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RESEARCH ARTICLE

Expression of *HvCslF9* and *HvCslF6* barley genes in the genetic background of wheat and their influence on the wheat β-glucan content

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Abstract

The dietary fibre (1,3;1,4)-β-D-glucan (β-glucan), is a major quality parameter of cereals. Grain β-glucan content is the most important factor from the aspect of human health maintenance. The grain of barley (*Hordeum vulgare*) is one of the most important β-glucan sources having a β-glucan content 10 times higher than that of wheat (*Triticum aestivum*). Winter wheat/winter barley 'Mv9kr1'/'Igri' 1HS ditelosomic and 'Mv9kr1'/'Igri' 7H disomic addition lines carrying the *HvCslF9* and *HvCslF6* barley genes, respectively, were used to investigate the additive effect of barley cellulose synthase-like genes on the wheat β-glucan content. A significantly higher β-glucan level was detected in the leaves and grains of the wheat/barley 1HS and 7H addition lines compared to the control wheat line. The expression of the *HvCslF9* and *HvCslF6* genes in the genetic background of wheat was also determined by quantitative RT-PCR. The expression pattern of the *HvCslF9* gene transcript showed a gradual increase throughout grain development, while the *HvCslF6* gene was normally transcribed at relatively high levels. In leaves, the transcript of the *HvCslF9* gene could not be detected at the end of tillering, while the *HvCslF6* gene was still strongly expressed at this time in the 7H addition line. This study mapped the *HvCslF9* gene to the short arm of the 1H chromosome. The *HvGlb1* barley gene, encoding (1,3;1,4)-β-D-glucan endohydrolase isoenzyme EI, is possibly involved in the regulation of the β-glucan level during grain development. Previously this was also mapped to the barley 1H chromosome, and this study suggested that it was located on the 1HL chromosome arm. It was also concluded that the independent expression of the *HvCslF9* gene in the wheat background resulted in a slight increase in the β-glucan content. The results provide new insights into the expression and regulation of the *HvCslF* genes in the genetic background of wheat and indicate that cisgenesis can be used to increase the leaf and grain β-glucan content in wheat.

Introduction

Interspecific and intergeneric hybridization provide an opportunity to transfer agronomically useful traits from related species into hexaploid wheat (*Triticum aestivum* L., 2n = 6x = 42, AABBDD). One related species that has been considered is barley (*Hordeum vulgare* L., 2n = 2x = 14, HH), as it carries genes for abiotic stress tolerance and good nutritional parameters.

Nearly 80% of cultivated bread wheat is used in the human diet and provides a substantial portion of our dietary fibre consumption, which is generally recognised as providing health benefits (Quraishi *et al*., 2011). The non-starch cell-wall polysaccharides of cereal grains comprise cellulose, (1,3;1,4)-β-D-glucan (β-glucan) and arabinoxylans as major components (Taketa *et al*., 2012). The endosperm cell walls of barley, Brachypodium and oat are rich in β-glucan, in contrast to wheat grains,

which accumulate much lower amounts. Gebruers *et al*. (2008) reported that the mean β-glucan content in the wholemeal of the 150 HEALTHGRAIN wheat lines ranged from 0.5% to 0.9%, indicating very low genetic variability for increasing the β-glucan content with conventional breeding techniques.

Barley β-glucans are beneficial to human health, as they are a major source of soluble dietary fibres and have been recognised both as potential cholesterol-lowering polysaccharides (Kerckhoffs *et al*., 2003; Shelat *et al*., 2011) and as non-specific immune-activators (Allendorf *et al*., 2005). Dietary fibres such as β-glucan can play an important role in preventing diseases such as type2 diabetes and cardiovascular disorders (Brownlee, 2011). On the other hand, they have undesirable effects in cereal processing applications such as malting and brewing (Brennan & Cleary, 2005).

Cellulose synthase-like (*Csl*) genes are candidates to encode the enzymes that synthesise the backbone of various non-cellulosic linked cell wall polysaccharides (Doblin *et al*., 2009). They have been classified into nine gene families, designated *CslA* to *CslJ*, of which the *CslF*, *CslH* and *CslJ* families are restricted to cereals, though the *CslJ* group is not found in rice (*Oryza sativa* L.) or *Brachypodium distachyon* L. (Doblin *et al*., 2010).

