Bundle sheath cells of small veins in maize leaves are the location of uptake from the xylem

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Received 7 June 2000; Accepted 5 October 2000

Abstract

Rb⁺ as a tracer for K⁺ was used to test the hypothesis that uptake of K⁺ from xylem vessels of small veins into the symplast of maize leaves occurs at the xylem bundle sheath cell interface. 22.5 min after immersing cut leaves into 20 mM RbCl + 1 mM KCl, Rb⁺ appeared in the cells of the leaves. Sections of these leaves were freeze-dried. In cryo-thin sections (5 μm), ⁸⁶Rb⁺ and ⁴¹K⁺ content was determined by laser microprobe mass analysis with a large resolution of about 1 μm. Determining the ratio of ⁸⁶Rb⁺ to ⁴¹K⁺ in the cell walls and cytosols of bundle sheath cells, mesophyll cells, and in the cells between the xylem elements resulted in the following picture: In small veins, Rb⁺ entered the symplast directly at the xylem bundle sheath cell interface.

Key words: Apoplast, LAMMA, rubidium, transport, veins.

Introduction

Nutrients are transported from the roots to the leaves inside the veins in the xylem. In maize leaves, large veins (Fritz et al., 1983) contain large xylem vessels and take care of the longitudinal transport. Intermediate and small longitudinal veins have relatively small xylem vessels with lower flow rates. They distribute sap locally. Transverse veins connect all three types of longitudinal vein (Altus and Canny, 1985).

In maize, there are two apoplastic regions, separated by the suberin layer surrounding the bundle sheath cells (Evert et al., 1977; Hattersley and Browning, 1981). Thus, the nutrients from the roots cannot enter the mesophyll cells via an apoplastic pathway, but have to migrate symplastically through the plasmodesmata between bundle sheath cells and mesophyll cells, after being taken up into the symplast from that apoplast which contains the xylem vessels. Whereas in roots transport from the symplast to the xylem vessels via xylem parenchyma cells has been verified (De Boer, 1999; Wegner and De Boer, 1999; Wegner and Raschke, 1994; Wegner et al., 1994), there is no information on the pathway of ions from the xylem vessels to the symplast of leaves. In order to get some ideas of what pathways are possible, results obtained with other substances are inspected. Some transport studies were based on dyes as transport markers (Altus and Canny, 1985; Canny, 1986, 1988, 1990). Accumulation of Prussian Blue crystals (Evert et al., 1985) and sulphorhamidine (Canny, 1990) within the cell wall of the xylem/bundle-sheath interface indicated a direct flow of water from the xylem to the bundle sheath cells. It was assumed that uptake of ions occurred via the same pathway. This putative role in ion uptake is in line with the name parenchymatic bundle sheath cells (Esau, 1965).

For sugars, Fritz et al. found indications for another pathway (Fritz et al., 1983). Tips of leaves were fed with [¹⁴C] sucrose for 2–8 min. Accumulation was found first in the xylem, then in vascular parenchyma and then in the thick-walled sieve elements. But there was no accumulation in the bundle sheath cells, indicating uptake of sucrose via the vascular parenchyma cells without involvement of bundle sheath cells. However, uptake of sucrose from the xylem is not a major pathway under physiological conditions.

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A clearer picture has been obtained for the export of photosynthates. Feeding $^{14}$CO$_2$ from the air resulted first in an unspecified overall increasing radiation. Then a specific increase was found in the parenchyma cells followed by an increase in the thick-walled sieve elements. Russin et al. raised the question of whether import and export between the apoplast in the veins and bundle sheath cells made use of different pathways or not (Russin et al., 1996). This question was initiated by findings in the sedl mutant of maize that had disfunctional plasmodesmata between bundle sheath cells and vascular parenchyma cells. In the sink state, the plants grew normally, but after sink-to-source transition starch was found to be accumulated in the parenchyma cells, and finally plasmolysis occurred indicating that export of carbohydrates did not work. One hypothesis was that only export occurred via the parenchyma cells, whereas import for growth (and this would include ion uptake which is investigated here) seemed to be independent of the parenchyma cells.

