Structural and Functional Vein Maturation in Developing Tobacco Leaves in Relation to AtSUC2 Promoter Activity


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Transgenic tobacco (Nicotiana tabacum) plants expressing green fluorescent protein (GFP) from the AtSUC2 promoter were used to study the function of different vein classes in developing leaves. In sink leaves, unloading capacity occurred acropetally, with the class I (midrib) and class II veins becoming functional in phloem unloading before the maturation of the class III veinal network. In contrast, in developing cotyledons and source leaves, loading capacity occurred in a basipetal direction. There was a strong correlation between loading capacity, as assessed by $^{14}$C Suc uptake and companion cell expression of AtSUC2-GFP. Developing cotyledons were shown to utilize all available vein classes for loading. A second line of transgenic plants was produced in which GFP, expressed from the AtSUC2 promoter, was targeted to the endoplasmic reticulum instead of the cytoplasm. In these AtSUC2-GFP-ER plants, GFP was unable to traffic into the sieve element and was restricted solely to the companion cells of source leaf tissues. Partial shading of leaves undergoing the sink-source transition demonstrated that the activation of the AtSUC2 promoter in tobacco was influenced by light. Functional and structural maturation of the minor veins required light or a product of light. The activation of the AtSUC2 promoter within major veins appears to be regulated differently from that in the minor veins. The relationship between AtSUC2 activation and the activity of endogenous tobacco Suc transporters is discussed.

The growth of a leaf from emergence to final size involves periods of cell division, tissue differentiation, and cell expansion. After germination, the stored reserves in the cotyledons are immediately mobilized to provide a source of carbon for the growing plant. In contrast, all other immature leaves on the plant function as sinks for assimilate. Little is known concerning the structural development of the various vein classes that make up the phloem network of cotyledons and source leaves in relation to their function in phloem loading and, similarly, it is not clear how the capacity to unload solutes proceeds with respect to phloem development in immature sink leaves (Esau, 1960; Turgeon, 1989).

In tobacco (Nicotiana spp.), a developing sink leaf has three functional vein classes: class I (the midrib), class II (arising as single branches from the midrib), and class III (which form “islands” between adjacent class II veins; Avery, 1933; Roberts et al., 1997). In the expanding sink leaf, assimilate unloading (Turgeon, 1987; Roberts et al., 1997) occurs from the class III veinal network. This vein class also has the capacity to unload macromolecules such as green fluorescent protein (GFP; Imlau et al., 1999; Oparka et al., 1999), and systemic viruses (Roberts et al., 1997). Before the leaf is fully expanded, it undergoes a transition from a sink (net carbon importer) to a source (net carbon exporter). As the leaf expands, unloading from the major veins becomes reduced and eventually ceases (Turgeon, 1987; Roberts et al., 1997; Imlau et al., 1999; Oparka et al., 1999). The cessation of unloading has been attributed to the loss of symplastic continuity that occurs as intercellular spaces enlarge and cells are pulled apart, decreasing plasmodesmal connectivity (Ding et al., 1988; Roberts et al., 2001).

In a number of species, the termination of import coincides with the structural maturation of the minor veins (Ding et al., 1988). Minor veins have been defined arbitrarily as those without midribs of parenchyma projecting below the lower leaf surface (Esau, 1965). On a functional basis, they have been defined in tobacco as the class IV and V veins, which are immature in sink leaves, are nonfunctional before the sink-source transition, but are later responsible for phloem loading in a mature source leaf (Haritatos et al., 2000). It has recently been demonstrated that the galactinol synthase promoter (CmGAS1) is activated in the minor veins of tobacco leaves even though galactinol is not synthesized in this tissue. Thus, it appears that the minor vein system is developmentally defined and regulated and that the factors controlling the genes expressed in photoassimilate ex-
port are also able to activate this foreign promoter (Haritatos et al., 2000). In some species, the structural development of the minor veins is synchronized with export (Issebrands and Larson, 1973), but in others it appears that minor vein maturation is a preparatory step, but not necessarily the trigger, that leads to export (Fellows and Geiger, 1974; Turgeon, 1989).

