A Plasma Membrane-Anchored Fluorescent Protein Fusion Illuminates Sieve Element Plasma Membranes in Arabidopsis and Tobacco1[W][OA]

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Rapid acquisition of quantitative anatomical data from the sieve tubes of angiosperm phloem has been confounded by their small size, their distance from organ surfaces, and the time-consuming nature of traditional methods, such as transmission electron microscopy. To improve access to these cells, for which good anatomical data are critical, a monomeric yellow fluorescent protein (mCitrine) was N-terminally fused to a small (approximately 6 kD) membrane protein (AtRCI2A) and stably expressed in Arabidopsis thaliana (Columbia-0 ecotype) and Nicotiana tabacum (‘Samsun’) under the control of a companion cell-specific promoter (AtSUC2p). The construct, called by its abbreviation SUmCR, yielded stable sieve element (SE) plasma membrane fluorescence labeling, even after plastic (methacrylate) embedding. In conjunction with wide-field fluorescence measurements of sieve pore number and position using aniline blue-stained callose, mCitrine-labeled material was used to calculate rough estimates of sieve tube-specific conductivity for both species. The SUmCR construct also revealed a hitherto unknown expression domain of the AtSUC2 Suc-H+ symporter in the epidermis of the cell division zone of developing root tips. The success of this construct in targeting plasma membrane-anchored fluorescent proteins to SEs could be attributable to the small size of AtRCI2A or to the presence of other signals innate to AtRCI2A that permit the protein to be trafficked to SEs. The construct provides a hitherto unique entrance into companion cell-to-SE protein targeting, as well as a new tool for studying whole-plant phloem anatomy and architecture.

Recent theoretical work has highlighted the lack of good quantitative, geometrical measurements of sieve tubes in the phloem vasculature of plants (Thompson and Holbrook, 2003, 2004; Thompson, 2006). The demand for such measurements is acute because quantitative anatomy determines fluid mechanical conductivity, which, in turn, sets the osmotic context for the molecular control of local solute exchange throughout the phloem. A complete understanding of phloem function is unlikely to emerge without attention to multiscale integration of processes operating at both long and short distances; anatomy is a key element of this integration.

The phloem is seldom accurately visualized from organ surfaces and is not always easily identified or measured with transmission light microscopy (Esau, 1969). Transmission electron microscopy is a potential alternative (Kühn et al., 1997; Ehlers et al., 2000; Turgeon et al., 2001) but lacks the three-dimensional perspective needed for good quantitative measurements, it requires more time to implement than light microscopy, and it is inappropriate for statistical sampling of large amounts of tissue. Likewise, exogenously applied fluorochromes (Oparka et al., 1995; Knoblauch and van Bel, 1998; Knoblauch et al., 2001; Martens et al., 2006; Bauby et al., 2007) and sieve element (SE)-specific antibodies, like RS6 (Khan et al., 2007), have proven their utility, but none of the fluorochromes is specific to the phloem and the use of antibodies is expensive and time consuming, lessening the opportunity for fast and direct observation of phloem cells alone.

Despite the time and cost of the development of stably transgenic plants, the study of phloem anatomy and architecture is likely best and ultimately most easily served by plants that express markers in phloem cells under the control of developmentally or physiologically significant promoters (Imlau et al., 1999; Stadler et al., 2005; Bauby et al., 2007). For present purposes, an ideal marker is one that fluorescently labels the plasma membranes of conductive SEs and sieve pores with an endogenous marker and allows quick measurements of the quantitative geometry of SEs (their radius and length) and sieve plates (the radius and length and number of sieve pores in each sieve plate) down to the resolution limits of light microscopy. Designing SE-specific markers in mature phloem represents a significant challenge. Membrane proteins expressed in immature SEs that are still...
nucleate, such as the early nodulin (ENOD)-like protein of Arabidopsis (*Arabidopsis thaliana*; Khan et al., 2007), are likely diluted or degraded as the SEs expand and mature, making them potentially poor targets for fluorescence tagging. Mature SEs lose the capacity for protein expression, delegating it to their sister cells, the companion cells (CCs; van Bel and Knoblauch, 2000). Thus, CC-expressed membrane-anchored proteins remain the strongest candidate for continuous SE membrane labeling in mature phloem.

To date, however, the only fluorescent proteins (FPs), FP fusions, or virus-associated FPs (Casper and Holt, 1996; Cheng et al., 2000) known to traffic from nucleate CCs to enucleate SEs are soluble, whereas membrane-anchored FPs fail to traffic at all (Imlau et al., 1999; Lalonde et al., 2003; Stadler et al., 2005; Martens et al., 2006). Past attempts at SE membrane targeting may have failed for the following reasons, which at this time remain speculative: (1) the FP interfered with signal peptides necessary for CC-to-SE plasmodesmatal trafficking (Lalonde et al., 2003; Stadler et al., 2005); (2) the Stokes radius of the FP membrane protein fusion was greater than the size exclusion limit of the CC-to-SE plasmodesmata (Stadler et al., 2005); (3) the promoter driving expression was not strong enough to reach a threshold for plasmodesmatal trafficking (Lalonde et al., 2003); (4) cDNA clones were used that lacked the full genomic intronic and 3'-untranslated region (UTR) sequences, some of which might be important for intra- and intercellular targeting (Lalonde et al., 2003); or (5) membrane proteins were used that are not normally targeted to SEs, such as the SUT1/ SUC2 Suc-H⁺ symporters (Lalonde et al., 2003; Stadler et al., 2005).

