Acta Physiol Plant (2013) 35:2407–2419
DOI 10.1007/s11738-013-1275-0

ORIGINAL PAPER

Characterization and gene mapping of a brittle culm mutant of diploid wheat (*Triticum monococcum* L.) with irregular xylem vessels development

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Received: 18 December 2012 / Revised: 14 March 2013 / Accepted: 19 March 2013 / Published online: 2 April 2013

Abstract Diploid wheat, *Triticum monococcum* L. (einkorn) is an ideal plant material for wheat functional genomics. Brittle culm mutant was identified by screening of the ethyl methane sulphonate-treated M_2 progenies of a *T. monococcum* accession pau14087 by banding the plant parts manually. The brittle culm mutant with drooping leaves, early flowering, reduced tiller numbers and susceptible to lodging had also exhibited brittleness in all plant parts than the wild-type parents. Comprehensive mechanical strength, histological, biochemical, scanning electron microscopy, and Fourier transform infrared spectroscopy analyses of brittle culm mutant supplemented and complemented the findings that the mutant had defective cellulose biosynthesis pathway and deposition of cell wall materials on secondary cell wall of mechanical tissues. Microscopic studies demonstrated that the decrease in cellulose contents resulted in the irregular cell wall organization in xylem vessels of leaf vascular bundles. To map the *brc5* mutant, mapping populations were developed by crossing the brittle culm mutant with wild *Triticum boeoticum* acc. pau5088, having non-brittle characters. The brittle culm mutation was mapped between SSR markers, *Xcfd39* and *Xgwm126* on $5A^mL$ chromosome of *T. monococcum*, with genetic distances of 2.6 and 4.8 cM, respectively. The *brc5* mutant mapped on $5A^mL$, being distinct from a previously mapped brittle culm mutant in wheat, has been designated as *brc5*. The work on fine mapping and map-based cloning of *brc5* gene regulating synthesis and deposition of cellulose on the secondary cell wall is in progress.

Keywords *brc5* mutant · Cellulose · Gene mapping · Irregular xylem vessels · SSR marker · *Triticum monococcum* (L.)

Abbreviations

BSA	Bulk segregant analysis
brc5	Brittle culm mutant 5
EMS	Ethyl methane sulfonate
IIT	Indian Institute of Technology
M_2	Second generation after mutagenesis
F_2	Second filial generation
PCR	Polymerase chain reaction
RILs	Recombinant inbred lines
SEM	Scanning electron microscopy
FTIR	Fourier transform infrared spectroscopy
VBs	Vascular bundles

Communicated by P. Sowinski.

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TS Transverse section
WT Wild-type

Introduction

Mechanical strength of plants is an important agronomic trait and directly related to the crop lodging thus yield losses and grain quality (Ching et al. 2006; Wang et al. 2012). The intrinsic mechanical strength of plants is mainly controlled by cell wall and any changes in cell wall structure or composition affect the mechanical strength of plants (Cosgrove 2000). Sclerenchymatous tissues of plants having both primary cell wall and secondary thick cell wall provide the maximum mechanical strength to plants (Carpita and McCann 2000). Cellulose constitutes 20–30 % of dry weight of primary cell walls and 40–90 % of the secondary cell walls (Taylor et al. 1999).

Mutations in the genes of cellulose biosynthesis or related pathway result in cellulose-deficient mutants or mutants defective in the stem strength. These mutants are ideal material to understand the mechanisms that involved in the mechanical strength of the plant and the biosynthesis of plant cell wall materials. Several mutants defective in stem strength were isolated and characterized. Several brittle culm mutants have been identified in higher plants and considered excellent material to study the process of secondary cell wall formation (Aohara et al. 2009). In our previous study, three EMS-induced brittle culm mutants, *brc1*, *brc2* and *brc3* have been reported in *T. monococcum*. These diploid wheat brittle culm mutants exhibit 47–57 % reduced α-cellulose in the secondary cell walls than that of WT (Ansari et al. 2012). In *Arabidopsis*, several mutants with reduced mechanical strength have been reported (Turner and Somervilles 1997; Zhong et al. 2005; Wu et al. 2012). *Arabidopsis* xylem-collapsed mutants, *irregular xylem* (*irx*) *1*, *3*, and *5* have defects in their cellulose synthase catalytic subunits (CesAs), and exhibited collapsed xylem phenotype due to negative pressure developed by water transport in thin cell wall xylem cells (Taylor et al. 1999, 2000, 2003). In barley, brittle culm (bc) mutant was first described with reduced mechanical strength, and had up to 80 % reduction in cellulose content with reduced breaking strength as compared with those of WT (Kokubo et al. 1989, 1991). In rice, several brittle mutants with reduced mechanical strength have been characterized (Li et al. 2003; Xu et al. 2008; Aohara et al. 2009). The rice classic mutant, brittle culm 1 (*bc1*) has a defect in COBRA-like protein that may function in the formation of secondary cell walls in the developing sclerenchyma cells and in vascular bundles. Mutation in bc1 not only cause reduced cellulose content and secondary cell wall thickness but also increased lignin (Li et al. 2003). All rice bc1, 2, 3, 4, 6 and 7 show brittleness in culm and leaves (Nagao and Takahashi 1963; Singh et al. 1994; Yan et al. 2007).

Diploid wheat (*T. monococcum* L., A^mA^m) is one of the model plants of Poaceae family that can be used to study the genetics of cell wall biosynthesis (Ansari et al. 2012). The diploid wheat is an ideal material for induced mutations and variability which could be characterized and transferred to cultivated wheat. The chemical mutagen, EMS, is a potential mutagen that has been successfully applied in mutation-based research of wheat and rice (Wang et al. 2009; Ansari et al. 2012, 2013). *T. monococcum* ($2n = 14$) has a small genome size (5,700 Mb) compared with that of hexaploid wheat (17,300 Mb), and the existence of a high level of polymorphism for DNA-based markers with its wild progenitor *T. boeoticum* (Singh et al. 2007), conservation of colinearity and synteny with other cereal crops, availability of a BAC libraries and high resistance against various wheat diseases make this species an attractive diploid model for gene discovery in wheat (Wicker et al. 2001).

