Overexpression of a BAHD Acyltransferase, OsAt10, Alters Rice Cell Wall Hydroxycinnamic Acid Content and Saccharification

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Grass cell wall properties influence food, feed, and biofuel feedstock usage efficiency. The glucuronoarabinoxylan of grass cell walls is esterified with the phenylpropanoid-derived hydroxycinnamic acids ferulic acid (FA) and para-coumaric acid (p-CA). Feruloyl esters undergo oxidative coupling with neighboring phenylpropanoids on glucuronoarabinoxylan and lignin. Examination of rice (Oryza sativa) mutants in a grass-expanded and diverged clade of BAHD acyl-coenzyme A-utilizing transferases identified four mutants with altered cell wall FA or p-CA contents. Here, we report on the effects of overexpressing one of these genes, OsAt10 (LOC_Os06g33390), in rice. An activation-tagged line, OsAt10-D1, shows a 60% reduction in matrix polysaccharide-bound FA and an approximately 300% increase in p-CA in young leaf tissue but no discernible phenotypic alterations in vegetative development, lignin content, or lignin composition. Two additional independent OsAt10 overexpression lines show similar changes in FA and p-CA content. Cell wall fractionation and liquid chromatography-mass spectrometry experiments isolate the cell wall alterations in the mutant to ester conjugates of a five-carbon sugar with p-CA and FA. These results suggest that OsAt10 is a p-coumaroyl coenzyme A transferase involved in glucuronoarabinoxylan modification. Biomass from OsAt10-D1 exhibits a 20% to 40% increase in saccharification yield depending on the assay. Thus, OsAt10 is an attractive target for improving grass cell wall quality for fuel and animal feed.
by cell wall composition and structure. Cell wall content influences the efficiency with which animals digest grass forages and feed (Lam et al., 2003; Casler and Jung, 2006). In human foods, the cell wall components of whole grains, such as in whole wheat and brown rice, have beneficial effects on health and various impacts on food processing (Fincher, 2009). Furthermore, grass root and leaf litter compositions affect soil carbon storage (Pendall et al., 2011; Zhou et al., 2012).

In contrast to the primary cell walls of dicotyledonous plants (type I walls), primary cell walls of grasses and other commelinid monocots (type II walls) consist of up to 40% dry weight of the matrix polysaccharide glucuronoarabinoxylan (GAX; for review, see Carpita, 1996; Vogel, 2008; Scheller and Ulvskov, 2010). Grass GAX is composed of a backbone of β-1,4-linked Xyl residues substituted mostly at the O3 position with arabinofuranose residues and infrequently with GlcA residues (Obel et al., 2006). In contrast, the xylan of dicots is present mostly in secondary cell walls that accumulate after cessation of growth, is infrequently substituted with Ara, and possesses numerous GlcA and methylglucuronic acid residues. Another unique aspect of the GAX of grasses and other monocots with type II walls is that a fraction of the Ara residues are substituted at the O3 position with the hydroxycinnamic acid ferulic acid (FA; Fig. 1A; for review, see Buana, 2009). Dehydrodimers of ferulate (diferulates) form through oxidative coupling likely mediated by peroxidases (Takahama and Oniki, 1994; Allerdings et al., 2005). Furthermore, the observation of ether linkages between ferulate and monolignols suggests that FA on GAX may nucleate lignin polymerization (Bunzel et al., 2004).

Another hydroxycinnamate, para-coumaric acid (p-CA; Fig. 1A), is also ester linked to components of grass cell walls. p-Coumaroyl esters are abundant on lignin but also esterify grass GAX (Mueller-Harvey et al., 1986; Ishii et al., 1990; Saulnier et al., 1995; Faulds et al., 2004; Ralph, 2010). Although p-CA is readily oxidized to its radical, p-CA dimers have not been observed in planta (Ralph et al., 1994). Rather, p-coumaroyl substituents may act as “radical catalysts,” rapidly passing the radical to sinapyl alcohols and facilitating lignin polymerization (Takahama and Oniki, 1994; Ralph, 2010).

FA on GAX, and especially diferulates, are thought to act to strengthen primary and secondary cell walls. Diferulate accumulation correlates with the cessation of grass leaf elongation, and the addition of FA to rice internodes blocks their expansion (MacAdam and Grabber, 2002; Sasayama et al., 2011). Several researchers have observed that ferulate esters in grass biomass block digestibility. FA is inversely correlated with enzymatic sugar-release parameters in vitro (Grabber et al., 1998a, 1998b; Lam et al., 2003; Casler and Jung, 2006). Digestibility inversely correlates with FA and

Figure 1. A, Structures of relevant hydroxycinnamic acids. B, Inferred Bayesian phylogeny of the rice Mitchell clade BAH-D acyl-CoA-utilizing enzymes including the following: the rice acyltransferases (OsAT); the Arabidopsis protein AT3G62160, which allowed this clade to be identified by Mitchell et al. (2007); biochemically characterized BAH-D-IV and BAH-D-III proteins as an outgroup (ACT and VAAT; Burbenne et al., 2003; Beekwilder et al., 2004); Arabidopsis proteins that use hydroxycinnamoyl-CoA adducts as substrates (HCT and SFT; Hoffmann et al., 2003; Molina et al., 2009); and the rice genes that cluster with the Arabidopsis HCT (HCT-like). Proteins are identified by the locus identifier that encodes them or their GenBank identifier as well as by proposed names. Clade credibility values are 100 unless shown. The two major subclades are designated clade i and ii. The shading density of the circles on the tree branches indicates the level of RNA expression in terms of counts of Sanger ESTs and representation in massively parallel signature sequence data. [See online article for color version of this figure.]
diferulic acid amounts in canarygrass (*Phalaris canariensis*) and ryegrass (*Lolium spp.*) accessions (Lam et al., 2003; Casler and Jung, 2006). In addition, cell wall-associated diferulates and free and cell wall-associated FA and p-CA appear to deter fungal pathogens and insect pests of grasses (Santiago et al., 2007, 2008; Lanoue et al., 2010).

Despite their importance, the proteins that incorporate hydroxycinnamates into grass cell walls have only begun to be characterized. Mitchell et al. (2007) proposed that a subclade of proteins with the Pfam domain, PF02458, for which transcripts are more abundant in grasses relative to dicots, might incorporate FA into grass walls. PF02458 domain-containing proteins are CoA-acyl-dependent acyltransferases present in plants, fungi, and a few bacteria. In plants, these enzymes have been named BAHD acyltransferases, based on the first biochemically characterized family members. They catalyze the addition of an acyl group from the thioester of CoA to oxygen and nitrogen nucleophiles of diverse acceptor molecules in specialized plant metabolism, including volatile esters, anthocyanins, and flavonoids (for review, see Dudareva and Pichersky, 2000; D’Auria, 2006).

There are well over 50 BAHD members in most sequenced vascular plants (Table I). The BAHD enzymes group robustly into five clades (D’Auria, 2006), although more recently subclades have been proposed (Tuominen et al., 2011). Several characterized members use hydroxycinnamoyl-CoA as substrates, including the hydroxycinnamoyl-coenzyme A:shikimate/quinate hydroxycinnamoyltransferase (HCT) involved in the synthesis of lignin precursors (Hoffmann et al., 2003) and sinapoyl and coumaroyl spermidine transferases (Luo et al., 2009). Other recent reports have described Arabidopsis (*Arabidopsis thaliana*) suberin and cutin feruloyl transferases and a wax fatty alcohol caffeoyl transferase, all of which transfer hydroxycinnamoyl-CoAs to ω-hydroxy fatty acid acceptors that accumulate in the extracellular matrix (Molina et al., 2009; Kosma et al., 2012; Rautengarten et al., 2012). Other BAHD enzymes catalyze the addition of esters to sugar acceptors, such as those that are part of anthocyanins (Unno et al., 2007).

Recent work has provided support for the hypothesis that the BAHD acyltransferase subclade identified by Mitchell et al. (2007), hereafter referred to as the “Mitchell clade,” is involved in cell wall modification. Withers et al. (2012) described the biochemical characterization of one member of the Mitchell clade, PMT, or here referred to as OsAT4, which possesses p-coumarate monolignol acyltransferase activity in vitro. Furthermore, Piston et al. (2010) found that rice plants with reduced expression of genes from the Mitchell clade exhibited reduced FA in leaves. In that study, plants were engineered with a long inverted repeat-silencing construct that targeted conserved regions of the genes that we refer to here as OsAt6 through OsAt10. The authors observed 2- to 3-fold reductions in gene expression and an average 20% reduction in cell wall FA content in the leaves of progeny from two independent transgenic events. Because of the small effect on FA accumulation and the nonspecific nature of the construct, Piston et al. (2010) were not able to determine the function of the silenced gene products.