Here, a variety of constructs were developed and tested in Arabidopsis with these potential limitations in mind. The strong CC-specific promoter *AtSUC2* promoter (Truernit and Sauer, 1995; Imlau et al., 1999) was used to drive FP fusions (Cutler et al., 2000) of proteins that are known to be targeted to plasma membranes (PIP subfamily of aquaporins; Frayssse et al., 2005) or are potentially small enough to sneak through the plasmodesmata as an FP fusion (*AtRCI2A* and *AtRCI2B*; Medina et al., 2001; Nylander et al., 2001). N-terminal FP fusions were employed to avoid interruption of potentially important 3'-signaling sequences in the mature mRNA, and a monomeric, environmentally stable form of yellow fluorescent protein (YFP; mCitrine) was used to avoid FP dimerization (Griesbeck et al., 2001; Zacharias et al., 2002). Where possible, cDNA clones (including 3'-UTR) and genomic clones (introns plus 3'-UTR) of each membrane protein gene were tested. At least one of these constructs was found to unequivocally and vigorously label SE plasma membranes in both Arabidopsis and tobacco (*Nicotiana tabacum*).

### RESULTS

#### Isolation of Successful Transgenic Lines

Sixteen N-terminal mCitrine fusion constructs, all under the control of the *AtSUC2* promoter (Table I; Fig. 1, A and B), were generated and tested in Arabidopsis (Columbia-0 [Col-0] ecotype). At least 20 T0 plants were screened for each of the 16 constructs, and representative plants from each were found to express mCitrine to varying degrees in the veins of mature source leaves (data not shown). Proper transformation of each construct was confirmed by reverse transcription (RT)-PCR and sequencing (Fig. 1C). In general, those constructs that contained genomic clones generated a stronger vein-level mCitrine signal, but not consistently. Both *mCitrine*-SUC2 lines failed to label SE membranes, which is consistent with previous results (Lalonde et al., 2003; Stadler et al., 2005). The only construct that yielded strong and consistent SE labeling in Arabidopsis was *AtSUC2p::mCitrine-AtRCI2A*-genomic (Figs. 2, B and E, and 3, A and B).

*AtRCI2A* is a gene with no known phloem function, but which appears to be generally important for drought, cold, and salt tolerance, and for abscisic acid

### Table I. Constructs generated for SE plasma membrane mCitrine labeling

<table>
<thead>
<tr>
<th>Family</th>
<th>Name</th>
<th>AKA</th>
<th>TAIR Locusa</th>
<th>Type of Cloneb</th>
<th>Predicted Fusion Lengthc</th>
<th>Fusion Massd</th>
<th>TMDrd</th>
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<td>6</td>
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<tr>
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<td>PIP1B</td>
<td>At2g45960</td>
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<td>12</td>
<td></td>
</tr>
</tbody>
</table>

aRhee et al. (2003). bClones of each gene were generated from either genomic or cDNA templates, or both, beginning with the start codon and including the full 3'-UTR. cPredicted fusion length and mass include Ala-10 linker (Supplemental Fig. S1). Soluble mCitrine (239 amino acids) has a predicted mass of 27 kD. dThe evidence for two transmembrane domains in the RCI2 protein family is still circumstantial (Nylander et al., 2001; Kroemer et al., 2004).

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response signaling (Nylander et al., 2001; Kroemer et al., 2004; Medina et al., 2005; Mitsuya et al., 2006; Dai et al., 2007). The gene possesses orthologs in a variety of plant species as well as three Pseudomonas species, Neurospora crassa, and Caenorhabditis elegans (Kroemer et al., 2004). For this study, the gene was selected for the small size of the protein it expresses (Fig. 1B; Table I) and because it is known to target the plasma membrane (Cutler et al., 2000). The mCitrine-RCI2A fusion is predicted to have two transmembrane domains (Nylander et al., 2001; Kroemer et al., 2004) with the relatively large barrel-can mCitrine on the cytoplasmic face (Fig. 1D). Two strongly expressing homozygous seed lines, 19.07 and 11.07, were isolated at the T2 generation under phosphinothricin selection. Two CC-only control lines, one expressing the construct \( \text{AtSUC2} \text{p}\text{TmCitrine-AtPIP1;3-cDNA} \) (line 08) and the other the construct \( \text{AtSUC2} \text{p}\text{AtmCitrine-AtPIP2;2-genomic} \) (line 33), both of which yielded strong CC expression but no SE expression, were isolated at the T1 generation.

