An Early Nodulin-Like Protein Accumulates in the Sieve Element Plasma Membrane of Arabidopsis\textsuperscript{1}[OA]

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Membrane proteins within the sieve element-companion cell complex have essential roles in the physiological functioning of the phloem. The monoclonal antibody line RS6, selected from hybridomas raised against sieve elements isolated from California shield leaf (\textit{Streptanthus tortuosus}; Brassicaceae) tissue cultures, recognizes an antigen in the Arabidopsis (\textit{Arabidopsis thaliana}) ecotype Columbia that is associated specifically with the plasma membrane of sieve elements, but not companion cells, and accumulates at the earliest stages of sieve element differentiation. The identity of the RS6 antigen was revealed by reverse transcription-PCR of Arabidopsis leaf RNA using degenerate primers to be an early nodulin (ENOD)-like protein that is encoded by the expressed gene \textit{At3g20570}. Arabidopsis ENOD-like proteins are encoded by a multigene family composed of several types of structurally related phytocyanins that have a similar overall domain structure of an amino-terminal signal peptide, plastocyanin-like copper-binding domain, proline/serine-rich domain, and carboxy-terminal hydrophobic domain. The amino- and carboxy-terminal domains of the 21.5-kD sieve element-specific ENOD are posttranslationally cleaved from the precursor protein, resulting in a mature peptide of approximately 15 kD that is attached to the sieve element plasma membrane via a carboxy-terminal glycosylphosphatidylinositol membrane anchor. Many of the Arabidopsis ENOD-like proteins accumulate in gametophytic tissues, whereas in both floral and vegetative tissues, the sieve element-specific ENOD is expressed only within the phloem. Members of the ENOD subfamily of the cupredoxin superfamily do not appear to bind copper and have unknown functions. Phenotypic analysis of homozygous T-DNA insertion mutants for the gene \textit{At3g20570} shows minimal alteration in vegetative growth but a significant reduction in the overall reproductive potential.

Sieve elements are terminally differentiated cells that undergo exceptional cytoplasmic reorganization to become the functional living conductive cells of the phloem. During differentiation, the lumen of sieve elements becomes a low-resistance pathway for mass flow by selective degradation of the nucleus, ribosomes, dictyosomes, and vacuoles, as well as undergoing changes in the endoplasmic reticulum forming the uniquely structured sieve element reticulum. The junction between the sieve element reticulum and the sieve element plasma membrane (SEPM) ultimately forms a tightly coupled parietal membrane complex that includes P protein, mitochondria, and plastids held together by ultrastructurally defined clamp-like structures in mature sieve elements (Sjölund and Shih, 1983; Ehlers et al., 2000). High-resolution electron microscopy detected minute actin and profilin-like structures that could anchor the organelles in a parietal position (Ehlers et al., 2000). The intact plasma membrane becomes contiguous from one sieve element to another through the connecting sieve plate pores, creating a functional syncytium that allows for the long-distance transport of water, ions, photosynthates, and macromolecules.

A cohesive picture of protein function in the sieve element-companion cell complex is just beginning to develop. Such a comprehensive understanding of signaling and metabolic events that occur within the phloem requires the integration of the soluble, integral membrane, and membrane-associated proteins in combination with their ligands, substrates, and modification status. Functionally, the soluble proteins in the sieve element have been categorized as structural proteins, proteins that mediate the redox status of the phloem sap, RNA-binding proteins that could be involved in signal transduction, and proteins involved in putative stress and defense responses (Hayashi et al., 2000; Kehr, 2006). Enzymes have been identified for complex biosynthetic reactions such as the production of ascorbic acid and jasmonic acid (Hancock et al., 2003; Hause et al., 2003), and furthermore, it has been shown that alkaloid biosynthesis and secondary

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metabolism occur within the parietal region of sieve elements (Bird et al., 2003).

Integral membrane proteins have been characterized that transport a variety of compounds, ions, and water across the plasma membrane of sieve elements and/or companion cells of the phloem. Specific Suc transporters in the SEPM are involved in Suc loading, recovery during transport, and unloading. Suc transporters are encoded by multigene families and are assigned to the SUT1/SUT3, SUT2, and SUT4 subgroups on the basis of their sequence homology, substrate affinity, and function. SUT1 in potato (Solanum tuberosum) tubers was localized to the sieve elements of phloem, and its mRNA has been found in both companion cells and sieve elements. In Arabidopsis (Arabidopsis thaliana), AtSUC2 was localized in companion cells and is believed to be responsible for phloem loading of Suc. Similarly, in Plantago major, PmSUC2 is the primary Suc transporter involved in phloem loading, whereas PmSUC1 is also localized to the sieve elements but appears to function in Suc unloading. The SUT1 (wheat [Triticum aestivum] and tomato [Lycopersicon esculentum]) and SUT2 (potato and tomato) and SUT4 (tomato) proteins were localized to the SEPM of the transport phloem (for review, see Kühn, 2003, and refs. therein). Similarly, a putative member of a monosaccharide transporter family was identified in the plasma membrane of sieve elements and in phloem parenchyma cells (Zhang et al., 2004).

