AtCSLA7, a Cellulose Synthase-Like Putative Glycosyltransferase, Is Important for Pollen Tube Growth and Embryogenesis in Arabidopsis[1]

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The cellulose synthase-like proteins are a large family of proteins in plants thought to be processive polysaccharide β-glycosyltransferases. We have characterized an Arabidopsis mutant with a transposon insertion in the gene encoding AtCSLA7 of the CSLA subfamily. Analysis of the transmission efficiency of the insertion indicated that AtCSLA7 is important for pollen tube growth. Moreover, the homozygous insertion was embryo lethal. A detailed analysis of seed developmental progression revealed that mutant embryos developed more slowly than wild-type siblings. The mutant embryos also showed abnormal cell patterning and they arrested at a globular stage. The defective embryonic development was associated with reduced proliferation and failed cellularization of the endosperm. *AtCSLA7* is widely expressed, and is likely to be required for synthesis of a cell wall polysaccharide found throughout the plant. Our results suggest that this polysaccharide is essential for cell wall structure or for signaling during plant embryo development.

Plant cell walls are composed mainly of the matrix pectic and hemicellulosic polysaccharides and cellulose (Brett and Waldron, 1996; Fry, 2000). The matrix polysaccharides are diverse and complex in structure, with α- or β-linked sugars in long backbones often decorated with short side chains. Xyloglucan, which has a β-1,4-glucan backbone, is the most abundant hemicellulose found in the primary cell wall of dicotyledonous plants, and is thought to cross-link cellulose microfibrils. Hemicellulosic polysaccharides with backbones of β-1,3-glucan (callose), β-1,4-mannan, or β-1,4-xylan are also abundant in certain cell types (Brett and Waldron, 1996; Fry, 2000). Classical arabinogalactan proteins (AGPs), consisting of up to 90% polysaccharide, are often also considered hemicellulosic polysaccharides (Fry, 2000). The arabinogalactan chains on AGPs consist of β-1,3-galactan with β-1,6-galactan branches that are further decorated, mostly with Ara (Fry, 2000; Majewska-Sawka and Nothnagel, 2000). These arabinogalactan chains can probably be found on a wide variety of cell wall proteins (Borner et al., 2002). In contrast to these polysaccharides with β-linked sugars in the backbones, the main backbone of pectin is α-1,4-polygalacturonan or, in rhamnogalacturonan I (RG-I), alternating α-1,4-rhamnosyl and α-1,2-galacturonosyl residues. The backbone of RG-I is modified by the addition of side chains of α-1,5-arabinan and β-1,4-galactan. Other polysaccharides such as mixed linkage β-glucans are found in certain species or tissues (Fry, 2000). Therefore, cell walls contain a rich array of different polymers, but the role and the extent of functional redundancy between these different polysaccharides are unknown.

For many years, researchers have attempted to purify transferases involved in the synthesis of cell wall polysaccharides to isolate the corresponding gene. However, just two have been successfully purified because of difficulties both in retaining activity after solubilization and assaying the transferases has limited the success of this approach. A galactomannan galactosyltransferase was purified from developing fenugreek (*Trigonella foenum-graecum*) cotyledons (Edwards et al., 1999) and a xyloglucan fucosyl transferase was purified from pea (*Pisum sativum*) seedlings (Perrin et al., 1999). These two transferases are predicted to have a single transmembrane domain with the transferase activity within the lumen of the Golgi apparatus.

In contrast, the plant cellulose synthase genes were first identified based on the high homology of the gene family with cellulose synthases (CELA) of bacteria (Pear et al., 1996). Second, studies of Arabidopsis mutants have been invaluable in identifying and characterizing the cellulose synthase genes. Recently, callose synthase of Arabidopsis (Hong et al., 2001), tobacco (*Nicotiana talata*; Doblin et al., 2001), and cotton (*Gossypium hirsutum*; Cui et al., 2001) have also been identified by a sequence homology-based approach. Both the cellulose and callose synthases are plasma membrane proteins with multiple mem-

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brane-spanning domains. Although there are 12 cellulose synthase (*CESA*) genes in Arabidopsis (Richmond and Somerville, 2000; Saxena and Brown, 2000), characterization of the various Arabidopsis mutants has indicated that the genes are not redundant (Williamson et al., 2001). This could be because they form hetero-oligomeric complexes (Taylor et al., 2000), and because the genes are expressed at different growth and development stages.

The cellulose synthases are part of a large family of inverting processive β-glycosyltransferases. The family includes mammalian hyaluronan synthases and fungal chitin synthases, and belongs to the glycosyltransferase superfamily GT2 (Henrissat and Davies, 2000). In silico analysis suggests that a large number of relatively uncharacterized genes in plants, called the *CELLULOSE SYNTHASE-LIKE* (*CSL*) genes (Richmond and Somerville, 2000; Saxena and Brown, 2000; Hazen et al., 2002) encode glycosyltransferases in this family. These proteins have been divided into between six and eight different subfamilies (depending on the plant), and they show varying degrees of sequence similarity to CESA proteins. It was initially speculated that each subfamily might be involved in synthesizing the backbones of the abundant polysaccharides, namely callose, α-1,4-polygalacturonan, RG-I, RG-II, xyloglucan, and xylan (Richmond and Somerville, 2000). However, the recently identified callose synthase is not a member of the CSL family (Doblin et al., 2001; Hong et al., 2001). Furthermore, the pectin backbones contain α-linkages, which are unlikely to be synthesized by a member of the GT2 glycosyltransferase family. Thus, the CSL subfamilies might synthesize the backbone of the remaining β-linked polysaccharides such as β-1,4-galactan, xylan, mannan, xyloglucan, and the β-1,3- and β-1,6-galactan of AGPs. In this hypothesis, type II transferases would be used to synthesize the α-linked backbones of arabinan and pectin and the short side chains on the β-linked polysaccharide backbones.

