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Characterization and molecular mapping of EMS-induced brittle culm mutants of diploid wheat (*Triticum monococcum* L.)

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Abstract Diploid wheat (*Triticum monococcum* L, A^mA^m) is an ideal material for induced mutations which can be easily characterized and transferred to polyploid wheats. The EMS-induced brittle culm mutants, *brc1, brc2*, and *brc3* used in the present investigation, were isolated from *T. monococcum*. All the brittle mutants had brittle roots, leaves, leaf sheaths, culms, and spikes, and were also susceptible to lodging. The mutants had 47–57% reduced α-cellulose in the secondary cell walls than that of *T. monococcum* indicating that all of them had defective synthesis of cellulose. All the mutants were monogenic recessive. Bulk segregation analysis of the mutants, using A^m genome anchored SSR markers in their F_2 populations with *T. boeoticum*, located the mutants, *brc1, brc2*, and *brc3* on chromosome 6A, 3A, and 1A of *T. monococcum,* respectively. Molecular analysis of the putatively linked markers showed that *brc1* mapped on chromosome 6AS between Xbarc37 and Xbarc113 markers*, brc2* on chromosome 3AL between Xcfd62 and Xcfa2170 markers whereas *brc3* mapped on chromosome 1AL between Xgwm135 and Xwmc470 markers. Isolation and mapping of three different brittle culm mutants in wheat for the first time shows that there might be many more genes in wheat which affect synthesis and deposition of cellulose.

Keywords Brittle culm mutants · Bulk segregant analysis · Cellulose · Diploid wheat · Microsatellite markers · *Triticum monococcum*

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Introduction

Mechanical strength of plants is an important agronomic trait for yield stability and harvest index. The plant cell wall is a major component of mechanical support to cells, tissues, and the entire plant body. The plant cells can be grouped into three basic types, parenchyma, collenchyma, and sclerenchyma, on the basis of their wall thickening. Both parenchyma and collenchyma cells, having primary cell wall, provide

the main support in the growing regions of the plant tissues, whereas the sclerenchyma cells having both primary and secondary walls provide major strength in non-elongating parts of plant (Carpita and McCann 2000). Cell walls contain different substances to perform their specific functions where cellulose usually constitutes 20–30% of the dry weight of the primary cell wall and 40–90% of the secondary cell wall (Taylor et al. 1999). Besides cellulose, lignins, and hemicelluloses are the other two important constituents of the cell wall.

The mechanisms involved in the mechanical strength of a plant and the biosynthesis of plant cell walls are still not fully understood. Some mutants defective in plant culm strength have been isolated and characterized. The brittle culm mutant of barley showed reduced cellulose content and mechanical strength (Kokubo et al. 1989, 1991), indicating the role of cellulose in the plant mechanical strength (Li et al. 2003). Similarly, some mutants with reduced mechanical strength and alterations in cell shapes and cell elongation have been characterized in *Arabidopsis* and the corresponding genes have been cloned. The irregular xylem mutants (*irx1* to *irx3*) had defective cellulose synthesis in secondary cell walls and decreased strength of mature stems (Turner and Somerville 1997; Jones et al. 2001). The mutation in *root hair defective3* (*RHD3*) gene of the *fragile fiber 4* (*fra4*) mutant of *Arabidopsis* caused a dramatic reduction in the wall thickness of fibers, vessels, pith cells in the inflorescence stems and mechanical strength of stems (Hu et al. 2003). The changed crystalline cellulose biosynthesis in *rsw1* (radial swelling) mutant of *Arabidopsis thaliana* caused changes in the cell shape, indicating the direct role of cellulose in maintaining cell morphology (Arioli et al. 1998). The synthesis of cellulose microfibrils requires the presence of a membrane bound endo-1, 4-β-D-glucanase (*kor1*). Mutation in *kor1* caused abnormal cell wall structure and aberrant cell plate formation. Cells in the *kor1* mutants could not elongate normally, resulting in an extremely dwarf phenotype (Stéphanie et al. 2005). The Brittle Culm 1 (*BC1*) of rice, encoding a COBRA-like protein, is expressed mainly in the developing sclerenchyma cells and in vascular bundles. Mutations in *BC1* not only reduced cellulose content and cell wall thickness but also increased lignin (Li et al. 2003).

This article deals with the characterization and mapping of EMS-induced three brittle culm mutants in diploid wheat, *T. monococcum*. These mutants, isolated for the first time in wheat, could have far reaching implications and application in the investigation of biosynthetic pathway and utilization of cellulose in wheat and other cereals.

Materials and methods

Plant materials

The EMS-induced brittle culm mutants (*brc1, brc2* and *brc3*) used in the present study were isolated at the Punjab Agricultural University, Ludhiana from diploid wheat *T. monococcum* accession pau14087 in M_2 generation after seed treatment with 0.25% EMS. A brittle mutant was noticed during manual weeding in the M_2 EMS-treated population in the field. The other brittle mutants were screened after twisting of leaves and culms of all the M_2 progenies manually. The seeds of the mutants, the wild type parent and an accession pau 5088 of *T. boeoticum* (the wild and tall progenitor of *T. monococcum*) were planted at the Indian Institute of Technology, Roorkee in November, 2005. Each of the brittle culm mutants was crossed with both wild type parent *T. monococcum* and wild progenitor *T. boeoticum* for developing F_2 populations for inheritance and mapping studies. The F_1 hybrids were selfed to obtain F_2 populations. The F_2 populations were planted at IIT Roorkee in 2007 and 2008 in 2 m rows with row-to-row distance of 30 cm and plant-to-plant distance of 10 cm following the standard package of agronomic practices for wheat cultivation.

Brittleness of mutants

The culms, leaves, and spikes of all the three brittle mutants break easily on slight bending. The data on brittleness of individual plants of the three F_2 populations was recorded at different stages of plant development viz., after 30 days of sowing, during flowering and harvesting by bending the plant parts manually with hands.

Measurements of physical properties

The breaking force and elongation ratio of culms/flag leaves of the brittle mutants and the wild type *T. monococcum* parent were measured with a universal

strength testing device (Model 7001; ZhongKai, China). To reduce sampling error, the first internodes of culms were used for the measurements. In each of the mutants, ten culms were dried at 30°C for 3 days before the measurements. The elongation ratio (%) was defined by the formula (L1 − L2)/L2 × 100, where L1 is the length of the culm segment at breaking and L2, the original length of the culm segment.