The mapping of the genetic determinants of phenotypic variation is often a key step in the characterization of mutants and QTLs. In complex genomes like wheat, mapping remains a non-trivial process. Bulked segregant analysis (BSA) is more efficient, time and cost-effective method to rapidly identify genetic markers linked to a genomic region associated with the selected mutant phenotypes than traditional gene mapping where each informative marker is genotyped in every F_2 individuals. BSA requires access to quantitative genetic markers that are polymorphic in the mapping population. This strategy is expected to be widely adopted for mapping in many species (Liu et al. 2012). Using the diploid wheat mapping populations, high-density molecular maps have already been developed (Dubcovsky et al. 1996; Taenzler et al. 2002; Singh et al. 2007) and a number of mutations have been mapped (Kuraparthy et al. 2007; Sood et al. 2009; Ansari et al. 2012, 2013) and genes for disease resistance have been introgressed into bread wheat cultivars (Chhuneja et al. 2008; Singh et al. 2010).

This article deals with the characterization and mapping of a novel, EMS-induced brittle culm mutant (*brc5*), in which the walls of mature xylem vessels in leaves appear to spontaneously collapse inwards and result in irregular xylem vessels in diploid wheat, *T. monococcum*. This mutant also has greatly reduced cellulose content in secondary cell walls of mechanical tissues. The results will not only promote map-based cloning of *brc5* gene but also could have far reaching implications and applications in the investigation of cellulose biosynthetic pathway and xylem vessels development in wheat and other cereals.

Materials and methods

Plant materials

The EMS-induced brittle culm (*brc5*) mutant used in the present study was isolated from diploid wheat *T. monococcum* accession pau14087 at the Punjab Agricultural University, Ludhiana, after seed treatment with 0.25 % EMS. This mutant was identified during manual screening in the EMS-treated M_2 population in the field. The seeds of *brc5*, the WT parent and an accession pau5088 of *T. boeoticum* (the wild and tall progenitor of *T. monococcum*) were planted at the Indian Institute of Technology (IIT), Roorkee in November 2005. The *brc5* mutant was crossed with both WT parent, *T. monococcum* and its wild progenitor *T. boeoticum* for developing F_2 populations for inheritance and mapping studies, respectively. 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.

Effect of TOPIK herbicide on *brc5* mutant

TOPIK (Syngenta, India) herbicide is a foliar acting grass weed killer and used post-emergence on bread wheat, durum wheat and some other crops. To see the effect of TOPIK herbicide on *T. monococcum* and *brc5* mutant, a solution was prepared according to the manufacturer instruction and foliar sprayed using a conventional fan nozzle before flag leaf sheath extending stage.

Mechanical strength of *brc5* mutant

The data on brittleness of *brc5* mutant and F_2 population were recorded at 30 days of sowing, during flowering and harvesting by banding the plant parts manually. The breaking force and elongation ratio of culm second internode and flag leaf of both *brc5* mutant and its WT, *T. monococcum* were measured with a digital force tester (5948 Micro Tester, Instron) as reported previously (Wu et al. 2012). These two parameters are important to define the elasticity of plant parts and force required to break the flag leaf and culm internodes, respectively. To reduce the sampling error, the second internodes of culm and flag leaf with equal lengths and width were used for the measurement and placed immediately in the digital force tester to test the mechanical force required to break the samples.

Cell wall composition assay

The second internodes of WT and *brc5* plants were harvested after flowering. The collected samples were shade dried for 2 days and ground into fine powder by TissueLyser II (Qiagen). Alcohol insoluble residues (AIR) were prepared as previously described (Harholt et al. 2006). Two milligram of each de-starched AIR samples was hydrolyzed in 2 M trifluoroacetic acid (TFA) at 121 °C for 90 min. The silylated sugars from supernatant were prepared as described previously (Li et al. 2009). The cell wall derivatives were analyzed using an Agilent 7890A Series GC system with a 5975C MSD instrument. The total dry matter in the *brc5* mutant and WT was measured in the leaves and culm. Crystalline cellulose was analyzed by anthrone assay (Updegraff 1969). Briefly, the leaves and second 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 % (v/v) 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).

Scanning electron microscopy

The samples were prepared for scanning electron microscopy (SEM) according to Mou et al. (2000). The WT and mutant tissue samples were immersed in 2.5 % glutaraldehyde for 2 h at room temperature and dehydrated in ascending ethanol concentrations (v/v) of 50 % for 5 min, 70 % for 30 min (twice), 90 % for 30 min (twice), and 100 % for 30 min (twice). Finally, in ethanol-amyl acetate series (v/v) of absolute ethanol:amyl acetate (3:1) for 30 min, absolute ethanol:amyl acetate (2:2) for 30 min, absolute ethanol:amyl acetate (1:3) for 30 min and amyl acetate for 30 min. The samples were kept for critical point drying for 40 min, and mounted onto metal stubs with double-sided carbon tape. For sputter coating, a thin layer of gold metals was applied over the samples using an automated sputter coater. These samples were then analyzed under SEM (*Leo 435*, Cambridge, USA) and the surface images were taken at 150, 250, 500, and 1,500× magnification.

Histological observations

In order to determine whether the mutation in *brc5* affects the histology of mutant and biochemical composition of

cell walls, transverse section of culm second internode and flag leaf from WT and *brc5* plants were histochemically stained with phloroglucinol–HCl, a diagnostic strain for lignin. Phloroglucinol–HCl is known to react with cinnamaldehyde residues in lignin, and the color intensity approximately reflects the total lignin content. The flag leaf and second internode of stems were excised to fix in formalin–acetic acid–alcohol (FAA) fixative (50 % ethanol, 5 % glacial acetic acid, 10 % formalin, 35 % ddH_2O) overnight. Following FAA fixation, samples were dehydrated in a graded ethanol series and finally xylene. For sectioning, the tissues were embedded in paraffin wax (Sdfine, Mumbai, India) 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 the graded ethanol series. The tissues were stained with phloroglucinol (2 % in 95 % ethanol) dye and mounted in 30 % HCl for histological localization of lignin, as suggested by Wu et al. (2012). Transverse sections of the stems of the *brc5* mutant and *T. monococcum* were observed under a light microscope (Axiostar plus 1169-151, Carl Zeiss Co., Oberkochen, Germany) at different magnifications.

Fourier transform infrared spectroscopy

The *brc5* and WT culm were analyzed by an IR spectrometer (Thermo Electron Corp., Waltham, MA, USA) using the KBr pellet technique. Small piece of culm was powdered and added to KBr to form a pellet that contained 1 % test material. The spectrum was taken in the wavenumber range of 500–1,800 cm^{-1} at a 4 cm^{-1} resolution in absorbance mode. Each final spectrum was the average of 48 scans.

