Calcium transport and delivery to the xylem in onion (*Allium cepa* L.) roots

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Biology

Waterloo, Ontario, Canada, 2000

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Abstract

This study concerns the pathway of Ca^{2+} transport in onion (*Allium cepa* L.) roots. Ca^{2+} is an essential macronutrient, which must be present in ionic form in the soil solution to be absorbed by plant roots. To sustain their growth, plants require a continuous supply of Ca^{2+}, as it is not redistributed from mature organs to the meristems. The pathway(s) and mechanism(s) of Ca^{2+} uptake and transport across the root have not been previously resolved. Because of low level of Ca^{2+} in the cell cytosol, the symplastic pathway may be insufficient to meet the Ca^{2+} requirements of the developing shoot. The apoplastic pathway, however, is obstructed by the deposition of hydrophobic materials (Casparian bands) in the walls of the endodermis and exodermis. It is commonly believed that Ca^{2+} follows an apoplastic pathway on its route to the xylem by entering the root tip, where the wall modifications in the endodermis and exodermis are not yet developed. In the present study, onion roots were used as a model system. These roots did not possess any measurable capacity for apoplastic transport, as determined by exclusion of 8-hydroxy-1-3-6-pyrenetrisuphonate (PTS) from the transpiration stream. Direct application of $^{45}Ca^{2+}$ to discrete zones of the root revealed that Ca^{2+} applied to the root tip was not translocated to the shoot, ruling out the root tip as a point of entry for this ion. Further, an antimonate precipitation study ruled out the possibility that Ca^{2+} was delivered to the transpiration stream by maturation of tracheary elements. $^{45}Ca^{2+}$ applied to more mature zones indicated that the ion moved radially through the mature endodermis and exodermis. The permeability of the exodermal Casparian bands to Ca^{2+} was tested by means of compartmental elution, and the results indicated that these wall modifications hindered apoplastic Ca^{2+} transport. Since Ca^{2+} was translocated from the oldest

root zone, but could not pass through the exodermis apoplastically, its uptake into the symplast was investigated. Results of experiments with a number of inhibitors led to the hypothesis that lanthanum-sensitive Ca^{2+} channels in the plasma membranes of the cells at the root margin mediate Ca^{2+} uptake while, at the other end of the radial path, P-type Ca^{2+}-ATPases load Ca^{2+} into the xylem. A new model of radial Ca^{2+} transport is proposed, in which Ca^{2+} enters the cytoplasm of the cells at peripheral layer, is then extruded into the walls of the central cortex, where its transport occurs by cation exchange reaction, crosses the endodermis via the symplastic pathway, and is actively delivered to the apoplast of the xylem.

Acknowledgements

The overwhelming help that I received during preparation of this thesis from many people is going to bring joyful tears into my eyes for years to come. Thank you all!

First of all, I would like to thank Professor Carol A. Peterson for her invaluable guidance and endless patience. The professionalism of her work is going to be my model for the rest of my carrier. Her warmth and carrying made all the years a pleasure of being a student in her laboratory.

The fellow researchers in the lab provided a wonderful work environment. Many thanks Daryl Enstone for being able to point to the references in a fraction of second; Fengshan Ma for his expertise in plant biology and philosophy; Jeff Taylor for sharing his life optimism; Jeanine West for singing songs while working together. I was very fortunate having you around!

The members of my advisory committee, Dr. R. L. Legge, Dr. W. Dheil-Jones and W. Rauser provided a source of knowledge and support which I needed during the struggle. Thank you for your time!

Sincere thanks to the members of the staff and faculty of the Department of Biology, University of Waterloo for their support, especially Dr. Marilyn Griffith for her guidance.

Finally, I would like to thank my sons: Paul, Peter and David for their love, help and support. It was fun to study together. I am blessed having you!

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Chapter 1
General introduction

In his contribution to the book "Plant Roots. The Hidden Half (1991)" Clarkson states, "The major nutrient whose movement is the most poorly understood is calcium" (Clarkson, 1991). This statement did not change in the second edition of the book in 1996 (Waisel et al. 1996). Given the diversity and importance of Ca^{2+}-mediated functions in plants, it is astonishing that the pathway of Ca^{2+} transport within roots is not yet clear. It is still not firmly established whether Ca^{2+} movement occurs primarily through the apoplast, the symplast, or some other, as yet undiscovered pathway. It is clearly important to know how the roots absorb and translocate Ca^{2+}.