Experiments using transgenic *Arabidopsis thaliana* L. plants revealed that the *CslF* and *CslH* families are probably involved in β-glucan synthesis. The expression of rice *CslF* genes in Arabidopsis, which has no *CslF* genes and no (1,3;1,4)-β-D-glucan in its cell walls, led to the appearance of (1,3;1,4)-β-D-glucan in the leaf epidermal walls (Burton *et al*., 2006). Similar results were obtained when the barley *CslH1* gene was expressed in Arabidopsis, but in this case the transcript level remained very low and was restricted to cells having either mature primary walls or secondary wall thickening, suggesting that *CslH* is involved in β-glucan production during secondary wall synthesis (Doblin *et al*., 2009).

The cellulose synthase-like F gene subfamily of barley was analysed by Burton *et al*. (2008) and seven *HvCslF* genes (*HvCslF3*, *HvCslF4*, *HvCslF6*, *HvCslF7*, *HvCslF8*, *HvCslF9* and *HvCslF10*) were isolated, sequenced and placed on a genetic map. Among the *HvCslF* genes, *HvCslF6* and *HvCslF9* were found to have the highest levels of mRNA transcripts in developing barley endosperms (Burton *et al*., 2008). The *HvCslF9* and *HvCslF6* genes were mapped to loci near the centromeres of chromosomes 1H and 7H, respectively, which were close to quantitative trait loci (QTLs) for barley grain β-glucan content (Burton *et al*., 2008; Igartua *et al*., 2002; Molina-Cano *et al*., 2007). Using betaglucanless mutants Taketa *et al*. (2012) demonstrated that, among the seven *CslF* and one *CslH* genes present in the barley genome, the *HvCslF6* gene had a unique role and was the key determinant controlling the biosynthesis of β-glucan. All these findings, together with the recent characterisation of genes encoding putative β-glucan synthase in wheat (*TaCslF6*) (Nemeth *et al*., 2010), provide an opportunity to increase β-glucan synthesis in wheat by trans- and cisgenesis (Zhao & Shewry, 2011).

A major QTL for the β-glucan content in barley grain has been reported across a broad centromeric region of barley chromosome 1H, which cover both the *HvCslF9* and *HvGlb1* locus (Woodward & Fincher, 1982; Slakeski *et al*., 1990; Han *et al*., 1995). This suggested that the expression of *HvGlb1* gene, encoding one of the barley (1,3;1,4)-β-D-glucan endohydrolase isoenzymes, might also affect the β-glucan level during the grain development (Burton *et al*., 2008).

The expression of barley genes in wheat was confirmed using wheat/barley addition lines (Islam & Shepherd, 1990; Cho *et al*., 2006). Addition lines developed by crossing wheat and alien species are excellent genetic resources to study the effect and expression of alien genes in the genetic background of wheat. Moreover, these disomic and ditelosomic additions facilitate the definition of the physical position of genes and DNA markers within particular chromosomal regions (Bilgic *et al*., 2007).

The aim of this study was to investigate the effect of the *HvCslF6* and *HvCslF9* barley genes on the β-glucan content of wheat leaves and grain with the help of the winter wheat/winter barley 7H disomic- and 1HS ditelosomic addition lines, respectively. In addition, the position of the *HvCslF9* and *HvGlb1* genes was pinpointed to 1HS and 1HL, respectively. Increases in β-glucan content were detected both in the leaves and grains of the wheat/barley 1HS and 7H addition lines compared to the control wheat, but different levels were observed for the different tissues and addition lines. The expression of the *HvCslF9* and *HvCslF6* genes in the wheat genetic background was also determined by quantitative RT-PCR and the results were compared to the β-glucan levels.

Materials and methods

Plant materials

The 1HS ditelosomic and 7H disomic wheat/barley addition lines were developed from the 'Mv9kr1' × 'Igri' wheat/barley hybrid produced in Martonvásár (Molnár-Láng *et al*., 2000, 2007; Szakács & Molnár-Láng, 2010).

