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RESEARCH PAPER

The heterologous expression in *Arabidopsis thaliana* of sorghum transcription factor *SbbHLH1* downregulates lignin synthesis

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Abstract

Basic helix–loop–helix (*bHLH*) genes are important regulators of development in plants. *SbbHLH1*, a *Sorghum bicolor bHLH* sequence, was isolated from a suppression subtractive hybridization library constructed using 13 independent *brown midrib* (*bmr*) mutants as the tester and wild-type sorghum as the driver. The gene was upregulated in at least five of the mutants at the five- to seven-leaf stage. Using a yeast expression system, the N-terminal portion of SbbHLH1 was shown to be required for proper transactivation. Its heterologous expression in *Arabidopsis thaliana* markedly reduced the plant's lignin content. It downregulated the lignin synthesis genes *4CL1*, *HCT*, *COMT*, *PAL1*, and *CCR1*, and upregulated the transcription factors *MYB83*, *MYB46*, and *MYB63*. The hypothesis is proposed that *SbbHLH1* has stronger effect on the regulation of lignin synthesis than the various MYB transcription factors, with a possible feedback mechanism acting on the MYB transcriptional regulators.

Key words: bHLH transcription factor, bioenergy, genetic engineering, *SbbHLH1*, sorghum, repressor of lignin synthesis.

Introduction

Lignocellulosic biomass, mainly composed of cellulose, hemicellulose, and lignin (Hendriks and Zeeman, 2009), is an important feedstock for biofuel production. One of its major constituents is lignin (the remainder is a mixture of cellulose and hemicellulose), a material that provides plants with structural support, a mechanical barrier against pest attack, and the raw material for constructing the vascular system (Koch *et al*., 2004; Bhuiyan *et al*., 2009). Unlike cellulose and hemicellulose, lignin is not readily to be degraded, so its presence retards the fermentation of biomass. The hydrolysis of biomass with strong acids does improve fermentation efficiency, but this step both adds to the cost of the whole process and creates environmental problems (Boerjan *et al*., 2003; Hamelinck *et al*., 2005). As a result, significant interest has been shown in developing genetic strategies to decrease the lignin content of plant biomass without compromising plant productivity, development, and pest resistance. A genetic engineering-based solution has already been successfully demonstrated in both eucalyptus and poplar (Pilate *et al*., 2002; Baucher *et al*., 2003; Huntley *et al*., 2003), while an engineered low lignin switch grass has been shown to require ~30% less cellulase to be successfully fermented, and the yield of ethanol per unit mass of biomass was raised by 30% (Fu *et al*., 2011).

Sorghum is a major warm season cereal crop, providing both grain and fodder. Its centre of origin lies in north-east Africa, but it is now distributed widely from the tropics to the temperate zone. It is renowned for its tolerance of high

Abbreviations bHLH, basic helix–loop–helix; GFP, green fluorescent protein; GUS, β-glucuronidase; ORF, open reading frame; SSH, suppressive subtractive hybridization; sqRT-PCR, semi-quantitative reverse transcription-PCR; TF, transcription factor; WT, wild type.

temperature and moisture deficit. Its potential as a source of biofuel for ethanol production may be higher than that of its chief competitors, maize, sugarcane, and switch grass (Rooney *et al*., 2007). The cell walls of the *Brown midrib* (*bmr*) class of mutant, which is already known in maize, sorghum, and pearl millet, are low in lignin, for example, *bm1–bm5* in maize (Kuc and Nelson, 1964; Ali *et al*., 2010), *bmr1–bmr38* in sorghum (Xin *et al*., 2009), and three mutants in pearl millet (Sattler *et al*., 2010). Some of these mutants have been shown to involve genes in the phenylpropanoid pathway, through which the monolignols are synthesized (Zhao and Dixon, 2011). The maize *bm3* mutant, for example, is induced by the insertion of a retrotransposon in the second exon of the caffeic acid *O*-methyltransferase (*COMT*) gene (Vignols *et al*., 1995). Among the sorghum *bmr* mutants, nonsense mutations create a premature stop codon in the cinnamyl alcohol dehydrogenase (*CAD*) (Saballos *et al*., 2009; Sattler *et al*., 2009) and *COMT* (Bout and Vermerris, 2003) genes in the *bmr6* and *bmr12* mutants, respectively; missense mutations of the 4-coumarate CoA ligase (*4CL*) and *COMT* genes were also found in, respectively, *bmr2* (Saballos *et al*., 2012) and *bmr12* (Sattler *et al*., 2012).

In addition to genes involved in lignin synthesis, certain transcription factors (TFs) have also been implicated in the regulation of lignin content. To date, most belong to the *NAC* (NAM, ATAF1 and -2, and CUC2) and *MYB* (myeloblastosis) families (Mitsuda *et al*., 2007; Zhong *et al*., 2007*a*,*b*, 2008; Shen *et al*., 2009; Zhou *et al*., 2009; Zhao and Dixon, 2011). The NAC secondary wall thickening promoting factor genes *NST1–3* all regulate the expression of a series of *MYB* TFs, which interact with the AC-rich promoters present in a number of lignin synthesis genes. An exception is the ferulic acid 5-hydroxylase (*F5H*) gene, whose promoter has no AC-rich elements. Instead, this gene is directly regulated by *NST* homologues (Zhao and Dixon, 2011). Much of the transcriptional network determined so far relates to the model dicotyledonous species *Arabidopsis thaliana*, with few, if any, transcriptional regulators for lignin synthesis known in the cereals.

Here, we report the isolation of the basic helix–loop–helix (*bHLH*) TF *SbbHLH1* from a suppressive subtractive hybridization (SSH) library formed from a collection of sorghum *bmr* mutants (Yan *et al*., 2012). This gene was the most significantly upregulated in the microarray analysis of SSH clones, and is upregulated in at least five of these sorghum *bmr* mutants (Yan *et al*., 2012). It also appear with the highest frequency in the SSH library. We showed that its heterologous expression in *A. thaliana* decreased lignin content by repressing the expression of lignin synthesis genes. The functional mechanism underlying the activity of SbbHLH1 is discussed.