Here, we report that the Mitchell clade of BAHD acyltransferases is expanded and diverged in grasses relative to eudicots and nonsporophyte species. We find that rice mutants with altered expression of four of these genes have altered cell wall hydroxycinnamate content. In-depth characterization of ectopic expression lines for one gene, OsAt10, revealed that this modification increases matrix polysaccharide-associated ester-linked p-CA while simultaneously decreasing matrix polysaccharide-associated FA. OsAt10 overexpression plants exhibit increased in vitro saccharification, with no discernible effects on vegetative development. Thus, this gene is a useful target for improving biofuel and feed production.

### Table I. BAHD acyl-CoA transferases encoded in selected sequenced plant genomes

<table>
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<th>Species</th>
<th>Putative BAHD Proteins: Total No. (No. Perfect HXXXD)a</th>
<th>Clade V: Total No. (No. Perfect HXXXD) b</th>
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<th>AT Clade i1</th>
<th>AT Clade i2</th>
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<td>38 (38)</td>
<td>12</td>
<td>4</td>
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</tbody>
</table>

aConsists of nonredundant predicted protein sequences identified via HMMER3.0 based on Pfam version 25 and that contain the region surrounding the conserved active-site motif HXXXD, although proteins with single amino acid variations in the H are included. The number in parentheses is the number with the strict HXXXD motif. For justification, see “Results.”
bClade identified by D’Auria (2006).
cAcyltransferase (AT) protein clades delineated in Figure 1B and Supplemental Figure S1.
RESULTS

The Mitchell Clade of BAHD Aciyltransferases Is Expanded and Diverged in Grasses

Mitchell et al. (2007) identified what we term the Mitchell clade of BAHD acyl-CoA-dependent acetyltransferases on the basis of high gene expression in grasses relative to dicots. To refine the hypothesis that these enzymes might be involved in grass-diverged cell wall synthesis, we systematically characterized the distribution of this clade in selected plant species and compared the clade with other characterized BAHD proteins. We identified BAHD proteins from the genomes of a diverse set of sequenced plant species available at the time of the analysis and examined the phylogenetic relationships among them and a reference set of BAHDs (Table I). To gain higher sensitivity relative to local sequence alignment (i.e. BLAST) for recognizing sequences with low, but potentially still significant, homology, we used a hidden Markov model to identify putative BAHD proteins (Finn et al., 2011). We then inferred an initial model of the phylogenetic relationships among the putative BAHD proteins from each genome and the set of biochemically characterized BAHD proteins cataloged by D’Auria (2006). While we are aware that recent analyses have included the presence of a strict HXXXD motif as indicative of whether the protein is an active BAHD (Banks et al., 2011; Tuominen et al., 2011), we have included proteins with single amino acid alterations to this motif, since one of the known biochemically active proteins for the family involved in taxol biosynthesis, BAPT (National Center for Biotechnology Information identifier AAL92459; Walker et al., 2002), possesses a variation of this motif in which the His is replaced by a Ser.

As observed by Tuominen et al. (2011), the distribution of BAHD proteins varies among species (Table I; Supplemental Fig. S1). The Mitchell clade is embedded within clade V, or clade Va of Tuominen et al. (2011). Furthermore, we find that the Mitchell clade includes a biochemically characterized banana (Musa spp.) alcohol CoA acyltransferase, BanAAT (Beekwilder et al., 2004), and is related to a group of BAHD proteins that participate in taxol biosynthesis (Fig. 1B; Supplemental Fig. S1).

We also conducted a more in-depth analysis of clade V BAHD proteins. We found that multiple proteins with similarity to the rice Mitchell clade are present in the grasses sorghum (Sorghum bicolor) and Brachypodium distachyon (Table I; Supplemental Fig. S1). In contrast, the annotated proteomes of the dicots Arabidopsis, soybean (Glycine max), and Medicago truncatula encode only one or two proteins closely related to this clade. Similar sequences are entirely absent from the annotated proteomes of poplar (Populus trichocarpa) and the nonpermatophyte plants Selaginella and Physcomitrella spp. Among characterized Arabidopsis proteins, the most closely related biochemically characterized proteins are the spermidine hydroxycinnamoyl transferases, coumaroyl spermidine transferase and sinapoyl spermidine transferase (Supplemental Fig. S1; Luo et al., 2009). The recently discovered cutin, wax, and suberin hydroxycinnamoyl transferases (Molina et al., 2009; Kosma et al., 2012; Rautengarten et al., 2012), although part of clade V, are not part of, or even closely related to, the Mitchell clade. In summary, the Mitchell clade appears to be conserved and expanded in grasses relative to dicotyledonous and nonpermatophyte plants. This is consistent with this clade functioning in aspects of commelinid metabolism that diverge from the metabolism of other plants, such as the synthesis of type II cell walls.

The analysis described above also revealed that the Mitchell clade of BAHD acyltransferases included more proteins than originally recognized. Instead of comprising 12 members in rice (Mitchell et al., 2007; Piston et al., 2010), the group consists of 20 closely related members that are further subdivided into two subclades (i and ii; Fig. 1B). In rice, the 10 genes in subclade i are all supported by EST evidence and are relatively highly expressed; whereas only seven of the 10 members of subclade ii have been EST validated, and they are relatively weakly expressed compared with subclade i members (Fig. 1B). In addition, the multispecies tree reveals that most proteins of subclade i are represented in all three grass species examined and are more similar to the nongrass proteins (Supplemental Fig. S1). In contrast, subclade ii contains more species-specific expansions and/or contractions. To facilitate communication about the Mitchell clade acyltransferases, we have given the clade members of rice preliminary names with the format Oryza sativa acyltransferase, OsAT1 through OsAT20. As mentioned previously, OsAT4 was recently named PMT and found to be capable of esterifying monolignols (Withers et al., 2012).

Screen of Rice Mutants for Altered Cell Wall Hydroxycinnamic Acid Content

To test the hypothesis that members of the Mitchell clade of BAHD CoA acyltransferases are involved in the incorporation of FA into grass cell walls, we screened rice mutants with altered expression of these genes and assessed their cell wall characteristics. Supplemental Table S2 describes the mutant lines we characterized. To complement the work of Piston et al. (2010), we particularly, but not exclusively, focused on putative activation-tagged lines from the South Korean collection (An et al., 2005; Jeong et al., 2006). We used line-specific PCR primers designed to distinguish the presence and absence of each transfer DNA (T-DNA) insert to screen 17 putative T-DNA mutant lines putatively targeting 12 of the 20 acyltransferase genes. We did not detect an insert in four lines, and for two lines, we did not identify any segregants that were homozygous for the insert out of 20 analyzed offspring (Supplemental Table S2).
For the remaining 11 lines, we characterized the alkali-labile hydroxycinnamoyl ester content of cell wall alcohol-insoluble residue (AIR) from leaf blades and sheaths of side tillers. We compared homozygous, mutant, and wild-type segregant plants 7 or 10 weeks after planting. The screen revealed four mutants with possible cell wall hydroxycinnamic acid phenotypes (Fig. 2; Supplemental Table S2). All four lines showed changes in the expression of the nearest acyltransferase gene to the T-DNA insertion site via quantitative reverse transcription (qRT)-PCR (Supplemental Table S2). Three of the phenotypes were in putative mutants of subclade i proteins, and one was a mutant in a subclade ii protein. Specifically, homozygous mutant progeny of 2A-20021, which increases the expression of OsAt5, exhibited a significant increase in the ratio of FA to p-CA. In addition, homozygous mutant progeny of 2A-40095, which carry an insertion that interrupts the 5' end of the coding sequence for OsAt7, exhibited reduced FA in leaf sheaths (approximately 60% less). The location of the T-DNA insertion truncates the OsAt7 mRNA and, consequently, despite the presence of transcriptional activator sequences, reduces OsAt7 mRNA expression. In line 2A-40095, the 5' end of the transcript was reduced by approximately 4-fold, and the 3' end was reduced more than approximately 5,000-fold compared with the wild-type segregant. Furthermore, homozygous mutant progeny of 4A-03423 (hereafter referred to as OsAT10-D1), which has increased expression of OsAt10, exhibited reduced FA (approximately 60% less) and an increase in p-CA (approximately 300% more) in sheaths and leaves. Lastly, homozygous mutant progeny of 1B-00523, which has increased expression of OsAT15, a subclade ii member, exhibited reduced FA in leaves relative to wild-type segregants (approximately 60% less). The other lines we examined showed no significant changes in FA or p-CA in the developmental stages and organs examined, and we did not test gene expression in those lines.

