Genetic Complexity of Cellulose Synthase A Gene Function in Arabidopsis Embryogenesis

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The products of the cellulose synthase A (CESA) gene family are thought to function as isoforms of the cellulose synthase catalytic subunit, but for most CESA genes, the exact role in plant growth is still unknown. Assessing the function of individual CESA genes will require the identification of the null-mutant phenotypes and of the gene expression profiles for each gene. Here, we report that only four of 10 CESA genes, CESA1, CESA2, CESA3, and CESA9 are significantly expressed in the Arabidopsis embryo. We further identified two new mutations in the RADIALLY SWOLLEN1 (RSW1/CESA1) gene of Arabidopsis that obstruct organized growth in both shoot and root and interfere with cell division and cell expansion already in embryogenesis. One mutation is expected to completely abolish the enzymatic activity of RSW1(CESA1) because it eliminated one of three conserved Asp residues, which are considered essential for β-glycosyltransferase activity. In this presumed null mutant, primary cell walls are still being formed, but are thin, highly undulated, and frequently interrupted. From the heart-stage onward, cell elongation in the embryo axis is severely impaired, and cell width is disproportionally increased. In the embryo, CESA1, CESA2, CESA3, and CESA9 are expressed in largely overlapping domains and may act cooperatively in higher order complexes. The embryonic phenotype of the presumed rsw1 mutant indicates that the RSW1(CESA1) product has a critical, nonredundant function, but is nevertheless not strictly required for primary cell wall formation.

Plant cell shape is a key determinant in plant morphogenesis and is in turn strongly influenced by the organization of the cell wall. Within the cell wall, cellulose microfibrils constitute the major load-bearing structures, and their oriented deposition confers differential extensibility to the wall (Carpita and Gibeaut, 1993; McCann and Roberts, 1994). Presumably under the influence of the microtubular cytoskeleton, microfibrils in the primary wall of growing cells are deposited perpendicularly to the prospective elongation axis (Wyatt and Carpita, 1993; Wymer and Lloyd, 1996). Once wrapped around a cell in parallel hoops, the largely inelastic microfibrils are thought to efficiently restrict cell expansion to a single dimension. In organs along the apical-basal axis, such as stems and roots, oriented microfibrils allow cells to expand anisotropically, and in young meristematic tissues, they may even play a role in establishing new growth directions and forming new organs (Green, 1994). Because of the central role of cellulose microfibrils in shaping plant morphology at the cellular and at the organismal level, their formation is probably subject to complex developmental controls.

Cellulose, a paracrystalline form of H-bonded β-(1,4)-Glc chains (McCann and Roberts, 1991; Carpita and Gibeaut, 1993), is thought to be synthesized by membrane-bound complexes. Although these complexes can readily be visualized as rosette-terminal complexes, cellulose synthesis activity is difficult to assay in vitro. This problem has hampered biochemical approaches to identify cellulose-synthesizing enzymes (Delmer, 1999). Cellulose synthase genes have been eventually discovered genetically in bacteria, and divergent homologs in plants have been identified (for review, see Cutler and Sorm-erville, 1997; Saxena and Brown, 2000). Members of the cellulose synthase A (CESA) gene family in higher plants are believed to encode isoforms of the cellulose synthase catalytic subunit, but for most of...
these genes, the precise role in developing plants remains to be characterized (Delmer, 1999).

