Evidence for Symplastic Involvement in the Radial Movement of Calcium in Onion Roots

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The pathway of Ca$^{2+}$ movement from the soil solution into the stele of the root is not known with certainty despite a considerable body of literature on the subject. Does this ion cross an intact, mature exodermis and endodermis? If so, is its movement through these layers primarily apoplastic or symplastic? These questions were addressed using onion (Allium cepa) adventitious roots lacking laterals. Radioactive Ca$^{2+}$ applied to the root tip was not transported to the remainder of the plant, indicating that this ion cannot be supplied to the shoot through this region where the exodermis and endodermis are immature. A more mature zone, in which the endodermal Casparian band was present, delivered 2.67 nmol of Ca$^{2+}$ mm$^{-1}$ treated root length d$^{-1}$ to the transpiration stream, demonstrating that the ion had moved through an intact endodermis. Farther from the root tip, a third zone in which Casparian bands were present in the exodermis as well as the endodermis delivered 0.87 nmol Ca$^{2+}$ mm$^{-1}$ root length d$^{-1}$ to the transpiration stream, proving that the ion had moved through an unbroken exodermis. Compartmental elution analyses indicated that Ca$^{2+}$ had not diffused through the Casparian bands of the exodermis, and inhibitor studies using La$^{3+}$ and vanadate (VO$^{4-}$) pointed to a major involvement of the symplast in the radial transport of Ca$^{2+}$ through the endodermis. It was concluded that in onion roots, the radial movement of Ca$^{2+}$ through the exodermis and endodermis is primarily symplastic.

The pathway whereby Ca$^{2+}$ moves radially from the soil solution to the tracheary elements of a root has long been a subject of debate (for reviews, see Clarkson, 1991; White, 2001; White and Broadley, 2003). To reach the root stele (the location of the tracheary elements), an ion must pass through the epidermis, exodermis (if present), central cortex, and endodermis. Because in typical roots the walls of the epidermis and central cortex cells are permeable to ions, the current debate concerns the passage of ions through the endodermis and exodermis.

One idea is that the Ca$^{2+}$ delivered to the xylem may move predominantly or entirely apoplastically. This has been considered by a variety of authors (White, 1998; McLaughlin and Wimmer, 1999; White et al., 2000; White, 2001, White and Broadley, 2003). In some studies, the amount of Ca$^{2+}$ translocated was positively correlated with the transpiration rate, as would be expected if its movement were apoplastic (Hylmö, 1953; Bell and Biddulph, 1963; Lazeroff and Pitman, 1966). The lack of selectivity between Ca$^{2+}$, Ba$^{2+}$, and Sr$^{2+}$ uptake by roots has also been cited as evidence for the presence of an apoplastic radial pathway for Ca$^{2+}$ (Bowen and Dymond, 1956; White, 2001). The argument against this view is that Casparian bands of the endodermis and exodermis have proved impermeable to several ions (de Ruzf de Lavison, 1910; Baker, 1971; Robards and Robb, 1972; Peterson, 1987; Peterson et al., 1993) including Ca$^{2+}$ (Singh and Jacobson, 1977, Kuhn et al., 2000, Bückling et al., 2002) within the limits of the tests employed.

A second idea is that the Casparian bands in the endodermis and exodermis are circumvented by a symplastic step. In this case, the overall radial movement of Ca$^{2+}$ to the stele would show features of symplastic transport. In support of this idea, there are some reports that Ca$^{2+}$ movement to the shoot is not correlated with the speed of the transpiration stream (Atkinson et al., 1992; Jarvis and House, 1970). Drew and Biddulph (1971) found that the passage of Ca$^{2+}$ into the transpiration stream of bean (Phaseolus vulgaris) was reduced by cold temperatures and several metabolic inhibitors. Also, when the bathing solution contained a relatively low concentration of Ca$^{2+}$, the ion was concentrated in the transpiration stream. In a correlative study of Ca$^{2+}$ movement into the stele of marrow (Cucurbita pepo) roots, Harrison-Murray and Clarkson (1973) found that the entry of Ca$^{2+}$ into the stele was inversely correlated with suberin lamella development in the endodermis. Their interpretation of the results was that Ca$^{2+}$ moved through the endodermal symplastically, entering the cytoplasm by means of Ca$^{2+}$ channels located in the membranes lining the outer tangential walls of the endodermal cells, and exiting the cytoplasm by means of Ca$^{2+}$-ATPases located on the membranes lining the inner tangential walls of these cells. Suberin lamellae would interfere with these changes of...
compartment, inhibiting \(Ca^{2+}\) movement to the stele. For a full discussion, see Clarkson, 1991.) The counter-argument to a path involving a symplastic step(s) is that a radial flow of \(Ca^{2+}\) would make it difficult for the cytoplasm to maintain its normal low concentration of the ion (Flowers and Yeo, 1992). However, Clarkson (1984) pointed out that appropriate cytosolic concentrations could be maintained by rapid fluxes through the endodermal cells. After reviewing the evidence on the subject, White and Broadley (2003) state “Although the relative contributions of the apoplastic and symplastic pathways to the delivery of \(Ca\) to the xylem are unknown, it is likely that a functional separation of apoplastic \(Ca^{2+}\) fluxes (for transfer to the shoot) and symplastic \(Ca^{2+}\) fluxes (for cell signaling) would enable the root to fulfill the demand of the shoot for \(Ca\) without compromising intracellular \([Ca^{2+}]_{cyt}\) signals.”