The additions lines, the parental wheat and barley genotypes were grown in a greenhouse in Martonvásár. Vernalisation was carried out at 4°C for 6 weeks, after which the vernalised plants were grown in 2 L pots filled with a 2:1:1 mixture of garden soil, humus and sand. The plants were grown until tillering under an initial regime

of 15°C day : 10°C night temperature, 12 h light : 12 h dark photoperiod (Tischner *et al.*, 1997). The temperature was raised by increments of 2°C after tillering (day length 14 h), stem elongation (15 h illumination), flowering and 2 weeks after fertilisation.

Leaves and finely ground grains of 10 plants each of the wheat line 'Mv9kr1', the barley cultivar 'Igri', the 1HS ditelosomic addition line and the 7H disomic addition line were used for β-glucan analysis.

In situ hybridisation

Root tips of germinating seeds of the wheat/barley addition lines (1HS, 7H) were used for mitotic chromosome preparation as described by Jiang *et al.* (1994).

The genomic *in situ* hybridization (GISH) experiment was carried out as described earlier by Cseh *et al.* (2011). Barley total genomic DNA was labelled with digoxigenin-11-dUTP (Roche) with a nick translation mix and used as a probe. Unlabelled wheat genomic DNA was used as blocking DNA at a ratio of 30:1.

The slides were screened using a Zeiss Axioskop-2 fluorescence microscope equipped with filter sets appropriate for 4′,6-diamidino-2-phenylindole (DAPI) (filter set 1) and Rhodamin (filter set 15). Images were captured with a Spot Charge-Coupled Device (CCD) camera (Diagnostic Instruments, Sterling Heights, MI, USA) and processed with IMAGE PRO PLUS software (Media Cybernetics, Rockville, MD, USA).

DNA extraction, PCR amplification

Genomic DNA was extracted from fresh young leaves of wheat line Mv9kr1, barley cultivar 'Igri' (B) and the 'Mv9kr1'/'Igri' 1HS and 7H disomic addition lines using Quick Gene-Mini80 (FujiFilm, Osaka, Japan) with a QuickGene DNA tissue kit (FujiFilm) according to the manufacturer's instructions.

The following set of barley chromosome arm-specific SSR markers and gene-specific STS markers were selected: Bmac0213-1HS, Bmag0345-1HL (Ramsay *et al.*, 2000), HvCslF9 (Burton *et al.*, 2008), HvGlb1 P5 (Jin *et al.*, 2011), Bmag0021-7HS, HvID-7HL (Ramsay *et al.*, 2000) and HvCslF6-7HL (Burton *et al.*, 2008; Cseh *et al.*, 2011). The primer sequences and annealing temperatures used in this study are presented in Table S1. The PCR amplification was performed under the conditions described by Cseh *et al.* (2011). All the primer pairs were tested on the DNA templates of the parental wheat and barley and on the addition lines. A negative control was used when running PCR with each primer pair. The PCR products were separated and analysed using a Fragment Analyzer™ automated capillary electrophoresis system (Advanced Analytical Technologies, Ames, IA, USA).

RNA extraction and quantitative RT-PCR

RNA was extracted from leaves of the 'Mv9kr1'/'Igri' 1HS and 7H addition lines at the end of tillering (ET) and 6 days after pollination (6 DAP). Leaflet samples were harvested from 10 plants of each genotype and RNA isolation was performed in two independent biological replicates.

Spikes were hand pollinated and bagged at anthesis, and the developing caryopses were collected at 6, 10, 14, 18, 22 and 26 DAP. Grain were collected from at least six different spikes and combined before RNA extraction in two independent biological replicates. Before RNA isolation the embryos were cut from the grain with a scalpel blade.

The RNA isolation procedure was carried out using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and the RNA was cleaned up with an RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands). The RNA samples were digested with DNase I (Qiagen) and reverse-transcribed with a High Capacity RNA-to-cDNA Kit (Life Technologies).