Materials and methods

Plant materials

A set of 13 sorghum *bmr* mutants (*bmr*, *bmr6*, *bmr12*, *bmr29*, *bmr30*, *bmr31*, *bmr32*, *bmr33*, *bmr34*, *bmr35*, *bmr36*, *bmr45*, and *bmr49*) were all derived by ethyl methanesulfonate mutagenesis of the cultivar BTx623 (Xin *et al*., 2009; Yan *et al*., 2012). The phenotypes including whole-plant morphology, brown midribs, decreased lignin content, and so on, have been well described in previous reports (Xin *et al*., 2009; Sattler *et al*., 2010; Yan *et al*., 2012).

Wild type (WT) cv. BTx623 and the *bmr* mutants were grown in a greenhouse at ambient temperature under a 14 h photoperiod. *A. thaliana* ecotype Columbia plants were grown either *in vitro* on half-strength MS medium containing 20 g l^{-1} of sucrose, or in soil. The temperature was maintained at 22–23°C and the photoperiod was 16 h.

Identification of SbbHLH1 and analysis of its transcription in bmr mutants

The construction of an SSH library and the subsequent microarray analysis have been described by Yan *et al*. (2012). Based on the outcome of the microarray analysis, a *bHLH* gene (named *SbbHLH1*) was selected for further analysis because it showed the most significant upregulation. Its entire open reading frame (ORF) was obtained by conducting a BLAST search of the sorghum genome sequence (http://www.phytozome.net). *SbbHLH1* homologues were identified from searches of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and Phytozome databases, and a phylogeny was constructed using DNAMAN v6.0 software (Lynnon Corp.). The transcription of *SbbHLH1* in the *bmr* mutants was initially monitored using semi-quantitative reverse transcription-PCR (sqRT-PCR) (the primers directed at *SbbHLH1* and the reference gene sorghum tubulin are listed in Supplementary Table 1 at *JXB* online). After an initial denaturation step (95 °C for 5 min), the program comprised 30–35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 120 s, and was completed with a final extension step of 72 °C for 10 min.

Subcellular localization and transcriptional activity assay

The *SbbHLH1* ORF without a termination codon was inserted in frame into the *Xba*I and *Bam*HI sites of the transient expression vector p326GFP to create the *SbbHLH1–GFP* fusion gene driven by the cauliflower mosaic virus 35S (CaMV35S) promoter (Supplementary Fig. 1 at *JXB* online) (Lee *et al*., 2001). The transgene was transiently expressed in onion epidermal cells as reported by Scott *et al*. (1999), with the green fluorescent protein (GFP) signal being monitored by confocal microscopy (LSM700, Carl Zeiss AG, Oberkochen, Germany).

The entire *SbbHLH1* ORF, two separate N-terminal portions (residues 1–75 and 1–128) and two separate C-terminal portions (residues 76–245 and 129–245) were inserted into pGBKT7 (Clontech) (Supplementary Fig. 2A at *JXB* online) and transformed into *Saccharomyces cerevisiae* strain AH109 (Supplementary Fig. 2B), as described previously (Zaragoza *et al*., 2004; Lu *et al*., 2009; He *et al*., 2012). Transformants were grown on SD medium lacking tryptophan (Trp) for positive clone selection and then on SD medium lacking Trp, histidine (His) and adenine (Ade) for the transactivation assay, according to the manufacturer's instructions.

Transformation of A. thaliana

SbbHLH1 was inserted into pSTART (De Amicis *et al*., 2007) by replacing the β-glucuronidase (*GUS*) gene, and the vector was then transformed into *A. thaliana* using the floral dip method (Clough and Bent, 1998). A segregation test conducted in the T_2 generation was used to identify lines carrying a single expressed copy of the transgene, by culturing the plants on half-strength MS medium containing 50 mg l^{-1} of kanamycin. T_3 lines that did not segregate for kanamycin resistance were assumed to carry the transgene in the homozygous state. RT-PCR was used to monitor the transcription of *SbbHLH1* (relevant PCR primer sequences given in Supplementary Table 1).

Analysis of lignin, cellulose, and hemicelluloses content

The distribution of lignin of these transgenic *A. thaliana* lines was measured at the five- to seven-leaf stage by staining stem sections using

Wiesner's method (Pomar *et al*., 2002). Stem lignin content was quantified using the acetyl bromide method reported by Yan *et al*. (2012). Stems of transgenic *A. thaliana* were finely ground in liquid nitrogen, filtered through a 425 µm screen to remove large particles and vaccum freeze dried. Each 10 mg of dried materials was treated with pure ethanol, 95% ethanol, and water to remove the soluble components. After drying, the product was dissolved in acetyl bromide:acetic acid (1:3), and treated sequentially by adding 0.9 ml 2 M NaOH, 3 ml acetic acid, and 0.1 ml 7.5 M hydroxylamine HCl. The supernatants were obtained by centrifugation and mixed with acetic acid, and the absorbance at 280 nm was recorded for the calculation of lignin content.

Cellulose content was determined as following. Stem powder (100 mg) was centrifuged after being incubated with acetic acid and nitric acid (8:1) in boiling water. The sediments were washed with water and then hydrolysed with 10% (w/w) H_2SO_4 and 0.1 M potassium bichromate. The solution was mixed with 20% (w/w) KI and 0.5% (w/w) starch solution, and titrated with 0.2 M sodium hyposulfite. Cellulose content was calculated based on the volume of sodium hyposulfite for titration. For hemicellulose content determination, 10 ml 80% (w/w) calcium nitrate was added to 200 mg powder and boiled for 5 min. Sediments obtained by centrifugation were washed with hot water and hydrolysed with 2 M HCl in boiling water for 45 min. After centrifugation, the supernatants were mixed with one drop of phenothalin and neutralized with NaOH to a rose pink colour. The volume of the solution was made up to 100 ml with water and filtered through filter paper. Two millilitres of the solution was mixed with 1.5 ml of dinitrosalicylic acid and incubated in boiling water for 5 min. Absorbance was measured at 540 nm, and the content was determined by referring to a standard glucose curve.