Atomic force microscopy

Mature wheat straw was collected, cleaned and dried and cut into small pieces of about 1 cm in length. The wheat straw pieces were disencrusted thoroughly with a purification method (Liu et al. 2005) to remove pectin, polysaccharides, lignin, and other non-cellulosic substances completely. The samples were used for atomic force microscopy (AFM). The AFM images were obtained on a Nanoscope III Multi Mode AFM.

Scanning electron microscopy before and after delignification

For scanning electron microscopy, the samples were prepared as described previously (Mou et al. 2000). The mutant tissue samples were immersed in 2.5% glutaraldehyde for 2 h at room temperature and transferred to 50% ethanol for 5 min, 70% ethanol for 30 min (twice), 90% ethanol for 30 min (twice), 100% ethanol for 30 min (twice), absolute alcohol:amyl acetate (3:1) for 30 min, absolute alcohol:amyl acetate (2:2) for 30 min, absolute alcohol:amyl acetate (1:3) for 30 min, and amyl acetate for 30 min. The samples were kept for critical point drying for 40 min. The samples were mounted onto metal stubs with double sided carbon tape. A thin layer of gold metal was applied over the sample using an automated sputter coater. These samples were then analyzed under scanning electron microscope and the surface images were taken at 200, 300, 500, and 1,500× magnification.

The culms of brittle culm mutants along with the wild type parent were first oven dried at 50°C for 24 h. The hydrothermally pretreated culms were delignified by treating approximately 25 g of dried culms in 800 ml MilliQ water with 40 ml of 98% glacial acetic acid and 20 g of sodium chlorite ($NaClO_2$). The mixture was placed in a water bath at 80°C for 1 h. The sodium chlorite and acetic acid additions were repeated twice followed by the addition of glacial acetic acid only. The reaction was terminated by cooling to 10°C. The holocellulose was isolated by filtration through a glass filter and rinsing with ice-cold MilliQ water, followed by oven-drying at 50°C for 24 h. For SEM, the culms were lyophilized without prior oven-drying (Kristensen et al. 2008).

Dry matter composition

The total dry matter in the brittle culm mutants was measured in the leaves and culms. Crystalline cellulose was assayed according to Updegraff (1969) method. Briefly, the leaves and third internode of the culms were ground into a fine powder in liquid nitrogen and washed thrice in phosphate buffer (50 mM, pH 7.2) and extracted twice with 70% methanol at 70°C for 1 h and dried under vacuum. The dried cell wall material was used for estimation of cellulose content with the anthrone reagent with Whatman 3MM paper as the standard. Klason lignin was estimated by incubating the ground culms and leaves with 72% (w/w) sulfuric acid for 1 h, and washing twice with a 1:20 dilution of 72% sulfuric acid in water, heating at 65°C for 30 min, washing once with water, and drying the residue at 80°C overnight. The cell wall materials were used for assays of hemicellulose and silicon according to Van Soest and others (Van Soest and Robertson 1985; Van Soest et al. 1991).

Histological observations

For histological examination, the leaves and stems were excised and fixed in FAA fixative. To support the tissues for sectioning, the tissues were embedded in paraffin wax (Sdfine) at 60°C, and sectioned to 10 μm thickness on a rotary microtome. The ribbons obtained from paraffin sections were mounted on slides, hydrated, and dehydrated in a graded ethanol series. The tissues were stained for 2 min in Phloroglucinol–HCl solution according to the standard protocol (Srivastava 1966) and toluidine blue O (0.05%, w/v) dye as per the method suggested by Johansen (1940). Transverse sections of the stems and leaves of the brittle mutants and wild type *T. monococcum* were observed under a light microscope (Axiostar plus 1169-151, Carl Zeiss Co., Oberkochen, Germany) at different magnifications.

Genomic DNA isolation and genotyping for SSR markers

The genomic DNA of the mutant plant leaves was extracted by CTAB method as described by McCouch et al. (1988). A number of high density molecular maps have been developed in wheat (Somers et al. 2004) including a RIL population between *T. monococcum* acc. pau14087, used for mutant isolation, and *T. boeoticum* acc. pau5088 used for making mapping populations with the brittle mutants (Singh et al. 2007). The primers for anchored SSR markers at about 10 cM interval form each of the diploid wheat chromosomes and polymerase chain reaction (PCR) protocols were carried out according to the details and methods described by Singh et al. (2007). The PCR products were separated on 2.5% agarose and 8% polyacrylamide gels according to the length of the amplified fragments and stained using ethidium bromide.

Bulk segregant analysis (BSA)

The data on brittleness of all the F_2 plants was recorded in the three F_2 mapping populations of the brittle mutants in crosses with *T. boeoticum*. Positive bulks were made for each of the brittle culm mutants from the homozygous mutant plants from the respective F_2 mapping populations with *T. boeoticum.* A common negative bulk was prepared from 15 RILs of *T. boeoticum* pau5088 × *T. monococcum* pau14087 cross without any mutant in the parents. An equal concentration of DNA from the mutant plants was pooled. These two sets of bulks along with the parents were used to identify putative SSR markers (Supplementary Table S1) linked to the three brittle culm mutants. The microsatellite markers polymorphic between the parents and bulks were used to genotype individual plants constituting the particular bulks and the F_2 mapping populations.

Inheritance and molecular mapping of mutants

To determine the inheritance of the brittle culm mutants, the mutants were crossed with its wild parent *T. monococcum* and as well as *T. boeoticum* and the F_1s' were advanced to F_2. The F_2 mapping populations derived from *T. boeoticum* acc. pau 5088/*brc1, brc2* and, *brc3* mutants, consisting of 250, 250, and 185 F_2 plants, respectively were used for inheritance studies and mapping. Only the data on brittleness through bending of culms and leaves was recorded on each of the F_2 plants of the three different mapping populations. Chi-square test was applied to test the goodness of fit to a particular segregation ratio. Linkage analysis of polymorphic marker loci was done with the computer program 'MAPMAKER/EXP version 3.0' (Lander et al. 1987; Lincoln et al. 1990) using the data on brittleness and the polymorphic SSR markers in the respective F_2 mapping populations.

Comparative genomics

After mapping the brittle culm mutants on various *T. monococcum* chromosomes, their syntenic rice chromosomes were searched for cellulose synthase (*CesA*) and cellulose synthase like (*CSL*) genes in order to identify the wheat EST sequences orthologous to the rice genes (SI Table 2) involved in cellulose biosynthesis. The sequences of rice genes were BLASTed against the wheat ESTs available at http://www.compbio.dfci.harvard.edu/cgi-bin/tgi/Blast/index.cgi. Wheat ESTs showing maximum homology (e^{-50} and bit score $\geq$ 200) with each of the rice genes were further blasted against the bin-mapped wheat ESTs/contigs available at http://www.wheat.pw.usda.gov/GG2/blast.shtml with a view to identify the location of orthologous wheat ESTs on the wheat EST physical maps.