The various sections of phloem have been described according to their function (van Bel, 1996). Thus, in the expanding sink leaf, the class III veins involved in unloading are referred to as “release” phloem, the class I and II veins are the “transport” phloem, and after the sink-source transition, the minor veins become the “collection” phloem (van Bel, 1996). In contrast with the single function carried out by either the collection or release phloem, the transport phloem must maintain the pressure flow necessary to ensure that assimilate reaches the terminal sinks while also supplying assimilate to the heterotrophic tissues along the phloem pathway.

Investigations into the development and functioning of leaf phloem frequently have been limited by the destructive nature of the autoradiographic methods used (Turgeon, 1984, 1987). Recently, fluorescent probes have been used to trace functional phloem transport using noninvasive approaches (Roberts et al., 1997). A complementary approach to studying the functional maturation of the phloem has been to localize the activity of specific Suc transport proteins that may participate in active vein loading (Imlau et al., 1999; Oparka et al., 1999; Haritatos et al., 2000). In the present work, we utilized the Arabidopsis Suc transporter promoter, AtSUC2, to drive the expression of GFP within tobacco leaves (Imlau et al., 1999; Oparka et al., 1999). In wild-type Arabidopsis leaves, this promoter regulates the expression of the companion cell-specific AtSUC2 Suc-H\(^+\) symporter gene, which is located predominantly in source tissue (Truernit and Sauer, 1995; Stadler and Sauer, 1996). However, this protein is not restricted to minor vein companion cells but may also be found in the major veins of leaves and within the vascular bundles of sink tissues such as stamens, siliques, and roots (Truernit and Sauer, 1995; Stadler and Sauer, 1996), suggesting a role in the retrieval of Suc that leaks out of sieve elements during long-distance transport (Stadler and Sauer, 1996). Arabidopsis plants expressing AtSUC2-GFP (AtSUC2 promoter-driven GFP expression) in the companion cells are able to traffic the 27-kD GFP protein through plasmodesmata into the sieve elements and ultimately into sink tissues, where it is unloaded symplastically into the mesophyll cells from the major veins (Imlau et al., 1999; Oparka et al., 1999).

The expression of AtSUC2-GFP in transgenic tobacco in relation to the source or sink status of the tissue shows a similar pattern to that in Arabidopsis (Imlau et al., 1999; Oparka et al., 1999). In tobacco leaves undergoing the sink-source transition, the unloading of GFP is restricted to basal class III veins, whereas in the apical (source) region of these leaves, GFP unloading ceases. As leaf development progresses, unloaded GFP disappears from epidermal and mesophyll tissues as the tissue becomes a source, and in the mature leaf, GFP is found exclusively in the companion cells of the minor veins where AtSUC2-GFP is being expressed. This specific localization of GFP in source leaves gives the minor vein network a punctate appearance (Oparka et al., 1999; Roberts et al., 2001).

Because GFP expressed from the AtSUC2 promoter is phloem mobile (Imlau et al., 1999; Oparka et al., 1999), we produced a second line of transgenic plants in which GFP was directed by a signal peptide into the companion cell endoplasmic reticulum (ER; AtSUC2-GFP-ER). In these plants, GFP was unable to traffic from companion cell to sieve element, and the GFP was retained exclusively in the companion cells of source leaves.

In this paper, we use AtSUC2-GFP and AtSUC2-GFP-ER transgenic plants to identify vein classes in tobacco leaves where unloading and loading, respectively, are taking place. Specifically, we have followed the developmental progression of AtSUC2 activity in source leaves to determine the direction of vein maturation and solute loading capacity in developing cotyledons and source leaves. We show that the activation of the AtSUC2 promoter is closely correlated with Suc loading, as shown by \(^{13}\)C autoradiography, and demonstrate that the sink-source transition can be influenced by partial shading of sink leaves, identifying a number of factors that influence minor vein maturation and phloem loading.

