixation.* Bacterium species of *Azospirillum* are able to form close associations with roots of grasses and cereal crops (*Poaceae*) such as wheat, corn, oats, barley and rice; and by this way, they release nitrogen (as ammonia NH_3) in the rizosphere. The optimal soil pH for *Azospirillum* growth is pH 6.8-7.8, and at temperature above 15 ºC with the optimum 32-40 ºC.

- *Cyanobacterial N-fixation* ***(Symbiosis I)***. Cyanobacteria (*e.g.* species of *Anabena* and *Nostoc*) fix nitrogen in their specialized cells called heterocysts. *Anabaena azollae* colonizes cavities which are formed at the base of fronds of *Azolla*, an aquatic fern, which is the only fern that can fix nitrogen by symbiotic association. Species of *Nostoc,* another genus of cyanobacteria, can fix nitrogen by symbiotic association with *Porella navicularis*, a liwerwort. *Cyanobacteria* can also live on the leaf surface of Angiosperms such as rain forest palm *Welfia regia,* and provide nitrogen to the host plant.

(F.1.) N-fixation by root nodule bacteria of Rhizobium and Frankia (Symbiosis II)

- *Rhizobial* N-fixation. Soil bacteria became specialized for developing and living in root nodules of only legume (*Fabaceae*) species of both woody and herbaceous, and fix aerial N_2. These species are *Rhizobium legumninosarum* of beans (*Phaseolus*) and peas (*Pisum*); *Sinorhizobium melilotii* of alfalfa (*Medicago*), *Mesorhizobium loti* of bird's-foot trefoil (*Lotus*), *Bradyrhizobium japonicum* of soybean (*Glycine*), and *Azorhizobium caulinodans* of sesbania (*Sesbania* ssp).

Figure 8. Frankia root nodules (*left and middle*) on seabuckthorn (*Hippophae rhamnoides ssp. Carpatica*) (Rosales!) (*right*). Plotting mm-paper for sizing (*Photo by BG Kerti*).

- *Frankial* N-fixation. Filamentous *Actinomycete* of *Frankia* strains, similar to legume *Rhizobia*, also fix nitrogen by living in root hair nodules (*Fig 8*).

The genus *Frankia* was described first as filamentous fungus and named by *J. Brunchorst (1886)* to honor the biologist *AB Frank*, and only recently (1970), and it was classified to prokaryotic Actinobacteria (*syn.*: *Actinomycetales*). Complete circular genome sequences of *Frankia* strains are available with size rage from 5.433.628 bp (*NCBI # CP000249.1*) to 8.982.042 bp (*NCBI # CP000820*), with very high GC ratio (72.8 %), low gene (6.782) and protein numbers (6.707) (*NCBI # CP000249.1*). In total, three main *Frankia* strains were isolated from species of three plant orders of Fagales (all *Alnus* species, some species of *Casuarina*, *Allocasuarina, Coriaria, Morella, Myrica, Gymnostoma)*, two genera of Cucurbitales (*Coriaria* and *Datisca*), and numerous genera of Rosales (*Elaeagnus, Hippophae, Shepherdia, Colletia, Discaria, Kentrothamnus, Retanilla, Trevoa, Caenothus, Dryas, Purshia, Chamaebatia* and *Cercocarpus*). The 16S ribosomal DNA sequences of the root nodules of these species indicated that the nodules are formed by *Frankia*.

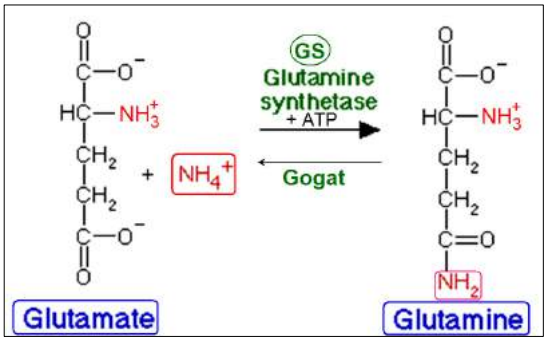

Figure 9a. The metabolic function of GS (glutamine synthase enzyme; EC 6.3.1.2)

(F.2.) Plant ammonification

As plants are not able to fix atmospheric N_2, they take up inorganic N-salts of NH_3/NH_4^+ and NO_3^- are fixed by bacteria (summarized above). When NH_3 is taken up, enzyme GS **(**glutamine synthases; EC 6.3.1.2; *syn*: gln-synthase**)** transfer it to amino acid (aa) glutamate, which became glutamine (this process is called ammonification) (*Fig. 9a,b*). The reverse reaction is catalyzed by enzyme GOGAT (glutamate synthase; EC 1.4.1.14). The 2-oxoglutarate can also bind NH_3, which transamination is catalyzed by enzyme GDH (glutamate dehydrogenases) (GDH1, EC 1.4.1.3), which reaction also produce glutamate aa (*Fig. 9b*). When plants take up anionic NO_3^- (from NH_4NO_3 or KNO_3) the process is catalyzed by NR (nitrate reductase; EC 1.7.99.4) ($NO_3^- \rightarrow NO_2^-$) followed by NiR (nitrite reductase; EC 1.7.2.1), which catalyzes the reaction of $NO_2^- \rightarrow NH_3$, and finally closed up also by GS.

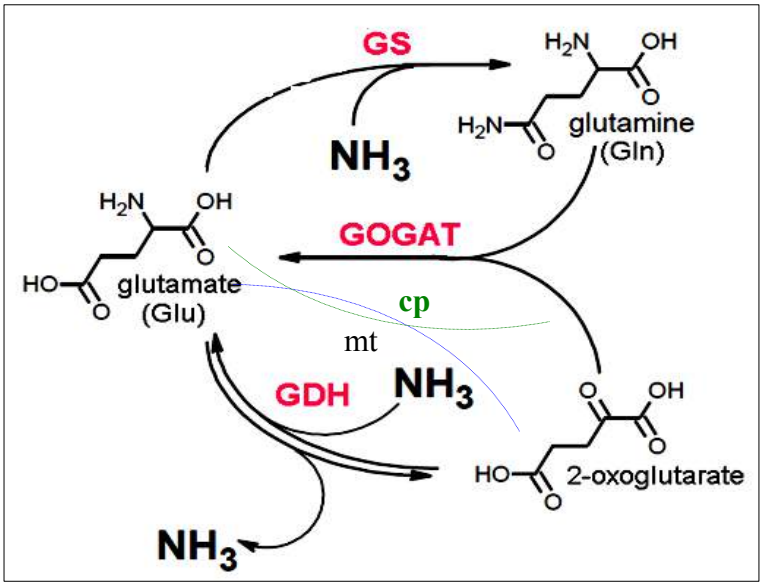

Figure 9b. Key metabolic routes of ammonium in plants. The reversible transaminase GS (glutamine synthase, EC 6.3.1.2; *syn.*: GLUL - glutamate-ammonia ligase), and GOGAT (glutamine oxoglutarate aminotransferase; EC 1.4.1.13; *syn.*: glutamate synthase) in the chloroplasts (*cp*); and the reversible enzyme GDH (glutamate dehydrogenase, EC 1.4.1.3) located in the mitochondria (*mt*) are indicated (*Kőmíves et al., 2015 in Bittsánszky et al., 2015*).

(F.3.) N-loss

Plant N-loss releases the nitrogen-monoxide (NO). NO is produced not

only from nitrite NO_2^- → NO (catalyzed by FdCo-NiR; the FerredoxinCopper-NiR; # EC 1.7.7.1) but also from the amino acid L-arginine (L-Arg), which is the main substrate for NO-synthesis through the reaction of arginine + O2 → citrulline + NO (*Fig. 9c*); and catalyzed by NOSs (nitric oxide synthases, 1433 aa; # EC 1.14.13.39).

The NO released (*Gyulai et al., 2014*) has several vital functions in animals (vasodilatator, see Viagra) and in plants. In the *in vitro* plant experiments, NaNP (Na-nitroferricyanide III, $Na_2Fe(CN)_5NO \times 2H_2O$, is used ($10^{-7}$ to 10^{-4} M) to release NO.

(G) Samples of other legume trees

Next to *Robinia*, there are several other important legume trees and shrubs growing in the Northern Hemisphere (*Fig. 10*). Next to *Gleditsia triacanthos* (2n = 28), there are several other *Gleditsia* species *e.g. Gleditsia amorphoides, G. aquatica, G. caspia, G. ferox, G. japonica, G. sinensis, G. fera, G. melanacantha, G. macrantha, G. heterophylla,* and *G. horrida.*

The evergreen, legume, thorny gorse (*Ulex europaeus*), with yellow flowers, is the main *hedge bushes* in England and Wales.

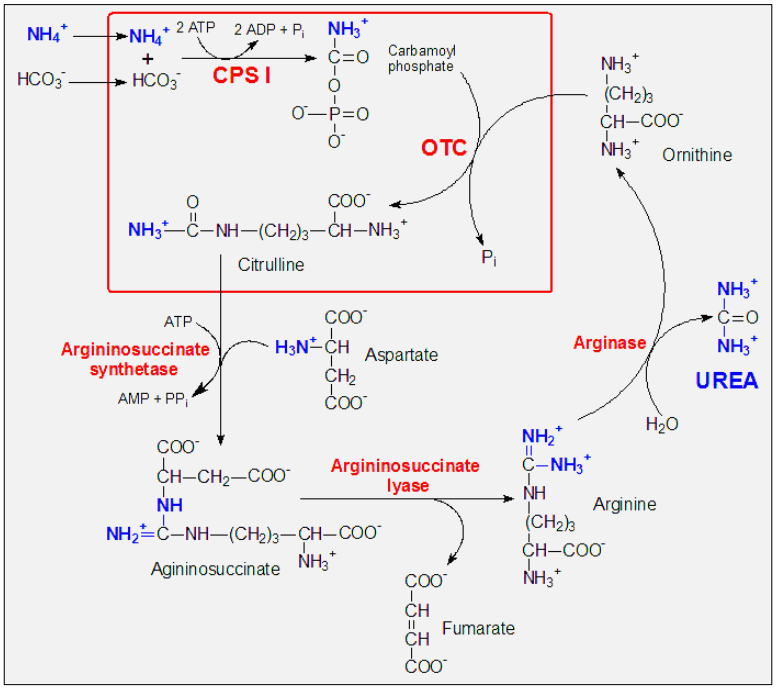

Figure 9c. Diagram of the *urea cycle*. Reactions taking place in the mitochondria are indicated in red rectangle. Enzymes including CPS-I (*Carbamoil Phosphate Synthetase*-I) and OTC (*Ornithine Transcarbamoylase*) are indicated.