Genomic DNA isolation and PCR amplification

The genomic DNA from parents and the F_2 population was extracted following the cetyltrimethyl ammonium bromide (CTAB) method, as described by Saghai-Maroof et al. (1984) by pooling leaf tissue from each plant of F_2 population. The anchored molecular markers from a map developed in a RIL population between *T. monococcum* acc. pau14087 and *T. boeoticum* acc. pau5088 (Singh et al. 2007) were used for mapping the brittle culm mutant. The primers for anchored SSR markers at about 10 cM from each of the diploid wheat chromosomes and polymerase chain reaction (PCR) protocols were carried out in a thermocycler (*Applied Biosystems*, Singapore) according to methods described by Singh et al. (2007) with some modifications. The reaction was conducted in a volume of 10 µl. The reaction mixture contained 1× PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.125 mM of each dNTPs, 0.5 U Taq DNA polymerase, 0.2 µM each primer, 20 ng DNA template and sterile deionized H_2O. Amplification conditions were 94 °C for 4 min; 29 cycles of 94 °C for 1 min, 50–63 °C (depending on the primer condition) for 1 min, 72 °C for 2 min; 72 °C for 7 min as the final extension. The PCR products were separated by electrophoresis on 8 % polyacrylamide gels for approximately 1 h, and the band patterns were visualized using ethidium bromide staining (Singh et al. 2007).

Bulk segregant analysis (BSA)

The phenotypic data on brittleness through bending of culms and leaves were recorded on each plant of F_2 population. For bulk segregant analysis (BSA), positive bulk of 12 brittle culm mutant plants was made from homozygous F_2 brittle culm mutant plants from the F_2 mapping population of *brc5* × *T. boeoticum* pau5088. A negative bulk was prepared from 12 non-brittle F_2 plants from the F_2 mapping population of *brc5* × *T. boeoticum* pau5088. 50 ng/µl of the DNA from every 12 plants of positive and negative bulk was pooled into two separate 1.5 ml Eppendorf tubes. These DNA samples from both bulks along with the DNA of both parents were used as a template to identify putative SSR markers linked to the *brc5* mutant. The putatively linked SSR marker *Xcfd39*, polymorphic between the parents and bulks were used to genotype individual plants constituting the particular bulks and the F_2 mapping populations. Two more nearby linked SSR markers *Xgwm126* and *Xgwm6* (as per the diploid wheat map of Singh et al. 2007) were also used for genotyping of the debulked F_2 plants. These three SSR markers together were used to genotype individual plants of the F_2 mapping population.

Linkage analysis and molecular mapping

To study the inheritance of *brc5* mutation, it was crossed with its WT parent *T. monococcum* and *T. boeoticum* for molecular mapping. The F_1 plants were self-pollinated to produce F_2 seeds and advanced to F_2 generations. A mapping population of 180 F_2 plants derived from *T. boeoticum* acc. pau5088/*brc5* was used for gene mapping and genotyping, as determined by phenotypic segregation of F_2 progenies. Phenotypic evaluations of F_1 and F_2 plants were performed by bending of culm and leaves manually for brittle and non-brittle character. Chi-square test was applied to test the goodness-of-fit to the segregation ratio. A linkage map was constructed with 'MAPMAKER/EXP version 3.0' (Lander et al. 1987, Lincoln et al. 1993) according to the linkage data of the *brc5* mutant and polymorphic SSR markers in the F_2 mapping population. Recombination frequencies were converted to

centi-Morgans (cM) using Haldane's mapping function (Haldane 1919). The LOD score of 3.0 was used for the linkage threshold. The genetic distance (centi-Morgan, cM) was calculated according to the Kosambi function (Kosambi 1944).

Statistical analysis

Data are the mean ± SE of three independent replicates. The analyses of variance were computed on statistically significant differences ($P < 0.05$) determined based on the appropriate F tests. The mean differences were compared utilizing Duncan's multiple range test.

Results

Phenotypic characterization and mechanical strength of *brc5* mutant

Phenotypically, *brc5* mutant plants were clearly distinguishable from *T. monococcum* as they were spreading with drooping leaves during flowering and more sensitive to a herbicide, TOPIK as compared to the WT parent *T. monococcum* (Table 1). The *brc5* mutant was characterized by brittle culms, leaves and spikes that can be easily broken by bending (Fig. 1a, b). As shown in Fig. 1c, the culm elongation ratio *brc5* mutant plant was nearly 2.5 times lower than of the WT plants. Meanwhile, the breaking force of the flag leaf and second internode of *brc5* mutant was also reduced by 50 and 62 %, respectively, compared with that of WT plants (Fig. 1d, e). The significant decrease in the elongation ratio and breaking force of *brc5* mutant suggested that mutation in the *brc5* strongly affects the mechanical strength of *T. monococcum*.

Table 1 Morphology of *T. monococcum* and *brc5* mutant

Trait	*T. monococcum*	*brc5*
Growth habit after flowering	Erect	Spreading
Days to 50 % flowering	~112 ± 6	~92 ± 3
Numbers of tillers per plant	~40 ± 3	~18 ± 2
Main culm height (cm)	~128 ± 7	~98 ± 3
One-week-old seedling root length (cm)	~1.2 ± 0.6	~0.9 ± 0.2
Flag leaf length (cm)	~36 ± 3	~18 ± 3
Spikelets per spike	~31 ± 3	~19 ± 2
Brittleness in all plant parts	Absent	Present
Susceptibility to herbicide (Topik)	No	Yes
Susceptibility to powdery mildew	No	No

A total of 12 plants were used for measurement of all the data

Histology of the flag leaf

In order to determine whether the mutation in *brc5* affects the histology of mutant leaves, transverse section of the mature flag from WT and *brc5* plants was histochemically stained with phloroglucinol–HCl. The results indicated that reduced cell wall thickness was not only present in culm parenchyma and cortical fibers but also in the vascular bundles. The *brc5* leaf cortical vascular bundles were found to exhibit collapsed metaxylem and protoxylem vessels (Fig. 2a–d). One possible cause of collapsed xylem vessels is by a lack of resistance by thin cell walls to the negative pressure developed in the leaves vascular bundles due to transpiration.

Bundle sheath cell wall of *brc5* mutant plants also exhibit reduced cell wall thickening compared to that of WT bundle sheath. Cell density (number of cells per unit area) of the flag leaf was similar in both the *brc5* mutant and *T. monococcum*.