Keunecke and Hansen studied K$^+$ transport across the plasmalemma of maize bundle sheath cells by means of the patch clamp technique, in particular, pH-dependence of K$^+$-fluxes (Keunecke and Hansen, 2000). Their results have been taken as evidence that the channels involved are adapted to K$^+$ uptake from an acid apoplast such as occurs during uptake of anions by cotransport. Such a mechanism has been suggested before (Canny, 1987, 1990) as mentioned above. However, these studies and also those of Russin et al. provided only indirect evidence (Russin et al., 1996). Thus, LAMMA (laser microprobe mass analysis) was employed to test the hypothesis that uptake of ions from xylem vessels in small veins occurs at the xylem/bundle sheath interface. Rubidium served as a tracer for potassium (Gierth et al., 1998; MacRobbie, 1995).

Materials and methods

Seeds of Zea mays L. cv. Helix (KWS Kleinwanzelbe Saatzaucht AG, Einbeck, Germany) were germinated between two layers of filter paper moistened with 0.2 mM CaSO$_4$ after soaking in tap water. After 4 d, the roots were immersed in aerated tap water for one week. Then, four plants were transferred to a 5.0 l pot with the following nutrient solution: 0.5 mM NH$_4$NO$_3$, 0.7 mM K$_2$SO$_4$, 0.1 mM KCl, 2 mM Ca(NO$_3$)$_2$, 0.5 mM MgSO$_4$, 0.1 mM KH$_2$PO$_4$, 1 mM H$_2$BO$_3$, 0.5 mM MnSO$_4$, 0.1 mM ZnSO$_4$, 0.2 mM SnSO$_4$, 1 mM FeEDTA, and 10 mM (NH$_4$)$_2$MoO$_4$. The nutrient solutions were aerated and changed twice a week. The plants were grown in a greenhouse (light/dark regime 15/9 h, 550 $\mu$mol cm$^{-2}$ s$^{-1}$) provided by Philips SON-T Agro 400, at 22/18 °C.

The sixth of seven leaves (counting from the bottom, coleoptile included) of 5-week-old plants was used. The age of plants was critical for obtaining very thin sections. Leaves were cut, and the last 1 mm of the leaf was inserted into a solution containing 20 mM RbCl+1 mM KCl, pH 7.2 (TRIS/MES). The adequate time of immersion of the leaf in the Rb$^+$-solution was determined by a narrow interval between the time when Rb$^+$ had not yet reached the region of interest (cells around the xylem vessels) and the time when the imported Rb$^+$ had already equilibrated with the resident K$^+$ in the cytosol, vacuole and in the cell walls. In a series of experiments, it was found that after an immersion time of 22.5 min the front of Rb$^+$ had reached the region of interest. For the following evaluation, those preparations were used which showed a non-uniform distribution of the $^{85}$Rb$^+/^{40}$K$^+$-ratio in different cells.

A sample of 3 mm width and 5 mm length was taken from the leaf 3 mm above the surface of the solution in order to exclude leaf sections which were in direct contact with the solution. The sample was immediately transferred into freon cooled with liquid nitrogen (77 K), and then embedded in freezing medium (batch no. W13135, Jung, Heidelberg) and cut to 5 μm thin cross-sections by means of a cryo-microtome. The sections were laid on EM-copper grids (G2150C, Plano W. Planet GmbH, Wetzlar) which were covered with a formvar film (Sigma-Aldrich Chemie GmbH, Steinheim), freeze-dried, and stored in a desiccator.

Laser microprobe mass analysis was performed with a LAMMA 500 instrument (transmission-type microprobe, Leybold-Heraeus, Cologne, Germany) on the cryo thin sections mounted on electron microscopy grids. LAMMA permits mass spectroscopic analysis of small volumes at high spatial resolution. A focused NdYAG laser pulse ($\lambda = 265$ nm, irradiance $10^5$ W cm$^{-2}$, spot size 1 μm) evaporates microvolumes of typically 5 μm$^3$ depending on the thickness of the sections. The penetration depth is equal to the thickness of the probe (5 μm). As the beam is in the direction of the axis of the xylem and the parallel cells, the penetration depth has only minor influence on the selection of the tissue. However, the thickness of the beam (1 μm) is crucial for the spatial resolution.