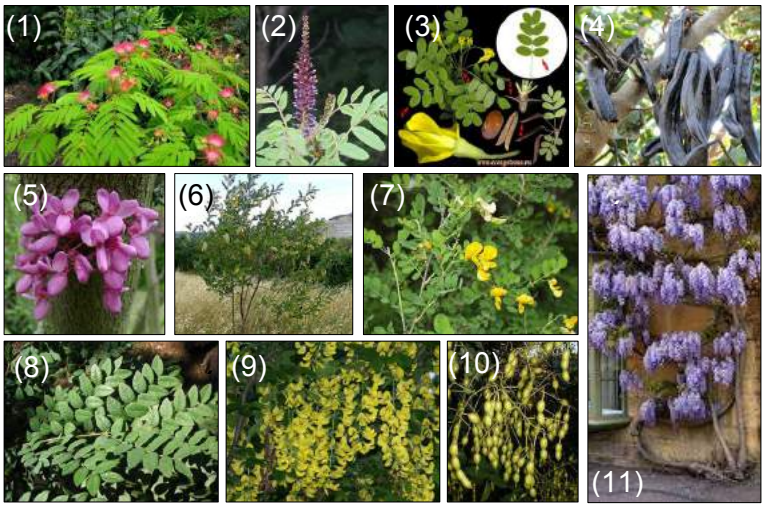

Figure 10. Samples of legume trees growing in the Northern Hemisphere including Hungary (except the 4th sample). (**1**) *Albizia julibrissin* (Persian silk tree); (**2**) *Amorpha fruticosa* (Desert false indigo; a dangerously invasive plant in Hungary); (**3**) *Caragana arborescens* (Siberian pea shrub); (**4**) *Ceratonia siliqua* (Carob tree, *syn.* St. John's bread); (**5**) *Cercis siliquastrum* (Judas tree); (**6**) *Colutea arborescens* (Bladder senna); (**7**) *Coronilla (Hippocrepis) emerus* (Scorpion senna); (**8**) *Gymnocladus dioicus* (Kentucky coffeetree); (**9**) *Laburnum anagyroides* (Golden rain); (**10**) *Sophora japonica* (Japanese pagoda tree); (**11**) *Wisteria sinensis* (Chinese Wisteria) *(Láposi and Gyulai, 2011).*

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Woody grasses - Temperate bamboos (genus *Phyllostachys*)

(A) Introduction

Bamboos are *woody grasses* of family *Poaceae*, comprising six tribes of *Bambuseae, Buergersiochloeae, Guaduelleae, Olyreae, Parianeae* and *Puelieae* with about 111 genera and over 1,600 species, with sizes from *small herbaceous* to *giant timber bamboos*. Genus *Phyllostachys,* with about 50 species (*Fig. A*), *belongs to* subtribe *Shibateinae*. Bamboos are the fastest-growing woody plants in the world, they can grow up to one meter a day (3.8-5.0 centimetres per hr).

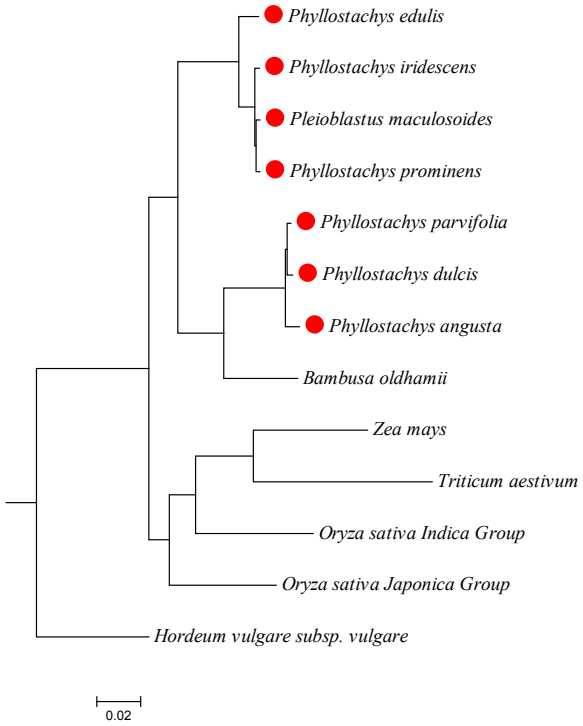

Figure (A) Genetic phylogram (*Fast Minimum Evolution*) of *Phyllostachys* species and the closest relatives of monocots based on the sequence *PAL*1 gene (*Phenylalanine Ammonia-Lyase*) (complete cds; *NCBI Sequence ID: FJ594468.1*; Length: 2260 nt).

There is a rising global interest in the use of bamboos as they are regarded as renewable resources with strong regeneration potential. *Phyllostachys* is a temperate bamboo genus belonging to *Poaceae,* (subfamily *Bambusoideae,* tribe *Bambuseae,* subtribe *Shibataeinae*), and its species are mainly native in warm temperate regions of SE China (*Ma et al., 2014*). Since centuries they have additionally been grown in neighbouring countries including Korea and Japan (*Wang and Stapleton, 2006*), where they have become naturalised. There are a total of 60 species growing in China alone. The natural distribution of bamboos is greatly affected by the amount and yearly distribution of precipitation. Sufficient and evenly distributed precipitation during the spring-summer-autumn growing season is extremely important for bamboos (*Kleinhenz and Midmore, 2001*). They are characterised by high canopy transpiration regardless of culm age (*Tsuruta et al., 2016*), which is comparable to coniferous forests. *Phyllostachys edulis (syn. P. pubescens)* is the most important species covering 30% of bamboo forests covering a total area of over 7.2 million hectares in China.

Similarly to other monocarpic, monopodial genera of *Bambuseae* (*Abe and Shibata, 2012, 2014a,b; Matsuo et al., 2014*) *Phyllostachys* are also classified as monocarpic (some taxa proven to be polycarpic), and reproduce mainly vegetatively (*Isagi et al., 2016*) from nodal shoots developing from the underground rhizome system, which comprises 39-57 % of the total biomass. This means there is an underground rhizome system with buds on each node, which have the capability to develop into either above ground culms or continue as a side branch of the underground rhizome system (*Ueda, 1960*).

Phyllostachys species are among the fastest growing plants. *Ueda (1960)* reported 119 cm and 121 cm of longitudinal growth in 24 hours of culms of *P. edulis (syn. P. pubescens)* and *P. bambusoides (syn. P. reticulata)* respectively in Kyoto, Japan.

Phyllostachys species are greatly versatile in their agricultural use, and as such are important in the agriculture of East-*Asian* countries.

In Europe they are important mainly in ornamental horticulture, but their agricultural potential other than as ornamentals has not been fully investigated and exploited.

Japan's long history of bamboo usage is evidenced by bamboo's appearance in ancient literature. People in Japan have traditionally used naturally growing species of the genus *Phyllostachys* and other genera. Their usage has been handed down to the present day in the form of traditional products the creation of which requires skillful techniques. Some of the many historical and new modern uses of bamboo in Japan include (*Shibata, 2015*) as:

Sacred plants. Bamboo came to be regarded as a highly sacred plant under the influence of Chinese culture. Culms and/or branches of bamboo are used as god lodging parts in religious services and as tools in religious ceremonies.

Materials for primary industry. Bamboo culms are ideal material for use as poles and similar in agriculture, aquaculture and other primary industries.

Materials for woven products. Some bamboo species are easy to split, which makes them useful for the manufacture of woven baskets, sieves, blinds and the like.

Materials for tools used in the tea ceremony and ikebana (Japanese art of flower arrangement). Without bamboo these typical Japanese cultural activities would not have been established. Particularly it would have not been possible to produce the tea whisk - *Chasen*.

Materials for musical instruments. Some traditional Japanese flutes and some castanets.

*Materials for traditional and everyday application:*Due to bamboo's excellent material characteristics precise rulers, knitting needles, and rubbing pads.

Materials for food and wrapping: Bamboo shoots are very important as vegetables, and suitable for fermentation and drying. Bamboo leaves and culm sheaths have long been known for their antibacterial properties, and have environmentally been used in Japan to wrap a variety of food types. Such usage continues today for traditional foods like sushi and Japanese cakes. Bamboo leaves are also used in Japan to make tea, alcohol and other beverages. As the green surface part of bamboo culm is considered to have a stronger antibacterial effect, it is sold today as a high quality product.

Materials for modern application: The bamboo filaments facilitated Thomas Edison's invention of the light bulb around 136 years ago. The use of bamboo in the construction of reinforced-concrete buildings as the alternative to steel is still regarded as important today in some developing countries.

Horticultural usage. In Japan, bamboo is regarded as a sacred plant due to the Chinese cultural influence, so is an important material in gardening. Bamboo's temperate climate zone preference and growth vigor make it ideal even in tight spaces. The large number of species of various sizes (from 0.5 m to 20 m tall) and ornamental characteristics (culm shape, leaf variegation, culm color etc.), all adds to its versatility.

Woody materials resources. Lamination techniques must be applied to process the bamboo material for use in construction. In Japan, kitchens and other parts of houses prone to water exposure have laminated bamboo timber flooring for its antibacterial and deodorant properties. Split bamboo is used in the construction of wind-resistant greenhouses.

Charcoal resources. Bamboo charcoal is as a high-quality, versatile material, due to its high density very fine pores creates high absorbency, making it suitable for a variety of applications including under floor humidity control and deodorization. Bamboo charcoal powder is also extensively used in soil improvement, cosmetics, audio equipment, an energy resource in industry and bamboo active charcoal for use in water purification.

Fiber resources. Bamboo fiber is known for its high quality, with usages depending on its fineness. Coarse fiber is used for purposes such as making compost and road pavement materials, while fine fiber from bamboo culms and sheaths is regarded as a superior material for the production of traditional Japanese paper (*Washi*). Bamboo culms are today used industrially for pulp in Japan, while fine fiber is also used for clothes, food, industrial product reinforcement and efforts have recently been made toward its use on a nanoparticle level, also with potential for use in the production of bio-plastics and bio-glass.

