Targeted mutation of barley (1,3;1,4)-β-glucan synthases reveals complex relationships between the storage and cell wall polysaccharide content

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SUMMARY

Barley (Hordeum vulgare L.) grain is comparatively rich in (1,3;1,4)-β-glucan, a source of fermentable dietary fibre that protects against various human health conditions. However, low grain (1,3;1,4)-β-glucan content is preferred for brewing and distilling. We took a reverse genetics approach, using CRISPR/Cas9 to generate mutations in members of the Cellulose synthase-like (Csl) gene superfamily that encode known (HvCslF6 and HvCslH1) and putative (HvCslF3 and HvCslF9) (1,3;1,4)-β-glucan synthases. Resultant mutations ranged from single amino acid (aa) substitutions to frameshift mutations causing premature stop codons, and led to specific differences in grain morphology, composition and (1,3;1,4)-β-glucan content. (1,3;1,4)-β-Glucan was absent in the grain of cslf3 knockout lines, whereas cslf9 knockout lines had similar (1,3;1,4)-β-glucan content to wild-type (WT). However, cslf9 mutants showed changes in the abundance of other cell-wall-related monosaccharides compared with WT. Thousand grain weight (TGW), grain length, width and surface area were altered in cslf6 knockouts, and to a lesser extent TGW in cslf9 knockouts. cslf3 and cslh1 mutants had no effect on grain (1,3;1,4)-β-glucan content. Our data indicate that multiple members of the Csl/F/H family fulfill important functions during grain development but, with the exception of HvCslF6, do not impact the abundance of (1,3;1,4)-β-glucan in mature grain.

Keywords: barley, cell walls, gene editing, CRISPR/Cas9, (1,3;1,4)-β-glucan.

INTRODUCTION

Cell walls are a distinctive structural feature of plant cells. They are composed of polysaccharide-rich layers that determine cell size and shape, and impact plant growth and development. The natural diversity of inter- and intra-species cell wall composition has an impact on the processing properties of plant-derived materials, both for industrial applications and human nutrition. For example, non-cellulosic polysaccharides are a key source of soluble dietary fibre that influence the nutritional quality and digestibility of plant-based foods (Doblin et al., 2010; Burton and Fincher, 2012). (1,3,1,4)-β-Glucan is a non-cellulosic polysaccharide found in the small grain cereals. Barley (Hordeum vulgare L.) has higher grain (1,3,1,4)-β-glucan content (4–10% w/w) compared with other cereals such as wheat (Triticum aestivum L.; 1% w/w) and rice (Oryza sativa L.; < 0.06% w/w; Burton and Fincher, 2012). A wide range of natural variation for grain (1,3,1,4)-β-glucan content (2.5–8% w/w) has been described in elite barley cultivars (Houston et al., 2014; Izydorczyk et al., 2000), and this influences their end-use properties. The brewing and distilling industries require barley cultivars with low grain (1,3,1,4)-β-glucan content for efficient malting and brewing. This is because (1,3,1,4)-β-glucan content has a direct impact on the viscosity of the mash and, if too high, leads to filtration problems during brewing or to the formation of undesirable hazes in the final product (Wang et al., 2004; Gupta et al., 2010). Barley
cultivars with high grain (1,3;1,4)-β-glucan content are preferred by the functional food sector based on its beneficial effect on human health (Cavallero et al., 2002; Keenan et al., 2007; Collins et al., 2010). (1,3;1,4)-β-Glucan is not digested in the small intestine of humans, acting as fermentable dietary fibre that reduces the risk of diet-related conditions such as cardiovascular disease, type II diabetes and colorectal cancer (Brennan and Cleary, 2005; Wood, 2007; Cosola et al., 2017; Ames et al., 2018).

Members of three Cellulose synthase-like (CslF/H/J) subfamilies within the glycosyltransferase (GT) 2 family (Lombard et al., 2014) synthesise (1,3;1,4)-β-glucan. However, the functions of many members of each of these subfamilies are yet to be characterised (Little et al., 2018). HvCslF6, HvCslH1 and HvCslJ1 have been shown to be directly involved in (1,3;1,4)-β-glucan synthesis by either mutant knockout/down of Csf6 (Nemeth et al., 2010; Vega-Sanchez et al., 2012), transgenic overexpression of HvCslF6 in barley grain via an endosperm-specific promoter (Burton et al., 2011), or heterologous expression of HvCslF6, HvCslH1 or HvCslJ1 in either Arabidopsis or tobacco leaves (Little et al., 2018). A genome-wide association study (GWAS) on grain (1,3;1,4)-β-glucan content (Houston et al., 2014) confirmed that a QTL identified previously by Han et al. (1995) incorporates a cluster of genes including HvCslF3, HvCslF4, HvCslF8, HvCslF10, HvCslF12 and HvCslH1 on chromosome 2H, while a QTL on 1H co-locates with HvCslF9 and HvGlb1, the latter being one of two (1,3;1,4)-β-glucan-specific endoglucanases. These QTL regions support the hypothesis that multiple Csf genes may act as mediators of (1,3;1,4)-β-glucan synthesis (Burton et al., 2006) and contribute to natural variation in (1,3;1,4)-β-glucan content.

Although HvCslF6 transcripts are the most abundant of all putative (1,3;1,4)-β-glucan synthase genes in developing grain and many vegetative tissues, HvCslF9 is expressed during early grain development and in root tips (Burton et al., 2008). HvCslF3 is also highly expressed in root tips and coleoptiles. Due to the presence of (1,3;1,4)-β-glucan in the tissues where they are transcribed, both HvCslF3 and HvCslF9 are considered potentially capable of synthesising (1,3;1,4)-β-glucan (Burton et al., 2008; Aditya et al., 2015). In addition, the expression of HvCslF17 in leaf tissue supports previous findings that this gene contributes to synthesis of (1,3;1,4)-β-glucan in this tissue (Burton et al., 2008, 2011; Doblin et al., 2009). Despite distinct expression profiles, in planta functions of the HvCslF3, HvCslF9 and HvCslH1 genes have yet to be confirmed in barley by loss-of-function mutants. This contrasts with the barley Csf6 gene, where several cslf6 alleles containing amino acid substitutions (bgla, bglb and bglc) have been described that exhibit reduced levels of (1,3;1,4)-β-glucan, susceptibility to chilling and alterations in grain morphology (Taketa et al., 2012). Loss-of-function alleles for members of the HvCslF/H families would provide an opportunity to assess their role in (1,3;1,4)-β-glucan accumulation as well as plant growth and development.

