The *jiaoyao1* Mutant Is an Allele of *korrigan1* That Abolishes Endoglucanase Activity and Affects the Organization of Both Cellulose Microfibrils and Microtubules in *Arabidopsis*

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In higher plants, cellulose is synthesized by plasma membrane–localized cellulose synthase complexes (CSCs). *Arabidopsis thaliana* GH9A1/KORRIGAN1 is a membrane-bound, family 9 glycosyl hydrolase that is important for cellulose synthesis in both primary and secondary cell walls. Most previously identified *korrigan1* mutants show severe phenotypes such as embryo lethality; therefore, the role of GH9A1 in cellulose synthesis remains unclear. Here, we report a novel A577V missense mutation, designated *jiaoyao1* (*jia1*), in the second of the glycosyl hydrolase family 9 active site signature motifs in GH9A1. *jia1* is defective in cell expansion in dark-grown hypocotyls, roots, and adult plants. Consistent with its defect in cell expansion, this mutation in GH9A1 resulted in reduced cellulose content and reduced CSC velocity at the plasma membrane. Green fluorescent protein–GH9A1 is associated with CSCs at multiple locations, including the plasma membrane, Golgi, trans-Golgi network, and small CESA-containing compartments or microtubule-associated cellulose synthase compartments, indicating a tight association between GH9A1 and CSCs. GH9A1*A577V* abolishes the endoglucanase activity of GH9A1 in vitro but does not affect its interaction with CESAs in vitro, suggesting that endoglucanase activity is important for cellulose synthesis. Interestingly, *jia1* results in both cellulose microfibril and microtubule disorganization. Our study establishes the important role of endoglucanase in cellulose synthesis and cellulose microfibril organization in plants.

INTRODUCTION

Plant endo-β-1,4-glucanases (EGases) belong to glycosyl hydrolase family 9, which consists of three distinct structural subclasses. *Arabidopsis thaliana* encodes at least 25 EGases, comprising members that are membrane anchored (class A), secreted to the cell wall (class B), or secreted and have a CBM49 carbohydrate binding module (class C) (Urbanowicz et al., 2007). While certain microbial EGases degrade crystalline cellulose, plant EGases are unable to hydrolyze crystalline cellulose. However, endoglucanase activity for class C EGases has been observed with various substrates, including xyloglucan, xylan, glucomannans, mixed-linkage glucans, and soluble cellulose derivatives such as carboxymethyl cellulose (CMC) and noncrystalline phosphoric acid–swollen cellulose (Master et al., 2004; Yoshida et al., 2006; Urbanowicz et al., 2007). *Arabidopsis* GH9A1/KORRIGAN1 (KOR1) belongs to class A type II integral membrane EGases, consisting of a cytoplasmic domain, a single transmembrane domain, and an extracellular catalytic domain. Although AtGH9A1 does not have a CBM domain, it has been shown to hydrolyze CMC and amorphous cellulose in vitro (Mølhøj et al., 2001a; Master et al., 2004; Liebminger et al., 2013). N-Glycosylation is important for KOR1 enzymatic activity, as demonstrated for both Ptt-Cel9A, the *Populus tremula × tremuloides* KOR1 homolog, and AtGH9A1 (Master et al., 2004; Liebminger et al., 2013).

In *Arabidopsis*, there are three class A EGases: GH9A1, GH9A2, and GH9A3. GH9A1 is ubiquitously expressed, whereas GH9A2 and GH9A3 are expressed in restricted cell types (Mølhøj et al., 2001b). The *kor1* mutants, first identified in a genetic screen for short hypocotyls, display primary cell wall defects such as irregular cell wall separations, abnormal cytokinesis, reduced cellulose content, and inhibition of tissue elongation (Nicol et al., 1998; Zuo et al., 2000; His et al., 2001; Lane et al., 2001; Mølhøj et al., 2001a; Sato et al., 2001). Furthermore, the *irregular xylem2* mutants, identified to be allelic to *kor1*, have a cellulose deficiency specific to the secondary cell wall (Szyjanowicz et al., 2004).

The tomato (*Solanum lycopersicum*) GH9A1 homolog Sl-GH9A1 was localized to both the Golgi and the plasma membrane by Suc gradient fractionation of tomato root microsomal membranes (Brummell et al., 1997). Partially consistent with this observation, AtGH9A1-GFP (for green fluorescent protein) showed a punctate pattern in interphase cells and was observed at the cell plate during cytokinesis of tobacco (*Nicotiana tabacum*) BY2 cells (Zuo et al., 2000). Overexpression of GFP-AtGH9A1 partially rescued...
kor1-1 hypocotyl and root growth phenotypes and was localized to an intracellular compartment composed of the Golgi, early endosomes, and tonoplast, but not to the plasma membrane (Robert et al., 2005). These discrepancies in the subcellular localization of GH9A1 have been met with three proposed functions: (1) recognition and removal of disordered glucan chains in cellulose; (2) generation of short primers upon which cellulose polymerization is initiated; and/or (3) termination of cellulose synthesis (Malheq et al., 2001a, 2002; Peng et al., 2002). To address whether GH9A1 interacts with or is an integral part of cellulose synthase complexes (CSCs), coimmunoprecipitation experiments were performed in which it was shown that GH9A1 does not stably interact with either primary (CESA3 or CESA6) or secondary (CESA7 or CESA8) CESA6s in detergent-solubilized extracts (Szyjanowicz et al., 2004; Desprez et al., 2007). These results cast doubt on whether GH9A1 and CSCs interact directly; however, GFP-GH9A1 is expressed under the control of its endogenous promoter localized to the plasma membrane and is proposed to be a part of primary CSCs (Crowell et al., 2010).

In a screen for seedlings that displayed a root-swelling phenotype, we identified a missense kor1 allele that we call jia1-1, named after a diminutive human race documented in Confucianism. A nucleotide substitution (C to T) in jia1 resulted in an amino acid substitution (A577V) within the second of its glycosyl hydrolase family 9 active site signature motifs, a sequence that is highly conserved among this family of EGases. The characterization of jia1 revealed that both cellulose microfibril ordering and cortical microtubule organization are reliant on the function of AtGH9A1.