Histology of the second internode

Culm sections from WT plants and *brc5* mutant plants show light staining and dark staining with phloroglucinol–HCl, respectively. The color differences between WT and *brc5* mutant in the mechanical or sclerenchymatous tissues, especially the sclenenchymatous tissues below epidermis and vascular bundles (Fig. 3) have been observed. These hypodermal tissues provide the main mechanical strength to plant body. Light microscope observation revealed that the WT sclerenchyma cells were heavily thickened in culm and almost closed with cell wall materials (Fig. 3a, c); in contrast, the *brc5* sclerenchyma cells were much thinner and hollow with little cell wall material deposition (Fig. 3e, g). We also compared the epidermal cell wall structure and thickness in *brc5* culm with that of WT epidermis. The lateral walls of epidermal cells become thickened due to deposition of cell wall materials; in contrast, *brc5* epidermal cells did not have such type of deposition (Fig. 3b, f). These cells also presumed to provide mechanical strength to plant body. Parenchymatous cells of *brc5* mutant culm also showed apparently reduced cell wall thickness, although to a lesser extent than that seen in sclerenchymatous tissues. Some parenchymatous cells in *brc5* mutant culm showed irregular shapes in contrast to round WT parenchymatous cells (Fig. 3d, h). In addition to the reduction in the wall thickness, some additional factors such as disturbance of cell wall assembly were also involved and were responsible for irregular parenchymatous cells. These results suggested that plant brittleness and reduction of mechanical strength of *brc5* mutant plants were due to defect in the cell wall thickening and deposition of cell wall materials on cell walls of mechanical tissues.

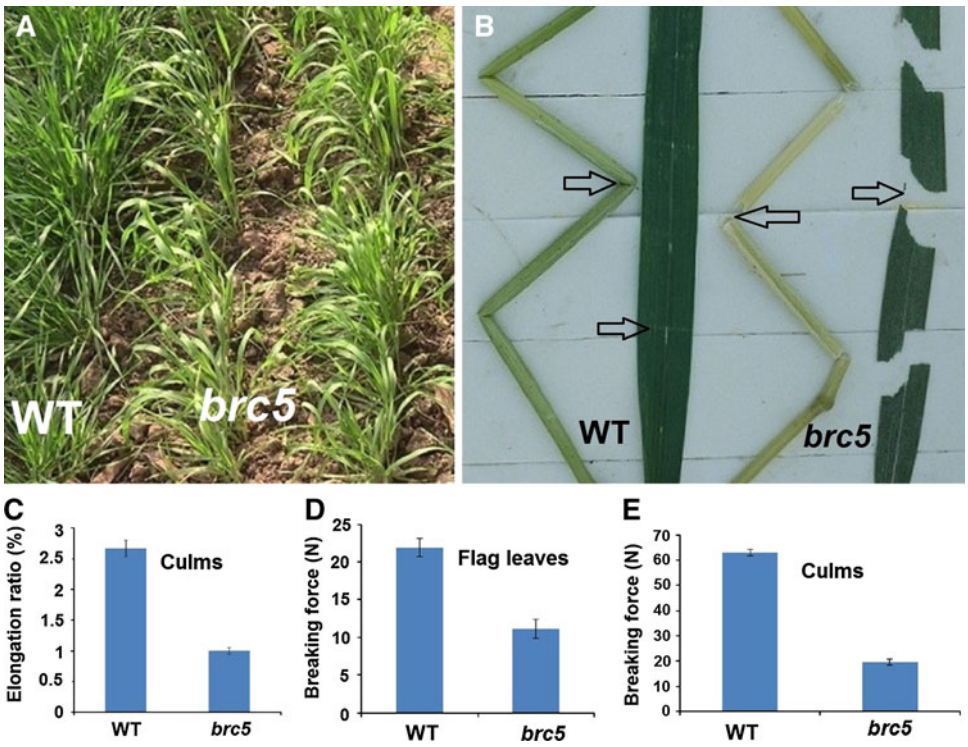

Fig. 1 Phenotypes and physical properties of wild-type (WT) and *brc5* mutant plant. **a** Field view of 45 days old WT plants (*left*) and *brc5* mutant plants (*right*). **b** Culm and flag leaf mechanical strength of WT (*left*) and *brc5* mutant (*right*) plants. Culm and leaves are easily broken in *brc5* mutant plant compare to its WT. **c** The elongation ratio of culm between WT and *brc5* mutant plant. **d** The force needed to break the flag leaf of WT and *brc5* mutant plant. **e** The force needed to break the culm of WT and *brc5* mutant plant

Scanning electron microscopy

The alteration of secondary cell wall in *brc5* mutant plants was further analyzed by SEM, confirming the significant reduction in secondary cell wall thickness in the mechanical tissues of *brc5* mutant. In WT plants, sclerenchymatous tissues present below the epidermis provide the main mechanical strength to plant body. SEM study revealed that cell walls of sclerenchymatous tissues become thickened and cell cavity completely filled by cell wall materials (Fig. 4a, b). In contrast, cell walls of sclerenchymatous tissues in *brc5* were much thinner and become hollow, without the deposition of cell wall materials. It indicated the pleiotropic effect of *brc5* locus which not only affects the synthesis of cell wall materials but also play a significant role in deposition of cell wall materials in mechanical tissues including xylem vessels, which provides the mechanical strength to plant body (Fig. 4c, d).

brc5 mutant exhibit alterations in chemical composition of cell walls

To determine whether the cellular morphology and the reduced mechanical strength in the *brc5* mutant plants resulted from alterations in cell wall biosynthesis materials, we analyzed cell wall materials of flag leaf and culm second internodes of mature plants. Because α-cellulose is the major component of plant secondary cell walls, we compared the α-cellulose of *brc5* mutant and WT. As shown in Table 2, the amount of α-cellulose in the flag leaf and in the culm cell walls of *brc5* mutant reduced to ~49 and ~45 %, respectively, in comparison to the WT, suggesting that *brc5* mutation might have directly or indirectly played an important role in cellulose biosynthesis. After cellulose, lignin also contributes to mechanical strength in some cells, therefore, the Klason lignin content also determined and found to increase by ~34 and ~22, respectively, in flag leaf and culm cell walls in comparison to the WT (Table 2), indicating that plants have the mechanism to balance cellulose and lignin contents in plant tissues to control mechanical strength. At the same time, *brc5* also had increased hemicellulose, ash content, silica and silicates and extractives (Table 2).