Studies of two genes in the *CSLD* subfamily, the subfamily most similar to the *CESA* genes, have been published recently (Favery et al., 2001; Wang et al., 2001). Two Arabidopsis mutants (*kojak* and *csld3*) have been produced by T-DNA or *dissociation* element (*Ds*) insertions into *AtCSLD3*. The mutants have fewer root hairs than the wild type (WT). By genetic analysis, it appears that the CSLD3 gene acts early in the process of root hair outgrowth (Favery et al., 2001; Wang et al., 2001). Although this protein is expressed in all parts of the plant (Wang et al., 2001), it is only in the root hair that a phenotype has been observed in the mutant. In contrast, N*aCSLD1* of tobacco is only expressed in anther and in vitro-grown pollen tubes, and it has been predicted that this might be a cellulose synthase in pollen (Doblin et al., 2001).

In the work described here, we identified a mutation in A*tCSLA7* of the CSLA subfamily of putative processive β-glycosyltransferases. The gene is ubiquitously expressed, and is important for pollen tube growth and essential for embryogenesis, suggesting a requirement for a specific β-linked polysaccharide in plant development.

RESULTS

Isolation of AtCSLA7 cDNA

As part of an ongoing program to screen for Arabidopsis insertion mutants in genes encoding polysaccharide synthases, we selected SGT4425 in the collection of *Ds* transposon insertion mutants with flanking sequences generated by Parinov et al. (1999). The sequence flanking the insertion in this line indicated that a Ds element had inserted in a gene encoding a putative glycosyltransferase. Before further analysis of this insertion mutant line, we confirmed by reverse transcriptase (RT)-PCR on RNA isolated from WT Arabidopsis callus that this gene was expressed. Preliminary sequence alignments suggested that the annotation of the gene AAD15455.1 by The Institute for Genomic Research was not correct because nucleotide sequence upstream of the proposed initiator Met appeared to encode amino acid sequence conserved in homologous genes. Using Netplantgene2 (Brunak et al., 1991; Hebsgaard et al., 1996), we identified a potential upstream exon, and confirmed the existence of this exon by amplification of a longer cDNA by RT-PCR. The cDNA amplified contains an in-frame upstream stop codon; therefore, we are confident that this sequence is full length. The intron/exon structure of the gene is shown in Figure 1A. The protein contains 556 amino acids (Fig. 1B), and has a predicted molecular mass of 63,795 D and a pI of 9.0. We predict that the protein has six transmembrane domains (Fig. 1B) with N and C termini in the cytosol (Fig. 1C).

Homology searches indicated that the encoded protein is a member of the processive β-glycosyltransferase superfamily (GT2) that includes plant and bacterial cellulose synthases (Henrissat and Davies, 2000). The *CSL* genes of Arabidopsis have been grouped into six subfamilies by Richmond and Somerville (2000). Using this nomenclature, the cDNA isolated corresponds to the gene *AtCSLA7* in the CSLA subfamily containing 15 members in Arabidopsis. The "D,D,D,QXXRW" characteristic motifs of processive β-glycosyltransferases (Karnezis et al., 2000; Saxena and Brown, 2000; Williamson et al., 2001) are also found in AtCSLA7 (Fig. 1B, boxed). By alignment with CESA and CSL subfamily proteins, we found many residues conserved in all members (Fig. 1B, bold), or conserved within the CSLA subfamily (Fig. 1B, shadowed). Therefore, AtCSLA7 contains all the characteristics expected of a processive β-glycosyltransferase.