**RESULTS**

**jia1 Is a KOR1 Missense Allele**

To identify novel factors involved in cellulose biosynthesis, we screened ethyl methaneisulfonate–mutagenized Arabidopsis seedlings (Columbia-0 [Col-0]) for altered root phenotypes. Approximately 100,000 ethyl methaneisulfonate–mutagenized seedlings were screened on half-strength Murashige and Skoog (MS) medium supplemented with 1% Suc. Seedlings with short and swollen roots were rescued and their progeny were tested. We identified 15 mutant alleles, which we designated jia. All jia mutants were recessive, following a 3:1 wild type:jia segregation pattern in the F2 generation of an outcross with the wild type. Allelic tests with known cellulose-deficient mutants, such as kor1 (rsw2-1, kor1-3), procuste1 (cesa6proc1-1), cesa3ces2, cobra2ces2-4, and cesa1ces1, revealed that two jia1 mutant alleles (jia1-1 and jia1-2) were allelic to kor1kor1-3. Although independent isolates, sequencing of the Arabidopsis GH9A1/KOR1 gene in the corresponding jia alleles showed that both contained the identical nucleotide substitution (C to T) in GH9A1, which resulted in the amino acid substitution A577V in GH9A1. The jia1-1 mutant phenotype was rescued by a construct containing the coding region of wild-type GH9A1 driven by its native promoter (Supplemental Figure 1A). The allelism of jia1 and kor1 was further supported by partial phenotypic complementation of jia1-1 by kor1-3 (Supplemental Figure 1B). Alignment of GH9A1 from monocot, dicot, moss, and green alga species indicated that residue Ala-577 is conserved between various plant species (Supplemental Figure 2).

**jia1 Shows Defects in Cell Expansion**

jia1-1 mutants exhibited short and swollen seedling roots. Seven-day-old, light-grown jia1-1 roots were ~67% shorter and ~23% wider than wild-type roots (Figures 1A, 1B, 1D, and 1E). Hypocotyls of dark-grown jia1-1 mutants were ~40% shorter than those of wild-type plants (Figures 1C and 1F). Additionally, adult jia1-1 mutants were dwarfed and had shorter siliques and smaller rosette leaves than wild-type plants (Supplemental Figure 3).

To assess the effect of the jia1 mutation on cellulose synthesis, we quantified the hypocotyl length of 5-d-old, dark-grown mutant and wild-type seedlings and the primary root length of 7-d-old, light-grown mutant and wild-type seedlings on increasing concentrations of the cellulose synthesis inhibitor isoxaben (Scheible et al., 2001; Desprez et al., 2002). We found that jia1-1 seedlings were hypersensitive to isoxaben as compared with the wild type (Figures 1G and 1H), suggesting that jia1-1 may be defective in cellulose biosynthesis. Crystalline cellulose analysis using the Updegraff method (Updegraff, 1969) showed that dark-grown hypocotyls of jia1-1 had ~22% less cellulose than those of the wild type (Figure 1I). The isoxaben hypersensitivity and reduced crystalline cellulose are consistent with the anisotropic growth defects observed for jia1-1 seedlings (Figures 1C and 1F).

**GH9A1 Colocalizes with CSCs**

To examine the subcellular localization of GH9A1, a ProGH9A1:GFP-GH9A1 construct containing ~2 kb of native GH9A1 promoter, the coding sequence of GH9A1, and an N-terminal GFP tag was transformed into kor1-3 plants (Paredes et al., 2008). GFP-GH9A1 fully rescued the kor1-3 mutant phenotype of 7-d-old, light-grown seedlings (Figures 2A and 2B), suggesting that the GFP-GH9A1 fusion protein was functional in plants. In dark-grown hypocotyls, GFP-GH9A1 localized to the plasma membrane with a punctate distribution and to larger intracellular compartments (Figure 2C; Supplemental Movie 1). Time-series imaging showed that plasma membrane–localized GFP-GH9A1 moved bidirectionally and followed linear tracks over time (Figure 2C; Supplemental Movie 1). The average velocity of membrane particles was 343 ± 133 nm/min (range, 0 to 700 nm/min; n = 1167 particles), which is similar to what has been reported previously for CSCs and proteins associated with CSCs (Paredes et al., 2006; Desprez et al., 2007; Gutierrez et al., 2009; Gu et al., 2010; Bischoff et al., 2011; Li et al., 2012).

To examine whether GH9A1 associates with CSCs in vivo, we generated a line expressing both mCherry-CESA3 and GFP-GH9A1. Two-channel confocal imaging revealed that the GFP-GH9A1 signal overlapped extensively with mCherry-CESA3 at the plasma membrane (Figure 3A; Supplemental Movie 2). GFP-GH9A1 and mCherry-CESA3 particles moved along linear tracks, as shown by time-averaged projections of 61 frames from a 5-min time series of images (Figure 3A; Supplemental Movie 2). The linear tracks traveled by mCherry-CESA3 coincided with those of GFP-GH9A1, as shown by the merged image (Figure 3A). Similar to mCherry-CESA3, GFP-GH9A1 particles traveled bidirectionally, and the average velocity of GFP-GH9A1 particles (268 ± 119 nm/min; n = 671 particles for both signals) was similar to that of mCherry-CESA3 particles (268 ± 119 nm/min; n = 671 particles for both signals) in the same cells (Figure 3B).
Figure 1. jia1-1 Seedlings Exhibit Aberrant Growth in Both Light-Grown Roots and Dark-Grown Hypocotyls.

(A) Seven-day-old, light-grown seedlings of the Col-0 wild type and jia1-1. Bar = 1 cm.
(B) Primary roots of 7-d-old, light-grown seedlings of the wild type and jia1-1. Bars = 50 μm.
(C) Five-day-old, dark-grown seedlings of the wild type and jia1-1. Bar = 1 cm.
(D) and (E) Quantification of root length (D) and width (E) of light-grown wild type and jia1-1 plants.
(F) Quantification of hypocotyl length of dark-grown wild-type and jia1-1 plants.

In (D) to (F), n = 50 seedlings per genotype. Double asterisks indicate significant differences from the wild type at the same data point (P < 0.01, Student’s t test).

(G) and (H) jia1-1 is hypersensitive to the cellulose synthesis inhibitor isoxaben. Seedlings were grown vertically for the indicated number of days on solid MS plates supplemented with increasing concentrations of isoxaben. The effect of isoxaben on primary root length of 7-d-old, light-grown wild-type and jia1-1 plants (G) and the hypocotyl length of 5-d-old, dark-grown wild-type and jia1-1 plants (H) were quantified. n = 50 seedlings per genotype.