A third idea, one that reconciles the opposing views on the pathway (apoplastic versus symplastic) of radial \(Ca^{2+}\) movement through the endodermis, is that entry into the stele occurs near the root tip where the endodermis is immature (Flowers and Yeo, 1992). Additional points of entry in more mature zones would be through discontinuities in the Casparian bands associated with the growth of lateral root primordia (Ferguson, 1979; Peterson and Moon 1993, White and Broadley, 2003).

In this study, the passage of \(Ca^{2+}\) from the soil solution into the transpiration stream of onion (Allium cepa) roots was studied with a view to answering two questions: (1) Does this ion move radially through an intact exodermis and endodermis, or is the ion provided to the plant by the root tip (a region devoid of Casparian bands)? (2) If the ion does pass through an intact exodermis and/or endodermis, is its pathway predominantly apoplastic or symplastic? Young, adventitious roots sprouted from bulbs were chosen for this study because these roots lack laterals, the formation and emergence of which leads to discontinuities in the Casparian bands of the endodermis and exodermis, respectively (Esau, 1940; Peterson and Moon, 1993). Thus, all areas except for the root tip have a complete Casparian band in the endodermis, and the exodermal Casparian band is also complete in older root regions. To answer the first question, \(^{45}Ca^{2+}\) was applied to discrete zones of roots and the amounts moved to the xylem were estimated from radioactivity in all parts of the plants proximal to the treated areas. To answer the second question, different methods proved appropriate for the endodermis and exodermis. For the former, the permeability of the Casparian band to \(Ca^{2+}\) was tested by compartmental elution, a technique that can be used to measure the quantities of ions in various compartments of plant cells (Cram, 1968; Jeschke 1982; Peterson 1987; Maclon et al., 1990). The permeability of the exodermal Casparian bands to \(Ca^{2+}\) was tested by measuring the amount of the ion eluted from the cell wall compartments of “intact” segments with sealed ends and “dissected” segments that had been longitudinally bisected and had their steles removed. If exodermal Casparian bands do not restrict the apoplastic diffusion of \(Ca^{2+}\), the amounts eluted from the cell walls in intact and dissected segments should be the same. If, however, these bands do restrict apoplastic diffusion, the amount of \(Ca^{2+}\) eluted from the cell wall compartment in the intact segments will be substantially smaller than that from the dissected ones. For the endodermis, the effects of La\(^{3+}\) (an inhibitor of \(Ca^{2+}\) channels) and VO\(_4^{3-}\) (an inhibitor of \(Ca^{2+}\)-ATPases) on the movement of \(Ca^{2+}\) to the stele were tested in a system where exudate was collected from excised roots. If the radial movement is entirely through the apoplast, the inhibitors will have no effect on ion delivery to the stele. However, if movement is proceeding with a symplastic step (which necessitates the ion moving into and out of the endodermal cells through their membranes), the amount of \(Ca^{2+}\) in the stele should be substantially reduced. This will be true even if membrane effects other than inhibition of \(Ca^{2+}\)-channels and \(Ca^{2+}\)-ATPases are elicited by the inhibitors.

RESULTS

Sites of \(Ca^{2+}\) Uptake and Axial Transport Along Onion Roots

Adventitious roots sprouted from onion bulbs developed uniformly when grown in the favorable conditions provided. Such roots were without laterals, either emerged or in the form of primordia. The roots were divided into three anatomical zones of interest with reference to the maturation of the endodermis, early metaxylem vessels, and exodermis (Fig. 1). The tip zone was the apical 5 mm of the root where functional xylem and Casparian bands were absent. The young zone, 15 to 40 mm from the tip, had functional xylem and Casparian bands in the endodermis. The old zone, which began 100 mm from the root tip and extended to the root base, possessed functional xylem, and Casparian bands in all cells and suberin lamellae in most cells of both the endodermis and exodermis. Thus, passage cells were present in both of the latter layers. For the experiments, the tip or the central regions of the young and old zones were sealed into a treatment chamber (Fig. 2).

When 1 mM \(^{45}Ca^{2+}\) was applied to the tip zone, no measurable radioactivity was detected in the rest of the plant (Table I). During the 24 h treatment time, the root tips had grown an average of 3 mm, which resulted in a final length of 8 mm being treated. In some cases, Casparian bands had formed in the endodermis in the proximal part of the treated zone, but the early metaxylem was always immature. Otherwise, the anatomy of the relevant structures did not change during treatment. When the young zone was treated, radioactivity was detected in the plant parts proximal but not distal to the treated site. The amount of \(^{45}Ca^{2+}\) extracted from the plant was equivalent to a delivery
to the transpiration stream of 2.67 nmol mm\(^{-1}\) treated root length (Table I). Treatment of the old zone resulted in transport of 0.87 nmol mm\(^{-1}\) treated root length (Table I). As was the case for the young zone, \(\text{Ca}^{2+}\) movement from the old zone was only in an upward direction (i.e. toward the shoot). No positive correlation between the amount of the ion transported and transpiration rate was evident (Table I).