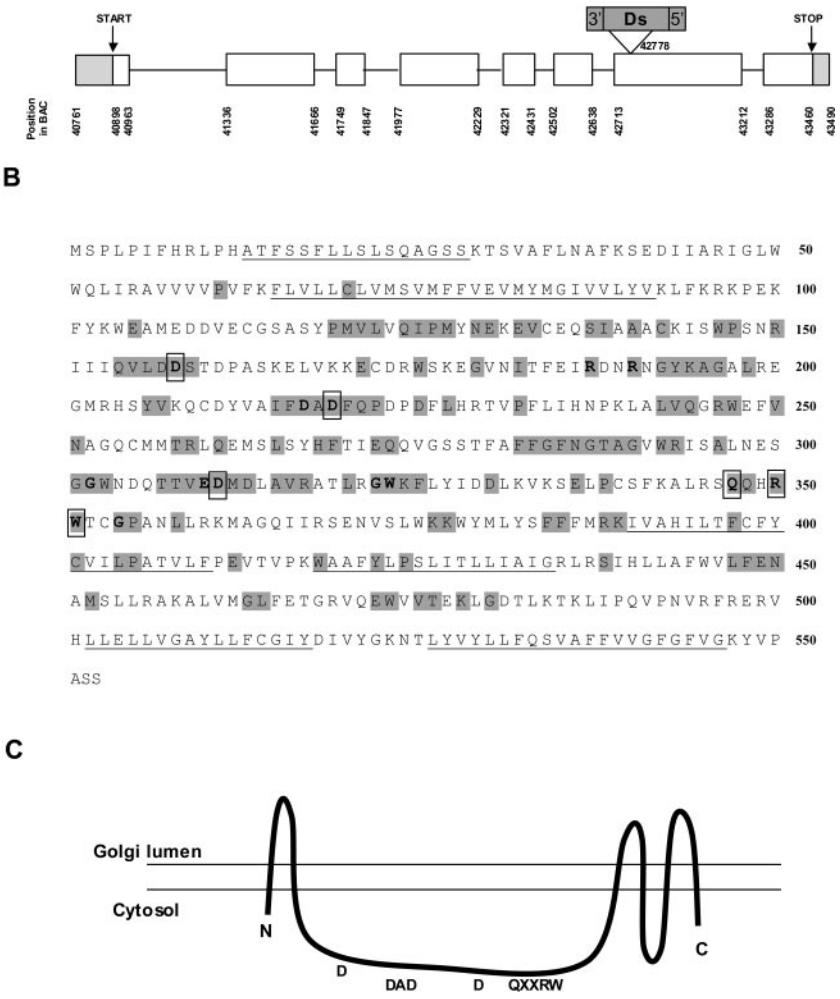

Figure 1. Structure of the predicted *AtCSLA7* gene and AtCSLA7 protein. A, Position of introns, exons, and *Ds* insertion in *AtCSLA7*. Rectangular boxes represent the exons and the lines represent introns in the gene. Light-gray rectangles are untranslated regions. Start and stop codons are indicated. The dark-gray rectangle represents the *Ds* insertion. Numbers refer to nucleotide position in the BAC T20F21. B, AtCSLA7 protein. Underlined amino acids correspond to the putative transmembrane domains of the protein. Shaded characters are the amino acids conserved in most members of the CSLA family. Bold characters are highly conserved amino acids in CSL, CELA, and CESA families. Boxes represent the D,D,D,QXXRW motif characteristic of β-glycosyltransferases. C, Topology model of AtCSLA7.

AtCSLA7 Expression

To investigate any organ-specific expression of *AtCSLA7*, RT-PCR was used to amplify the cDNA from RNA isolated from a range of plant tissues and organs. We found that the gene was expressed in all tissues examined, including old and young leaves, roots, callus, and pollen. Some examples are shown in Figure 2.

Identification of an Insertion Mutant

The transposon insertion line SGT4425 was analyzed for potential insertion in *AtCSLA7*. The position of the *Ds* insertion in exon 7 (Fig. 1A) was confirmed by direct PCR amplification of both ends of the *Ds* element and associated flanking genomic DNA. Analysis of over 300 plants from seven generations showed that all kanamycin-resistant (kan^r) plants contained the insert at this site, showing tight linkage between kanamycin resistance and this insertion. We also screened these plants for any homozygous individuals that would not yield PCR amplification of the gene with a pair of gene-specific primers. However, all the kan^r plants were heterozygous for the insertion. This indicated that the *AtCSLA7* gene is an essential gene.

Transmission of the Insertion in AtCSLA7

If homozygous plants die, we would expect a segregation ratio of 2:1 kan^r:kanamycin-sensitive (kan^s) plants in the surviving progeny of plants heterozygous for the *Ds* insertion. However, plants from four generations consistently produced progeny that segregated approximately 1.3:1 kan^r:kan^s seedlings (Table I), indicating reduced transmission of the *Ds* insertion. This analysis also demonstrated tight linkage of the *Ds* insertion and the reduced transmission phenotype. The reduced genetic transmission of *Ds* in SGT4425 suggested a gametophytic role for *AtCSLA7*.

Male and female transmission of *Ds* was determined by performing reciprocal test crosses with WT Arabidopsis Landsberg *erecta* (L*er*) and analyzing progeny on kanamycin selection plates. The data from five separate experiments are shown in Table II.

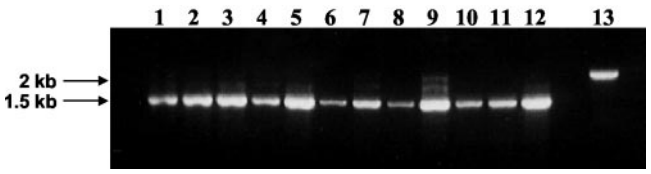

Figure 2. The expression of *AtCSLA7* in different plant tissues: analysis by RT-PCR using internal primers 1 and 4. 1 through 12, PCR products from RT-PCR reaction (approximately 1.5 kb); 13, PCR product from genomic amplification (approximately 2 kb). 1, Four-day-old callus; 2, 7-d-old callus; 3, 7-d-old plantlets; 4, roots; 5, leaves of rosettes; 6, young stems before flowering; 7, whole old stems including flowers, siliques, and leaves; 8, stems alone; 9, leaves of stem; 10, flowers; 11, pollen; 12, young siliques; 13, genomic DNA.

Male transmission was reduced to 29% of WT. In contrast, the female transmission efficiency (TE) was not affected. These data suggested an important gametophytic role for *AtCSLA7* in pollen development or function.

Embryo Lethality of Homozygous Ds Insertion in AtCSLA7

Given the reduced, but significant, male transmission of the *Ds* insertion in SGT4425, homozygous progeny were predicted to occur at a frequency of 11%. However, as described above, no homozygous progeny were detected. Moreover, no evidence was obtained for a seedling lethal phenotype, suggesting that homozygotes might be embryo lethal. Examination of developing seed in mature green siliques of hemizygous SGT4425 mutants revealed that all siliques contained a proportion of aborted seeds (Fig. 3A). The proportion of aborted seeds was found to be 14.7% (total no. of seeds scored = 2,301) in plants from several different generations. Siliques of WT L*er* did not show aborted seeds and none were observed when SGT4425 pollen was used to pollinate L*er* pistils. When SGT4425 was used as the female parent in a cross to L*er*, aborted seeds were observed infrequently (approximately 2%, $n = 393$). These data indicated that the homozygous *Ds* insertion in *AtCSLA7* is a recessive seed lethal mutation.

Transformation of SGT24425 with a 4-kb genomic region including *AtCSLA7* allowed recovery of plants homozygous for the *Ds* insertion in *AtCSLA7*. There was approximately doubled male TE and one-half the proportion of aborted seeds in plants hemizygous for the complementing DNA, indicating complementation of the phenotypes by *AtCSLA7* (not shown).