Here we utilised a reverse genetics approach to provide insight into the contribution of each of four members of the HvCslF/H subfamilies to barley grain morphology and composition. We used CRISPR/Cas9-based gene-editing technology to generate site-specific double-strand breaks leading to the introduction of targeted mutations via the non-homologous end-joining (NHEJ) repair mechanism. We analysed plant and grain morphology/development, grain monosaccharide composition, and the structure, distribution and content of (1,3;1,4)-β-glucan in the grain to provide insight into the function of each of the four genes. Our findings suggest that each of the genes contributes to the phenotype of barley grain, thereby providing opportunities to modify composition and morphology via HvCslF/H genes other than HvCslF6.

RESULTS

CRISPR/Cas9-induced mutations in members of the HvCslF/H gene family

We catalogued the mutations in each of the target genes over three generations (T0, T1 and T2). In the T0 plants all CRISPR/Cas9-induced mutations were in the heterozygous state. The frequency of mutations ranged from 35.3% for HvCslF3, to 9.3% for HvCslF6, 22% for HvCslF9 and 4.7% for HvCslH1 (Table S1). In putative T0 mutants, 83% of the plants carried short InDels (ranging from 1 bp deletions to 2 bp insertions; Table 1; Figure S1). An exception to this trend was identified for HvCslF9 where a 39-bp deletion was detected in the heterozygous state in a single T0 plant. Homozygous lines were detected in the T1 (14 out of 58 for HvCslF3, 8 out of 169 for HvCslF9 and 2 out of 58 for HvCslH1). These lines contained InDels of varying sizes, some of which had the potential to change the protein sequence. Screening for the presence of Cas9 by polymerase chain reaction (PCR) indicated a high prevalence of transgene retention in HvCslF6 and HvCslH1 genotypes (92% and 97%, respectively) compared with HvCslF3 (55%) and HvCslF9 (56%) in T1 plants (Table S2). A range of T1 alleles were selected to maximise the possibilities of identifying potential phenotypic and allelic changes in the subsequent generation. A subset of 10 T2 alleles (Table 1; Figure 1; cslf3, cslf6, cslf9 and cslh1) was used to characterise the effect of these mutations on grain morphology and composition.

Mutations in HvCslF/H genes influence grain morphology

Using grain from a subset of 10 T2 lines of cslf3 (2), cslf6 (2), cslf9 (3) and cslh1 (3), we assessed the effect of each mutation on thousand grain weight (TGW), grain area, grain length and grain width, and compared these with wild-type (WT) grain of cv. Golden Promise. Cas9-free T2
Table 1 Collection of csflh mutants (T3) used for phenotypic characterisation in this study, and their predicted protein effect

| Gene     | InDel size | Allele | Allelic state | Type of mutation | Mutation location | Predicted protein effect |
|----------|------------|--------|---------------|------------------|-------------------|--------------------------|
| HvCslF3  | –2 bp      | csfl3-1| Homozygous    | Frameshift       | 148_150del        | Multiple aa changes      |
|          | +3 bp      | csfl3-2| Homozygous    | Frameshift       | 149_152ins        | 1 aa insertion           |
|          | +1 bp      | csfl6-2| Homozygous    | Frameshift       | 177_178ins        | Premature stop codon     |
|          | +1 bp      | csfl6-2/+| Heterozygous | Frameshift       | 177_178ins        | (29 aa downstream PAM)   |
| HvCslF6  | +3 bp      | csfl9-1| Homozygous    | Frameshift       | 117_156del        | 13 aa deletion           |
|          | +5 bp      | csfl9-2| Homozygous    | Frameshift       | 126_131del        | Premature stop codon     |
|          | +1 bp      | csfl9-3| Homozygous    | Frameshift       | 130_131ins        | (2 aa downstream PAM)    |
| HvCslH1  | –21 bp     | cslh1-1| Homozygous    | Frameshift       | 25_46del          | 7 aa deletion            |
|          | +1 bp      | cslh1-2| Homozygous    | Frameshift       | 48_49ins          | Multiple aa changes      |
|          | +13 bp     | cslh1-3| Homozygous    | Frameshift       | 52_65ins          | Multiple aa changes      |

The mutation location was described with respect to the cDNA start. PAM represents proto adjacent spacer motif, aa represents amino acid. Mutation locations are provided as nucleotides from the start codon followed by ‘ins’ for an insertion or ‘del’ for a deletion.

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lines were identified for csfl3, csfl9 and cslh1; however, multiple Cas9 insertions were suspected in csfl6-2 lines based on a preliminary segregation test for the selectable marker (Figure S2).

Grain from the csfl6-2 mutants containing a premature stop codon (Table 1) in the first exon exhibited a significantly lower TGW (P = 0.002, Tukey’s test) compared with WT (Figure 2b). In addition, the grain was significantly longer (P = 0.006, Tukey’s test) compared with WT. In heterozygous mutants (csfl6-2/+, TGW was affected to a lesser extent, due to higher phenotypic variation for this trait. Grains were significantly longer (P = 0.002, Tukey’s test) than WT, whereas grain width was not significantly different, possibly leading to larger grains in terms of overall grain area. Significant differences in grain from csfl6 homozygous and heterozygous plants compared with WT indicate there may be some dosage dependency of HvCslF6 in sporophytic (maternal) tissues, similar to that reported previously for substitution alleles (Taketa et al., 2008).

The csfl9 homozygous mutant (csfl9-3) that carries a premature stop codon in the first exon (due to a 1-bp insertion) had significantly lower TGW compared with WT (P = 0.021, Tukey’s test; Figure 2b), whereas no significant differences were observed in csfl9-1 carrying a 39-bp in-frame deletion and csfl9-2 carrying a 5-bp deletion leading to a premature stop codon also in the first exon (P = 0.310; csfl9-1 and P = 0.154; csfl9-2, Tukey’s test; Figure 2b). No statistically significant differences were identified for grain length (P = range: 0.169–0.887, Tukey’s test, csfl9 mutants versus WT) or overall area (P = range: 0.087–0.116, Tukey’s test) compared with WT. However, grain from csfl9 homozygous mutants had significantly smaller overall area compared with either csfl6-2 (P = 0.038; csfl9-1, P = 0.041; csfl9-2, P = 0.030; csfl9-3, Tukey’s test) or csfl6-2/+ mutants (P = 0.002; csfl9-1, P = 0.003; csfl9-2, P = 0.002; csfl9-3, Tukey’s test).