Tobacco ('Samsun') was also transformed with the \( \text{SUmCR} \) construct (an abbreviation of \( \text{AtSUC2} \text{p}\text{mCitrine-AtRCI2A-genomic} \)). Over 20 independent transgenic lines were screened, and one line (17), which strongly expressed mCitrine in the leaf vasculature and also in the roots (Fig. 2, D and F), was selected. This line is currently in the T1 generation.

### mCitrine Labeling of SE Membranes

Live-cell imaging of Arabidopsis \( \text{SUmCR-19.07 and \text{tobacco SUmCR-17}} \) demonstrated a strong mCitrine signal in SE plasma membranes (Figs. 3, A–D, and 4, A–E and H–O). The same was demonstrated in the SE plasma membranes of thin-sectioned, methacrylate-embedded Arabidopsis inflorescence stem phloem (Fig. 3, E, F, I, and J), as well as in the membranes lining the sieve pores (Fig. 4G). SE labeling was confirmed by aniline blue counterstaining against sieve plate callose (Fig. 3, A–D). In control transgenic lines (\( \text{AtPIP1;3-cDNA line 08 and AtPIP2;2-genomic} \))...
line 33), mCitrine label was absent or undetectable in SE plasma membranes, despite the presence of strong CC labeling (e.g. Figs. 3, G and H, and 4F).

A space between the opposing membrane faces of individual sieve plates was regularly discernible (Figs. 3, A, C, D, and J, and 4, A–E, G, and K–N). The scattering inherent to imaging through intact, entire Arabidopsis root tissue made it impossible to resolve double membranes in root phloem sieve plates (Fig. 4, H–J). Unidentified, nonplasmalemma, mCitrine-labeled membrane structures were also labeled in the SEs of both species (Figs. 3C and 4, A, D, E, G, and L–O). The increased brightness often observed in one face of a sieve plate relative to the other could be due to surging (Knoblauch and van Bel, 1998) of these free mCitrine-labeled membrane structures following fixation or hand sectioning (Fig. 4, A, C, and M).

The faces of transversely or obliquely oriented Arabidopsis sieve plates were observed in both longitudinal and cross sections in methacrylate-embedded material (Fig. 3, E and I). These sieve plates were often textured in appearance, but there was no clear evidence of unoccluded or open sieve pores, possibly due to the accumulation of free, mCitrine-labeled membrane structures at the face. Thus far, the only unequivocal evidence of sieve pore mCitrine labeling comes from anti-GFP antibody AlexaFluor 555 staining of longitudinal, thin-sectioned, methacrylate-embedded Arabidopsis stem material (Fig. 4G). Such images are rare, however, making precise, quantitative measurements of sieve pore shape and size difficult to replicate in this species using this method.

The Survival of mCitrine Label in Methacrylate

Thin sectioning of methacrylate-embedded SUMCR material was first undertaken to allow immunofluorescent labeling of the membrane-bound mCitrine with an AlexaFluor 555-conjugated anti-GFP antibody, under the assumption that the mCitrine signal would be quenched during fixation, dehydration, and embedding. However, in addition to preserving antigenicity (Figs. 3, F and H, and 4G), the methacrylate-embedded material yielded a strong mCitrine fluorescence signal (Fig. 3, E, G, I, and J). The mCitrine identity of this signal is confirmed by the strong overlap between the mCitrine and AlexaFluor 555 signals (Fig. 3, E–H). Specificity of the antibody for mCitrine is demonstrated by the absence of AlexaFluor 555 label in the SEs of the AtSUC2p::mCitrine-PIP1;3-cDNA transgenic line, which yields an mCitrine label only in CCs (Fig. 3, G and H, as compared to Fig. 3, E and F).

Methacrylate-embedded material was mounted in Vectashield mounting medium (see “Materials and Methods”). With wide-field fluorescence microscopy, the mCitrine signal in methacrylate-embedded material appeared to be at least as photostable as the mCitrine signal in fresh material (tens of minutes). With confocal microscopy, mCitrine was bleached much more quickly in methacrylate (seconds) than in fresh material (minutes).

mCitrine Signal in Root Tips

Stadler et al. (2005) showed that GFP-membrane protein fusions under the control of the AtSUC2 promoter accumulated no further than the metaphloem CCs of developing roots (their constructs: tmGFP2 and tmGFP9). In contrast, the SUMCR Arabidopsis and tobacco plants described here demonstrate significant root tip mCitrine accumulation (Figs. 2 and 5A). Furthermore, accumulation of the mCitrine signal is specific to the epidermis of the cell division zone of the root tip (Fig. 5, B and C). This pattern of expression was observed in all SUMCR primary root tips regardless of the presence or absence of Suc in the growth medium, and was evident in emerging lateral root tips prior to the appearance of a strong lateral root phloem signal.