Amino acids and other transporters also have been localized in the plasma membranes of sieve elements (Hirner et al., 1998). A proton-amino acid cotransporter (AAP3) localizes to the plasma membrane of the phloem in roots, whereas a Pro transporter (AtProT1) and an ATP-binding cassette transporter-like protein (ALS3) localizes to the plasma membrane of phloem in all parts of Arabidopsis (Okumoto et al., 2004; Grallath et al., 2005; Larsen et al., 2005). Calcium and photosynthetic-induced potassium ion channels have been localized to the SEPM (Lacombe et al., 2000; Volk and Franceschi, 2000), and a sulfate transporter (Sultr1; 3) from Arabidopsis localizes to the sieve element–companion cell complex of phloem in cotyledons, hypocotyls, and roots (Yoshimoto et al., 2003). SoPIP1.2, a spinach (Spinacia oleracea) aquaporin, has been localized to the SEPM of leaves, roots, and petioles (Fraysse et al., 2005), whereas ZmTIP1, an aquaporin from maize (Zea mays), and AHA3, a H+-ATPase of Arabidopsis, are found in the plasma membrane of the companion cells (DeWitt and Sussman, 1995; Barrieu et al., 1998).

Identifying the cellular and organ-specific accumulation of proteins in the sieve element–companion cell complex of the phloem is necessary to develop a full physiological understanding of this complex vascular tissue. Little is known about proteins that accumulate at the SEPM-cell wall interface. In other tissues, such proteins interact with the cell surface and play a role in cell wall synthesis, morphogenesis, and signaling (Kohorn, 2000; Svoboda, 2004; Gillmor et al., 2005). In this study, a monoclonal antibody line, RS6, was used to identify a 15-kD protein with structural and sequence characteristics of the Arabidopsis family of early nodulin (ENOD)-like proteins, which localizes to the plasma membrane of differentiating and mature sieve elements. The sieve element-specific ENOD-like protein (SE-ENOD) appears to be expressed and processed through the endomembrane system in nucleate sieve elements prior to selective autophagy of the organelles and is ultimately glycosylphosphatidylinositol (GPI) anchored in the SEPM. T-DNA insertion mutants of At3g20570 encoding the SE-ENOD show a minimally altered growth phenotype under normal growth conditions with a significant reduction in the reproductive potential of the plant.

RESULTS

The RS6 Antigen Localizes to the SEPM

A series of monoclonal antibodies (mab) were generated from sieve element–enriched fractions collected from California shield leaf (Streptanthus tortuosus; Brassicaceae) callus cultures. The mab line RS6 was initially screened to detect phloem-specific antigens by immunocytochemistry of free-hand sections of cauliflower (Brassica oleracea) stems and subsequently visualized by confocal microscopy of fresh tissue vibratome sections (Fig. 1, A–C). The RS6 mab specifically recognizes a sieve element–specific antigen (Fig. 1, B and C) and has been previously used as a sieve element marker (Meyer et al., 2004). High-resolution immunolocalization by transmission electron microscopy shows that the RS6 monoclonal antibody recognizes an antigen on the SEPM (Fig. 1, E–J) but does not label adjacent companion cells or the surrounding parenchyma cells (Fig. 1E) of Arabidopsis stems at any stage of sieve element development. The RS6 antigen can readily be detected in the plasma membrane of both the immature nucleated sieve elements (Fig. 1, F, G, and I) and mature enucleated sieve elements (Fig. 1H). Immature sieve elements, which were morphologically identified by the presence of the idiosyncratic P-protein bodies (Fig. 1, F, G, and I), showed dense labeling of the plasma membrane (Fig. 1, I and J) that becomes more distributed as the cell expands and differentiates (compare Fig. 11 with Fig. 1, E–H).

The Sieve Element–Specific Streptanthus RS6 Antigen Is Encoded by the Arabidopsis Gene At3g20570

The RS6 mab identified a single protein of approximately 15 kD on immunoblots of proteins isolated from enriched Streptanthus callus–derived sieve elements (data not shown). To isolate the gene encoding the RS6 antigen, two sets of degenerate oligonucleotide primers were designed from the amino acid sequences of the N terminus (REFAVGAGKWTIPS) and an internal tryptic fragment (TSFLTHSGPYFYIFGK) obtained from the RS6-immunopurified Streptanthus protein. The 17-nucleotide oligomers, designated as RS6-N5’ and RS6-I3’, were synthesized with a 96-fold degeneracy,
Figure 1. Confocal and high-resolution transmission electron microscopy immunolocalization of the SE-ENOD. A, Brightfield image showing the phloem (P) and xylem (X) in fresh tissue vibratome sections (50–100 μm) of cauliflower stem. B, The RS6 monoclonal antibody labeled only the phloem in the vascular bundles of cauliflower stem sections (bar = 200 μm). C, Immunolabeling was specific to the sieve elements (SE) of the phloem in the midrib of an Arabidopsis leaf section (bar = 10 μm). D, Transmission electron micrograph of the normal serum control treatment (no RS6 mab) shows minimal background immunogold labeling of the sieve element-companion cell (CC) complex of Arabidopsis shoot tips. E, Immunogold labeled SE-ENOD was observed only in the periphery of the sieve element and not in the companion cell. F, G, I, and J, Immunogold labeled SE-ENOD was detected in immature sieve elements, which were identified by the presence of a nucleus (N) and nondispersed P-protein bodies (PPB). H, Labeling in the mature sieve elements demonstrates that the SE-ENOD is also present in translocating sieve elements. J, High magnification of the cell wall between two immature sieve elements (see I) show the RS6 antigen localizes to the SEPM. Black arrowheads point out examples of the labeling of the plasma membrane-bound RS6 antigen with the gold particles, which can easily be observed around the entire periphery of the sieve elements.