WT and aborted seeds from mature SGT4425 green siliques were examined by differential interference contrast (DIC) microscopy. WT seeds contained cotyledonary stage embryos (Fig. 3B), but all aborted seeds ($n = 76$) contained small, undeveloped embryos with a distinct suspensor that were arrested at globular stage, or were elongated along the apical-basal axis (Fig. 3C). Mutant embryos were globular or elongate structures showing no evidence of cotyledon development. Cell proliferation was severely reduced such that the terminal phenotype of most mutant embryos was to arrest with 16 to 48 cells. Similarly, the endosperm remained uncellularized in aborted seeds and peripheral free nuclear endosperm was clearly visible (Fig. 3C).

To investigate further the developmental progression, embryos from a series of developing siliques of WT and hemizygous SGT4425 plants were categorized into stages of development from four to 16 cells to cotyledon. This revealed that embryo development was by and large synchronous in WT siliques, with sibling embryos spanning two successive stages (Fig. 4, A–D; Table III). In contrast, SGT4425 siliques contained embryos of a wider range of developmental stages. A proportion (13%–23%) of embryos with delayed development (four–16-cell stage) was apparent when the majority of WT embryos (Fig. 4D) were at heart stage (Table III). This difference was already apparent at globular stage with delayed embryos at the one- to 16-cell stage.

It was not possible to distinguish most WT and mutant embryos at one- to eight-cell stages. However, some abnormal eight-cell embryos were observed in which the axial and transverse division planes were rotated by 45° (Fig. 4E). Delayed globular embryos also showed abnormal division patterns that often involved an incomplete set of protoderm divisions (Fig. 4F). In siliques containing WT embryos at the late heart stage (Fig. 4D), mutant embryos were often elongated along the apical basal axis (Fig. 4H). In most mutant embryos, the protoderm layer was incomplete and aberrant cell division patterns were observed in the basal region of the embryo (Fig. 4, E–H). A common phenotype involved the formation of two additional cell tiers resulting from additional transverse divisions (Fig. 4, G and H). Thus, SGT4425 mutant embryos show delayed development and abnormal cell patterning. No evidence was obtained for incomplete cell divisions. However, we cannot rule out subtle effects on cytokinesis not detectable with the DIC microscopy procedure used.

We investigated whether the effects of insertion in *AtCSLA7* were restricted to the embryo or were also seen in endosperm development. The mean number of endosperm nuclear divisions was determined in whole-mount seeds by DIC microscopy. Seeds con-

Table I. *Segregation of kan^r and kan^s progeny of SGT4425 plants heterozygous for a Ds insertion in AtCSLA7*

Pooled data for sibling plants are shown for four successive generations (F_1–F_4).

Generation	kan^r	kan^s	kan^r:kan^s
F_1	666	517	1.3
F_2	1,329	885	1.5
F_3	3,610	3,009	1.2
F_4	1,290	971	1.3

Table II. *Segregation of kan^r and kan^s seedlings in reciprocal test crosses of SGT4425 heterozygotes and WT (Ler) in five separate experiments*

Experiment	SGT4425 × WT			WT × SGT4425		
	kan^r	kan^s	Transmission efficiency female	kan^r	kan^s	Transmission efficiency male
			%			%
1	78	82	95	49	147	33
2	90	91	99	50	136	37
3	79	79	100	43	140	31
4	127	132	96	60	172	35
5	263	250	105	57	288	20
Mean	637	634	100	259	883	29

taining mutant embryos at approximately the 16-cell stage contained 60.9 nuclei per seed, which was comparable with 52.9 in WT seeds containing embryos at the 16-cell stage (number of nuclei > 600). In terminally arrested seeds, endosperm nuclei showed a small increase to 76.4 nuclei per seed, whereas nuclei in seeds containing WT globular embryos continued to increase beyond 175 nuclei per seed, when the number of nuclei could be reliably counted. Thus, endosperm proliferation is not maintained in mutant SGT4425 seeds and is associated with failure of the endosperm to cellularize. The nuclei present in the endosperm of the arrested mutant seeds were relatively uniform and of comparable size with those in WT seeds at the coenocytic endosperm stage (see Fig. 3C). However, in some mutant seeds, larger nuclei were observed at the micropylar pole (Fig. 4G), suggesting continued cycles of endoreduplication after failed cellularization.

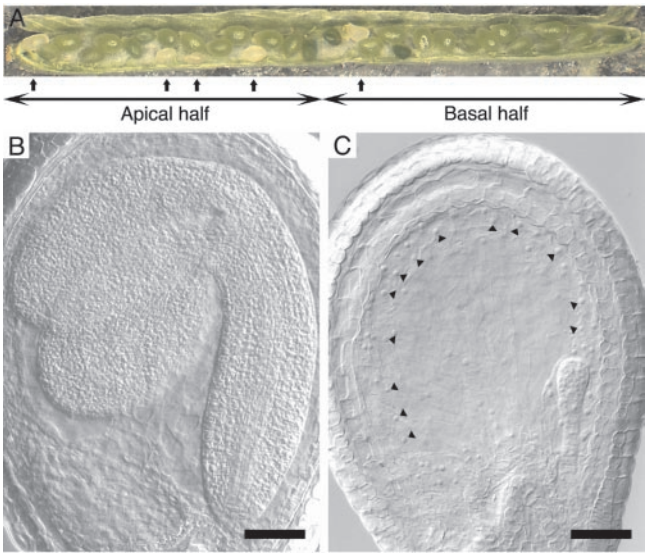

Figure 3. Seed and embryo morphology in hemizygous SGT4425 plants. A, Dissected silique at cotyledonary stage showing WT (green) and aborted (white) seeds. Aborted seeds (arrows) are unequally distributed within the silique, biased toward the apical half. B and C, Phenotype of a normal (B) and an aborted (C) seed from a cotyledonary stage silique. The mutant embryo is arrested at globular stage and peripheral free nuclear endosperm is still visible (arrowheads). Bars = 50 μm in B and C.