Figure 2. Cell wall hydroxycinnamate composition of leaf blades (left column) and leaf sheaths (right column) of T-DNA mutant rice lines. Data are for homozygous wild-type segregant plants (gray bars) and homozygous mutant plants (hatched bars). Each plant line is designated by the repository identifier and the putative target gene. A and D, Average FA content from an AIR preparation. B and E, p-CA content from AIR. C and F, The ratio of FA to p-CA.

For T-DNA mutant lines 1B-00523 and 2D-40243 were harvested 10 weeks after germination. All other lines were harvested 7 weeks after germination. Averages from samples from two to three plants for each genotype were measured independently. Error bars indicate so. *Significant difference at P < 0.05, **significant difference at P < 0.01 (Student’s t test).
Gene Expression and Developmental Phenotypes of OsAT10-D1

The T-DNA insertion site for the line we refer to as OsAT10-D1 (PFG_4A-03423) is approximately 8.5 kb downstream of the transcriptional start site for OsAt10 (Fig. 3A). The insert is oriented with the activating sequences proximate to OsAt10 and in the range observed to activate expression (Jeong et al., 2006). As mentioned above, qRT-PCR indicated that, indeed, the expression of OsAt10 was increased by more than 100-fold in the leaves of homozygous OsAT10-D1 plants (Fig. 3B). In OsAT10-D1, besides OsAt10 the expression of other genes proximate to the site of the T-DNA insertion does not vary significantly relative to the wild type (Fig. 3B). Similarly, the expression of related OsAt genes does not vary significantly in OsAT10-D1 (Supplemental Fig. S2), reducing the possibility that the observed phenotype is due to compensation at the level of gene expression of a related acyltransferase. Of the acyltransferase transcripts examined in this survey, OsAt6 appears to vary the most, although not significantly. However, OsAt6 (LOC_Os01g08380) is expressed near the lower limit of our detection and, in fact, was reported as undetectable in a previous qRT-PCR study (Piston et al., 2010).

OsAT10-D1 lines show no change in size and dry mass at maturity (Fig. 4, A and B). However, we did measure an approximately 20% to 30% decrease in total seed mass per plant for the mutant compared with the wild type (Fig. 4C).

Cell Walls of OsAt10 Overexpression Lines Have Heritable Alterations in Ester-Linked Hydroxycinnamic Acids

For OsAT10-D1, we confirmed the inheritance of the altered cell wall hydroxycinnamate phenotype in young leaves and mature tillers of plants from two additional generations (Fig. 5; Supplemental Fig. S3). This line reliably exhibits an approximately 50% decrease in ester-linked FA in young leaf tissue (Fig. 5A). The same tissue shows an approximately 300% (i.e. 3-fold) increase in ester-linked p-CA (Fig. 5B). The change in both components is most clearly displayed as a change in the ratio of FA to p-CA (Fig. 5C), which is independent of potential variation in the absolute amounts due to variation in sample mass and extraction efficiency. In addition, we found similar trends, but less extreme changes, in pools of total aerial tissues harvested after senescence for the plants from each genotype (Fig. 5). These mature straw samples possess approximately 40% less FA and approximately 80% more p-CA compared with the wild type. The difference in the magnitude of the effect on p-CA in juvenile versus mature organs may be due to an accumulation of p-CA in a cell wall fraction of mature tissues, such as lignin (Ralph, 2010), that is unaffected by increased OsAT10 expression.

We also quantified the four most abundant diferulates. Although signals were near the level of detection for most dimer species, the most abundant dimer, 8-O-4, clearly decreases significantly compared with wild-type amounts (Fig. 5D). Moreover, the sum of the dimers shows a highly significant decrease relative to the sum of all the ester-linked hydroxycinnamates in both mutant families examined (Fig. 5D). We found that the sum of the diferulates decreases proportionally to the decrease in FA, so that there is no change in the ratio of FA to dimer in the mutant relative to the wild type (Fig. 5D).

To gather further evidence that the phenotype in the activation-tagged line was due to ectopic
expression of OsAt10, we generated additional OsAt10 overexpression lines utilizing the maize ubiquitin1 promoter (Ubipro; Fig. 6B; Supplemental Fig. S4). Contrary to our typical experience for high-efficiency transformation with the japonica cultivar Kitaake (Jung et al., 2008), we were only able to regenerate from tissue culture two independent transformants that possess the transgene (Fig. 6B). This result suggests that the OsAt10 construct interferes with transformation efficiency. In young leaf blades of plants that were harvested approximately 1 month following tissue culture regeneration, both of the confirmed transgenic lines showed increased expression of OsAt10, a dramatic change in the ratio of FA to p-CA, and no change in the ratio of FA dimers to FA compared with the nontransgenic “escape” lines (Fig. 6B). The low hydroxycinnamate levels in these plants (approximately 10- to 30-fold less than young leaves in Fig. 5) may be due to the recent calli regeneration. We also characterized segregating progeny of Ubipro:OsAt10-4 (Supplemental Fig. S4). Young leaf tissue from Ubipro:OsAt10-4 expressed OsAt10 approximately 3,000-fold more than wild-type segregants. Mature straw from the Ubipro:OsAt10-4 progeny displayed a similar cell wall phenotype to OsAT10-D1 mature straw. Specifically, Ubipro:OsAt10 mature straw exhibits a 45% increase in p-CA and a 20% decrease in FA compared with straw from wild-type segregants (Supplemental Fig. S4).

Figure 4. OsAT10-D1 has normal vegetative development. A, OsAT10-D1 plants (4A-03423.5 progeny) compared with the wild-type (WT) segregants (4A-03423.1 progeny) at senescence, 7 months after planting. B, Average dry biomass at senescence. C, Average seed mass per plant at senescence. Gray bars indicate the wild type (WW; 4A-03423.1 progeny), and hatched bars indicate mutants (TT; 4A-03423.5 and 4A-03423.12 progeny). n = 12. Error bars represent 2 × se. *Significant difference at P < 0.05 via Student’s t test.

Figure 5. OsAT10-D1 shows alterations in cell wall hydroxycinnamic acids. Data are for wild-type segregants lacking the insert (gray bars, progeny of 4A-03423.1) and progeny of homozygous mutants (cross-hatched bars, progeny of 4A-03423.5; horizontally hatched bars, progeny of 4A-03423.12) for young leaves and a pool of mature straw. A, FA content. B, p-CA content. C, The ratio of FA to p-CA. D, Ferulate dimer amounts, the ratio of FA to dimer, and the ratio of dimer to total hydroxycinnamates (HCA) for AIR from young leaf samples in A to C. Error bars represent 2 × se of three to five biological replicates for young leaves and 2 × se of technical duplicates for mature straw. *Significance via Student’s t test at P < 0.05, **significance at P < 0.01, ***significance at P < 1 × 10⁻⁶, which applies to both mutant samples.
The Difference in OsAT10-D1 Hydroxycinnamates Is Predominantly Trifluoracetate Soluble and Is Linked to a Five-Carbon Sugar

In grass cell walls, hydroxycinnamates are predominantly esterified to GAX and to lignin. To determine which cell wall fraction is altered in the OsAT10-D1 mutant, we subjected AIR from mutant and wild-type mature rice straw to a mild, 50 mM trifluoracetate (TFA) treatment to cleave acid-labile glycosidic bonds within the matrix polysaccharides (Saulnier et al., 1995; Obel et al., 2003). Within 30 min, 50 mM TFA treatment of maize bran cleaved 70% of the α(1-3)-Xyl-arabinosyl bonds, including Ara residues esterified to ferulate (Saulnier et al., 1995). Cleavage of other glycosidic bonds, such as those of the xylan backbone, proceeded with slower kinetics. Following acid hydrolysis, we saponified the TFA supernatants and remaining pellets and analyzed the products with HPLC. The results demonstrate that the alteration in FA and p-CA amounts in OsAT10-D1 is primarily in the matrix polysaccharide fraction of the cell wall (Fig. 7). For both the wild type and OsAT10-D1, the FA is predominantly associated with the TFA fraction, with less than 20% of FA remaining in the pellet for both genotypes (Fig. 7A). The reverse is observed for the p-CA for the wild type, for which approximately 70% of the p-CA remains in the pellet (Fig. 7B). The mutant has a similar absolute amount of p-CA in the pellet but a lower percentage (approximately 55%). Instead, the additional p-CA and reduced FA amounts in the cell wall of OsAT10-D1 are in the TFA-soluble matrix polysaccharide fraction. This can be clearly seen in the ratio plot, in which the ratio of FA to p-CA is most drastically different in the supernatant after prolonged TFA treatment (Fig. 7C). Indeed, the change in p-CA in the matrix polysaccharide fraction for mature rice straw approaches the approximately 300% increase observed for young leaves. Because the lignin-associated p-CA fraction is unaffected by the polysaccharide modification, the observation that the modified cell wall constituent is part of the polysaccharide fraction is consistent with the “dilution” of the mutant effect in mature straw for OsAT10-D1 and Ubi_{pCA}:OsAT10-4 plants that have accumulated p-coumaroylated lignin relative to young leaves (Fig. 5; Supplemental Fig. S4).