and total RNA isolated from Arabidopsis leaves served as the template for the oligo(dT)-primed reverse transcription (RT) reaction. A diffuse band of amplified cDNA from the initial PCR of the single-stranded cDNA template with RS6-N5' and oligo(dT) primers was gel purified and subjected to a second round of PCR with RS6-N5' and RS6-I3' primers. A distinct band of approximately 400 bp was cloned and sequenced but was larger in size than expected. The 3’ degenerate primer had misprimed on a repeated nucleotide sequence, increasing the size of the partial cDNA clone from the expected 113 bp at the RS6-I3' target site to 422 bp at the repeated sequence. TBLASTX analysis (The Arabidopsis Information Resource [TAIR]) of the 422-bp sequence matched a hypothetical protein encoded by the gene At3g20570. The sequence of this partial cDNA, as well as five Arabidopsis expressed sequence tags (At996405, AU227049, AV531349, AV549656, and AV553691) and a full-length cDNA (U23621) indicate that At3g20570 is a correctly annotated expressed gene composed of two exons separated by an 88-bp intron.

The At3g20570 gene encodes a deduced protein sequence of 203 amino acids with a calculated molecular mass of 21,509 D (Fig. 2). The empirically derived sequences for the N-terminal and internal amino acids of the protein immunopurified from Streptanthus matched all but three amino acids in each sequence with the deduced amino acid sequence from the Arabidopsis gene. SignalP V3.0 (Nielsen et al., 1997) predicted a 27-amino acid signal sequence, and the predicted amino acid sequence of the processed N terminus agrees with the position of the N-terminal sequence of the immunopurified RS6-specific protein from Streptanthus. Processing of the signal sequence would result in
The RS6 Antigen Has Structural Similarity with the ENOD5 Class of ENODs

The four-domain organization (signal peptide, plastocyanin domain, Pro/Ser-rich domain, and hydrophobic domain) of the Arabidopsis RS6 antigen (Fig. 2) is similar to the precursors of the plastocyanin subclass that includes stellacyanins and uclacyanins and the subclass of ENODs that are typified by the pea ENOD5 proteins. Both classes of plastocyanins and ENODs share a domain of approximately 100 amino acids, corresponding to the plastocyanin copper-binding domain. Plastocyanins are members of a distinct family of cupredoxins that bind copper in a type 1 conformation through four conserved ligands: His-39, Cys-79, His-84, and Met-89 or Gln-89 (Greene et al., 1998). The ENODs MiENOD16/20 and GmN315/ENOD55 have amino acid substitutions at three of the four ligands (His-39 → Glu, Cys-79 → Ser, Met-89 → Leu), and structural models indicate that members of this plastocyanin subfamily do not bind copper (Greene et al., 1998). All four of the conserved amino acid ligands are substituted in the RS6 antigen (His-39 → Gln, Cys-79 → Ser, His-84 → Asn, Met-89 → Leu; Fig. 2). In contrast, the position of two invariant Cys residues forming a disulfide bond to create the structural copper-binding center is retained within the ENODs and the RS6 antigen, as is the location of the single intron in the genes encoding these proteins (Fig. 2).

A BLASTP (TAIR) query for proteins with similarity to the Arabidopsis RS6 antigen resulted in 37 Arabidopsis ENOD-like, uclacyanin-like, and stellacyanin-like proteins that have significant matches (E values ranging from 6 × 10^{-9} to 4 × 10^{-5}). Twenty eight of these proteins had the structural features in common with the Arabidopsis RS6 antigen and produced a minimum of gaps in a CLUSTALX analysis (Table I). CLUSTALX pairwise and multiple alignment of the 29 sequences was used for phylogenetic analysis using distance-based method Neighbor Joining (NJ), the character-based Parsimony analysis, and the MrBayes program for the Bayesian inference of phylogeny. An unrooted tree was generated from the NJ interior branch test with 1,000 bootstrap performed by MEGA 2.1 using the poisson correction (pairwise distances) model (Fig. 4). The topology of the trees showed that the At3g20570 sequence was clustered within a clade of six ENOD-like protein sequences that was part of a larger clade of 13 ENOD-like proteins. A phylogenetic tree displaying similar topology was obtained for NJ test of 1,000 bootstrap replicates with heuristic search using PAUP 4.0. Character-based parsimony analysis for 1,000 bootstrap replicates with PAUP 4.0 also gave a similar topology for a 50% majority-rule consensus tree with heuristic search. The phylogenetic analyses establish the RS6 antigen as a member of the subfamily of ENOD-like proteins in Arabidopsis, and the antigen is designated as the SE-ENOD protein.

The expression patterns for the five most closely related genes from a single clade containing SE-ENOD
in the phylogenetic tree were examined to determine if closely related ENOD-like genes are expressed in vascular tissues. Gene promoter::GUS cassettes were constructed to analyze their expression patterns in leaves, stems, roots, flowers, and reproductive organs. In contrast to the phloem-specific expression directed by the promoter from At3g20570 that encodes the SE-ENOD (Fig. 3, B–D), the promoters from four closely related genes directed GUS expression to floral tissues and reproductive organs. In the flowers, SE-ENOD GUS expression was detected in the vascular tissue of the petals, sepals, stamens, and in the gynoecium (Fig. 3, E–G). Figure 3E shows a complete flower with GUS expression localized to the veins of petals and the vasculature of filament and style, and Figure 3G shows GUS expression in a flower at the junction of the filament and anther where the vascular tissues from filament are juxtaposed with those of the anther. GUS activity was not observed in pollen within the anthers. In contrast, the GUS expression directed by the promoter of At4g30590 was detected in the anthers, pollen, and developing ovules (Fig. 3, H–L). A similar pattern of GUS expression directed by the At4g31840 promoter was seen in the reproductive tissue, whereas GUS expression directed by the promoters of At2g23990 and At5g25090 could only be detected in the developing ovules and not in the pollen (data not shown). The promoter construct from At2g25060, which is the fifth member of the clade, failed to show any GUS expression in flowers, leaves, or seedlings.