Impermeability of Exodermal Casparian Bands to \(\text{Ca}^{2+}\)

The data from the compartmental elution of \(^{45}\text{Ca}^{2+}\) from both intact and dissected segments (Fig. 3) fitted a triple exponential function (Fig. 4) with average half-times ranging from 47 to 66 s, 5.6 to 7.5 min, and 102 to 210 min corresponding to wall, cytoplasm, and vacuole, respectively (although the identity of the last compartment is not known with any certainty; Table II elution at 22°C). The amount of \(\text{Ca}^{2+}\) in the wall compartment of the intact segments (2.0 µmol g\(^{-1}\) segment fresh weight [fw]) was substantially smaller than that in the dissected segments (8.8 µmol g\(^{-1}\) segment fw), indicating that the exodermal Casparian bands are not freely permeable to the ion (Table II, Fig. 3). Identification of the cell wall compartment, critical for this study, was validated by results of elutions at 4°C. Here the efflux from the wall compartment was unaffected (average half-time of 63 s), but that from the next (cytoplasmic) compartment could not be distinguished from the slowest (vacuolar) compartment (Table II). The half-time of elution from the latter combined compartment was not significantly different from that of the vacuole at 22°C in either whole or dissected segments (Table II).

Effect of Inhibitors on \(\text{Ca}^{2+}\) Transport to the Stele

In the young zone of the root, treatment with inhibitors substantially reduced the flux of \(\text{Ca}^{2+}\) to the stele, as measured by the amount of the ion appearing in the root exudate. Addition of 1 mM La\(^{3+}\) to the treatment chamber (Fig. 5) resulted in 73% less \(\text{Ca}^{2+}\) in the root exudate compared to the control. Similarly, prior treatment of the cells in the stele with VO\(^{3-}\) fed into the transpiration stream resulted in a 73% inhibition of \(\text{Ca}^{2+}\) translocation (Table III). Neither inhibitor affected the volumes of exudates produced by the roots (Table III).

![Figure 1](Image)

**Figure 1.** Median longitudinal section of an onion root. Major developmental landmarks are indicated, as well as zones of interest in this study. Not drawn to scale. A to C, Cross sections of onion root stained with berberine-aniline blue and viewed with UV light. A, Section taken from the old zone of the root (150 mm from tip). Casparian bands and suberin lamellae (asterisks) are present in the exodermis and Casparian bands (arrowheads) and suberin lamellae in the endodermis. Late metaxylem vessels are mature and lignified. B, Section taken from young zone of the root (30 mm from root tip). Casparian bands (arrowheads) are present in endodermis only. Early metaxylem vessels are mature. C, Section taken from tip zone of the root (5 mm from tip). No Casparian bands were detected; xylem vessels are immature. Bars = 500 µm.

![Figure 2](Image)

**Figure 2.** Sectional diagram of the apparatus used to apply \(^{45}\text{Ca}^{2+}\) to discrete zones of onion root. An entire onion with its numerous adventitious roots (only two of which are shown) was placed in a large tray so that the leaves projected into the atmosphere. One root was sealed into a multi-chambered plexiglass box. Flanking (f) and bordering (b) chambers were filled with 1 mM Ca SO\(_4\), and the treatment chamber (t) with radioactive \(\text{Ca}^{2+}\) (see text). The cover and sides of the large tray were lined with wet paper towels, and the untreated roots were covered with moist vermiculite (not shown).
Table I. Translocation of $^{45}\text{Ca}^{2+}$ from developmentally different zones of onion roots, and transpiration rates

<table>
<thead>
<tr>
<th>Distance from Root Tip mm</th>
<th>Transpiration g water lost during 24 h</th>
<th>$^{45}\text{Ca}^{2+}$ Translocation nmol mm$^{-1}$ treated root length</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–8 (tip)</td>
<td>13.5 ± 5.8</td>
<td>0.0</td>
</tr>
<tr>
<td>15–30 (young)</td>
<td>8.7 ± 0.9</td>
<td>2.67 ± 0.74</td>
</tr>
<tr>
<td>&gt;100 (old)</td>
<td>18.1 ± 4.3</td>
<td>0.87 ± 0.36</td>
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</table>

The total length of root tip (8 mm) at the end of experiment was considered in calculations. *, statistically significant differences in the two values analyzed ($n = 4$, ± SD, Student’s $t$ test, $P = 0.06$).

DISCUSSION

Onion proved to be an ideal species for testing the permeability of the endo- and exodermis to Ca$^{2+}$ as the roots did not develop laterals and, therefore, the Casparian bands of these layers, once formed, remained intact. Moreover, these roots did not develop root hairs, facilitating a clear distinction of the wall compartment in compartmental elution experiments.

Sites of Ca$^{2+}$ Uptake and Axial Transport Along Onion Roots

When $^{45}\text{Ca}^{2+}$ was applied to the root tip, the only location in the root not invested with at least one complete Casparian band, no measurable amount was transported away in the transpiration stream. Perhaps this is not surprising as this zone lacked mature xylem vessels. The general apoplastic impermeability of the tip noted in other studies (Lüttge and Weigl, 1962; Rasmussen, 1968; Wierzbička, 1986; Enstone and Peterson, 1992; Cholewa and Peterson, 2001) also applies to Ca$^{2+}$ ions since there was no detectable movement through parenchyma to the point where the xylem elements were functional. Enstone and Peterson (1992) postulated that the apoplastic impermeability of the root apex may be a consequence of an absence of sufficiently large intermicrofibrillar channels in the walls of the cells to allow tracer movement. In view of the fact that the endodermis matures closer to the tip than the xylem (Peterson and Lefcourt, 1990), one would expect that the root tip would not supply Ca$^{2+}$ to the transpiration stream. On the contrary, Huang et al. (1993) found that Ca$^{2+}$ transport could occur from the tip zone in wheat (Triticum aestivum). However, it is unknown whether or not some of the tracheary elements were mature in the treated zone.