Analysis of SE Geometry and Estimates of Fluid Mechanical Conductivity

Thompson and Holbrook (2003) derived an equation that yields a rough estimate of the conductivity of sieve tubes, called sieve tube-specific conductivity or...
$k \ (\mu m^2)$, based on basic fluid mechanical principles for stationary, laminar, low Reynolds number flow:

$$k = \left[ \frac{8N_p r_p^4 l}{8N_p r_p^4 (l - l_p) + (8l_p + 3\pi r_p)r^4} \right] r^2,$$

where $N_p$ is the number of sieve pores per sieve plate, $r \ (\mu m)$ is SE radius, $l \ (\mu m)$ is SE length, $r_p \ (\mu m)$ is sieve pore radius, and $l_p$ is sieve plate thickness ($\mu m$; Fig. 6C).

One goal of this study was to provide image data that could be used to measure each of the geometric parameters included in Equation 1. SE radius, plate thickness, and SE length are easily measured with confocal and wide-field fluorescence images of Arabidopsis and tobacco SEs (Table II), but because sieve pores were either too small or occluded to be measured with an mCitrine signal, it was necessary to measure sieve pore number and radius by alternative means.

Aniline blue-stained callose in sieve plates (Fig. 6A) provided a rough means of estimating both the number and radius of sieve pores. In longitudinal sections, individual callose-filled or callose-coated pores are regularly visible as banding patterns in side views of both Arabidopsis and tobacco sieve plates, although more clearly in those of Arabidopsis. Assuming several things relevant to wide-field fluorescence microscopy—that the visible pores are those that cross the focal plane, that the depth of field is of roughly the same order as pore diameter if not thinner, that all pores are of even size and distribution, that the largest visible pore provides a measure of typical pore radius, and that the pores and plate are circular in circumference—a rough estimate of the number of pores per sieve plate can be derived in terms of parameters easily measured from the UV-excited sieve plate images (Fig. 6B):

$$N_p \approx \frac{\pi D_{\text{plate}} N_{p, \text{vis}}}{4 D_{\text{pore}}}.$$

**Figure 3.** Wide-field fluorescence microscopy of the mCitrine-ARCI2A probe in SEs of Arabidopsis (A, B, and E–J) and tobacco (C and D). mCitrine is shown in false-color yellow, aniline blue-stained callose in false-color magenta, and the anti-GFP AlexaFluor 555 conjugate in false-color red. A to D, Live phloem of hand-sectioned Arabidopsis inflorescence stems (A and B) and tobacco source leaf petioles (C and D), showing CCs (dotted outline), SEs, sieve plates (arrowheads), and mCitrine-labeled, nonplasmalemma, SE membrane structures (arrows). The SE and CC on the right side of the sieve plate in B were excised during sectioning and are not visible here. E and F, Methacrylate-embedded cross section of anti-GFP-AlexaFluor 555-stained, SLmCR-transformed Arabidopsis inflorescence stem phloem showing intact mCitrine signal (E) and overlapping AlexaFluor 555 signal (F). Clear SE outlines are visible in both images. G and H, Same as for E and F, but using Arabidopsis plants transformed with the mCitrine-PIP1;3-cDNA construct (line 08). No SE outlines are visible. I, Longitudinal section of a minor vein from a methacrylate-embedded Arabidopsis source leaf. J, Composite image of a longitudinal section of a complete SE from a methacrylate-embedded Arabidopsis inflorescence stem. All bars = 5 $\mu m$. 

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Figure 4. Confocal microscopy of the mCitrine-ArRCl2A probe in SEs of Arabidopsis (A–J) and tobacco (K–O). See Figure 3 legend for false-coloring and symbol scheme. A to E, mCitrine signal from longitudinally hand-sectioned, SUmCR-transformed Arabidopsis inflorescence stems. Bars = 5 μm. F, mCitrine signal from a longitudinally hand-sectioned inflorescence stem of Arabidopsis transformed with the mCitrine-PIP2:2-genomic construct (line 33). Bar = 5 μm. G, AlexaFluor 555 signal from a longitudinal section of anti-GFP-AlexaFluor 555-stained, methacrylate-embedded, inflorescence stem of SUmCR-transformed Arabidopsis showing labeled sieve pores. Bar = 5 μm. H to J, mCitrine signal of root phloem in intact SUmCR-transformed Arabidopsis roots. Bars = 3 μm. K to O, mCitrine signal from longitudinally hand-sectioned internal phloem of SUmCR-transformed, tobacco source leaf petioles. Bars = 10 μm, except O = 20 μm.
DISCUSSION

Mapping Anatomy and Architecture of the Phloem

The goal of this work was to provide a fluorescent, SE plasma membrane marker for rapid studies of sieve tube anatomy and whole-plant architecture. The SUmCR construct meets this goal in both Arabidopsis and tobacco. The label is easily visualized in hand-sectioned material under wide-field fluorescence and confocal microscopy, is nonmobile (i.e. the root tip signal is limited to specific cell layers), and requires no treatment or incubation prior to visualization.