Transcription of genes involved in lignin synthesis and the transcription regulation pathway

The transcription of various lignin synthesis genes and transcription regulators was initially monitored by sqRT-PCR. The former group all belonged to the phenylpropanoid pathway (Besseau *et al*., 2007); they were *PAL1* (phenylalanine ammonia lyase), *C4H* (cinnamic acid 4-hydroxylase), *4CL1*, *HCT* (hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase), *C3H1* (coumaric acid 3-hdroxylase), *CCoAOMT* (caffeoyl-CoA *O*-methyltransferase), *CCR1* (cinnamoyl-CoA reductase), *COMT*, *CAD6*, *F5H1*, and *F5H2*. The latter group comprised *NST1–3* and the *MYB* factors *MYB46*, *MYB58*, *MYB63*, *MYB83*, and *MYB85*. More precise quantification of transcript abundance was obtained for some of these genes (*PAL1*, *4CL1*, *HCT*, *COMT*, *CCR1*, *F5H2*, *MYB46*, *MYB83*, *MYB63*, *NST1*, and *NST2*) using real-time PCR (qRT-PCR) (Eppendorf realplex^2; Eppendorf AG, Hamburg, Germany). The primer sequences for the sqRT-PCR (Sonbol *et al*., 2009) and qRT-PCR are given in Supplementary Table 1.

Accession numbers

The accession numbers of the genes were: *SbbHLH1* (Sb03g046090), *PAL1* (At2g37040), *C4H* (At2g30490), *4CL1* (At1g51680), *HCT* (At5g48930), *C3H1* (At2g40890), *CCoAOMT* (At4g34040), *CCR1* (At1g15950), *COMT* (At5g54160), *CAD6* (At4g34230), *F5H1*(At4g34050), *F5H2* (At5g04330), *NST1* (At2g46770), *NST2* (At3g61910), *NST3* (At1g32770), *MYB46* (At5g12870), *MYB83* (At3g08500), *MYB58* (At1g16490), *MYB63* (At1g79180), *MYB85* (At4g22680).

Results

The SbbHLH1 sequence, its transcription, and its subcellular localization

Of the 96 SSH clones analysed, six contained the *SbbHLH1* sequence, and no other sequence was recovered as frequently. It was also the most significantly upregulated (Yan *et al*., 2012). Its full 5015 bp genomic sequence comprised three exons and two introns, and its 735 bp cDNA encoded a 244-residue protein of predicted molecular weight ~6.7 kDa and pI 6.08. The protein included the signature bHLH domain of two α-helices connected by a loop, starting at residue 76 and ending at residue 128 (Fig. 1A). It lacked both a transmembrane domain and a secretory peptide region. The gene was present as a single copy in sorghum, and its sequence shared considerable similarity to two homologues present in foxtail millet and to three present in each of rice, barley, and *Brachypodium distachyon*. The degree of this homology ranged from 62 to 92% (Fig. 1B). *SbHLH1* was found to be upregulated in sorghum *bmr* mutants with sqRT-PCR (Yan *et al*., 2012). In the present research, its expression was further investigated, and was found in the root, stem, and leaf of sorghum (Fig. 1C). Transcript abundance was highest in the leaf and lowest in the stem (the tissue that is most lignified) (Fig. 1C). The product of the *CaMV35S::SbbHLH1–GFP* transgene, when transiently expressed in onion epidermal cells, localized in the cytoplasm and nucleus (Fig. 2).

Transactivation test of SbbHLH1 with a yeast expression system

In this assay, two N-terminal (aa 1–75 and 1–128) and two C-terminal (aa 76–245 and 129–245) truncated forms of *SbbHLH1* (Fig. 3) were expressed independently in yeast AH109 by integration into pGBKT7 (carrying the *TRP1* gene as a selection marker) to generate fusion proteins with GAL4. If the tested full-length gene or the truncations have transactivation activity, the fusion protein could activate the expression of *HIS3* and *ADE2* genes in AH109 after interacting with GAL4 binding domains in the promoters, and allow the yeast to grow on medium lacking Trp, His and Ade (Supplementary Fig. 2). Cells carrying any one of these four transgenes or the full sequence or an empty vector all grew well on SD–Trp medium. However, on SD–Trp–His–Ade medium, while cells carrying either the entire *SbbHLH1* sequence or either of its N-terminal truncated forms survived, those carrying the empty vector or either of the two C-terminal truncations did not. This observation demonstrated that the gene has transactivation activity, which relies on the proximal part (aa 1–75) of the gene and not on its bHLH domain.

Heterologous expression of SbbHLH1 in A. thaliana

Transcription of the *SbbHLH1* gene in the *A. thaliana* genome was assayed in two transgene homozygous T_3 selections (OE1 and OE2) (Fig. 4A). There was no discernible effect of the transgene on the phenotype during the vegetative growth stage (data not shown), but once the switch to reproductive growth had been made, the stems of the transgenics lost their rigidity, unlike those of the wild type (Fig. 4B). Measurement of the lignin content with Wiesner's staining showed that the transgenic plants contained less lignin than the wild type (Fig. 4C, D). The acetyl bromide analysis data were consistent with this conclusion (Fig. 4E). Thus, the

