

Distinct deposition of ester-linked ferulic and *p*-coumaric acids to the cell wall of developing sorghum internodes

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Abstract Sorghum is important as a cereal crop, and also as livestock feed and a renewable energy crop because it produces a large amount of biomass. In grass plants like sorghum, hydroxycinnamates such as ferulic acids (FA) and *p*-coumaric acids (*p*CA) are characteristically ester-linked to the cell wall, and are believed to affect cell wall digestibility. Genetic manipulation of the esterification of FA and *p*CA to the cell wall appears to be one of the solutions to increase the digestibility of the cell wall so as to utilize sorghum biomass effectively. In this study, we measured esterified FA and *p*CA in each stage of internode elongation and determined the accumulation pattern of each hydroxycinnamate. The results revealed that FA were mainly accumulated during the cell elongation stage, and that *p*CA accumulation was increased after the cell elongation stage. Furthermore, 6 of the 12 sorghum BAHD acyltransferase genes were significantly expressed in the elongating internodes, suggesting that these genes might be involved in the feruloylation and/or *p*-coumaroylation of the cell wall in sorghum internodes.

Key words: BAHD acyltransferase, ferulic acid, *p*-coumaric acid, sorghum.

Introduction

Sorghum is an important cereal crop that is mainly cultivated in Asian and African countries. It adapts well to arid areas that are not suitable for growing other major cereals. Sorghum has the capacity to produce a large amount of biomass, and is therefore also used as livestock feed. Sorghum has recently received attention as a source of renewable biofuel energy in many countries, including Japan. Because sorghum is the first C4 crop with a full genome sequence available (Paterson et al. 2009), there is potential to improve the digestibility of biomass toward the production of nutritious feed and highly efficient energy crops through gene manipulation.

The main source of plant biomass is the cell wall. Grass cell walls such as those in sorghum have Type II cell walls that differ from those of dicotyledonous plants.

One of the characteristic features of Type II cell walls is that they are rich in low molecular hydroxycinnamates, which are ester-linked to the primary and secondary cell walls (Iiyama et al. 1994; Mueller-Harvey et al. 1986; Vogel 2008). The two major hydroxycinnamates are ferulic acid (FA) and *p*-coumaric acid (*p*CA). These hydroxycinnamates, especially FA, are ester-linked to arabinoxylan (AX) (Faulds et al. 2004; Hatfield et al. 1999; Ishii et al. 1990; Ralph 2010; Saulnier et al. 1995; Vogel 2008), and are believed to provide grass cell wall indigestibility (Barros-Rios et al. 2012; Ishii 1997). It has been suggested that feruloylated AX can form cross-linkages between cell wall materials such as between hemicelluloses, and between hemicellulose and lignin (Grabber et al. 2000; Iiyama et al. 1990; Ishii 1991; Ishii 1997; Ralph et al. 1995; Saulnier et al. 1999; Vogel 2008). Such cross-linking would influence the properties of

Abbreviations: AIR, alcohol-insoluble residue; AX, arabinoxylan; BSA, bovine serum albumin; FA, ferulic acid; FW, fresh weight; GC, gas chromatography; IgG, immunoglobulin G; *p*CA, *p*-coumaric acid; PBS, phosphate buffered saline; RT-PCR, reverse transcription polymerase chain reaction; SD, standard deviation; TUB, tubulin.

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grass cell walls, particularly terminating cell extensions, cell wall rigidity, and defense responses towards pests (Barros-Rios et al. 2012, 2015; Brisson et al. 1994; Iiyama et al. 1994; Kamisaka et al. 1990; Lee et al. 1997). In contrast, *p*-coumaroyl esters are abundant in lignin. It has been proposed that *p*CA is incorporated into the cell wall as a conjugate with monolignol, especially sinapyl alcohol (Hatfield et al. 2009; Ralph et al. 1994), and may facilitate lignin polymerization by passing radicals to sinapyl alcohol (Chabannes et al. 2001; Ralph 2010; Takahama and Oniki 1994).