The SE-ENOD Is GPI Anchored in the SEPM

Borner et al. (2003) identified the SE-ENOD (At3g20570) as one of 28 Arabidopsis phytocyanins predicted to be GPI anchored. GPI anchors attach proteins to the plasma membrane by cleaving the carboxy-terminal hydrophobic domain and covalently attaching a phosphoethanolamine and a conserved glycan to phosphatidylinositol or a ceramide at the carboxy-terminal amino residue. The SE-ENOD has two potential cleavage ω sites (Asn-178 or Ser-179) at the amino end of the hydrophobic domain and an Ala at the ω + 2 sites (Ala180,181) consistent with many GPI-anchored proteins (GAPs; Fig. 2). Cleavage of the carboxy-terminal hydrophobic domain of SE-ENOD at the ω site would result in a peptide with a calculated $M_r$ of 15,019.4 D.

GAPs are typically confirmed by treating membrane preparations with phosphatidylinositol-specific phospholipase C (PIPLC) followed by Triton X-114...
detergent phase partitioning; GAPs phase shift from the hydrophobic detergent to the hydrophilic aqueous phase upon cleavage of the GPI anchor. Phase-shift experiments were impractical to confirm GPI anchoring of the SE-ENOD due to the limited amount of sieve element membranes that can be isolated from intact plants or tissue cultures and the potential for contamination with other cell types. To determine if the SE-ENOD is GPI anchored, fresh stem sections from young cauliflower plants were treated with PIPLC followed by immunofluorescent detection of the SE-ENOD in situ with the RS6 mab. Figure 5 shows micrographs of the results that were typical of three replicated experiments. Sections were visualized by brightfield (Fig. 5, B, E, I, M, and Q) or stained with calcofluor white and visualized by UV light (Fig. 5, F, J, N, and R) to reveal the overall cell wall structure. The RS6-plus positive mab controls showed bright immunofluorescence of the sieve elements in the typical peripheral pattern (Fig. 5, A, D, and G), whereas the RS6-minus negative mab controls showed minimal background due to nonspecific binding of the secondary antibody labeled with ALEXA 488 (Fig. 5, H and K). The PIPLC-minus sections showed a pattern of bright immunofluorescence in the sieve elements similar to the RS6-plus antibody control (Fig. 5, L and O).

In sharp contrast, the PIPLC-plus enzyme-treated sections showed only minimal fluorescence, indicating that the SE-ENOD is GPI anchored to the plasma membrane and is not cross-linked with the sieve element cell wall (Fig. 5, P and S).

### T-DNA Insertion Mutants of *At3g20570*

Three T-DNA insertion mutants of *At3g20570* (SALK 105873, 126416, and 044182) were identified in the Salk Institute T-DNA insertion library database (http://signal.salk.edu/cgi-bin/tdnaexpress) and one was identified from the GABI-Kat mutant collection (Max Planck Institute for Plant Breeding Research, Köln, Germany; Rosso et al., 2003). Plants from the three SALK lines and the one GABI-Kat line were self-pollinated and the progeny screened by PCR to select plants that were homozygous for the insertions. Homozygotes were identified for SALK 105873 (insert in the 5′ untranslated region [UTR]), SALK 044182 (insert in the promoter), and GABI-Kat (insert in exon 1), whereas only heterozygotes could be identified for SALK 126416 (insert in the 3′ UTR) in the initial and subsequent generations. Two SALK 105873 homozygotes were selected for the initial study (M1 and M2),
and M1 was subsequently used as the donor parent for three successive backcrosses to the wild-type Col-0 with progeny selection for the presence of the 5’ UTR T-DNA insertion in At3g20570 by PCR.

RT-PCR amplification of the At3g20570 mRNA in wild-type Col-0 plants showed that the gene is expressed at low levels in floral stems, leaves, and whole seedlings (Fig. 6A). In contrast, At3g20570 mRNA was detected neither in the SALK 105873 M1 and M2 mutant lines by ethidium bromide visualization of the RT-PCR products (Fig. 6B) nor in a Southern blot of the M1 PCR product (Fig. 6C). The SE-ENOD protein cannot be readily detected on immunoblots without sieve element enrichment. Thus, the SALK 105873 M1 line and both a homozygote and heterozygote for the T-DNA insertion in GABI 37108 were analyzed by immunolocalization of fresh tissue sections of floral stems using the RS6 mab. Fluorescence microscopy of the wild-type Col-0 plant (Fig. 6D) and the GABI 37108 heterozygote (Fig. 6E) showed the typical sieve element-specific labeling of the SE-ENOD protein with the RS6 mab. In contrast, sieve element-specific labeling was not detected in the SALK 105873 M1 (Fig. 6F) or GABI 371E08 homozygous lines (Fig. 6G).