Extract resources. In Asia some *Phyllostachys* taxa have long been used in phytotherapy due to their phytochemical content and antioxidant properties. Bamboo shoots contain dietary fiber, protein, carbohydrates, amino acids, minerals, flavonoids, phenolic compounds, and sterols. Shoot or culm extracts have high antioxidant, anti-inflamatory, anti-hypertensive, antibacterial and anti-malassezia properties. Shoot or culm extracts have also been shown to have immuno-stimulating, anticarcinogenic, in ischemic injury treatment anti-apoptotic properties. Other beneficial health effects include: use as a source of dietary fiber improving lipid profile and bowel function, as therapeutic agent for atopic dermatitis, allergic airway diseases including asthma, for the prevention of hypercholesterolemia, high blood pressure, pulmonary inflammation and lung injury, and to relieve lipotoxicity (*Neményi et al., 2015*). The *herba* of leaves have high antioxidant properties and contain flavonoids, phenolic acids, chlorogenic acid derivatives, phenolic and flavone glycosides. Leaf extracts have been shown to have anticarcinogenic, cardioprotective effects, relieve lipotoxicity, protect against oxidative stress-induced glaucoma. Leaf extracts can synergistically be used in combination with other compounds in the treatment of immune/allergic diseases related to mast cells, in the treatment of leukaemia, in the prevention and treatment of diabetic complications and autoimmunity. Bamboo leaves are also used as food additives, and bamboo leaf extracts also exhibit antimicrobial activity during food storage (*Neményi et al., 2014*).

Energy resources. Bamboo charcoal has traditionally been used as an energy resource in Japan producing more heat than common timber charcoal. The use of woody resources including bamboo in pellet form as an energy source is currently being reviewed in Japan. The combined combustion of bamboo chips at electric power plants and the use of bamboo chips for bio-gas power generation has been implemented and trials involving the use of bamboo for bio-ethanol production is also ongoing.

Environmental capital resources. Bamboo forests account for less than 1% of forest area in Japan, but they are regarded as important in the resolution of global warming issues. They are recognized as supporting specific biodiversity and helping to maintain favorable environmental conditions. Plans are in place to evaluate these properties toward environmental capital resumption in bamboo forest management in many parts of Japan.

The first plants were introduced to Europe in the United Kingdom in 1720. From 1856 Eugéne Mazel played an important role in introducing several taxa from Japan and China to his park in Prafrance, Anduze, France, propagules of which were distributed to other countries including Hungary, where *Phyllostachys aurea*, *P. bambusoides*, *P. flexuosa, P. nigra* var. *nigra* and *P. viridiglaucescens* were growing in Botanical Gardens, mansion and Royal palace parks in the 1880-ties. From the beginning of the 20th century vegetative propagating material of many taxa have been imported from Japan and more recently from China and redistributed in Europe

(B) Perclinal chimeric mutations in many taxa produce similar culm forms

Many *Phyllostachys* taxa have phenotypically similar culm variations which appeared as spontaneous mutations in groves of the basic species. They were described as either *varietas, forma* or cultivar and are propagated vegetatively.

They include green core with yellow surface periclinal chimera mutation type of culm (g'GG) where LI turns yellow and LII and LIII remains green creating a yellow culm with green stripe in the canal or *sulcus* (*Okamura and Tanaka, 1986*). This is characteristic of *Phyllostachys aurea* f. *koi, P. aureosulcata* f. *spectabilis, P. bambusoides* f. *castillonis, P. edulis* f. *bicolor,* while *P. edulis* f. *nabeshimana,* and *P. vivax* f. *viridivittata* have a high ratio of the mutable yellow gene (g') in LI and also a mutable green gene (G') creating many green longitudinal stripes on the yellow culm surface.

The other is a yellow core green surface periclinal mutation type of culm (Gg'g'), where LI remains green and LII and LIII turns yellow, creating a green culm with yellow stripe in the canal or *sulcus* (*Okamura and Tanaka, 1986*). This is typical of *Phyllostachys aurea* f. *flavescens-inversa, P. aureosulcata* var. *aureosulcata, P.angusta* f. *flavosulcata, P. arcane* f. *luteosulcata, P. bambusoides* f. *castilloni-inversa, P. edulis* f. *luteosulcata, P. hirtivagina* f. *luteovittata, P. nidularia* f. *mirabilis, P. sulphurea* f. *houzeauana, P. violascens* f. *notata,* and *P. vivax* f. *huangwenzhu*.

The third is when there is a mutable yellow gene (g') in each germlayer (LI, LII, LIII) creating an almost purely yellow culm (g'g'g') with very few occasional green stripes (*Okamura and Tanaka, 1986*). This type of mutation characterizes *Phyllostacys aurea* f. *holochrysa, P. aureosulcata* f. *aureocaulis, P. bambusoides* f. *holochrysa, P. edulis* f. *holochrysa, P. sulphurea* var. *sulphurea,* and *P. vivax* f. *aureocaulis.* There are also other mutants with distorted internodes *(P. edulis* f. *heterocycla, P. edulis* f. *subconvexa)* or anthocyanin pigmentation *(P. nigra* var. *nigra,* and *P. nigra* f. *boryana)* (*Fig. 1*).

(C) Single clone, no seedling regeneration in the wild

Phyllostachys species reproduce sexually every 60-120 years depending on species, two clones of *P. edulis (syn., P. pubescens)* have been determined to have flowering intervals of 67 to 69 years in Japan (*Shibata, 2002*). Though flowering plants are greatly depleted by the successive flowering event lasting 2-3 years, they probably do not die under natural forest conditions, but regenerate vegetatively from the rhizome system similar to which was observed in the case of *P. bambusoides (syn., P. reticulata,* Madake*)* and *P. nigra* var. *henonis* (Hachiku) in Japan. When available *Phyllostachys* taxa can be propagated in nurseries from seeds, but there is a long nursery raising period of 4 years (often under greenhouse conditions depending on local climate), before seedlings can be transplanted out into the field and one to two decades to reach maximum mature size. *Isagi et al., (2016)* studied, the genetic diversity of *P. edulis (syn., P. pubescens)* for the entire distribution range of the species in Japan to a vast area of its range in China. They used 16 microsatellite markers for the analysis. They used 246 leaf samples of adult *P. edulis (syn., P pubescens)* collected from 28 populations spanning the entire ranges in Japan (n = 20) and a large area in SE China (n = 8).

They also analyzed the patterns of *pollination* (selfing or outcrossing) from leaf samples that were collected from seedlings (32 and 30 for each flowering event respectively)

resulting from the flowering events in 2005 and 2006, planted in the Wakayama Farm, Tochigi Prefecture, Japan. Among these, they found that 12 loci fixed by a single allele, whereas they detected only 2 alleles for each of the remaining 4 loci. The observations indicated that all samples they collected from Japan and China are ofsspring of an identical single clone. This clone distributed over more than 2,800 km and they estimated its biomass to be approximately 6.6×10^{11} kg, which is exceptionally large (*Isagi et al., 2016*). They also report that 20 different genets were generated among seedlings from flowering events of 2005 and 2006, by recombination through selfing of a single flowering genet/colony. (*term genet refers for a colony of plants, fungi or bacteria that come from a single genetic source*). *Isagi et al., (2016)* reported that the diverse composition of genets among seedlings and the predominance of a single clone in the wild suggest that the intermittent flowering of *P. edulis (syn., P. pubescens)* in the wild has possibly produced a variety of clones through recombination, but the single clonality caused the breeding system to perform selfing, but in the wild, they did not find any adult plants with genotypes generated by selfing of the dominant clone. *Inbreeding depression* might be one of the reasons for the low viability of *P. edulis* seedlings in the wild. Also, according to their theory the resulting seedlings possibly cannot compete with other tree species or adult *P. edulis (syn., P. pubescens).* So, sexual reproduction does not contribute to the remarkable expansive range of this species for almost all adult *P. edulis (syn., P. pubescens)* plants growing in Japan and China, whivh are likely the vegetative offspring of a single clone propagated by human transplantation, and its subsequent expansion through the rhizome-culm system (*Isagi et al., 2016*). The flowering pattern of *P. edulis*, which was characterized by partial flowering of a clone (*polycarpic*), was similar to that of the *Sasa* species. Thus, they suggest it is unlikely that all culms of the single genet distributed in Japan and China flower and die simultaneously. In their opinion the relatively small size of the flowering area and rapid reproduction of culms has led to the stability of *P. edulis (syn., P. pubescens)* communities (by *polycarpy*). Genetic diversity contributes to *disease tolerance*, evolutionary potential, ecosystem function, and adaptation to the changing environment. So, the very low genetic diversity is an important consideration for the long-term management of this widely distributed temperate bamboo species (*Isagi et al., 2016*).

Figure 1. ***P. edulis*** f. *bicolor* in Japan (*top*). Middle (*left to right*): ***P. nigra*** f. *boryana;* ***P. aurea;*** ***P. edulis*** f. *nabeshimana;* ***P. nigra*** var. *henonis;* ***P. edulis*** f. *bicolor;* and ***P. edulis*** f. *heterocycla.* Bottom (*left to right*): ***P. nigra*** var. *nigra;* ***P. violascens;*** ***P. vivax;*** ***P. vivax*** f. *viridivittata;* ***P. vivax*** f. *huangwenzhou;* and ***P. aureosulcata*** f. *aureocaulis*

(D) Bamboo seed morphometric analysis

Computer-assisted digital morphometry (*Fig. 2*) provides a new powerful array of objective measurements of size, shape, tomography, texture, fractals, *etc*. of significantly large populations of objects rapidly, with accuracy and precision (*Rovner and Gyulai, 2007; Gyulai et al., 2015*). Bellow we present, an analysis of bamboo seeds of *P. edulis (syn. P. pubescens)* by using *Fovea Pro 4.0 Computer Program* coupled with *Adobe Photoshop* and *IBM SPSS* data analyses (18 parameters of 33 measured were used).

We also aimed to determine the evolutionary stage of the bamboo plant by estimating the levels of variation (driven by '*Force of Mutation*') *vs*. selection (driven by '*Force of Selection*') based on histogram analysis (*MS Excel*) of digital seed parameters.

Phyllostachys edulis (syn. P. pubescens) seed lots commercially available in 2013, 2014 and 2015 from China were analysed. For the analysis 200 seeds per lot were used.

The following 18 seed morphometric parameters were analysed: *Area (cm^2):* If pixels are interpreted as little squares, the area of a feature is determined by counting the connected thresholded pixels and applying a calibration factor based on the size of the pixels. *Convex Area (cm^2)*: a shape is convex if there are no "dents" or indentations in it. *Length (cm)*: maximum caliper dimension. *Breadth (cm)*: minimum caliper dimension. *Equiv.Diam (cm):* the diameter of a circle with the same area as the feature. *Inscrib.Rad. (cm):* Radius of the inscribed circle. *Circum.Rad. (cm):* Radius of the circumscribed circle. *Perimeter (cm):* distances between the centres of pixels on the object side of the boundary. *Convex Perim (cm):* perimeter of the convex bounding polygon. *FormFactor:* 4π•Area / Perimeter. *Roundness:* 4 • *Area* / π • M*ax Dimension. Aspect Ratio: Length / Breadth. Solidity*: *Area / Convex Area.* Convexity: *Perimeter / Convex Perimeter. Symmetry:* The symmetry of a shape is encoded in the Fourier transform of its boundary. *Radius Ratio*: *Inscribed Radius / Circumscribed Radius. Elongation: Area / ConvexHull Area. Skeleton Length (cm):* total lineal distance down the centre line of an object (*see Chapter 15*).