In young zones of onion roots (between 15 and 40 mm from the root tip), where the endodermis and early metaxylem vessels were mature (Fig. 1), radioactive Ca$^{2+}$ moved to the shoot. In fact, the largest amount was transported from this zone (Table I). In this region, no suberin lamellae are as yet developed in endodermal cells (Barnabas and Peterson, 1992) so that all cells comprising this layer would be available for import of Ca$^{2+}$ into the symplast and export again on the stele side of the Casparian bands. Robards et al. (1973) correlated the anatomy of barley (Hordeum vulgare) roots with Ca$^{2+}$ (using $^{85}\text{Sr}$ as a tracer) transport to the stele and found that the zone of maximum uptake was 20 mm from the root tip. As in onion, this is in the zone in barley where the endodermis is mature but suberin lamellae have not yet formed. The maximum amount of Ca$^{2+}$ that entered the stele in barley was 0.13 nmol mm$^{-1}$ treated root length (calculated from Robards et al., 1973, Fig. 29; where a maximum of 260 pmol of Ca$^{2+}$ mm$^{-1}$ root was reported, and assuming 0.4 mm root radius from Aloni et al., 1998, Fig. 13). This is much less than the average value of 2.7 nmol mm$^{-1}$ in onion. Clearly, movement through an intact endodermis in onion did not result in less ion delivery to the stele than in barley roots where lateral root primordia were presumably developing.

In the most mature (old) zone of the root (greater than 100 mm from the root tip), Casparian bands were present in the endodermis as well as in the exodermis (Fig. 1). Despite the presence of two sets of Casparian bands on the radial path, this zone was capable of transferring Ca$^{2+}$ to the stele (Table I). This ion did, therefore, pass through an intact, mature exo- and endodermis. The less efficient radial transport in the old zone (compared to that of the young zone) could be a consequence of the presence of suberin lamellae in both the endo- and exodermis, as similar results were previously obtained with marrow and barley (Harrison-Murray and Clarkson, 1973; Robards et al., 1973; Ferguson and Clarkson, 1976).

From the above comparison of Ca$^{2+}$ transport through various root zones it is clear that, in this study, the ion does not enter the stele apoplastically at the root tip where Casparian bands are not yet formed, nor apoplastically in older regions through discontinuities

Figure 3. Cross sections of onion roots used for compartmental elution. Shading indicates hypothetical areas from which Ca$^{2+}$ could be eluted from the walls. A, Section from a whole segment with wax-capped ends. Elution occurs through the epidermis. Left half of root, assuming the exodermal Casparian band is permeable to Ca$^{2+}$; right half of root, assuming the exodermal Casparian band is impermeable to Ca$^{2+}$. B, Section from a dissected segment, i.e. one that had been bisected and had its stele removed. Elution occurs through the epidermis and cortex. For the diagram, it is assumed that Ca$^{2+}$ is not eluted from the anticlinal walls of the exodermis.

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in the Casparian bands of the endodermis and exodermis brought about by lateral root production. Rather, Ca\(^{2+}\) moved through an intact endodermis and exodermis on its way to the stele.

In all zones tested, there was a consistent lack of Ca\(^{2+}\) transport toward the root tip, supporting the general view that Ca\(^{2+}\) is immobile in the phloem and is not circulated within plants (Marschner, 1995). Most previous reports agree with the exclusive basipetal translocation of this ion, e.g. in onion (Maclon, 1975) and maize (Zea mays, Marschner and Richter, 1973). But in one study (Huang et al., 1993), Ca\(^{2+}\) transport toward the tip of wheat roots and its redistribution to other nontreated seminal roots was detected.

**Pathway of Ca\(^{2+}\) Movement Through the Exodermis**

The technique of compartmental elution was used to discern the path of Ca\(^{2+}\) as it moved through the mature exodermis, a layer with Casparian bands near the root surface. With this technique, it is imperative to identify the compartment of interest (i.e. the wall) correctly. In this study, when \(^{45}\text{Ca}^{2+}\) was eluted from either intact or dissected segments, three compartments were distinguished. The fastest eluting of these was identified as the cell wall based on a comparison of its half-time (about 1 min) with literature data (Drew and Biddulph, 1971; Maclon, 1975; Rygiewicz et al., 1984; Peterson, 1987; Rauser, 1987; White et al., 1992; DiTomaso et al., 1993; Devienne et al., 1994; Kronzucker et al., 1995a, 1995b) and its lack of change during elution at cold temperature. A surface film, which would have eluted more rapidly than the wall compartment, was not evident. Perhaps the Ca\(^{2+}\) in such a film had associated with the wall. The second fastest eluting compartment could be unequivocally identified as the cytoplasm based on its half-time of elution and extreme sensitivity to cold temperature. The third compartment may have been the vacuole or some other very slowly eluting compartment. The wall compartment was identified with certainty, but it included both the water free space and the Donnan free space. Most of the Ca\(^{2+}\) (99.6%) in this compartment was in the Donnan free space (calculations not shown).