AtCSLA7 Is Important for Pollen Tube Growth

The reduced male transmission indicated a role for AtCSLA7 in pollen development or during pollen function. We used fluorescein diacetate, Alexander, and 4′,6-diamino-phenylindole staining to test pollen for plasma membrane integrity, cytoplasmic density, and nuclear constitution, respectively. In all tests, we found that pollen development and viability appeared normal in SGT4425 (data not shown).

To investigate whether pollen tube growth was affected, the distribution of aborted seeds in siliques was examined in an in vivo competition experiment. If mutant pollen grew more slowly than WT, less transmission would be expected in ovules fertilized toward the base of the silique (Meinke, 1982). Siliques were divided into equal halves, and the proportion of aborted seeds counted in the basal and apical halves (Fig. 3A; Table IV). In selfed SGT4425, the proportion of aborted seeds in the apical half was close to the expected maximum of 25% (Table IV), supporting the notion that pollen development and germination were not significantly affected. However, in the basal half, there were significantly fewer aborted seeds (8.2%, a 3-fold decrease), indicating that further growth of the *AtCSLA7* mutant pollen tubes was impaired.

DISCUSSION

We have isolated an insertional mutant of A*tCSLA7*, a gene predicted to encode a processive β-glycosyltransferase. The mutant shows embryo lethality and pollen tube growth is impaired. The results suggest that a cell wall polysaccharide synthesized by AtCSLA7 is essential for aspects of growth and development in Arabidopsis.

AtCSLA7 Is a Member of the Superfamily of Processive β-Glycosyltransferases

AtCSLA7 is a member of the large GT2 family of inverting processive β-glycosyltransferases that in-

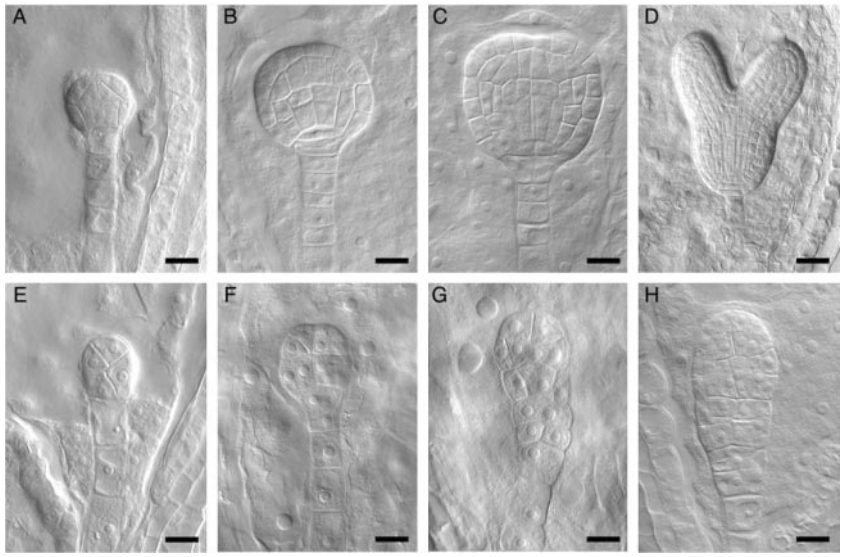

Figure 4. Embryo development in WT (A–D) and SGT4425 mutant (E–H) embryos present within siliques of hemizygous SGT4425 plants at different developmental stages. A and E, Sixteen-cell embryo proper stage; B and F, mid-globular stage; C and G, late globular transition stage; D and H, late heart stage of WT embryos. E, Abnormal eight-cell embryo proper with altered axial and transverse divisions. F, Abnormal early globular embryo. G and H, Abnormal embryos containing 28 to 46 cells showing abnormal transverse divisions and incomplete protoderm formation. Bars = 10 μm in A through C and E through H. Bar = 20 μm in D.

cludes hyaluronan and cellulose synthases (Henrissat and Davies, 2000; Saxena and Brown, 2000; Nobles et al., 2001). Like the other members of this family, AtCSLA7 is predicted to contain several transmembrane domains. In comparison with other members of the family, we predict a topology with six transmembrane domains (Fig. 1B). The large cytosolic loop would contain the conserved glycosyltransferase domain, often known as the D,D,D,QXXRW motif (Saxena and Brown, 2000; Nobles et al., 2001). The first and second D residues correspond to the DDS and DAD motifs, respectively (Fig. 1B), both conserved in all the CSLA members. These residues are thought to be important in binding the donor NDP-sugar and Mn^{2+} (Wiggins and Munro, 1998; Karnezis et al., 2000). The third Asp and the QXXRW motif are found in the acceptor domain of CSLA7 and these residues are conserved in all the processive glycosyltransferases (Davies and Henrissat, 2002). By aligning AtCSLA7 with CELA, CESA, and CSL proteins from different organisms, we found that the Asp and the QXXRW motif lie within a widely conserved sequence: $G(X)_8ED(X)_{10}G[W/Y/F](X)_{23–25}$ $QXXRW(X)_2G$ (Fig. 1B), suggesting that these residues are essential for the activity of the proteins. There are also further conserved regions in all members of the CSLA subfamily. Therefore, AtCSLA7 contains all the characteristics expected of the processive β-glycosyltransferases.