(I) Crystalline cellulose content of 4-d-old, dark-grown wild-type (Col-0), prc1-1, and jia1-1 plants. Double asterisks indicate significant differences from the wild type at the same data point (P < 0.01, Student’s t test). Data were collected from five technical replicates for each tissue sample. Error bars represent SD.

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These observed velocities for both mCherry-CESA3 and GFP-GH9A1 were lower than what had been observed for either CESA3 or GH9A1 alone (Figure 2D), possibly due to the effect of multiple tags on the motility of CSCs. Nevertheless, the frequency distribution of velocities for mCherry-CESA3 was similar to that of GFP-GH9A1 (Figure 3B), further supporting the dynamic similarity of these two molecular components.

CSCs were observed in various cellular locations, including the plasma membrane, the periphery of the Golgi apparatus, and small CESA-containing compartments (SmaCCs) or microtubule-
associated cellulose synthase compartments (MASCs) (Haigler and Brown, 1986; Crowell et al., 2009; Gutierrez et al., 2009). Isoxaben treatment is known to deplete CSCs from the plasma membrane, with the subsequent appearance of numerous SmaCCs/MASCs (Crowell et al., 2009; Gutierrez et al., 2009). After dark-grown hypocotyls were treated with 100 nM isoxaben for 2 h, GFP-GH9A1 punctae moved simultaneously with SmaCCs/MASCs that were colabeled with mCherry-CESA3 (Figure 3C; Supplemental Movie 3). In addition to SmaCCs/MASCs, GFP-GH9A1 also localized to donut-shaped intracellular compartments that exhibited cytoplasmic streaming and colocalized with mCherry-CESA3 (Figure 3C; Supplemental Movie 3); thus, we predict their identity to be Golgi bodies. These results suggest that GH9A1 associates with primary CSCs at the plasma membrane and various intracellular compartments.

To further examine the identity of the GH9A1-labeled intracellular compartments, we crossed GFP-GH9A1 to a subset of Arabidopsis mCherry-labeled organelle marker lines (Geldner et al., 2009). Two putative markers for the Golgi apparatus and the trans-Golgi network, mCherry-SYP32 and mCherry-SYP61, respectively, show significant overlap with GFP-GH9A1 (Supplemental Figures 4A and 4B). Similar to structures positive for these Golgi markers, GFP-GH9A1 displayed rapid, nonlinear cytoplasmic streaming (Supplemental Movie 4). Partial colocalization was observed between GFP-GH9A1 and mCherry-VTI12, a putative early endosome/trans-Golgi network marker (Uemura et al., 2004). Most often, mCherry-VTI12 was found to label the center of a Golgi stack surrounded by GFP-GH9A1 signal at the periphery (Supplemental Figure 4C). Some small compartments labeled by mCherry-VTI12 were not colocalized with GFP-GH9A1. Signals from GFP-GH9A1 and the post-Golgi/endosome marker mCherry-RabC1 partially overlapped (Supplemental Figure 4D), while GFP-GH9A1 did not colocalize with the endosome/recycling endosome marker mCherry-RabA1g or the late endosome/vacuole

**Figure 3.** GH9A1 Colocalizes with CESA.

Arabidopsis seedlings expressing both mCherry-CESA3 and GFP-GH9A1 were grown in the dark for 3 d before imaging.

(A) Single optical sections and time averages of 61 frames (5-min duration in 5-s intervals) of plasma membrane–localized mCherry-CESA3 and GFP-GH9A1. The mCherry channel, GFP channel, and merged image of mCherry and GFP are shown. Bar = 5 μm.

(B) Histogram of particle velocities. The mean velocity is 268 ± 119 nm/min for mCherry-CESA3 and 260 ± 113 nm/min for GFP-GH9A1 (n = 671 particles for both).

(C) Single optical sections of mCherry-CESA3 and GFP-GH9A1 in intracellular compartments after a 2-h exposure to 100 nM isoxaben. Large circles denote Golgi compartments, and small circles denote SmaCCs/MASCs. Bar = 5 μm.

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marker mCherry-RabG3f. These results suggest that, similar to CSCs (Paredez et al., 2006; Crowell et al., 2009; Gutierrez et al., 2009), the trafficking of GH9A1 occurs mainly between the Golgi apparatus, the trans-Golgi network, and the early endosomes.

**GH9A1 Interacts with Both Primary and Secondary CESAs**

The association of GH9A1 and CSCs prompted us to test for direct interaction between CESA and GH9A1. Using in vitro affinity chromatography, interaction was detected for both the *Escherichia coli*–expressed, His-tagged, full-length GH9A1 and the His-tagged version of the predicted jia1-encoded GH9A1<sup>AST7V</sup> with the *E. coli*–expressed, glutathione S-transferase (GST)–tagged central domains (CD) of two representative primary CESAs (GST-CSA1CD and GST-CSA3CD) and one secondary CESA (GST-CSA8CD) (Figures 4A and 4C). The amounts of His-tagged GH9A1 and His-tagged GH9A1<sup>AST7V</sup> proteins bound by GST-CESACD proteins were similar, suggesting that the AST7V amino acid substitution does not affect the binding of GH9A1 to CESAs.

**The jia1 Mutation Abolishes the Endoglucanase Activity of GH9A1 in Vitro**

*Arabidopsis* GH9A1 contains two glycosyl hydrolase family 9 active site signature motifs, with the signature 1 motif comprising residues Ser-499 to Arg-515 and the signature 2 motif comprising residues Tyr-559 to Ala-577 (Figure 4A; Supplemental Figure 2). The amino acid substitution (Ala-577 to Val) in jia1-1 maps to the second signature motif of GH9A1. To test whether the amount of GH9A1<sup>AST7V</sup> protein expressed in jia1-1 is similar to GH9A1 expression levels in wild-type plants, total protein extracts from 10-d-old, light-grown seedlings were compared on immunoblots probed with anti-GH9A1 antiserum. The blots were also probed with an anti-α-tubulin monoclonal antibody as a loading control. The amount of GH9A1 protein detected in jia1-1 extracts did not differ from that in wild-type plants (Figure 4B), indicating that the single AST7V substitution affects a particular function of GH9A1 and that the phenotypes of jia1-1 are not due to reduced levels of GH9A1.