Further analysis of the TFA-solubilized cell wall hydroxycinnamates with electrospray ionization liquid chromatography-mass spectrometry (LC-MS) strongly supports the hypothesis that GAX is modified by OsAT10 activation. For this experiment, we compared TFA-hydrolyzed AIR with and without saponification. The TFA-solubilized extract had two major new ion peaks that were absent in the saponified extract and in hydroxycinnamate standards (Fig. 8A). The mass spectra of these peaks are consistent with the major unknown peak in the mutant (unknown peak 1) consisting of p-CA esterified to a five-carbon sugar (mass-to-charge ratio [m/z] of major ion = 295.0285; Fig. 8B),
whereas the predominant peak in the wild type (unknown peak 2) contains FA esterified to a five-carbon sugar (m/z of major ion = 325.093; Fig. 8C). Because Ara and Xyl have the same Mr, they are indistinguishable in this experiment; however, our strong expectation is that the esterified sugar is Ara. Relative quantification of the ion counts of each of these peaks in the mutant versus the wild type is consistent with the results measured via UV detection. That is, compared with the wild type, OsAT10-D1 has approximately 4.6-fold more p-CA-sugar and 2.5-fold less FA-sugar (Fig.

**Figure 8.** The modified hydroxycinnamates in OsAT10-D1 are esterified to a five-carbon sugar. A, LC-MS shows the total ion abundances in the ethyl acetate extracts for wild-type (a) and mutant (b) plants after 50 mM TFA and 2 M NaOH and for wild-type (c) and mutant (d) plants after 50 mM TFA treatment only. Labeled peaks were consistent both with standards, when available, and with their masses. trans-Cinnamate was added as an extraction control. B and C, Electrospray ionization mass spectra from total ion chromatograms. “M” denotes the major ion, and the other masses are as labeled. These compounds may be present in solution, such as due to reaction with TFA, or formed by interaction with the mobile phases. Although electrospray ionization is a “soft ionization” method in which compounds are typically not fragmented, sometimes fragmentation can occur during the electrospray process if weak bonds are present. B, The major ion in the mass spectrum for unknown peak 1 is consistent with a p-coumaroylated five-carbon sugar. C, The major ion in the mass spectrum for unknown peak 2 is consistent with a feruloylated five-carbon sugar. [See online article for color version of this figure.]
The relative amounts of FA and p-CA after saponification are also consistent with the HPLC data (Fig. 8A). The relative amounts of FA and p-CA after saponification are also consistent with the HPLC data (Fig. 8A).

The OsAT10-D1 Line Has an Increase in Cell Wall Glc Content

Compensatory changes are often seen among the components of the cell wall (Humphrey et al., 2007). Quantification of sugars released by acid treatment of destarched AIR preparations from mature straw suggests that the Glc content is increased by approximately 20% (w/w) for the mutant relative to the wild type (Fig. 9A). We observed the difference both with TFA treatment, which liberates monosaccharides derived from matrix polysaccharides and amorphous cellulose, and when the TFA residue was further treated with sulfuric acid, which breaks down crystalline cellulose (Fig. 9A). The difference in the mutant compared with the wild type is most apparent when the products of both treatments are summed together, which gives an increase in Glc in the mutant compared with the wild type of 19% ± 11%. By mass, we did not observe any other significant changes in sugar amounts in the mutant compared with the wild type (Supplemental Fig. S5). We also observed no change in the total mass percentage of sugars in AIR. When the TFA-solubilized sugars are expressed in terms of mol %, the data also indicate an increase in Glc content of 11% ± 5% (Fig. 9B). The sum of the mol % of other measured sugars (i.e., Xyl, Ara, and the sum of minor sugars) decreases proportionally to the Glc increase (7% ± 7%). This balance in mol % change suggests that the change in polysaccharide content in the mutant is restricted to the Glc-containing polymers.

The OsAT10-D1 Line Shows No Alterations in Lignin Content or Composition

To further explore the extent of cell wall changes in OsAT10-D1 relative to the wild type, we measured lignin content and composition. We hypothesized that alteration in pools of hydroxyphenyl-CoA adducts in the OsAT10-D1 line might lead to alterations in lignin amount or content, in terms of syringyl (S), guaiacyl (G), and hydroxyphenyl (H) subunits. Due to the presence of H residues in grass lignin, all methods of lignin analysis are not equally accurate for grasses relative to dicots. For our analysis, we used two methods suitable for grasses: acetylbromide solubilization (Grabber et al., 1996; Fukushima and Hatfield, 2004) and pyrosylation-molecular beam mass spectrometry (py-MBMS; Evans and Milne, 1987; Agblevor et al., 1994). “Lignin” analyses of whole tissue or AIR typically include all classes of phenylpropanoids, both esterified and nonesterified, although many hydroxycinnamates are esterified to polysaccharides. Since our previous analysis had established a difference in ester-linked phenolics in the mutant, we quantified lignin content and composition with and without removing esterified hydroxycinnamates with saponification.

OsAT10-D1 mature straw samples show no significant differences in the content of acetylbromide-soluble lignin after saponification relative to the wild type (Table II). We obtained a similar result via py-MBMS for mature straw and separate, young leaf and sheath samples. The py-MBMS also revealed no difference in the syringyl-guaiacyl (S:G) lignin ratio in the mutant compared with the wild type after saponification (Table II). We also collected py-MBMS data for unprocessed straw and AIR of OsAT10-D1. Separate analyses of the saponified and unsaponified samples reveals distinctions between the wild type and mutant in the unsaponified samples (Fig. 10A). Principal component 1 explains the alcohol extraction (30% of the variation), and principal component 2 explains the differences between wild-type and mutant samples (19% of the variation). The loadings for principal component 2 show that the major ions that distinguish wild-type and mutant samples are phenolics (Fig. 10B). The mass spectrometry fragmentation pattern is consistent with an interpretation in which there is an increase of p-CA, as reflected by peaks 120, 94, and 91, and a decrease in FA, as reflected in the reduction in the coniferyl ion, peak 150 (Evans and Milne, 1987). Because principal component analysis no longer distinguishes the samples after saponification (Fig. 10C), the observed differences in phenylpropanoids between OsAT10-D1 and the wild type are likely associated with ester-linked hydroxycinnamates and not lignin, consistent with the other results.

Figure 9. Destarched AIR from OsAT10-D1 mature straw (hatched bars; 4A-03423.5 progeny) has increased Glc content relative to that of the wild type (gray bars; 4A-03423.1 progeny). A, Mass analysis shows significant increases in Glc after TFA treatment and after additional treatment with sulfuric acid (TFA→H2SO4) as well as with the sum of the two treatments. B, Analysis of the molecular fraction (mol %) of monomeric sugars released by TFA in the mutant relative to the wild type shows an increase in Glc equal to the decrease in the sum of the decreases in Xyl, Ara, and other sugars. Error bars show 2×SE of three replicates. **Difference at P < 0.01, *difference at P < 0.05 via Student’s t test.
A limitation of the pyrolysis method for determining lignin composition is that it inaccurately measures H-lignin, which volatilizes poorly and instead turns to char upon heating. Because of the increase in p-CA, a precursor of H-lignin, in OsAT10-D1 cell walls relative to the wild type, we sought to determine whether there is a change in the char content of OsAT10-D1 using a thermogravimetric pyrolysis instrument. Duplicate runs per genotype of the thermogravimetric instrument did not detect a difference in the mass remaining from mature straw after pyrolysis (Supplemental Fig. S6), again consistent with there being no difference in core lignin composition or content between OsAT10-D1 and the wild type.