Mutants in the Processive β-Glycosyltransferase Family

The best studied Arabidopsis mutants in processive β-glycosyltransferases are those in the cellulose synthase CESA family (Saxena and Brown, 2000; Williamson et al., 2001). Interestingly, despite the existence of 12 *CESA* genes, often expressed in the same tissue, single-gene defects have been found to lead to clear phenotypic alterations. However, unlike the *Atcsla7* mutant, none have yet been found to be essential. A model has been proposed in which the cellulose synthase subunits are active as a protein complex, and an absence of one subunit might inhibit the function of all the subunits of the complex (Taylor et al., 2000; Dhugga, 2001). There may be some overlap in expression and function of the different cellulose synthase complexes, such that some cellulose is synthesized in the absence of any one complex.

Very few mutants in any *CSL* gene have been described yet. Two groups have recently characterized a mutant in *AtCSLD3* (Favery et al., 2001; Wang et al., 2001). The plants have weakened root hair walls because they burst as they begin to extend. Despite the expression of this gene in every tissue examined, the phenotype was observed only in the root hairs. This suggests that some redundancy may exist among the five *AtCSLD* genes. The only other previously discussed *CSL* mutant is *rat4*, which contains an insertional disruption in *AtCSLA9* (described in a review by Richmond and Somerville, 2001). The *rat4* mutant is resistant to *Agrobacterium tumefaciens* transformation. This bacterium binds to plant cell walls at an early stage of the infection (Nam et al., 1999). Interestingly, *rat4* is dominant, suggesting the heterozygous mutant has insufficient of a certain cell wall component that is essential for *A. tumefaciens* infection. The recessive embryo lethality of the *Atcsla7* mutation demonstrates that *AtCSLA7* is not redundant to the other 14 family members, at least in early embryos. Thus, the AtCSLA7 protein might work in a complex with other CSLA family members, as has been suggested in the CESA family. Alternatively, it might synthesize a variant of a polysaccharide with a specific function, or be part of the only CSLA complex expressed in pollen and in seed development.

Table III. *Embryo development in WT and SGT 4425*

Embryo developmental stages correspond to the cell no. or morphology of the embryo proper.

Silique	No. of Seeds at Each Embryo Developmental Stage					
	1–4 Cells	8–16 Cells	Globular	Heart	Torpedo	Cotyledon
WT (L*er*)						
1	46	22	–	–	–	–
2	30	27	–	–	–	–
3	1	44	20	–	–	–
4	–	10	48	–	–	–
5	–	–	33	37	–	–
6	–	–	3	70	–	–
7	–	–	–	46	21	–
8	–	–	–	–	5	68
9	–	–	–	–	2	64
SGT4425						
1	35	19	–	–	–	–
2	17	48	3	–	–	–
3	7	7	44	–	–	–
4	4	6	26	17	–	–
5	5	11	3	39	–	–
6	1	14	1	44	6	–
7	2	11	–	39	9	–
8	2	5	2	7	51	–
9	–	4	5	–	–	44
10	–	3	5	–	–	50

AtCSLA7 Is Required for Normal Pollen Tube Growth

Mutant *Atcsla7* pollen developed normally, but its TE was reduced by 71% compared with the WT. This suggested a defect during progamic (postpollination) development, which involves a number of distinct steps including adhesion, cell polarization and germination, pollen tube growth, guidance, and fertilization (Franklin-Tong, 1999; Wilhelmi and Preuss, 1999). The efficient fertilization of ovules positioned toward the apical end of the pistil suggests that early events are not affected and that mutant pollen tubes are correctly guided. Similarly, defects in fertilization can be excluded because failed ovules that could result from occupancy of the micropyle by mutant pollen tubes were not observed in *Atcsla7* siliques. However, mutant pollen tubes clearly do not compete effectively with WT pollen tubes in the basal region of the pistil. This suggests a late defect in either in the rate of pollen tube growth, or termination of pollen tube extension resulting in pollen tubes being unable to reach the most basal ovules. The incomplete penetrance of the *Atcsl7* mutation on pollen tube growth could support a role for other family members, or may suggest that glycans synthesized by AtCSL7 have a quantitative role in pollen tube extension.

The specialized tip growth mechanism of the pollen tube is associated with dynamic changes in cell wall structure and composition (Hepler et al., 2001). The pollen tube wall has an inner callosic layer and an outer fibrillar layer containing predominantly pectic polysaccharides, cellulose, xyloglucan, and arabinogalactan (Li et al., 1999). During pollen tube extension, calcium-mediated cross-linking of de-esterified pectins in the flanks of the apical pollen tube wall is thought to reinforce the pollen tube wall and focus cell expansion at the apex (Franklin-Tong, 1999). The defect in pollen tube growth in *Atcsl7* could result from changes in cell wall properties, including its extensibility and/or stability as a result of the absence of a specific polysaccharide. Alternatively, AtCSLA7 could affect pollen tube growth though disruption of signaling events that are wall mediated. Such interactions between the stylar environment and the pollen tube are clearly significant in tube growth and guidance. For example, nonclassical

Table IV. *In vivo pollen competition*

The no. of aborted seeds was determined in the apical and basal halves of the siliques of selfed SGT4425 from different generations of plants.

Seeds	Apical Half		Basal Half	
	Aborted	Total	Aborted	Total
Generation F_3	39	156	11	147
Generation F_7	115	522	45	505
% Average	23.5	–	8.2	–

AGPs present in the transmitting tissue of the style have been shown to be important for pollen tube growth (Cheung et al., 1995; Wu et al., 2000).

AtCSLA7 Is Required for Embryo Development and Endosperm Proliferation

By studying the development of mutant and sibling WT seeds in individual siliques, we found that the rate of development of *AtcslA7* mutant embryos was severely impaired, and that simultaneously the endosperm failed to proliferate. Although the embryos continued to increase in cell number throughout the normal developmental period, they finally arrested with terminal phenotypes that were morphologically pro-embryo or early globular. Patterning was generally normal until the octant stage, but early defects were observed in the orientation of the first or second axial divisions. The most common phenotype involved defects at the dermatogen stage, when octant embryos undergo eight asymmetric periclinal cell divisions to form the protoderm layer. The protoderm was frequently incomplete and abnormal transverse divisions in the basal region of the pro-embryo resulted in axially elongated globular embryos.