Phenotypic analysis of the SALK 105873 M1 mutant and the wild-type Col-0 plants grown under a short-day photoperiod (10 h light, 14 h dark) at 22°C revealed few differences in the growth characteristics during the first 5 weeks of vegetative growth. No differences were observed for the time to seedling emergence or the appearance of true leaves. The number of leaves per plant was not significantly different between the mutant and wild type at 3, 5, or 9 weeks after emergence (Fig. 7A), although the mutant plants accumulated significantly more ($P = 0.0137$) leaf biomass than the wild-type plants (Fig. 7C). Several of the wild-type plants had bolted in the 5th week, and bolting overlapped with the mutants for a period of 6 d; most of the mutant plants bolted in the 6th week. The inflorescence biomass was not significantly different between the mutant and wild-type plants. In contrast, significant differences in both the number ($P = 0.0080$; Fig. 7A) and biomass (fresh weight, $P = 0.0070$, Fig. 7B; dry weight, $P = 0.0002$, Fig. 7C) of the

![Phylogenetic tree](image-url)
siliques were recorded, with almost twice as many siliques with 3 times the biomass on wild-type plants as compared to the mutant. The reduced number of siliques paralleled the significant ($P = 0.0041$) reduction in the number of seeds measured as dry weight produced by the mutant plants. However, the number of seeds per milligram was not different between the mutant and wild type, indicating that seed size was not significantly altered by the mutation (Fig. 7A).

DISCUSSION

ENOD-Like Proteins Have Functions beyond Nodulation

The SE-ENOD and other ENOD-like proteins of Arabidopsis are members of the superfamily of cupredoxins, which are typified by active copper centers that function in electron transport. Members of the cupredoxin superfamily include the well-known plastocyanins that participate in electron transport during the light reactions of photosynthesis and a subclass of phytochromes (plantacyanins, stellacyanins, and uclacyanins) that are involved in electron transfer during redox reactions (Adman, 1991; Ryden and Hunt, 1993; Nersissian et al., 1998). Alignment and prediction algorithms showed that the greatest sequence and structural similarity occurs between the Arabidopsis ENOD-like proteins and members of another subclass of phytochromes that are expressed very early in developing nitrogen-fixing root nodules of legumes. This structurally related group of ENODs includes the pea (Pisum sativum)/vetch (Vicia sativa) ENOD5 (PsENOD5/VsENOD5), Medicago truncatula MtENOD16 and MtENOD20, and soybean (Glycine max) N315/ENOD55. A common feature of the ENOD subclass of phytochromes is the loss or substitution of the key
amino acid ligands for binding copper (Greene et al., 1998). The four residues that are required for copper coordination in plastocyanins are substituted in the Arabidopsis SE-ENOD such that the protein is unlikely to bind copper and would have functions other than electron transport.

In legumes, the spatial and temporal expression pattern during symbiosis suggests that ENODs participate in the initial interactions that occur between rhizobium and root cortical cells leading to cell differentiation and cell wall reorganization during nodulation (Scheres et al., 1990). ENOD5 proteins identified from root cortical cells of pea, vetch, and broad bean (Vicia faba) have characteristics of arabinogalactan proteins with predicted GPI-membrane anchor attachment sites. Due to the presence of arabinogalactan modifications and the GPI-anchor attachment, this group of ENODs has been proposed to function in cell-to-cell signaling, cell differentiation, tissue development, and signal transduction pathways (Fruhling et al., 2000). Furthermore, the expression of ENOD genes in both nodulating and nonnodulating plants suggests a broader function for some ENODs in cell division, dedifferentiation, and development of different organs (Govers et al., 1991; Reddy et al., 1998; Varkonyi-Gasic and White, 2002).

Phytocyanins and ENOD genes that have been localized to the floral tissue of Arabidopsis and other plants are thought to function in gametophytic development (Kim et al., 2003; Scutt et al., 2003; SzczYGlowski and Amyot, 2003; Lalanne et al., 2004). Phylogenetic analysis of 12 phytocyanin-like and 16 ENOD-like proteins in Arabidopsis showed a clear separation between the uclacyanin-like/stellacyanin-like subclass and the ENOD-like subclass of phytocyanins. Thirteen of the ENOD-like proteins formed a single clade that separates into three well-supported lineages. The SE-ENOD is an outlier to its five most closely related ENOD-like proteins, which are encoded by At2g25060, At4g31840, At5g25090, At2g23990, and At4g30590. GUS localization directed by the promoters of these genes showed that four of the five genes are expressed in the female or both the female and male gametophytes. The promoter of the fifth gene, At2g25060, failed to show GUS expression in any part of Arabidopsis plants, even though the identification of a cDNA indicates that the gene is expressed. In an array analysis of developing ovules and embryo sacs, Yu et al. (2005) detected the expression of two ENOD-like genes (At4g30590 and At2g23990) of the five that are closely related to the SE-ENOD. The expression of the more distantly related ENOD-like Pup2 (At5g53870) gene was up-regulated in the embryo sac within the gynoeicum according to a reverse genetic analysis (Scutt et al., 2003). Two ENOD-like proteins (At3g18590 and At1g48940) and a stellacyanin-like protein (At5g20230) are embedded in the pollen membrane (Lalanne et al., 2004). Recently, plantcyanins, which
Figure 7. Comparative analysis of the growth and reproductive potential between wild-type Arabidopsis Col-0 and SALK 105873-M1 homozygous mutant plants. The bars in all graphs illustrate the mean ± se of the respective measurements of 16 plants in each data set. Paired Student’s t test was performed for all data sets to determine the statistical significance. A, The average number of leaves at 3, 5, and 9 weeks after the appearance of the true leaves, the number of siliques, and the number of seeds per milligram of seeds. The difference in number of siliques between wild-type Col-0 and SALK 105873-M1 was significant (P = 0.0080). B, The fresh weight (milligrams) of tissues taken at 25 d after bolting showed no significant difference for the leaves and inflorescence between the wild-type Col-0 and SALK 105873-M1, whereas the fresh weight of the siliques on mutant plants was significantly (P = 0.0007) reduced. C, The dry weight (milligrams) of tissues for wild-type Col-0 and SALK 105873-M1 was determined at the end of experiment. Leaves on the mutant plants accumulated significantly (P = 0.0137) more biomass than wild-type plants, whereas no difference was observed between the dry weights of the inflorescence. The dry weights of the siliques (P = 0.0002) and seeds (P = 0.0041) showed significant differences between the two groups of plants with a reduced reproductive potential for the SALK 105873-M1 mutant.