The OsAT10-D1 Line Shows an Increase in Saccharification

Ferulate in grass biomass is inversely correlated with digestibility across diverse grass accessions (Lam et al., 2003; Casler and Jung, 2006). The phenotype of the OsAT10-D1 line provided the opportunity to determine whether there is also an increase in enzymatic digestibility with reduced FA content when comparing two near-isogenic plant lines. We found that destarched AIR after mild pretreatment followed by incubation with a cellulase cocktail resulted in the release of approximately 20% more reducing sugar from the mutant with a cellulase cocktail resulting in the release of approximate 40%. Cellulase and b-glucosidase enzymatic treatment, with a total sugar yield increase of approximately 40%. Cellulase and b-glucosidase enzymatic activities in the slurry are unchanged on the mutant straw (Fig. 11C; Supplemental Fig. S7), suggesting that the fungus grew similarly on both. In contrast, and of relevance to the nature of the change caused by the increased expression of OsAT10, xylanase activity is dramatically enhanced, especially at later time points (Fig. 11C).

DISCUSSION

Hydroxycinnamoyl esters in cell walls influence basic disease resistance, and food and feed quality (Santiago et al., 2007; Buanafina, 2009). The molecular details of the incorporation of hydroxycinnamimates into cell walls remain largely obscure. Here, we present results showing that overexpression of OsAT10 in the OsAT10-D1 line decreases FA and increases p-CA in leaf blades, leaf sheaths, and mature straw (Fig. 5). We have also confirmed that overexpression of OsAT10 in two other independent lines, Ubipro-OsAT10-4 and Ubipro-OsAT10-5, alters the ratio of FA to p-CA in a manner similar to OsAT10-D1 (Fig. 6).

Uneven Distribution of BAHD CoA Acyltransferases across Plants

OsAT10 belongs to a grass-diverged and expanded clade of BAHD acyl-CoA-utilizing acyltransferase proteins...
that were originally identified due to their high expression in grasses relative to dicots (Mitchell et al., 2007). Our phylogenetic analysis shows that, with the exception of that of poplar, the genomes of the angiosperms we examined have annotated proteins within the Mitchell clade. However, compared with zero to two members in dicotyledonous and non-spermatophyte species, the grasses examined possess genes encoding 12 to 20 of these proteins (Table I; Supplemental Fig. S1). The presence and expression of a protein in the Mitchell clade in banana, BanAAT (Beekwilder et al., 2004), is consistent with the presence of hydroxycinnamates in cell walls of banana, a commelinid monocot (Carpita, 1996).

Mutant lines for the most closely related Arabidopsis protein, AT3G62160, were recently examined for changes in hydroxycinnamate content of the cell wall and other extracellular polymers; no differences were identified (Rautengarten et al., 2012; C. Rautengarten and H.V. Scheller, unpublished data). Although the function of the Arabidopsis protein remains to be determined, that result is consistent with a model of neofunctionalization or subneofunctionalization of the Mitchell clade of BAHD acyltransferases in commelinid monocots (He and Zhang, 2005). In this model, a duplicated Mitchell clade member in the progenitor of commelinids acquired the ability to modify a cell wall-related substrate. Additional gene duplication events then led to further increases in the number of members of the Mitchell clade, especially those in subclade i. The conservation of subclade i Mitchell clade members across the grasses (Table I; Supplemental Fig. S1) is consistent with selection for the retention of their function(s). As will be discussed further below, the different overexpression phenotypes of mutants for different Mitchell clade members as well as evidence from the literature are consistent with members of this clade having evolved different specificities.

The Cell Wall Target of OsAT10 Is GAX

Previous results have found that p-coumaroyl esters are predominantly bonded to lignin, whereas FA is largely esterified to GAX. However, enzymatic release experiments have provided some prior evidence that p-CA is also incorporated into the polysaccharides of grass cell walls (Mueller-Harvey et al., 1986; Ishii et al., 1990; Faulds et al., 2004). Hydroxycinnamoyl esters are also present in other less abundant components of the cell wall fraction, including suberin, cutin, and waxes (Molina et al., 2009; Schreiber, 2010; Kosma et al., 2012; Rautengarten et al., 2012). We find that mature rice straw has approximately 20% of p-CA associated with matrix polysaccharide (Fig. 7). This is likely the major location of the p-CA in the young leaf tissue, which we expect to have very low lignin amounts (Fig. 5) and incompletely developed cutin and wax layers (Richardson et al., 2007). We found that the hydroxycinnamic acid changes in OsAT10-D1 are predominantly on the TFA-soluble matrix polysaccharide fraction, not the acid-resistant lignin fraction (Fig. 7). Indeed, we were able to confirm that hydroxycinnamoyl moieties in the TFA-released fraction are ester linked to a five-carbon sugar (Fig. 8). Since the sugar-hydroxycinnamoyl species

![Figure 10. Principal component analysis of py-MBMS data for OsAT10D-1 corroborates the change in extractable phenolics but shows no difference in lignin composition. A, The first two components of technical replicates of total biomass (negative in principal component 1 [PC1]) and AIR (positive in PC1) for wild-type straw (WT; diamonds and squares; progeny of 4A-03423.5) and mutant straw (Mut; triangles and X's; progeny of 4A-03423.1). B, Loadings plot for PC2 of A. The m/z values of the four most differentially abundant ions are shown. The ions that are overrepresented in mutant tissue are 120 (4-vinylphenol or 2,3-dihydrobenzofuran), 91 (fragment of 2,3-dihydrobenzofuran and most phenols), and 94 (phenol). The ion that is most underrepresented in the mutant is 150 (coumaroyl alcohol/coniferyl alcohol). C, Principal component analysis for mutant and wild-type residue after 2 N NaOH extraction. Samples are poorly distinguished, indicating that the principal components of variation are extractable and, therefore, are not polymeric lignin. [See online article for color version of this figure.]
migrate in narrow windows in the LC-MS analysis, it is most likely that the fractions represent only Ara esters, which have been frequently described (Mueller-Harvey et al., 1986; Saulnier et al., 1995; Buana, 2009). Although a formal possibility, it is unlikely that this peak also contains feruloylated Xyl, which has only been described from bamboo (Leleba oldhami) xyloglucan (Ishii et al., 1990). Furthermore, we have no evidence to suggest that OsAT10 functions as a p-CA monolignol acyltransferase, which has been described in recent publications (Hatfield et al., 2009; Withers et al., 2012). Rather, it seems likely that the native function of OsAT10 is to incorporate p-CA into a precursor of GAX.

How OsAT10 can mediate the transfer of p-CA to xylan is a conundrum, since all the BAHD enzymes to date have been found to be cytoplasmic (D’Auria, 2006), whereas, xylan synthesis takes place inside the Golgi lumen (Oikawa et al., 2013). One possibility is that OsAT10 transfers p-CA to UDP-arabinofuranose, which is formed by UDP-Ara mutase located on the cytoplasmic side of the Golgi (Rautengarten et al., 2011). Buana (2009) proposed such a reaction mechanism, but no direct biochemical evidence has been demonstrated. Biochemical analysis of wheat lysates revealed the transient feruloylation of a 40-kD protein (Obel et al., 2003). Although the protein was not identified, the observation is consistent with UDP-Ara mutase functioning as an intermediate.

Another observation for which the mechanism remains to be determined is the decrease in sugar feruloylation that accompanies the increase in polysaccharide p-coumaroylation in OsAt10-overexpressing plants. Specificity in the frequency of substitution of Ara, such that FA and p-CA substituents are in partial competition, may account for this observation. Alternatively, or in addition, since p-CA is a precursor of FA, the increased activity of the p-CA transferase, OsAT10, may reduce the amount of feruloyl-CoA available for the modification of matrix polysaccharide. The absence of an effect on lignin composition or content makes the latter hypothesis less likely, since the pool of lignin precursors is also cytoplasmic (Achnine et al., 2004). Further experiments will be needed to test these various models.

**Mitchell Clade Acytransferases Have Different Effects on Cell Wall Hydroxycinnamoyl Esters**

Both our data and those of Piston et al. (2010) provide genetic evidence that changing the expression of Mitchell clade CoA acyltransferases alters the amounts of cell wall hydroxycinnamoyl esters. Superficially, our result that OsAT10 overexpression decreases cell wall ferulate appears to conflict with that of Piston et al. (2010), who reported that simultaneously reduced expression of OsAt6 through OsAt10 (i.e. construct pAF7-B) causes an approximately 20% reduction in the amount of FA in mature leaves. One possibility is that in OsAT10-D1 a change in expression of other related acyltransferases causes the observed phenotype. However,
that there is no measurable change in the expression of related acyltransferases in OsAT10-D1 makes this model less likely (Supplemental Fig. S2).