This study was designed to determine the pathway(s) of Ca^{2+} movement in onion roots, and to contribute to the understanding of the mechanism of Ca^{2+} transport. In subsequent chapters, experiments are reported which were designed to: establish if there is a substantial apoplastic bypass in onion roots that would allow unrestricted Ca^{2+} transport from the soil solution to the xylem (Chapter 2); locate the most efficient site for Ca^{2+} uptake and translocation along the root axis (Chapter 3); determine whether or not exodermal Casparian bands pose a barrier to apoplastic Ca^{2+} transport (Chaper 4); locate free Ca^{2+}at the subcellular level within the root (Chapter 5); and test whatever or not Ca^{2+} transporters are involved in its acquisition and release into the xylem (Chapter 6).

In all experiments, unbuffered 1 mM $CaSO_4$ in distilled water, with pH adjusted to 5.5, was used. This external concentration of Ca^{2+} was chosen to allow the detection of an apoplastic pathway, as it has been reported that at this concentration, membrane transport and cell wall

exchange capacity may be saturated and the excess Ca^{2+} would move apoplastically with the transpiration stream (Yeo et al. 1987). This concentration reflects the natural concentration in the soil solution, and is the average Ca^{2+} concentration sufficient for adequate growth (Epstein, 1965).

1.1 Importance of Ca^{2+} uptake and transport in roots

Understanding the mechanisms controlling the uptake and long distance transport of Ca^{2+} has become increasingly important because of the widespread occurrence of Ca^{2+} deficiency and Ca^{2+}-related disorders in plants (Marshner, 1986). Processes that regulate Ca^{2+} availability and influence its effects on plant production are also of interest in view of the changes that are occuring in today's ecosystems. Acidification of the soils by natural processes and removal of basic cations can lead to destabilization of the normal cycles of Ca^{2+} uptake and conservation by plants. This process is rapidly accelerated by a variety of anthropogenic activities including grazing, burning, and harvesting. Atmospheric decomposition of strong acids is considered to have approximately doubled the natural leaching rates for basic cations (Joslin et al. 1992). All of above processes will have a long-term effect on Ca^{2+} supply rates to the vegetation, as Ca^{2+} levels have declined significantly in the soils in many parts of the world in the past few decades (McLaughin and Whimmer, 1999). Despite the possibility of global Ca^{2+} deficiency, the pathway of its acquisition and transport by roots is poorly understood.

1.2 Calcium as a macronutrient

Higher plants contain large amounts of Ca^{2+}, ranging 1-50 mg Ca^{2+}/g dry matter (Kirkby and Pibleam, 1984). All plants require Ca^{2+} in an ionic form in their extracellular medium and this cation is involved in variety of structural and regulatory roles. As little as 2.5 to 100 μM Ca^{2+} in a flowing nutrient solution is sufficient to sustain normal plant growth and development (Russell, 1977; Hanson, 1984). At the cellular level, Ca^{2+} deficiency leads to increased vacuolation of the meristematic cells, multiple nuclei, chromosomal abnormalities,

and cell wall distortion (Kirkby and Pilbeam, 1984: Roux and Slocum, Roux SJ, Slocum RD. 1982 1982). Other important Ca^{2+}-deficiency related disorders are reviewed by Kirkby and Pilbeam (1984). Such disorders occur predominantly in crop plants grown in intensive agriculture and horticulture. Recently discovered Ca^{2+}-related disorders include inhibition of lignification (Eklund and Eliason, 1990), susceptibility to low temperature damage (DeHayes, 1997), and a general decrease in disease resistance (McLaughlin and Whimmer, 1999).

1.3 Functions of Ca^{2+}

There are three primary functions of Ca^{2+} in plants: signaling – a role as a second messenger (enzyme activation and stabilization), maintenance of membrane form (liquid crystaline) and activity, and stabilization of the cell wall structure.

The second messenger function of Ca^{2+} has been established with unequivocal evidence presented in many reviews (Hepler and Wayne, 1985; Kauss, 1987; Poovaiach and Reddy, 1987; Bush, 1995; Trewavas and Malho, 1997). This aspect of Ca^{2+} function is not discussed further in the present study.