Results

Identification of *brc1*, *brc2*, and *brc3* mutants

The brittle culm mutants, *brc1*, *brc2*, and *brc3* were semi-dwarf with shorter stems, smaller drooping leaves and spreading growth habit as compared with the wild type parent *T. monococcum.* The leaves, culms and spikes of *brc1*, *brc2*, and *brc3* mutants were brittle whereas the plant parts of *T. monococcum* did not break on bending. The *brc1* and *brc2* expressed brittleness earlier during plant development as compared to *brc3*. Some of the brittle mutants were more sensitive to certain herbicides and wheat powdery mildew disease as compared to the wild type *T. monococcum* (Table 1).

Table 1 Morphological characteristics of *T. monococcum* and the brittle culm mutants

Trait	Wild type	Brittle culm mutants		
	T. monococcum	*brc1*	*brc2*	*brc3*
Growth habit after flowering	Erect	Spreading	Spreading	Spreading
Days to 50% flowering	110	90	115	120
Main culm height (cm)	130	75.4	92.1	98.3
Flag leaf length (cm)	40.6	35.5	36.5	37.5
Numbers of tillers per plant	30	25	27	28
Spikelets per spike	32	22	26	28
Seedling root length (cm)	1.3	1.2	1.2	1.5
Brittleness in leaves, culms, roots and spikes	Absent	Present	Present	Present
Susceptibility to herbicide (Topik)	No	Yes	Yes	No
Susceptibility to powdery mildew	No	Yes	Yes	No

Mechanical strength of brittle mutants

Morphologically, *brc1*, *brc2*, and *brc3* mutant plants were clearly distinguishable from *T. monococcum* as they were spreading with drooping leaves during flowering and had brittle culms, leaves and spikes that could be easily broken by bending (Fig. 1). The culm elongation ratio of the wild type parent was nearly 2.5 times higher than of the brittle mutants indicating higher brittleness during pulling in the mutants. (Fig. 2a) The force required to break the culms of the mutant plants was reduced to 66, 72, and 70% for *brc1*, *brc2*, and *brc3*, respectively as compared to the wild type (Fig. 2b), whereas the force required to break the mutant leaves was decreased by 13 to 59% as compared to the wild type (Fig. 2c).

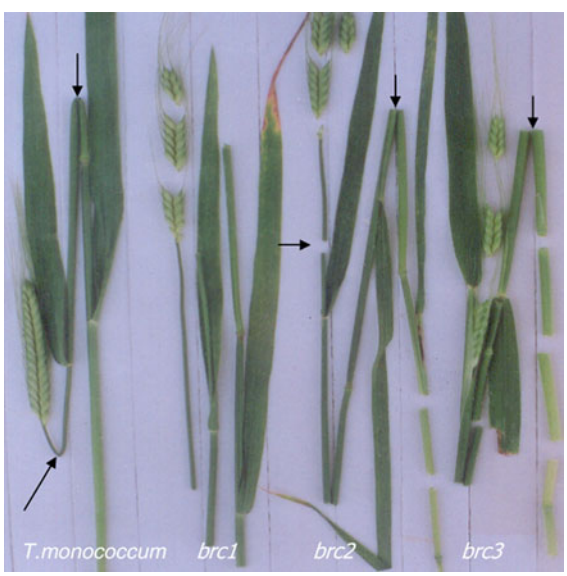

Fig. 1 Brittleness of culms and spikes of brittle culm mutants *brc1*, *brc2*, and *brc3* of *T. monococcum*

Atomic force microscopy of stem surface

Atomic force microscopy of the mutant culms indicated that the stem surface of the mutants were more rough than that of the wild type (Supplementary Fig. S1). It also indicated that the mutants had some abnormality in cellulose deposition on stem surfaces.

SEM of sclerenchymatous cell wall of brittle mutants

In the normal plants, several layers of sclerenchymatous cells, especially those around the peripheral vascular tissues and under the epidermal layer in culms and leaf veins, provide mechanical support to plants. The cell wall morphology of second internodes and flag leaf midrib of 90 days old plants of *T. monococcum* and brittle culm mutants *brc1*, *brc2*, and *brc3* was examined with scanning electron microscopy (Fig. 3 and Supplementary Fig. S2). Scanning electron microscopy observations revealed that the wild type sclerenchyma cell walls were heavily thickened and filled up with cell wall materials (Fig. 3, left column), in striking contrast to those of *brc1*, *brc2*, and *brc3* mutant plants where the cells had no secondary cell wall thickening. However, no differences in cell length and width were found among *brc1*, *brc2*, and *brc3* mutants and the wild type plants.

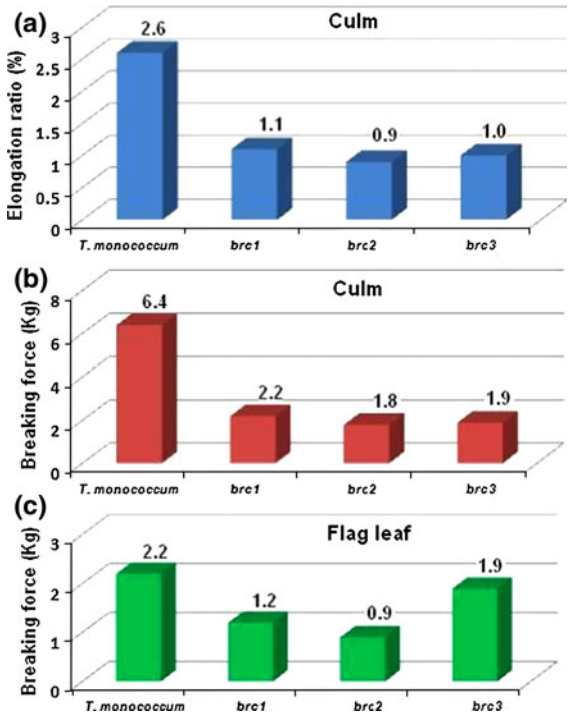

Fig. 2 Elongation ratio and breaking force of brittle culm mutants and wild type *T. monococcum*. **a** Elongation ratio of *T. monococcum* and mutants *brc1*, *brc2*, and *brc3*. **b** Breaking force needed to break culms of *T. monococcum*, *brc1*, *brc2*, and *brc3*. **c** Breaking force needed to break flag leaves of *T. monococcum*, *brc1*, *brc2*, and *brc3* mutants

The transverse sections of mature flag leaves through mid rib under scanning electron microscope showed that the sclerenchymatous cells above and around the vascular bundles had hollow cavities and thin cell walls in *brc1*, *brc2*, and *brc3* mutants whereas in the wild type, the sclerenchymatous cells were completely filled with cell wall material (Supplementary Fig. S2). These results suggest that reduction in the mechanical strength of *brc1*, *brc2*, and *brc3* mutant plants very likely resulted from some defects in thickening of cell wall of the mechanical tissues, such as sclerenchyma.