The csfl3 mutants csfl3-1 and csfl3-2, which carry a 2-bp deletion and a 3-bp insertion that cause a frame shift and the addition of a single amino acid, respectively (Table 1), showed a significant reduction in TGW (P = <0.022 and 0.002, Tukey’s test) but to a lesser extent than the csfl6 and csfl9 mutant lines (Figure S4). csfl3-2 had significantly narrower grains compared with WT (P = 0.008, Tukey’s test), although no significant differences were observed in grain area between csfl3 mutants and WT grain (P = 0.261; csfl3-1 and P = 0.157; csfl3-2, Tukey’s test; Figure S3). A lower TGW was observed for cslh1 homozygous frameshift mutants (cslh1-2 and cslh1-3) compared with WT (P = 0.012; cslh1-2 and P = 0.044; cslh1-3, Tukey’s test; Figure S5). No differences were observed in relation to overall area (including grain width and length) for csfl1 mutants compared with WT grain (P = range: 0.243–0.648, Tukey’s test).

Based on BLAST searches, the sgRNA sequences for HvCslF3 and HvCslH1 have potential off-targets that could result in mutations being introduced into additional genes (Table S3). To assess if these off-target mutations were present in the lines characterised here, we amplified the putative off-target amplicons in the same 10 lines (four csfl3 lines and six cslh1 lines) used for phenotypic characterisation of the grain (Table S4). For HvCslF3, the sgRNA could potentially bind to HORVU2Hr1G042350 (encoding a putative Cellulose synthase-like D2 based on gene annotation), and for HvCslH1 the potential off-target site was in HORVU2Hr1G074940 (encoding a putative Cellulose synthase-like B4); however, no mutations were observed in these genes. There were no putative off-targets for the
sgRNAs designed to HvCslF6 and HvCslF9 because any potential matches identified contained mismatches within the PAM (Figure S6). Therefore, we conclude that for all the lines characterised that the phenotypes observed are due to mutations in the targeted genes.

Impact of the induced mutations on plant development

The same lines used for phenotypic characterisation of grain development were analysed for changes in whole plant growth under glasshouse conditions. Due to the small number of seed available for some lines it was not feasible to carry out a full replicated study of gross plant morphology during development. However, several phenotypes were striking, and therefore worth describing here.

We observed a delay in development and a reduction in plant height in both cslf6-2 (homozygous) and cslf6-2/+ (heterozygous) mutants compared with WT (cv. Golden Promise). cslf6-2 mutants had fewer tillers than WT after 10 weeks (Figure 2a). Five weeks later, spikes were developing in the cv. Golden Promise control, whereas the cslf6-2 mutant line was still in the vegetative phase. At maturity, only two to three spikes set seed in the cslf6-2 mutants, limiting further grain phenotypic studies (Figure S3). The phenotypic appearance of cslf6-2 homozygous grain was vastly different to other csl mutants (~50% reduction in TGW; Figures 2 and 3c). No major whole plant phenotypic changes were detected in cslf9 mutants compared with WT (Figure 2a). A similar plant phenotype to WT was observed in cslf3 and cslh1 gene-edited lines (Figures S4a and S5a).

Mutations in HvCslF6 influence grain (1,3;1,4)-β-glucan content

Grains from cslf6-2 (homozygous) mutants almost completely lack (1,3;1,4)-β-glucan (0.11% w/w C6 0.04), whereas grains from cslf6-2/+ (heterozygous lines) have intermediate levels of (1,3;1,4)-β-glucan (1.45% w/w C6 0.31) compared with the WT control (5.00% w/w C6 0.03; Figure 3a). There was no significant difference between (1,3;1,4)-β-glucan content of grain from cslf9 homozygous mutants (4.73% w/w C6 0.37, on average) compared with WT grain regardless of the mutation present (Figure 3a). We observed a consistent DP3:DP4 ratio ranging from 2.6 to 3.1 in mature grain flour samples across all lines analysed. This DP3:DP4 ratio was detected despite differences in the predicted effects of the CRISPR/Cas9-induced mutations on protein length and structure (Figure 3b). The method was sensitive enough to analyse a single cslf6-2 (knockout) mutant with 0.20% (w/w) C6 0.01 (1,3;1,4)-β-glucan content, showing a 3.1:1 C6 0.01 DP3:DP4 ratio. Other cslf6-2 knockout lines were below the threshold for (1,3;1,4)-β-glucan detection, therefore DP3:DP4 ratio analysis was not possible. Three cslf6-2/+ (heterozygous) mutants carrying the same mutation as cslf6-2 had an average DP3:DP4 ratio of...
Figure 2. Whole plant and grain morphology of cslf6 and cslf9 alleles. 
(a) Plant phenotype of cslf6 (cslf6-2 and cslf6-2+/+ and cslf9 (cslf9-1, cslf9-2 and cslf9-3) gene-edited lines 10 weeks post-germination. Each gene-edited line (left) was compared with cv. Golden Promise (WT; right). Below, mature grains harvested from the same cslf6 and cslf9 gene-edited lines. 
(b) Phenotypic assessment of grain characteristics: thousand grain weight (TGW), overall grain area, grain width and grain length for cslf6 and cslf9 lines. Error bars represent SD from two–four independent genotypes per mutation. Asterisks indicate statistically significant differences compared with the control (WT) at \( P < 0.05 \) (\*\*) and \( P < 0.01 \) (\**\*) using Tukey’s post hoc test in GraphPad Prism v.8.4.2.

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Expression of this gene (Burton et al., 2011). Developmental stages for analysis were selected based on the transcript abundance of the target genes; for HvCslF6, we sampled mature grain; and for HvCslF9, we sampled grain at 15 DPA, as this follows the peak of HvCslF6 expression (Burton et al., 2019). In thin sections of mature WT grain, (1,3;1,4)-β-glucan was detected in the starchy endosperm and cell walls of aleurone layers. In contrast, and as expected based on the (1,3;1,4)-β-glucan assay, labelling was completely absent in the endosperm of the cslf6-2 (homozygous) grain. We observed weak fluorescence in the cell walls of sub-aleurone cells and outer endosperm cells (Figures 3c and 4b), but this may be partially due to autofluorescence of phenolic acids (Figure S8).

(cslf6-2/+) (heterozygous) grain showed intermediate levels of (1,3;1,4)-β-glucan labelling across the aleurone and endosperm cell walls compared with WT grain (Figures 3c and 4). These results are consistent with the quantification of (1,3;1,4)-β-glucan in these lines (Figure 3a). Differences in grain shape and structure between cslf6 mutants and WT were apparent based on calcofluor counterstaining (Figure 3c,d). Grain sections were also stained with iodine to detect starch granules revealing that both homozygous and heterozygous mutants showed more even, compact staining than WT in mature endosperm tissues. Additionally, a potential difference in polysaccharide distribution was observed in the sub-aleurone and young endosperm tissue of the cslf6-2 (homozygous) mutant as revealed by intense staining with Toluidine blue (TB; Figure 3c).