To test whether the jia1 mutation affects the endoglucanase activity of *Arabidopsis* GH9A1, wild-type GH9A1<sub>ΔN</sub> and GH9A1<sup>AST7V,ΔN</sup> fragments (Figure 4A) were expressed in *Spodoptera frugiperda* S121 cells using a baculovirus expression system, which was recently shown to be suitable for the production of enzymatically active GH9A1 (Lieberminger et al., 2013). Immunoblot analysis of cell extracts and culture supernatants revealed that recombinant wild-type GH9A1<sub>ΔN</sub> and the mutant GH9A1<sup>AST7V,ΔN</sup> were successfully expressed in S121 cells and secreted into the culture medium. The secreted His6-tagged GH9A1<sub>ΔN</sub> and GH9A1<sup>AST7V,ΔN</sup> proteins could be purified by means of nickel-chelate affinity chromatography in comparable yields. It was shown previously that CMC4M is a suitable substrate to assess the enzymatic activity of GH9A1 variants (Lieberminger et al., 2013). Consistent with previous findings (Lieberminger et al., 2013), wild-type GH9A1<sub>ΔN</sub> showed a specific activity of 317 ± 25 milliunits/mg with CMC4M. By contrast, the activity of GH9A1<sup>AST7V,ΔN</sup> was barely detectable (4 ± 2 milliunits/mg; Figure 4D), suggesting that the jia1 mutation abolishes the endoglucanase activity of *Arabidopsis* GH9A1.

**CSC Velocity Is Reduced in jia1-1**

To monitor the dynamics of CSCs in jia1-1 mutants, we introduced GFP-CESA3, a marker for CSCs (Desprez et al., 2007), into a homoygous jia1-1 line. Although CSCs in the plasma membranes of jia1-1 epidermal cells were organized into linear arrays as they are in wild-type seedlings, the velocity of CSCs was reduced in the mutant. In control cells, GFP-CESA3 had an average velocity of 312 ± 129 nm/min (n = 882). In jia1-1 mutants, however, the average velocity of GFP-CESA3 was reduced to 149 ± 72 nm/min (n = 863; Figure 5A). This reduced velocity of GFP-CESA3 in jia1-1 is evident in compiled kymographs as steeper CSC trace slopes from jia1-1 images versus those of the wild type (Figure 5B). The reduced velocity is also reflected in the reduced mean frequency distribution of the velocities of GFP-CESA3 particles in jia1-1 mutants, where more GFP-CESA3 particles were observed in jia1-1 to have a velocity below 150 nm/min compared with those in control cells (Figure 5C). The reduction of CSC velocity in the jia1-1 mutant suggests that GH9A1 influences the dynamics of CSCs, which reflects the reduced catalytic activities of jia1-1 CSCs in vivo.

**Cellulose Microfibril Organization Is Altered in jia1-1**

Atomic force microscopy (AFM) was used to examine the impact of defective GH9A1 on cellulose microfibril organization in the primary cell walls of jia1-1 mutants. One prominent feature of the primary wall of *Arabidopsis* hypocotyls is that the cellulose microfibrils are arranged in multilayer sheets of varying angles (Figure 6A) (Chan et al., 2010). At the innermost wall surface, cellulose microfibrils appeared to have a nearly parallel alignment or to be bundled together, and multiple layers of cellulose microfibrils were not apparent in jia1-1 plants (Figure 6A). In addition, cellulose microfibrils were less dispersed in jia1-1 than those in the wild type, although the individual microfibril diameters were indistinguishable between the jia1-1 mutant and the wild type (3.1 ± 0.2 nm). Cellulose microfibrils in wild-type cells displayed a wavy texture, which is similar to that observed in onion (*Allium cepa*) epidermis and cucumber (*Cucumis sativus*) hypocotyls (Marga et al., 2005; Zhang et al., 2013). By contrast, cellulose microfibrils of jia1-1 had extensive abrupt kinks (45° ± 15°), which were rarely seen in the wild type (Figure 6B). Much larger kinks or discontinuities in cellulose microfibrils on the scale of micrometers were occasionally seen in onion epidermis that suffered mechanical damage during sample preparation (Supplemental Figure 5), leading us to speculate that the kinked cellulose microfibrils in jia1-1 are likely not the result of mechanical damage during sample preparation. The abrupt kinks in jia1-1 suggest a discontinuity of cellulose crystallinity, which may be induced by frequent disruptions during cellulose microfibril synthesis or aberrant microfibril mechanical strength.

To investigate the orientation and packing of cellulose microfibrils through the entire depth of the cell wall, vibrational sum frequency generation (SFG) spectroscopy was used. SFG can selectively detect the coherence of crystalline cellulose over several hundred nanometers within intact plant cell walls and thus is a nondestructive probe of the mesoscale ordering or packing of cellulose microfibrils (Barnette et al., 2011; Park
et al., 2013; Lee et al., 2014). The general features of the SFG peak shape of the wild-type and jia1-1 spectra were similar to each other. Both showed a broad peak centered at 2920 cm\(^{-1}\) in the alkyl (C-H and C-H\(_2\)) stretching region with a shoulder at 2968 cm\(^{-1}\) and a strong peak centered at 3320 cm\(^{-1}\) with a shoulder at 3450 cm\(^{-1}\) in the hydroxyl (O-H) stretching region (Figures 6C and 6D). These are characteristics of primary cell walls and much different from cellulose microfibrils in secondary cell walls (Park et al., 2013; Kafle et al., 2014; Lee et al., 2014). Since the SFG intensity in the alkyl and hydroxyl regions varies depending on the orientation of the laser incidence plane and the orientation of cellulose alignment, the hydroxyl:alkyl ratio is one metric that can show the relative orientation of cellulose in the plant cell wall (Kafle et al., 2014). In SFG spectra of wild-type hypocotyls (Figure 6C), it is noticeable that the hydroxyl:alkyl SFG intensity ratio did not vary significantly (P > 0.05, Student’s t test) when the sample was rotated with respect to the laser incidence plane. In SFG spectra of jia1-1 hypocotyls (Figure 6D), the hydroxyl:alkyl ratio in the transverse direction (3.03 ± 0.56) was significantly higher than that in the longitudinal direction (1.68 ± 0.44). These results suggest that the cellulose microfibrils were preferentially arranged transverse to the long axis of the hypocotyl in jia1-1 compared with the wild type. This is consistent with the AFM images of the innermost layer of cellulose microfibrils in jia1-1, which are more uniformly aligned than those in the wild type (Figures 6A and 6B). Since SFG is a nonlinear optical process, the intensity is sensitive not only to the cellulose amount in the probe volume but also to the pack density, orientation, and size of aggregated cellulose microfibrils within the coherence length, which is hundreds of nanometers to micrometers. Thus, the higher SFG intensity for the jia1-1 mutant containing slightly less cellulose implies a better packing or ordering of cellulose microfibrils in the cell wall compared with the wild type (Supplemental Figure 6). Together, AFM and SFG spectra analysis suggest that the jia1-1 mutant contains more transversely oriented cellulose microfibrils and better mesoscale packing or ordering than those in the wild type.