Knowledge of the functions of individual CESA genes in specific tissues or cell types will probably provide invaluable analytical and biotechnological tools for a better understanding and control of parameters underlying plant morphogenesis and physiology. However, no loss-of-function mutants are presently available for most CESA genes, and for some of the available mutants, it is not clear to what extent they reduce the gene function. For example, two mutations have been reported for RADIA LLY SWOLLEN1, RSW1(CESA1), one of two CESA genes implicated by mutation in primary cell wall formation (Arioli et al., 1998; Gillmor et al., 2002). One of these mutations is temperature sensitive and, at high temperatures, is associated with reduced cellulose synthesis, disassembly of cellulose synthase complexes, and radial expansions in young shoot and root organs (Arioli et al., 1998; Williamson et al., 2001). A presumably stronger mutation has very recently been reported to affect embryo shape at room temperature, but in this mutant as well, it is not clear whether the RSW1(CESA1) gene function is completely eliminated (Gillmor et al., 2002). A second CESA gene in primary cell wall formation, PRO CUST1, PRC1(CESA6), is required specifically in roots and dark-grown hypocotyls (Desnos et al., 1996; Fagard et al., 2000). CESA genes in secondary cell wall formation were identified through mutations associated with xylem defects in inflorescence stems and define the IRREGULAR XYLEM (IRX) genes IRX1(CESA8) and IRX3(CESA7) (Taylor et al., 1999, 2000). Finally, mutations conferring resistance to the herbicide isoxaben (ixr) were found in the CESA3 and PRC1(CESA6) genes (hence also referred to as IXR1 and IXR2 genes, respectively; Scheible et al., 2001; Desprez et al., 2002).

Given the size of the gene family and the likely functional overlap among its members, it will take considerable effort to sort out the roles of individual genes. The complexity of this analysis can be reduced through the isolation of null mutations in individual CESA genes and through the identification of stages, in which only a subset of the gene family is expressed. Here, we report the isolation of new, strong mutations in the Arabidopsis RSW1(CESA1) gene, one of which seems to completely abolish the enzymatic activity of the gene product and is associated with extreme defects in primary cell wall and cell shape already in embryos. In this presumed null mutant, residual cellulose synthesis will probably reflect the activity of other CESA genes. In this context, we show that only three other CESA genes, CESA2, CESA3, and CESA9, are significantly expressed in the embryo. These genes are expressed in vastly overlapping domains, suggesting that none of them has a function restricted to specific embryonic tissues or stages.

RESULTS

Identification of New Mutations in RSW1(CESA1)

In a screen for embryo cell shape mutants, we isolated two allelic mutations that were associated with dramatic distortions in seedling morphology (Fig. 1A). Upon outcrossing to wild type, abnormalities were observed in approximately 25% of the F2 individuals (176 and 90 of 525 and 297, respectively) consistent with the recessive inheritance of the seedling morphology trait. Phenotype and map position (flanking RFLP markers g8300 and mi431) suggested that the mutations correspond to new alleles of the RSW1(CESA1) gene, which was confirmed in complementation tests with the temperature-sensitive allele rsw1-1 (Arioli et al., 1998). To identify the molecular lesions in the two mutants (rsw1-20 and rsw1-45), we determined the genomic sequence of both mutant alleles along with the Landsberg erecta (Ler) wild-type strain in four independent DNA pools and localized single-point mutations in each of the two mutant alleles. Sequence analysis of the deduced mutant gene products revealed that the mutation in the phenotypically stronger rsw1-20 mutation converted the third of three Asp residues within the conserved glycosyltransferase “D,D,D,QXXRW” motif to Asn (Fig. 2, A and C). Moreover, secondary structure analysis predicted a disruption of the local α-helix made up of residue 779 to 785 in the rsw1-20 mutant (Fig. 2B).

These structural features strongly suggest complete loss of enzymatic activity of the mutant gene product (Saxena and Brown, 1997; Saxena et al., 2001; see “Discussion”). The mutation in allele rsw1-45 affected an adjacent, yet less conserved, position and presumably

Figure 1. Seedling phenotype of wild-type and rsw1 mutants 5 d after germination. A and B, Seedlings germinated in the light at 22°C (A) and 31°C (B). Genotypes from left to right are Ler wild type, rsw1-45, and rsw1-20. C, Ler wild-type and rsw1-20 mutant seedlings germinated at 22°C in the dark.
did not disrupt the local α-helical structure (data not shown). The fact that both mutants display similar phenotypic features at different levels of severity provides genetic evidence that all features described below are attributable to loss of RSW1(CESA1) function.