Developing grains (15 DPA) from cslf9 mutants (all homozygous) exhibited a similar (1,3;1,4)-β-glucan labelling pattern (i.e. distribution) compared with WT grain (Figures 3d and 4). However, this was accompanied by a reduction in calcofluor staining intensity in the cslf9-2 mutant, the outer layers of the cslf9-3 mutant (both of which contain a premature stop codon) and to a lesser extent in the cslf9-1 mutant (which contains a 39-bp deletion; Figure 3d). Further characterisation of cslf9 alleles by linkage analysis (Table S5; Method S3) showed a 24.11 ± 1.56% reduction in cellulose content compared with WT grain, which was also verified by a crystalline cellulose biochemical assay (18.27 ± 0.62% reduction). Moreover, cslf9-2 and cslf9-3 knockout mutants appear to have a different distribution of starch and cell wall polysaccharide staining in transverse grain sections compared with WT grain. A stronger TB stain in the outer endosperm cells of mutants was observed, whereas in WT grain a darker blue colour was seen around the central grain area, indicating a potential difference in polysaccharide distribution (Figure 3d).

Mutations in HvCslF6 and HvCslF9 alter grain polysaccharide composition

The differences in staining intensity indicated that polysaccharide distribution, amount and/or cell structure may be altered between cslf6 and cslf9 mutant grain and WT. To assess the monosaccharide composition of these mutant lines, mild acid hydrolysis was used on mature grain flour. Standard conditions were followed such that only non-cellulosic polysaccharides (but not cellulose) would be hydrolysed. Consistent with previous studies, the most abundant non-cellulosic monosaccharides detected in WT non-starched mature grain flour samples were glucose, followed by xylose, arabinose and galactose (Table 2). These represent the building blocks of the three most abundant non-cellulosic polysaccharides reported in barley grain; starch, (1,3;1,4)-β-glucan and arabinogalactan (Wilson et al., 2012). Significant differences in non-cellulosic monosaccharide composition were identified between WT and mutants, particularly in glucose levels between WT (54.04 ± 0.93% w/w) and cslf6-2 (43.37 ± 1.77% w/w) and cslf9-3 (40.18 ± 2.13% w/w) alleles (Table 2). The reduced levels of glucose in cslf6-2 mirrored the reduction in (1,3;1,4)-β-glucan content (Figure 3a). The reduction of glucose observed in cslf9-2 and cslf9-3, both of which carry a premature stop codon, was not detected in cslf9-1 that carries the 39-bp deletion. Moreover, no significant difference was detected in (1,3;1,4)-β-glucan content (% w/w) in any of the cslf9 mutant lines compared with WT (Figure 3a), suggesting that the reduction in glucose may reflect a change in starch abundance (Table S5). Small but significant increases in xylose and arabinose content were identified in cslf6-2 and cslf9-3 lines compared with WT (Table 2). Due to the altered non-cellulosic monosaccharide composition of grain from cslf9 alleles compared with WT, total starch content was analysed in these lines (Table S5). A significant reduction in total starch was observed across all cslf9 alleles (50.90 ± 0.61% w/w cslf9-1; 45.62 ± 0.19% w/w cslf9-2; and 40.24 ± 0.45% w/w cslf9-3) compared with WT grain (59.32 ± 0.97% w/w, P < 0.001, Tukey’s test; Table S5).

Taken together, these findings suggest that several of the CRISPR-induced mutations in HvCslF6 and HvCslF9 appear to have an effect on the abundance of cellular carbon stores in the barley grain.

DISCUSSION

Multiple genes have been reported to play a role in (1,3;1,4)-β-glucan biosynthesis. These include genes from the CslF family, of which CslF6 has a prominent role
Gain of function assays in tobacco and Arabidopsis indicate that CslF2, CslF4, CslH1 and CslJ may also contribute to (1,3;1,4)-\(\beta\)-glucan biosynthesis (for review, see Amos and Mohnen, 2019), while QTL and GWAS studies in barley have hypothesised roles for other CslF family members (Han et al., 1995; Molina-Cano et al., 2007; Houston et al., 2014). Despite this, loss-of-function mutants have only confirmed the function of CslF6 in barley and rice (Taketa et al., 2012; Vega-Sanchez et al., 2012; Cu et al., 2016), while functional evidence supporting a role for the other genes has been lacking. In this study a collection of mutants was successfully generated for several members of the HvCslF/H subfamilies of GT2 enzymes, and a subset of these lines was characterised at both the phenotypic and molecular levels. This pool of gene-edited mutants represents a resource for the functional assessment of these and other cell-wall-related genes.

To initially test our gene-editing system, we targeted the HvCslF6 gene. Taketa et al. (2012) previously reported cslf6 alleles containing amino acid substitutions in barley betaglucanless (bgl) mutants. Here we aimed to generate a true knockout allele by targeting the first exon of HvCslF6. Although relatively few lines were recovered, we successfully generated the cslf6-2 allele that contains a premature stop codon and was therefore of interest for further characterisation. Grain (1,3;1,4)-\(\beta\)-glucan quantification in cslf6-2 mutants (homozygous for a 1-bp insertion leading to a premature stop codon in the first exon) confirmed the severe reduction of this polysaccharide in mature grain (0.11 \(\pm\) 0.04% w/w), while intermediate (1,3;1,4)-\(\beta\)-glucan levels were detected in cslf6-2/+ heterozygous lines (1.45 \(\pm\) 0.31% w/w). Low (1,3;1,4)-\(\beta\)-glucan content has traditionally been desired in malting barley breeding programmes to ensure efficient filtration processes during brewing via decreased