Real-time PCR was performed with an Applied Biosystems Fast 7500 instrument using SYBR Green detection chemistry (QuantiFast SYBR Green PCR Kit, Qiagen), as described by Soós *et al.* (2010). The wheat actin gene (GenBank accession number AB181991) with the primer sequences 5′-GCCACACTGTTCCAATCTATGA-3′ and 5′-TGATGGAATTGTATGTCGCTTC-3′ was used as an internal control (Mackintosh *et al.*, 2007). All the reactions were performed in quadruplicate. The relative ratio of threshold cycle (Ct) values between the endogenous control and the specific genes (*HvCslF9* and *HvCslF6*) was calculated for each sample and the abundance of the transcripts of genes of interest are represented by arbitrary units normalized against the control gene.

Analysis of the (1,3;1,4)-β-D-glucan content of leaves and grain

Leaves were collected from the plants at the ET and 6 DAP stages, frozen overnight at −80°C and freeze-dried using a BenchTop 6K (VirTis, Warminster, PA, USA) instrument. Dry leaves were milled in a Retch M400 ball laboratory mill (Qiagen, Venlo, Netherlands) for β-glucan analysis.

Mature seeds were also collected of the same plants and milled in a Retch M400 ball laboratory mill to produce wholemeal.

Samples of finely ground grains or freeze-dried leaves of the plants (100 mg) were used for mixed-linkage β-glucan analysis in four parallels according to the AACC Method 32-23 (McCleary & Codd, 1991) using a Megazyme kit (Megazyme, Bray, Ireland). The samples were suspended and hydrated in a buffer solution (pH 6.5) and then incubated with purified lichenase enzyme and

filtered. An aliquot of the filtrate was then hydrolysed to completion with purified β-glucosidase. The D-glucose produced was assayed using a glucose oxidase/peroxidase reagent. Chemical data from the β-glucan analysis were evaluated using single-factor analysis with the help of the Microsoft Excel program. Values of $P < 0.05$ were considered to be statistically significant.

Results

Cytogenetic identification

The presence of two telocentric barley chromosomes was confirmed using GISH in 10 plants of the progenies of the 'Mv9kr1'/'Igri' 1HS isochromosome addition line (Fig. S1A, Supporting Information).

The presence of two added barley chromosomes was confirmed using GISH in 10 plants of the 'Mv9kr1'/'Igri' 7H disomic addition line (Fig. S1B).

SSR and STS marker analysis

DNA was isolated from the selected plants. The presence of the 1HS chromosome arm and the absence of the 1HL arm in the 1HS ditelosomic addition line was confirmed with the help of SSR markers specific for 1HS (Bmac0213) and for 1HL (Bmag0345) specific SSR marker (Table S1). The *HvCslF9* gene-specific PCR primers gave PCR products of the expected size when using DNA from the 1HS line as a template (Fig. S2). Moreover, the presence of the HvCslF9 STS marker in the 1HS line confirmed the position of the *HvCslF9* gene on the 1H chromosome and placed it on the short arm. The *HvGlb1* gene was mapped previously to the chromosome 1H (Loi *et al.*, 1988; Burton *et al.*, 2008). Primer pairs (P5) specific to the *HvGlb1* gene gave PCR products on the parental barley cultivar ('Igri') and did not amplify PCR products in the 1HS line, thus locating the position of this gene on the 1HL arm.

Two 7H-specific SSR markers (Bmag0021, HvID) were tested in order to confirm the presence of the 7H chromosome in the progenies of the 7H disomic addition line (Table S1). The 7H-specific SSR primer pairs and the *HvCslF6* gene-specific STS marker gave PCR products of the expected size using DNA from the 7H addition lines as a template (Fig. S2).

Gene expression

In the 'Mv9kr1'/'Igri' 1HS ditelosomic line, the abundance of the *HvCsIF9* gene transcript showed a gradual increase with the onset of grain development (Fig. 1A). Transcripts of the *HvCslF6* gene (Fig. 1B) peaked at 6 DAP. The expression of this gene fluctuated only slightly around a relatively high level in the 'Mv9kr1'/'Igri' 7H addition line and showed no great change compared