Seedling Phenotypes

Seedlings of light-germinated rsw1-20 and rsw1-45 mutants appeared short and stout with an uneven surface because of irregularly shaped and swollen cells in the epidermis of both hypocotyl and cotyledons (Figs. 1A and 4F). In rsw1-20 mutants, hypocotyl length was dramatically reduced (27% of that of wild type; Table I), and the diameter was increased by approximately one-third at the basal end. In rsw1-45 mutants, the reduction in hypocotyl length was somewhat less pronounced (42% of that of wild type; Table I). Cotyledon size was also dramatically reduced, particularly in rsw1-20 mutants. Barely any root growth was observed in either mutant. All other analyzed defects, including internal cellular defects at various stages, were less severe in rsw1-45 than in rsw1-20 mutants (data not shown), suggesting a residual gene activity in rsw1-45 mutants. Unless otherwise noted, we will restrict the description of mutant phenotypes to the presumed null mutant rsw1-20.

We assessed hypocotyl elongation in rsw1-20 mutants germinated in the dark. Hypocotyls of wild-type seedlings expand to a greater length when germinated in the dark than in the light. At least one cellulose synthase isoform is selectively required in this expansion process (Desnos et al., 1996; Fagard et al., 2000). Hypocotyls of both light- and dark-germinated mutant seedlings remained extremely short (Fig. 1C; Table I). Therefore, RSW1(CESA1) gene function is stringently required under both conditions, indicating that no gene activated specifically in the dark can substitute for missing RSW1(CESA1) activity (see “Discussion”).

The two new mutations in the RSW1(CESA1) gene can assist in classifying the previously identified rsw1-1 allele. This allele being temperature sensitive, the more severe defects seen at high temperature (31°C) could be attributable to temperature-sensitive properties of the mutant gene product. As an alternative, however, it is possible that the increased demand for cellulose synthesis at higher temperatures enhances abnormalities whenever the RSW1(CESA1) function is reduced. Additional alleles of the RSW1 gene allow us to distinguish between these possibilities. At 31°C, rsw1-45 mutant seedlings resemble rsw1-1 mutant seedlings (Williamson et al., 2001), suggesting approximately similar levels of residual gene activity in both mutants. However, whereas rsw1-1 mutants become largely normalized and grow long roots at room temperature (Williamson et al., 2001), the morphology of rsw1-20 and rsw1-45 was nearly identical at 22°C and 31°C (Fig. 1, A and B; Table 1).

### Table 1. Effects of temperature and light on seedling phenotype

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Hypocotyl Length ± s.e.</th>
<th>n.d., Not detected.</th>
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<tbody>
<tr>
<td></td>
<td>Ler No.</td>
<td>rsw1-20 No.</td>
</tr>
<tr>
<td>22°C Light</td>
<td>3.12 ± 0.35</td>
<td>0.84 ± 0.17</td>
</tr>
<tr>
<td>31°C Light</td>
<td>3.03 ± 0.40</td>
<td>0.98 ± 0.40</td>
</tr>
<tr>
<td>22°C Dark</td>
<td>8.90 ± 1.79</td>
<td>1.00 ± 0.14</td>
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Note: The length of the hypocotyl is measured in millimeters (mm).
Table I). This result implies that the temperature sensitivity of the rsw1-1 allele is largely, if not entirely, attributable to a temperature-dependent activity of the rsw1-1 mutant gene product.