**Figure 3.** (a) Quantification of (1,3;1,4)-\(\beta\)-glucan concentration in mature grain of cslf6 and cslf9 mutants. (b) DP3:DP4 ratio of (1,3;1,4)-\(\beta\)-glucan in mature grain of the same lines. Error bars represent SE from two–four independent genotypes per mutation, except for a single cslf6-2 (homozygous) allele whose error bar for DP3:DP4 ratio was derived from two technical replicates. Other cslf6-2 plants were below the threshold for (1,3;1,4)-\(\beta\)-glucan detection, therefore DP3:DP4 ratio analysis was not possible. Asterisks indicate statistically significant differences compared with the control (WT) at \(P < 0.05\) (\(*\)) and \(P < 0.001\) (****) using Tukey’s test in GraphPad Prism v.8.4.2. (c) Confocal images of transverse mature grain sections (10 \(\times\)) labelled with BG1 antibody in cslf6 alleles (cslf6-2 and cslf6-2/+ and cv. Golden Promise (WT)). (1,3;1,4)-\(\beta\)-Glucan was detected in green fluourescence using Alexa Fluor 488 (BG-1). Cell wall structure was observed by calcofluor (CF) counterstaining, in blue. Below, light microscope images (5 \(\times\)) of the same lines. Starch content was detected by Lugol’s (LG) iodine staining. Toluidine blue O (TB) was used for differentially staining primary and secondary cell walls. (d) Confocal images of developing grain (15 DPA) in cslf9 alleles (cslf9-1, cslf9-2 and cslf9-3) and wild-type (WT; transverse grain sections, 10 \(\times\)). Below, light microscope images (5 \(\times\)) of the same cslf9 lines. (1,3;1,4)-\(\beta\)-Glucan and other cell wall components were detected as described for cslf6 mutants above. Scale bars: 1 mm.
mash and wort viscosity. Although the gene-edited csf6 mutants reported here show extremely low levels of grain (1,3;1,4)-β-glucan, they are unsuitable for malting due to poor agronomic traits, including decreased TGW, flatter and longer grains, and low germination rate. Future gene-editing efforts might either focus on targeting specific residues in the HvCslF6 coding sequence closer to the 3’ end (Jobling, 2015; Dimitroff et al., 2016) or modulating transcription of the HvCslF6 gene via regulatory regions to specifically control (1,3;1,4)-β-glucan content in the grain. Regardless, our results demonstrate the effectiveness of CRISPR/Cas9-based gene-editing technology and confirm the major role of HvCslF6 in grain (1,3;1,4)-β-glucan biosynthesis, as previously reported in barley (Burton et al., 2011; Takeshi et al., 2012; Hu et al., 2014), wheat (Nemeth et al., 2010) and rice (Vega-Sanchez et al., 2012).

Despite the differences in alleles generated and genetic background used, a similar phenotype to the naturally occurring barley betaglucanless (bgl) mutant (Taketa et al., 2012) was observed in our csf6-2 knockouts; a reduction in plant height, vigour and spike development. Such extreme phenotypes are to be expected given that HvCslF6 is expressed in a wide range of tissues and developmental stages (Burton et al., 2008). Moreover, HvCslF6 is the main gene responsible for the synthesis of (1,3;1,4)-β-glucan, which has an integral role in the primary cell wall of barley. When DP3:DP4 ratios were quantified, no differences were observed in csf6 homozygous and heterozygous mutant grain compared with WT. Burton et al. (2011) described a reduced DP3:DP4 ratio in grain of HvCslF6 over-expressing lines, affecting (1,3;1,4)-β-glucan solubility. Although a higher DP3:DP4 ratio was noticed in csf6-2/- heterozygous mutants, this was not significantly different from controls (P = 0.285, Tukey’s test). Immunohistochemical detection of (1,3;1,4)-β-glucan confirmed the absence of this polysaccharide in mature grain of csf6-2 homozygous mutants, whereas a weak labelling was detected across aleurone and endosperm tissues in heterozygous lines. It is known that an inverse relationship exists between (1,3;1,4)-β-glucan and starch content in grains of several grass species.
including barley, based on comparative studies across cereal species with contrasting levels of this polysaccharide (Trafford et al., 2013; Lim et al., 2019). The precise mechanism(s) that regulate this relationship remain unknown. Sections of cslf6 mutant grain (homozygous and heterozygous) analysed for starch distribution suggest a stronger and more compact staining in sub-aerulone layers of knockout and heterozygous lines, potentially due to lower levels of (1,3;1,4)-\(\beta\)-glucan.

In addition to confirming the utility of the gene-editing system, the cslf6-2 lines described here provide an important ‘null’ background for future experiments. These lines can be used to either investigate the molecular, spatial and temporal details of HvCslF6 or other gene function in grain (1,3,1,4)-\(\beta\)-glucan synthesis. Previous studies of wheat addition lines carrying HvCslF6 revealed a 60% increase in grain (1,3,1,4)-\(\beta\)-glucan content although, when compared with barley controls, this only reached 20% of the (1,3,1,4)-\(\beta\)-glucan level in the grain (Cseh et al., 2013). This indicates that in a heterologous system, HvCslF6 alone is insufficient to replicate the complete (1,3,1,4)-\(\beta\)-glucan biosynthetic activity of barley, and other activities must be present that support (1,3,1,4)-\(\beta\)-glucan synthesis. Further analysis of cslf6-2 knockout lines could potentially reveal regulatory proteins affecting this gene. It is known that HvCesA1, HvCesA2 and HvCesA6, members of the Cellulose synthase gene subfamily within the GT2 family, are co-expressed with HvCslF6 (Burton and Fincher, 2009; Wilson et al., 2012). It currently remains unclear whether their function or regulation is compromised through lack of functional HvCslF6 in cslf6-2 lines. The cslf6-2 knockout alleles also provide an opportunity to create double mutants, for example with cslh1 lines carrying frameshift mutations, to assess the contribution of multiple members of the Csl gene family to cell wall composition in leaf, grain and other tissues.

Unlike HvCslF6, previous studies have not reported loss-of-function phenotypes for the HvCslF9 gene. HvCslF9 expression in developing grain is generally low but shows a prominent peak at about 8 DPA (cv. Sloop; Burton et al., 2008), although this varies depending on the cultivar (Garcia-Gimenez et al., 2019). In cv. Sloop, this peak coincides with initiation of (1,3,1,4)-\(\beta\)-glucan synthesis, which by 10 DPA is uniformly distributed throughout the cellulosified endosperm (Wilson et al., 2006), and accumulation continues until late grain development (Wong et al., 2015). One possibility is that HvCslF9 contributes to (1,3,1,4)-\(\beta\)-glucan synthesis in a temporally and/or spatially restricted manner. Our data confirm that HvCslF9 has a role in barley grain development. Knockout alleles exhibited pleiotropic changes in grain morphology, monosaccharide, polysaccharide and starch composition, but only minor changes in grain morphology, monosaccharide, polysaccharide and starch composition, but only minor changes in grain (1,3,1,4)-\(\beta\)-glucan content. This was confirmed in four independent knockout mutants, two lines carrying a 5-bp deletion (cslf9-2) and another two carrying a 1-bp insertion (cslf9-3), all leading to premature stop codons. Similar results were obtained for cslf9-1 mutants carrying a 39-bp deletion in the first exon.