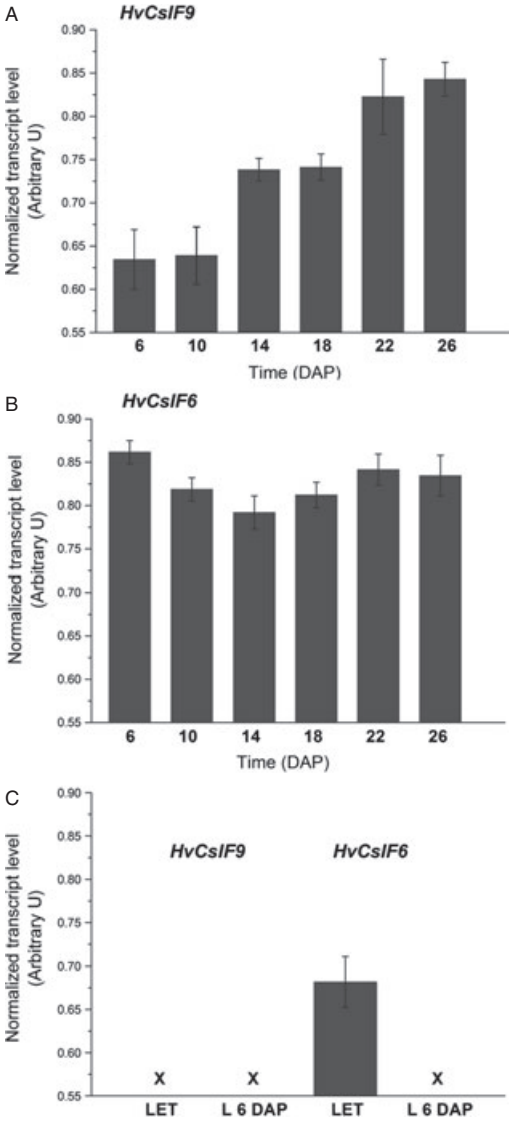

Figure 1 (A) Normalized transcript levels (arbitrary units) of the barley *HvCslF9* gene in developing grain of the 'Mv9kr1'/'Igri' 1HS ditelosomic addition line at 6, 10, 14, 18, 22 and 26 days after pollination (DAP). Real-time PCR data were obtained from two independent experiments with similar results (included three technical replicates); reactions were performed in quadruplicate. Bars on all Q-PCR plots indicate standard deviations. (B) Normalized levels of *HvCslF6* gene transcripts (arbitrary units) in developing grain of the 'Mv9kr1'/'Igri' 7H disomic addition line at various times after pollination. Real-time PCR data were obtained from two independent experiments with similar results (included three technical replicates); reactions were performed in quadruplicate. Bars indicate standard deviations. (C) Normalized levels of *HvCslF9* and *HvCslF6* gene transcripts (arbitrary units) in leaves using leaflet samples (L) of the 'Mv9kr1'/Igri' 1HS ditelosomic addition line and the 'Mv9kr1'/'Igri' 7H disomic addition line (7H) at the end of tillering (ET) and 6 days after pollination (6 DAP). Real-time PCR data were obtained from two independent experiments with similar results (included three technical replicates); reactions were performed in quadruplicate. Bars indicate standard deviations.

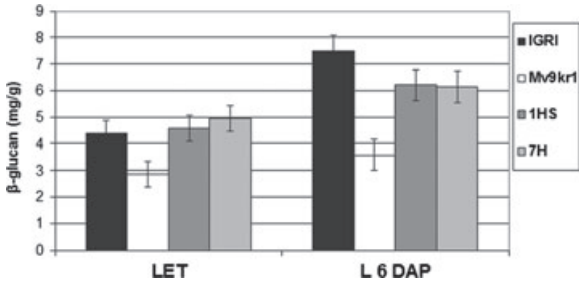

Figure 2 Histogram of the (1,3;1,4)-β-D-glucan content in the leaves (L) of barley ('Igri'), wheat ('Mv9kr1'), the 'Mv9kr1'/'Igri' 1HS ditelosomic addition line (1HS) and the 'Mv9kr1'/'Igri' 7H disomic addition line (7H) at the end of tillering (ET) and 6 days after pollination (6 DAP). Mixed-linkage β-glucan analysis was performed in four parallels according to the AACC Method 32-23. The LSD5% values at ET and 6 DAP, respectively, were 0.48 and 0.58.

to the *HvCsIF9* in the 1HS line. These data suggest that either *HvCslF9* expression is highly regulated by trans acting regulatory elements found in the wheat genome or the barley cis elements are not located on the 1HS chromosome arm. In addition, the relatively high expression and the pattern of the *HvCslF6* gene indicates that *HvCslF6* is not induced in parallel with the onset of grain development, either due to the lack of specific trans acting elements in the 7H chromosome or to the presence of a stronger one in the wheat genome.