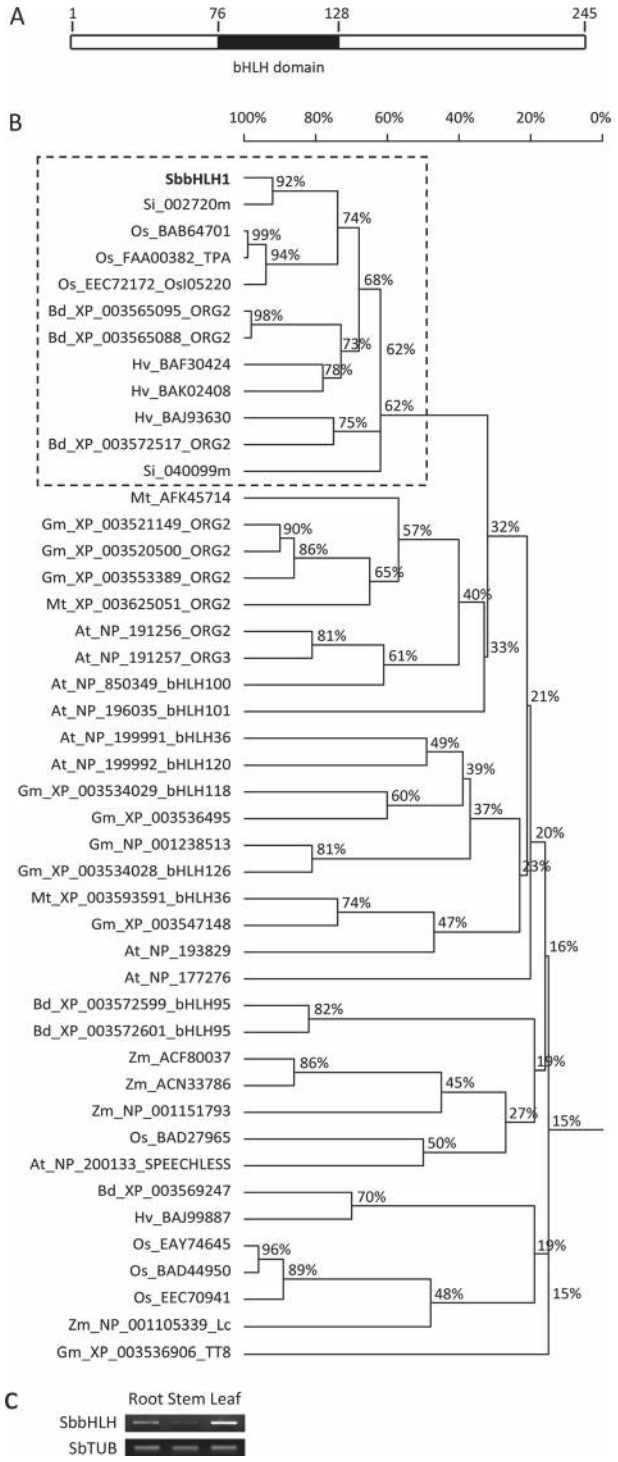

Fig. 1. Structure and transcription profile of *SbbHLH1* and the phylogeny of bHLH proteins. (A) The predicted structure of the *SbbHLH1* product, a 245-residue protein. Its bHLH domain extends from aa 76 to 128. (B) Phylogeny of SbbHLH1 with other plant bHLH proteins. SbbHLH1, indicated in bold, is similar to two foxtail millet, three rice, three *B. distachyon*, and three barley bHLH proteins. (C) Transcription of *SbbHLH1* in various tissues of the sorghum variety BTx623. Transcript abundance was lowest in the stem. At, *A. thaliana*; Bd, *B. distachyon*; Hv, barley; Gm, soybean; Mt, *Medicago truncatula*; Os, rice; Si, foxtaial millet; Zm, maize.

reduced stem strength of the transgenic lines was probably a direct effect of a lower level of lignification, so that the heterologous expression of *SbbHLH1* had the effect of repressing lignin synthesis. Combined with decreased lignification, the cellulose (Fig. 4F) and hemicellulose (Fig. 4G) content of the transgenic *A. thaliana* was increased.

Regulation of lignin synthesis and signalling pathways in transgenic A. thaliana

The transcription profiles of a number of genes associated with lignin synthesis and the relevant signalling pathways were derived using sqRT-PCR. Of these, *PAL1*, *HCT*, *CCR1*, and *COMT* were all clearly downregulated in the transgenic *A. thaliana* lines (Supplementary Fig. 3A at *JXB* online). However, while the transcript abundance of *F5H1* was unaffected by the presence of the transgene, that of *F5H2* was increased (Supplementary Fig. 3A), as were those of the TFs *MYB46*, *MYB83*, *MYB58*, and *MYB63* (Supplementary Fig. 3B). The sqRT-PCR data were verified by the use of qRT-PCR (Fig. 5), which confirmed that *4CL1*, *HCT*, and *COMT* were all downregulated in the two transgenic lines, *PAL1* was particularly strongly repressed, and *F5H2* was upregulated. *CCR1* was downregulated in one of the transgenic lines, but not in the other. *MYB83* was clearly upregulated, and both *MYB46* and *MYB63* significantly so. *NST1* and *-2* were not changed.

Discussion

Overexpression of SbbHLH1 reduces lignin content

bHLH proteins are an important class of plant TFs (Riechmann *et al.*, 2000; Ledent and Vervoort, 2001). The first *bHLH* gene to be characterized was *Lc* in maize, which regulates flavonoid/anthocyanin synthesis (Ludwig *et al.*, 1989). Other members of the gene family have proven to be involved in the signalling of light (Roig-Villanova *et al.*, 2007; Leivar *et al.*, 2008), hormones (Abe *et al.*, 2003; Yin *et al.*, 2005), wounding and drought (Smolen *et al.*, 2002; Kiribuchi *et al.*, 2004), branching (Komatsu *et al.*, 2001), reproductive development (Rajani and Sundaresan, 2001; Gremski *et al.*, 2007), and the growth of stomata (Kanaoka *et al.*, 2008) and roots (Menand *et al.*, 2007; Ohashi-Ito and Bergmann, 2007). bHLH proteins typically form dimers and recognize E- or E-like boxes in their target gene promoters, and some function by interacting with other TFs (Ramsay and Glover, 2005). An involvement of the bHLH TFs with the regulation of lignin synthesis was suggested by an analysis of the promoter sequences of white spruce and loblolly pine *CAD*, which included elements associated with MYB, WRKY, and bHLH TFs (Bedon *et al.*, 2009). Similarly, Ehlting *et al.* (2005) showed that the large number of TFs upregulated during stem development in *A. thaliana* included some members of the bHLH family.