Both CSC Tracks and Microtubule Organization Are Altered in jia1-1

To explore how microtubule organization might relate to changes in cellulose microfibril organization in jia1-1, we examined...
microtubules in 3-d-old, dark-grown hypocotyl epidermal cells expressing GFP-MAP4 (Marc et al., 1998). The orientation of microtubules in epidermal cells showed spatial and temporal variation in jia1-1 as compared with the wild type. To more easily describe the patterns we observed, the seedling hypocotyls were conceptually divided into three regions, upper (approximately six to eight cells), middle (approximately eight cells), and lower (approximately eight cells), termed zones 1, 2, and 3, respectively (Supplemental Figure 6A). Zone 1 is closer to the apical hook of dark-grown hypocotyls, and zone 1 cells are the most actively elongating of the three zones. Conversely, zone 3 is closer to the primary root, and most of the cells in this lower region have elongated completely. The orientation of epidermal cortical microtubules in zone 1 was mostly transverse, while that in zone 3 was longitudinal; zone 2 showed an oblique orientation of cortical microtubules, where a gradual transition from transverse to longitudinal was observed in both wild-type and jia1-1 cells (Figure 7A; Supplemental Movie 5). In the wild type, 7% of the cells observed had wavy, spaghetti-like microtubules in zone 1, as compared with 23% in the same region of jia1-1 (Figure 7B). These wavy microtubules were more frequently seen in jia1-1 in zones 2 (~42%) and 3 (~62%). Fragmented microtubules were rarely observed in zones 1 and 2, and the frequency of fragmented microtubules in zone 3 was below 5% in wild-type hypocotyls. jia1-1 had an increased frequency of fragmented microtubules in all zones, with the highest increases in zones 2 (13%) and 3 (18%). Collectively, these results indicate that microtubule organization is affected in the jia1-1 mutant.

The trajectories of CSCs in the plasma membrane mirror the orientation of underlying cortical microtubules (Paredez et al., 2006; Crowell et al., 2009; Gutierrez et al., 2009; Li et al., 2012; Lei et al., 2013). To examine whether the jia1 mutation affects the coalignment of CSCs and microtubules, jia1-1 was crossed with a transgenic line expressing both GFP-CESA3 and mCherry-TUA5 (Gutierrez et al., 2009). The widespread coalignament between GFP-CESA3 and microtubules in jia1-1 was evident, as highlighted by color tracks in two-channel confocal images (Figures 7C and 7D). In zone 3 jia1-1 cells, where microtubule organization switched from transverse to longitudinal organization, the trajectory of an overlying GFP-CESA3 particle had a concomitant transverse-to-longitudinal reorientation (Figure 7D). Further supporting the coalignament between CSCs and microtubules, in jia1-1 cells where disorganized microtubules were present, the trajectories of CSCs mirror the underlying disorganized microtubules (Supplemental Movie 6).

**DISCUSSION**

GH9A1 is an essential Arabidopsis gene whose conditional mutants show a collection of defects in several cellular functions, including cellulose synthesis in both primary and secondary cell walls, cytokinesis, microtubule organization, and hormonal responses (Nicol et al., 1998; Zuo et al., 2000; Szyjanowicz et al., 2004; Paredez et al., 2008). The proposed hypothetical cellulose proofreading, priming, and terminating functions of AtGH9A1 (Mølhøj et al., 2001a, 2002; Peng et al., 2002) are based on the presence of a luminal catalytic domain, which shares homology with a well-characterized bacterial EGase. While AtGH9A1 and its ortholog in Brassica have been reported to degrade amorphous cellulose in vitro, the precise role of GH9A1 is still unknown. Our study provided evidence that the endoglucanase activity of GH9A1 is important for proper cellulose biosynthesis in planta. The association of GH9A1 and CSCs is widespread in various intracellular compartments and at the plasma membrane, suggesting that GH9A1 might be an integral part of CSCs.

**Is the Endoglucanase Activity of GH9A1 Essential for Cellulose Synthesis in Plants?**

jia1, encoding an A577V substitution in the second active site signature motif, is the only reported GH9A1 mutation that abolished...
its endoglucanase activity in vitro. Considering the relatively mild phenotypes in both plant development and cellulose biosynthesis resulting from the jia1 mutation, we propose that the endoglucanase activity of GH9A1 is an important, but nonessential, function for in vivo cellulose synthesis and plant viability. Although unlikely, we cannot exclude the possibility that the very low residual activity of GH9A1A577V is enough to sustain cellulose biosynthesis and plant viability, which might explain the difference between kor1 null lethality and jia1-1. Alternatively, the physiological substrates for GH9A1 may not be

Figure 6. Cellulose Morphology Is Altered in jia1-1.

(A) AFM images of the inner cell wall of 3-d-old, dark-grown hypocotyls show different morphology of the cell wall for the wild type and jia1-1. Arrows indicate the long axis of the cells. Bars = 0.5 μm.

(B) Multiple layers of cellulose microfibrils were apparent in the wild type but not in jia1-1. Images are magnified from the highlighted squares in (A). At least three layers of cellulose microfibrils were highlighted in green, red, and blue (innermost) dashed lines in wild-type samples. However, only one layer of cellulose microfibrils was observed in jia1 samples, and these displayed multiple abrupt kinks highlighted by blue dashed lines. Bars = 0.1 μm.

(C) and (D) SFG spectra were taken from zone 1 (a region of hypocotyl close to the apical hook) of 4-d-old, dark-grown hypocotyls of the wild type (C) and jia1-1 (D). The samples were rotated so that the laser plane of incidence was along the long axis of the hypocotyl (L) and transverse to the long axis of the hypocotyl (T). The hydroxyl:alkyl ratios were calculated using the SFG intensities at 3320 and 2944 cm⁻¹ with the average and SD from n = 4 locations on the sample. Double asterisks indicate significant differences in hydroxyl:alkyl ratio (P < 0.05, Student’s t test). Spectra were normalized with respect to the peak at 2944 cm⁻¹.