Potential candidate genes for feruloylation and *p*-coumaroylation to the cell wall have been identified in rice based on bioinformatics study (Mitchell et al. 2007). The 12 identified genes encode Pfam PF02458 proteins (which contain the PFAM domain) that belong to the CoA-acyl transferase family and were specifically expressed in grasses. These proteins have been named BAHD acyl transferases based on the four biochemically characterized enzymes of this family: BEAT (benzylalcohol *O*-acetyltransferase), AHCT (anthocyanin *O*-hydroxycinnamoyltransferase), HCBT (anthranilate *N*-hydroxycinnamoyl/benzoyltransferase), and DAT (deacetylvindoline 4-*O*-acetyltransferase) (D'Auria 2006). Subsequently, several analyses supporting the function of the BAHD genes identified by Mitchell et al. (2007) have been reported. Piston et al. (2010) reported that the expression levels of four genes (LOC_Os01g09010, LOC_Os06g39390, LOC_Os06g39470 and LOC_Os05g08640) belonging to the BAHD acyl-transferase in rice were correlated to the FA contents of rice cell walls. In contrast, it has been proposed that one of the four genes (LOC_Os06g39390) is involved in the esterification of *p*CA to AX based on the phenotype of overexpression of this gene (Bartley et al. 2013). For other genes, the enzymes encoded by LOC_Os01g18744 (OsPMT) and LOC_Os05g19910 (OsFMT1) have been shown to possess enzymatic activity to esterify *p*CA and FA to monolignol, respectively (Karlen et al. 2016; Withers et al. 2012). Furthermore, the homologous genes of LOC_Os01g09010 in *Setaria viridis* and LOC_Os01g42880 in *Brachypodium* have been proposed to be involved in the synthesis of feruloylated AX (Buanafina et al. 2016; de Souza et al. 2018). Therefore, the 12 genes identified by Mitchell et al. (2007) are very likely to be involved in the esterification of hydroxycinnamates to the cell wall.

Here, we measured esterified FA and *p*CA in each stage of elongating internodes of sorghum variety Tx430, and showed the accumulation pattern of each hydroxycinnamate. Our results demonstrated that FA were mainly accumulated during the cell elongation stage and that *p*CA accumulation progressed after the cell elongation stage. Among the 12 sorghum BAHD genes that are homologous to rice genes identified by Mitchell et al. (2007), 6 genes were significantly expressed

in the elongating sorghum internodes, suggesting that they might be involved in the feruloylation and/or *p*-coumaroylation of the cell wall component in sorghum.

Materials and methods

Plant materials and growth conditions

Seeds of the sorghum inbred line Tx430 were obtained from the genebank at the National Agriculture and Food Research Organization (Ibaraki, Japan). Tx430 was cultivated for approximately 3 months in a temperature-controlled greenhouse at 28°C during the day and 23°C during the night under natural light conditions.

Histochemical and immunohistochemical observation of sorghum internodes

Samples of the first internodes were harvested during internode elongation when the ear emerges from the leaf sheath and fixed in formalin : acetic acid : 50% ethanol (1:1:18) overnight at room temperature. Each fixed segment was embedded in paraffin wax according to the previously described method (Sato et al. 2011). For anatomical analysis, 10- μ m-thick sections were cut using a stainless steel knife on a microtome, de-waxed with xylene, and hydrated through an ethanol series (50–100%). The sections were stained with 0.25% Toluidine Blue O and observed using a LEICA DM6 B light microscope (LEICA, Wetzlar, Germany). For lignin observation, 60- μ m-thick sections were stained with 50 μ l of phloroglucinol ethanol solution (20 mg ml⁻¹) for 1 min on slide glass and observed immediately after adding 20 μ l of hydrochloric acid. For immunohistochemical observation of feruloylated polymer, 20- μ m-thick sections were blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, adjusted to pH 7.3) for 60 min at room temperature, and then treated with rat monoclonal antibody LM12 (Pedersen et al. 2012) that had been diluted 1:10 with 1% BSA in PBS for 60 min at room temperature. The sections were washed five times with PBS and incubated for 60 min at room temperature with Alexa Fluor 488-conjugated antibody against rat immunoglobulin G (IgG) that had been diluted 1:50 with 1% BSA in PBS. The immunolabeled samples were washed five times with PBS, stained with Calcofluor White M2R (Fluorescent Brightener 28; Merck, Darmstadt, Germany) for 10 min, and then washed three times with PBS. The sections were observed using a Carl Zeiss LSM710 confocal laser-scanning microscope under 448 nm and 405 nm lasers (Carl Zeiss, Oberkochen, Germany). Fluorescent images generated with Calcofluor White (blue) and Alexa Fluor 488 (green) were merged using ZEN software (Carl Zeiss).