Here, the sorghum homolog *SbbHLH1* emerged as a prime candidate associated with the brown midrib phenotype, as it

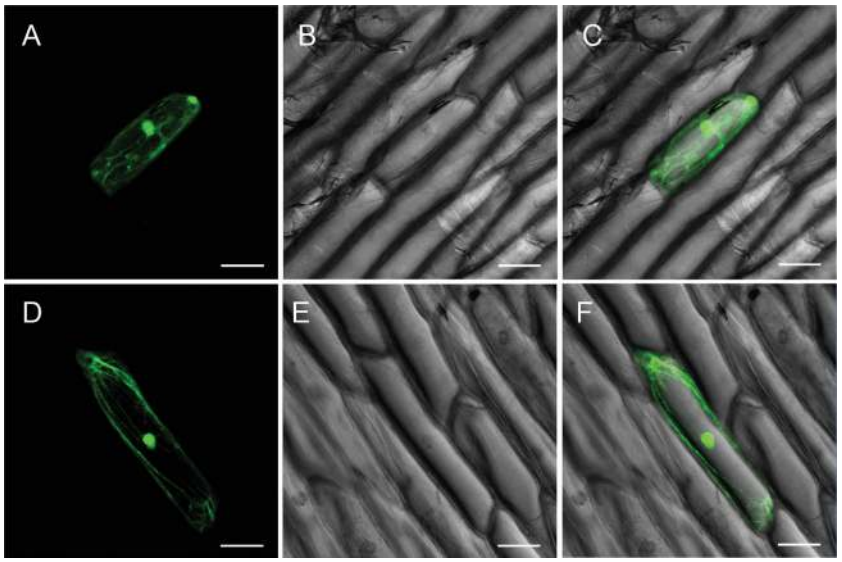

Fig. 2. Localization of SbbHLH1 deposition. (A–C) Expression of *GFP* only. Fluorescence was distributed throughout the whole cell. (D–F) Expression of the SbbHLH1–GFP fusion protein. Fluorescence was also broadly distributed in the cytoplasm and nucleus. Results are shown as GFP signal (A, D); differential interference contrast field (B, E) and merged images (C, F). Bars, 100 μm.

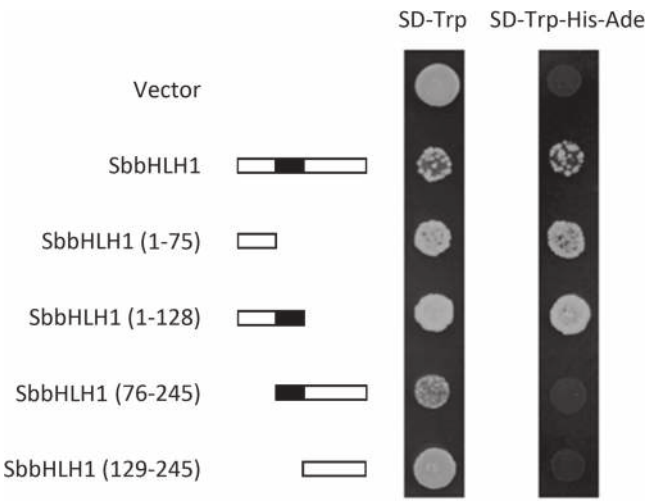

Fig. 3. Transactivation of SbbHLH1. The whole *SbbHLH1* ORF and four truncated fragments were each introduced into pGBKT7, and transformed yeasts selected on both SD–Trp and SD–Trp–His–Ade. Constructs containing SbbHLH1 residues 1–75 were functional and supported survival on the multiple amino acid-deficient medium. The filled bar indicates the SbbHLH1 bHLH domain. Trp, tryptophan; His, histidine; Ade, adenine.

was so frequently identified and was the most upregulated in the SHH library based on 13 *bmr* mutants. Transcription analysis showed that *SbbHLH1* was upregulated in the majority of these mutants. Its transcript abundance was least in the stem, the site of highest lignin content, and its product was deposited in the nucleus and cytoplasm. A transactivation assay of this gene produced positive results (Fig. 3), suggesting that it could work as a TF. The part of SbbHLH1 essential for its transcriptional activity proved to be within the first 75 residues from the N terminus.

Heterologous expression of *SbbHLH1* in *A. thaliana* did indeed reduce lignin content and stem strength (Fig. 4). Lignin has been shown to be essential for mechanical support of plants (Jones *et al.*, 2001; Zhao and Dixon, 2011). In a study of an *Arabidopsis irx4* mutant, a mutation was found in the first enzyme CCR1 of the lignin biosynthetic pathway and caused significantly decreased lignin content, while the cellulose and hemicelluloses content was remained unchanged. With this mutant and a population of transgenic *A. thaliana*, Jones *et al.* (2001) found there was a significant positive correlation between lignin content and stem strength by measuring the physical properties and lignin content of the stems. Besides the decreased lignin content (Fig. 4C–E), we also detected increased cellulose and hemicellulose content in the *SbbHLH1* transgenic *A. thaliana* (Fig. 4F, G). One explanation could be the compensatory mechanism, through which decreased lignin synthesis combines with increased cellulose and hemicellulose levels (Hu *et al.*, 1999; Vogler *et al.*, 2009; Ambavaram *et al.*, 2011). Thus, the lodging phenotype of *SbbHLH1* transgenic *A. thaliana* (Fig. 4B) could be explained by the reduced lignification level (Fig. 4C–E). These observations are all consistent with the role of *SbbHLH1* as a TF heavily implicated in the sorghum *bmr* phenomenon, and active as a repressor of lignin synthesis.