are extracellular matrix proteins in the phycocyanin family, have been shown to play a role in reproduction by affecting anther development and pollination (Dong et al., 2005). In contrast, GLU1 expression in flowers directed by the SE-ENOD promoter was limited to the vasculature of all floral organs, including the gynoecium and in the filaments of the stamens, where an intense staining occurred at the junction with the anther but not within the anther.

What Is the Role of the SE-ENOD in Vascular Physiology?

The phloem-specific accumulation of the SE-ENOD, which is different from the other Arabidopsis ENOD-like proteins, makes it an interesting candidate to study the functional role of ENOD-like proteins in a highly specialized cell type. The SE-ENOD accumulates specifically in the plasma membrane of differentiating sieve elements prior to selective autophagy of the organelles and persists in functioning sieve elements. The presence of SE-ENOD in the SEFM throughout the vascular system indicates a persistent, widespread role for this protein in phloem physiology. Analysis of the SE-ENOD sequence shows distinct amino acid motifs that are characteristic of posttranslational glycosylation and GPI anchor attachment sites. Johnson et al. (2003) provided evidence that a closely related ENOD-like protein, which contains similar AG-glycomodules, is N-glycosylated. Localization studies suggest that the SE-ENOD is embedded in the outer leaflet of the plasma membrane by a GPI anchor where the protein could mediate interactions between the SEFM and cell wall. Many GAPs are glycoproteins, and, as such, the lipid modification could attach the protein to the plasma membrane and the carbohydrate moiety could interact with the cell wall components (Gillmor et al., 2005). However, phospholipase C treatment of tissue sections in situ was sufficient to remove the SE-ENOD from the tissue, indicating that the protein was GPI anchored but not embedded in the sieve element cell wall. Borner et al. (2002) proposed that GAPs could be released by regulated phospholipase cleavage to rapidly mediate interactions or cotarget proteins at discrete extracellular locations. Although cytosolic or membrane-bound classes of phospholipase C occur in plant cells (Dowd et al., 2006), there is currently no evidence
to support the idea that GAPs such as the SE-ENOD are cleaved from the plasma membrane of enucleate sieve elements.

The homozygous SALK 105873 and GABI 371E08 T-DNA insertion mutants of the gene At3g20570 that encodes SE-ENOD eliminated the expression of At3g20570 and SE-ENOD accumulation. An analysis of mutant plant growth and reproduction resulted in a phenotype that can be distinguished from a wild-type plant. Although the SALK 105873 M1 mutant line revealed few differences in the growth characteristics as compared with the wild-type Col-0 plants during the first 9 weeks of vegetative growth, the mutant plants accumulated significantly more leaf biomass and, in general, bolted 3 to 7 d later than the wild-type plants. In addition, there were highly significant differences in both the number and biomass of the siliques, with almost twice as many siliques with 3 times the biomass on wild-type plants as compared to the mutant. The mutant plants also appeared to develop fewer lateral branches as compared to the wild-type Col-0. The accumulation of biomass can be attributed to photosynthetic partitioning during the phase transition from the vegetative phase to the reproductive phase. Carbohydrate signaling is important for the transition of vegetative phase to reproductive phase and known to affect floral initiation by activating or inhibiting genes involved in floral transition (Levy and Dean, 1998; Ohto et al., 2001). The control of flowering time in Arabidopsis is important in determining the reproductive potential of the plant. The transition to flowering is influenced by both endogenous and environmental signals and is regulated by a complex network involving several genetic pathways (Moon et al., 2003; Bouveret et al., 2006).

Phytocyanins and ENODs have been implicated in a number of developmental processes, including the generation of a specialized cell surface. The localization and structural studies of the SE-ENOD presented in this article are consistent with the hypothesis that the SE-ENOD is involved in cell surface interactions within the extracellular region of the SEPM. In this context, the accumulation of more biomass and a delayed flowering response by SALK 105873 M1 mutant plant could mean that the knockout of gene At3g20570 directly or indirectly causes either a defect in the process of starch mobilization during the transition phase or affects a signaling process. It is also possible that a more extreme mutant phenotype is masked by the expression of other ENOD-like proteins within the vascular tissues. While the five ENOD-like proteins that are most closely related to the SE-ENOD were expressed in gametophytic tissues, other more distantly related ENOD-like proteins could be expressed in vascular tissues. Further analysis of the genes encoding the Arabidopsis ENOD-like proteins and their respective mutants could provide a mechanistic understanding of the physiological role of the SE-ENOD and give insight into the broader functions of the ENOD-like proteins from Arabidopsis.

MATERIALS AND METHODS

Tissue Cultures

Phloem (+) tissue cultures of California shield leaf (Streptanthus tortuosus) were grown as previously described by Toth et al. (1994). Cultures were maintained in the dark at room temperature on a Murashige and Skoog medium containing 2% Suc, 0.9% agarose, 4.65 μM kinetin, and 0.54 μM naphthalene acetic acid.