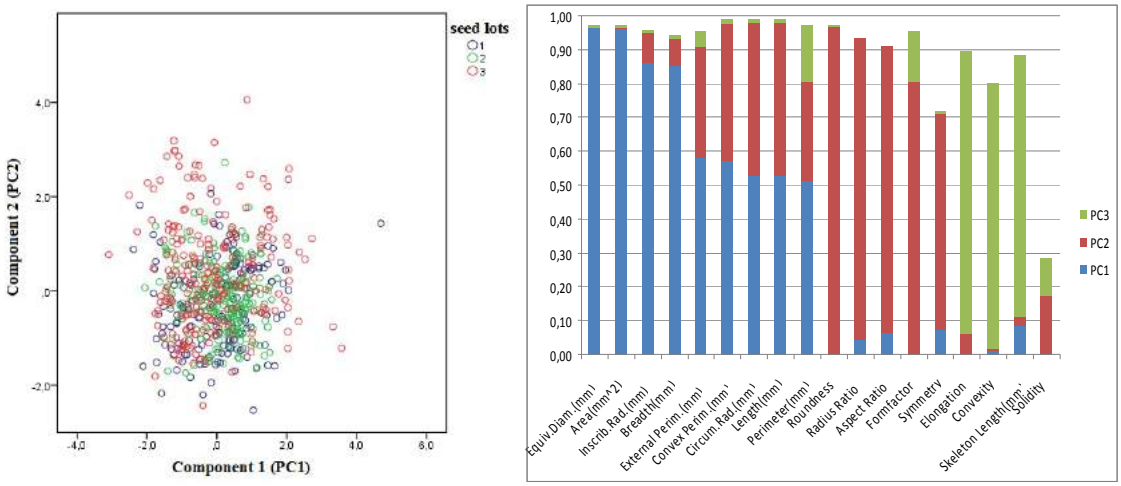

Figure 2. Principal Component Analysis (PCA) (*IBM SPSS*) of the first two principal components of seed parameters of the three seed lots (2013 - blue, 2014 - green, 2015 - red of *P. edulis (syn. P. pubescens)* (*left*). Shares of variances (%) explained by the first three Principal Components (IBM SPSS) for 18 seed parameters of the of the three seed lots of *P. edulis (syn., P. pubescens)* studied. Seed 'Solidity' was found not correlative (*right*).

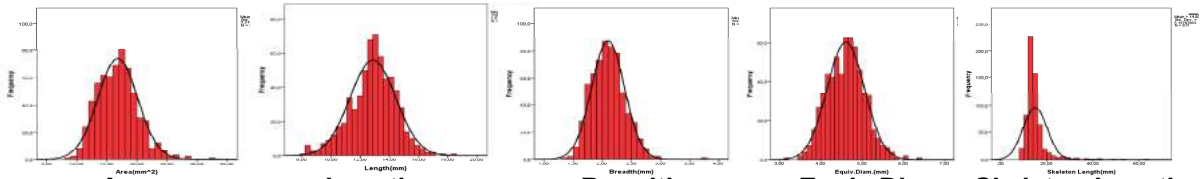

Figure 3. Samples of histogram analysis (*MS Excel*) for five of the 18 seed parameters (Y-axis) based on digital seed morphometry data of the three seed lots (2013, 2014, 2015) of *P. edulis (syn., P. pubescens)*.

Histograms of the seed parameters from the analysis, revealed that *P. edulis (syn., P. pubescens)* seeds show a close distribution to a normal distribution for the 18 parameters (*Fig. 3*). Histogram statistics (by SPSS) proved the visual observations of histograms (*Fig. 3*). In histogram statistics, skewness shows the level of symmetry (*i.e.,* the lack of symmetry), and its value is zero in the case of normal distribution. Negative skewness indicates right-modal distribution, which is the result of high number of seeds with higher seed parameters than the mean, and with small number of seeds with lower parameters than the mean; and *vice versa* in positive skewness. Most of the distributions showed statistically normal distribution, however, the measures of skewness for Solidity confirmed significant negatively skewed distributions.

The *Radius Ratio, Roundness, Formfactor* and *Symmetry* of seeds showed significant positively skewed distributions. In the case of kurtosis, only three parameters (*Skeleton Length, Elongation,* and *Convexity*) of *P.edulis (syn., P. pubescens)* seeds showed significantly peaked and skewed distribution with high values. The following 8 parameters do not deviate from a normal distribution: *Area, Breadth, Equiv. Diam., Inscrib. Rad., Perimeter, External Perim., Convex Perim.,* and *Aspect Ratio*. The study of *Rovner and Gyulai (2007)* revealed that, unlike previous assumptions, seed parameters of wild plant species are not normally distributed. Rather, they typically show a multimodal distribution, and the cultivated plants show normal (*unimodal*) distribution. Values of X- (*i.e., skewness*) and Y-axis (*i.e., kurtosis*) of histograms of seed parameters were also postulated as the *level of diversity* (along X-axis), which shows the level '*Force of Mutation*'; and the *level of selection* (along Y-axis with high/low protrusions), which shows the '*Force of Selection*' (*Gyulai et al., 2015*).

In our study here, *P. edulis* showed high Y-values (*i.e., 'Force of Selection'*) in three - Inscrib. Rad, Length and Area - of the previously determined three most discriminative CRT parameters (*Gyulai et al., 2015*). Seed Diversity Index (SDI) of the three seed lots of *P. edulis*, calculated as mean values of the *Relative Standard Deviations* (RSD%) of the 18 seed parameters were as follows: 12.9 (2013), 14.39 (2014), 16.56 (2015), and total sample (3 seed lots) 15.75. This result may indicate a tendency of narrowing variation which, consequently, increases the vulnerability of this species to environmental change. This is confirmed in practice by the authors since the single clone of *P. edulis (syn., P. pubescens)* imported from Japan is the least adaptable, observed among the more than 60 *Phyllostachys* taxa under trial in the Gödöllö Botanical Garden of Szent István University, Hungary. Also an observation of the seedlings derived from the sowing of the 3 seed lots showed 3-5% of seedlings were affected by albinism which can also be regarded as a sign of the selfing of the single clone of *P. edulis (syn., P. pubescens)*.

(E) Conclusions

Phyllostachys taxa are vegetatively propagated for centuries, and as such, globally distributed, resulting in mainly single clonal plants with low genetic diversity. There is no natural regeneration of seedlings in groves of *P. edulis* and *P. bambusoides* as a possible result of competition with other plants, and the vegetatively growing 'mother' plants from rhizomes. A single clone of *P. edulis* planted over centuries ago in Japan and SE China was proven by DNA analysis. Seed morphometry also shows low variability of seed morphometric characters in *P. edulis* (*syn., P. pubescens*). Annual generative propagation from seeds is only possible in the case of *P. edulis* (*syn., P. pubescens*), but it is a result of *selfing* of the single clone which produces seedlings with limited genetic diversity. Efforts should be made during the establishment of new *bamboo forests* to use generative planting material (seedlings) to improve genetic diversity of the species.

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Forest buffer zones, and hedgerows in agro-ecosystems

Industrialized agriculture has become more and more dominant during the second half of the 20th century, and the trend keeps going on into the 21st century as well. One of the most prominent consequences of this change is the unfavourably altering landscape. Diversity, which once was a general characteristic across vast areas, has diminished with the spread of industrialized production. Patches of forests and shrubs, hedgerows were and are being eliminated, and the small arable plots are merged into huge blocks of homogenous fields measuring well over hundreds of hectares. However, it is not monotony and repetitiveness that makes us worry. The increase in field size on the long run contributes to decreasing soil fertility: the lack of hedges means the lack of protective shields against the erosive effects of wind. Abolishing hedges also means taking away habitats of beneficial organisms; those are arthropods, birds, and mammals that control pests.

1. The pros and cons of hedgerows: facts and myths
2. Planting and maintaining hedges

1. The pros and cons of hedgerows: facts and myths

The possibility of droughts is lower where there are forest strips or rows of bushes scattered within the field, as opposed to large, tree-less areas. The reason behind this general experience is not only that hedges slow the speed of winds. Hedges are also able to trap the various forms of aerial moisture such as mist, fog, hoarfrost or rime. Hedges, masses of organic material, are able to store the excess water of melting snow or downpours. Trees and bushes are also able to drain water from depth unavailable for herbs. In dry periods, the moisture evaporated by the forest patches reappears as morning dew on the surfaces of arable lands. Therefore fields bordered by hedges are less prone to desiccation.

Hedges buffer pesticide drift and it is extremely important in organic production. There is no need to explain the importance of keeping an area pesticide free: when the yield is no longer certified organic, the farmer can sell it only as conventional, that is, at a much lower price.

On the long run, forest patches offer additional products such as firewood, timber material, forest fruits, edible mushrooms, herbs and game. The collection, processing and management of these special side products are usually labour-intensive, but due to the immense demand, are highly marketable.

Hedges reduce production area, but there can be some other, less obvious disadvantages, too.

If not properly planned, hedges may leave crooked corners, small irregular pieces of land and thus present an obstacle to machinery. When planning and planting ignore local ventilation conditions, hedges can also create pockets of frost or heat.

Where the low level of shrubs and herbs is missing, the patch of trees rises immediately beside the field. These trees not only cast shadow on the field, but create an unfortunate whirl of wind as well, and these combined decrease the yield along the edges of the field.

All these disadvantages are avoidable with careful planning. And these are the reasons an already existent hedge should be reconstructed, replanted or removed.

Let us take a closer look on the role of hedges from the pest management point of view.

There are some issues labelled by experts as ‘disadvantages’, but when inspected thoroughly, most of these concerns can be dismissed.

One of the most important among them is weeds. Between the hedge and the field there is, inevitably, a strip of herbaceous plants. Although many express their worries about this strip increasing the number of weeds within the field, this is relevant only when the edge and the strip are being disturbed on a regular basis, that is, when machinery repeatedly treads the strip, or when the soil of the strip is broken up time and again. Ragweed (*Ambrosia artemisiifolia*), redroot pigweed (*Amaranthus retroflexus*) and lamb’s quarters (*Chenopodium album*), weeds so characteristic of arable fields not only tolerate, but require trampling and disturbance. Ragweed for example that thrives the next year after the soil has been disturbed will continue proliferating for a couple of years, but eventually, if the area is left unharmed, other herbaceous plants will take over the space. With proper weed management, these weed species are not viable within the field, nor they are on the undisturbed herb strips.