Instead, the various phenotypes observed in mutants for Mitchell clade members are consistent with a model in which differing OsATs have distinct functions. First, this model explains the apparent conflict between the results of Piston et al. (2010) and our own findings. We postulate that Piston et al. (2010) observed small or no effects because their two constructs (one simultaneously targeting OsAt6 through OsAt10, the other silencing OsAt1, OsAt2, OsAt11, and OsAt12) caused multiple small effects. Indeed, while several studies have found that BAHD acyltransferases often have promiscuous specificities (D’Auria, 2006), work with anthocyanin-malonyl transferases (Dm3MAT1), for example, has measured discrimination for a hydroxylated substrate over a malonylated one (Unno et al., 2007). Also, PMT/OsAT4 shows at least 100-fold discrimination for p-coumaroyl CoA over feruloyl CoA (Withers et al., 2012). Thus, it is probable that various Mitchell clade acyltransferases have differential affinities for the hydroxycinnamoyl-CoA adducts and candidate nucleophile acceptor molecules. This model is also consistent with the report of Withers et al. (2012) that OsAT4 (PMT) uses monolignol acceptors, rather than sugar acceptors, as the data suggest for OsAT10.

Glc Polysaccharide, But Not Lignin, Compensation in OsAT10-D1

The mature cell wall of OsAT10-D1 exhibits an approximately 10% to 20% increase by mass and mol % in both TFA-soluble Glc, corresponding to mixed linkage glucan and amorphous cellulose, and TFA-insoluble Glc, representing crystalline cellulose (Fig. 9). In contrast, chemical, mass spectrometry, and thermogravimetric assays did not detect alterations in lignin content or composition in the OsAT10-D1 mutant line (Table II; Fig. 10; Supplemental Fig. S5). This suggests that lignin amounts are not sensitive to changes in phenylpropanoid pathway flux that may be caused by increased OsAT10 activity. The observations that FA and Glc levels change but lignin does not might be due to the spatial and temporal separation of the incorporation of hydroxycinnamates into a GAX precursor and the synthesis of lignin. Generally, our results contribute to an emerging view that plants possess molecules that are able to sense and trigger responses to specific changes in cell wall composition and/or function; however, the identity of those sensors remains largely obscure (Humphrey et al., 2007; Wolf et al., 2012).

Cell Wall Hydroxycinnamoyl Esters May Contribute to Plant Reproduction

Correlative studies suggest that FA dimerization has a role in halting plant growth via the inhibition of cell wall elongation and expansion (MacAdam and Grabber, 2002; Obel et al., 2002; Sasayama et al., 2011). Thus, we might have expected plants with reduced FA dimer content to either be smaller, due to decreased ability to support themselves, or be larger, due to greater expansion of cells during growth. Indeed, the progeny of a maize line with reduced ferulate esters at the seedling stage has recently been found to increase biomass by approximately 8% in field trials (Jung and Phillips, 2010). However, we observed no consistent effect on vegetative growth for greenhouse-grown OsAT10 over-expression lines (Fig. 4).

OsAT10-D1 does exhibit reduced seed yields per plant by approximately 20% (Fig. 4). This observation correlates with the difficulty of isolating homozygous knockout lines for various OsAt genes (e.g. OsAt4 and OsAt13; Supplemental Table S2; J. Dubcovsky, personal communication). On the other hand, the low-ferulate maize lines did not exhibit a change in ear mass at the stage examined (Jung and Phillips, 2010). Further studies will be needed to determine if and how grass cell wall hydroxycinnamic acids act during reproduction. From a practical perspective, restoring grain yield in plants engineered to overexpress OsAT10 would be an important consideration for the development of a dual-use crop for both food and biofuel/feed purposes but would be less crucial for dedicated fuel and feed grasses. An obvious way to accomplish this would be to use a promoter with low expression in reproductive tissues.

Heightened Saccharification of OsAT10-D1

An impetus for this work was to understand if a specific genetic reduction in the amount of cell wall-associated FA would improve the digestibility of cell wall sugars from modified plants for biofuel and feed production. We found that the decreased amount of FA and a proportional decrease in diferulates in OsAT10-D1 straw did indeed lead to greater sugar yields in both enzymatic and fungal saccharification assays (Fig. 11). The quantitative similarity between the improvement in cellulase-mediated digestion and cell wall Glc content (approximately 20%) raises the caveat that the improvement in enzymatic digestibility may not be due to altered cellulose accessibility but rather cellulose amount. On the other hand, the larger improvement in Glc yield (approximately 40%) from incubation with the fungus Penicillium sp. YT02 is consistent with improvements in the accessibility of Glc-containing polysaccharides. Compared with the simple cellulase cocktail treatment, the pretreatment and synthesis by the fungus of a suite of enzymes might have accentuated the effects of the reduced ferulate content of the mutant.

The improvement in fungal Xyl release was particularly dramatic (85%) and was accompanied by a clear increase in fungal xylanase production (Fig. 11). These observations are consistent with the model that the
modifications in the mutants are on the xylan polymer, which is made more accessible by reduced ability to cross link via diferulates and ethers to lignin. We do not expect that there is a major improvement in the quality of the xylan of the OsAT10-D1 that permits higher xylanase activity due to a reduction in FA substitution (Gilbert et al., 2008). In fact, the xylan in the mutant has an approximately 50% higher total hydroxycinnamoyl ester substitution rate compared with the wild type (Fig. 7).

CONCLUSION

We find that altered expression of members of a grass-diverged and -expanded clade of BAHD acyl-CoA acyltransferases alters the amounts of hydroxycinnamic acids in grass cell walls. In particular, increased expression of OsAT10 increases p-CA content but decreases FA content of rice matrix polysaccharide, consistent with our tentative assignment of this enzyme as a p-coumaryl-CoA transferase. Together with the recent report that OsAT4 has p-CA monolignol transferase activity, this suggests that other members of the Mitchell clade of acyl-CoA acyltransferases likely possess feruloyl transferase activity(ies). This insight opens the possibility of a detailed examination of the biological functions of and selective basis for acylation of the different grass cell wall polymers with hydroxycinnamates. Of practical importance toward improving the efficiency of biofuel production from grass biomass and the nutritional value of forage grasses, we have found that the increased OsAT10 expression increases straw Glc content and improves in vitro digestibility. The fact that this is an overexpression effect will facilitate the rapid testing of this gene in other grass species.

MATERIALS AND METHODS

Acyltransferase Identification and Phylogenetic Analysis

To identify putative BAHD acyltransferases, we downloaded the hidden Markov model profile for PF02458 from the Pfam database and then searched the annotated protein sequences of diverse plant genomes to identify potential domains using HMMER version 3.0 (Finn et al., 2011). We used the following genome annotation sources and versions, which were current at the time of the analysis: Arabidopsis (Arabidopsis thaliana), The Arabidopsis Information Resource version 10; Brachypodium distachyon, Phytome version 7.0; soybean (Glycine max), Phytome version 7.0; Medicago truncatula, M13.5; rice (Oryza sativa), Michigan State University version 6.1; Physcomitrella patens, Phytome version 7.0; poplar (Populus trichocarpa), Phytome version 7.0; sorghum (Sorghum bicolor), Phytome version 7.0; Selaginella moellendorffii, Phytome version 7.0. To serve as a reference for phylogenetic analysis, we downloaded the sequences of 46 previously characterized BAHD enzymes from the National Center for Biotechnology Information and identified their conserved PF02458 domains as described above. We refer to these proteins as the D’Auria set (D’Auria, 2006). In addition, we randomly selected an outgroup of the Auria set and the outgroup sequences. Phylogenetic analysis was conducted in two stages. The first stage was a coarse analysis of all the BAHDs from each species relying on neighbor-joining algorithms, and the second consisted of a more robust Bayesian analysis. For the first stage, the goals were to identify proteins most closely related to the Mitchell clade for second-stage analysis and to analyze their context relative to other BAHDs. For the first stage, we used MEGAS 5.0 (Tamura et al., 2011) to infer and visualize neighbor-joining phylogenetic trees for each species’ BAHD proteins in conjunction with the D’Auria set and the outgroup sequences. Parameters were as follows: amino acid substitutions according to the Jones-Taylor-Thornton model, γ distribution of mutation rate among sites, distribution shape parameter of one, and gaps treated by pairwise deletion. Five hundred bootstraps were used to identify a consensus tree for each species. In these trees, the previously delineated BAHD clades unfailingly grouped together (D’Auria, 2006). From the phylogenetic tree made of the rice BAHDs and the D’Auria set, we identified proteins from the D’Auria set most closely related to the Mitchell clade. These proteins are included in Supplemental Figure S1 and consist of the taxol biosynthesis genes and BanAAAT. We used these characterized proteins as markers to identify the Mitchell clade and related proteins from each of the species-specific trees for further analysis of the relationships among Mitchell clade members from diverse species.