Production and Characterization of RS6 Mab

Mab were produced at the Tissue Culture and Hybridoma facility in the College of Medicine at the University of Iowa. A sieve element-enriched fraction was isolated from the phloem-induced tissue cultures of California shield leaf as previously described by Sijöland (1990) and Toth et al. (1994). The primary inoculation of 12-week-old BALB/c mice with sieve element-enriched fractions mixed with Freund’s complete adjuvant (1:1) was followed by two sory inoculations with Freund’s incomplete adjuvant at 3-week intervals. Spleen cells from mice showing a strong immunological response during prescreening of the sera were fused with myeloma cells to produce the mab. Sieve element-specific antibodies were identified using immunofluorescence microscopy of free-hand sections of California shield leaf plants as described by Toth et al. (1994). Monoclonal cell lines were established from hybridomas producing the sieve element-specific RS6 antibodies.

Immunolocalization

Free-hand cross sections of stems from Arabidopsis (Arabidopsis thaliana) Col-0 plants, SALK 105873 M1 mutant, heterozygous, and homozygous GABI 371E08 mutant plants were cut with a razor blade and collected in water. Sections of broccoli (Brassica oleracea) flower stalks were cut with a vibratome at 100 μm and collected in phosphate-buffered saline (PBS). Sections were washed twice in 10 mM PBS, pH 7.4 and then incubated for 30 min in PBS containing 3% nonfat dry milk. Sections were washed three additional times with PBS and incubated for 45 min with the RS6 primary monoclonal antibody in PBS containing 3% nonfat dry milk (1:100). After incubation with primary antibody, the sections were washed three times with PBS and then incubated in PBS containing ALEXA 488-nm (Molecular Probes, Invitrogen) fluorescently tagged secondary goat anti-mouse antibody (1:200). Finally, the labeled sections were washed twice with PBS and once with double distilled water and observed under an epifluorescence microscope (Nikon Eclipse 90i) with an excitation wavelength of 490 nm and an emission wavelength of 512 nm. Cauliflower was observed with a Leica TCS-SP2 confocal microscope with 488-nm excitation and spectrometer filtering of the green channel for Alexa 488-nm fluorescence (520–550 nm) and a red channel for chlorophyll autofluorescence (600–700 nm).

High-Resolution Immunolocalization

Arabidopsis tissues containing apical meristems were rapidly frozen in a Balzers high pressure liquid nitrogen freezing unit and subsequently processed by freeze substitution (1% glutaraldehyde, 0.1% uranyl acetate in 100% acetone) for 3 d at −80°C. The temperature was raised to −30°C and the freeze substitution medium was sequentially replaced at 8-h intervals with 100% acetone followed by a Lowacryl K4M series (25%, 50%, and 75% in 100% acetone) to 100% of the embedding medium. Tissues were placed in gelatin capsules and polymerized with UV light at −30°C for 24 h and then at room temperature for 48 h. Ultrathin sections were mounted onto grids and incubated at 4°C in blocking solution (0.8% bovine serum albumin, 0.1% gelatin, 5% normal goat serum in PBS). RS6 mab were diluted (1:100) in blocking solution and incubated with the tissue sections for 2 h at 4°C. Then washed (0.8% bovine serum albumin, 0.1% gelatin in PBS) and incubated with goat anti-mouse secondary antibody conjugated to 10-nm colloidal gold. Sections were fixed in 2.5% glutaraldehyde, stained with uranyl acetate and lead citrate, and examined in a Hitachi H-7000 electron microscope.

ENOD-Like Gene Promoter-Reporter Gene Expression in Transgenic Arabidopsis

Approximately 1,000 bp promoter sequences extending 5′ from, but not including, the translation start codon of six genes (At3g20570, At2g25060,
Early Nodulin-Like Protein in Phloem Sieve Elements

RS6 Protein Purification and Peptide Sequencing

Total proteins were extracted in a French pressure cell press (2,500 psi) from 40 g of 15-d-old phloem (+) culture and 40 ml of extraction buffer (10 mM Tris, pH 7.2, 10 mM EGTA, 150 mM NaCl, 20 mM dithiothreitol, 1% CHAPS, 10 mM KCl, 1% Sigma protease inhibitor cocktail). Cellular debris was removed by centrifugation at 18,000 rpm for 15 min at 4°C; 60 ml of the supernatant was collected and the volume reduced to 15 ml by dialysis against polyethylene glycol 20,000. The buffer was exchanged by dialyzing twice against PBS and 0.1% CHAPS. The RS6 monoclonal antibody (6 mL) was bound to PBS equilibrated rat-anti-mouse IgG Sepharose 4B (1 mL). The affinity column was washed with 10 volumes of PBS, loaded with the supernatant, and subsequently washed with 5 volumes of PBS plus 0.1% CHAPS followed by 5 volumes of 10 mM Tris, pH 8.0, plus 0.1% CHAPS. The protein was eluted with 0.1 M Gly, pH 2.5, plus 0.1% CHAPS, and fractions were neutralized with 1 M Tris, pH 8.0. Five fractions were collected, pooled, concentrated (Millipore Centricon-3), and resolved in a 12% SDS-PAGE minigel. Following electrophoresis, the purified protein was transferred onto polyvinylidene difluoride (Immobilon-P; Millipore) membranes by electroblotting (10 mM cleaved-amplified polymorphic sequence, pH 11, 10% methanol) using the semidry method (Harlow and Lane, 1988). The blots were washed, stained with 0.2% Ponceau S in 1% acetic acid, and destained in water. Proteins were eluted from the membrane and either analyzed intact or digested with trypsin prior to determining the N-terminal and internal peptide sequence with an ABI 473 protein sequencer utilizing Edman chemistry and HPLC C18 reverse-phase phenylthiohydantoin column.