Cell wall composition of brittle culm mutants

The cellulose content in the culm cell walls of *brc1*, *brc2*, and *brc3* mutants was reduced to ~46, ~56, and ~55%, respectively in comparison to the wild type (Table 2), suggesting that *brc1*, *brc2*, and *brc3* mutations might have directly or indirectly played an important role in the cellulose biosynthesis. As shown in Table 2, the Klason lignin of the *brc1*, *brc2*, and *brc3* mutant culms was increased by ~30, ~21, and ~26%, respectively compared with that of the wild type culms. At the same time they also had increased hemicellulose (5.86–17.94%), ash (53.65–129.26%), silica and silicates and extractives, waxes and lipids. Similar differences were found in the cell walls of leaves of wild type and the brittle mutants (Table 3). The cellulose content in the leaf cell wall was reduced approximately by 50% in the mutants whereas all the other components were increased to variable extent in all the mutants.

Histochemical analysis of leaves and culms of brittle mutants

To determine whether the alterations of cellulose and lignin were localized in particular cells, transverse sections of the culms of wild type and mutant plants were histochemically stained with Wiesner stain, phloroglucinol–HCl. Wiesner stain is known to react with cinnamaldehyde residues in lignin, and the color intensity approximately reflects the total lignin content. The color differences between wild type and the mutants in the mechanical tissues, especially in the sclerenchyma cells below and above the epidermis and around the vascular bundles, indicated an apparent increase in lignin quantity in the mutant plants (Supplementary Fig. S3).

Phloroglucinol staining of TS of flag leaves of *brc1*, *brc2*, and *brc3* mutants revealed that only a few sclerenchyma cells were present around the vascular bundle of midrib which were replaced by parenchymatous cells in *brc1*, *brc2*, and *brc3* whereas in *T. monococcum* all the cells around the vascular bundles and below the upper and lower epidermis were sclerenchymatous (Supplementary Fig. S4). In *brc1* and *brc2,* metaxylem vessels were deformed and protoxylem vessels completely collapsed which are the major water conducting tissues of the plants. Higher number of chloroplasts were observed in parenchymatous cells around the vascular bundles of leaves of *brc1*, *brc2*, and *brc3* than in *T. monococcum* which supports the darker green color of *brc1*, *brc2*, and *brc3* than the wild type. Chlorenchymatous cells were elongated in *brc1*, *brc2*, and *brc3* and were round in *T. monococcum*. Numbers of bulliforms cells were ~2 in *brc1*, *brc2*, and *brc3* and ~4 in *T. monococcum* (Supplementary Fig. S4).

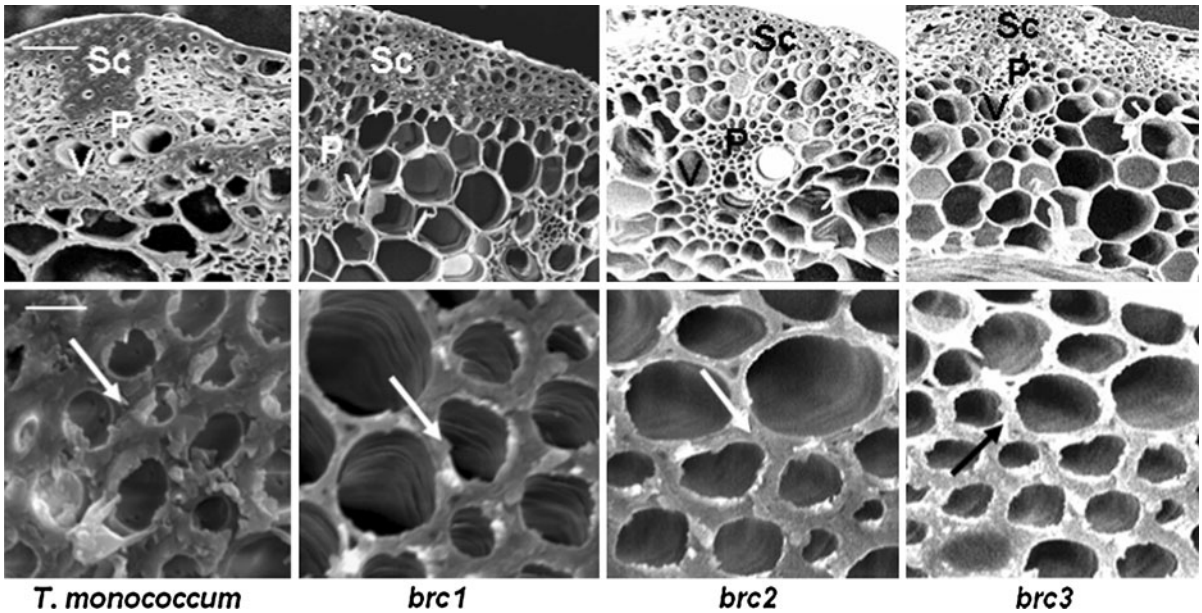

Fig. 3 SEM of sclerenchyma cells (*Sc*) of culm second internode of *T. monococcum* and brittle culm mutants *brc1*, *brc2*, and *brc3*. (*Upper row*): TS of second internode of *T. monococcum*, *brc1*, *brc2*, *and brc3*. (*Lower row*): Sclerenchyma fibers (*Arrows*) of *T. monococcum*, *brc1*, *brc2*, and brc3 magnified from the above row at sections indicated by Sc. Scale bars for upper row = 25 μm and lower row = 5 μm

Table 2 Chemical composition of culm internodal cell walls of the brittle culm mutants