Analysis of cell wall-linked hydroxycinnamoyl esters in the cell wall

The level of hydroxycinnamic acids linked to the cell walls via alkali-labile linkages, such as ester bonds, in cell wall residues

was measured following alkaline hydrolysis of the alcohol-insoluble residue (AIR). The AIR was prepared using the modified procedure described in a previous report (Pettolino et al. 2012). The elongating first internode was divided into five parts of 3 cm each from the basal part and were ground into powder using a multi-beads shocker (Yasui Kikai, Osaka, Japan) with liquid nitrogen. The samples were treated three times with 80% ethanol at 60°C for 20 min, acetone at room temperature for 10 min, and methanol at room temperature for 10 min, and then the residue was dried at 60°C for 1 to 2 days. The resultant powder was incubated in 10 mM Tris-maleate buffer (pH 6.9) at 100°C for 10 min to gelatinize the starch granules, and then starch was digested twice in α -amylase solution (2 U mg⁻¹ of carbohydrate in 10 mM Tris-maleate buffer) from porcine pancreas (Merck) at 40°C for 1 h. Next, four volumes of cold absolute ethanol were added and the temperature was maintained at -20°C for 1 h. The residue was washed three times with absolute ethanol, and then dried at 60°C for 1 to 2 days. The dried AIR (approximately 20 mg) was treated with 5 ml of 1 M sodium hydroxide at room temperature for 24 h, and subsequent experiments proceeded according to the previously described method (Nishikubo et al. 2000). Hydroxycinnamates were analyzed by gas chromatography (GC) on a capillary column DB-5 (Agilent Technologies, CA, USA).

Multiple alignment and phylogenetic tree construction

The amino acid sequences of BAHD acyltransferase from rice (Mitchell et al. 2007; Piston et al. 2010) and sorghum were aligned using CLUSTALW (Thompson et al. 1994). A neighbor-joining analysis (Saitou and Nei 1987) of the complete protein alignments was performed using MEGA X software (Kumar et al. 2018). The parameter gap/missing was set as 'Pairwise deletion' with the substitution models using the 'Poisson model' (Zuckerkanndl and Pauling 1965). The confidence of the different nodes was calculated using 1,000 bootstrap iterations (Felsenstein 1985).

Expression analysis of BAHD genes

Total RNA was extracted with an RNeasy plant mini kit (Qiagen, Hilden, Germany) from each stage of elongating internode. The first-strand cDNA was synthesized using ReverTra Ace[®] RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Reverse transcription polymerase chain reaction (RT-PCR) was performed using KOD One[®] PCR Master Mix under the following conditions: 94°C for 2 min; 37 cycles at 98°C for 10 s, 60°C for 5 s, 68°C for 30 s. Real-time PCR was performed using KOD SYBR[®] qPCR Mix (Toyobo) on a Thermal Cycler Dice[®] real time system III (TaKaRa Bio, Shiga, Japan) under the following conditions: 98°C for 2 min; 60 cycles at 98°C for 10 s, 63°C for 10 s, 68°C for 30 s; 95°C for 15 s, 60°C for 30 s, 95°C for 15 s. The primers used are listed in Supplementary Table 1.

Results and discussion

Developmental stage of elongating internodes

In grass plants such as sorghum, the internodes rapidly elongate during heading (Figure 1A, B). When the ear emerges from the leaf sheath, the first internode that is immediately below the panicles is just elongating (Figure 1B, C). Various biological processes, including cell elongation, cell elongation arrest, and secondary cell wall synthesis, occur during internode elongation. Generally, the basal part of the elongating internode in grass plants consists of young cells (including dividing and elongating cells), and these cells mature towards the apex parts by forming secondary cell walls (Kende et al. 1998; Lin et al. 2017). In order to characterize the cell development stage of the elongating internode in sorghum Tx430, we observed internode segments taken from a 1.5, 4.5, 7.5, 10.5, and 13.5 cm distance from node II (Figure 2).

The transverse section stained with Toluidine Blue O showed that many parenchyma cells have nuclei and cytoplasm in the section at 1.5 cm (Figure 2B), suggesting that these cells were in the pre- or early stage of cell elongation. In the transverse sections at 4.5 cm and 7.5 cm, the number of parenchyma cells containing nuclei and cytoplasm were decreased. In this stage, phloroglucinol staining of lignin (magenta) was observed only in protoxylem cell walls (Figure 2C). These observations indicate that cell elongation accompanied by vacuolar enlargement occurs in this stage. In the sections at 10.5 cm and 13.5 cm, the cell walls of the sclerenchyma and vascular bundle became thick (Figure



Figure 1. Growth stages of the sorghum plant (Tx430) used in this research. (A) Tx430 after internode elongation. (B) Tx430 during internode elongation in the heading stage. (C) Nodes (indicated by arrowheads) and internodes in the heading stage. The elongating first internode is indicated by an asterisk. Bars = 10 cm.