The transcript of the *HvCslF9* gene could not be detected in the leaves (ET and 6 DAP) (Fig. 1C). The *HvCslF6* gene was strongly expressed in the leaves of 7H addition lines during tillering (Fig. 1C), but transcripts could not be detected at 6 DAP.

(1,3;1,4)-β-D-Glucan content

The enzymatic analysis of β-glucan content in the leaves of 'Mv9kr1', 'Igri', the 'Mv9kr1'/'Igri' 1HS ditelosomic addition line and the 'Mv9kr1'/'Igri' 7H disomic addition line at the ET stage revealed that the leaves of the 1HS ditelosomic (4.58 mg g^{-1}) and 7H disomic (4.96 mg g^{-1}) addition lines contained significantly higher levels of β-glucan than those of control wheat (2.85 mg g^{-1}) (Fig. 2). Interestingly, at this stage the leaves of both addition lines contained a higher level of mixed-linkage β-glucan than the 'Igri' barley parent (4.40 mg g^{-1}).

At the 6 DAP stage the leaves of barley cultivar 'Igri' showed the highest level of β-glucan (7.49 mg g^{-1}) compared to the 1HS and 7H addition lines (both approximately 6.15 mg g^{-1}) and the control wheat (3.57 mg g^{-1}). The β-glucan content of both addition lines was 1.7-fold higher than that of control wheat.

Barley leaves accumulated the most β-glucan (3.09 mg g^{-1}) during the period between ET and 6 DAP,

Table 1 (1,3;1,4)-β-D-Glucan content in wholegrain samples and standard deviation (SD) of the studied genotypes

Genotype	*n*	Grain; β-Glucan, mg g^{-1} ± SD (d.m.)
Mv9kr1	4	6.43 ± 0.20
Igri	4	49.82 ± 0.56
1HS addition line	4	7.88 ± 0.41
7H addition line	4	10.21 ± 0.40

d.m., dry matter; *n*, number of parallel samples.

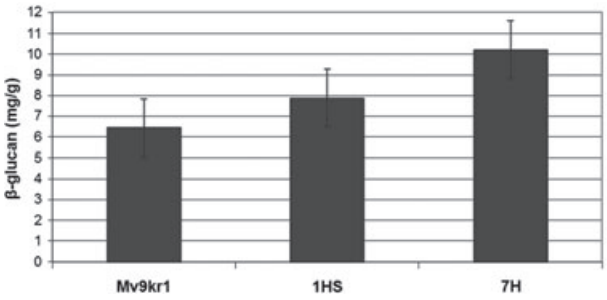

Figure 3 (1,3;1,4)-β-D-Glucan content in the grains of wheat ('Mv9kr1'), the 'Mv9kr1'/'Igri' 1HS ditelosomic addition line (1HS) and the 'Mv9kr1'/'Igri' 7H disomic addition line (7H). There was a statistically significant increase ($P < 0.05$) in (1,3;1,4)-β-D-glucan content in the grain of the 'Mv9kr1' line. Mixed-linkage β-glucan analysis was performed in four parallels according to the AACC Method 32-23. The bars show the LSD5% value: 1.4.

which was approximately twice as high as that detected in the addition lines (Table S2). A recovery of 1.62 mg g^{-1} was detected in the 1HS ditelosomic line, while the 7H addition line showed a rise of 1.18 mg g^{-1}. Both addition lines (1HS, 7H) showed a β-glucan accumulation approx. twice that detected in the control wheat line (0.72 mg g^{-1}).