For the second stage of the analysis, we analyzed the relationships among BAHDs closely related to the Mitchell clade. Selected proteins from the diverse plant species were aligned, and the alignments were manually edited. To generate Figure 1B, we used MrBayes3.1.2 (Huelsenbeck and Ronquist, 2001) with the WAG and for amino acid substitutions for variable sites and a rate distribution with some invariable residues. Analysis with a subset of the data showed that this was the most probable fixed-rate model for the data set. We ran the Markov Chain Monte Carlo simulation for 125,000 generations until the average SD of split frequencies stabilized below 0.01. The multiple-species tree in Supplemental Figure S1 was with the same parameters run for 1.5 × 10⁶ generations.

Plant Lines and Growth Conditions

We selected mutant lines for the target genes from RiceGE, the rice mutant flanking sequence database (An et al., 2005; Jeong et al., 2006). The initial screen was conducted on the segregating progeny of the primary transgenics. Seeds from the stock center were sterilized with a 40% commercial bleach solution and germinated on one-half-strength Murashige and Skoog medium containing 1.5% Suc, 0.55 mM myoinositol, and 0.2% phytagel at 28°C with continuous white light. After 7 d, the seedlings were transplanted into topsoil and grown in a greenhouse (20°C–30°C, 60–80% relative humidity). Natural day lengths less than 14 h were supplemented with artificial lighting. Fertilizer (14-14-14 slow release) was applied every 6 weeks.

For genotyping, 10- to 20-mg leaf samples from 14 to 20 segregating progeny were harvested, frozen in liquid nitrogen, and ground with a Qagen Tissue Disperser (17 Hz, 1 min). The samples were vortexed in 200 μL of DNA extraction buffer containing 100 mM Tris-HCl (pH 9.5), 1 × KCl, and 10 mM EDTA (pH 8.0), incubated at 65°C for 30 min, diluted with 1 mL of water, and centrifuged for 10 min at the maximum speed. The supernatant was used as the template for genotyping by PCR, with conditions as follows: 94°C, 5 min; 35 cycles of 94°C for 35 s, 55°C for 45 s, and 72°C for 45 s; concluding with 72°C for 5 min. Genotyping primers are listed in Supplemental Table S1. Three primers were used for each mutant line. Two primers recognized genomic sequences flanking the T-DNA insert, and one recognized a sequence near one of the T-DNA borders. Depending on the number of bands amplified, we were able to clearly distinguish heterozygous and homozygous plants. We further characterized the next two generations in selfed progeny of homozygous mutant and wild-type segregant plants of line 4A-03423, referred to herein as OsAT10-D1. Homozygous plants in subsequent generations were confirmed by spot-checking the genotype with PCR, and other molecular analyses, as described. Specifically, we characterized mutant progeny of 4A-03423.4, 4A-03423.12, and 4A-03423.12.9 and wild-type segregant progeny of 4A-03423.5.
and 4A-03423.5.6. In this notation, the period indicates each new generation and the numbers following the period indicate the parent plant of the analyzed progeny.

**Generation of Ubi<sub>pro</sub>-OsA101 Lines**

We amplified a 1,541-bp fragment encoding OsA101 (LOC_Os06g38300.1) including both the start and stop codons from Nipponbare seedling complementary DNA (cDNA) with the cloning primers listed in Supplemental Table S1. The PCR fragment was gel purified, cloned into pENTR-DTOPO (Invitrogen) and confirmed by sequencing. We then recombined the gene into the final pCAMBIA1300-Ubi-GW-Nos construct (Park et al., 2010). This binary vector contains a Gateway cassette, flanked by the maize Ubi promoter and the 3′ terminator of nopaline synthase from Agrobacterium tumefaciens, and the Hpt2 gene, which confers resistance to hygromycin. We used *A. tumefaciens* EHA105 to transform fresh calli from the rice *japonica* cultivar Kitaake, as described previously (Cheng et al., 1997). After regeneration of plantlets, plants were transferred to the greenhouse under the conditions described above and genotyped with primers for Hpt2 (Supplemental Table S1).

**qRT-PCR**

We measured gene expression in young leaf samples. The samples were harvested 5 weeks after transplanting to the greenhouse and consisted of the mature aerial part. Immature tissue was ground by milling with a Wiley Mill with a 5-mm screen followed by a Udy mill with a 1-mm screen. Ground tissue (500 mg) was extracted with 1 mL of Trizol reagent (Invitrogen) with subsequent processing for the following analysis: SQRT(E<sub>Cq</sub>Ub<sub>q5</sub>)/SQRT(E<sub>Cq</sub>Cc<sub>55</sub>) where the gene of interest was removed by centrifugation (10,000g, 10 min), and the residue was subsequently washed three to five times with 70% ethanol and dried at approximately 35°C under vacuum using a CentriVap. The dried powder obtained after 70% ethanol wash is designated as AIR. The AIR was destarched as described by ØBro et al. (2004). AIR was treated with amylase (TerraMy; Novozymes) at a loading of 0.3 units per 10 mg of biomass in MOPS buffer (50 mS, pH 7.0) at 85°C for 1 h followed by amylglucosidase (0.33 units per 10 mg of biomass) and pullulanase (0.04 units per 10 mg of biomass) in acetate buffer (200 mS, pH 4.5) for 2 h at 50°C. Amyloglucosidase and pullulanase were purchased from Megazyme. The reactions were stopped by adding 3 volumes of cold 95% ethanol, vortexed, and centrifuged at 10,000g for 10 min. The residue obtained after centrifugation was washed three times with 70% ethanol and dried at 32°C using a CentriVap Vacuum Concentrator (Labconco).

**Analysis of Hydroxycinnamic Acids**

To release esterified hydroxycinnamic acids from the cell wall, AIR (1–10 mg, depending on the experiment; typically 3 mg) was saponified with 500 mS of 2% NaOH for 24 h at 25°C with mixing at 300 rpm, similar to previous descriptions (Rautengarten et al., 2012). For analysis of later generations, we doped reactions with an extraction standard, trans-cinnamic acid, but this improvement had not yet been developed for the initial screening. After saponification, the supernatant was acidified to pH < 2 with 100 µL of concentrated HCl, vortexed, and extracted three times with 300 mS of ethyl acetate. The extracts were combined and evaporated to dryness using a CentriVap at 32°C. The samples were redissolved in 300 mS of ethyl acetate and used for HPLC analysis. Care was taken to shield the samples from light during the entire process of extraction to prevent the isomerization of hydroxycinnamates in light.

Quantification of hydroxycinnamic acids was carried out on a Dionex Ultimate 3000 HPLC system (Thermo Scientific-Dionex) with UV detection. Samples were separated on a reverse-phase C18 column (Synergy BD Fusion-RP 80 Å, 250 × 2 mm; Phenomenex) with a flow of 0.3 mLS min<sup>−1</sup> and a gradient of solvent A (0.2% [v/v] TFA) and solvent B (acetonitrile) as follows: 0 to 5 min, 10% B isocratic; 5 to 25 min, 10% to 30% B linear; 25 to 40 min, 30% B isocratic; 40 to 45 min, 30% to 35% B linear; 45 to 46 min, 35% to 100% B linear; 46 to 51 min, 100% B isocratic; 51 to 53 min, 100% to 10% B linear; 53 to 60 min, 10% B isocratic. The column temperature was maintained at 30°C, and detection was carried out at 320 nm. Des. J. Ralph and E. Lu provided the ferulate dehydromers, which were treated with 2 N NaOH prior to running as standards (Ralph et al., 1994). To confirm that the species with corresponding retention times were diterulates, we also collected the two major dimer peaks from the HPLC and verified their mass by LC-MS.