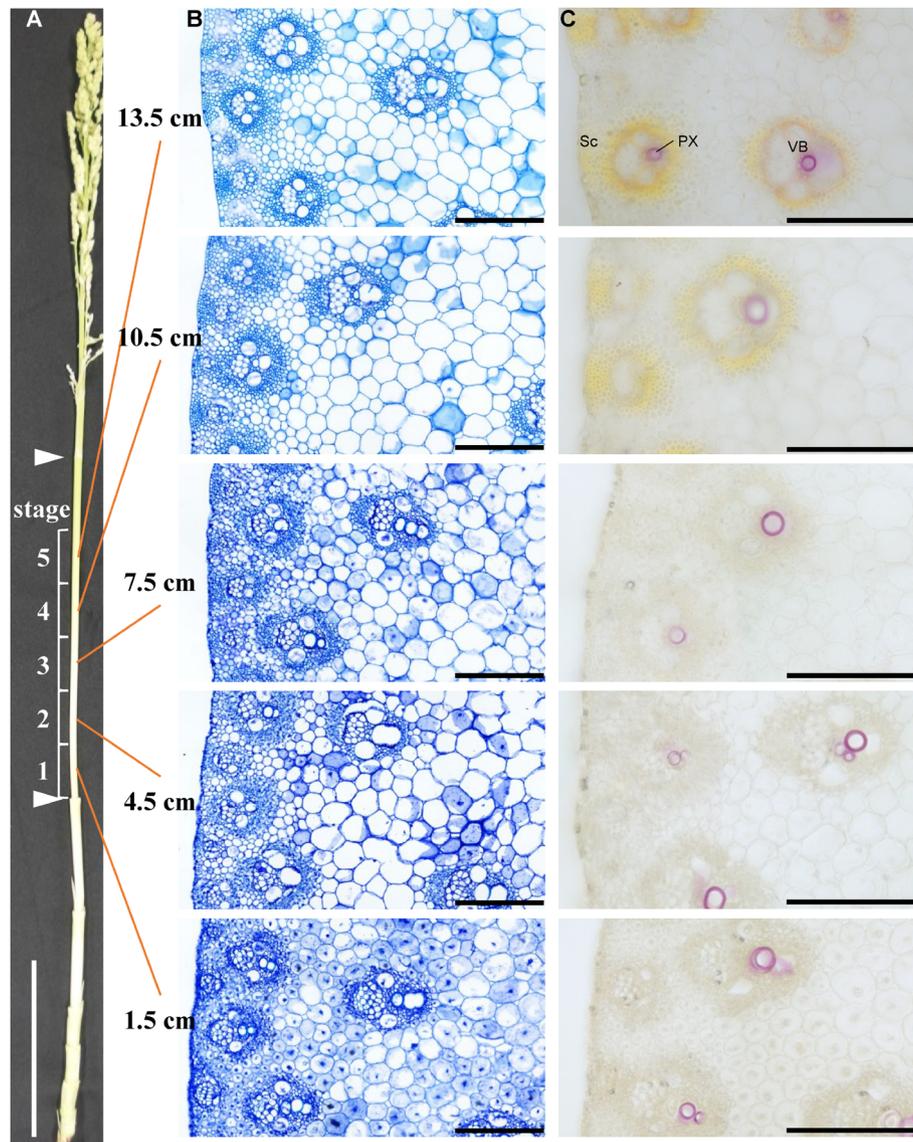


Figure 2. Histochemical observation of elongating sorghum internodes. (A) The positions of the sections for observation and segments of stage 1 to 5 for subsequent analysis are shown. Bar=10 cm. (B) Cross sections stained with Toluidine Blue O from the basal to the apical part. (C) Cross sections stained with phloroglucinol staining of cell wall lignin. In the mature apical part, the phloroglucinol staining was observed in the protoxylem and vascular bundles, indicating the progress of the secondary cell wall formation. Bars=200 μ m. PX, protoxylem; Sc, sclerenchyma; VB, vascular bundle.