The β-glucan contents of bulked grain of the studied genotypes are presented in Table 1. The β-glucan level (mg g^{-1} grain) was 6.43 mg g^{-1} in the 'Mv9kr1' wheat line and approximately eight times higher in the barley cultivar (49.82 mg g^{-1}). The 1HS addition line contained 7.88 mg g^{-1} β-glucan and the 7H addition line 10.21 mg g^{-1}. The β-glucan level of the grain was significantly higher in the 1HS and 7H addition lines than in the control wheat (LSD5% = 1.4) (Fig. 3).

Discussion

The *HvCslF6* and *HvCslF9* barley genes were previously reported to play a key role in barley β-glucan biosynthesis, but their expression in wheat and its effect on the grain and leaf β-glucan content remained unknown. This work describes significantly elevated amounts of β-glucan in

the leaves and grain of wheat/barley addition lines which carry the *HvCslF6* and *HvCslF9* barley genes in the wheat background. The data provide additional support for the role of the *CslF6* and *CslF9* genes in β-glucan biosynthesis in cereals and confirm the *CslF6* and *CslF9* genes as functional members of a β-glucan synthase complex.

Previous genetic mapping studies located *HvCslF6* on the centromeric region of the 7H chromosome. Cseh *et al*. (2011) physically mapped the *HvCslF6* gene in the centromeric region of the 7HL chromosome arm by combining *in situ* hybridization and STS and SSR marker analysis on a 4BS/7HL wheat-barley translocation line. Both *HvCslF9* and *HvGlb1* genes were mapped using a 'Clipper' × 'Sahara' population to the centromeric region of 1H (Burton *et al*., 2008). The present work on a 1HS ditelosomic line showed that *HvCslF9* was mapped on 1HS, while *HvGlb1* was positioned to 1HL.

Expression analysis on the *HvCslF6* and *HvCslF9* genes in the leaves of the 7H and 1HS addition lines, respectively, only revealed transcripts for *HvCslF6* at the ET stage, which disappeared by 6 DAP. This was in partial agreement with expression of *HvCslF6* and *HvCslF9* detected in barley by Burton *et al*. (2008), where the transcripts of both genes were observed, but in an earlier developmental stage: in the first leaf base. However, the increased β-glucan content in the 1HS line carrying *HvCslF9* strongly suggests the possibility that mRNA transcripts of *HvCslF9* were present in an earlier stage and disappeared before ET.

An increase in β-glucan content of approximately 70% was detected in the leaves of both addition lines (1HS, 7H) compared to the control wheat at the ET and 6 DAP stages, but barley showed the most rapid increase in the β-glucan level between ET and 6DAP (Table S2). Thus the β-glucan content increased even when the expression of the *HvCslF6* and *HvCslF9* genes was no longer detected. This might support the theory that the products of the *HvCslF6* and *HvCslF9* genes synthesise an intermediary oligoglucoside rather than the final polysaccharide. This alternative, two-phase process was proposed by Burton *et al*. (2010) for the assembly of (1,3;1,4)-β-D-glucans. It was suggested that the CslF and CslH enzymes synthesize (1,4)-β-oligoglucosides in the Golgi and that these are transferred to the plasma membrane via Golgi-derived vesicles. In the second phase (1,4)-β-oligoglucosides are joined through (1,3)-β-linkages by another enzyme, resulting in (1,3;1,4)-β-glucan.

A very high level of β-glucan was also detected in barley leaves when the *HvCslF6* transgene was over-expressed by the constitutive promoter (35S), but in grain the β-glucan level remained similar to the control (Burton *et al*., 2011). In this study the *HvCslF6* and *HvCslF9* genes significantly increased both the leaf and grain β-glucan content in the wheat background.

The transcript abundance for the *HvCslF6* gene was relatively high from 6 DAP to 26 DAP in grains of the 7H addition line. In contrast *HvCslF9* showed a significant gradual increase in mRNA level in grains of the 1HS addition line.