The area to be analysed is selected by means of an optical microscope and a pilot laser. The elemental and molecular ions are accelerated into a time-of-flight mass spectrometer (mass resolution m/Δm 650) and detected by an open electron multiplier linked to a transient recorder (LeCroy TR 8818, Heidelberg, Germany) at sampling rates of 10 ns. Further data processing (calibration, determination of peak area, averaging, etc.) is done by computer. All generated elemental ions are detected with a sensitivity down to $10^{-19}$ g (Seydel et al., 1992). Only relative elemental concentrations (normalized to the $^{12}$C$^+$ content) are presented in this paper.

Preparation of the cross-section of Fig. 1 was done as follows: a sample was fixed in 5% glutaraldehyde and 4% paraformaldehyde for 1 h, then post-fixed in 3% OsO$_4$ for 16 h, and dehydrated in ethanol. Samples were embedded in Spurr’s resin, and 2 μm thick sections were cut.

Results

These investigations were focused on longitudinal veins. Figure 1 shows a micrograph of a large and a small vein indicating the targets of the LAMMA shots. The beam with a diameter of 1 μm was focused onto the border between adjacent cells or vessels or into the middle of the cells or vessels. The following nomenclature is used: The abbreviation of a cell type alone denotes the centre of the respective cell, for example, X is the lumen of a xylem vessel; two symbols connected by a hyphen label the border between these cells or vessels.

Figure 2 shows the LAMMA spectrum obtained from a X-BS border of a small vein. The ion peaks of the following isotopes can be identified: $^{85}$Rb$^+$, $^{87}$Rb$^+$,
by these processes, it can serve as a reliable measure of the accumulation of Rb\(^+\). In the second normalization procedure, the ratio of \(^{85}\text{Rb}\(^+\)/\(^{41}\text{K}\(^+\)\) obtained from the xylem/bundle sheath (X-BS) interface was set to 1. This enables a better comparison of the ratios at different locations.

Averaging was done in two different ways: firstly, all experiments were averaged regardless of the assignment to a certain leaf; secondly the ratios from an individual leaf were averaged in a first step, and then the averages from each leaf were averaged. The higher scatter (SD) in the small veins can be explained by variations in the distance of these veins from the importing large veins, where transport should be faster due to larger diameters. The standard deviation SD is smaller in the ratios pre-averaged per leaf, as expected. However, in either case the scatters of the individual experiments are too high and would prevent any decision of where Rb\(^+\) accumulates first. In such a situation, the difference between different locations can only be obtained by statistical means, i.e. many repetitions of the experiments and calculation of SE, the standard error of the means.

Figure 3 shows averaged data from ‘one pool’ with SE used for the error bars. The targets of the LAMMA beam need some comments. If it was focused on the middle of a vessel or a cell, it was not known whether the material at that place had been lost during the cutting of thin sections. Thus, the reliability of the data from these locations is not high. If the beam was focused on the border of cells or vessels, the area of interest is non-homogenous including the cell wall, free apoplastic space and the adjacent cytosolic layer. Thus each location deserves a discussion of what the data means.
Table 1. Normalized ratios of $^{85}\text{Rb}^+/^{41}\text{K}^+$ (in the columns ‘mean’) obtained from small veins (upper table) and from large veins (lower table) at different locations

The ratio at the X–BS border was normalized to 1 as indicated by the asterisk behind the 1 (‘*’). The original values are shown in parentheses. The number of experiments or leaves are given in parentheses in the columns of the standard deviations (SD). Averaging was done in one step over all single experiments, or in two steps (first averaging over all experiments on one leaf, and then averaging over the data from all leaves).