Tree hedges are often accused of providing overwintering (hibernation) sites and shelter for arthropod pests. Apart from a few confirmed cases, these assumptions lack proof. The larvae of the most important pests of cereals, *eg.*, cereal ground beetle (*Zabrus tenebriodes*), turnip moth (*Agrotis segetum*), wheat chafers (*Anisoplia* spp.), and the pupae and larvae of cereal flies (*Chloropidae*) and cereal midges (*Haplodiplosis marginata* and *Mayetiola destructor*) overwinter within the soil of the field. These pests do not need margins or hedgerows for their survival. The same applies to some of the major pests of the corn as well. The maize leaf weevil (*Tanymecus dilaticollis*), the grubs (*Melolonthidae* larvae) and wireworms (*Elateridae* larvae) find underground shelters within the field, the eggs of the western corn rootworm (*Diabrotica virgifera virgifera*) are positioned into the soil of the field, and the leftover stalks of corn provide overwintering habitat for the larvae of the European corn borer (*Ostrinia nubilalis*).

Wheat bugs (*Eurygaster* and *Aelia* spp.) on the other hand, are worth mentioning among the species that seek overwintering shelters within the leaf litter on the southern, warmer slopes of forest hedges (*Fig. 3*). A thorough examination of the litter however reveals, that the majority of arthropods dwelling there are either predators (ground beetles - *Carabidae*, spiders - *Araneae* and rove beetles - *Staphylinidae*) or neither predators nor pests of any importance of arable production. But to see the whole picture about wheat bugs we have to note that being exceptionally good in

the air, they fly, search and find any cereal field regardless of the distance between the field and the soil of the forest patch.

Tree hedges are perfect refuges for the black bean aphid (*Aphis fabae*), a major pest of sugar beet (*Beta vulgaris* subsp. *vulgaris*. var. *altissima*), sunflower (*Helianthus annuus*), poppyseed (*Papaver somniferum*) and beans (*Phaseolus* spp.). To lay the overwintering eggs, female aphids seek the branches of the spindle tree (*Euonymus europaeus*), and this shrub is almost omnipresent in any hedgerow.

The extent tree hedges contribute to the proliferation of *chafer grubs* is still a matter of debate. We believe in the benefits of diversity, that is, the more diverse the composition of *sylvan species*, the higher the chance of having a large spectrum of *birds* and *small rodents* within the tree hedgerow to control the outbreak of chafer grubs.

While some argue that hedges contribute to the escalation of populations of the common vole (*Microtus arvalis*), this small rodent, being outnumbered by sylvan rodents, avoids bushy or forest patches, and prefers cultivated fields of *alfalfa* and *grasses,* where the soil is rich in nutrients due either to manure or fertilizers. Vole control should be based on making the habitat suitable for birds of prey (owls – *Strigidae*, and buzzards - *Acciptridae*), and predatory mammals (fox – *Vulpes vulpes*, polecats and weasels – *Mustela* spp.). A complex canopy of various trees and a dense undergrowth of bushes at the lower level are ideal, but until that phase is reached, at least some high standing observation and plucking poles should be erected for the birds.

Tree hedges however, also provide shelter for a range of beneficial arthropods, that is, natural predators of agricultural pests, including, but not limited to ladybugs (*Coccinellidae*), hover flies (*Syrphidae*), lacewings (*Chrysopidae*), and parasitoid wasps (*Aphidiidae*). The proximity of overwintering habitats is more important for these organisms than it is for the aphid community, because riding the wind, aphids can easily spread between host fields even in the lack of hedges.

The flowering patches of smaller herbaceous plants within hedges not only provide complex food sources for beneficial arthropods and pollinators, parasitoid wasps for example are unable to reproduce without them. These wasps lay their eggs inside the body of the larvae of adults of other arthropods (for example *aphids*). As the wasp larva hatches within, it consumes its host to pupate and develop into an adult individual. Beneficial wasps need to feed on nectars and pollens of wild flowers to lay viable eggs. Herbs also supply pests (*click beetles* and *noctuid moths*) with food, but as with overwintering, pests do not rely on hedgerows as heavily as beneficial arthropods do.

Fruits are a valuable source of food for predatory mammals, rodents and songbirds, so there should be endemic, fruit-bearing bushes such as blackthorn (*Prunus spinosa*), elder (*Sambucus* spp.), cornelian cherry (*Cornus mas*), rosehip (*Rosa canina*), hawthorn (*Crataegus* spp.), blackberry (*Rubus* spp.) and hazelnut (*Corylus* spp.) planted within the hedgerows. The fruit of these bushes attract certain rodents (*eg.,* wood mouse, *Apodemus sylvaticus*) that generally do not forage on the fields, but their presence lures a range of predators.

2. Planting and maintaining hedges

The ideal hedgerow fits into the landscape smoothly. The concept of ideal however, varies from location to location, depending on the actual landscape: exposure to sun and wind, slopes, the presence of wet or extremely dry habitats, the natural species composition of the flora and fauna of the region, to name a few conditions.

Where there is a hedge, or a system of hedges already present, the best a farmer can do is to examine the system and leave it as it is. Apart from a few exceptional cases, there are no reasons to reconstruct, replant or completely remove a useful and functional, endemic hedgerow.

The ideal hedge does not allow for the creation of frost or heat pockets, nor does it block everyday transport or field operations. On slopes, hedges are following the level lines. A hedge oriented north to south casts the smallest portion of shadows on the valuable arable field. An ideal hedge is connected to another hedge or at least to a patch of *natural landscape*, and that is connected to another hedge and so on, so as to provide for the undisturbed movement and safe shelter of game. At some locations, the local fauna is adjusted to certain heights of trees and are intimidated by higher ones. For the safety and preservation of wildlife, these are places for *shrubs-only hedgerows* with a maximum height of 3 m. An ideal hedge resembles the natural landscape and consists of groups of bushes, groups of trees and irregular patches of herbaceous areas as well in a relatively random fashion. On the contrary, a continuous single file of bushes or shrubs is not as valuable.

This simplified drawing of a cross section of a hedge is a suggestion, a draft, not a blueprint (*Fig. 1*).

The core of the hedge is the row or rows of trees. This zone is a fairly undulating strip of trees interrupted here and there by nearby bushes and patches of herbaceous plants (*Fig. 2*).

There are as many kinds of *tree species* in an ideal hedge as possible. The best arrangement is planting the same species in a smaller group, which is followed by another group of another species. When planting a new hedge, the farmer should look for using endemic species and trees that are commonly found in that particular area. Among fruitless species, some of the best suggestions are oaks (*Quercus* spp.), maples (*Acer* spp.), elms (*Ulmus* spp.) and poplars (*Populus* spp.).

A man-made hedge preferably should not include *invasive* and *non-native* species, but should have trees that attract bees and other pollinators (*Fig. 3*). These insectary trees may include *endemic* and *non-endemic* species as well. Lindens (*Tilia* spp.), black locust (*Robinia pseudoacacia*) and hackberry (mayday tree)

(*Celtis* spp.) are some of the most important nectar producing trees.

Figure 1. Recommended structure of protective hedgerows

The most important elements of the sylvan part of the hedgerows are those who bear fruits. Diversity is the key factor in selecting the species for a newly established hedgerow. Mulberries (*Morus* spp.), walnuts (*Juglans* spp.), filbert (*Corylus maxima*), common hackberry (*Celtis occidentalis*), rowans (*Sorbus* spp.), wild pear (*Pyrus pyraster*), wild apple (*Malus sylvestris*), Mirabelle plums (*Prunus domestica*), wild cherries (*Prunus avium*), common dogwood (*Cornus sanguinea*), and local conditions permitting, even almond (*Amygdalus communis*) or chestnut (*Castanea sativa*) are all suitable for hedges. These species are all easily available for propagation by the collection of seeds or by planting seedlings.

Adjusted to the specific conditions of the region, there should be local, resistant varieties of cultivated fruit species (plums, peaches – *Prunus persica* and apricots – *Prunus armeniaca*, cherries and sour cherries – *Prunus cerasus*, apples – *Malus* spp., pears – *Pyrus* spp. and medlar – *Mespilus germanica*) included in the hedge by sowing their seeds.

There is an ongoing debate about the nature of planting. While obtaining fruit of various *wild trees* and local varieties is relatively easy, and so is sowing the seeds, young seedlings require a lot of protection. The advantage of sowing fruit bearing trees by seeds, that is, by their fruit, is that the seedlings usually develop healthy, strong and resistant. Their life expectancy is high. Since only a minimum percent of seeds germinate, this procedure requires lots of propagation material. Small seedlings compete with herbaceous plants and once they have a shoots with a diameter of a human finger, deer (*Cervus* spp.) and hares (*Lepus* spp.) will find them attractive.

Hedges created with nursery raised seedlings lack the initial hardships, but the need for protection against game is inevitable, thus piles of trimmings of shoots of thorny trees and bushes is needed to ward off the hungry animals around the groups of freshly planted seedlings. These trimmings not only shield young trees, they also provide shelter for smaller *mammals* and *insects (Fig. 3, 4)*, and over time, they decompose to increase the organic content of the soil, and thus fertilize the hedge area.

Beside the zone of trees within an ideal hedge, stretch a strip of shrubs, with about 30 % of *thorny bushes* and shrubs as a shelter for the smaller birds. The principle of selecting species and positioning individuals is the same as with trees. Some of the most common shrubs of the Carpathian basin and Europe include rosehip, blackthorn, fly honeysuckle (*Lonicera canadensis*), bramble (*Rubus fruticosus*), dogwood and hazelnut.

The strip of shrubs is surrounded by a wavy, somewhat smaller section of undisturbed taller herbaceous plants for an ideal hedge formation, where umbellifers such as hemlock (*Conium maculatum*) rough chervil (*Chaerophyllum temulum*), wild carrot chervil (*Anthriscus cerefolium*), and cow parsley (*Anthriscus sylvestris*) are to attract parasitoid wasps. Patches of stinging nettle (*Urtica dioica*), false mayweed (*Tripleurospermum inodorum*), a couple of white mulleins (*Verbascum lychnitis*), wormwood (*Artemisia absinthium*), annual fleabane (*Erigeron annuus*) or yellow melilot (*Melilotus officinalis*) are sown to prevent the spreading of invasive pioneer species such as ragweed, common milkweed (*Asclepias syriaca*), cannabis (*Cannabis* spp.) and Canadian goldenrod (*Solidago canadensis*).

And finally, the hedge joins the field with a narrow strip of grass, clovers and trefoils (*Trifolium* spp.) or even, alfalfa (*Medicago sativa*). Depending actual precipitation, this section is mowed 1 to 3 times a year, so as not maintain the habitat for smaller organisms (*e.g.*, certain web builder spiders) that are sensitive to trampling and disturbance.