Having identified and characterized the wall compartment, it was possible to test the hypothesis presented in the introduction, namely that a smaller amount of Ca\(^{2+}\) eluted from the walls of the intact segments with sealed ends compared to the amount eluted from the dissected segments would indicate that the Casparian band is not freely permeable to Ca\(^{2+}\). (It is necessary to assume that the Donnan free space of the epidermal and outer exodermal walls is the same as that of the walls of the central cortex.) These results were, in fact, obtained in experiments run at 22°C and 4°C (Table II). The amounts of Ca\(^{2+}\) in the wall compartments based on weight (as reported in Table II) can be corrected for sample volumes by considering that the length of the intact segments

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Figure 4. Graphed results from one replicate of a typical elution experiment (whole segments). Beginning with graph A, the times of the elution steps on the abscissa (linear scale) are plotted against a log (ordinate) scale of the dpm from the \(^{45}\text{Ca}^{2+}\) that would have been present in the segments at the times indicated. The line of best fit was calculated for the linear part of the curve. The amount of Ca\(^{2+}\) indicated by the intercept of the line on the ordinate at time 0 (indicating the amount of the ion in the most slowly-eluting compartment) was subtracted from all higher values to obtain the set of data plotted in B. Again, a line of best fit to the linear portion of the curve was obtained, and the value at the ordinate (the amount of the ion in the second most slowly-eluting compartment) was subtracted from the remaining values to produce data for graph C. In graph C, the straight line of best fit includes the point on the ordinate, indicating that this is the fastest-eluting compartment of the root. The half-times of elution from the three compartments are obtained from the slopes of the straight lines. The elution of Ca\(^{2+}\) from onion root segments was resolved into three compartments: A, vacuole (or other slowly eluting compartment), B, cytoplasm, C, cell wall.
treated was 2.0 cm (not 2.5 cm), and that the steles were removed from the dissected segments. Then the average ratio of the amount of Ca\(^{2+}\) eluted from intact to dissected segments at 22°C is 1:3.3, and is near the range of those obtained earlier (1:3.9 to 1:6.3) by Peterson (1987) for SO\(_4^{2-}\) in onion roots. By a process of elimination, we are forced to conclude that the movement of Ca\(^{2+}\) through the onion root exodermis occurs primarily in the symplast.

### Pathway of Ca\(^{2+}\) Movement Through the Endodermis

The experiments designed to discover how Ca\(^{2+}\) moved through the endodermis on its way to the stele entailed measuring the amounts of this ion in root exudates, a method that has been used previously. For example, barley roots actively exuded externally applied \(^{45}\)Ca\(^{2+}\) from young (30 mm from tip) and mature (60 mm from tip) zones (Halperin et al., 1997). For onion, the ability of detopped roots to remain metabolically active and produce exudates for 19 h has been demonstrated by Hodgnes and Vaadia (1964). In this study, onion roots exuded steadily for at least 17 h after excision (data not shown), and the volume of exudate was not influenced by treating the roots with either of the inhibitors employed (Table III).

In this series of experiments, distinguishing a predominantly apoplastic pathway from a symplastic one rests on the effect of inhibitors of membrane function on delivery of Ca\(^{2+}\) to the root stele. If the entire route is apoplastic, the inhibitors should have no effect. If it is symplastic, however, a sharp reduction in the amount of Ca\(^{2+}\) delivered is expected. For this study, the inhibitors were chosen to give maximum effects on the delivery of Ca\(^{2+}\) to the stele. According to the model of Clarkson (1991), which is based on known mechanisms of Ca\(^{2+}\) passage through membranes (see Sanders et al., 1999), entry into the cytoplasm of the endodermis is mediated by Ca\(^{2+}\) channels located in plasmalemmae on the cortical side of the Casparian band, and exit from the cytoplasm is through Ca\(^{2+}\)-ATPases in the plasmalemmae on the stele side of the Casparian band. (The same explanation could be advanced to explain a symplastic step through the exodermis.) In this study, La\(^{3+}\) was chosen as an inhibitor of Ca\(^{2+}\) channels. La\(^{3+}\) is an antagonist of Ca\(^{2+}\) influx into cells, and does not penetrate the plasmalemma (DuPont and Leonard, 1977; Lonergan and Williamson, 1988; Perdue et al., 1988). La\(^{3+}\), a very potent Ca\(^{2+}\) channel blocker, has been used at a concentration of 1 mM (as in this study) to abolish Ca\(^{2+}\) increases in mesophyll tissue of tobacco leaves (Moyen et al., 1998). In maize root plasmalemma vesicles, the class of Ca\(^{2+}\) channels that is inhibited by La\(^{3+}\) mediates 70% of the Ca\(^{2+}\) influx; the remaining 30% is inhibited by Gd\(^{3+}\) but not La\(^{3+}\) (Marshall et al., 1994). It seems that La\(^{3+}\)-sensitive channels may be exclusive Ca\(^{2+}\) transporters in wheat roots (Huang et al., 1994; Sasaki et al., 1994). In addition to the classes of channels described above, others are present in roots (for reviews, see White, 1998; Demidchik et al., 2002); however, their involvement in radial Ca\(^{2+}\) transport has not been investigated. To inhibit Ca\(^{2+}\)-ATPases, the inhibitor VO\(_4^{3-}\) was selected. A number of plasmalemma Ca\(^{2+}\)-ATPases from different plant species have been characterized (reviewed in Bush, 1995; Sze et al., 2000). They belong to the P-type ATPase family, i.e., they form a phosphorylated intermediate during the catalytic cycle. VO\(_4^{3-}\) inhibits all these Ca\(^{2+}\)-ATPases (Sze, 1985; Serano, 1990; Sze et al., 2000).