[See online article for color version of this figure.]
identical to those tested in vitro; thus, the GH9A1A577V mutant protein may yet be able to exhibit some degree of activity in vivo.

The importance of endoglucanases in bacterial cellulose synthesis has been firmly established. In Gluconacetobacter xylinus, an open reading frame is located upstream of the bcs operon, which encodes the EGase CMCax. CMCax is secreted by G. xylinus, and its endoglucanase activity is essential for cellulose synthesis in vivo but not in vitro (Standal et al., 1994; Kawano et al., 2002). While CMCax is a single gene in G. xylinus, Arabidopsis encodes two GH9A1-like genes: GH9A2 and GH9A3. It is possible that GH9A2 and/or GH9A3 can functionally compensate for the lack of endoglucanase activity in GH9A1A577V, allowing the jia1-1 mutant to retain cellulose synthesis ability yet not at full capacity. However, gene expression data show that GH9A2 and GH9A3 are expressed in specialized cells/tissues, such as root hairs, trichomes, and leaf vasculature (Mølhøj et al., 2001b). Therefore, it is unlikely that GH9A2 and/or GH9A3 can effectively substitute for GH9A1 and accommodate the cellulose biosynthetic requirements for the entire plant, taking into account that cellulose synthesized in specific cells is not transported intercellularly. Functional characterization of a double or triple mutant will be valuable to determine whether the combined endoglucanase activities of GH9A1 and GH9A1-like proteins are essential for cellulose synthesis in plants.

KOR1 Is Associated with CSCs

A number of lines of evidence indicate that GH9A1 is important for cellulose biosynthesis, probably through a direct physical association between GH9A1 and CSCs (Nicol et al., 1998; His
GH9A1 and Microtubule Organization

Mounting evidence supports the hypothesis that the orientation of newly synthesized cellulose microfibrils is determined by the underlying cortical microtubules (Green, 1962; Hepler and Newcomb, 1964; Baskin, 2001; Paredez et al., 2006). However, the mechanistic details of how microtubules control the organization of cellulose microfibrils remained unclear until the recent discovery of CELLULOSE SYNTHASE INTERACTIVE PROTEIN1 (CSI1), a linker between CSCs and microtubules (Gu et al., 2010; Gu and Somerville, 2010; Li et al., 2012). CSI1 is indispensable for the guidance of CSCs along cortical microtubules and is required to maintain the normal motility of CSCs. Similar to CSI1, GH9A1 colocalizes with CSCs and travels in a speed indistinguishable from that of CSCs. However, the colocalization between CSCs and cortical microtubules was not affected in the jia1-1 mutant. Further supporting that GH9A1 is dispensable for the colignment of CSCs and cortical microtubules, the CSC tracks mirrored the disorganized microtubules in jia1-1. CSI1 is required to maintain the normal velocity of CSCs, a function that may be microtubule dependent (Lei et al., 2012, 2014; Li et al., 2012, 2014). Similarly, the jia1 mutation also reduced CSC velocity, a reduction that is more comparable to that of the cs1/csi3 double mutant than that of cs1 (Lei et al., 2013). We hypothesize that the reduction of CSC velocity in jia1 is most likely not through CSI1/microtubule function, since the colignment of CSCs and microtubules is normal in jia1. However, we cannot rule out the possibility that GH9A1 may assist the motility of CSCs at the membrane through a microtubule-dependent mechanism. Experiments to examine the CSC motility in the jia1 cs1 double or jia1 cs1 csi3 triple mutant are under way to test the above hypothesis.

The microtubule defect in jia1 may be due to feedback regulation between the cell wall and the cytoskeleton (Wyatt and Carpita, 1993; Fisher and Cyr, 1998). Elimination of CSCs by either pharmacological or genetic means supports that dynamic reciprocity exists between the intracellular microtubules and the extracellular cell wall (DeBolt et al., 2007a, 2007b; Paredez et al., 2008). For example, 2,6-dichlorobenzonitrile, an inhibitor of cellulose synthesis, disrupted microtubule organization at a concentration of 0.5 μM (Peng et al., 2013). At a much higher concentration (5 μM), 2,6-dichlorobenzonitrile caused a complete loss of CSC motility at the plasma membrane (DeBolt et al., 2007b). Another drug, morlin (7-ethoxy-4-methyl chromen-2-one), is able to disrupt both microtubule organization and CSC motility (DeBolt et al., 2007a, 2007b). korl-3 and a null allele of CESAl (cesa9rect-20) were identified in a screen for Arabidopsis mutants that are hypersensitive to oryzalin, a microtubule-depolymerizing drug, in which both mutants exhibited altered cortical microtubule orientation (Paredez et al., 2008). Interestingly, CSC motility is also reduced in korl-3 and cesa9rect-20. Together with the observations that cortical microtubule orientation was altered and CSC velocity was reduced in jia1, it is tempting to speculate that a reduced cellulose synthesis rate might be tightly associated with defects in microtubule organization. It is exciting that, in addition to CSI1, GH9A1 may represent a novel class of proteins involved in the intimate interaction between CSCs and microtubules. Further experiments are required to elucidate how the behavior of CSCs affects the underlying cortical microtubule organization.

GH9A1 and Cellulose Microfibril Organization

Recently, AFM was developed to visualize nascent cellulose microfibrils at the inner surface of cell walls in onion epidermal cells. This method allows the visualization of cellulose microfibrils with minimal disturbance and in near-native conditions (Zhang et al., 2013). Applying this method, microfibrils in intact Arabidopsis tissues are revealed in unprecedented detail. Two interesting observations of cellulose microfibril organization were made in jia1-1 mutant plants: abrupt kinks and loss of multilayer organization. Cellulose microfibrils often exist as bundles in both primary and secondary cell walls (Fernandes et al., 2011; Thomas et al., 2013; Zhang et al., 2013; Li et al., 2014). It is apparent that the kinks in jia1-1 are at the same scale as cellulose microfibril bundles. Based on the prevalence and the size of the kinks observed in jia1-1, they are unlikely to be artifacts resulting from sample preparation. As such, kinks were also observed in the microtubules of the jia1-1 mutant, so it is likely that the formation of kinks is attributable to defective microtubule organization. The defects in microtubule organization may result in disorganized secretion of noncellulosic cell wall materials such as hemicelluloses and pectins, which may result in an uneven coating of cellulose microfibrils by these materials, therefore influencing the local mechanical strength of cellulose microfibrils. Alternatively, the formation of these kinks may be independent of microtubule function. For example, kinks may be formed via the unsynchronized rate of individual elementary cellulose synthesis and the abrupt disruption of cellulose synthesis. Further research is needed to elucidate the exact mechanism for altered cellulose microfibril organization in jia1-1, possibly through a comparison of cellulose microfibril organization between jia1-1 and various cellulose-deficient mutants.