In *Atcsla7*, both embryonic cell patterning and cell proliferation are affected, yet in many mutants these phenotypes are not linked. In mutants that act early during embryogenesis to disturb embryo patterning such as *ton/fass*, *keule*, and *knolle* (Torres-Ruiz and Jurgens, 1994; Assaad et al., 1996; Lukowitz et al., 1996), abnormal embryos continue to develop. Similarly, the cell wall Hyp-rich glycoprotein RSH is essential for determination of division planes and cell shape in the embryo, but the cells continue to proliferate (Hall and Cannon, 2002). Second, a number of mutants including *rsp1-3* (Yadegari et al., 1994) and *edd1* (Uwer et al., 1998) arrest with terminal globular phenotypes, yet these mutants show normal globular embryo patterning, including a complete protoderm layer. The defects in *Atcsla7* of both cell proliferation and cell patterning might result from the metabolic dysfunction and chaotic failure of individual cells. However, given the prediction that AtCSLA7 is involved in cell wall synthesis, we favor a model where the phenotype arises from disturbed cell signaling that normally regulates cell proliferation and cell division patterning in the embryo. Although little is known about the role of cell wall components in signaling in embryos of higher plants, studies in *fucus* show that localized deposition of a sulfated polysaccharide is required to establish polarity of the egg cell, and this cell wall polysaccharide can determine cell fate (Belanger and Quatrano, 2000).

The effects of the *Atcsla7* mutation on seed development were not restricted to the embryo. Detailed analysis of the developing seeds revealed arrested proliferation of the endosperm nuclei without cellularization. This may reflect a shared requirement for AtCSLA7 in the embryo and the endosperm. Alternatively, AtCSLA7 may have a primary role in either, with defects in signaling between the two being responsible for the associated delay in embryo and endosperm development. Such signals could come from the embryo itself or the endosperm. The role of the endosperm in seed development is thought to involve the provision of both nutrients and signals to the developing embryo (Berger, 1999). In the *medea fis*, and *fie* mutants, endosperm development can proceed independently of embryo development (Vinkenoog et al., 2000). However, complete development of the endosperm does not occur, and it may depend on the presence of a normal embryo. Conversely, embryo development can occur in the absence of the endosperm during somatic embryogenesis (Berger, 1999), but the requirement of a secreted type IV endochitinase (EP3) derived from nonembryogenic cells may reflect a signaling role of the endosperm in embryogenesis (van Hengel et al., 1998, 2002). The nature of potential signals that control embryo development are unknown, although the involvement of oligosaccharides derived from chitin containing AGPs that are released by EP3 has been suggested (van Hengel et al., 1998, 2002; Berger, 1999).

The Function of AtCSLA7

The CSLs are likely to be β-glycosyltransferases that synthesize the backbones of cell wall polysaccharides. There are at least eight classes of β-glycan backbone-like chains in the dicot cell wall: cellulose, callose, mannans, xylans, the glucan of xyloglucan, the β-1,4-galactan of RG-I, and the β-1,3- and β-1,6-galactans of AGPs. Because cellulose synthase and callose synthases have been described, the six CSL families (CSLA–E, CSLG) identified in Arabidopsis on the basis of sequence similarity (Richmond and Somerville, 2000) could synthesize the six remaining classes. Two further families (F and H) have been identified in rice (*Oryza sativa*; Hazen et al., 2002), and one of these might synthesize the mixed-linkage glucan not found in dicots. It is also important to consider that one family as defined by sequence similarity could make more than one polysaccharide, and conversely, two families may synthesize the same polysaccharide. Indeed, it has been proposed that the CSLD family might be cellulose synthases (Doblin et al., 2001).

Which polysaccharide does AtCSLA7 synthesize? We clearly do not yet know. Cell wall polysaccharides have structural and signaling roles. We believe that the *AtcslA7* embryo phenotype is more consistent with a signaling role, and is more severe than that because of cellulose deficiency (Gillmor et al., 2002). A signaling role would suggest that xyloglucan or AGPs are possible candidates. *AtCSLA7* is ubiquitously expressed, and these polysaccharides

are present in most cell types. The structural xylans are thought to be essentially secondary wall polysaccharides, and, therefore, are likely to be essential only at a later developmental stage. The *cyt1* mutant, unable to synthesize GDP-Man, is likely to be deficient in mannans, glycoproteins, and GPI-anchored cell wall proteins (Lukowitz et al., 2001). This mutant has a less severe embryo development phenotype than *AtcslA7*; therefore, mannan synthesis is unlikely. In contrast, the β-1,4-glucan backbone of xyloglucan is a good candidate because the bacterial β-1,4-glucan (cellulose) synthases are more closely related to the CSLA subfamily than other plant glycosyltransferases. Alternatively, a clue may come from *rat4*, a mutant in *AtCSLA9* (described in a review by Richmond and Somerville, 2001). This mutant is resistant to Agrobacterial infection, like the AGP mutant *rat1* (Nam et al., 1999). Therefore, could the CslA family be involved in AG glycan synthesis? AGPs contain both β-1,3- and β-1,6-galactan backbones that could be synthesized by processive glycosyltransferases. Intriguingly, AGPs have been implicated both in embryo development and pollen tube growth. Because arabinogalactan glycosylation is predicted in a wide range of cell wall proteins (Borner et al., 2002), any defect leading to reduced AG glycosylation could impair the cell wall function of many proteins, leading to a broad spectrum of phenotypic changes including defective cell signaling during embryogenesis and pollen tube growth. We are currently investigating these possibilities.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The SGT4425 line (*Ds* insertion, Parinov et al., 1999) in Arabidopsis ecotype L*er* was provided by the Nottingham Arabidopsis Stock Centre (UK). Mutants were selected on solid medium containing Murashige and Skoog salts and 35 μg mL^{-1} kanamycin. After 3 weeks, plants were transferred to soil:sand (3:2 [v/v]) or Murashige and Skoog liquid medium and grown at 20°C under fluorescent white light in 16-h-light/8-h-dark cycles. The mutant line was backcrossed to WT L*er* grown in soil under identical conditions. Arabidopsis ecotype Columbia (Col0) liquid callus cultures were grown as described previously (Prime et al., 2000).