Internodal cell wall composition (mg/g)	Wild type	Brittle culm mutants					
	T. monococcum	*brc1*	Change (%)	*brc2*	Change (%)	*brc3*	Change (%)
α-Cellulose	387	206	−46.77	168	−56.58	172	−55.55
Hemicellulose	273	289	+5.86	314	+15.01	322	+17.94
Klason lignin	175	228	+30.28	212	+21.14	221	+26.28
Ash	82	126	+53.65	188	+129.26	172	+109.75
Silica & silicates	47	51	+8.51	49	+4.25	48	+2.12
Extractives	52	68	+30.76	65	+25.00	59	+13.46

Table 3 Chemical composition of cell wall of leaves of the brittle culm mutants

Leaf cell wall composition (mg/g)	Wild type	Brittle culm mutant					
	T. monococcum	*brc1*	Change (%)	*brc2*	Change (%)	*brc3*	Change (%)
α-Cellulose	306	147	−51.96	132	−56.86	138	−54.90
Hemicellulose	250	270	+8.00	265	+5.66	269	+7.07
Klason lignin	160	190	+18.75	162	+1.25	184	+15.00
Ash	122	160	+31.14	188	+54.09	172	+40.98
Silica & silicates	52	62	+19.23	65	+25.00	69	+32.69
Extractives	57	68	+19.29	92	+61.40	82	+43.85

Toluidine blue stains cellulose, callose, and other glucans into greenish yellow whereas it stains lignin as green. Greenish yellow color was observed in the sclerenchyma cells and vascular bundles in the wild type and green color was visible in sclerenchyma cells and vascular bundles of *brc1*, *brc2*, and *brc3* mutants, demonstrating a significantly high level of ordered cellulose in the sclerenchyma cells and vascular bundles in wild type plants as compared to the mutants (Supplementary Fig. S5). In addition the thickness of sclerenchymatous secondary cell walls was more in the wild type than in the mutants. This finding is consistent with the SEM observations indicating that the *brc1*, *brc2*, and *brc3* mutants were deficient in cellulose mainly in the secondary cell walls.

Cell wall strength in the mutants after removal of hemicellulose and lignin

To understand the changes in cell wall structure, cell walls were treated with sodium chlorite and examined by SEM. The SEM revealed that the walls of all mechanical tissue cells of the mutants were thinner than those of the wild type and deformed (Supplementary Fig. S6). In addition to the reduced wall thickness in cells of mechanical tissues, the mutants also exhibited alteration of structure of the vessel elements. Hemicellulose is the main component of the middle lamellae and its removal caused a drastic change in the organization of surface microfibrils. Uneven surface microfibrils were observed in the mutants. The wild type had smooth surface with parallel cellulose microfibrils running in a single direction whereas increased porosity was observed in *brc1*, *brc2*, and *brc3* due to disruption of surface cellulose microfibrils (Supplementary Fig. S7). These results indicated that the brittle culm mutations affected the organization of cellulose microfibrils.

Inheritance and mapping of the brittle mutants

The F_1s obtained from six crosses were non-brittle like the wild type parents indicating that all the brittle culm mutants were recessive. The F_2 plants segregated for brittleness. The brittle and non-brittle F_2 plants of crosses segregated in 3:1 ratio indicating that the brittle mutants were monogenic recessive (Table 4).

Bulk segregant analysis (BSA)

Hundred and thirty-three anchored SSR markers polymorphic between *T. monococcum and T. boeoticum* (Singh et al. 2007) were used for BSA. For BSA positive bulks were made by pooling DNA from 15 homozygous F_2 brittle culm plants from each of the F_2 populations directly along with a common bulk from 15 RILs from a *T. boeoticum* × *T. monococcum* cross without any mutant in the parents. The SSR markers Xbarc37 and Xbarc113 located on chromosome 6AS showed polymorphism between bulk of *brc1* and the common bulk, markers Xcfd62 and Xcfa2170 located on chromosome 3AL showed polymorphism between bulks of *brc2* whereas the markers Xgwm135 and Xwmc470 located on chromosome 1AL showed polymorphism between bulk of *brc3* and the common bulk (Fig. 4a–c). Genotyping of the debulked F_2 plants with the putatively linked markers from BSA confirmed their close linkage with *brc1*, *brc2*, and *brc3* mutants, Fig. 5a–c, respectively.

Mapping of the *brc1, brc2* and *brc3* by genotyping of F_2 populations:

To map the *brc1*, *brc2*, and *brc3* mutants, F_2 mapping populations were developed by crossing the brittle culm mutants (*brc1*, *brc2*, and *brc3*) with *Triticum boeoticum* acc. pau 5088, a non-brittle and tall wild

Table 4 Segregation of F_2 plants for brittleness in crosses of brittle culm mutants *brc1*, *brc2*, and *brc3* with *T. monococcum and T. boeoticum*

Cross	Segregation in F_2 population			χ^2 (3:1)
	Total plants	Non-brittle	Brittle	
brc1/T. monococcum	100	76	24	0.053*
brc2/T. monococcum	96	73	23	0.055*
brc3/T. monococcum	100	74	26	0.053*
brc1/T. boeoticum	250	188	62	0.941*
brc2/T. boeoticum	250	187	63	0.941*
brc3/T. boeoticum	185	139	46	0.966*

* Data gave a good fit to 3:1 ratio at $P = < 0.01$ and 1 d.f

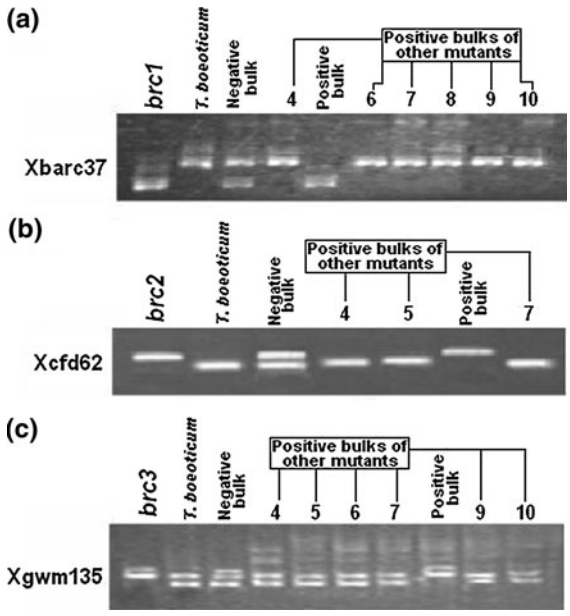

Fig. 4 Identification of putative linked SSR markers Xbarc37, Xcfd62, and Xgwm135 for three brittle culm mutants *brc1*, *brc2*, and *brc3*, respectively through Bulk segregant analysis (BSA); **a** *Lane 1*: *brc1*, *2*: *T.boeoticum 3*: Negative bulk and *5*: Positive bulk of *brc1; 4*, *6*, *7*, *8*, *9*, and *10* are the positive bulks of other mutants; **b** *Lane 1*: *brc2*, *2*: *T. boeoticum 3*: Negative bulk; *6*: Positive bulk of *brc2* and *4*, *5*, and *7* are the positive bulks of other mutants; **c** *Lane 1*: *brc3*, *2*: *T. boeoticum 3*: Negative bulk and *8*: Positive bulk of *brc3, 4*, *5*, *6*, *7*, *9*, and *10* are the positive bulks of other mutants