We further compared the monosaccharide compositions between *brc5* mutant and WT plants; the alcohol-insoluble residues (AIR) were prepared from the flag leaf and second internodes of *brc5* and WT plants, and alditol derivatives were analyzed by GC–MS for total sugar composition of cell walls. Glucose contents was reduced significantly in *brc5* flag leaf and culms (Table 3), consistent with the results obtained from α-cellulose assays. No significant differences were observed in the non-cellulosic cell-wall monosaccharides between WT and *brc5* mutant plants (Table 3). Slight variations were observed in the levels of D-xylose with higher proportion present in flag leaf and culm of *brc5* mutants. Because glucose and xylose are the

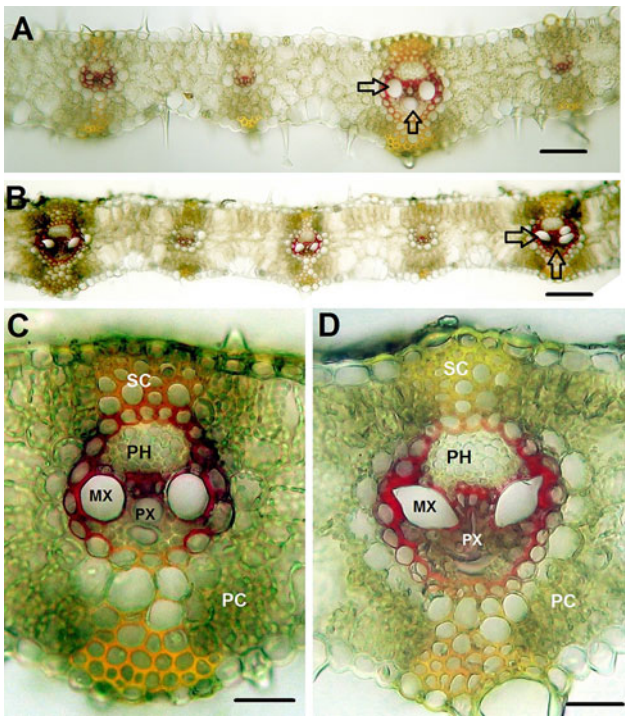

Fig. 2 Comparison of tissue morphology in flag leaf using phloroglucinol–HCl (lignin-specific) staining. Transverse sections of the flag leaf revealed collapsed protoxylem vessels and metaxylem vessels in leaf veins vascular bundles of *brc5* mutant plant compared to that of WT (*arrows*). **a** Transverse section of WT flag leaf (×20). **b** Transverse section of *brc5* mutant plant flag leaf (×20). **c** Cross-section of the WT flag leaf vascular bundle showing normal xylem vessels structure and development (×40). **d** Cross-section of the *brc5* flag leaf vascular bundle showing collapsed metaxylem vessels and protoxylem vessels (×40). *SC* sclerenchyma cells, *MX* metaxylem, *PX* protoxylem, *PH* phloem, *PC* parenchyma

two major monosaccharide's constituents of cellulose and hemicellulose, respectively, in the secondary cell walls, it is very likely that *brc5* mutation affects the secondary cell wall formation, which also supported by histochemical study (Fig. 3).

Comparison of FTIR spectra

To visualize the main spectral differences between WT and *brc5* culm second internode, averages of spectra from all three experiments were calculated and offset-corrected (Fig. 5). Results from the comparison of the FTIR spectra of WT and *brc5* culm showed that there were some differences in the wave number, shape, and the number of absorption peaks of their spectra within the same range of wave number. The spectra of WT and *brc5* mutant plants exhibited remarkable differences. Comparison of the WT spectrum with the *brc5* spectrum showed cellulose-specific peaks at 1,515, 1,465, 1,325, 1,235, 1,315, 1,155, 935, and

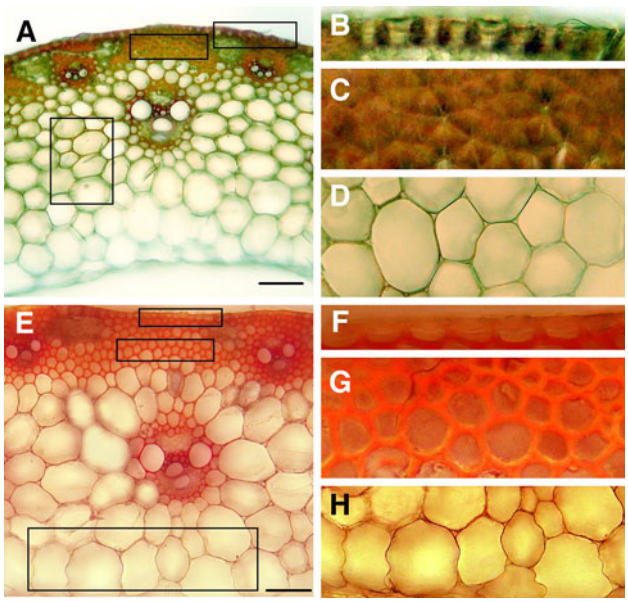

Fig. 3 Comparison of tissue morphology in culms using phloroglucinol–HCl (lignin-specific) staining. Transverse sections of the culm second internode of WT (**a**) and *brc5* (**b**) mutant plant. The regions marked by *lines* in **a** and **e** are enlarged in **b**, **c** and **d** and **f**, **g** and **h**, respectively. **b**, **f** Epidermal cells exhibit thick secondary cell wall thickening on the lateral cell walls in WT and absent in epidermal cells of *brc5* mutant plant. **c**, **g** Cortical fibers (sclerenchyma) secondary cell wall become thin in *brc5* mutant plant as compared to WT. **d**, **h** The *brc5* mutant plant parenchymatous cells show a thin cell wall with abnormal cell shape as compared to WT

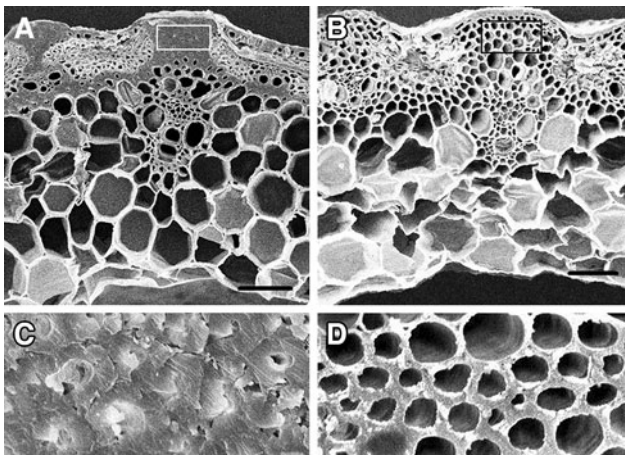

Fig. 4 Scanning electron microscopy micrographs of the transverse section of second internode revealed reduced thickness of the secondary cell wall of sclerenchymatous cortical fibers in *brc5* mutant plant compared to WT. **a** Cross-section of culm second internode of WT. **b** Cross-section of culm second internode of *brc5* mutant plant. **c**, **d** Sclerenchymatous cortical fibers of *T. monococcum*, and *brc5* magnified from the marker area of the TS from the *upper row*. *Scale bar* 25 μm