RESULTS
Development of Loading Capacity in Cotyledons

The cotyledons of AtSUC2-GFP plants were examined to investigate the structural development of the vein classes in relation to the functional activation of the AtSUC2 promoter. In cotyledons, no unloading of GFP was observed from any of the vein classes (data not shown). As the cotyledon emerges, two vein classes are present (Fig. 1B), but initially GFP is expressed exclusively within the companion cells of the midrib (Fig. 1A). As the cotyledon expands, additional vein classes are formed, and these, once mature, also express GFP (Fig. 1, C, E, and G). A leaf-cleaning technique was used to clearly identify lignified xylem vessels. Lignification was used to indicate structural maturation of the veins because xylem differentiation normally occurs after phloem structural maturation (Esau, 1965). This structural maturation (Fig. 1, B, D, F, and H) precedes the functional expression of AtSUC2-GFP. For example, class II veins near the base of the leaf are present (Fig. 1D) but do not express GFP (Fig. 1C). The expression
pattern of AtSUC2-GFP shows that functional maturation occurs basipetally, with the class II veins near the tip of the leaf becoming fluorescent and functional before those at the base (Fig. 1, C and D). Similarly, class III veins develop and mature basipetally until in the mature cotyledon there is a complete network of major veins, comprising the class I to III veins (Fig. 1, G and H). The progression of functional vein maturation of a pair of cotyledons over a period of 5 d is shown in Figure 1, J to P. GFP is expressed first in
the class I veins (Fig. 1J), followed by the class II veins (Fig. 1L), and finally the class III veins (Fig. 1N).

**AtSUC2-GFP Expression Is Correlated with Suc Uptake Capacity**

When a mature AtSUC2-GFP cotyledon was incubated on $^{14}$C-Suc, the radiolabel accumulated in all the vein classes expressing GFP (Fig. 1, Q and R).

**Development of Unloading Capacity in Sink Leaves**

In AtSUC2-GFP plants, the GFP synthesized in the companion cells is able to move into the sieve elements and be transported, along with photoassimilate, to developing leaves and other sink areas of the plant. When it first emerges, the sink leaf has only the midrib to facilitate the transport and unloading of assimilate (Fig. 2A). However, as the leaf expands, other vein classes are formed and begin to unload GFP (Fig. 2, C–F). Unlike phloem loading in the cotyledon, phloem unloading of GFP occurred in an acropetal direction, the class II veins at the base of the leaf (Fig. 2D) maturing in advance of those at the tip (Fig. 2F). After further leaf expansion, a network of class I to III veins had formed, and the unloading of GFP (Fig. 3A) took place predominantly from the class III veins.

**The Sink-Source Transition**

During the next stage of leaf development, there was a basipetal transition from sink to source, progressing from the apex to the base of the leaf (Turgeon, 1989). As demonstrated previously (Oparka et al., 1999), the unloading of GFP from the class III veins ceased (Fig. 3A), and the minor vein network, comprising class IV and V veins, started to mature (Fig. 3A). In AtSUC2-GFP leaves undergoing the sink-source transition, the uptake of Suc (Fig. 3D) occurred predominantly into the same minor vein networks expressing companion cell-specific GFP (Fig. 3C). However, at the basal end of the leaf, where GFP was being unloaded from the class III veins (Fig. 3A), the major veins were observed to accumulate radiolabeled carbon (Fig. 3B).

**The Sink-Source Transition in AtSUC2-GFP-ER Plants**

To identify the location of *AtSUC2* activation, without the complication of phloem transport of free GFP, a second line of transgenic plants was produced in which GFP, also expressed from the *AtSUC2* promoter, was targeted to the ER (Fig. 3, compare F with E). In these AtSUC2-GFP-ER plants, GFP is unable to traffic into the sieve element and, therefore, does not spread to sink tissues. This results in plants that show GFP fluorescence only in the ER of companion cells in source tissue. As expected, the pattern of expression of GFP in cotyledons of AtSUC2-GFP-ER plants was similar to that described for AtSUC2-GFP plants (data not shown). In contrast, in a leaf in which the sink-source transition had recently commenced, GFP was only seen in the veins at the tip of the leaf and was completely absent from veins near the base (Fig. 3G). As the transition progressed basipetally, all veins began to express GFP. In plants approximately 25 d post-sowing, the sink-source transition occurred in a 33-mm-long leaf with only the major veins (classes I–III) showing fluorescence (Fig. 3H). However, in older, larger plants (approximately 40 d post-sowing), the sink-source transition occurred in leaves up to 16 cm long and involved both major and minor vein classes as revealed by GFP fluorescence (see Fig. 5C).