2C). The section at 13.5 cm showed phloroglucinol staining in the vascular bundle, indicating that the secondary cell walls were actively formed in these stages. These observations showed that the elongating internodes of sorghum contain tissues in various stages of growth, namely, before cell elongation, during cell elongation, and in secondary cell wall formation, from the basal to the apical part. Therefore, it is possible to analyze cell walls following the growth stage using elongating internodes. In subsequent experiments, we divided elongating internodes into five parts of 3 cm each, from the basal to the apical part, that is, from the young to the mature part (stage 1–5, Figure 2A).

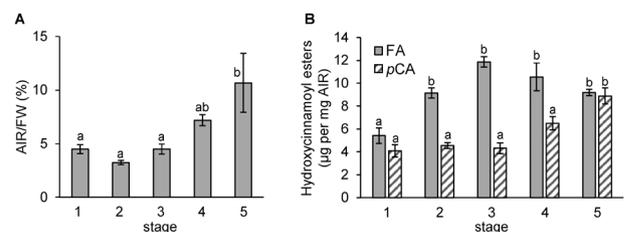


Figure 3. Cell wall component levels in stages 1 to 5 of the elongating internodes shown in Figure 2A. (A) Alcohol-insoluble cell wall residues (AIR) were expressed as a percentage of fresh weight (FW). The levels were increased from stage 3 to 5. (B) Alkaline-soluble ester-linked FA and pCA levels in each stage of internodes. Statistical analysis was performed by Tukey's test. Different letters indicate significant difference at $p < 0.05$ ($n = 3$). Values are means \pm standard error (SE).

Hydroxycinnamoyl ester contents in each stage of elongating internodes

Type II cell walls, found only in commelinoid monocotyledonous plants such as sorghum, are specifically rich in low-molecular-weight hydroxycinnamates (*p*CA and FA) that are ester-linked to AX (Faulds *et al.* 2004; Hatfield *et al.* 1999; Ishii *et al.* 1990; Ralph 2010; Saulnier *et al.* 1995; Vogel 2008). *p*CA has also been demonstrated to be esterified to lignin (Lu and Ralph 1999; Ralph 2010). We examined the content of cell wall-linked hydroxycinnamoyl esters in the cell

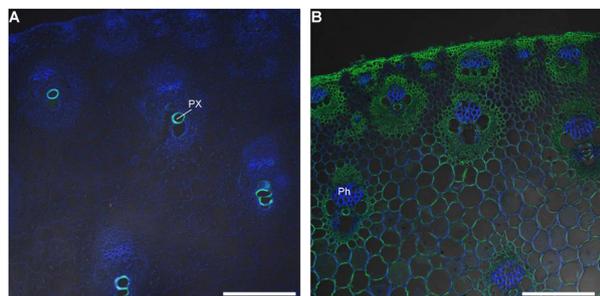


Figure 4. Immunohistochemical localization of feruloylated polymers using LM12 monoclonal antibody. (A) Stage 1 section 1.5 cm from the basal part, showing FA localized only in protoxylem. (B) Stage 3 section 7.5 cm from the basal part, showing FA localized in all tissues except for phloem. Bars=200 μ m. Ph, phloem; PX, protoxylem.

wall fraction from each developmental stage. The amount of α -amylase-treated AIR/fresh weight (FW) represents the overall quantity of cell walls synthesized by a plant (Sakamoto *et al.* 2018). The AIR/FW of internodes in stage 1 to 3 had no significant difference (4.49%, 3.24% and 4.49%, respectively; Figure 3A). In contrast, the AIR/FW in stage 4 and 5 were 7.19% and 10.68%, respectively, indicating significant increase (Figure 3A). These data were consistent with the results of phloroglucinol staining (Figure 2C), and showed that the secondary cell walls are being actively synthesized in stage 4 and 5.