SbbHLH1 reduces lignin content by repressing lignin synthesis

Upstream repressors of the phenylpropanoid pathway have been characterized from several kinds of plants. Both the heterologous expression in *A. thaliana* of the maize TFs *ZmMYB31* (Fornale *et al.*, 2010) and *ZmMYB42* (Sonbol *et al.*, 2009) and the heterologous expression of *AmMYB308* and *AmMYB330* in tobacco (Tamagnone *et al.*, 1998) result in downregulation of lignin synthesis. Ambavaram *et al.*

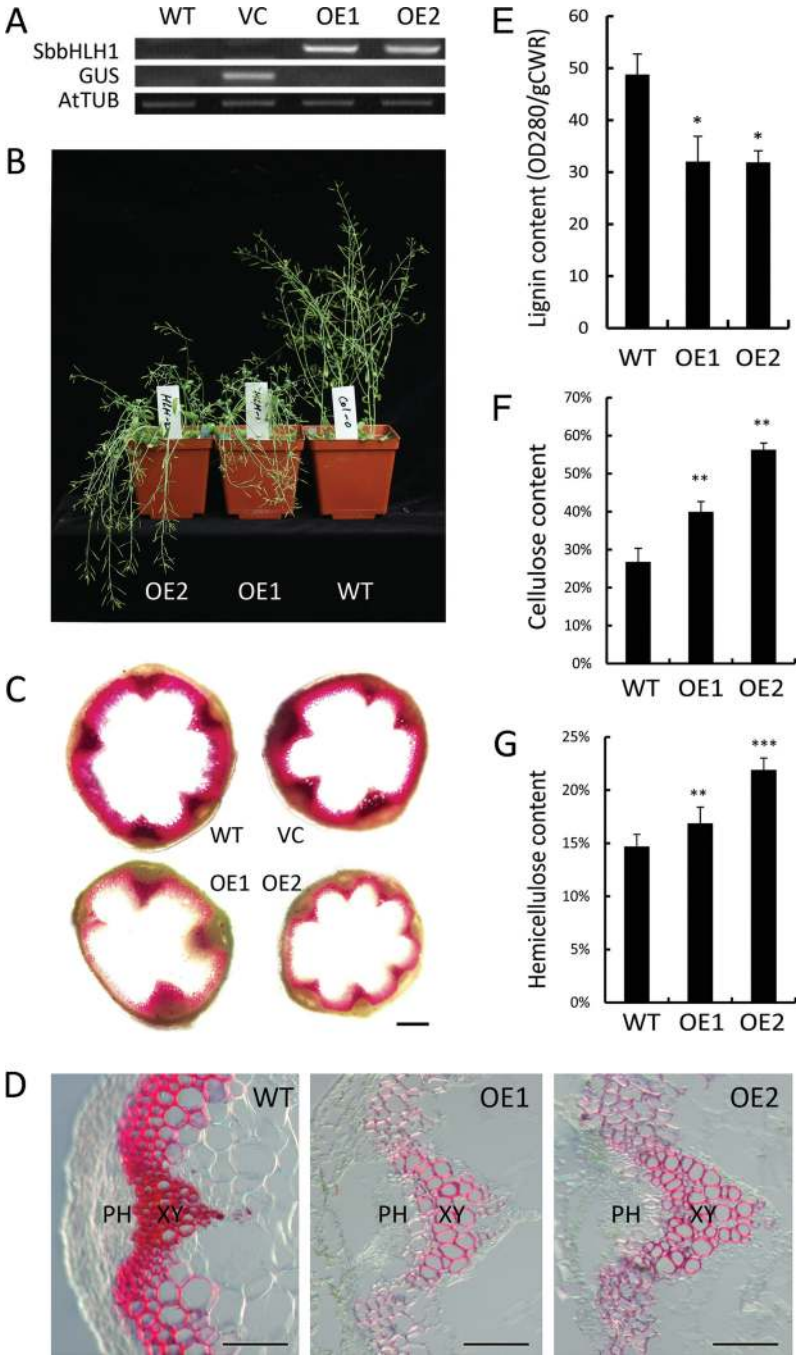

Fig. 4. Heterologous expression of *SbbHLH1* in *A. thaliana*. (A) sqRT-PCR analysis of T_3 lines OE1 and OE2. VC, pSTART vector control; WT, wild-type *A. thaliana* ecotype Columbia; OE1, OE2: transgenic *A. thaliana* lines carrying *SbbHLH1.* (B) The phenotype of OE1 and OE2 at flowering showed that expression of the *SbbHLH1* transgene weakened the stem's strength. (C) Lignin content as shown by Wiesner's staining of a stem section. Both the wild-type and the GUS vector control plants stained more intensely than the two transgenic lines. Bar, 200 μm. (D) One vascular bundle in the frozen sections of the stems stained with Wiesner's method. The lignin content of the transgenic plants was markedly lower than that of the wild type. PH, phloem; XY, xylem. Bars, 100 μm. (E) Quantification of lignin content. Lignin content was calculated by cell-wall residue. The lignin content of OE1 and OE2 was markedly less than that in the wild type. (F, G) Cellulose (F) and hemicellulose (G) content in wild-type and transgenic *A. thaliana*. Both were higher in the OE lines. Data are the mean value ±standard deviation of more than three biological repeats. Significance between transgenic lines and controls was evaluated with Student's *t*-test (*P <0.05; **P <0.01; ***P <0.001).