The mCitrine label can also be used in methacrylate-embedded material. It is not yet known how the mCitrine fluorophore survives fixation, dehydration, infiltration, and embedding, although it is possible that the dithiothreitol (DTT) included in each dehydration and infiltration step ameliorates quenching of the already fairly stable mCitrine barrel-can structure. GFP has been shown to survive paraformaldehyde fixation in Xenopus oocytes (Hughes et al., 2001), as well as glycol methacrylate embedding in mice (Ohta et al., 2000) and LR White embedding in zebrafish (Luby-Phelps et al., 2003).

The complete overlap of the AlexaFluor 555-conjugated anti-GFP antibody signal confirms the origin of the putative mCitrine signal as a GFP derivative (Fig. 3, E and F), and the absence of the AlexaFluor 555 signal from SEs in a transgenic line that lacks a SE mCitrine signal (Fig. 3, G and H) confirms that the anti-GFP antibody is unbiased toward SEs. The AlexaFluor 555 conjugate further confirms that mCitrine is localized to sieve pore membranes (Fig. 4G).

The image data presented here provide a useful platform for analyzing SE quantitative anatomy and for providing the datasets necessary to generate reasonable first-order estimates of sieve tube-specific conductivity ($k$). However, good data for pore radius and pore number continue to depend, at the moment, on the use of aniline blue as a pore-staining proxy (Fig. 6, A and B) because sieve pores are only occasionally discernible using the mCitrine and AlexaFluor 555 signals. This is an optical limitation made worse by the possibility that pores are occluded by free mCitrine-labeled membrane structures (e.g. Figs. 3, E and I, and 4, A and C). Available data for sieve pore radius (Esau, 1969; Thompson and Holbrook, 2003) has long indicated that the pores of most plants are quite small ($<0.5$ $\mu m$). Notable exceptions include the sieve pores of the cucurbits and some legumes. For most species, then, pore number and radius data must be supplemented by secondary methods, such as electron microscopy or aniline blue staining. Higher resolution imaging of aniline blue-stained plates using a UV laser on a single-photon confocal microscope leads to rapid aniline blue bleaching, but imaging could be possible with lower energy multiphoton microscopy (A. Schulz, personal communication), deconvolution methods, and the pore number calculation introduced in Figure 6. The

(See supplemental data for derivation.) This method, applied to the cartoon, 40-pore plate in Figure 6B, yields an estimate of 35 pores. The principal anticipated bias of this method is that more pores are visible than actually cross the focal plane, making Equation 2 an overestimate of $N_p$. A single sieve pore diameter ($D_{sieve}$) measurement is taken from each image. Sieve pore radius is similar between the species (Table II), whereas tobacco has twice the number of pores as Arabidopsis (despite having approximately 4 times the sieve plate area).

Even the most limited datasets (Table II) can provide useful estimates of $k$ (Eq. 1). Arabidopsis has a $k$ value on the order of 0.28 $\mu m^2$, whereas tobacco has a $k$ value on the order of 0.45 $\mu m^2$. For both species, these estimates are most sensitive to error in our measurement of sieve pore radius, $r_p$ (Fig. 6, D and E). In Arabidopsis, $k$ is secondarily most sensitive to errors in our measurement of SE radius, $r$, whereas in tobacco errors in estimates of $r$ are less important.
importance of improving estimates of pore radius is highlighted by the fact that it is this variable to which our calculation of conductivity is most sensitive (Fig. 6, D and E).

An additional source of error is the loss of turgor and subsequent reduction in sieve tube radius that inevitably follows fixation or hand sectioning. Sensitivity analysis of our conductivity estimate (Fig. 6, D and E) suggests that radial contraction of the sieve tube will not be as strong a source of error in tobacco as it is in Arabidopsis, but the importance of this error in either species will depend ultimately on the degree of expected radial contraction. Thompson (2005) showed that, given the high values of the drained pore modulus (or elasticity) of phloem tissue and the fact that SEs are bounded by far thicker walls than are cells in the surrounding tissue (Esau and Cheadle, 1958; Esau, 1969), the geometric expansion of sieve tubes following a change in turgor is expected to be relatively small. However, the unnatural conditions imposed by fixation and sectioning impose a far larger drop in turgor than would be expected under the physiological range of changes explored by Thompson (2005). The drained pore modulus of the phloem is known to be high relative to other tissues (in part due to the small size of most phloem cells), but values as low as 2 MPa have been reported for bulk phloem tissue (Wright and Fisher, 1983; Kallarackal and Milburn, 1985). A sieve tube with this low a modulus operating at a turgor of 1 MPa that experienced a sudden drop of turgor to 0 MPa would undergo a radial contraction of up to 40% (assuming linear elastic behavior). It is unlikely, however, given the greater thickness of SE walls, that a modulus of 2 MPa is representative of the SEs themselves. If the SE operated with a modulus of 18 MPa (which is at the high end of measured values for bulk phloem tissue), a sudden 1 MPa drop in turgor would result only in a 5% drop in radius. This implies that a 40% reduction in SE radius in Arabidopsis would mean a 50% drop in k, whereas a 5% reduction would have a negligible effect. In tobacco, where k is less sensitive to changes in r, a 40% reduction in r would only result in a 10% reduction in k. The effect on k of a 5% drop in r would also be negligible. As such, the effect of turgor reduction during tissue preparation appears to be important, but it is one-tail. Thus, any error in our measurement of r due to turgor loss during tissue preparation is expected to yield an underestimate of k.