diploid progenitor of *T. monococcum*. A total of 250, 250, and 185 individual F_2 plants were used for genotyping of *brc1*, *brc2*, and *brc3* F_2 populations (Supplementary Figs. S8, S9, and S10). Co-segregation analysis of individual markers Xbarc37 and Xbarc113 for *brc1* located on chromosome 6AS, Xcfd62, and Xcfa2170 for *brc2* on chromosome 3AL and Xgwm135 and Xwmc470 for *brc3* located on chromosome 1AL using the marker and mutant genotypes of F_2 plants was carried out with the help of recombination frequency between markers at each locus. Based on the data on recombination frequency, the marker Xbarc37 and Xbarc113 were mapped at distance of 1.9 and 10.3 cM, respectively from the *brc1* mutant on chromosome 6AS, marker Xcfd62, and Xcfa2170 mapped at distance of 0.8 and 2.9 cM, respectively from the *brc2* mutant on chromosome 3AL and marker Xgwm135 and Xwmc470 were mapped at distance of 2.1 and 3.9 cM, respectively from the *brc3* mutant on chromosome 1AL (Fig. 6).

BLAST search of rice genes and wheat ESTs

The homology of wheat chromosome 6A, 3A, and 1A with rice chromosome 2R, 1R, and 3R has already been known (Salse et al. 2009). On the basis of this homology we searched orthologous genes of cellulose synthase and related genes on corresponding rice chromosome as the details of these genes have been already reported (Sado et al. 2009; Joshi and Mansfield 2007). Six genes of cellulose synthesis pathway were reported on syntenic 1, 2, and 5 rice chromosomes in NCBI database. Six individual rice genes involved in cellulose synthesis pathway BLASTed against the available wheat ESTs, led to the identification of one wheat EST each showing high similarity (bit score $\geq$ 200) against each of the six rice genes (Supplementary Table S2). The identified wheat ESTs were used as query sequences in BLAST search against the bin-mapped wheat EST/contigs. However, none of the six EST sequences showed similarity with the bin-mapped wheat ESTs/contigs.

Discussion

In the present study, three independent EMS-induced brittle culm mutants of *T. monococcum* namely *brc1*, *brc2* and *brc3* were used for their phenotypic and molecular characterization. All the three mutants were found to be monogenic recessive. Such mutants have not been reported in polyploid wheats as the orthologous loci on its three genomes suppress the newly induced recessive mutants unless and until multiple mutants are induced at all the loci or certain loci have been silenced during evolution of polyploidy wheats.

All the three brittle culm mutants, *brc1*, *brc2*, and *brc3* were defective in cellulose synthesis and its deposition in the secondary cell walls of sclerenchyma cells. In all the brittle mutants cellulose of secondary cell wall was reduced by 47–57% compared to that of the wild type *T. moncoccum* with slight increase in lignin and significant changes in hemicellulose, silicates, and ash content etc. Comprehensive mechanical strength, histological, biochemical, SEM, and AFM analyses of culms and leaves of all the brittle mutants supplemented and complemented the findings that the mutants had defective cellulose synthesis and deposition on the secondary cell walls. All the plant parts of the mutants were highly brittle indicating that the

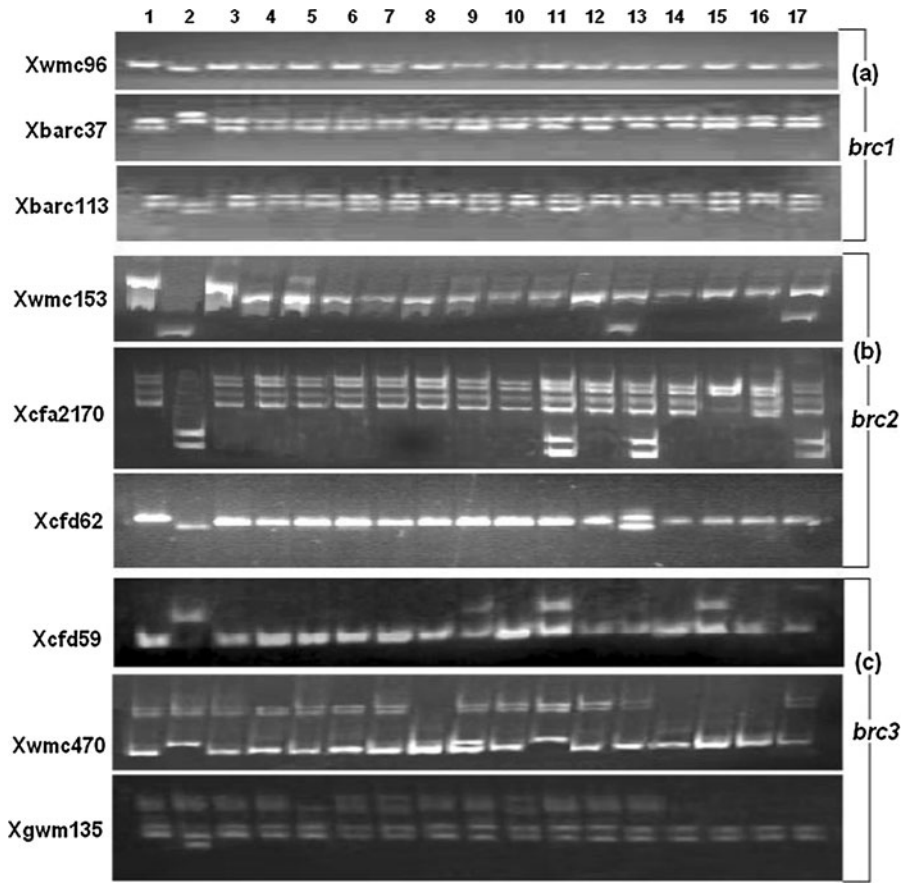

Fig. 5 PCR amplification of debulks of positive bulks of F_2 plants of various brittle culm mutant using putatively linked markers identified in BSA; *a* Xwmc96, Xbarc37, and Xbarc113 with *brc1* : *Lane 1*: *brc1*, *2*: *T. boeoticum*, *3–17*: debulks; *b* Xwmc153, Xcfd62, and Xcfa2170 with *brc2*; *Lane 1*: *brc2*, *2*: *T. boeoticum*, *3–17*: debulks; *c* Xcfd59, Xwmc470, and Xgwm135 with *brc3*; *Lane 1*: *brc3*, *2*: *T. boeoticum*, *3–17*: debulks