**Effects of Partial Shading of an AtSUC2-GFP Sink Leaf**

To investigate the effect of light on the progression of the sink-source transition, an area near the tip of an AtSUC2-GFP sink leaf was sandwiched between discs of opaque plastic to produce localized areas of shading (see Fig. 5, A and B). The leaf was allowed to continue growth for a further 10 d before examination, during which time it increased in size to at least twice its original length (between 12 and 16 cm). During this time, the unshaded area of the leaf underwent the transition from sink to source. In unshaded regions of this leaf, the minor veins had ma-
tured structurally (data not shown), and GFP was expressed in both the major and minor veins (Fig. 4B), showing that Suc transporters were functional at this stage. Within the shaded area, GFP was expressed in all the major veins (Fig. 4C), but the minor veins did not express GFP (Fig. 4C), although they were structurally mature (Fig. 4D).

A similar shading treatment of a fully expanded source leaf (19 cm long) did not result in any difference in the expression of GFP between the unshaded and shaded regions of the leaf. GFP was expressed in all the vein classes within both areas (data not shown).

**Effects of Partial Shading of an AtSUC2-GFP-ER Leaf**

In AtSUC2-GFP plants, GFP is already present in the major vein network of the sink leaf due to unloading (see Figs. 2 and 3). Therefore, it is possible that the presence of GFP within the major veins of the shaded area of an AtSUC2-GFP transition leaf could be due to its retention within the veins after import, rather than its specific companion cell expression after the sink-source transition. Therefore, we investigated the effect of partial shading on an AtSUC2-GFP-ER sink leaf (Fig. 5, A and B). In this case, the leaf increased substantially in length during the treatment period of 11 d (from approximately 5 to between 17 and 24 cm in length). Before shading, GFP could not be seen in any of the veins (data not shown), but after 11 d of treatment, GFP was observed in both the major and minor veins of the unshaded regions of the transition leaf (Fig. 5C). Once again, GFP was observed within the major veins of the shaded area of the leaf (Fig. 5D), demonstrating that the presence of GFP within these veins is due to expression of GFP under the \textit{AtSUC2} promoter, rather than phloem transport of GFP into the shaded area.

After removal of the shading discs, the leaf remained on the plant for a further 6 d in the greenhouse. GFP was subsequently expressed in all the vein classes throughout the leaf, indicating that the lack of expression of GFP in the minor veins of shaded areas of leaf was not irreversible and given the right conditions, the \textit{AtSUC2} promoter is activated (data not shown).

Leaf tissue taken from unshaded and shaded areas of AtSUC2-GFP-ER transition leaves was incubated on \(^{14}C\)-Suc. In both unshaded (Fig. 5E) and shaded areas (Fig. 5F), radiolabel was accumulated in both the major and minor veins.

**Discs Isolated from AtSUC2-GFP-ER Sink Leaves**

To identify whether communication with the whole leaf is required to initiate AtSUC2-GFP-ER expression within the major and minor veins, leaf
discs were isolated from sink leaves and floated on water in either the light or the dark for 8 d. Discs maintained in the light increased in diameter from 13 to 17 mm, whereas those in the dark increased to only 15 mm in diameter. GFP was expressed only within the major veins of leaf discs maintained in the light and not in the minor veins (Fig. 6A), although the latter were shown to have matured structurally (Fig. 6C). When incubated on 14C-Suc, radiolabel was accumulated by the major but not the minor veins of these discs (Fig. 6B).

In contrast, in discs maintained in the dark, GFP was not expressed in any of the veins (Fig. 6D), and the minor veins remained immature (Fig. 6F). However, radiolabeled Suc was accumulated within the major veins of these discs (Fig. 6E).

DISCUSSION

In this work, we utilized transgenic tobacco plants expressing GFP under the control of the AtSUC2 promoter to identify a number of features relating to leaf development, vein function, and the sink-source transition.

Structural Development Precedes Functional Maturation

In cotyledons, we have shown that the structural development of the different major vein classes precedes the expression of AtSUC2-GFP and that the activation of this promoter indicates a basipetal progression of functional vein maturation. In the developing leaf, the expression of AtSUC2-GFP supports the basipetal functional maturation of the veins involved in loading as demonstrated previously (Turgeon and Webb, 1976; Turgeon, 1989).