Figue 2. Protective hedgerows bordering smaller production areas in Hungary (*Photo by F. Tóth*)

Conclusion

Monotonous species composition, monoculture is favourable for pests, whereas diversity, a mixture of multi-species habitats favours the natural enemies of pests. To obtain the advantages of these semi-natural habitats, only 5-7 % of the territory of the farm should be converted into hedgerows. When tailored to the needs and conditions of the actual land, an ideal hedge not only serves the purposes of the farmer by reducing the possibility of a potential pest outbreak, but the advantages outweigh the disadvantages.

The gains on the short term are higher yields, decreased pest control costs and a landscape that is more pleasing to the eye (*Fig. 2*). These are significant justifications, when compared to that sacrifice of 5-7 % of acreage. But on the long run, our grandchildren may be able to thank us for preventing desertification, for inserting and maintaining fruitful and flowery patches into the already thick and heavily industrialized fabric of modern life.

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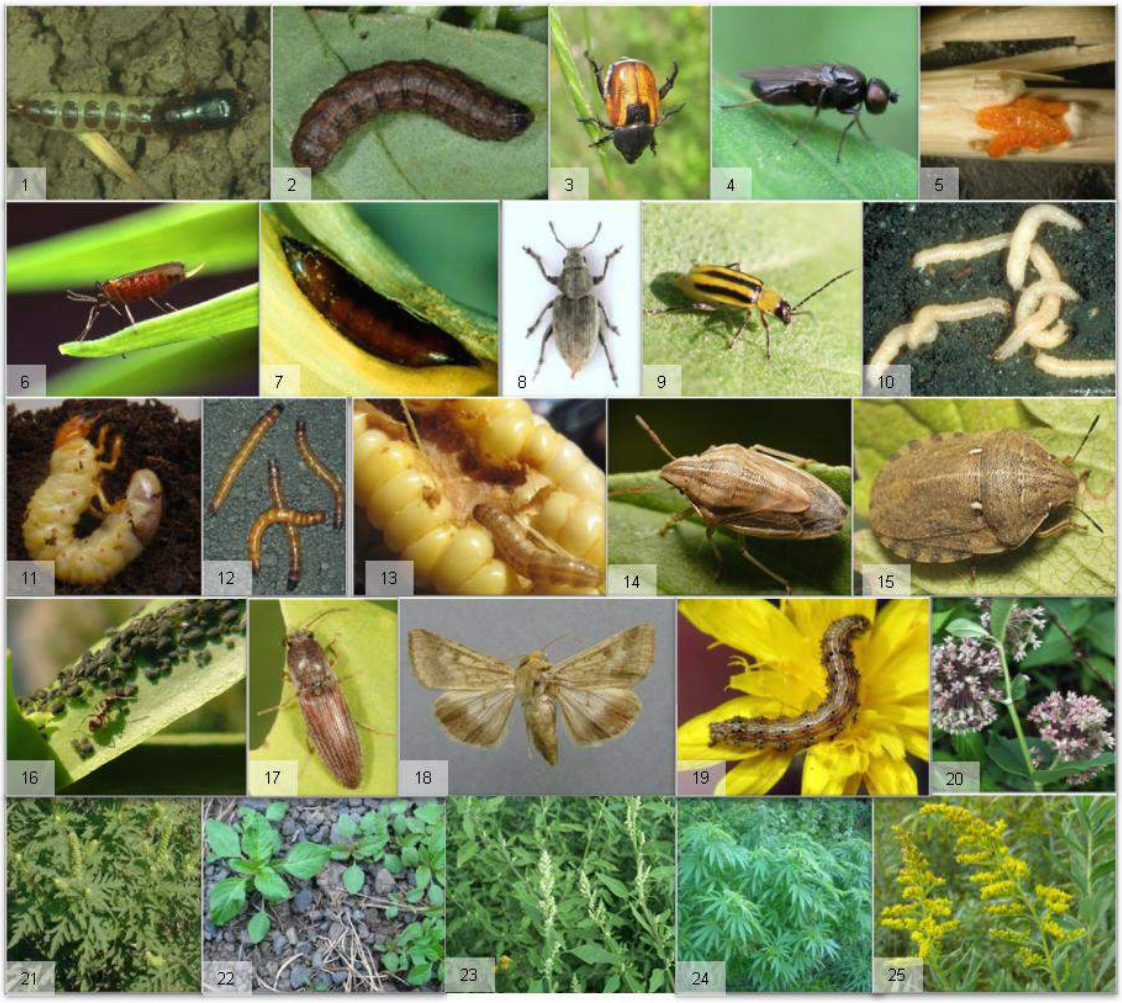

Figure 3. Insect Pests and Invasive Weeds. *(A)* Some of the most important pests were observed overwinter in the soil of the cultivated field or within the leftover crop residues on the surface of the field (1-13): 1. Cereal ground beetle (*Zabrus tenebrioides*), 2. Turnip moth (*Agrotis segetum*) larva, 3. Wheat chafer (*Anisoplia austriaca*), 4. Frit fly (*Oscinella frit*), 5. Saddle gall midge (*Haplodiplosis marginata)* larva, 6. Hessian fly (*Mayetiola destructor)* adult, 7. Hessian fly (*Mayetiola destructor)* pupa, 8. Maize leaf weevil (*Tanymecus dilaticollis*), 9. Western corn rootworm (*Diabrotica virgifera virgifera*) adult, 10. Western corn rootworm (*Diabrotica virgifera virgifera*) larvae, 11. European cockchafer (*Melolontha melolontha*) grub, 12. Wireworms (*Elateridae* larvae), 13. European corn borer (*Ostrinia nubilalis*), larva. *(B)* Certain pests of cereals and other major crops including sugar beet, sunflower and beans overwinter outside the field, within the leaf litter of warmer southern slopes of hedges (14-16): 14. Bishop Mitre's Shieldbug (*Aelia acuminata*), 15. Tortoise bug (*Eurygaster maura*), 16. Black bean aphid (*Aphis fabae*). *(C)* Some pests feed on the pollen of herbs (17-19): 17. Click beetle (*Agriotes lineatus)*, 18. Noctuid moth (*Helicoverpa armigera)* adult, 19. Noctuid moth (*Helicoverpa armigera*) larva. (D) Invasive Weeds (20-25): 20. Common milkweed (*Asclepias syriaca*), 21. Ragweed (*Ambrosia artemisiifolia*), 22. Redroot pigweed (*Amaranthus retroflexus*), 23. Lamb's quarters (*Chenopodium album*) 24. Cannabis (*Cannabis* spp.) 25. Canadian goldenrod (*Solidago canadensis)*

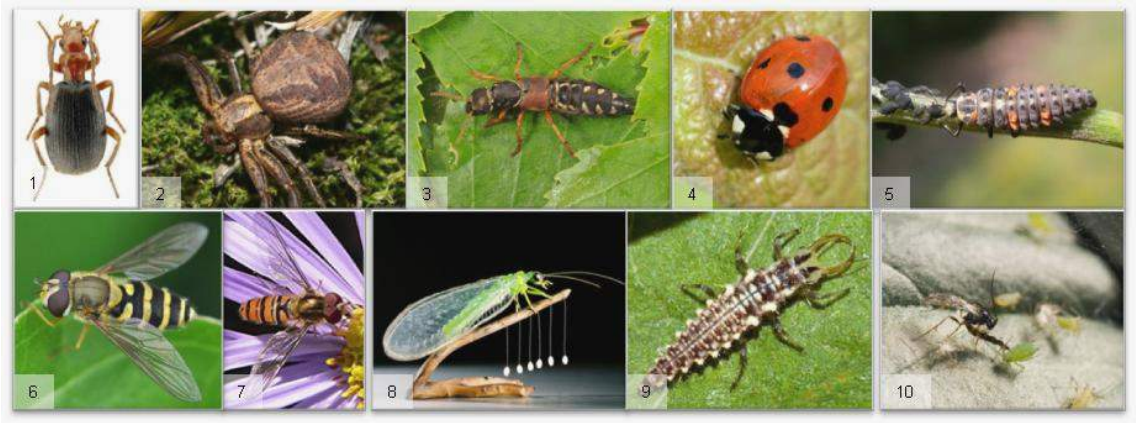

Figure 4. Hedges provide shelter for beneficial arthropods as well: 1. Bombardier Beetle (*Brachinus crepitans*), 2. Crab spider (*Xysticus kochi*), 3. Rove beetle (*Staphylinus caesareus*), 4. Seven-spot ladybird (*Coccinella septempunctata*) adult, 5. Seven-spot ladybird (*Coccinella septempunctata*) larva feeding on aphids, 6. Common banded hoverfly (*Syrphus ribesii*), 7. Marmalade hover fly (*Episyrphus balteatus)*, 8. Common green lacewing (*Chrysoperla carnea)* adult and egg, 9. Common green lacewing (*Chrysoperla carnea)* larva, 10. Parasitic wasp (*Aphidius gryi)* adult parasiting an aphid

Stock estimation and environmental monitoring of a fish population with reserve area

Abstract

For sustainable exploitation of renewable resources, the separation of a reserve area is a natural idea. In particular, in fishery management of such systems, dynamic modelling, monitoring and control has gained major attention in recent years. In this Chapter, based on the known dynamic model of a fish population with reserve area, the methodology of mathematical systems theory and optimal control is applied. In most cases, the control variable is fishing effort in the unreserved area. Working with illustrative data, first a deterministic stock estimation is proposed using an observer design method. A similar approach is also applied to the estimation of the effect of an unknown environmental change.

Keywords: stock estimation, fishery resource management, reserve area, observer system, ecosystem monitoring

1. Introduction

Recently, also qualitative properties of population systems, such as controllability and observability have been studied, see *e.g., Shamandy (2002,) López et al. (2004), (2007a, b)*. For an overview of the applications of mathematical systems theory in this context, we refer the reader to the review *Varga (2008).*

Over the last decades, the problem of sustainability of marine fisheries, the study the effects of a reserve area has played an important role in the management of fish resources. In fact, the the protection of a portion of the fishery stocks agains future overfishing, can be realized in a reserve (or no-take) area where fishing is prohibited, see *e.g., Agardy (1997), Pauly et al. (2002)* and a recent overview of the ecological effects of marine reserves a *Lester et al. (2009).* For a survey of criticism of reserve areas we refere to *Sale et al. (2005).*

To our knowledge, *Dubey et al. (2003)* was the first paper where the effect of a reserve area on the exploitation of a fishery resource has been modelled and analyzed in terms of a continuous-time logistic dynamics. The authors derive sufficient conditions for the existence of equilibrium in the dynamic model, and they also analyze its stability properties. In Bischi and *Lamantia (2007)*, based on a single-species discrete time logistic model with reserve area, the game-theoretic conflict of several fishing agents is studied, where the harvested fish is sold on a Cournot-type oligopolistic market. *Cartigny et al. (2008)* analyze the problem of designing the access of small- and large-scale fishermen to a protected fishing reproductive area.