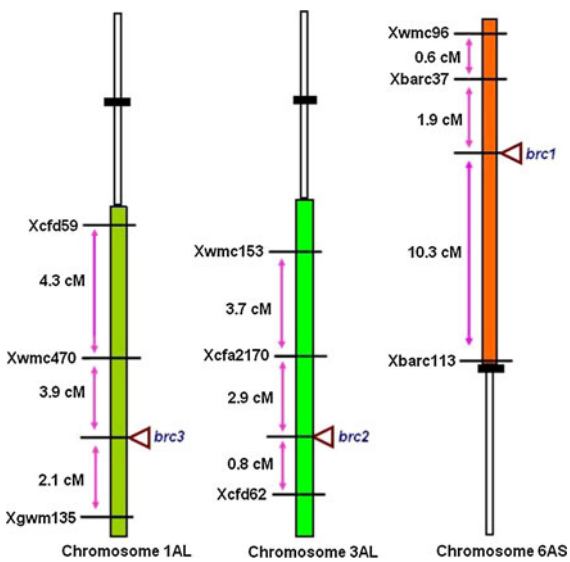

Fig. 6 Mapping of three brittle culm mutants *brc1*, *brc2*, and *brc3* on chromosome *6AS*, *3AL*, and *1AL* of *T. monococcum*, respectively

cellulose in secondary cell wall was the main component of straw strength and not the lignin in *T. monococcum* as often thought of.

The plant cell wall is the major component of mechanical support to cells, tissues, and the entire plant body. Sclerenchyma cells having both primary walls and thick secondary walls provide major mechanical support in non-elongating regions of the plant body (Carpita and McCann 2000). Many mutants defective in plant strength have been isolated and characterized. The barley brittle culm showed reduced mechanical strength and cellulose content, indicating a correlation between the cellulose content and the plant mechanical strength (Kokubo et al. 1989, 1991). The rice classic mutant *bc1* causes the altered biosynthesis of cellulose, hemicellulose, and lignin, leading to a reduction in the secondary cell wall thickness and the mechanical strength of rice plant (Li et al. 2003). In maize, Maize *Brittle stalk 2* also had decreased level of cellulose with a defect in COBRA-like protein (Sindhu et al. 2007). Similarly, in *Arabidopsis*, the irregular xylem mutants

(*irx1* to *irx3*) showed defects in cellulose synthesis in secondary walls, and decreased stiffness of mature stems indicating the direct role of cellulose in maintaining cell morphology (Turner and Somerville 1997).

The brittle culm mutants *brc1*, *brc2*, and *brc3* have been mapped to three different chromosomes 6A, 3A, and 1A of diploid wheat *T. monococcum*, respectively suggesting that there are multiple genes in diploid wheat for cellulose synthesis and deposition like that of rice and *Arabidopsis* (Sado et al. 2009). As per the microsatellite consensus map of bread wheat by Somers et al.(2004), Xbarc37 marker on 6AS tightly linked to *brc1* mutant was closest to the centromere whereas in the diploid wheat map (Singh et al. 2007) with additional molecular markers, *brc1* mapped 10.3 cM away from the centromere on 6AS. Similarly, the marker Xgwm135 tightly linked to *brc3* (2.1 cM) and closest to the centromere on 1AL in the consensus map (Somers et al. 2004), mapped more than 57 cM away from the centromere in the diploid wheat map (Singh et al. 2007) with the additional markers. It is, therefore, evident that with the application of additional markers all the three brittle mutants mapped away from the centromeres of their respective chromosome arms thus facilitating their fine mapping and map-based gene cloning.

In the complete genome sequence of *A. thaliana* it has been estimated that, among 27,000 *Arabidopsis* genes, approximately 15% are dedicated to the processes of cell wall synthesis, modification, assembly, and degradation, and correct regulation of these processes during growth and development (Carpita et al. 2001). The cell wall is one of the most complicated and the least-understood plant cell structures. Mutants defective in mechanical strength have been found to be valuable for identifying genes involved in the biogenesis and modification of cell walls (Li et al. 2003; Tanaka et al. 2003; Taylor et al. 2003; Zhong et al. 2002). The cell wall polysaccharides and glycoproteins have tremendous structural complexity, and therefore the plants require large families of glycosyl transferases (GTs) to facilitate their biosynthesis. Based on sequence similarities and the existence of certain motifs, hydrophobic clusters and their catalytic specificity (Rosen et al. 2004), GTs have been divided into 91 families (http://www.cazy.org/CAZY/). Among these, cellulose synthase active subunits (CESA) (Somerville, 2006) are responsible for synthesizing cellulose and CESA-like proteins (CSL), are believed to be responsible for the formation of glycan backbones in the endoplasmic reticulum (ER) or Golgi apparatus (Burton et al. 2006; Dhugga et al. 2004).

Straw strength is one of the most important agronomic traits that affects not only harvest index and stability of production but also the usefulness of cereal straws as animal forage. The cloning and manipulation of genes for these brittle culm mutants *brc1*, *brc2*, and *brc3* and their orthologs in other genomes could make a significant contribution to further improvement of wheat. It will be desirable to over express the wild type *CesA* or *CSL* genes to get higher cellulose synthesis for stronger straw and higher cellulose/lignin ratio for commercial exploitation in animal feed, pulp, and paper industry as well as for feedstock in biofuel industries.