As vibrational SFG spectroscopy can selectively detect the signal from crystalline cellulose within intact plant cell walls
(Barnette et al., 2011; Park et al., 2013), cellulose content, orientation, and mesoscale ordering of cellulose microfibrils can be reflected in the intensity and hydroxy:alkyl ratio of SFG spectra. jia1-1 shows a reduced crystalline cellulose content as determined by the Updegraff method (Updegraff, 1969). However, the SFG intensity was slightly stronger in jia1-1 than in the wild type. The apparent discrepancy can be attributed to the compensation of the signal from the increased ordering of cellulose microfibrils as compared with that in the wild type. The altered intensity and the hydroxy:alkyl ratio of cellulose microbrils are oriented transversely in the innermost plant epidermal cell walls, thus favoring longitudinal expansion (Roelofsen and Houwink, 1951; Roelofsen et al., 1953). However, during cell elongation, layers of cellulose microbrils rotate as sheets of varying angles, which establish the basis of the multinet growth hypothesis (Green, 1960; Roelofsen, 1965; Roland et al., 1975). It has been hypothesized that multinet cellulose organization may relate to the features of the primary cell walls, such as extensibility and rigidity (Li et al., 2014). It is likely that the loss of multinet cellulose organization in jia1-1 may affect the mechanical strength of the cell wall. It is also possible that the localized incorporation of other wall polymers might be affected in jia1, thus affecting the overall mechanical strength of cell wall. Reduced overall crystalline cellulose content in hypocotyls and abrupt kinks in jia1-1 mutants support the idea that the mechanical strength of the cell wall might be affected.

Consistent with the multinet hypothesis, the underlying cortical microtubules as well as CSC trajectories undergo relative rotary movements in the successive layers of cellulose in Arabidopsis hypocotyl epidermal cells (Chan et al., 2007; Zhang et al., 2013; Bashline et al., 2014). Pharmacological disruption of microtubule rotation inhibited the rotation of CSC trajectories, leading to the hypothesis that the rotation of cellulose microfibrils is dependent on microtubule organization (Chan et al., 2007, 2010, 2011; Chan, 2012). The loss of multiple-angle layers of cellulose microfibrils in jia1-1 may be attributed to the defect in microtubule organization. However, the realignment of microtubules and cellulose microfibrils was not affected in jia1-1. Alternatively, the jia1 mutation might induce hormonal response defects, thus affecting cell elongation. One severe mutant of GH9A1, tsd1, was reported to have defects in response to auxin and cytokinin, two hormones that are involved in coordinating plant growth (Krupková and Schmülling, 2009). Other factors, such as biophysical forces and turgor-driven expansion, may also influence cellulose microfibril reorientation. Further characterization of jia1 will shed light on the mechanism(s) underlying microtubule organization and cell wall architecture in plants.

METHODS

Transgenic Lines

The 35S promoter in pGWB2 was replaced with a 2-kb CESA3 promoter using HindIII and XbaI to create the construct pYG114 using the primers indicated in Supplemental Table 1. The full-length coding sequences of CESA3 and mCherry were amplified using the primers indicated in Supplemental Table 1, ligated together at an introduced XbaI site, and cloned into the pCR8/GW/TOPO vector (Life Technologies) to create the construct pYG115. The sequence-verified mCherry-CESA3 fragment was cloned into the pGWB2 vector that contained the native CESA3 promoter using Gateway LR Clonase II (Life Technologies) to produce pYG116. pYG116 was transformed into E. coli by Agrobacterium tumefaciens–mediated transformation. mCherry-CESA3 transgenic lines selected for further analysis were designated YG108 and YG109. To generate GFP-GH9A1 transgenic lines, the 3SS promoter in pH7FWG2 (Karimi et al., 2002) was replaced by a 2-kb GH9A1 promoter using the primers indicated in Supplemental Table 1 to create pYG117. The full-length coding sequence of GH9A1 was introduced into pYG118 using Gateway LR Clonase II. The sequence-verified construct pYG119 was introduced into kor1-3 using Agrobacterium-mediated transformation. GFP-GH9A1 transgenic lines selected for further analysis were designated YG110 and YG111. An mCherry-CESA3–expressing Arabidopsis thaliana line was crossed with one expressing GFP-GH9A1 to create the double-labeled transgenic lines.

Protein Purification and In Vitro Binding/Affinity Chromatography Assay

The coding sequences of the central domains of CESA1, CESA3, and CESA8 were cloned into the pGEX-KG vector in frame with an N-terminal GST tag and expressed in BL21Star-pLysS Escherichia coli. The GH9A1 wild-type coding sequence and GH9A1A577V coding sequence were cloned into YG201, which provides an N-terminal His tag protein fusion, and expressed in BL21-CodonPlus (DE3)-RiPL. E. coli. Protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 15°C for 20 h. Protein purification and an in vitro pull-down assay were performed as described previously (Bashline et al., 2013).

Immunoblot Analysis

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunostaining was performed by blocking the nitrocellulose membrane with PBS and 0.1% Tween 20 (PBS-T) containing 5% nonfat milk followed by incubation for 2 h at room temperature with the anti-GH9A1 antiserum diluted 1:2000 or anti-α-tubulin monoclonal antibody, clone B-5-1-2 (Sigma-Aldrich), diluted 1:8000 in PBS-T. After extensive washing with PBS-T, membranes were incubated for 2 h at room temperature with goat anti-rabbit or goat anti-mouse antibodies conjugated to peroxidase (Santa Cruz) diluted 1:10,000 in PBS-T containing 5% milk. Detection of antibody binding was performed using the ECL-Select protein gel blotting reagent and the ImageQuant LAS 4000 digital imaging system (GE Healthcare Life Sciences).