Immunolabelling was used to assess whether loss of HvCslF9 leads to differences in (1,3,1,4)-\(\beta\)-glucan deposition during earlier stages of grain development. BG1 labelling at 15 DPA confirmed the presence of (1,3,1,4)-\(\beta\)-glucan in the endosperm and aleurone layers of cslf9 knockout lines with similar distribution compared with the WT. The detection of (1,3,1,4)-\(\beta\)-glucan in mid and mature grain samples suggests that HvCslF9 might not contribute to mature grain (1,3,1,4)-\(\beta\)-glucan content. However, as we did not characterise (1,3,1,4)-\(\beta\)-glucan chain length or fine structure, we cannot rule out a role for HvCslF9 in these aspects of this polysaccharide. Various QTL and GWAS reports (Han et al., 1995; Molina-Cano et al., 2007; Houston et al., 2014) identified a region on chromosome 1H involved in (1,3,1,4)-\(\beta\)-glucan content, which encompasses HvCslF9 and a (1,3,1,4)-\(\beta\)-glucan endohydrolase (HvGibl) involved in the hydrolysis of this polysaccharide. This QTL

Table 2 Non-cellulosic monosaccharide mean values (% w/w ± SE) in gene-edited cslf/h mutants (T3)

| Genotype | Glucose | Xylose | Arabinose | Galactose |
|----------|---------|--------|-----------|-----------|
| WT       | 54.04 ± 0.93 | 4.66 ± 0.08 | 2.21 ± 0.01 | 0.25 ± 0.01 |
| cslf3-1  | 50.98 ± 0.92 | 4.46 ± 0.06 | 2.24 ± 0.01 | 0.26 ± 0.01 |
| cslf3-2  | 45.33 ± 0.78* | 4.72 ± 0.04 | 2.45 ± 0.02 | 0.26 ± 0.00 |
| cslf6-2  | 36.85 ± 0.89*** | 5.73 ± 0.20*** | 2.61 ± 0.06*** | 0.35 ± 0.02*** |
| cslf6-3  | 41.28 ± 1.23*** | 5.04 ± 0.17 | 2.51 ± 0.04** | 0.31 ± 0.01 |
| cslf9-1  | 51.40 ± 1.01 | 4.74 ± 0.12 | 2.30 ± 0.02 | 0.28 ± 0.01 |
| cslf9-2  | 43.37 ± 1.77*** | 5.06 ± 0.09 | 2.39 ± 0.02 | 0.29 ± 0.02 |
| cslf9-3  | 40.18 ± 2.13*** | 6.17 ± 0.35*** | 2.84 ± 0.13*** | 0.29 ± 0.01 |
| cslf1h-1 | 47.36 ± 1.56 | 4.99 ± 0.03 | 2.52 ± 0.01 | 0.25 ± 0.00 |
| cslf1h-2 | 48.60 ± 1.10* | 4.92 ± 0.10 | 2.44 ± 0.04* | 0.30 ± 0.01 |
| cslf1h-3 | 46.46 ± 0.92 | 5.13 ± 0.02 | 2.53 ± 0.02 | 0.29 ± 0.02 |

Asterisks indicate statistically significant differences compared with the control (WT) at \(P < 0.05\) (*), \(P < 0.01\) (**) and \(P < 0.001\) (***).
could also have an impact on malt (1,3;1,4)-β-glucan modification (Stuart et al., 1988; Slakeski and Fincher, 1992), and has been reported in QTL studies that quantified malt (1,3;1,4)-β-glucan content (Han et al., 1995, 1997; Ullrich et al., 1997; Szücs et al., 2009). Evidence presented in the current study suggests that HvCslF9 is unlikely to influence mature grain (1,3;1,4)-β-glucan content in the Golden Promise cultivar and, hence, HvCslF9 may not be the gene underlying this 1H QTL. Alternatively, HvCslF9 may contribute to (1,3;1,4)-β-glucan biosynthesis only in selected genotypes, at an earlier stage of grain development and/or in a defined grain tissue compartment not investigated here.

Despite there being no significant difference in the amount of grain (1,3;1,4)-β-glucan present compared with WT, the cslf9 mutants exhibited a lower TGW compared with WT grain. In the case of the two lines containing premature stop codons in HvCslF9, this reduction in TGW was coupled with a decrease in total starch, cellulose and arabinoxylan compared with WT. Subtle spatial differences in staining intensity were identified in the same lines using iodine (starch), TB (general cell walls) and calcofluor white (general cell walls including cellulose, callose and (1,3;1,4)-β-glucan) stains. These changes suggest that HvCslF9 may contribute to polysaccharide synthesis in specific regions of the grain, and/or specific time points during development, and this warrants further investigation via molecular and biochemical assays. A recent publication using transient heterologous expression in Nicotiana benthamiana leaves indicated that the HvCslF3 and HvCslF10 genes may be involved in the synthesis of a novel linear (1,4)-β-xylloglucan that consists of (1,4)-β-linked glucose and xylose residues (Little et al., 2019). The same study investigated activity of HvCslF9, but failed to identify any clear biosynthetic activity in the heterologous system. Combined with the results described by Little et al. (2018, 2019), our data agree with the hypothesis that some members of the CslF family genes could either influence or be involved in the synthesis of polysaccharides other than (1,3;1,4)-β-glucan.

The final two genes examined were HvCslF3 and HvCslH1, which are expressed at very low levels during grain development (Burton et al., 2008; Doblin et al., 2009). Therefore, as expected, frameshift mutations affecting HvCslF3 and HvCslH1 did not significantly alter either grain monosaccharide abundance or (1,3;1,4)-β-glucan content (Tables 2 and S4). The highest mRNA abundance is found for HvCslF3 in roots (Aditya et al., 2015) and for HvCslH1 in leaves (Doblin et al., 2009). However, while a clear role for CslH1 in (1,3;1,4)-β-glucan synthesis was demonstrated by Doblin et al. (2009) using heterologous expression in tobacco leaves, when Burton et al. (2011) overexpressed HvCslF3 in barley grain there was no detectable change in (1,3;1,4)-β-glucan content. Loss-of-function mutations in either gene impacted mature grain morphology, suggesting they are likely to have a function during plant development, but changes in mature grain composition were relatively small and statistically insignificant. Further phenotypic characterisation of additional plant tissues and developmental stages will be required to determine the precise contribution of these genes to cell wall composition; whether they have an organ- or tissue-specific role in (1,3;1,4)-β-glucan biosynthesis, are impacted by compensatory mechanisms (Pérez et al., 2019), or synthesise other polysaccharides such as glucoxylan (Little et al., 2019).