The effect of Ca^{2+} on membranes is universal in biology. In the beginning of the last century it was recognized that external Ca^{2+} is necessary to support the ion selectivity function of root cell membranes (True, 1914). Later it was shown that Ca^{2+} prevents sucrose leakage from corn scutellum slices. This led to the conclusion that Ca^{2+} binds certain anionic groups of the membrane to form bridges between structural components, thereby maintaining selective permeability and membrane integrity (Garrard and Humprey, 1967; Legge et al. 1982; Nur et al. 1986). Physical and essentially non-metabolic binding effects of Ca^{2+} on membrane structure have been demonstrated in experiments where addition of Ca^{2+} prevented and reversed cold-induced leakiness of the cells. Since the ambient temperature was too low to support metabolism, it was proposed that Ca^{2+} prevented the phase change of the membrane from liquid crystal to gel phase by means of physical binding to the membrane (Cristiansen and Foy, 1979).

A considerable part of Ca^{2+} is sequestered in the cell walls. It is generally accepted that the major substance that binds Ca^{2+}within the walls are pectic acids (Christiansen and Foy, 1979). Ca^{2+} pectate is a principle substance of the middle lamella and acts as"cement", binding walls of adjacent cells together (Aspinal, 1980; Bacic et al., 1988; Baron-Epel et al., 1988).

1.4 Uniqueness of calcium

An understanding of the unique chemical properties of Ca^{2+} is fundamental for an appreciation of how this ion fulfills its diverse biochemical functions. The "fitness of Ca^{2+}" and its chemical attributes are outlined by Hepler and Wayne (1985). The diversity of binding conditions that Ca^{2+} can occupy is a consequence of its large nuclear size, charge density, and the availability of the outer-shell electrons. These electrons allow Ca^{2+} to participate in multiple configurations, including both covalent and ionic bonding. Because of its size and low charge density, Ca^{2+} is specifically favoured for bonding with neutral oxygen sites in biological ligands such as polysaccharides and lipids. It forms relatively weak bonds at multiple bonding sites. When compared to Mg^{2+}, Ca^{2+} has a lower affinity for water and nitrogen atoms, which is advantageous in biological systems; namely because of an enhanced capacity to bind to less charged ligands and faster reaction times. The reaction time depends on the rate at which an ion sheds its hydrated shell, and the release of water by Ca^{2+} is approximately 100 times faster than by Mg^{2+}. The capacity to occupy multiple binding sites also allows Ca^{2+} to be associated in more complex configurations, and to act as site-specific enzyme co-factor (Roberts and Harmon 1992). Ironically, the metabolism-regulating role of Ca^{2+} coincides with its toxicity to cytoplasmic processes. This toxicity is a consequence of precipitation of inorganic phosphate with Ca^{2+}, impairing phosphorus-based metabolism of the cell. Thus, living cells maintain very low, nanomolar ranges of free Ca^{2+} in the cytosol against millimolar concentrations outside and in the intracellular organelles (Felle and Hepler, 1997). As a result, Ca^{2+} can serve as a second messenger ater entering the cytosol (along its electro-chemical gradient) in response to various stimuli.

1.5 Advantages of onion as a model system

Onion roots have been used as a model system in many physiological studies for several reasons. Firstly, it is relatively easy to obtain large quantities of roots from one bulb, which ensures genetically uniform samples for each experiment. Secondly, their anatomy is very well known (Peterson and Perumalla, 1984). Thirdly, they are exodermal as are the majority of tested angiosperm species (Perumalla et al. 1990; Peterson and Perumalla, 1990). Lastly, onion roots are ideal for the estimation of amounts of ions present in the cell walls, as they do not develop root hairs or lateral roots, when well watered. The growing onion root varies morphologically along its axis (Fig. 1.1). The apical meristem is surrounded by the root cap. After cell division and differentiation, Casparian bands are deposited in the cell walls of the endodermis at a distance of 5 to 10 mm from the root tip. This part of the root is referred to as meristematic zone throughout this study (Fig. 1.1). The next zone, referred to as the young zone (10 to 50 mm from the root tip) is characterized by the presence of both Casparian bands in the endodermis and conducting protoxylem vessels. In the mature zone, in addition to endodermal Casparian bands, there is a second set of Casparian bands in the exodermis (Fig. 1.1).

Onion develops a dimorphic exodermis with alternating long and short cells. Long cells deposit suberin lamellae simultaneously with the development of Casparian bands, while the short cells tend to remain without lamellae. Fig. 1.2 is a simplified diagrammatic representation of a cross section from a mature zone with mature exodermis. The protoplast of the long cells of the exodermis degenerates upon deposition of suberin lamellae, and these cells are dead (Ma, *personal communication*). The short cells remain alive and well connected to the epidermis and cortex with plasmodesmata, and the central cortex is composed of elongated parenchyma cells with thin walls. This layer is thicker than indicated on the diagram, typically having nine cells between the exodermis and endodermis (Barrowclough and Peterson, 2000). The innermost layer of the cortex (i.e. endodermis), has modifications in the cell walls in the form of Casparian bands and suberin lamellae. The endodermal cells facing the xylem poles do not develop suberin lamella, and are called

Figure 1.1 Diagram of the median longitudinal section of onion root. Major developmental differences (arrows) are indicated, as well as zones of the interest in this study. Not drawn to scale.