Cell wall-linked hydroxycinnamoyl esters (FA and *p*CA) were examined by mild alkaline hydrolysis with subsequent analysis by GC. The FA content rapidly increased from stage 1 to stage 3 and was slightly decreased in stage 4 and 5 (Figure 3B). In contrast, the content of *p*CA was almost constant in stages 1 to 4 and was subsequently increased in stage 5. These results show that FA esters were actively synthesized and deposited during the cell elongation stage prior to secondary cell wall formation and that *p*CA esters were actively deposited during secondary cell wall formation. The content of cell wall-linked hydroxycinnamoyl esters in different growth stages is similar to that previously reported in rice (Bartley *et al.* 2013; Lin *et al.* 2017). The ester-linked ferulate has been shown to form cross-links between cell wall polysaccharides such as AX,

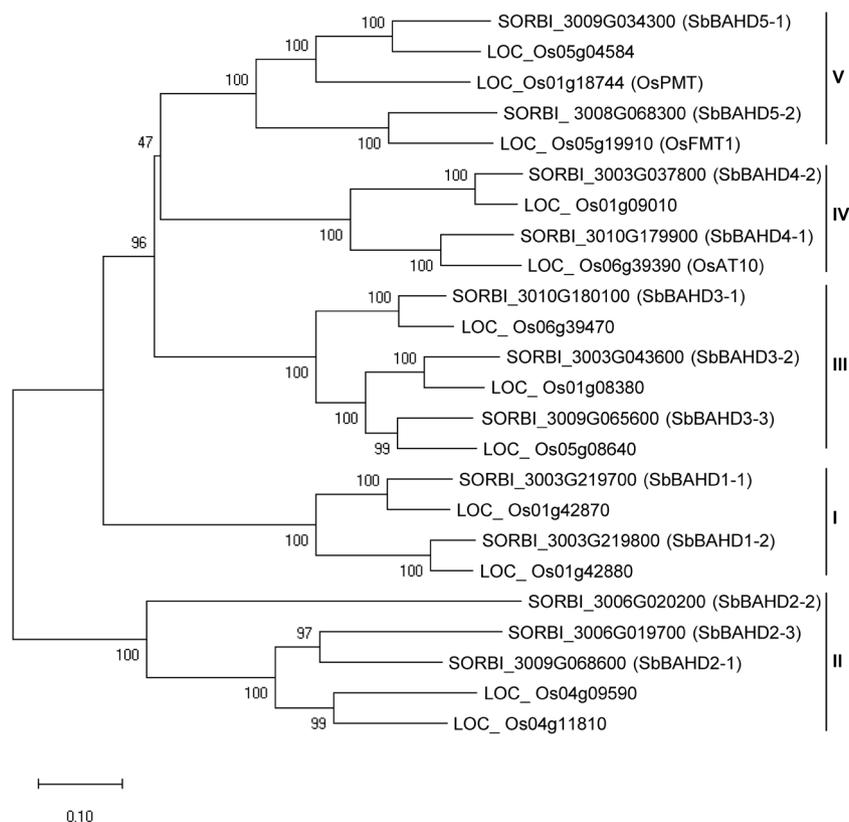


Figure 5. Phylogenetic tree of BAHD acyltransferases in sorghum and rice. Percentage bootstrap values of 1000 replicates are given at each branch point. The proteins were grouped into five clades (I-V). The scale bar is an indicator of genetic distance based on branch length.

and between polysaccharide and lignin (Grabber et al. 2000; Iiyama et al. 1990; Ishii 1991, 1997; Ralph et al. 1995; Saulnier et al. 1999; Vogel 2008). These cross-links between cell wall compositions are thought to hold biological roles, such as in cell wall maturity after cell extension (Kamisaka et al. 1990; Kondo et al. 1990) and cell wall protection against pests (Barros-Rios et al. 2012, 2015; Iiyama et al. 1994; Lee et al. 1997), and chemical properties that are resistant to enzymatic digestibility (Barros-Rios et al. 2012; Ikegawa et al. 1996; Ishii 1997). The function of *pCA* is not as well understood as that of FA. Ester-linked *pCA* is known to be rich in lignin, and some studies have suggested that it has the capacity to transfer radicals to other monolignols to facilitate lignin polymerization (Ralph 2010). In this study, *pCA* was increased in stage 4 to 5, suggesting that it plays a role in lignin polymerization during secondary cell wall formation.