Mutant Screen

Primer 1 (TGTAGTTGTAC CTGTCTTCAAG), primer 2 (TGCAGGAACTGCTGGCGTCTG), primer 3 (AGCCCATTTCG GAACTGTGAC), primer 4 (CTGAAACATAT TGAGACTCTATG), primer 5 (GGTTCCCGTCC GATTTCGACT), and primer 6 (ACGGTCGGGAA ACTAGCTCTAC) were used to screen by PCR for plants with insertions using DNA prepared from a single leaf according to Weigel and Glazebrook (2002). Primers 1 to 4 are gene specific and primers 5 and 6 are *Ds* specific.

Cloning of a Full-Length cDNA

Total RNA samples were prepared from WT Arabidopsis ecotype Col0 callus as described previously (Brusslan and Tobin, 1992). The RT-PCR reaction was performed using Superscript II (Life Technologies/Gibco-BRL, Cleveland) as described by the manufacturer in two steps. In the first step, primer 4 was used with 5 μg of total RNA in a total volume of 20 μL. The cDNA (1 μL of RT-PCR solution) was amplified by PCR using primers 4 and 7 (CACTTGGCCGA TTGAAAGA). The sequence was deposited in EMBL/GenBank (accession no. AJ488284). The AGI annotation number for this gene is At2g35650.

Expression of CSLA7

RNA samples were prepared from tissues of Col0 or L*er* plants using a protocol described previously (Brusslan and Tobin, 1992). Callus (4 and 7 d old), 7-d-old plantlets, roots produced from liquid growth of old plants, leaves of rosettes (2, 4, 6, 8, and 10 weeks old), all parts of floral stems (before or after flowering), stems alone, leaves of stems, flowers (including flower buds), pollen, and siliques (young and old) were used. Duplicate samples of RNA were prepared, except for pollen and roots. In all cases, at least three plants at a similar stage of development were used to make the RNA sample.

RT-PCR and PCR were as described above except that primers 1 and 4 were used to amplify the cDNA from an aliquot of 0.1 to 5 μL of the RT reaction. The quantity used for different tissues in the PCR was equivalent to 10 to 500 ng of RNA. Controls without RT were performed.

DNA Sequencing and Sequence Analysis

The DNA sequence analysis was performed at the sequencing facility of the Department of Biochemistry (University of Cambridge, UK) using sequencers (models 377 and 373, Applied Biosystems, Foster City, CA) and big dye termination reactions. Sequences were analyzed by the Wisconsin package version 10.3 (Accelrys Inc., San Diego) and hydrophobic sequences determined using the program Protscale (http://www.expasy.org).

Phenotypic Analysis of Pollen and Embryos

Analysis of mature pollen with 4′,6-diamino-phenylindole was carried out as previously described (Park et al., 1998). Pollen was treated with aniline blue solution to detect callose staining as previously described (Park and Twell, 2001). Fluorescein diacetate staining was carried out by incubation of freshly isolated pollen in 0.3 M mannitol solution containing 2 μg mL^{-1} fluorescein diacetate according to Heslop-Harrison and Heslop-Harrison (1970). Alexander staining of pollen was carried out on pollen released from flowers fixed in ethanol:acetic acid (3:1 [v/v]) for 30 min. After washing flowers in 50 mM Tris-HCl buffer (pH 6.8), released pollen grains were mixed directly with Alexander stain (Alexander, 1969).

For phenotypic characterization of mutant embryos, siliques of different lengths were dissected on a slide using a syringe needle (0.4 × 12 mm) under a STEMI SV8 dissecting microscope (Zeiss, Jena, Germany) and embryos were cleared with a drop of clearing solution (240 g of chloral hydrate and 30 g of glycerol in 90 mL of water) for 30 min at room temperature. Preparations were examined with a microscope (model BHS, Olympus, Tokyo) equipped for DIC microscopy. Images were captured and processed as described previously (Park et al., 1998).

Genetic Transmission Analysis

To determine gametophytic transmission of the transposon insertion, reciprocal test crosses were performed between the WT (L*er*) and mutant SGT4425. Harvested seed from individual siliques was sown on kanamycin-containing plates and the resistance phenotype was scored. The TE of the T-DNA through each gamete (TE male and TE female) was calculated as described previously (Howden et al., 1998).

Complementation of the CslA7 Mutant

The gene including 1,300 bp upstream of the start codon and approximately 500 bp downstream of the stop codon was amplified by PCR using the primers ACGCGTCGACA AGTTGATGATT AGTTGCTTAG and CGGAATTCAGC AGAACAGACA TGGCCACG. The approximately 4,000-bp fragment was cloned into the BinAR vector (a BIN19 derivative conferring hygromycin resistance in plants, and a gift of L. Willmitzer, MPI, Golm, Germany), and transformed into *Agrobacterium tumefaciens* C58C1. The mutant plants were transformed by the floral dipping protocol of Clough and

Bent (1998). Seeds were sown on Murashige and Skoog agar plates containing kanamycin (35 μg mL^{-1}) and hygromycin B (20 μg mL^{-1}).

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