Anatomy of rsw1-20 Mutants

Abnormal development in rsw1-20 mutant embryos occurred often as early as at the first division of the embryo proper. However, abnormalities before the heart stage were observed only in a portion of the mutants. For example, irregular horizontal divisions of apical cells were observed in early embryos (13.3% embryos from heterozygous parent, n = 90), but overall, the sequence of early cell divisions remained unchanged (Fig. 3, F–H). From the heart stage onward, cell shape alterations and cell wall interruptions were observed in approximately 25% of the progeny of heterozygous plants (21.2% embryos from heterozygous parent, n = 151), suggesting that the RSW1(CESA1) function became crucial in each mutant individual. A slight, but significant radial expansion of cells in hypocotyls of mutant embryos was observed at heart stage (Fig. 3, H and I) and became more pronounced during torpedo and bent-cotyledon stages (Fig. 3, K and M). At bent-cotyledon stage, radial cell expansions in the stele were recognizable, but rather subtle, whereas the radial expansion of the ground tissue and epidermal layers was rather extreme (Fig. 3M). Moreover, cells in these layers were often highly vacuolated and separated by incomplete cell walls. Interestingly, the overall cell arrangement in the hypocotyl and radicle, by contrast, the number of cells in the longitudinal dimension is nearly normal, whereas the length to width ratio of cells in nonvascular tissues is extremely distorted (compare wild-type and mutant cell number and cell shape in the outer cortex layer, enlarged in inlets). Arrow in inlet points at one of many incomplete cell divisions in the outer layers of the mutant hypocotyl. Scale bars = 20 μm in A, B, F, and G, same magnification; 50 μm in C, D, H, and I, same magnification; 75 μm in E and K; and 100 μm in M and L, same magnification.
guard cells were not produced at all (Fig. 4, C and D). On vegetative leaves, no trichomes were observed (data not shown). In contrast to trichomes, root hairs are exclusively generated by tip growth, and the mutant remarkably produced root hairs of approximately one-half the normal length (Fig. 4, G and H). Also transmission of the rsw1-20 allele through mutant pollen was not reduced. Together, these two observations may reflect a reduced requirement for RSW1(CESA1) in tip-growing cells.

In the mutant shoot apical meristem, cell divisions appeared highly irregular and were often incomplete (Fig. 4, I and K). Except for a discernable epidermal layer, no reproducible cell layer or internal zonation was apparent. Within 19 d of culture, seedlings produced an average of 7.6 extremely small vegetative leaves (sd = 1.6, n = 28). In the mutant primary root meristem, cell arrangements were normal, but cell dimensions were extremely distorted (Fig. 3M). Primary root growth was extremely limited, and no lateral roots were formed.

Cellular Defects in rsw1 Mutant Embryos

Differences in primary cell wall texture could be visualized by staining of the cell wall polysaccharides. As shown in Figure 5, A and B, mutant cell walls had a granular appearance with short projections to the inside. When viewed with the transmission electron microscope, markedly thinner, but again uneven, cell walls were observed in all analyzed tissues of the mutant, particularly in cells of the epidermis (Fig. 5, C–F). Most conspicuously, the size of intercellular spaces was strongly reduced in all tissues (Fig. 5, A and B). The intercellular spaces in between tissue layers and particularly at wall junctions were partially filled with excess wall material (Fig. 5, B and F).

To explore cell wall composition, we chose calcofluor staining to visualize biochemically wall composition across the entire embryo. Calcofluor specifically stains weakly or non-substituted β-glucans, which are probably represented by cellulose in these sections (Hughes and McCully, 1975). As shown in Figure 5, G and H, mutant walls again varied in thickness, and the staining was correspondingly non-uniform, in contrast to the even staining of wild-type cell walls. Furthermore, far less stainable material accumulated in the mutant. Quantification of the relative calcofluor fluorescence in identically treated sections from mutant and wild-type bent-cotyledon-stage hypocotyl indicated that mutant cell wall β-glucan content was reduced to 22.5% ± 4.2% of the wild-type value (Fig. 5I). We measured the corresponding β-glucan content in material extracted from mutant seedlings as 30% of that of the wild-type value (Fig. 5K). Importantly, parallel measurements of available rsw1 mutants grown at 31°C under identical conditions indicated that β-glucan content is lowest in rsw1-20 mutants (Fig. 5K).
Expression Profiles of CESA Genes

Residual cellulose synthesis in the presumed null-mutant rsw1-20 may reflect the activity of cellulose synthase isoforms, encoded by other CESA genes. To identify candidate CESA genes with overlapping functions, we assessed the expression of 10 Arabidopsis CESA genes in embryos at three postembryonic stages by semiquantitative reverse transcription (RT)-PCR. As shown in Figure 6, all 10 CESA genes were expressed at the three postembryonic stages (young plant, stem, and flower), whereas only CESA1, CESA2, CESA3, and CESA9 were significantly expressed in the embryo. In addition, very weak embryonic transcript levels were reproductively detected for CESA5.