836 cm^{-1} (Liu et al. 2005). Some transmittance at these cellulose-specific bands was also observed in the *brc5* spectrum. However, these cellulose-specific peaks were not

Table 2 Polysaccharides and other residue composition of flag leaf and culm intermodal cell walls between of wild-type and *brc5* plant (mg/g of dry matter)

Sample	α-Cellulose	Hemi cellulose	Klason lignin	Ash	Silica and silicates	Extractives
WT (flag leaf)	306 ± 4.8*	250 ± 4.7*	160 ± 4.6*	122 ± 5.7*	52 ± 2.4*	57 ± 3.3*
brc5 (flag leaf)	158 ± 3.6*	262 ± 5.4*	214 ± 7.6*	144 ± 6.2*	61 ± 3.2*	69 ± 2.7*
WT (culm)	387 ± 6.2*	273 ± 7.6*	175 ± 3.6*	82 ± 3.6*	47 ± 2.8*	52 ± 3.0*
brc5 (culm)	213 ± 5.8*	291 ± 4.8*	233 ± 6.4*	124 ± 4.7*	54 ± 2.9*	61 ± 2.8*

Each wall component was calculated as milligram per gram of cell wall residue

* Significantly different (*t* test at $P < 0.01$) when compared with wild-type ($n = 3$) ± SE

Table 3 Monosaccharide compositional analysis of cell wall residues of flag leaf and mature culms between of wild-type and *brc5* plant (mg/g of dry matter)

Sample	D-Glucose (D-Glu)	L-Arabinose (L-Ara)	D-Xylose (D-Xyl)	D-Mannose (D-Man)	D-Galactose (D-Gal)	Galacturonic acid (GalUA)	Glucuronic acid (GlcUA)
WT (flag leaf)	338 ± 7.4*	36 ± 1.3*	223 ± 7.8*	3.8 ± 0.2	10.8 ± 0.4*	19.8 ± 0.5*	5.6 ± 0.3
brc5 (flag leaf)	198 ± 4.7*	39 ± 1.8*	281 ± 1.6*	3.9 ± 0.2	14.8 ± 1.2*	23.8 ± 0.6*	6.1 ± 0.2
WT (culm)	394 ± 9.2*	27 ± 0.8*	229 ± 9.0*	1.9 ± 0.0	5.9 ± 0.2*	21.6 ± 0.6*	3.2 ± 0.0
brc5 (culm)	254 ± 6.2*	33 ± 0.9*	272 ± 1.3*	1.8 ± 0.0	6.8 ± 0.2*	24.2 ± 0.4*	3.3 ± 0.1

Cell wall monosaccharide residues prepared from the second internode of the wild-type and *brc5* mutant plant at maturity were used for compositional analysis. Each wall component was calculated as milligram per gram of alcohol-insoluble cell wall residue

* Significantly different (*t* test at $P < 0.01$) when compared with wild type ($n = 3$) ± SE

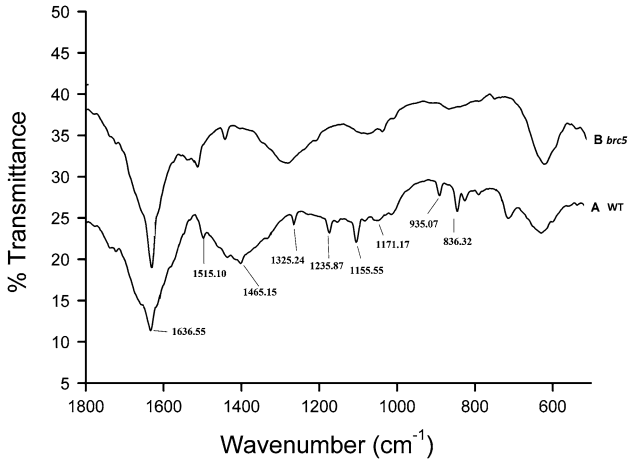

Fig. 5 FTIR spectra of cell walls from cortical vascular bundles of WT culm second internode and *brc5* mutant plant

as clearly discernible in *brc5* culm, apart from the peaks at 1,515 and 1,465 cm^{-1}. At the highest cellulose peak at 1,155 cm^{-1}, the transmittance in WT was about 45 % higher than in *brc5* (Fig. 5). The major differences of spectra in this region might result from the differences in cell wall composition of WT and *brc5* mutants.

Inheritance and mapping of the *brc5* mutant

To examine the inheritance pattern of the brittle culm phenotype, a F_2 mapping population was developed by crossing the brittle culm mutant (*brc5*) with *T. monococcum* acc. pau14087. The F_1 plants obtained from the cross of *T. monococcum* × *brc5* mutant was like the wild-type parent, indicating that the mutant was recessive. For genotyping of *brc5* mutant, a F_2 mapping population of 180 plants was developed by crossing the *brc5* mutant with *T. boeoticum* acc. pau5088, a non-brittle and tall wild diploid progenitor of *T. monococcum*. Out of 180 F_2 plants, 132 plants were WT and 48 had brittle culm due to cellulose deficiency in secondary cell walls. Chi-square value (0.072) gave a good fit ($P \leq 0.05$) to 3:1 ratio indicating that the *brc5* mutant was monogenic recessive. A set of 129 anchored polymorphic SSR markers from molecular linkage map of diploid A genome wheat developed using RIL population of the cross between *T. monococcum* and *T. boeoticum* (Singh et al. 2007) were used for bulk segregant analysis (BSA). The marker *Xcfd39* located on chromosome $5A^mL$ showed polymorphism between the positive and negative bulk of brittle culm mutant (Fig. 6a). Debulking of positive bulk plants also showed close linkage of the brittle culm mutant