cDNA Cloning by Degenerate PCR

Total RNA was extracted from fully expanded Arabidopsis leaves using the method of Carpenter and Simon (1998). Two sets of degenerate oligonucleotide primers were designed from the N-terminal and internal amino acid sequences obtained from immunopurified California shield leaf RS6 protein. Each of the 17-nucleotide oligomers, designated as RS6-N5 (5'-AARGGNTG-GACNATHCC-3') and RS6-13 (5'-GADATARTANGS-3'), was synthesized with 96-fold degeneracy. For first-strand synthesis, total RNA (18 μg) was denatured for 10 min at 70°C, placed on ice, and reverse transcribed using an oligo(dT) primer (Sigma) according to the Superscript II (BRL) manufacturer’s instructions. The single-stranded cDNA template was PCR amplified (94°C, 3 min: 40 cycles of 94°C, 30 s, 50°C, 1 min, 72°C, 2 min; 72°C, 10 min) using RS6-N5 and oligo(dT) primers. The double-stranded cDNA template was reamplified as above using the RS6-N5 and RS6-13 degenerate primers. The second round PCR product was electrophoresed in a 1.5% Tris-acetate EDTA agarose gel, gel purified with the Qagen Gel Extraction kit (Qagen), cloned into the pCR-2.1 vector (Invitrogen), and sequenced.

Phylogenetic Analysis of Full-Length Protein Sequences of Arabidopsis

BLASTP (TAIR) query of the Arabidopsis genome for proteins with similarity to the Arabidopsis RS6 antigen was performed. Twenty-nine sequences with significant E scores were aligned with ClustalX pairwise and multiple alignment and were subjected to phylogenetic analysis using distance-based method NJ, the character-based Parsimony analysis, and MrBayes program for the likelihood method Bayesian inference of phylogeny. NJ interior branch test with 1,000 bootstrap was performed by MEGA 2.1 using the poisson correction (pairwise distances) model. NJ test of 1,000 bootstrap replicates with heuristic search and character-based parsimony analysis for 1,000 bootstrap replicates using PAUP 4.0 was also performed on the same data set for a 50% majority-rule consensus tree with heuristic search. Several trees of similar topology and quality were obtained but only single tree from distance method is shown.

PIPLC Digestion of the SE-ENDO GPI Anchor

To determine if the SE-ENDO is GPI anchored in the plasma membrane, fresh stem sections from young cauliflower plants were treated with phosphatase C followed by detection of the protein or its absence with immunocolocalization (Sigma). In each experiment, eight, 100-μm thick cross sections were cut using a Leica vibrotome and collected in water. The sections were split into halves with a razor blade; the halves from a single section served as the positive RS6’ mab and negative RS6’ mab controls, while the halves from a serial whole were split into wells and reagent/rinin treated. Toh-RNA (5 μg) was denatured for 10 min at 70°C, placed on ice, and reverse transcribed by SuperScript II RNase H (Invitrogen) from the 3’ gene-specific primer. The single-stranded cDNA template was PCR amplified (94°C, 2 min: 35 cycles of 94°C, 30 s, 50°C, 1 min, 72°C, 2 min: 72°C, 10 min) using the 5’ and 3’ gene-specific primers. The PCR product was electrophoresed in a 1.5% Tris-acetate EDTA agarose gel and blotted to a 0.45-μm MagnaGraph nylon transfer membrane (Invitrogen). Gene-specific nucleotide probes were labeled using DIG DNA Labeling and Detection kit (Roche Diagnostics) according to the manufacturer’s instructions. Southern hybridization was performed in tubes at a temperature of 60°C. Immunodetection of the DIG-labeled probes was performed using an anti-DIG Fab fragments conjugated to alkaline phosphatase and visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indol phosphate substrates. Gene-specific primers for SALK 105873: RP 5’-ACGGCATGACCGCTGACTA-3’; LP 5’-CACAGAGAGCATCAACTTGGAA-3’. Gene specific primers for At4g00736: 5’ open reading frame, 5’-ATGGCGACAGAATTACGAGGAG-3’. The sample (Pisum sativum) actin control primers: 5’ actin: 5’GAGTTCAATGTTCTC-3’ and 3’ actin: 5’GGATCTGTTCACTGTTATAGAG-3’. Twenty replicates of individual seeds of wild-type Arabidopsis ecotype Col-0 or the homozygous mutant SALK 105873 m1 were sown in 3 × 2-inch round pots containingScotts Metro-Mix soil and cold treated for 4 d in dark. The pots were then transferred to a Conviron (model CMP 4030) growth chamber and the plants were grown at 22°C with a photoperiod of 10 h of light (130–150 μmol m−2 s−1) and 14 h of dark. Leaf numbers were measured at 3, 5, and 7 weeks postemergence. At 9 weeks, fresh and dry weights and the number of siliques were recorded for one-half of plants, and water was withheld for 2 weeks on the other one-half of the plants to mature the seeds. Seed germination rates were recorded on agar plates.

Sequence data from this article can be found in the GenBank/EMBL databases under accession numbers 816933, 817044, 817213, 817657, 817791.
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