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References

Arioli T, Peng LC, Betzner AS, Burn J, Wittke W, Herth W, Camilleri C, Höfte H, Plazinski J, Birch R, Cork A, Glover J, Redmond J, Williamson RE (1998) Molecular analysis of cellulose biosynthesis in *Arabidopsis*. Science 279: 717–720

Burton RA, Wilson SM, Hrmova M, Harvey AJ, Shirley NJ, Medhurst A, Stone BA, Newbigin EJ, Bacic A, Fincher GB (2006) Cellulose synthase-like CslF genes mediate the synthesis of cell wall (1, 3;1, 4)-b-D-glucans. Science 311: 1940–1942

Carpita N, McCann M (2000) The cell wall. In: Buchanan BB, Gruissem W, Jones RL (eds) Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville MD, pp 52–108

Carpita N, Tierney M, Campbell M (2001) Molecular biology of the plant cell wall: searching for the genes that define structure, architecture and dynamics. Plant Mol Biol 47: 1–5

Dhugga KS, Barreiro R, Whitten B, Stecca K, Hazebroek J, Randhawa GS, Dolan M, Kinney AJ, Tomes D, Nichols S, Anderson P (2004) Guar seed b-mannan synthase is a member of the cellulose synthase super gene family. Science 303:363–366

Hu Y, Zhong RQ, Herbert Morrison III W, Ye ZH (2003) The *Arabidopsis* RHD3 gene is required for cell wall biosynthesis and actin organization. Planta 217:912–921

Johansen DA (1940) Plant microtechnique Ist Ed. 182. McGraw-Hill, New York

Jones L, Ennos AR, Turner SR (2001) Cloning and characterization of irregular xylem 4 (irx4): a severely lignin-deficient mutant of *Arabidopsis*. Plant J 26:205–216

Joshi CP, Mansfield SD (2007) The cellulose paradox—simple molecule, complex biosynthesis. Curr Opin Plant Biol 10: 220–226

Kokubo A, Kuraishi S, Sakurai N (1989) Culm strength of barley correlation among maximum bending stress, cell wall dimensions, and cellulose content. Plant Physiol 91: 876–882

Kokubo A, Sakurai N, Kuraishi S, Takabe K (1991) Culm brittleness of barley (*Hordeum vulgare* L.) mutants is caused by smaller number of cellulose molecules in cell wall. Plant Physiol 97:509–514

Kristensen JB, Thygesen LG, Felby C, Jorgensen H, Elder T (2008) Cell-wall structural changes in wheat straw pretreated for bioethanol production. Biotechnol Biofuels. doi:10.1186/1754-6834-1-5

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newberg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Li YH, Qian Q, Zhou YH, Yan MX, Sun L, Zhang M et al (2003) BRITTLE CULM1, which encodes a COBRA-Like protein affects the mechanical properties of rice plant. Plant Cell 15:2020–2031

Lincoln SE, Daly MJ, Lander ES (1990) Constructing genetic linkage maps with MAPMAKER: a tutorial and reference manual. Technical report. Whitehead Institute for Biomedical Research, Cambridge

Liu RG, Yu H, Huang Y (2005) Structure and morphology of cellulose in wheat straw. Cellulose 12:25–34

McCouch SR, Kochert G, Yu ZH, Wang ZY, Khush GS, Coffman WR, Tanksley SD (1988) Molecular mapping of rice chromosomes. Theor Appl Genet 76:815–820

Mou ZL, He YK, Dai Y, Liu XF, Li J (2000) Deficiency in fatty acid synthase leads to premature cell death and dramatic alteration in plant morphology. Plant Cell 12:405–417

Rosen ML, Edman M, Sjostrom M, Wieslander A (2004) Recognition of fold and sugar linkage for glycosyl transferases by multivariate sequence analysis. J Biol Chem 279: 38683–38692

Sado PE, Tessier D, Vasseur M, Elmorjani K, Guillon F, Saulnier L (2009) Integrating genes and phenotype: a wheat-*Arabidopsis*-rice glycosyltransferase database for candidate gene analyses. Func Integr Genomics 9(1):43–58

Salse J, Abrouk M, Bolot S, Guilhot N, Courcelle E, Faraut T, Waugh R, Close TJ, Messing J, Feuillet C (2009) Reconstruction of monocotelydoneous proto-chromosomes reveals faster evolution in plants than in animals. Proc Natl Acad Sci 1106(35):14908–14913

Sindhu A, Langewisch T, Olek A, Multani DS, McCann MC, Vermerris W, Carpita NC, Johal G (2007) Maize brittle stalk2 encodes a COBRA-like protein expressed in early organ development but required for tissue flexibility at maturity. Plant Physiol 145(4):1444–1459

Singh K, Ghai M, Garg M, Chhuneja P, Kaur P, Schnurbusch T, Keller B, Dhaliwal HS (2007) An integrated molecular linkage map of diploid wheat based on a *Triticum boeoticum* x *T. monococcum* RIL population. Theor Appl Genet 115:301–312

Somers DJ, Isaac P, Edwards K (2004) A high -density microsatellite consensus map for bread wheat (*Triticum aestivum* L.). Theor Appl Genet 109:1105–1114

Somerville C (2006) Cellulose synthesis in higher plants. Annu Rev Cell Dev Biol 22:53–78

Srivastava LM (1966) Histochemical studies on lignin. Tappi 49:173–183

Stéphanie R, Adeline B, Olivier G, Daniel K, B′eatrice SJ, Sandra P, Hauser MT, Höfte H, Vernhettes S (2005) An *Arabidopsis* endo-1, 4-ß-DGlucanase involved in cellulose synthesis undergoes regulated intracellular cycling. Plant Cell 17:3378–3389

Tanaka K, Murata K, Yamazaki M, Onosato K, Miyao A, Hirochika H (2003) Three distinct rice cellulose synthase catalytic subunit genes required for cellulose synthesis in the secondary wall. Plant Physiol 133:73–83

Taylor NG, Scheible W, Cutler S, Somerville CR, Turner SR (1999) The irregular xylem3 locus of *Arabidopsis* encodes a cellulose synthase required for secondary cell wall synthesis. Plant Cell 11:69–779

Taylor NG, Howells RM, Huttly AK, Vickers K, Turner SR (2003) Interactions among three distinct CesA proteins essential for cellulose synthesis. Proc Natl Acad Sci 100: 1450–1455

Turner SR, Somerville CR (1997) Collapsed xylem phenotype of *Arabidopsis* in the secondary cell wall. Plant Cell 9:689–701

Updegraff DM (1969) Semimicro determination of cellulose in biological materials. Anal Biochem 32:420–424

Van Soest PJ, Robertson JB (1985) Analysis of Forages and Fibrous Food, vol 74–75, pp 80–82. In: A laboratory manual for animal science 613. Cornell University, Ithaca, New York

Van Soest PJ, Robertson JB, Lewis BA (1991) Method for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 74: 3583–3597

Zhong R, Burk DH, Morrison WH III, Ye ZH (2002) A kinesin like protein is essential for oriented deposition of cellulose microfibrils and cell wall strength. Plant Cell 14:3101–3117