Regardless of these difficulties, it is clear that tobacco petiole phloem possesses a slightly higher value of k, as indicated by the sensitivity analysis in Fig. 6, D and E.
of $k$ than the inflorescence stem phloem of Arabidopsis. This has important implications for the distance over which the phloem can maintain certain fluid mechanical properties, such as a small source-sink turgor difference, and the capacity to rapidly transmit changes in local turgor pressure or concentration throughout the rest of the phloem (Thompson, 2006). Plants with higher values of $k$ can translocate sap over longer distances while still maintaining these important control properties; a plant with too low a value of $k$ for a given translocation distance would be required to scale its local molecular control of sap solute exchange in a position-dependent manner (Thompson and Holbrook, 2004; Thompson, 2006).

### Expression of the SUmCR Construct Yields an mCitrine Signal in SE Membranes of Both Arabidopsis and Tobacco

Past attempts to modify plants transgenically to express FP membrane protein fusions in SEs (Lalonde et al., 2003; Yoshimoto et al., 2003; Stadler et al., 2005) have resulted in retention of the fluorescence signal in CCs. At the same time, of course, a wide variety of integral membrane proteins are known and expected to be found in SEs. The target of the SE-specific antibody RS6, raised against the isolated SEs of *Streptanthus tortuosus* (Brassicaceae), was recently found in Arabidopsis to be an ENOD-like integral protein (Khan et al., 2007). Other proteins have been immunolocalized to SEs, such as the Suc-H\(^+\) symporter SUT1 in potato (*Solanum tuberosum*; Kühn et al., 1997), a putative monosaccharide transporter and a proton-ATPase in developing apple (*Malus domestica*) fruit (Zhang et al., 2004), a proton-amino acid transporter in Arabidopsis roots (Okumoto et al., 2004), a calcium channel-like protein in tobacco and *Pistia stratiotes* leaves (Volk and Franceschi, 2000), and a plasma membrane aquaporin in spinach (*Spinacia oleracea*; Fraysse et al., 2005).

It is unknown why the SUmCR construct labels SE membranes intensely and exclusively while others do not. At first blush, the strength of the mCitrine signal generated by the SUmCR construct relative to the larger PIP1, PIP2, and SUC2 fusions (Fig. 1; Table I), which demonstrated no SE labeling or labeling that was perhaps too faint to detect (e.g. Figs. 3, G and H, and 4F), would suggest that the principal factor is the size of the protein. Indeed, SUmCR is a smaller fusion than has been developed previously for this purpose; however, while size may be an important factor for the trafficking of soluble proteins, it is unclear how the same argument could be made for membrane-anchored protein fusions. Stadler et al. (2005) found that, whereas relatively large soluble GFP-protein fusions were capable of trafficking between CCs and SEs in Arabidopsis, GFP-integral protein fusions of any size—and even one with a smaller mass (tmGFP9)—were incapable of trafficking between SEs and CCs (Imlau et al., 1999; Lalonde et al., 2003; Yoshimoto et al., 2003; Stadler et al., 2005) in contrast to the 2,141-bp fragment used here.

The high metabolic demands of these cells—cells that are many cell lengths away from developing protophloem—could partially explain why Suc transporters are needed near the root tip. Enhanced Suc loading in root tip epidermal cells could enhance Suc uptake in those regions that need it most, such as the first 200 to 300 $\mu$m of the Arabidopsis root tip where cell division rates are highest (Beemster and Baskin, 1998). The expression pattern was observed regardless of whether the growth medium contained Suc, suggesting that the promoter’s expression pattern is independent of rhizosphere Suc concentration.

### Table II. Calculating sieve tube-specific conductivity, $k$

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<thead>
<tr>
<th>Variable (Eq. 1)</th>
<th>Value ± stderr ($N$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r$ ($\mu$m)</td>
<td>2.0 ± 0.1 (7)</td>
</tr>
<tr>
<td>$l$ ($\mu$m)</td>
<td>107 ± 7 (13)</td>
</tr>
<tr>
<td>$r_l$ ($\mu$m)</td>
<td>0.26 ± 0.01 (7)</td>
</tr>
<tr>
<td>$l_l$ ($\mu$m)</td>
<td>1.0 ± 0.1 (14)</td>
</tr>
<tr>
<td>$N_p$</td>
<td>52 ± 6 (7)</td>
</tr>
<tr>
<td>$k$ ($\mu$m$^2$)</td>
<td>0.28</td>
</tr>
</tbody>
</table>

These data were derived from images of aniline-blue stained callus (Fig. 6).
be largely irrelevant up to a certain limit (Lough and Lucas, 2006). Another possibility is that the SUmCR construct simply expresses protein at a much higher level than do the other constructs; however, the others, which yield no expression in SEs, regularly exhibit the same degree of expression in CCs (e.g. Fig. 4F).