Treatment of onion roots with either La\(^{3+}\) or VO\(_4^{3-}\) substantially reduced the amount of Ca\(^{2+}\) in the root exudate (Table III), providing unequivocal evidence that the majority if not all the Ca\(^{2+}\) delivered to the stele passed through membranes. Thus, at least one symplastic step in the radial path is indicated. Treatment of onion roots with La\(^{3+}\) resulted in a 73% inhibition of Ca\(^{2+}\) transport to the stele (Table III). This incomplete inhibition may reflect the complex mechanism of Ca\(^{2+}\) transport by roots. It is apparent that other classes of Ca\(^{2+}\) channels might be present in the

### Table II. Amounts of Ca\(^{2+}\) present in the compartments and their half-times of elution from intact and dissected segments taken from an old zone of onion roots

<table>
<thead>
<tr>
<th>Compartment (Elution Temperature)</th>
<th>Whole Segments</th>
<th>Dissected Segments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca(^{2+}) μmol g(^{-1}) f.w.</td>
<td>Half-time</td>
</tr>
<tr>
<td>Wall (22°C)</td>
<td>2.0 ± 0.4*</td>
<td>66 ± 10 s</td>
</tr>
<tr>
<td></td>
<td>1.9 ± 0.1</td>
<td>69 ± 14 s</td>
</tr>
<tr>
<td>Cytoplasm (22°C)</td>
<td>3.1 ± 1.2</td>
<td>7.5 ± 0.8 min</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Vacuole (22°C)</td>
<td>3.4 ± 0.7</td>
<td>210 ± 30 min</td>
</tr>
<tr>
<td></td>
<td>3.6 ± 0.5</td>
<td>350 ± 60 min</td>
</tr>
</tbody>
</table>

The difference between the amounts of Ca\(^{2+}\) eluted from the cell wall compartment from whole segments compared to dissected segments is statistically significant from elutions at 22°C (*, n = 7, ±SD, Student’s t test, P = 0.004) and from elutions at 4°C (†, n = 3, ±SD, Student’s t test, P = 0.01). ND denotes compartment not detected.
vesicles were involved in the radial transport of apoplastic solutes, one would expect that such dyes applied to the outside of the root would be carried through the endodermis and exodermis and be visible in the more centrally located tissues. This does not occur (Peterson et al., 1982). Is it possible that cytosolic levels of free Ca\(^{2+}\) can remain low in the endodermal and exodermal cells as required by its role as a second messenger? White (1998) calculated that the Ca\(^{2+}\)-ATPase content would need to be greater than the average total protein content of the root plasma membrane to supply sufficient Ca\(^{2+}\) to the shoot. Such a problem would be even more severe for onion roots, since the measured flux of Ca\(^{2+}\) at the endodermis is greater than in White’s model (calculations not shown). The problem of Ca\(^{2+}\) transport across the onion exodermis is even more serious, as this layer is dimorphic (Kroemer, 1903) so that only the membranes of the short cells can be considered. These occupy 13% of the surface area of the layer (Ma and Peterson, 2001). A consideration of the protein contents of the endodermal and exodermal membranes, and the observed radial transport of Ca\(^{2+}\) will be the subject of a subsequent paper.

It should be noted that this work concerns the endodermis in two stages of development, i.e. with Casparian bands (in the young zone) and with the addition of suberin lamellae in most but not all cells (in the old zone). Similarly, passage cells were always present in the exodermis. The question of Ca\(^{2+}\) movement through an endodermis (or exodermis) with tertiary walls and without passage cells could not be approached with this experimental material. It is most likely that in this study, Ca\(^{2+}\) movement into the old zone was predominantly apoplastic in the epidermis and central cortex and was through the cytoplasm of only the passage cells of the exodermis and endodermis. Such symplastic steps would not involve Ca\(^{2+}\) transport through plasmodesmata.

The results of this study clearly show that in onion roots, most or all of the Ca\(^{2+}\) that enters the root stele moves through the symplast at some point(s). Due to the impermeability of the endodermal Casparian band to ions (see introduction) and of the exodermal

| Movement of Ca\(^{2+}\) through the Symplast |

The results of this study support the model of Clarkson (1991) in which a symplastic step through the endodermis occurs during radial Ca\(^{2+}\) transport in roots. His model can now be extended to include the exodermis. The mechanism(s) of Ca\(^{2+}\) movement through the symplast of such layers, however, are not clear. Transport of apoplastic fluid across the cytoplasm of onion root cells by means of endocytotic vesicles was ruled out by earlier studies. Such vesicles occurred in the cells of the central cortex but these emptied their contents into the cells’ vacuoles (Cholewa and Peterson, 2001). Further, if endocytotic

Plasmalemmae of roots (White and Davenport, 2002). For example, Marshall et al. (1994) provided evidence for the presence of La\(^{3+}\)-insensitive Ca\(^{2+}\) channels in maize roots. It is likely that onion roots possess such channels in their plasmalemmae. VO\(_4^{3-}\), a well-known inhibitor of P-type ATPases, was also effective in reducing the amount of Ca\(^{2+}\) movement into onion roots. This ion is not toxic to the roots, since treatment of onion roots with 1 mM VO\(_4^{3-}\) for 6 h induced a reversible inhibition of root growth (Navas and Gracia-Herdugo, 1988). The observed 73% inhibition of Ca\(^{2+}\) exudation upon treatment of onion roots with VO\(_4^{3-}\) (in this study, Table III) strongly supports the hypothesis that P-type Ca\(^{2+}\) pumps are involved in release of Ca\(^{2+}\) into the stelar apoplast.

### Table III. Effects of La\(^{3+}\) and VaO\(_4^{3-}\) on root exudation and Ca\(^{2+}\) translocation from young zones of onion roots

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ca(^{2+}) in Exudate</th>
<th>Exudate Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.01 ± 0.84</td>
<td>1.2 ± 0.5*</td>
</tr>
<tr>
<td>+ La(^{3+})</td>
<td>0.54 ± 0.12</td>
<td>1.3 ± 0.4*</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.92 ± 1.97</td>
<td>1.1 ± 0.4*</td>
</tr>
<tr>
<td>+ Vanadate</td>
<td>0.51 ± 0.08</td>
<td>1.1 ± 0.4*</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>73</td>
<td></td>
</tr>
</tbody>
</table>

\(^{45}\text{Ca}^{2+}\) translocation was measured in exudates after 17 h (n = 3, ±SD, Student’s t test, P = 0.05, *, no significant difference).