The absence of detectable embryonic expression for CESA4, CESA6, CESA7, CESA8, and CESA10 suggests that they are not required for embryonic primary wall formation (see “Discussion”). For CESA2, CESA3, CESA5, and CESA9 genes expressed along with RSW1(CESA1) in the embryo, no embryonic mutant defects have been reported. To gain insight into the roles of these four genes in embryogenesis, we determined their embryonic mRNA expression domains by in situ hybridization to sectioned embryos. No CESA5 transcripts were detected at any
stage (data not shown), indicating that CESAs expression was below the detection threshold in all analyzed stages and tissues (see “Discussion”). As shown in Figure 7, CESAs1 was expressed throughout the embryo in the late-heart and torpedo stage, and was still visible at the bent-cotyledon stage. CESA2 expression had a very similar spatial and temporal pattern but at a somewhat reduced expression level, whereas expression of CESA3 and CESA9 appeared stronger in cotyledons than in hypocotyls and roots. The expression of all four genes decreased toward the bent-cotyledon stage, at which CESA9 was barely detectable. Overall, the expression of none of the four genes was restricted to specific cell types or stages, suggesting that they may collectively contribute to cell wall formation throughout the embryo.

DISCUSSION

Great efforts are currently being made to identify the role of individual CESAs genes (for review, see Delmer, 1999; Richmond, 2000; Richmond and Somerville, 2001). The results of these investigations could be relevant for many crop species, because the high degree of conservation among orthologs from different plant species compared with paralogs within a given species suggests conserved functional specialization within the gene family (Holland et al., 2000). The identification of null mutations in CESAs genes and of developmental stages with reduced genetic complexity can greatly facilitate this analysis. Here, we have identified a highly probable null mutation in the RSW1(CESA1) gene and show that only a subset of CESAs genes is expressed along with RSW1(CESA1) in the Arabidopsis embryo. Therefore, we suggest embryogenesis as a suitable stage of reduced CESAs functional complexity to study the genetics of primary cell wall formation. As a first step, we have recorded CESAs gene expression patterns during embryogenesis.

Loss of Gene Function in rsw1-20 Mutants

The structural properties of processive β-glycosyltransferases have been studied extensively (for summary, see Saxena and Brown, 1997; Saxena et al., 2001). Three Asp residues within the “D,D,D,QXXRW” motif are conserved from bacteria to plants. Although it is not finally resolved which of the Asp residues serve as bases during the catalytic reaction, two should be required for two glycosidic linkages formed simultaneously or sequentially during the synthesis of cellulose. Moreover, only the third Asp residue, which is affected in rsw1-20 mutants, is located within “do-

Figure 6. CESAs gene expression in embryos, young plants, and mature plants. Semiquantitative RT-PCR of total RNA prepared from embryos, seedlings, inflorescence stems, and flowers. Lanes 1 to 10, CESAs1 to CESAs10 RT-PCR products; R, approximately evenly expressed ROC1 gene (Lippuner et al., 1994); numbers on the right indicate the size of the PCR products in base pairs.

Figure 7. Expression pattern of CESAs gene mRNA in wild-type (Col-0) embryos at heart/early torpedo (A–D), late torpedo (E–H), and bent-cotyledon (I–M) stage. Hybridization with antisense (A–M) and sense probes (N–Q). A, E, I, and N, RSW1(CESA1); B, F, K, and O, CESAs2; C, G, L, and P, CESAs3; and D, H, M, and Q, CESAs9.
main B,” the characteristic domain of processive \( \beta \)-glycosyltransferases (Saxena et al., 2001). Finally, site-directed mutagenesis in bacteria has directly demonstrated that the exchange of any of the three Asp residues results in a reduction of \( \beta \)-glycosyltransferase activity to less than 1% of the wild-type level (Saxena et al., 2001). We conclude that \textit{rsw1-20} mutants lack all \( \beta \)-glycosyltransferase activity provided by the \textit{RSW1(CESA1)} locus and can therefore serve as a suitable genetic background to assess the contribution of other cellulose synthase genes. The presumed null-mutant phenotype supports a pivotal role of \textit{RSW1(CESA1)} in primary wall formation but also indicates that loss of \textit{RSW1(CESA1)} gene function does not obstruct embryogenesis and even allows for early stages of vegetative development.