<table>
<thead>
<tr>
<th></th>
<th>Mean (over all single values)</th>
<th>Mean (of the mean values for the veins)</th>
<th>SD (over all single values)</th>
<th>SD (of the mean for the veins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small veins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X–BS</td>
<td>1* (0.431)</td>
<td>1* (0.407)</td>
<td>0.254 ($n = 177$)</td>
<td>0.235 ($n = 35$)</td>
</tr>
<tr>
<td>X–VP</td>
<td>0.915</td>
<td>0.923</td>
<td>0.444</td>
<td>0.301</td>
</tr>
<tr>
<td>X–X</td>
<td>1.055</td>
<td>1.072</td>
<td>0.434</td>
<td>0.261</td>
</tr>
<tr>
<td>VP–BS</td>
<td>0.880</td>
<td>0.891</td>
<td>0.430</td>
<td>0.362</td>
</tr>
<tr>
<td>BS</td>
<td>0.930</td>
<td>0.923</td>
<td>0.466</td>
<td>0.453</td>
</tr>
<tr>
<td>M</td>
<td>0.827</td>
<td>0.956</td>
<td>0.500</td>
<td>0.386</td>
</tr>
<tr>
<td>BS–BS</td>
<td>0.994</td>
<td>0.924</td>
<td>0.734</td>
<td>0.259</td>
</tr>
<tr>
<td>BS–M</td>
<td>0.785</td>
<td>0.809</td>
<td>0.226</td>
<td>0.122</td>
</tr>
<tr>
<td>Large veins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X–BS</td>
<td>1* (0.758)</td>
<td>1* (0.747)</td>
<td>0.344 ($n = 126$)</td>
<td>0.301 ($n = 19$)</td>
</tr>
<tr>
<td>X–I</td>
<td>1.222</td>
<td>1.173</td>
<td>0.664</td>
<td>0.169</td>
</tr>
<tr>
<td>BS–BS</td>
<td>1.025</td>
<td>1.030</td>
<td>0.327</td>
<td>0.331</td>
</tr>
<tr>
<td>BS</td>
<td>0.932</td>
<td>0.900</td>
<td>0.376</td>
<td>0.268</td>
</tr>
<tr>
<td>M</td>
<td>0.831</td>
<td>0.804</td>
<td>0.332</td>
<td>0.255</td>
</tr>
</tbody>
</table>

(Mühling and Sattelmacher, 1995, 1997) gives little weight to the contribution from the apoplast and the cell wall and leads to the assumption that the ratio obtained from borders is mainly determined by the ratio in the cytosol. Thus, the finding of equal ratios in X–BS and BS–BS in Fig. 3A as well as in Fig. 3B has two messages: the above assumption about the cell walls seems to be correct, and Rb$^+$ has rapidly equilibrated within the bundle-sheath cell.

The important finding in Fig. 3A is that only a slightly higher value is found in the border X–X. As the free space of the apoplast inside the suberin ring can be assumed to be equilibrated (no barriers), the only difference between X–BS and X–X is the involvement of the cytosolic layer in X–BS. Figure 3A shows that this has already equilibrated with the xylem vessels. That the front of Rb$^+$ is just caught in the correct moment is indicated by the fact that the BS–M border has the lowest ratio of $^{85}\text{Rb}^+/^{41}\text{K}^+$. In contrast, the low value of M (even though it is welcome for the conclusions here) may be of less significance as it is not known what has happened to the material inside the cells.

The values of X–VP and VP–BS are lower than those of BS–BS. This is of special significance as this rules out a putative objection. It may be argued that the information obtained from the ratios is falsified by a putative longer loading time for large cells as compared to small cells. However, the VP cells are smaller than the BS cells, and the inverse result as shown in Fig. 3A should be caused by cell volume.

Figure 3B deals with large veins. The highest value is found for X–I. I stands for inner region, that is the region between the xylem elements. This region consists of tracheary elements which have a diameter of about one-fifth or less of the large vessels on either side of them.

In both parts of Fig. 3, the ratio of $^{85}\text{Rb}^+/^{41}\text{K}^+$ is normalized to one. This includes the cell wall of the bundle sheath cells and the adjacent cytosolic layer, probably mixed with part of the vacuole and part of the xylem. The about 10-fold lower concentration of cations in the apoplast with respect to the cytosol and the vacuole.
Volume flow through them will therefore be of the order of 500 times less than in the large vessels. They probably constitute a practically stationary volume of xylem sap lying between the fast moving sap in the two large vessels. Furthermore, these tracheary elements are connected to the vessels and to each other by pits and thus diffusive exchange of Rb\(^+\) into this sap from Rb\(^+\)-labelled sap in the two vessels will be rapid. They will form a trap for Rb\(^+\), and quickly show a large Rb\(^+\)/K\(^+\) ratio, as indicated by the high X-I value in Fig. 3B. This is not investigated in more detail here, since the data of Fig. 3B are only presented in order to show that the situation described in Fig. 3A for small veins cannot be generalized.