(2011) have shown in rice that the AP2/ERF family TF *SHN* decreases lignin synthesis by repressing *NST* and *MYB* activators. *EgMYB1* from eucalyptus (Legay *et al*., 2010), *PvMYB4* from switchgrass (Shen *et al*., 2012), and *LlMYB1* from *Leucaena leucocephala* (Omer *et al*., 2013) were also found to be lignin synthesis repressors. The promoters of most

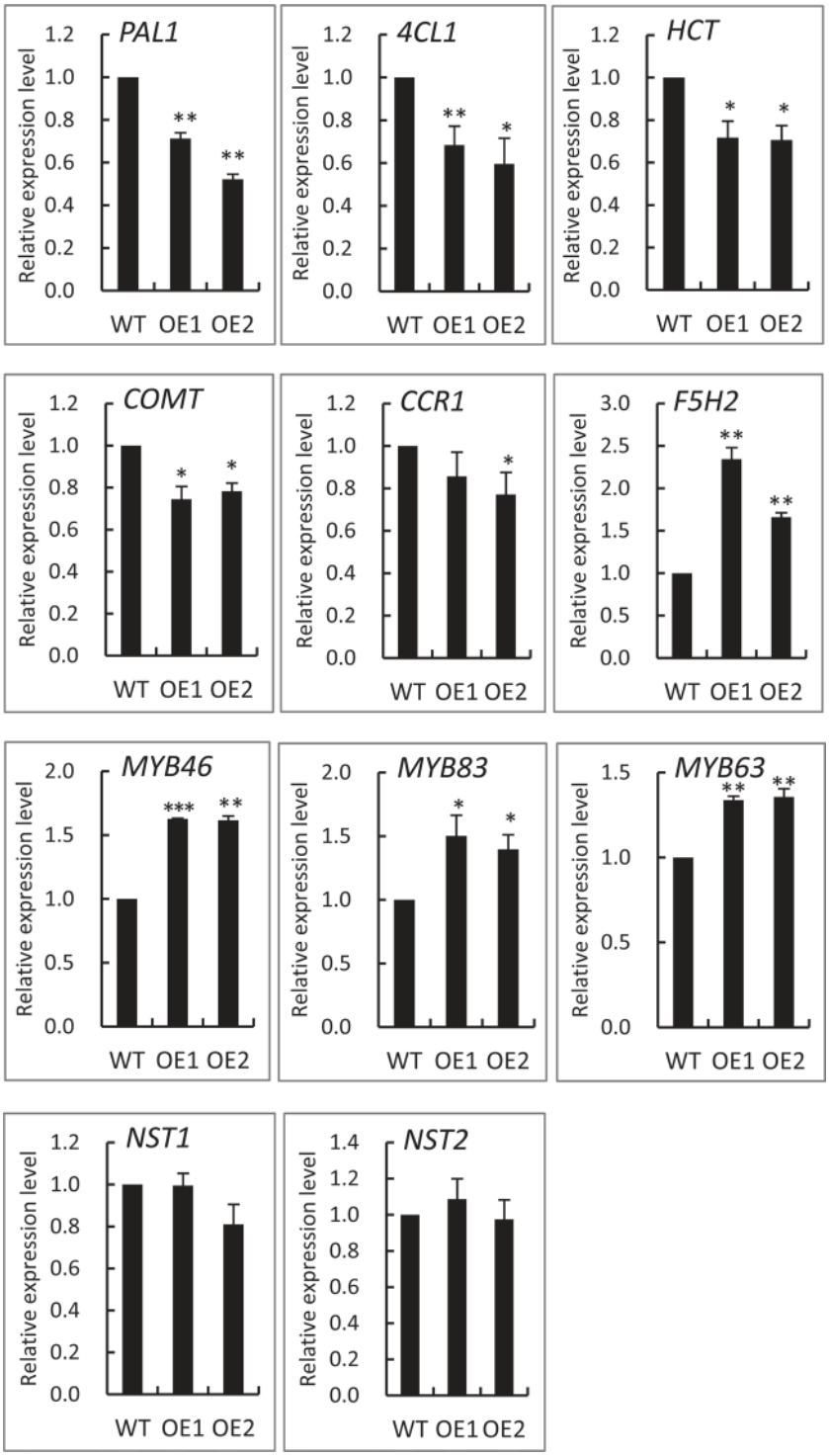

Fig. 5. qRT-PCR analysis of transcription in *A. thaliana* caused by the presence of the *SbbHLH1* transgene. The lignin synthesis genes were broadly downregulated, but the MYB TFs were upregulated. WT, wild- type *A. thaliana* ecotype Columbia; OE1, OE2, transgenic *A. thaliana* lines carrying *SbbHLH1.* Data are the mean value ±standard deviation of more than three biological repeats. Significance between transgenic lines and wild type was evaluated with Student's *t*-test (*P <0.05; **P <0.01; ***P <0.001).

phenylpropanoid pathway genes include AC-rich elements, which are diagnostic for MYB binding (Zhao and Dixon, 2011). The present experiments have shown that the lignin synthesis- associated genes *4CL1*, *HCT*, *COMT*, *PAL1*, and *CCR1* were all downregulated in transgenic *A. thaliana* plants expressing *SbbHLH1* (Fig. 5 and Supplementary Fig. 3), which explains the decreased lignin content.