\section*{RSW1(CESA1) Function in Cell Division and Cell Expansion}

Cell wall defects in \textit{rsw1-20} embryos are extremely severe in late-stage embryos and in apical meristems. By contrast, cell elongation in germinating mutants does not seem to be associated with additional wall disruptions. Therefore, cell wall integrity seems to be stringently dependent on \textit{RSW1(CESA1)} activity in rapidly dividing cells but less critical in the elongation of nondividing cells. This differential requirement could reflect the fact that \textit{rsw1} mutations do not interfere with cell division but strongly restrict cell expansion and thereby reduce the requirement for cellulose synthesis selectively in expanding cells.

Mutations in \textit{RSW1(CESA1)} restrict cell expansion during germination in light and in darkness, indicating that no germination-specific cellulose synthase activity can substitute for the \textit{RSW1(CESA1)} function. It has previously been noted that the activities of the \textit{RSW1(CESA1)} product and the dark-growth-specific cellulose synthase isoform \textit{PRC1(CESA6)} are not redundant (Desnos et al., 1996; Fagard et al., 2000). Because both \textit{rsw1} and \textit{prc1} homozygous single mutants are impaired in cell expansion and double heterozygous individuals look normal, both proteins could possibly constitute necessary components of a higher order complex (Fagard et al., 2000). Our observations are consistent with this interpretation for the action of both gene products in elongating hypocotyls. However, \textit{RSW1(CESA1)} must also be able to act in the absence of \textit{PRC1(CESA6)}, because \textit{PRC1(CESA6)} is not expressed in the embryo. Therefore, the strongest argument for a simultaneous non-redundant action of the two gene products is the inability of \textit{RSW1(CESA1)} to substitute for the \textit{PRC1(CESA6)} function in the elongating hypocotyl. The reciprocal inability of \textit{PRC1(CESA6)} to substitute for the \textit{RSW1(CESA1)} function could simply reflect the irreversibility of cell wall defects in \textit{rsw1} mutants.
specific defects in the epidermis may result from a general weakening of the wall structure.

**Dissecting Overlapping CESA Functions**

Assigning functions to individual members of large gene families eventually requires the analysis of multiple loss-of-function mutants. Expression studies cannot replace, but potentially facilitate, this genetic analysis. For genes such as cellulose synthase genes that are expected to act locally, probably cell autonomously, expression studies can reduce the complexity of future mutant analysis, because only genes co-expressed at the same stage or tissue need to be examined for potentially redundant functions.

Expression profiles of CESA genes have been studied or deduced from expressed sequence tag frequencies in several species, but CESA expression in Arabidopsis embryos has not been reported yet (Fagard et al., 2000; Holland et al., 2000; Dhugga, 2001; Richmond and Somerville, 2001; Scheible et al., 2001). We noticed that transcripts of only five of 10 classified Arabidopsis CESA genes were detected by RT-PCR in RNA from embryos, of which one had only an extremely low expression level. By contrast, transcripts of all 10 genes were readily detectable at all postembryonic stages. We observed similar expression profiles in three independent assays, but rather than focusing on the relative transcript abundance of CESA genes at various stages, we aimed at detecting minute amounts of embryonic expression. No transcripts of CESA4, CESA6, CESA7, CESA8, and CESA10 were found even after extending the number of amplification cycles up to 32 in RT-PCR of embryonic RNA. The absence of CESA4, CESA6, CESA7, CESA8, and CESA10 transcripts from the embryonic RNA pool is consistent with previously assigned roles for the IRX3(CESA7) and IRX1(CESA8) genes in secondary wall formation and for PRC1(CESA6) in cell expansion at germination (Taylor et al., 1999, 2000; Fagard et al., 2000). On the basis of their expression pattern, we propose that all five genes (CESA4, CESA6, CESA7, CESA8, and CESA10) are not involved in embryonic cell wall formation, which also may be the case for CESA5. Expression levels of CESA5 are far below those of any other embryonically expressed CESA gene in all cell types and could be gratuitous.