**Discussion**

Large and small veins of maize show different ion accumulation pathways as indicated by the present LAMMA measurements of rapidly frozen leaf sections and as expected from their different morphology. The high X-I value in Fig. 3B accounts for the fact that the inner region consists of tracheary elements which, as mentioned above, are connected to the rapid moving sap in the large xylem elements. These large veins are not studied here. They are designed to forward the sap through transverse veins into small veins, and small veins are responsible for the radial exchange. The investigations here seek to answer the question of whether there is transport via the xylem/bundle sheath interface of small veins as they have been the locus of the patch clamp studies of Keunecke and Hansen (Keunecke and Hansen, 2000). In small veins, ions from the xylem seem to approach bundle sheath cells preferentially. However, the validity of these conclusions needs some comments.

The first problem arises from the question of whether the cell membranes involved are permeable to rubidium. This question has been settled in separate patch-clamp studies indicating that permeability for rubidium is at least as high as that for potassium (M Keunecke and U-P Hansen, unpublished results).

The second question deals with the changes in concentration during the preparation procedure, i.e. the question of what happens to the sap inside the cells during freezing and cutting. Since the sections are dried, there is no solvent lost. The solutes are assumed to migrate to the walls or the underlying formvar film. However, this problem is accounted for by calculating the ratios of \(^{85}\text{Rb}^{+}/\text{K}^{+}\). If it is assumed that the rate-limiting step is uptake via membrane transporters or plasmodesmata between adjacent cells, and that within a cell Rb\(^+\) is uniformly distributed by cytosolic streaming and diffusion, then the \(^{85}\text{Rb}^{+}/\text{K}^{+}\) ratio is insensitive to a concentration effect or loss of part of the material.

The third question is related to the different sizes of the regions of interest. Filling a large cell takes longer than filling a small cell. Fortunately, the results in Table 1 and in Fig. 3A show lower values for the small cells (VP) than for the large cells (BS). Thus, if such a volume effect would play a role, the differences in Fig. 3A would be even greater, and the evidence for the hypothesis that there is a direct uptake from the xylem into the bundle sheath cells even stronger. Nevertheless, it is not priori clear that such a volume effect occurs: If the tonoplast provides a diffusion barrier, and if the cytosolic layer has equal thickness in different cells, then the (cytosolic) volume/surface ratio is constant.

In Fig. 3A and B, there is some experimental support for the arguments raised for questions two and three: The X–BS and BS–BS values are of the same order of magnitude. This seems to indicate that Rb\(^+\) has equilibrated rapidly within the cytosolic phase of the bundle sheath cells. With respect to this, the finding is important that all other borders (BS–M, VP–BS, X–VP in Fig. 3A) show smaller \(^{85}\text{Rb}^{+}/\text{K}^{+}\) ratios.

The descending values from X–X via X–BS, BS–BS to BS–M in Fig. 3A in small veins confirm other suggestions (Canny, 1990; Russin et al., 1996): ions enter the bundle-sheath directly from the xylem and then they move to the mesophyll cells via the plasmodesmata (Evert et al., 1977; Botha, 1992), thus circumventing the suberin lamella which surrounds the bundle sheath (Evert et al., 1977, 1985, 1996; Hattersley and Browning, 1981; Botha et al., 1982; Canny, 1995). The vascular parenchyma cells are loaded later (Fig. 3A). This means that the bundle sheath cells in contact with the xylem are of special interest for transport studies from the apoplast (xylem) to the symplast and confirms that the patch-clamp studies described previously (Keunecke and Hansen, 2000; Keunecke et al., 1997) deal with the major pathway of ion uptake into the symplast of the leaf.

**Acknowledgements**

This work was supported by the Deutsche Forschungsgemeinschaft (Ha 712/11–2). We are grateful to Dr I Dörr, Kiel, who did the preparation for Fig. 1, and to Dr M Canny, Canberra, for very helpful suggestions.

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