Distribution of ferulic acid in elongating internodes

To confirm the deposition of FA in the sorghum stem, we performed immunohistochemical observation using LM12 antibodies that detect feruloylated polymers (Pedersen et al. 2012). At the lowest internodes in stage 1 (1.5 cm from the bottom), green fluorescence of LM12 was only observed in protoxylem vessels (Figure 4A). In contrast, for sections in stage 3 (7.5 cm from the bottom), internodes showed green fluorescence of LM12 in all tissues except for phloem tissues (Figure 4B). The protoxylem had thick secondary cell walls even in the early stage of internode development, suggesting that FA did not accumulate in young cells before cell elongation, but actively deposited during cell elongation—this is consistent with the results of cell wall-linked hydroxycinnamoyl esters analysis (Figure 3B). Although the FA accumulation in all tissues except for phloem is a very interesting phenomenon, the reason remains unknown. Cao et al. (2014) and Xue et al. (2013) observed the internodes of *Miscanthus* by various cell wall polysaccharide monoclonal antibodies and showed that cell wall polysaccharide distributed differently in each genotype, tissue, and cell type. Because FA is ester-linked to AX, which is one of the cell wall polysaccharides, the absence of feruloylated polymers detected by LM12 in phloem may reflect the distribution of AX.

Identification of putative sorghum feruloyl-transferase sequences and their expression

Amino acid sequences of rice BAHD acyltransferase genes (Mitchell et al. 2007; Piston et al. 2010) were obtained from the Rice Genome Annotation Project Database (Kawahara et al. 2013; Sakai et al. 2013) and were Blastp searched against the sorghum database (http://ensembl.gramene.org/Sorghum_bicolor/

Info/Index). Twelve sequences were identified and the phylogenetic tree of the putative BAHD genes of sorghum and rice was developed using MEGA X. These sorghum genes were grouped into five clades (group I–V, Figure 5), as were the rice genes previously reported (Piston et al. 2010). Amino acid sequences within groups had 75 to 99% similarity whereas sequences from different groups had 67 to 82% similarity.

All putative BAHD genes from sorghum were tested for expression by RT-PCR with cDNA derived from elongating internodes and seedlings. SbBAHD2-1, 2-2, 2-3, 3-2, 4-1 and 5-2 were not amplified in cDNA from elongating stems (Figure 6). The expression of genes belonging to group II was not detected, which is consistent with the results of the above ground organs of rice reported by Piston et al. (2010). Therefore, it is suggested that genes in group II are not involved in the synthesis of cell wall-bound hydroxycinnamoyl esters in sorghum internodes. The other six genes, SbBAHD1-1, 1-2, 3-1, 3-3, 4-2 and 5-1, showed amplification of each specific band, and we thus performed real-time PCR of these genes. The results showed that every gene was

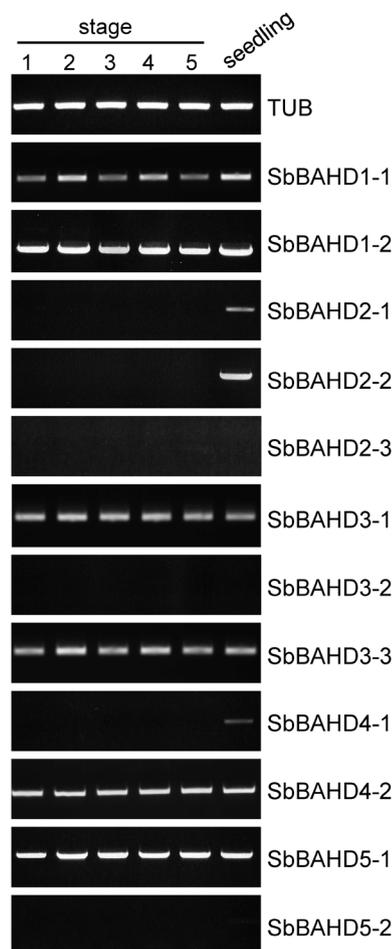


Figure 6. Reverse transcriptional PCR of SbBAHD genes amplified from cDNA derived from each stage of elongating internodes and seedlings. Alpha-tubulin (TUB) was used as an RT-PCR control.