In situ hybridization was used to further dissect expression profiles of embryonically expressed CESA genes. These expression profiles were found to largely overlap, and all four genes were generally expressed throughout the embryo up to the bentcotyledon stage, which could reflect their collective requirement throughout the embryo. Cellulose synthase complexes are presumably heteromeric at any stage, and a minimum of at least two types of subunits has been proposed (Scheible et al., 2001). Our results suggest that no more than four CESA gene products are required for the formation of a functional complex in the embryo and that even a complex made up of only the active products of CESA2, CESA3, and CESA9 could retain limited functionality. This conclusion is based on the observation that despite marked cell wall defects, primary cell walls are formed in rsw1-20 mutants. The recessive segregation of the rsw1-20 mutant phenotype indicates that the mutant RSW1(CESA1) product is either not incorporated or tolerated as an inactive component in the cellulose synthase complex.

Because CESA2, CESA3, and CESA9 are co-expressed with RSW1(CESA1) in virtually all embryonic tissues, their inability to substitute for RSW1(CESA1) function in these tissues, indicates functional divergence at the level of the gene products. By contrast, the degree to which CESA2, CESA3, and CESA9 can substitute for each other’s function is unclear. The products of CESA2 and CESA9 share extensive sequence similarity and the absence of identified mutants as well as of dramatic defects in CESA2 antisense lines (Burn et al., 2002) could be attributable to extensive functional overlap between these two genes. Both genes are also related to PRC1(CESA6), which in turn has been shown to act nonredundantly with RSW1(CESA1) during germination (Fagard et al., 2000). The fourth coexpressed gene, CESAl3(IRX1) seems to have a unique function in higher order complexes, because CESAl3 antisense plants have recently been shown to be impaired in cell elongation in the postembryonic shoot and because overexpression of CESAl3 did not normalize the rsw1-1 mutant phenotype (Burn et al., 2002). Taken together, at least three of the four CESA products coexpressed in most cells of the embryo seem to have unique functions in cellulose synthase complexes, and one possible scenario is therefore that ideally all four gene products are present in a complex, but that the loss of CESA2 and CESA9 would have relatively minor consequences. Given the reduced complexity of CESA function in the highly reproducible cell pattern in Arabidopsis embryogenesis, this stage seems to be best suited to genetically trace the requirement for each subunit in primary cell wall formation.

**MATERIALS AND METHODS**

**Plant Growth Conditions**

Mutants rsw1-20 and rsw1-45 were isolated from ethyl methyl sulphonate mutagenesis of Ler seeds of Arabidopsis (Jürgens et al., 1991). Seedlings were germinated and grown on one-half Murashige and Skoog (1962) medium supplemented with 1.5% (w/v) Suc at the indicated temperatures. For germination in the dark, seeds were cold-treated for 48 h and then exposed to fluorescent white light (80 μm m⁻² s⁻¹) for 2 h to synchronize germination. Subsequently, plates were wrapped in three layers of aluminum foil. For culture in the light, seeds were cold-treated for 48 h and then placed under continuous fluorescent white light (80 μm m⁻² s⁻¹). Embryos were isolated from plants that were grown under long-day (16 h) light cycles in growth chambers (Conviron, Manitoba, Canada) at the indicated temperatures. Unless stated otherwise, wild type refers to the Ler line.
Measurement of Hypocotyl Length

Growth of seedlings was arrested by adding an aqueous solution of 0.4% (w/v) formaldehyde. hypocotyls and roots were spread on agar plates, and hypocotyl lengths were measured on digitally captured images.

Light Microscopy

Samples for light microscopy were processed as described by Beeckman et al. (2000). To quantify the β-glucan content of cell walls, transverse sections (2 μm) of seeds embedded in LR White (London Resin, Basingstoke, UK) were made from wild type and mutant. The sections were stained with 0.07% (w/v) calcofluor white M2R (Sigma-Aldrich, St. Louis) in phosphate-buffered saline for 10 min, rinsed in phosphate-buffered saline, and mounted in Vectorshield (Vector Laboratories, Burlingame, CA). Computer images of well-oriented cross sections through the hypocotyl were captured using an inverted (Axiovert 100M) confocal microscope (LSM510, Zeiss, Jena, Germany) equipped with an UV (364 nm) laser (Coherent Enterprise, LPS-Lasersysteme, Mössingen, Germany) and HDFT UV 375/BP 385-470 as filter combination. Average fluorescence of cell walls of cortical cells from 10 independent mutant and wild-type embryos was determined by measuring the average pixel density of selected cell walls using the ImageJ analysis software version 1.26 (developed by W. Weijer, Research Branch, National Institute of Mental Health, Bethesda, MD). The same software was used to determine the cross-sectional area of selected cell walls. Relative fluorescence was reported as integrated density, which is the product of the cross-sectional area and the average fluorescence of cell walls. Polysaccharides in cell walls were visualized through periodic acid-thiocarbohydrazide silver proteinate staining (PATAg; Roland and Côté, 1989).