The lack of ribosomes in SEs (although there is ER) implicates two pathways for the trafficking of the mCitrine-AtRCI2A fusion between CCs and SEs. Both necessarily require the expression of the mature, membrane-anchored fusion protein in the CC followed by trafficking to the SE (Schulz, 1999). In the first case, the fusion protein diffuses from the CC plasma membrane through the membrane sleeve of the plasmodematal pore unit (or PPU) directly to the SE plasma membrane. In the second case, the mature protein moves to the PPU via the CC endoplasmic reticulum (ER) and diffuses in the desmotubule membrane to the SE ER, followed by additional vesicular transport to the SE plasma membrane. The ER route seems more likely given the observed mCitrine labeling of nonplasmalemma membrane structures, but such labeling cannot explicitly rule out the plasma membrane sleeve as an important route for CC-to-SE mCitrine-RCI2A trafficking.

In either case, it is conceivable that gating of some sort is involved (even though lipids freely move between CC and SE via PPUs; Martens et al., 2006). Additional layers of selectivity could exist in the CC ER prior to PPU trafficking. For instance, ER membrane proteins diffuse faster in the membrane than can be explained by passive diffusion alone (Runions et al., 2005), suggesting that the CC ER could be capable of actively shepherding certain membrane proteins to specific ER domains near PPUs. A determination of which suite of trafficking mechanisms predominates is beyond the scope of this study, but experimental modifications to the SUmCR construct could provide a useful entree for learning more.

MATERIALS AND METHODS

Additional details of all of the methods presented below can be found in Supplemental Documentation S1.

Plant Strains and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotype Col-0, unless otherwise noted, was cultivated in growth chambers under a 14-h day, 200 μmol m⁻² s⁻¹ illumination with cool-white fluorescent bulbs, 22°C, 50% relative humidity, and in individual cells on Scott’s LCI Sunshine soil mix. Plants were watered as needed, and when seedlings had at least six true leaves, they were fertilized and in individual cells on Scott’s LC1 Sunshine soil mix. Plants were watered as needed, and when seedlings had at least six true leaves, they were fertilized with 5 mg/L phosphinothricin (i.e. Basta; Crescent Chemical Company; molecular weight 195 g/mol) and Silwet L-77, 0.02% (Lehle Seeds) four times at 4-d intervals following stratification, germination, and the emergence of the first true leaves. Selected plants were then screened for leaf minor vein-specific mCitrine expression under low magnification with a Zeiss Axioskop using a YFP-specific filter set (as above) for mCitrine. Fragments were TA-cloned and sequenced to confirm construct identity.

Plants were transformed following standard methods (Horsch et al., 1985; Bent et al., 1994), with modifications detailed in Supplemental Documentation S1. At least 20 Arabidopsis plants were selected per transformation, spraying with 5 mg/L phosphinothricin (i.e. Basta; Crescent Chemical Company; molecular weight 195 g/mol) and Silwet L-77, 0.02% (Lehle Seeds) four times at 4-d intervals following stratification, germination, and the emergence of the first true leaves. Selected plants were then screened for leaf minor vein-specific mCitrine expression under low magnification with a Zeiss Axioskop using a YFP-specific filter set (41028; Chroma Technology). Briefly labeled known homozygote T1 seedlings were selected and bulked into the T2 generation using the same phosphinothricin/Silwet solution.

Total RNA was isolated from each of the expressing Arabidopsis lines. First-strand cDNA was generated from each using a poly-T primer and SuperScript III (Invitrogen) reverse transcriptase. PCR primers were designed to amplify fragments between 200 and 250 bp spanning the 3' end of each line using a gene-specific reverse primer against a mCitrine-based forward primer, mC-5'FW(mtpcr). Fragments were TA-cloned and sequenced to confirm construct identity.

Transformed tobacco plants were selected on 8 mg/L phosphinothricin in medium (as described in Supplemental Documentation S1), cultivated as above, and screened with wide-field fluorescent microscopy using mature source leaves as with Arabidopsis.

Imaging

Arabidopsis stem inflorescence stems were glued to a glass microscope slide with dental adhesive (Hollister), and then finely hand sectioned with a sharp blade. Stem strips were stained with 0.1% aqueous aniline blue for 10 min in the dark, and then rinsed, mounted in 10 mL KCl, 10 mL CaCl₂, and 5 mL NaCl, and imaged. The petioles and midribs of mature tobacco source leaves were treated as with Arabidopsis stems, but care was taken to keep track of external versus internal petiole phloem. Undisturbed Arabidopsis root phloem could be imaged through intact tissue with inevitable scattering. Fresh and sectioned material was imaged using wide-field fluorescence with a Zeiss Axioskop using a YFP-specific filter set (as above) for mCitrine labeling, a narrow-band DAPI/Hoechst/AMCA filter set (3103v2; Chroma) for aniline blue, or a TRITC (Rhodamine)/Dil/Cy3 filter set (31022; Chroma) for the AlexaFluor 555 conjugate dye.