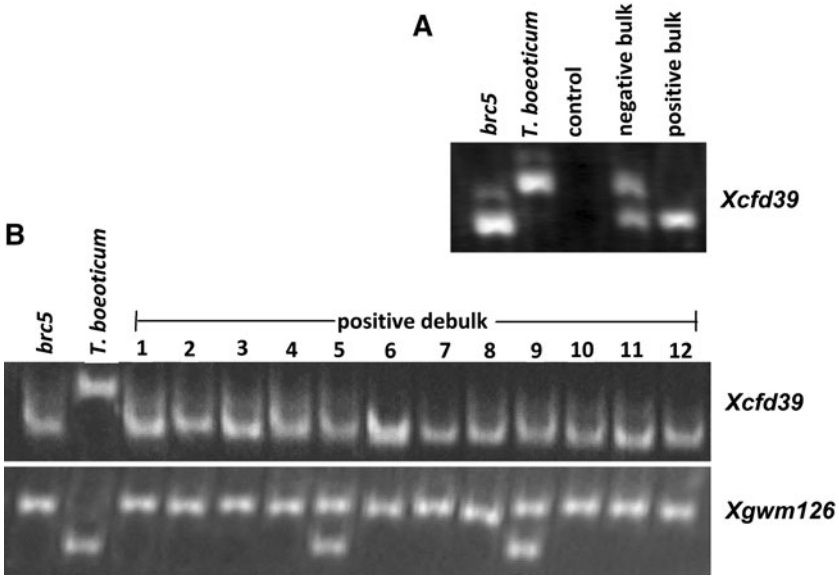

Fig. 6 Identification of putative SSR marker *Xcfd39* linked with *brc5* mutant through bulk segregant analysis. **a** *Lane 1 brc5*, *2 T. boeoticum*, *3* control without DNA template, *4* negative bulk, *5* positive bulk of homozygous *brc5* plants; **b** PCR amplification of debulks of positive bulks (*1–12*) of F_2 plants using putatively linked marker Xcfd39 identified in BSA and its closely linked marker *Xgwm126*; *Lane 1 brc5*, *2 T. boeoticum* (Singh et al. 2007)

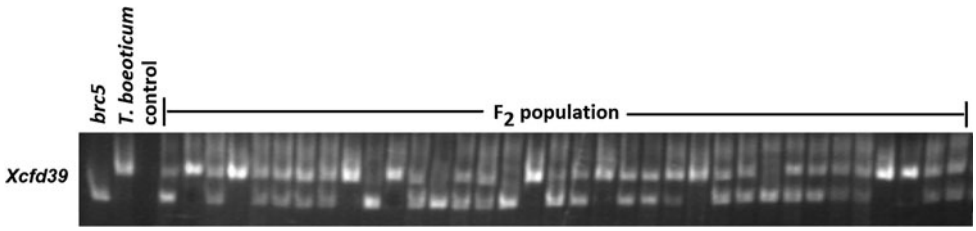

Fig. 7 The segregation of SSR marker *Xcfd39* in representative 35 F_2 plants of *brc5* × *T. boeoticum* cross. *Lane 1 brc5*, *2 T. boeoticum*, *3* control, *4–39* portion of the F_2 plants

with markers *Xcfd39* as none of the 12 plants had its WT allele followed by *Xgwm126* with 2/12 plants with its WT (Fig. 6b). To map the brittle culm mutant, F_2 mapping population developed from the cross of the mutant with *T. boeoticum* acc. pau5088, a non-brittle and tall wild progenitor of *T. monococcum*, was used. A total of 180 individual F_2 plants were phenotyped for brittle culm and genotyped for the putatively linked markers (Fig. 7). Based on the recombination frequency between the mutant and the markers *Xcfd39*, *Xgwm126* and *Xgwm6*, the *brc5* mutant was mapped on chromosome $5A^mL$ at 2.6, 4.8 and 10.4 cM from the markers, respectively (Fig. 8).

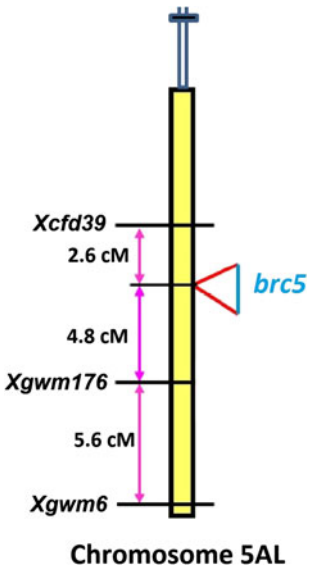

Fig. 8 Mapping of *brc5* mutant on chromosome 5AL of *T. monococcum*

Discussion

Several important genes and QTLs have been mapped on 'A' genome of *T. boeoticum*/*T. monococcum* and transferred to 'A' genome of hexaploid wheat cultivars (Chhuneja et al. 2008; Singh et al. 2010). EMS mutagenesis in *T. monococcum* can give a wide spectrum of mutants with distinct phenotypes (Dhaliwal et al. 1987) which can be used for mapping and gene cloning (Kuraparthy et al. 2007; Sood et al. 2009; Yan et al. 2003, Ansari et al. 2012, 2013). With the availability of advanced genomic resources and International Wheat Genome Sequencing Consortium (http://www.wheatgenome.org/news.php), importance and demand

for these mutants will further increase in wheat as the valuable tools in reverse genetics analysis to identify novel gene function and expression.

Mechanical strength is an important agronomic trait in wheat production. Mechanical failure of the stem tissues is a cause for crop lodging, which results in significant yield losses, particularly in cereals (Zuber 1973). Secondary cell wall of mechanical tissues is the main determinant of mechanical strength of plant tissues (Appenzeller et al. 2004). During the formation of secondary cell wall, three main polymers, namely cellulose, hemicellulose and lignin, synthesized and deposited to make secondary cell walls through a number of complex metabolic pathways. Assembly of these cell wall polymers should be strictly regulated during cell wall formation to form highly ordered cell wall structure (Carpita et al. 2001; Brown et al. 2005). However, the mechanisms of regulation of secondary cell wall formation remain largely unknown. So far, many brittle mutants with reduced mechanical strength have been isolated in *Arabidopsis*, wheat, barley, maize, rice and rye (Brown et al. 2011; Ansari et al. 2012; Kokubo et al. 1991; Ching et al. 2006; Wu et al. 2012; Kubicka and Kubicki 1988). Biochemical and molecular characterization of these mutants showed that the brittle culm phenotype was due to defect in cellulose biosynthesis pathway.