The efficiency of CRISPR/Cas9-induced mutations in the primary transformants ranged from 5% to 35% in this study. Previous studies in barley reported CRISPR/Cas9-induced mutation frequencies of 10-23% (Lawrenson et al., 2015), 44% (Holme et al., 2017) and ~80% (Kapusi et al., 2017; Gasparis et al., 2018) of plants after Agrobacterium-mediated transformation. In other cereals such as wheat, mutant production efficiency was reported from 0.3% to 5% using biolistic bombardment of CRISPR/Cas9 components (Zhang et al., 2016; Gil-Humanes et al., 2017; Liang et al., 2017; Sánchez-León et al., 2018) and Agrobacterium-mediated transformation (Okada et al., 2019). In the T0 plants described here, all mutants were heterozygous (monoallelic) across the targeted genes, with 83% of the lines carrying short InDels 3-4-bp upstream the PAM sequence. This location of the Cas9-induced mutation relative to the PAM sequence agrees with Cas9 cleavage pattern and error-prone NHEJ. We did not detect any homozygous mutations in primary transformants (T0) unlike other studies in rice (Zhang et al., 2014; Zhou et al., 2014).

Taken together, our collection of gene-edited alleles for HvCslF/H with knockout and frameshift mutations represents a valuable genetic resource for studying barley cell walls, and demonstrates the effectiveness and potential of CRISPR/Cas9 genome-editing technology for the analysis of gene function. Importantly, our results show that members of the CslF/H family other than HvCslF6 have an in planta function in growth and development, providing greater opportunities to understand and optimise cell wall composition for specific downstream applications.

EXPERIMENTAL PROCEDURES

Plant material and Agrobacterium-mediated transformation

Barley cv. Golden Promise was used for embryo transformation via Agrobacterium tumefaciens following Bartlett et al. (2008) at the Functional Genomics (FUNGEN) facility, The James Hutton Institute (UK). Plants were grown in glasshouse conditions (16-h light/8-h dark photoperiod) until maturity in T0, T1 and T2 generations. At harvest, spikes were collected and manually threshed to obtain seed for subsequent generations.
sgRNA design and construct assembly

sgRNA sequences were manually designed using the criteria stated on ‘Addgene CRISPR Guide’ website (Addgene, 2018) to the 5’ end of the first exon for all four genes. This was to maximise the possibility of obtaining lines containing mutations that caused a knockout of gene function induced by CRISPR/Cas9. To prevent off-target mutations, a BLAST search of sgRNA plus PAM sequences was performed on Barlex (Colmsee et al., 2015) to determine sequence specificity to HvCasF3, HvCasF6, HvCasF9 and HvCasH1, avoiding sgRNAs with potential matches to non-target genes and conserved protein motifs (Figure S4). Final sgRNA sequences were selected based on their target location and BLAST scores (Tables S3 and S6). Each sgRNA was cloned into the pC95-sequences were selected based on their target location and BLAST HvCslH1 numbers of grain, average grain area, length and width were measured with Lugol’s iodine solution and TB O were collected using a Zeiss cent labelling when neither the primary antibody nor secondary antibodies were included as this motif is vital for Cas9 to recognise the sequence. DNA sequencing was carried out as described in Houston (2012), were inserted into the pBract214m-HvCsl9-HSPT expression vector, which contains a barley codon optimised Cas9, and independently transformed into cv. Golden Promise (Method S1; Figure S7).

Screening of CRISPR/Cas9-induced mutations

Mutations were identified by a nested PCR method combined with InDel Detection by Amplicon Analysis (Yang et al., 2015). Genomic DNA was isolated from a leaf disc (2 mm diameter) taken from individual barley seedlings using the Phire Plant Direct PCR Kit (Thermo Fisher Scientific, Waltham, USA). An aliquot of 0.5 µl crude extract was used as a template for cas9 and InDel PCR detection in a reaction containing: 10 µl x Phire Plant PCR Buff-fer, 1 µl of each external forward and reverse primer at 10 mm (Table S8), 0.4 µl Phire Hot Start II DNA polymerase and 7.1 µl sdH2O in a total volume of 20 µl. External PCR amplicons, about 1.5 kb for each targeted gene, served as a DNA template (1 µl aliquot) for the nested (internal) PCR containing: 2.5 µl x Hot Start Taq buffer, 2.5 µl dNTPs, 0.12 µl Hot Start Taq DNA polymerase (Qiagen, Hilden, Germany) and 1 µl each primer: external forward and reverse primer at 10 mm (Table S8), fluorescent-labelled primer amplicons were processed using an ABI3730 DNA Analyzer (Applied Biosystems, Foster City, USA). The resulting chromatograms were visualised and analysed using GeneMapper® (Applied Biosystems, v4.1), which allowed InDel identification based on size differences compared with cv. Golden Promise control. Mutations were confirmed by Sanger sequencing after reaction clean up and preparation as described in Houston et al. (2012). Resulting sequences were manually trimmed, aligned and analysed with Geneious V.9 (Kearse et al., 2012) to identify sequence variations. Potential off-target effects were checked by PCR using primers described in Table S8, amplifying using Hot Start Taq as described in Table S7. Only putative off-targets with zero mismatches in the PAM, i.e. HvCasF3 and HvCasH1, were included as this motif is vital for Cas9 to recognise the sequence. DNA sequencing was carried out as described above.

Phenotypic assessment of grain characteristics

Mature spikes were collected and hand threshed. A subset of ~30 bulked grains per genotype was used for phenotypic studies. All phenotypic data were collected from T2 plants (T3 grain). The number of grain, average grain area, length and width were measured with Marvin Seed Analyzer (GTA Sensorik GmbH, 2013).

The grains were weighed to combine with the grain number estimate and derive TGW.