Vector Construction and Plant Transformation

Vectors were prepared following standard protocols (Sambrook et al., 2001) using Escherichia coli strains DH5a (Hanahan, 1983) and DB3.1 (Invitrogen), depending on the absence or presence of a GATEWAY (Invitrogen) cassette, respectively. Agrobacterium tumefaciens strain GV3101 (Holsters et al., 1980) was used for transformation of both plant species.

pGREENII-0229, a plant expression vector carrying the nos-bar (phosphinothricin resistance) selection marker (Hellens et al., 2000a, 2000b), was modified to contain a 2.141-bp fragment of the AtSUC2 promoter region upstream of a GATEWAY (Invitrogen) destination cassette and a t3SS terminator (Fig. 1A). A GATEWAY entry vector containing a modified YFP reading frame (called mCitrine; Griesbeck et al., 2001; Zacharias et al., 2002; Shaner et al., 2005) was developed from a YFP clone provided courtesy of David Ehrhardt (Carnegie Institution of Washington) upstream of a 10-mer ALa linker flanked by a small multiple cloning site (Supplemental Fig. S1). Genomic and cDNA clones of each of the test genes were amplified from genomic DNA and first-strand cDNA, respectively, and subcloned into the multiple cloning site of the entry vector (Fig. 1B). All clones contained the 3'-UTR of each gene and the genomic clones also contained the full set of introns. Entry vectors were then LR recombined using GATEWAY LR clonase II (Invitrogen) with the destination vector to make mCitrine fusion constructs under the control of the AtSUC2 promoter, which subsequently introduced into GV3101 cells for plant transformation. Detailed cloning steps are provided in Supplemental Documentation S1.

Plants were transformed following standard methods (Horsch et al., 1985; Bent et al., 1994), with modifications detailed in Supplemental Documentation S1. At least 20 Arabidopsis plants were selected per transformation, spraying with 5 mg/L phosphinothricin (i.e. Basta; Crescent Chemical Company; molecular weight 195 g/mol) and Silwet L-77, 0.02% (Lehle Seeds) four times at 4-d intervals following stratification, germination, and the emergence of the first true leaves. Selected plants were then screened for leaf minor vein-specific mCitrine expression under low magnification with a Zeiss Axioskop using a YFP-specific filter set (41028; Chroma Technology). Briefly labeled known homozygote T1 seedlings were selected and bulked into the T2 generation using the same phosphinothricin/Silwet solution.

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Transformed tobacco plants were selected on 8 mg/L phosphinothricin in medium (as described in Supplemental Documentation S1), cultivated as above, and screened with wide-field fluorescent microscopy using mature source leaves as with Arabidopsis.
Fresh and sectioned material was imaged with confocal laser-scanning microscopy using a Zeiss LSM 510 confocal microscope with a 488-nm argon excitation band with a 505- to 550-nm emission band-pass filter for mCherry imaging, and a 543 He-Ne excitation band with a 650- to 615-nm emission band-pass filter for AlexaFluor 555 imaging. No UV laser was available on this microscope for aniline blue confocal imaging.

Material for methacrylate embedding (Baskin et al., 1992, 1996; Baskin and Wilson, 1997) was fixed in a pH 7.0, 4% paraformaldehyde buffer, dehydrated in ethanol with 1× DTT, and infiltrated in a methacrylate/catalyst (12,100, 18,800, and 11,280, respectively; Electron Microscopy Sciences), and 10 mM DTT. The series was performed in flat-bottomed embedding capsules (7002; Electron Microscopy Sciences). The methacrylate resin was cured with UV light at 4°C for 5 h. Sections from embedded material were chloroform and acetone etched (Baskin et al., 1992; Klink and Wolniak, 2001) and mounted in Vestacast (Vector Labs) under cover glasses sealed with nail polish, or etched and stained with a 1:20 dilution of anti-GFP antibody AlexaFluor 555 conjugate (A-11851; Molecular Probes) in phosphate-buffered saline for 2 h at 37°C in the dark, and then mounted.

Supplemental Data

The following materials are available in the online version of this article.
Supplemental Figure S1. Diagram of Al-a10 linker region used in each expression vector.
Supplemental Table S1. List of oligonucleotides used in this study.
Supplemental Documentation S1. Detailed description of vector construction and plant transformation, and a derivation of the sieve pore number equation (Eq. 2).

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Macromolecular trafficking indicated by localization and turnover of sucrose transporters in enucleated sieve elements. Science 275: 1298–1300