Major Veins Are Able to Load Assimilate in Small Leaves

In previous studies, it was shown that in source leaves the minor veins are primarily involved in assimilate loading, although other vein classes have the capacity for Suc retrieval (Turgeon and Webb, 1976; Turgeon, 1989; Haritatos et al., 2000). The present work emphasizes that in cotyledons, which act solely as sources, and in small leaves, a functional minor vein network has not formed by the time the

Figure 4. The effect of shading on AtSUC2-GFP leaves. A scanned image of a leaf (A) after its shading for 10 d indicating the unshaded area (B) and shaded area (C) imaged using the CLSM. GFP is seen in the major and minor veins (darts) in the unshaded region (B) but only in the major veins (darts) of the shaded region (C). An electron microscopy section (D) taken from the shaded area demonstrates the maturity of the minor veins as indicated by the absence of cell contents in the sieve elements (S). CC, Companion cell; Pa, phloem parenchyma. Scale: A = 1 cm, B and C = 0.1 cm, and D = 2 μm.
tissue becomes a source. Therefore, it is the major veins that are involved in assimilate loading in these small leaves. This corroborates the observation of Haritatos et al. (2000) that in Cucurbita maxima, activation of the galactinol synthase promoter (CmGAS1), which is indicative of vein loading, occurs in the veins of cotyledons and in most of the veins of the first formed leaves.

Acropetal Vein Maturation in Sink Leaves

In sink tobacco leaves, it has been suggested that the earliest differentiation takes place in the external phloem at the tip of the midrib and that maturation progresses basipetally (Avery, 1933). However, other reports claim that maturation of the midrib and major veins occurs acropetally (Esau, 1960; Turgeon and Webb, 1973). Although the present work does not provide any information regarding the structural maturation of the major veins, it does demonstrate an acropetal progression in the functionality of these veins with respect to their ability to unload GFP. Unloading from the phloem frequently has been observed to take place from the class III veins (Turgeon, 1987; Roberts et al., 1997; Imlau et al., 1999; Oparka et al., 1999). However, it is clear that the midrib and class II veins may be involved in phloem unloading when class III veins have not yet differentiated. Therefore, it is apparent that, as a leaf develops, the class I and II veins change from release phloem into transport phloem. Also, as the sink-source transition progresses, the class III veins change from release phloem into transport phloem and, finally, the minor veins become the collection phloem. The flow of assimilate in the class I and II veins must change direction, and it remains to be determined whether

Figure 5. The effect of shading on AtSUC2-GFP-ER leaves. Photographs of AtSUC2-GFP-ER plants were taken at the start of the shading treatment (A) and after 11 d of treatment (B). The applied disc is 19 mm in diameter. CLSM images indicate that GFP is expressed in both the major and minor veins (darts) in the unshaded regions (C) but only the major veins (darts) in the shaded region (D). Very faint traces of some class IV veins can be seen in the shaded region (D), but this is due to xylem autofluorescence. Autoradiographs of tissue taken from unshaded (E) and shaded (F) areas of the leaf and incubated with [14C]Suc show the accumulation of isotope in the major (arrow) and minor (darts) vein classes of both treatments. Scale: C to F = 0.2 cm.
the same sieve elements are utilized for both import and export (see also Roberts et al., 1997).

**AtSUC2 Activation during the Sink-Source Transition**

The cessation of unloading of GFP in AtSUC2-GFP plants (Oparka et al., 1999), and the appearance of GFP in the companion cells of AtSUC2-GFP plants undergoing the sink-source transition, suggests that activation of the *AtSUC2* promoter is an accurate marker for the sink-source transition in tobacco leaves. By examining the effect of partial shading of both AtSUC2-GFP and AtSUC2-GFP-ER plants, we have been able to identify a number of factors controlling *AtSUC2* activation and vein maturation, thereby indicating some putative control points in the sink-source transition.