F5H1, in contrast, is active in a different pathway, and is directly activated by *NST* rather than by *MYB* TFs (Zhao and Dixon, 2011). The *A. thaliana* genome carries two copies of

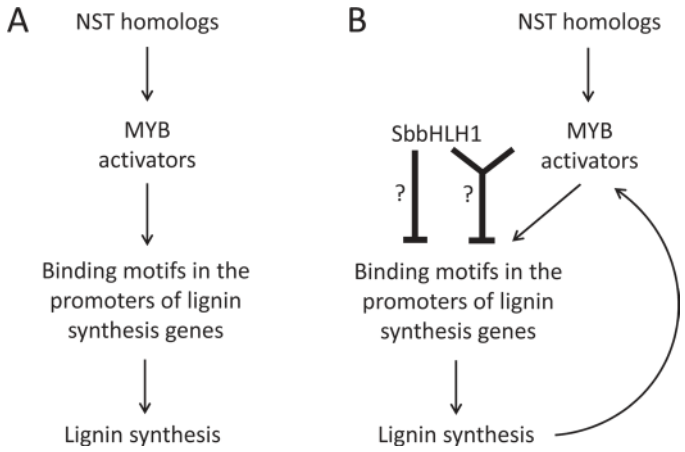

Fig. 6. Alternative models to explain how *SbbHLH1* represses lignin synthesis. (A) In wild-type *A. thaliana*, NST homologues activate certain MYB activators, which promote lignin synthesis by recognizing key AC-rich elements present in the synthesis gene promoters. (B) When the *SbbHLH1* transcript is present, it interacts with lignin synthesis gene promoters more strongly than the MYBs, thereby suppressing lignin synthesis. Alternatively, SbbHLH1 and the MYBs form a complex that represses lignin synthesis. The decreased lignin content feeds back to upregulate the expression of the MYB activators. Bolder lines indicate stronger effects.

F5H, one on chromosome 4 (*F5H1*) and the other on chromosome 5 (*F5H2*) (Costa *et al*., 2003; Raes *et al*., 2003; Xu *et al*., 2009; Zhao *et al*., 2010); the latter is 76.6% similar and 67.6% identical to the former (Costa *et al*., 2003). *F5H1* is known to be involved in lignin synthesis (Meyer *et al*., 1998; Reddy *et al*., 2005; Ehlting *et al*., 2006), and *MYB103* is needed for its function (Öhman *et al*., 2013). Expression of it is strongest in leaves and roots, and increases during inflorescence stem development (Raes *et al*., 2003). Expression of *F5H2* is different with *F5H1*: it is only strong in the young inflorescence stem and is weak in other tissues (Raes *et al*., 2003). *F5H2* cannot complement the *fah1* mutant (defective in *F5H1*) phenotype (Ehlting *et al*., 2006; Weng *et al*., 2012). All these data suggest that the two copies of the *F5H* gene in *A. thaliana* have different functions, which was shown by Weng *et al*. (2012), who found that *F5H2* (also named *CYP84A4*) and *F5H1* (*CYP84A1*) are not redundant. F5H2 use *p*-coumaraldehyde as a substrate and initiates a newly evolved metabolic pathway producing the previously unknown metabolites arabidopyrones, while the substrates of F5H1 are coniferaldehyde and coniferyl alcohol (Weng *et al*., 2012). *F5H1* transcription was not noticeably altered by the presence of the *SbbHLH1* transgene, but *F5H2* transcript abundance was markedly increased (Fig. 5 and Supplementary Fig. 3), suggesting that *SbbHLH1* could regulate this secondary metabolic pathway in *A. thaliana*.

A scenario for the action of SbbHLH1 in the regulation of lignin synthesis

In addition to repressors, transcriptional activators of lignin synthesis are known. For example, NST homologues can activate both *MYB46* and *MYB83* (McCarthy *et al*., 2009; Zhong *et al*., 2007*a*), the effect of which is to enhance lignin synthesis via the activation of *MYB58*, *MYB6*, and *MYB85* (Zhou *et al*., 2009). NST homologues can also promote S-lignin synthesis via the direct regulation of *F5H1*, and this promotion helps to adjust the S/G lignin ratio in the cell wall (Zhao *et al*., 2010). In the present experiments, the transcription of *NST1*, *NST2*, and *NST3* was not affected by the presence of the *SbbHLH1* transgene, but a number of downstream factors (*MYB46*, *MYB83*, *MYB58*, and *MYB63*) were clearly upregulated. However, the higher level of transcription expression of these activators did not drive up lignin content, so it would appear that *SbbHLH1* is more important than any of the *MYB* activators in the context of regulating lignin synthesis. As *bHLH* products have TF functionality, this effect could be realized through repression of the promoters of the lignin synthesis genes. It has been reported that some bHLH proteins can complex with MYB and WD-repeat TFs and are involved in the regulation a broad range of biological processes, including hormone signalling (Qi *et al*., 2011), trichome development (Ramsay and Glover, 2005; Zhao *et al*., 2008, 2012; Qi *et al*., 2011), root hair patterning (Zhao *et al*., 2012), and secondary metabolism pathways, such as alkaloid biosynthesis (Yamada *et al*., 2011), flavonoid synthesis (Hichri *et al*., 2011*a*,*b*, 2010; Matousek *et al*., 2012) and anthocyanin accumulation (Appelhagen *et al*., 2011; Paolocci *et al*., 2011; Qi *et al*., 2011). Thus, it is also possible that SbbHLH1 combines with one or other of the upregulated MYB activators to form part of a repressive complex. As the transcription of the various *MYB* activators was increased in the *A. thaliana* plants synthesizing a lower amount of lignin, a feedback regulation mechanism may also be operating.

Two possible models to address the function of *SbbHLH1* are presented in Fig. 6. In the absence of *SbbHLH1*, NST homologues upregulate the MYB activators, which promote lignin synthesis by binding to the promoters of various lignin synthesis genes (Fig. 6A). When *SbbHLH1* transcript is present, expression of the lignin synthesis genes is suppressed, thereby reducing the lignin content. This in turn upregulates expression of the MYB activators (Fig. 6B). Alternatively, SbbHLH1 combines with MYB activators, thereby suppressing their activation and hence leading to a repression of lignin

synthesis gene expression (Fig. 6B). This repression may also involve other protein factors, for example, WD-repeat proteins.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table 1. Sequences of primers used for transcription analysis.

Supplementary Fig. 1. Vectors used in the subcellular localization assay of *SbbHLH1*.

Supplementary Fig. 2. Diagram of pGBKT7 and AH109 for the transactivation assay.

Supplementary Fig. 3. sqRT-PCR of the transcription in *A. thaliana* caused by the *SbbHLH1* transgene.

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