In our paper, the stock estimation and monitoring of the considered population system will be based on the observability theory of nonlinear systems of *Lee and Markus (1971),* and on the observer design methodology of *Sundarapandian (2002)*. We call the attention to the fact that the observer system also provides a deterministic stock estimation method for the reserved area, as well; see *Guiro et al. (2009)*. In the latter a *global* observer was constructed for the same model of *Dubey et al. (2003)*, with a different methodology, and a compact survey of observer design methods was also given. Although our observer is only *local*, *i.e.,* provides stock estimates only near the equilibrium state, it may be more efficient than the global one, as shown in *Gámez et al. (2011)*. In the latter paper the monitoring problem in the fishing effort model with reserve area has been studied, in particular, observer has been also constructed for the system under the effect of a seasonal change in the abiotic environment. In the present work we complete this with the estimation of an unknown environmental parameter, using the same observer design methodology.

Sample of marine reserve: Milford Sound, New Zealand is a marine reserve. https://en.wikipedia.org/wiki/Marine_protected_area

The paper is organized as follows: In Section 2 first, from *Dubey et al. (2003)* we recall the dynamic model of a fish population with reserve area, and sufficient conditions for the stable coexistence of both subpopulations under the effect of a constant fishing effort. Then, based on a systems theoretical approach, a deterministic stock estimation method is proposed. Section 3 is devoted to the estimation of the effect of an unknown environmental change, applying the same observer design methodology of the previous section.

A Discussion section completes the paper. For the reader's convenience, certain concepts and *theorem*s of mathematical systems theory applied in the main body of the paper are shortly summarized in the *Appendix.*

2. Deterministic stock estimation by observation in the fishing area

First, from *Dubey et al. (2003)* we recall the dynamics of the fish population moving between two areas, an unreserved one (1) where fishing is allowed, and a reserved one (2) where fishing is prohibited. At time t, let $x_1(t)$ and $x_2(t)$ be the respective biomass densities of the same fish species inside the unreserved and reserved areas, respectively. Assume that the fish subpopulation of the unreserved area migrates into reserved area at a rate m_{12}, and there is also an inverse migration at rate m_{21}. Let E be the fishing effort applied to harvesting in the unreserved area and let us assume that in each area the growth of the fish population follows a logistic model. The dynamics of the fish subpopulations in the unreserved and reserved areas are then assumed to be governed by the following system of differential equations (2.1)-(2.2):

$$\dot{x}_1 = r_1 x_1\left(1-\frac{x_1}{K_1}\right) - m_{12}x_1 + m_{21}x_2 - qEx_1 \quad (2.1)$$

$$\dot{x}_2 = r_2 x_2\left(1-\frac{x_2}{K_2}\right) + m_{12}x_1 - m_{21}x_2, \quad (2.2)$$

where r_1 and r_2 are the intrinsic growth rates of the corresponding sub-populations, K_1 and K_2 are the carrying capacities for the fish species in the unreserved and reserved areas, respectively; q is the catchability coefficient of in the unreserved area. All parameters r_1, r_2, q, m_{12}, m_{21}, K_1 and K_2 are positive constants. In *Dubey et al. (2003)*, it is checked that for a unique positive equilibrium $x^* = (x_1^*, x_2^*)$ of the dynamic model (2.1)-(2.2) the following set of inequalities are sufficient:

$$\frac{r_2(r_1 - m_{12} - qE)^2}{K_2 m_{21}} < \frac{(r_2 - m_{21})r_1}{K_1} \quad (2.3a)$$

$$(r_2 - m_{21})(r_1 - m_{12} - qE) < m_{12}m_{21} \quad (2.3b)$$

$$\frac{r_1 x_1^*}{K_1} > r_1 - m_{12} - qE. \quad (2.3c)$$

Furthermore, the *Lyapunov* function

$$V(x) := \left(x_1 - x_1^* - x_1^* \ln\frac{x_1}{x_1^*}\right) + \frac{x_2^* m_{21}}{x_1^* m_{12}}\left(x_2 - x_2^* - x_2^* \ln\frac{x_2}{x_2^*}\right)$$

also implies asymptotic stability of equilibrium x^* for system (2.1)-(2.2), globally with respect to the positive orthant of $\mathbf{R}^2$. Throughout the paper we shall suppose conditions (2.3a)-(2.3c) to guarantee the stable coexistence of the system applying a constant reference fishing effort.

Now, let us consider the problem of stock estimation in the reserve area on the basis of the biomass harvested in the free area. (For technical reason, its difference from the equilibrium value is supposed to be observed.) To this end, in addition to dynamics (2.1)-(2.2) we introduce an observation equation

$$y = h(x) := qE(x_1 - x_1^*), \quad (2.4)$$

representing the observation of the biomass harvested in the free fishing area. Then linearizing observation system (2.1)-(2.2)-(2.4) near the equilibrium, we get the Jacobian of the right-hand side of (2.1)-(2.2)

$$A := \begin{bmatrix} r_1 - 2r_1\frac{x_1^*}{K_1} - m_{12} - qE & m_{21} \\ m_{12} & r_2 - 2r_2\frac{x_2^*}{K_2} - m_{21} \end{bmatrix},$$

and the observation matrix

$$C := h'(x^*) = \begin{pmatrix} qE & 0 \end{pmatrix}.$$

Now, for the linearized system we obviously have $\operatorname{rank}[C \mid CA]^T = 2$. Hence *Theorem* A.2 of *Appendix* implies local observability of the system near the equilibrium in the sense of Definition A.1 of *Appendix*. In other words, in principle, the whole system state (in particular the stock of the species in the reserve area) as function of time can be uniquely recovered, observing the biomass harvested per unit time. In the following illustrative example we will see how the state of the system (and hence the total stock) can be effectively calculated from the catch realized in the fishing area, applying the methodology of *Sundarapandian (2002)*, see *Appendix*.

Example 2.1. For a possible comparison, in this numerical example we use the same parameters as *Guiro et al. (2009)*: r_1=0.7, r_2=0.5, K_1=10, K_2=2.2, m_{12}=0.2, m_{21}=0.1, q=0.25 and E=0.9,

$$\begin{aligned}\dot{x}_1 &= 0.7x_1\left(1-\frac{x_1}{10}\right)-0.2x_1+0.1x_2-0.25\cdot 0.9x_1\\ \dot{x}_2 &= 0.5x_2\left(1-\frac{x_2}{2.2}\right)+0.2x_1-0.1x_2.\end{aligned} \tag{2.5}$$

Now the positive equilibrium is $x^* = (4.85, 3.12)$ and with

$$K := \begin{pmatrix} 0 \\ 10 \end{pmatrix},$$

matrix $A - KC$ is *Hurwitz*; therefore by *Theorem* A.4 of *Appendix* we have the following observer system

$$\begin{aligned}\dot{z}_1 &= 0.7z_1\left(1-\frac{z_1}{10}\right)-0.2z_1+0.1z_2-0.25\cdot 0.9z_1\\ \dot{z}_2 &= 0.5z_2\left(1-\frac{z_2}{2.2}\right)+0.2z_1-0.1z_2+10[y-0.25\cdot 0.9(z_1-x_1^*)].\end{aligned} \tag{2.6}$$

If we take an initial condition $x^0 := (30, 120)$ for system (2.5), and similarly, we consider another nearby initial condition $z^0 := (35, 100)$ for the observer system (2.6), then the corresponding solution z of the observer approaches the solution x of the original system, as shown in *Figure 1*. We note that in this particular case the convergence is much faster than that of the global observer constructed in *Guiro et al. (2009)*.

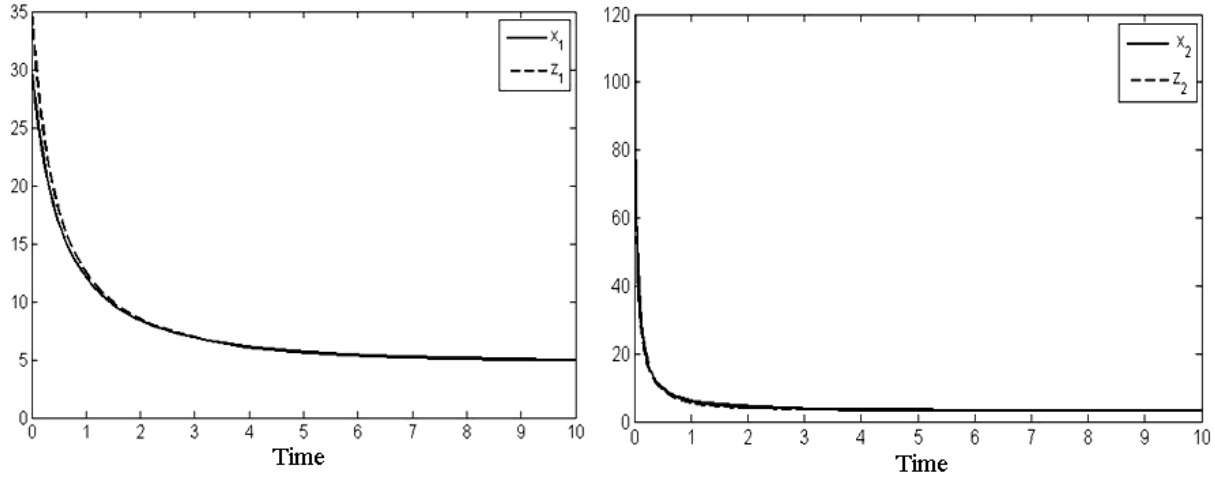

Figure 1. Solution of observer (2.6), approaching the solution of system (2.5)

3. Estimation of the effect of an unknown environmental change

Assume that the considered ecosystem consists, on the one hand, of a system of several interacting populations living in the given habitat, and the abiotic environment on the other. The latter may also be exposed to climatic (*e.g.,* seasonal) changes and/or human intervention, such as *e.g.,* pollution, described by certain abiotic parameters (*e.g.,* temperature or concentration). In this section, considering the model (2.1)-(2.2), we suppose that the reference values of certain abiotic parameters change to unknown constant values. The effect of this change will be described by a small additive term (disturbance) $w \in \mathbf{R}$ in certain model parameters. In our illustrative numerical example it will be shown how we can recover the whole state process of the population system and estimate the unknown disturbance at the same time, by constructing and solving the corresponding observer system. Let us suppose, for example, that a disturbance takes place in the migration rates. Let us consider first the corresponding fishery system, completed with a trivial equation for the unknown constant parameter *w*,

$$\dot{x}_1 = r_1 x_1\left(1-\frac{x_1}{K_1}\right)-(m_{12}+c_1 w)x_1+(m_{21}+c_2 w)x_2 - qEx_1 \tag{3.1}$$

$$\dot{x}_2 = r_2 x_2\left(1-\frac{x_2}{K_2}\right)+(m_{12}+c_1 w)x_1-(m_{21}+c_2 w)x_2 \tag{3.2}$$

$$\dot{w} = 0, \tag{3.3}$$

where c_1 and c_2 are positive constants. Since equilibrium $x^* = (x_1^*, x_2^*)$ is asymptotically stable for system (2.1)-(2.2), it is not hard to prove that equilibrium $(x_1^*, x_2^*, 0)$ is Lyapunov stable for system (3.1)-(3.3). Therefore *Theorem* A.5 of *Appendix* can be applied for the observer design.