**Hydroxycinnamate Fractionation**

To determine whether the changes in hydroxycinnamate content were associated with the matrix polysaccharide or the lignin fractions, 6 mg of destarched AIR was mixed with 600 mS of either 0.05% TFA or water, similar to a previously described method (Saulnier et al., 1995). Samples were incubated with 300 mS of cold 95% ethanol, vortexed, and extracted three times with 300 mS of ethyl acetate. Combined extracts were dried with a CentriVap without heat and resuspended in 50:50 methanol for HPLC analysis. The multiple time points show that the reaction that liberates the matrix polysaccharide goes approximately to completion. Longer times or higher acid concentrations caused degradation of the hydroxycinnamates.

**HPLC-Electrospray Ionization-Mass Spectrometry**

HPLC separation of the supernatant (5-mg samples) treated for 4 h with 50 mS TFA was performed using an Agilent 1290 HPLC system equipped with a Phenomenex Kinetex reverse-phase column (ODS-18; 100 mm × 2.1 mm, 2.6-µm particle size). Mobile phase A consisted of 5% HPLC-grade acetonitrile (Fisher Scientific) and 0.1% formic acid in HPLC-grade submicron filtered water (Fisher Scientific). Mobile phase B consisted of 0.1% formic acid in 100% HPLC-grade acetonitrile. These mobile phase solutions were filtered and vacuum degassed prior to use. A binary gradient at 0.3 mLS min<sup>−1</sup> flow rate was applied as follows: 90% solvent A and 10% solvent B from 0 to 4 min, linear gradient to 30% solvent B from 4 to 8 min, linear gradient to 50% solvent.
from 8 to 9 min, 50% solvent B from 9 to 12 min, linear gradient from 12 to 13 min to 100% solvent B, 100% solvent B from 13 to 15 min, and linear gradient to return the mobile phase to 90% solvent A and 10% solvent B from 15 to 16 min, which was maintained for an additional 5 min before the next sample was injected. The HPLC column eluent was introduced into an Agilent 6538 UHPLC Accurate Mass QTOF device equipped with an electrospray ionization source operated in negative ion mode. Nitrogen was used as a nebulizer gas (40 pounds per square inch) and a drying gas (325°C and 10 L min⁻¹ flow rate). Fragmentor, skimmer, and capillary voltages were 160, 65, and 750 V, respectively, and the capillary voltage was 3,500 V. Data were collected with Mass Hunter Acquisition (B.04.00) and analyzed with Mass Hunter Qualitative (B.04.00).

**Monosaccharide Composition by High-Performance Anion-Exchange Chromatography**

Destarched AIR (2–5 mg) was treated with 2 ml TFA at 120°C for 1 h. Next, the hydrolysate was dried using a CentriVap at 32°C. Monosaccharides produced by TFA hydrolysis were then redissolved in nanopure water and analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection on a Dionex ICS-3000 system equipped with an electrochemical detector and a 4 × 250-mm CarboPac PA20 column (O’Broo et al., 2004). The monosaccharides used as the external standards were obtained from Sigma-Aldrich and Alfa Aesar.

**Lignin Quantification Using Acetylbromide**

Lignin was quantified via acetylbromide solubilization (Fukushima and Hatfield, 2004), followed by quantification on a 96-well plate. Briefly, AIR (5 mg) was saponified in 2 N NaOH at 25°C for 24 h, then the supernatant was removed and the pellet was incubated with 300 μl of freshly prepared acetylbromide (25% [v/v] in acetic acid; Alfa Aesar) in screw-cap Eppendorf tubes (VWR International no. 16466-044) at 50°C for 3 h in a thermomixer at 1,050 rpm, with vortexing every 15 min for the last 1 h. After centrifuging, 100 μl of the solution was transferred to a fresh tube, followed by the addition of 400 μl of 2 N NaOH and 70 μl of freshly prepared 0.5 M hydroxylamine hydrochloride. Next, 57 μl of the solution was transferred to a UV-compatible 96-well plate, followed by the addition of 200 μl of glacial acetic acid. Absorption was measured at 280 nm with a SynergyHT (BioTek). The lignin content in the samples was determined with an extinction coefficient of 17.75 L g⁻¹ cm⁻¹ corresponding to average values for grass samples (Fukushima and Hatfield, 2004). Path length was determined by measuring the height of the plate.

**py-MBMS**

A commercially available molecular beam mass spectrometer designed specifically for biomass analysis was used for pyrolysis vapor analysis (Skyes et al., 2010). Approximately 4 mg of air-dried 20-mesh biomass was introduced into the quartz pyrolysis reactor via 80-μL deactivated stainless-steel Eco-Caps provided with the autosampler. Mass spectral data from m/z 30 to 450 were acquired on a Merlin Automation data system version 3.0 using 17-eV electron impact ionization. Lignin estimates and S:G ratios were determined by summing the intensities of peaks assigned to lignin compounds as described (Sykes et al., 2010). Several lignin peaks were omitted in the syringyl peaks described elsewhere (Gessesse and Gashe, 1997). The liberation of reducing sugars was quantified by 3,5-dinitrosalicylate (DNS) assay (Ghose, 1987).

**Enzymatic Saccharification Assay**

AIR (2–5 mg) was pretreated by shaking at 30°C in 500 μl of 100 mM citrate buffer (pH 5.0) followed by incubation at 100°C for 1 h. After cooling on the bench, final dilutions of 1:2,000 NS50013, which contains a cellulase cocktail, and 1:10,000 NS50010, which contains β-glucosidase, from the Novozymes Biomass Kit were added to the slurry. Reactions were incubated at 50°C with shaking with periodic removal of samples, which were stopped by freezing. Released reducing sugars were quantified by 3,5-dinitrosalicylate (DNS) assay (Ghose, 1987).

**Fungal Deconstruction and Enzymatic Assays**

**Pretreatment of Rice Straw**

Prior to pretreatment, wild-type and mutant rice straw was soaked in 1.2% H₂SO₄ overnight. Steam-based pretreatment was performed by loading the samples into an autoclave gun and treating them at 191°C with a residence time of 2 min. Pretreated materials were then released by rapid depressurization to allow the material to explode. The pretreated materials were collected, filtered, washed with distilled water, and stored at 4°C for subsequent degradation experiments using a modified Haggblad (1951) method.

**Rice Straw Degradation**

Pretreated wild-type and mutant rice straw was supplemented with 100 ml of Mandel’s medium in 500-ml flasks (Mandels et al., 1976). Spores from Penicillium sp. YT02 were collected from agar plates with a 0.9% NaCl solution, then adjusted to a concentration of 5.0 × 10¹⁲ spores ml⁻¹ and used as inoculum (10%, v/v) for fungal degradation.

**Protein Content**

Fungal growth was estimated by the protein content in the supernatant. Midlog fungal cell culture suspension was collected by centrifugation (14,000g) for 20 min at room temperature. The supernatant was collected, and protein content was determined via the Bradford method.

**Enzymatic Activities**

Total cellulase activity was determined against Whatman No. 1 filter paper (Sigma-Aldrich) using the DNS method (Xiao et al., 2004). Total endoglucanase activity was determined with carboxymethylcellulose (Clayesoms and Aerts, 1992) followed by reducing sugar measurements with DNS (Ghose, 1987). β-Glucosidase activity was determined with p-nitrophenyl-β-D-glucoside, and the liberation of p-nitrophenol was accompanied by absorption spectroscopy at 410 nm (Ghose, 1987). Xylanase activity was assayed as described elsewhere (Gessesse and Gashe, 1997). One international unit was defined as the enzymatic activity needed for the release of 1 mmol of sugar equivalents per unit of volume per minute. To improve accuracy, activity values are expressed relative to the protein concentration in the medium (IU mg⁻¹).

**Saccharides**

Fungal cultures were centrifuged, and the supernatants were analyzed for saccharide content by HPLC using an Aminex HPX-87H (Bio-Rad) organic acid column at 65°C. The mobile phase was 5 mM sulfuric acid at a flow rate of 0.5 ml min⁻¹. A refractive index detector was used.

**Statistical Analysis**

Two-tailed Student’s t tests were used to calculate the significance of differences in cell wall and growth parameters. Homoscedastic tests were used unless the difference between the variances of the two samples was greater than 3-fold, in which case a heteroscedastic test was used.

**Supplemental Data**

The following materials are available in the online version of this article.

Supplemental Figure S1. Mitchell clade-related BAHD phylogeny.

Supplemental Figure S2. qRT-PCR of related genes.


Humphrey TV, Bonetta DT, Goring DR (2007) Sentinelins at the cell wall: cell wall receptors and sensors. New Phytol 176: 7–21


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OsA10 Activation Alters Rice Cell Walls

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