---

Figure 5. Sectional diagrams of plexiglass boxes (also shown in Fig. 2) set up to test the effect of inhibitors on calcium delivery to the stele. Five excised roots (one of which is shown) are sealed into a box so that the cut (proximal) ends protrude into one of the flanking chambers (f). Both flanking (f) and bordering (b) chambers are filled with 1 mM CaSO\(_4\). Exudates from the cut ends of the five roots enter the same flanking chamber. A, Treatment with La\(^{3+}\). The treatment chamber (t) is filled with radioactive Ca\(^{2+}\) and 1mM LaCl\(_3\). B, Root segment pretreated with Na\(_3\)VO\(_4\) (see text). The distal end of the root (in the flanking chamber to the left in the diagram) is sealed shut; the treatment chamber (t) is filled with a solution containing radioactive Ca\(^{2+}\). Control data were generated from root segments set up as in A and B but without the inhibitors.
Casparian bands to Ca$^{2+}$ (this study), these layers should be included in any symplastic path.

MATERIALS AND METHODS

Plant Material

Adventitious roots of onion (Allium cepa L. cv Ebeneezer) were generated from bulbs kept for 12 d in vermiculite-filled pots in a greenhouse. The longest roots measured 16 cm and none had reached the bottoms of the pots. The bulbs were near the end of their storage time 6 months after harvest and sprouted leaves as well as roots.

Treatment of Various Root Zones

Zones to be treated with radioactive Ca$^{2+}$ were established by following the pattern of maturation of the endodermis, exodermis, and early metaxylem in the roots (Fig. 1) using the methods of Brundrett et al. (1988, 1991) and Peterson and Steudle (1993). With these techniques, Casparian bands, suberin lamellae, and functional xylem elements, respectively, were identified (Fig. 1). radioactive Ca$^{2+}$ in the form of a chloride salt (6CaCl$_2$) was applied to specific zones of the root (Fig. 2). An entire onion with its intact root system was placed in a tray that had been lined with wet, absorbent paper. The root to be treated was carefully positioned in a 45°C box divided by partitions to create a 15-mm-long treatment chamber, two narrow bordering chambers to trap any accidental leakage from the treatment chamber, and two flanking chambers to hold the adjacent parts of the root (Fig. 2). The root was sealed into place with a mixture of 90% lanolin and 10% wax (previously heated while mixed; Barrowclough et al., 2000), and the bounding and flanking chambers were filled with 1 mM CaSO$_4$ solution. To begin the treatment, 1 mM CaSO$_4$ labeled with 45CaCl$_2$ ($3.7 	imes 10^8$ Bq mL$^{-1}$) was carefully pipetted into the treatment chamber. The remaining roots (outside the plexiglass box) were sprayed with 1 mM CaSO$_4$ and covered with wet vermiculite. Then a tray lid, also lined with wet, absorbent paper was set into place so that only the leaves projected into the atmosphere. To promote transpiration, continuous light was supplied from a Tungston, General Electric (Fairfield, CT) bulb and the leaves, were heated at 500$^\circ$C for 5 min. After removal of dye, the experimental root was measured and divided into parts, i.e. the treated segment, the distal (apical) section, and the proximal (basal) section. All these parts, in addition to the bulb and the leaves, were heated at 500$^\circ$C for 17 h, cooled, and the resulting ash made up to 5 mL with 0.2 N HCl. A similarly processed, entire root from an untreated plant was used as a control. Radioactivity ($\beta$-emissions) in each plant part was measured with a liquid scintillation counter (Searle Analytic, model Mark III) after addition of scintillation liquid (Ecolite, ICN, Irvine, CA). Four replicates were included in each experiment. Data from the plant parts external to the treated root area were pooled and analyzed by Student’s $t$ test (2 tails, 3 types – two samples unequal variance).

Preparation of Root Segments for Compartmental Elution

Segments used to determine the permeability of the exodermal Casparian band were prepared according to the procedure of Peterson (1987). All root parts were taken at least 10 cm from the root tip to a location where the exodermis had a Casparian band. One set of 10 root segments was prepared for each replicate of each treatment. For “intact” specimens, 2.5-cm-long pieces were removed and their ends sealed with sticky wax (Kerr, Toronto) so that 2 cm of the root would be exposed to the treatment solution. “Dissected” segments were made by bisecting 2-cm-long pieces and removing their steles with the aid of fine forceps and a dissecting microscope (Fig. 3). To ensure that the expected maturation of the exodermis had occurred in the segments used for elution experiments, freehand cross sections were cut from the adjacent areas of the roots and assessed for the presence or absence of an exodermal Casparian band (procedure of Brundrett et al., 1988). Three roots from each bulb used for each replicate of each experiment were examined for unexamined lateral primordia by the clearing method of Hackett and Steward (1969).