In the present study, an EMS-induced brittle culm mutant (*brc5*) of diploid wheat *T. monococcum* acc. pau14087 was used for phenotypic and molecular characterization. Mechanical strength of *brc5* mutant leaf and culm was significantly reduced and exhibiting reduced cellulose contents with collapsed and irregular xylem vessels phenotype in leaf veins VBs. Histological and SEM observations of the flag leaf and culm second internodes revealed reduced cell wall thickness of mechanical or sclerenchymatous tissues (Figs. 3, 4). Cell wall thickness was also affected in epidermal and parenchymatous tissues. It was expected that any change in the cell wall composition should be detected by analyzing the chemical composition of cell walls, as demonstrated in *fp2*, *irx* and *bc15* mutants (Xu et al. 2008; Brown et al. 2011; Wu et al. 2012). In *brc5* mutant, α-cellulose of secondary cell wall in flag leaf and culm was reduced by ~49 and ~45 %, respectively, in comparison to the WT, *T. monococcum* with slight increase in lignin, hemicellulose, silica and silicates, and ash contents (Table 2). GC–MS study of cell wall monosaccharides revealed that glucose content decrease and D-xylose contents increase significantly in *brc5*. Because glucose and xylose are the two major monosaccharide's constituents of cellulose and hemicellulose, respectively, in the secondary cell walls, it is very likely that *brc5* mutation affects the secondary cell wall formation, which also supported by histochemical study (Fig. 3). Comprehensive mechanical strength, histological, biochemical, SEM, and FTIR analyses of culms and leaves of *brc5* mutant supplemented and complemented the findings that the mutant had defective cellulose synthesis and deposition on secondary cell walls of mechanical tissues and xylem vessels of leaf vascular bundles. *brc5* was found to be controlled by a single recessive gene. Such mutants have not been reported in polyploids wheat as the orthologous loci on their others genomes suppress the newly induced recessive mutations unless and until multiple mutations are induced at all the loci or certain loci have been silenced during evolution of polyploid wheat (Chen et al. 2012).

The plant cell wall is the major component of mechanical strength of cells, tissues, organs, and the entire plant body. Sclerenchyma cells having both primary cell walls and thick secondary cell walls provide major mechanical strength to non-elongating regions of the plant body (Carpita and McCann, 2000). Three mechanical strength defective mutants (*brc1*, *brc2* and *brc3*) have been identified and characterized in *T. monococcum* during our previous study (Ansari et al. 2012). The *brc5* mutant exhibits similar phenotypic characteristics as reported in previously characterized brittle culm mutants in *T. monococcum* like drooping leaf phenotype, semidwarf habit with shorter stem, root length, spreading growth habit, lower tiller numbers, as compared to WT parent *T. monococcum* (Fig. 1a) and reduced levels of cellulose in the secondary cell walls of mechanical tissues. This indicated that *brc5* mutation not only affects the cellulose content but also has a pleiotropic effect on leaf morphology, tiller numbers, plant height, and other morphological characters (Table 1). Similar mutants were also obtained in rice and *Arabidopsis* (Xu et al. 2008; Turner and Somerville 1997; Hirano et al. 2010; Li et al. 2003).

The barley brittle culm mutant showed reduced mechanical strength due to reduced level of cellulose, indicating a correlation between the cellulose content and plant mechanical strength (Kokubo et al. 1989, 1991). The rice classic mutant *bc1* has defect in cellulose biosynthesis, leading to the reduction of secondary cell wall thickness and also has significant increase in hemicellulose, lignin, and xylose sugar contents (Li et al. 2003). The transverse section of flag leaf revealed along with collapsed xylem vessels phenotype in leaf vascular bundles in *brc5* mutant. These results were consistent with those of several irregular xylem (*IRX*) mutants that have been reported in *Arabidopsis* (Taylor et al. 1999, 2000, 2003). All these irregular xylem (*IRX*) mutants have defects in their cellulose synthase catalytic subunits (CesAs), and exhibit collapsed xylem caused by a lack of resistance to the negative pressure due to water transport. *IRX7*, *IRX8* and *IRX9*, which encode proteins belonging to glycosyltransferase (GT) families 47, 8 and 43, respectively (Bourne and Henrissat 2001), appear to be required for normal vessel morphology and the

accumulation of xylan and cellulose in inflorescence stems (Zhong et al. 2005; Lee et al. 2007; Peña et al. 2007; Persson et al. 2007). Recently, transcript profiling of the genes expressed during xylem differentiation in *Zinnia* and *Arabidopsis* has led to the identification of a plant-specific family of transcription factors containing a NAC domain which regulate the formation of secondary cell walls. *Arabidopsis vascular-related NAC domain* (*VND*) *6* and *VND7* are transcriptional switches for the differentiation of metaxylem and protoxylem vessels, respectively, which control morphogenetic events, including the formation of secondary cell walls in the tissues (Demura et al. 2002; Kubo et al. 2005).

In the F_2 population between the *brc5* mutant and its WT, WT phenotype and the mutant showed a segregation ratio of 3:1 (non-brittle: brittle plants) indicating that the *brc5* mutant was controlled by a single recessive nuclear gene. Such mutants probably could not have been recovered in polyploid wheat because of the orthologous loci on other genomes, unless multiple mutants were induced at all the loci or certain loci were silenced during evolution. Our previous studies on the three brittle culm mutants (*brc1*, *brc2* and *brc3*) in *T. monococcum* have shown that deficiency in cellulose biosynthesis results in little deposition of cellulose on the secondary cell wall of sclerenchymatous tissues (Ansari et al. 2012). These mutants showed cellulose reduction up to 57 % in secondary cell walls compared to that of WT with slight increase in lignin and other non-cellulosic cell wall materials. Consistent with the findings in *brc1*, *brc2* and *brc3* mutants, cellulose amount of *brc5* mutant was reduced to around ~49 and ~45 % in flag leaves and culms, respectively. The *brc5* mutant has been mapped to chromosome $5A^m$ L of diploid wheat, *T. monococcum*, which is different to the findings previously reported by us for brittle culm mutants (*brc1*, *brc2* and *brc3*) in *T. monococcum*, on $1A^m$ L, $3A^m$ L, and $6A^m$ S respectively (Ansari et al. 2012). This provides another evidence that there are multiples genes in diploid wheat for secondary cell wall formation and cellulose biosynthesis and deposition like that of *Arabidopsis* and rice (Sado et al. 2009), as *brc5* mapped on $5A^m$ L hence no orthologous to those.

Mechanical strength is one of the most important agronomic traits that affect not only harvest index and grain production but also the usefulness of cereal straws as animal forage. As an important locus regulating wheat cellulose synthesis, knowledge of *brc5* (and its orthologs in other cereals) could make a significant contribution to the future improvement of wheat for enhanced mechanical strength and reduced lodging. It will be desirable to over express the WT *brc5* gene to get higher cellulose synthesis for stronger straw and higher cellulose/lignin ratio for commercial exploitation in biofuel and pulp and paper industry.

Author contribution M. J. Ansari and H. S. Dhaliwal designed and instructed the research work and were also involved in paper preparing. M. J. Ansari, S. Usmani and R. Kumar performed experiments. A. Al-Ghamdi and A. Nuru was responsible for data analysis and result interpretation. K. Singh contributed to performed BSA and gene mapping experiments and result interpretation. M. J. Ansari and A. Nuru contributed to performed histological studies. All the authors were responsible for writing the paper.

Acknowledgments The authors are grateful to Deanship of Scientific Research and College of Food and Agriculture Science Research, King Saud University Riyadh for providing research support.

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