**Is AtSUC2 Activation Linked to Structural Vein Maturation?**

Because AtSUC2 is expressed within the companion cells of mature veins (Stadler and Sauer, 1996), it could be argued that its expression is linked to structural maturation of the minor veins. However, it is clear that this is not the case; in both shaded leaves, and in leaf discs maintained in the light, the minor veins were structurally mature but did not express GFP. During the sink-source transition, the minor veins became structurally mature (Turgeon and Webb, 1973; Turgeon, 1989). This structural maturation of the minor veins does not appear to require light because it occurs in completely shaded areas of transition leaves. It seems likely that structural vein maturation is developmentally programmed, and shading does not prevent this program. Alternatively, an external signal may be transported from the unshaded area of the leaf into the shaded region, and this signal might be generated in isolated leaf discs in response to light. However, it is clear that the signal to initiate minor vein maturation is not received in isolated leaf discs maintained in the dark (e.g. Fig. 6F).

**AtSUC2 Activation Is Regulated Differently in Major and Minor Veins**

The signal to initiate *AtSUC2* activation in the major veins appears to differ from that in the minor veins. Shading does not prevent the expression of
GFP within the major veins of transition leaves, suggesting that a signal to initiate activation is received from the adjacent, unshaded tissues. Because GFP is expressed in the major veins of discs kept in the light, but not those in the dark, it appears that the signal to initiate activation could be generated by light.

The activation of the CmGAS1 promoter within the minor veins of tobacco suggests that the promoter responds to a regulatory system, common to a number of species, that governs the loading function of the minor veins (Haritatos et al., 2000). The nature of these signals, and whether the AtSUC2 promoter responds to the same conserved signals, remains to be determined.

One possible candidate as a signal for AtSUC2 activation is Suc itself, which has frequently been implicated, albeit as a negative signaling molecule (Bush, 1999; Lalonde et al., 1999). Increased apoplastic Suc levels have been shown to decrease Suc symporter levels in sugar beet (Beta vulgaris) leaves (Chiou and Bush, 1998; Vaughn et al., 2002). Leaf discs maintained in the light continue to expand and are autotrophic. Therefore, it is possible that sufficient Suc is present in the apoplast surrounding the minor veins to act as a signal that prevents AtSUC2 activation. However, one might expect that Suc would also be likely to feedback on the activation of AtSUC2 within the major veins, and this did not appear to be the case.

It has been demonstrated that levels of both the LeSUT1 mRNA in tomato (Lycopersicum esculentum) and SiSUT1 protein levels from potato (Solanum tuberosum) leaves decrease in the dark (Kühn et al., 1997); therefore, darkness could potentially prevent AtSUC2 activation in the minor veins of shaded leaves. However, in tobacco, GFP remained present in the major veins of the shaded areas; therefore, there is no evidence that shading affected GFP fluorescence in source tissue over periods of up to 10 d, even though GFP is reported to have a short half-life in living cells of approximately 4 h, as can be seen by the rate of loss of fluorescence during the sink-source transition (Roberts et al., 2001).

**CONCLUSION**

Although AtSUC2 is not normally expressed in tobacco, we have shown that the activation of its promoter in developing veins correlates with the capacity of these veins to load Suc (see also Haritatos et al., 2000). The functional development of veins with respect to loading, as imaged by expression of AtSUC2-GFP, occurs basipetally, whereas the unloading capacity of veins develops acropetally.

During the sink-source transition, the expression of AtSUC2-GFP may be differentially controlled between the major veins and the minor veins. Although minor veins become structurally mature in shaded areas of a leaf, AtSUC2-GFP expression is not initiated.
MATERIALS AND METHODS

Plant Material and Growth Conditions

The construction of transgenic tobacco (Nicotiana tabacum) expressing GFP under control of the companion cell-specific promoter AtSUC2 has been described previously (Imlau et al., 1999). Transgenic tobacco plants expressing companion cell-specific, ER-localized GFP were constructed as follows.

To create the AtSUC2-GFP-ER transgenic plants, the mGFP5-ER coding sequence was amplified from pBINmGFP5-ER (kind gift of Jim Haseloff, Department of Plant Sciences, University of Cambridge, United Kingdom; see also Siemerling et al., 1996) using oligonucleotide primers containing BspH and SacI restriction sites. The PCR product was digested with BspH and SacI and cloned into pSPAIATmGFP5-ER, which had been digested with NcoI and SacI to remove the GFP. pSPAIATmGFP5-ER was a subclone of the HindIII-SacI fragment of pEPI, which contained the SUC2 promoter sequence and GFP (Imlau et al., 1999), in pSP65. The SUC2 promoter-mGFP5-ER fragment was excised from pSPAIATm-GFP5-ER with HindIII and SacI and cloned into HindIII-SacI-digested pEPI to create pAtSUC2-mGFP5-ER. Agrobacteria (strain LBA4404) were transformed with this construct by electroporation. The transformed bacteria were used to create transgenic tobacco cv MD609 expressing mGFP5-ER under the control of the AtSUC2 promoter using standard tobacco transformation procedures (Barker et al., 1993).