Compartmental Elution

Labeling and elution were performed following the method of Peterson (1987). Segments to be loaded with 4Ca$^{2+}$ were incubated for 17 h in 25 mL of an aerated solution of 1 mM CaSO$_4$, adjusted to pH 5.5 with dilute HCl, and amended with 10 mM CaCl$_2$ (final specific activity = $3.7 	imes 10^8$ Bq mole$^{-1}$). At the end of this period, the segments were gently removed from the bathing solution, blotted with absorbent tissue, and immediately transferred into plastic vials fitted with plastic micromesh at the bottom end and an aeration tube. Each vial contained 10 segments (intact or dissected). The vials (and the segments they contained) were immersed in a series of elution solutions containing 10 mL unlabeled 1 mM CaSO$_4$, adjusted to pH 5.5. Between each change, vials were lifted just above the solution and rinsed with 1 mL of unlabeled solution to minimize error due to ullage. Transfers to fresh washout solutions were carried out at the following time intervals (min): 0.5, 1, 1.5, 2, 3, 4, 5, 7, 9, 11, 15, 20, 25, 30, 40, 60, 90, 120, 150, 180, 240, 300, and 360. At the end of the elution series, the capsules were removed from the sealed segments, and the batches of segments were weighed. Any remaining 4Ca$^{2+}$ was extracted by a 20-min treatment with 0.2 N HCl for 20 min. This solution was made up to 5 mL with water; then scintillation liquid (Ecolite, ICN, catalog no. 882475) was added to achieve a total volume of 20 mL. From each of the washout solutions, a 1-mL aliquot was removed with a scintillation vial to a total volume of 6 mL. Samples were assessed for radioactivity as described above. Each reading was corrected for the background present in a root from an onion that had not been exposed to 4Ca$^{2+}$.

Each comparison (i.e. intact versus dissected) was replicated at least three times. Data were analyzed as previously described (Peterson, 1987). To avoid any error owing to subjectivity, a maximization of $R^2$ (square of the sample correlation coefficient, using Microsoft Excel; Redmond, WA) for linear regression (Rygielwicz et al., 1984) was used as the criterion for determining data points to be included in each regression line. Values for the halftime of elution and the amount of Ca$^{2+}$ in each compartment were obtained using Microsoft Excel.

To verify the identity of the cell wall compartment, three replicates of an experiment were eluted at 4°C. For this trial, intact and dissected segments were initially incubated in the 1 mM radioactive Ca$^{2+}$ treatment solution at 22°C for 17 h. At the end of this labeling period, segments were blotted and then eluted (as described above) at low temperature.

Treatment with Inhibitors

To distinguish between Ca$^{2+}$ transport in the apoplasm versus the symplast of the endodermis, young zones of the root (with Casparian bands in the endodermis but not in the immature exodermis) were tested. The effects of La$^{3+}$ and VO$_4^{3-}$ on the amounts of Ca$^{2+}$ in root exudates were assessed.

For each replicate of a La$^{3+}$ experiment, 10 roots were excised from one onion bulb. Each root was cut obliquely under water to obtain the distal 6 cm (i.e. the part of the root that included the tip). Five of the roots were sealed into a plexiglass box as described above so that a 15-mm length in the central region of the young zone could be treated (Fig. 5). Bordering and flanking chambers were filled with 1 mM CaSO$_4$ and a treatment solution of 1 mM LaCl$_3$ and radioactive Ca$^{2+}$ (as described above) was added to the treatment chamber. The remaining five roots were sealed into a second chamber and treated in the same fashion but without LaCl$_3$.

The membranes of the cells in the stele (including the membranes of the stele side of the endodermis) were exposed to VO$_4^{3-}$ by introducing a solution of sodium orthovanadate (Na$_3$VO$_4$, Aldrich, St. Louis) into the transpiration stream. The inhibitor solution was prepared according to Spanswick (1984). In short, a 5-mM stock solution was made by dissolving Na$_3$VO$_4$ in distilled water at 38°C for 3 h. The pH was adjusted to 7.2 with dry MES (Sigma, St. Louis) and this colorless solution stored in a sealed bottle at room temperature. For the experiments, aliquots were diluted with distilled water to 1 mM VO$_4^{3-}$ and a drop of 1% aq Safranine 0 was added to color the solution red. To introduce this mixture into the transpiration stream, the temperature and cm of five roots was excised and the cut ends to O’Neill, still attached to the plant, were placed in the solution. The plant was allowed to transpire (under the conditions described above) for 2 h, after which the treated roots were removed from the bulb, their proximal ends cut (as described above) and inspected for red coloration. In those roots in which the
treatment solution had passed through the segment in the xylem, the distal cut ends were sealed with sticky wax (Kerr). The segments were immediately sealed into the treatment chamber and exposed to radioactive Ca^{2+} as described above. Control roots were treated in the same way but without Na_{2}VO_{4}. At the conclusion of the experiments (17 h of exudation), the radioactivity in the solutions from the bounding and flanking chambers was determined as described above, exudates from the five roots having been pooled in one of the flanking chambers.

The potential effects of both inhibitors on the process of root exudation were tested. In the case of La^{3+}, roots were excised from an onion bulb as described above, and the proximal (cut) end of each root was inserted into a 100-μL microcapillary tube and sealed in place with sticky wax (Kerr). The entire root piece was immersed in a solution of 1 mM CaSO_{4} and 1 mM LaCl_{3}. In the case of VO_{2}^{--}, the inhibitor was introduced into the root xylem as described above. Roots were then severed from the bulb and sealed at the distal ends with sticky wax. The proximal ends of the roots pieces were then sealed into 100-μL microcapillaries as described above and the roots immersed in 1mM CaSO_{4}. Control (untreated) and inhibitor-treated roots were allowed to exude for 17 h, after which the volumes of the exudates were calculated from the lengths of fluid in the microcapillaries.

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