In Situ Hybridization

Blunt-end PCR products for CesA1, CesA3, CesA5, and CesA9 (primer sequences, see above) were inserted in both orientations into pBHLuescript (Promega, Madison, WI); for CesA2, a fragment bordered by the sequences gtaaggggcaagctggtt and ccaagaataattggtttgaca was chosen. Orientation of the inserts was determined by analytical PCR. Sense and antisense transcripts were generated by transcription from the T7 promoter. Antisense probes were linearized in the plasmid polylinker or at gtactgttgagttcaactac- gctaagggggaccagtgtt and caaaagaattaatttaggggtaacaaa was chosen. Orienta-

LITERATURE CITED


测光长度

种子被放入装有0.4% (w/v)甲醛的溶液中，然后被放在固体培养基上。种子的长度被测量在数字化捕捉的图像上。

光显微镜

样本进行光显微镜处理如Beeckman等（2000）所述。定量β-葡聚糖细胞壁含量，用切片（2 µm）的种子嵌入LR White（伦敦树脂，Basingstoke, UK）制备。切片用0.07% (w/v) 钙氯氟白色M2R（Sigma-Aldrich, St. Louis）在磷酸缓冲液中染色10 min，用磷酸缓冲液清洗，然后用Vectorshield（Vector Laboratories, Burlingame, CA）用UV（364 nm）激光（Coherent Enterprise, LPS-Lasersysteme, Mössingen, Germany）和HDFT UV 375/BP 385-470作为过滤组合。通过测量选定细胞壁的平均像素密度来定量细胞壁的相对荧光强度。平均荧光强度被报告为积分密度，它是细胞壁的横断面面积和平均荧光强度的乘积。细胞壁中的多糖通过酸-硫基卡马素银蛋白甲基化染色（PATAg; Roland和Côté, 1989）来可视化。

原位杂交

在CesA1, CesA3, CesA5, and CesA9（引物序列，见上述）的终止片段插入pBHLuescript（Promega, Madison, WI）；CesA2，则为由序列gtaaggggcaagctggtt和ccaaagaattaattggtttgaca组成的边界部分。插入序列的方向通过分析PCR确定。从T7启动子转录的模板。抗义探针在plasmid polylinker或gtactgttgagttcaactac-gctaagggggaccagtgtt和caaaagaattaatttaggggtaacaaa中制备。定向插入的序列被选择。对于反义探针，插入序列在10%的非离子洗涤中。所有探针被置于尼龙膜上（Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK）与标记的RNA探针（Roche Diagnostics, Brussels）相互作用。为了监测digoxigenin-labeled RNA的敏感性，对修饰的分枝长细胞转变方法（Sanger et al., 1997）的ab377自动序列化（PerkinElmer Instruments, Norwalk, CT）进行了单一碱基对变异。从Lr中在两个突变线中被测定在四个独立DNA样本的每个基因型。没有其他序列变动在突变线中被从独立DNA样品中复制。二次序列结构预测在蛋白序列性能中被与PREDECTOR程序（http://mips.gsf.de/mips/straf/irishman; Frishman和Argos, 1997）使用的默认参数。

半定量RT-PCR

胚芽的心形-角质层被从硅酸盐中提取。年幼植物在Murashige和Skog改良培养基上收获后，生长。

测量胚轴长度

种子的生长被用添加了0.4% (w/v) formaldehyde的溶液来阻止。胚轴和根系被放在固体培养基上，胚轴长度在数字化捕捉的图像上被测量。
Cellulose Synthase Function in Embryos


References


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