Site-Directed Mutagenesis of GH9A1

The GH9A1A577V-encoding construct was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and Phusion High-Fidelity DNA Polymerase (Life Technologies). Mutagenesis was performed following the manufacturer’s instructions. The wild-type pVTBacHis-GH9A1 construct (Liebminger et al., 2013) was used as a template for site-directed mutagenesis using the primers indicated in Supplemental Table 1. To confirm the introduced mutations, all constructs were subjected to DNA sequencing.
Expression of GH9A1 Variants in Insect Cells and GH9A1 Activity Assays

Cultivation of Spodoptera frugiperda S212 cells, baculovirus-mediated infection, and purification of the recombinant proteins were performed as described in detail previously (Liebmaier et al., 2013). At the end of the purification procedure, the GH9A1-containing fractions were pooled and concentrated by ultrafiltration and then subjected to diafiltration using assay buffer containing 50 mM MES, pH 6.0, 250 mM NaCl, and 30 mM CaCl₂.

Purified recombinant GH9A1 (0.3 to 1 µg, 10 µL) was incubated with 90 µL of 0.1% CMC4M (Megazyme) in assay buffer for 90 min at 30°C. The reaction was stopped by the addition of 400 µL of ice-cold 50 mM sodium borate, pH 10.0. Then, 500 µL of freshly prepared BCA solution (a mixture of equal volumes of reagent A containing 5 mM 2,2'-bicinchoninic acid, 512 mM Na₂CO₃, and 288 mM NaHCO₃ and reagent B containing 5 mM CuSO₄ and 12 mM L-Ser) was added, and the samples were incubated for 15 min at 95°C. The samples were cooled on ice and then centrifuged for 3 min prior to measurement of their absorbance at 562 nm. Samples containing heat-inactivated enzyme were used as controls. The amount of reducing ends generated was then deduced using a Glc standard curve (0 to 25 nmol). All assays were done in duplicate.

Live-Cell Imaging

Dark-grown hypocotyls were grown on vertical MS plates [half-strength MS salts, 0.8% agar, and 0.05% 2-(N-morpholino)ethylsulfonic acid monohydrate, pH 5.7] in the dark as described for 3 d before being collected. Hypocotyl walls were rinsed and rehydrated with 20 mM HEPES, pH 7.0. ScanAsyst and Imaris (Bitplane) software. CESA particle dynamics analyses were performed previously (Gu et al., 2010; Bashline et al., 2014). Image analysis was performed using Metamorph (Molecular Devices), ImageJ, and Imaris (Bitplane) software. CESA particle dynamics analyses were performed as described previously (Gu et al., 2010; Bashline et al., 2014).

AFM of Arabidopsis Hypocotyl Walls

Hypocotyls of wild-type Arabidopsis (Col-0) and jia1 mutants were grown in the dark as described above for 3 d before being collected and stored at −80°C for less than 1 week. Hypocotyls were ground in liquid nitrogen and rinsed with 20 mM HEPES buffer, pH 7.0, and 0.1% Tween 20 until the ltrate was clear. One droplet of well-resuspended hypocotyl walls was scanned at a 512 × 512 sampling rate. At least 30 field of view technical replicates for each tissue sample.

Cellulose Content Measurements

Crystalline cellulose was measured in 4-d-old, dark-grown seedlings using the Updegraff method (Updegraff, 1969). Data were collected from five technical replicates for each tissue sample.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: Arabidopsis thaliana GH9A1 (AtGH9A1), AT5G49720; Vitis vinifera GH9A1 (VvGH9A1), VSVIVG01006265001; Ricinus communis GH9A1 (RcGH9A1), 30,063.t000040; Populus trichocarpa GH9A1 (PtGH9A1), Potri.010G177300; Oryza sativa GH9A1 (OsGH9A1), LOC_Os04g41970; Brachypodium distachyon GH9A1 (BdGH9A1), Bradi1g63560; Selaginella moellendorffii GH9A1 (SmGH9A1), 26852; Physcomitrella patens GH9A1 (PpGH9A1), Pp1864_152V6; and Chlamydomonas reinhardtii GH9A1 (CrGH9A1), Cre06.g270500.
Supplemental Data

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

Supplemental Figure 1. jia1-1 Is Allelic to kor1-3.

Supplemental Figure 2. Homology between the Amino Acid Sequences of GH9A1 Shows That Ala-577 Is Highly Conserved.

Supplemental Figure 3. Morphology of jia1-1.

Supplemental Figure 4. GH9A1 Is Located in Various Intracellular Organelles.

Supplemental Figure 5. AFM Images Show Mechanical Damage during Sample Preparation.

Supplemental Figure 6. SFG Spectra of 4-d-Old Dark-Grown Hypocotyls of the Wild Type and jia1-1.

Supplemental Table 1. DNA Primers Used in This Study.

Supplemental Movie 1. GH9A1 Localizes to Distinct Particles at the Plasma Membrane.

Supplemental Movie 2. Dynamic Association between GH9A1 and CESA Complexes at the Plasma Membrane.


Supplemental Movie 5. Microtubule Organization in the Wild Type and jia1-1.

Supplemental Movie 6. Disorganized Microtubule Bundles Coalign with Disorganized CSC Tracks in the jia1-1 Mutant.

ACKNOWLEDGMENTS

We thank Barbara Svoboda for technical assistance with GH9A1 activity assays. We thank Ryan Gutierrez and David Ehhrhardt for providing the mCherry-TUA5 GV3101 strain. We thank Herman H"ote for providing the mCherry-SYP32, -SYP61, -VIT12, -RabC1, -RabA1g, and -RabG3f transgenic seeds. We thank Yong Burn Park for technical assistance on sample preparation for SFG analysis. We thank Colleen McMichael for critical reading of the article. This work was supported by the Center for LignoCellulose Structure and Formation, an Energy Frontier Research Center funded by the U.S. Department of Energy, Office of Science, Office of Basic Sciences (Grant DE-SC0001090), and by the National Science Foundation (Grant 1121375 for mCherry-CESA and GFP-KOR1 transgenic line production and CESA central domain expression).

AUTHOR CONTRIBUTIONS


REFERENCES


The *jiaoyao1* Mutant Is an Allele of *korrigan1* That Abolishes Endoglucanase Activity and Affects the Organization of Both Cellulose Microfibrils and Microtubules in *Arabidopsis*

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*Plant Cell*; originally published online June 1, 2014;
DOI 10.1105/tpc.114.126193

This information is current as of June 24, 2014

Supplemental Data  
http://www.plantcell.org/content/suppl/2014/06/16/tpc.114.126193.DC1.html

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