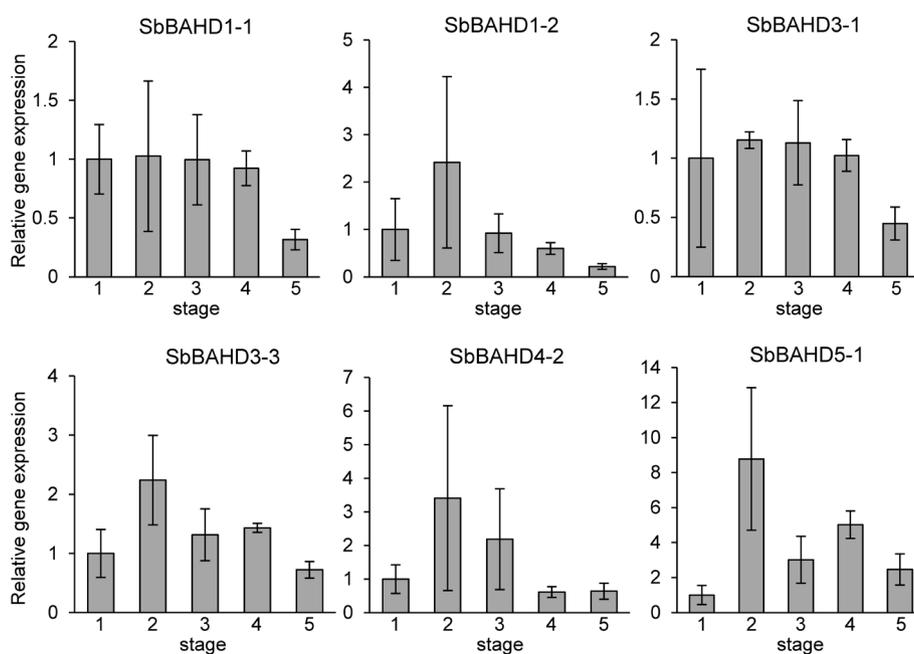


Figure 7. Expression levels of SbBAHD genes in each stage of elongating internodes measured by real-time PCR. The expression of α -tubulin was used as an internal control. Values are means of three biological replicates \pm SD ($n=3$).

expressed in the elongating internodes (Figure 7), although statistically significant differences were not detected among growth stages by Tukey's test.

Buanafina et al. (2016) reported that changing the expression of the *Brachypodium* BAHD gene Bradi2g43520, a homologue of rice LOC_Os01g42880 in group I, altered the levels of cell wall-esterified ferulates, suggesting that this gene could be a candidate for the feruloylation of AX in *Brachypodium*. Both sorghum genes belonging to group I were expressed in the elongating internodes, suggesting that these genes could be involved in the feruloylation of AX. For groups III and IV, Piston et al. (2010) suggested that the expression levels of four genes belonging to group III (LOC_Os05g08640 and LOC_Os06g39470) and IV (LOC_Os01g09010 and LOC_Os06g39390) were associated with the FA content of the cell walls. Furthermore, the LOC_Os01g09010 homologue of *S. viridis* has also been shown to play a key role in AX feruloylation (de Souza et al. 2018). In sorghum internodes, the homologues of these four genes except for BAHD4-1, a homologue of LOC_Os06g39390, were expressed. This result suggests that the expressed gene in group III and IV in sorghum might synthesize FA esters linked to cell wall components. Bartley et al. (2013) suggested that LOC_Os06g39390 in group IV is involved in the synthesis of esterified *p*CA bound to AX. Because the expression of SbBAHD4-1, a homologue of LOC_Os06g39390, was not detected in sorghum internodes, other expressed genes may be involved in the synthesis of *p*CA that is ester bound to AX. For group V, enzymes coded by LOC_Os01g18744 (OsPMT) and LOC_Os05g19910 (OsFMT1)

have been shown to possess enzymatic activity for *p*-coumarate and ferulate monolignol transferase, respectively (Karlen et al. 2016; Withers et al. 2012). SbBAHD5-1, a homologue of OsPMT, has been shown to be expressed in sorghum internodes, suggesting that this gene might be involved in the *p*-coumaroylation of monolignol in sorghum. A more detailed understanding of these expressed genes in the elongating internodes of sorghum will help to elucidate their physiological role and the importance of hydroxycinnamoyl esters.

Bartley et al. (2013) demonstrated that overexpression of OsAT10 in rice plants showed a 60% reduction in polysaccharide-bound FA and an approximately 300% increase in *p*CA in young leaf tissue. Furthermore, mature straw of OsAT10 overexpression plants exhibited an approximately 20% increase in enzymatic saccharification yield. Similarly, RNA interference silencing of the *S. viridis* BAHD01, a homologue of LOC_Os01g09010, showed an approximately 60% decrease in AX feruloylation and an increase in biomass saccharification efficiency of more than 40% (de Souza et al. 2018). Therefore, the functional elucidation and genetic manipulation of the BAHD acyltransferase in sorghum internodes is expected to contribute to improvement in the digestibility of cell walls and the resulting effective utilization of sorghum in livestock feed and plant biomass.

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