The β-glucan content measured in the leaves of the addition lines approached that of barley. Nevertheless, despite an increase of 60% compared to the control wheat, the β-glucan content in the grains of the 7H addition line only reached 1/5 of that of barley. In the grains of the 1HS line an increase of 20% was detected compared to the control wheat. In order to increase the grain β-glucan level, Burton *et al*. (2011) transformed barley with barley *CslF6* and *CslF9* cDNAs driven by an endosperm-specific oat globulin promoter (ASGLO). The transcript levels greatly increased for both the *CslF6* and *CslF9* transgenic lines, but significant increases (up to 50%) in grain β-glucan content were only found in the case of the CslF6 transgenic lines. At the same time the down-regulation of the wheat *CslF6* gene led to a lower level of (1,3;1,4)-β-D-glucan in wheat endosperms (Nemeth *et al*., 2010). These findings, together with the present results indicate that the cellulose synthase-like F gene subfamily is not responsible alone for the entire β-glucan synthesis in the developing grain, but that other genes, transcription factors or regulatory mechanisms might also participate in the process.

Coexpression analysis in barley revealed a very strong correlation between the *CesA* transcripts that have been assigned to primary cell wall cellulose synthesis in barley and the transcript abundance of the *CslF6* gene (Burton & Fincher, 2009). It was shown that natural siRNAs derived from the CesA/Csl cellulose synthase superfamily could also attenuate non-target RNAs, including the *HvCslF6* and *HvCslH* genes, implying that their expression could be affected in diverse ways, including silencing by the recipient genome (Held *et al*., 2008). The presence of natural siRNAs derived from the wheat genome and their silencing effect on the *HvCslF6* and *HvCslF9* genes could explain the observation that the β-glucan content in the 1HS and 7H additions was never the same as that of barley.

It is thought that in barley the *HvCslF9* and *HvGlb1* genes jointly affect β-glucan synthesis during grain development. In the 1HS line the effect of the *HvCslF9* gene could be studied independently of the expression of *HvGlb1* as the latter is located on the 1HL arm. The *HvCslF9* gene increased the β-glucan level by 20% compared to the control wheat. Their joint effect could only be investigated using transgenic methods, as 1HL and 1H addition lines cannot be developed due to the sterility effect of 1HL.

This study confirmed the fact that, within the cellulose synthase-like F gene subfamily, the C*slF6* gene has the greater influence on β-glucan synthesis. The *HvCslF6* and *HvCslF9* barley genes were found to be effective in increasing the β-glucan level in the wheat genetic background and are of potential relevance for the manipulation of wheat β-glucan levels. Both genes caused a considerable increase in the β-glucan content of the leaves, but further efforts will be needed to achieve an increase in the β-glucan content of the grain. The testing of the two-phase assembly hypothesis and the identification of further enzymes, transcription factors and other regulation elements with a role in β-glucan synthesis would be of great benefit for the manipulation of wheat β-glucan levels.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Barley chromosome arm-specific SSR and STS markers and gene-specific quantitative Real-Time PCR primer pars and optimal acquisition temperatures for PCR and RT-PCR analysis

Table S2. (1,3;1,4)-β-D-Glucan content and standard deviation (SD) of the studied genotypes

Fig. S1. (A) Genomic *in situ* hybridisation (GISH) on mitotic chromosomes of the 'Mv9kr1'/'Igri' 1HS ditelosomic addition line. Labelled genomic DNA of barley was

used as probe. The 1HS barley chromosome arm is highlighted in red. The chromosomes were counterstained with DAPI (blue). Scale bar represents 10 μm. (B) GISH on mitotic chromosomes of the 'Mv9kr1'/'Igri' 7H disomic addition line. Labelled genomic DNA of barley was used as probe. The 7H barley chromosome is highlighted in red. The chromosomes were counterstained with DAPI (blue). Scale bar represents 10 μm.

Fig. S2. Capillary gel electrophoresis pattern of the *HvCslF9* and *HvCslF6* gene-specific markers on the following DNA templates: negative control (NC), 'Mv9kr1'wheat line (Mv9), 'Igri' barley cultivar (I), 'Mv9kr1'/'Igri' 1HS ditelosomic addition line (1HS) and 'Mv9kr1'/'Igri' 7H disomic addition line (7H). Gene-specific bands are indicated by arrows. Primer pairs specific to the *HvCslF9* gene amplified fragments of the expected size on the 'Mv9kr1'/'Igri' 1HS ditelosomic addition line templates, indicating that the gene is located on chromosome arm 1HS.