Grain (1,3;1,4)-β-glucan quantification

For each genotype, five mature grains were milled and used to quantify (1,3;1,4)-β-glucan content using a modified version of the Mixed Linkage β-Glucan Assay Kit (K-BGLU, AACC Method 32-23.01, AOAC Method 995.16, Megazyme Int. (Wicklow, Ireland)), and based on the method described by McClernon and Codd (1991) for the analysis of small flour samples (15 mg) described in Burton et al. (2011). Grain (1,3;1,4)-β-glucan content from two–four independent genotypes was averaged for each type of CRISPR/Cas9-induced mutation and calculated as % of dry weight (w/w). Each batch of grain was assayed in triplicate with two cv. Golden Promise negative controls, no primary antibody with secondary and vice versa performed in parallel with the rest of the samples. Transverse grain sections were counterstained with 0.001% Calcofluor White (Sigma-Aldrich, St Louis, USA) and de-waxed prior to antibody labelling. For (1,3;1,4)-β-glucan detection, a 1:50 dilution of anti-mouse primary antibody, BGI (Sigma-Aldrich, St Louis, USA) was used. Goat anti-mouse secondary antibody conjugated to Alexa Fluor 488 (Sigma-Aldrich, St Louis, USA) was used for fluorescence detection, diluted 1:100. Each batch of immunolabelling assays contained two cv. Golden Promise negative controls, no primary antibody with secondary and vice versa performed in parallel with the rest of the samples. Transverse grain sections were counterstained with 0.001% Calcofluor White (Sigma-Aldrich, St Louis, USA). For starch staining, a 1:5 dilution of Lugol’s iodine solution (Sigma-Aldrich, St Louis, USA) was applied to the grain sections. Lignin and differential polysaccharide staining were detected using a 0.02% TB O solution (Sigma-Aldrich, St Louis, USA) as described in Method S2.

Imaging

Imaging of immunolabelled grain sections was performed on a Zeiss LSM 710 Confocal Laser Scanning Microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) using a PL APO 20×/0.7 water dipping objective (Zeiss). Calcofluor was excited using 405 nm light from a blue diode laser, and emission was collected between 420 and 460 nm. Alexa Fluor 488 dye (BGI) was excited using 488 nm laser light from an argon ion laser, and emission was collected between 504 and 543 nm. Grain autofluorescence was detected by excitation using 594–633 nm laser light from a red diode laser, and emission was collected between 504 and 543 nm. Grain autofluorescence was detected by excitation using 594–633 nm laser light from a red diode laser, and emission was collected between 504 and 543 nm. Grain autofluorescence was detected by excitation using 594–633 nm laser light from a red diode laser, and emission was collected between 504 and 543 nm. Grain autofluorescence was detected by excitation using 594–633 nm laser light from a red diode laser, and emission was collected between 504 and 543 nm. Grain autofluorescence was detected by excitation using 594–633 nm laser light from a red diode laser, and emission was collected between 504 and 543 nm. Grain autofluorescence was detected by excitation using 594–633 nm laser light from a red diode laser, and emission was collected between 504 and 543 nm. Grain autofluorescence was detected by excitation using 594–633 nm laser light from a red diode laser, and emission was collected between 504 and 543 nm. Grain autofluorescence was detected by excitation using 594–633 nm laser light from a red diode laser, and emission was collected between 504 and 543 nm. Grain autofluorescence was detected by excitation using 594–633 nm laser light from a red diode laser, and emission was collected between 504 and 543 nm.
DP3:DP4 analysis

The DP3:DP4 ratio was determined by high-performance anion-exchange chromatography (HPAEC) as described in Ermawar et al. (2015). Lichenase enzyme (Megazyme Int., Wicklow, Ireland) was used to release oligosaccharides for profiling by HPAEC. A no-enzyme treated control, a lichenase digest of barley flour, laminaribiose and laminaritriose were included as controls under the same chromatographic conditions as for CRISPR/Cas9-induced lines (Doblin et al., 2009; Pettolino et al., 2009).

Non-cellulosic monosaccharide analysis

Non-cellulosic monosaccharide analysis was carried out as described in Hassan et al. (2017) using reversed-phase high-performance liquid chromatography separation coupled to diode array detection (Agilent Technologies). Dehulled grain was ground, and resulting flour samples (20 mg) were prepared according to Pettolino et al. (2012). Acid hydrolysis of non-starched alcohol insoluble residue was undertaken by adding 1 M sulphuric acid, as described previously (Burton et al., 2011), to the insoluble material (~15 mg); Between 3 and 12 replicates were analysed for each genotype. The moisture content of the ground flour was determined as outlined in AOAC 925.10 (AOAC International, 2005). All analyses were performed using quadruplicate technical replication.

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AUTHOR CONTRIBUTIONS

KH, MRT, RAB and RW conceived this work. KH designed and generated the sgRNA constructs. JS carried out barley transformations. Abdellah Barakate adapted the genotyping method for mutation detection. GG-G and KH performed the genotypic characterisation of the CRISPR lines. GG-G carried out the grain phenotyping, (1,3;1,4)-β-glucan quantification, immunolabelling experiments and data analyses. PS processed the CRISPR lines/samples for subsequent assays. S FK and RAB carried out the grain monosaccharide and starch analyses. MSD and PH performed the linkage and celluose analyses. The manuscript was drafted by GG-G, KH and MRT, and reviewed by Abdellah Barakate, Antony Bacic, RAB, MSD, RW and GBF. All the authors read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials.

SUPPORTING INFORMATION

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

Figure S1. Frequency of mutations in T0, T1 and T2 generations across the target genes.

Figure S2. Determination of cas9 copy number by hygromycin segregation test.

Figure S3. Images of whole plant phenotype in the casf6-2 homozygous mutant.

Figure S4. (a) Plant phenotype of cslf3 mutants. (b) Assessment of grain characteristics.

Figure S5. (a) Plant phenotype of cslh1 mutants. (b) Assessment of grain characteristics.

Figure S6. Representation of sgRNA + PAM sequences in the context of BLAST hits.

Figure S7. Vector map of pBract214m-HvCas9-HSPT-sgRNA (destination vector).

Figure S8. Negative controls used for grain immunolabelling of (1,3;1,4)-β-glucan.

Methods S1. sgRNA construct assembly.

Methods S2. Tissue fixation, embedding and immunocytochemistry.

Methods S3. Starch and cellulose quantification.

Table S1 Summary of transformation results (T0) across HvCslF/H genes

Table S2 Allelic state of CRISPR/Cas9-induced mutations and Cas9 presence in T1 plants

Table S3 BLAST score results for HvCslF3 and HvCslH1 sgRNAs

Table S4 Subset of cslf3 and cslh1 mutants used for grain (1,3,4,4)-β-glucan quantification

Table S5 Grain composition of cslf9 alleles (T3) compared with WT

Table S6 sgRNA and PAM sequences for HvCslF3, HvCslF6, HvCslF9 and HvCslH1

Table S7 Phire and Hot Start Taq PCR conditions

Table S8 Primer sequences used in this study

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