Plants were grown from seed in a heated greenhouse (16.5-h day length at > 250 W m–2, 28°C day, and 22°C night temperature minima) and used for experiments between 30 and 40 d post-sowing.

Leaf Clearing and Staining

Whole leaves were incubated in 95% (v/v) glacial acetic acid:ethanol (1:3 [v/v]) at 60°C for approximately 60 min or until decolorized, changing the solution as necessary until the chlorophyll was extracted. They were then transferred to 20 volumes of hydrogen peroxide at room temperature for 48 h. The leaves were then stained in Safranin O (0.1% [w/v] aqueous solution) for a few minutes before washing to remove excess stain from the mesophyll tissue.

Shading Experiments

Nineteen-millimeter-diameter rubber washers were attached, using a 6-mm-long pin, to both the adaxial and abaxial surface of leaves that were 6-mm-long pin, to both the adaxial and abaxial surface of leaves that were transferred to 20 volumes of hydrogen peroxide at room temperature for 48 h. The leaves were then stained in Safranin O (0.1% [w/v] aqueous solution) for a few minutes before washing to remove excess stain from the mesophyll tissue.

Leaf Disc Isolation

Leaf discs were cut from 5-cm-long AtSUC2-GFP-ER sink leaves using a 13-mm-diameter cork borer. The discs were floated on sterile distilled water in petri dishes either in the light (16.5-h day length) or in the dark for 8 d before examination. These will be referred to as unshaded and shaded sink leaf discs, respectively.

Accumulation of Suc in Veins

This was conducted essentially as described by Turgeon (1984) on whole leaves from AtSUC2-GFP plants and tissue from unshaded and shaded transition AtSUC2-GFP-ER leaves. The abaxial leaf surface was abraded with carbondurum, keeping the surface wet with MES buffer (25 mM containing 20 mM CaCl₂ adjusted to pH 5.5 with KOH), and 13-mm-diameter leaf discs were removed with a cork borer. Precut discs taken from source and sink AtSUC2-GFP-ER leaves and maintained for 8 d with or without shading as described above were similarly abraded and incubated without further cutting. After rinsing in MES buffer for 10 min, the discs were incubated with [U-14C]Suc (25 mM, 9.25 × 10⁴ Bq ml⁻¹) for 30 min, maintaining the previous shading conditions as appropriate. The discs were washed for 30 min in MES buffer, changing the solution 5 times. The discs were then blotted dry, mounted between waxed card held between aluminum plates, fast frozen in powdered dry ice, and subsequently freeze dried. The dried tissue was compressed between polished metal plates before being mounted on card and exposed to autoradiography film (Kodak BioMax MR-1 film, Sigma, Poole, UK) at ~80°C for 4 to 7 d.

CLSM

To image GFP expression in leaf tissue, a confocal laser scanning microscope (MRC 1000, Bio-Rad, Hemel Hempsted, UK) attached to an Optiphot II microscope (Nikon, Tokyo) was used. GFP was excited using a 100-mW argon laser at 488 nm with an emission filter of 522 DF 32 nm. For clear images of the vein network, the abaxial epidermis was removed and the leaf mounted in sodium oil under a coverslip. Imaging and reconstruction of whole leaves was as described previously (Roberts et al., 1997). Safranin O was imaged using a 25-mW krypton argon laser at 568 nm with an emission filter of 605 DF 32 nm.

Electron Microscopy

Leaf samples from sink and transitional leaves or leaf discs were fixed and embedded essentially as described previously (Fasseas et al., 1989; Wright and Oparka, 1997), and 0.1-µm sections were cut with a Leica Ultracut UCT (Leica Mikrosysteme GmbH, Wein, Austria) to assess the maturity of the minor veins.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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LITERATURE CITED

Maturation of Phloem Function and the Sink Source Transition