Let us suppose that, similarly to Section 2, the biomass harvested in unit time is observed:

$$y = h(x,w) := qE(x_1 - x_1^*). \tag{3.4}$$

Now the linearization of observation system (3.1)-(3.4) gives

$$A = \begin{pmatrix} r_1 - 2r_1\frac{x_1^*}{K_1} - m_{12} - qE & m_{21} & -c_1 x_1^* + c_2 x_2^* \\ m_{12} & r_2 - 2r_2\frac{x_2^*}{K_2} - m_{21} & c_1 x_1^* - c_2 x_2^* \\ 0 & 0 & 0 \end{pmatrix}; C := h'(x^*) = \begin{pmatrix} qE & 0 & 0 \end{pmatrix}. \tag{3.5}$$

Hence we easily obtain that $\operatorname{rank}[C \mid CA \mid CA^2]^T = 3$, if $K_2 \neq 2x_2^*$ and $c_1 x_1^* \neq c_2 x_2^*$. Therefore, by *Theorem* A.2 (*see Appendix*), the system is locally observable near the equilibrium, and applying the method of *Sundarapandian (2002)* we can construct a corresponding observer system, as shown in the following

Example 3.1. Using the same system parameters as in Example 2.1, with the presence of an unknown environmental disturbance w and coefficients c_1=0.1, c_2=0.3, we have

$$\begin{aligned} \dot{x}_1 &= 0.7x_1\left(1-\frac{x_1}{10}\right)-(0.2+0.1w)x_1+(0.1+0.3w)x_2 - 0.25\cdot 0.9x_1 \\ \dot{x}_2 &= 0.5x_2\left(1-\frac{x_2}{2.2}\right)+(0.2+0.1w)x_1-(0.1+0.3w)x_2 \\ \dot{w} &= 0 \end{aligned} \tag{3.6}$$

System (3.6) has a nonnegative equilibrium $\bar{x} = (4.85, 3.12, 0)$, and with

$$K := \begin{pmatrix} 0 \\ 0 \\ 100 \end{pmatrix}$$

matrix $A - KC$ is *Hurwitz*, therefore by *Theorem* A.5 of *Appendix* we can construct the following observer system:

$$\begin{aligned} \dot{z}_1 &= 0.7z_1\left(1-\frac{z_1}{10}\right)-(0.2+0.1z_3)x_1+(0.1+0.3z_3)z_2 - 0.25\cdot 0.9z_1 \\ \dot{z}_2 &= 0.5x_2\left(1-\frac{x_2}{2.2}\right)+(0.2+0.1z_3)z_1-(0.1+0.3z_3)z_2 \\ \dot{z}_3 &= 100[y - 0.25\cdot 0.9(z_1 - 4.85)]. \end{aligned}$$

If we suppose that environmental perturbation corresponds to the value $w = 0.2$ and we take an initial condition $(x^0, w^0) := (10, 5, 0.2)$, of system (3.6), and similarly, we consider another nearby initial

condition, $z^0 := (15, 10, 0.3)$ for the observer system (3.7). *Figure 2* shows that the corresponding solution z approaches the solution x of the original system, and also correctly estimates the "unknown" parameter w.

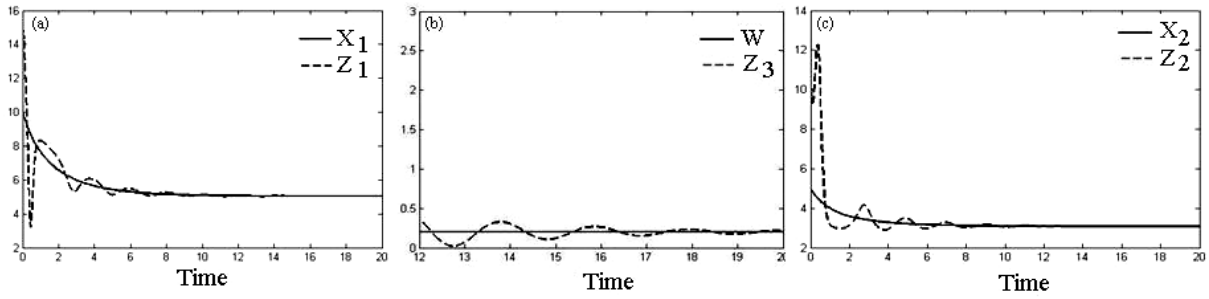

Figure 2. Simultaneous state and parameter estimation in system (3.6) with its observer (3.7). For a better scaling, in the graphical representation the graph of z_3 is plotted only after the relatively large transient values

4. Discussion

Over the last decade, tools of mathematical systems theory has been successfully applied to both density-dependent multi-species and structured single-species dynamic population models, for a survey of our results on the subject see *Varga (2008)*. The majority of stock assessment methods use a *statistical* approach, see *Cadrin et al. (2005).*

Recently *Guiro et al. (2009)* used a global observer system for *deterministic* stock estimation. Our observer system method proposed in this paper is local, but can not only perform better near equilibrium, but it also turned out to be able to estimate stock and environmental parameters simultaneously. This method can be also extended to general spatially structured populations, as well as to stage-, age- or size structured populations. For example, in a size-structured model only fish of cachable size are harvested and therefore observed, and the total state vector containing all size classes are estimated. Similarly, the state of a population system in time-dependent environment can be also estimated with an appropriate observer system.

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Appendix

A.1. Observability and observer of nonlinear systems

Given positive integers *m, n*, let

$$f : \mathbf{R}^n \to \mathbf{R}^n, \quad h : \mathbf{R}^n \to \mathbf{R}^m$$

be continuously differentiable functions and for some $x^* \in \mathbf{R}^n$ we have that $f(x^*) = 0$ and $h(x^*) = 0$.

We consider the following observation system

$$\dot{x} = f(x) \qquad \text{(A.1)}$$

$$y = h(x)\ , \qquad \text{(A.2)}$$

where y is called the *observed function*.

Definition A.1. Observation system (A.1)-(A.2) is called *locally observable* near equilibrium x^*, over a given time interval $[0,T]$, if there exists $\varepsilon > 0$, such that for any two different solutions x and $\bar{x}$ of system (A.1) with $|\, x(t) - x^* \,| < \varepsilon$ and $|\, \bar{x}(t) - x^* \,| < \varepsilon \ (t \in [0,T])$, the observed functions $h \circ x$ and $h \circ \bar{x}$ are different. ($\circ$ denotes the composition of functions. For brevity, the reference to $[0,T]$ is often suppressed).

For the formulation of a sufficient condition for local observability consider the linearization of the observation system (A.1)-(A.2), consisting in the calculation of the Jacobians

$$A := f'(x^*) \text{ and } C := h'(x^*)\ .$$

***Theorem* A.2** (*Lee and Markus, 1971*). Suppose that

$$rank[C \,|\, CA \,|\, CA^2 \,|\, ... \,|\, CA^{n-1}]^T = n. \qquad \text{(A.3)}$$

Then system (A.1)-(A.2) is locally observable near x^*.

Now, we recall the construction of an observer system will be based on Sundarapandian (2002). Let us consider observation system (A.1)-(A.2).

***Definition* A.3.** Given a continuously differentiable function $G : \mathbf{R}^n \times \mathbf{R}^m \to \mathbf{R}^n$, system

$$\dot{z} = G(z, y) \qquad \text{(A.4)}$$

is called a *local asymptotic (respectively, exponential) observer for observation system* (A.1)-(A.2) if the composite system (A.1)-(A.2), (A.4) satisfies the following two requirements.

i) If $x(0) = z(0)$, then $x(t) = z(t)$, for all $t \geq 0$.

ii) There exists a neighbourhood V of the equilibrium x^* of $\mathbf{R}^n$ such that for all $x(0), z(0) \in V$, the estimation error $z(t) - x(t)$ decays asymptotically (respectively, exponentially) to zero.

***Theorem* A.4** (*Sundarapandian, 2002*). Suppose that the observation system (A.1)-(A.2) is Lyapunov stable at equilibrium, and that there exists a matrix K such that matrix $A - KC$ is *Hurwitz* (*i.e.,* its eigenvalues have negative real parts), where $A = f'(x^*)$ and $C = h'(x^*)$. Then dynamic system defined by

$$\dot{z} = f(z) + K[y - h(z)]$$

is a local exponential observer for observation system (A.1)-(A.2).

Now, for the estimation of a change in the dynamical parameters of an ecosystem, we recall that Sundarapandian (2002) also considered the possibility of an "input generator" determined by an external system called *exosystem* $w' = s(w)$, in terms of which we can form a composite (nonlinear) system with inputs of the form

$$\begin{aligned} \dot{x} &= F(x, u(w)) \\ \dot{w} &= s(w) \qquad \text{(A.5)} \\ y &= h(x), \end{aligned}$$

where we suppose that $F : \mathbf{R}^n \times \mathbf{R}^k \to \mathbf{R}^n$, $s : \mathbf{R}^k \to \mathbf{R}^k$ are continuously differentiable and $F(x^*, 0) = 0,\ u(w^*) = 0, s(w^*) = 0$. Variable u is interpreted as a time-dependent vector of system parameters of the original system (A.1), corresponding to right-hand side f. For the construction of an observer for the composite system we can apply the following

***Theorem* A.5** (*Sundarapandian, 2002*). Suppose that observation system (A.5) is Lyapunov stable at equilibrium. If system (A.5) has a local exponential observer, and that there exists a matrix K such that matrix $A - KC$ is stable (its eigenvalues have negative real parts), where $A = F'(x^*, w^*)$ and $C = h'(x^*)$. Then dynamic system defined by

$$\dot{z} = F(z, u(w)) + K[y - h(z)]$$

is a local exponential observer for observation system (A.5).