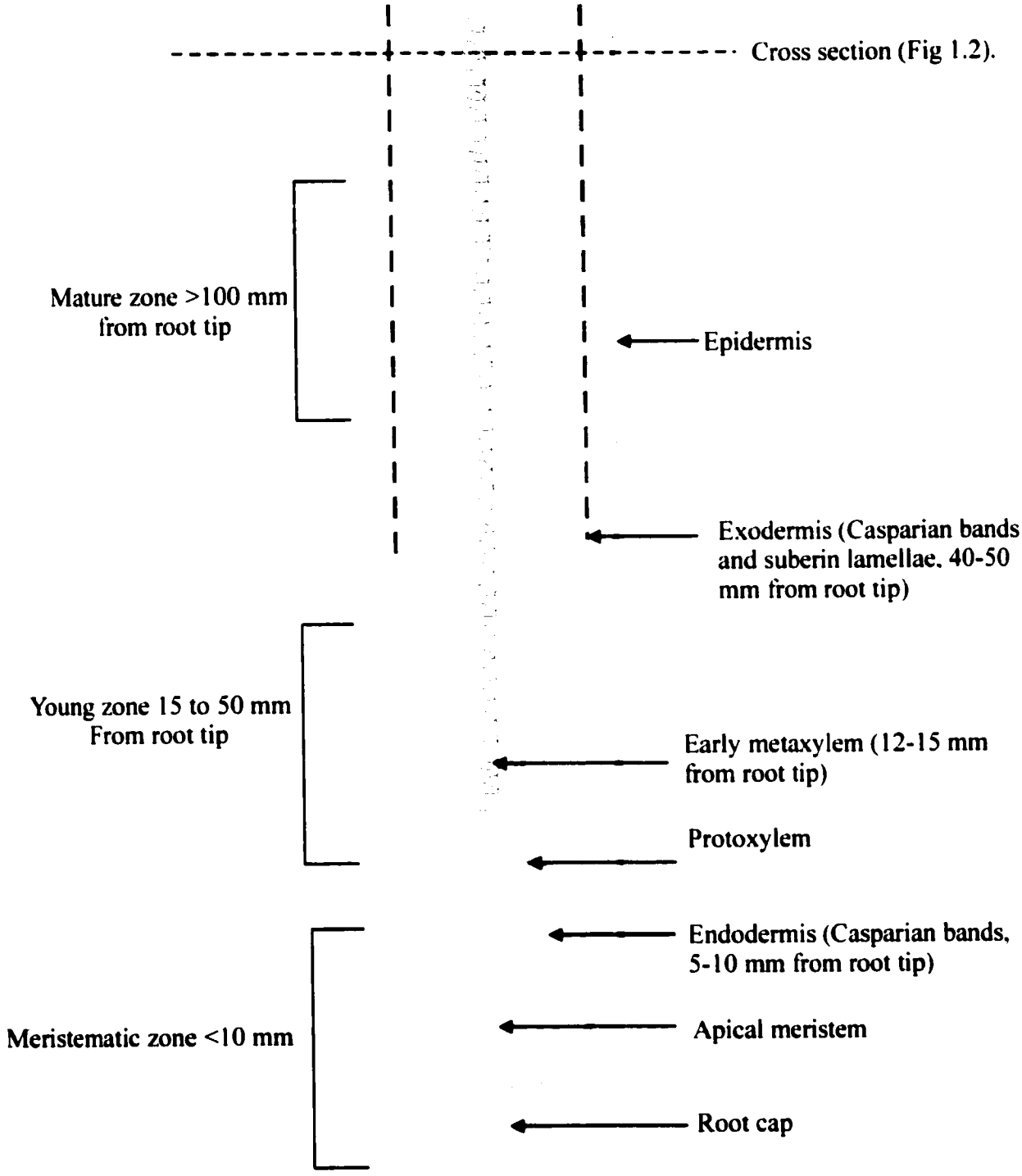
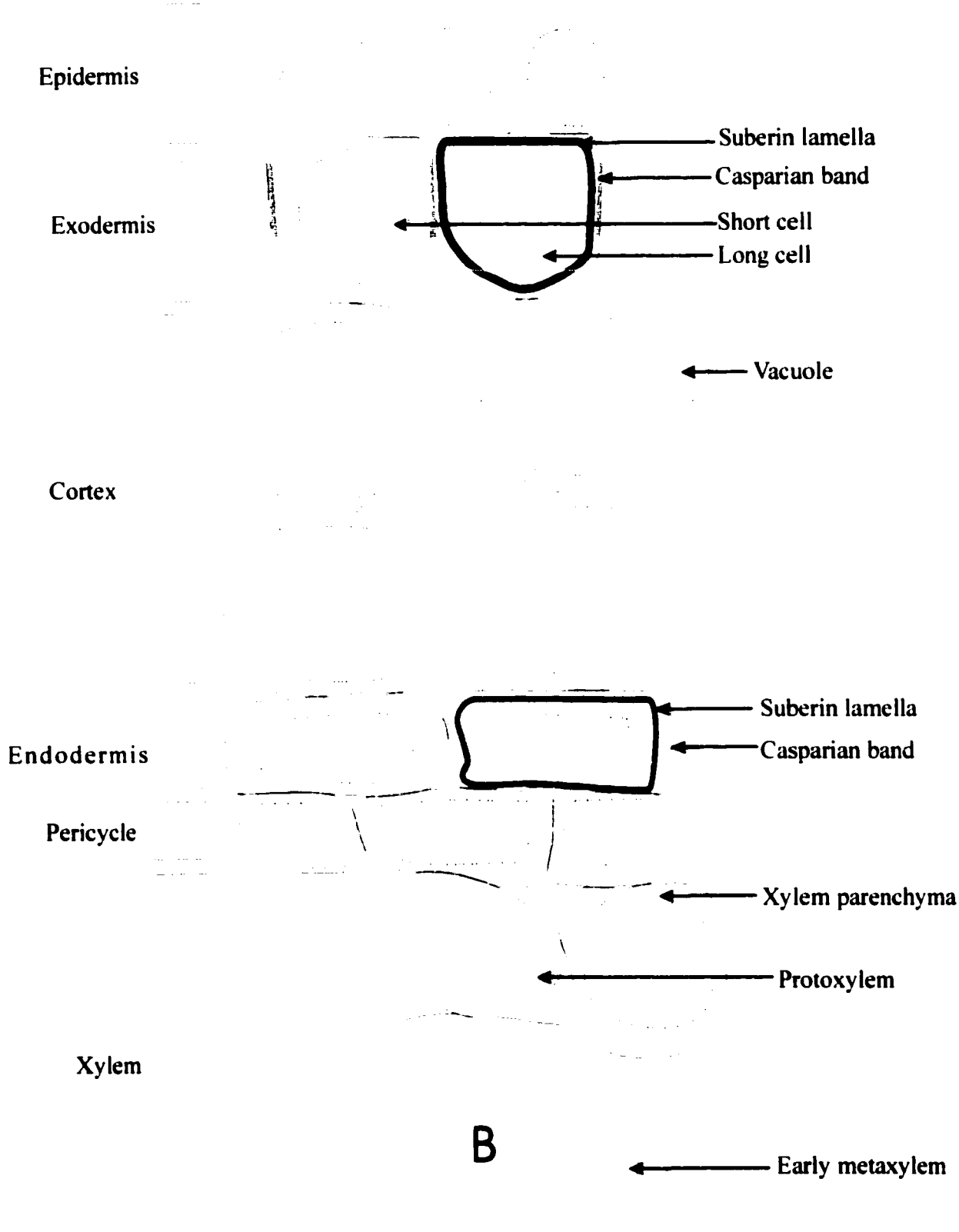

Cross section (Fig 1.2).
Mature zone >100 mm from root tip
Epidermis
Exodermis (Casparian bands and suberin lamellae, 40-50 mm from root tip)
Young zone 15 to 50 mm From root tip
Early metaxylem (12-15 mm from root tip)
Protoxylem
Endodermis (Casparian bands, 5-10 mm from root tip)
Meristematic zone <10 mm
Apical meristem
Root cap

Figure 1.2 Diagram of the onion root cells involved in the radial transport of Ca^{2+} from the soil solution (A) to the lumena of xylem vessels (B). Not drawn to scale. On average there are 10 cells in the central cortex between the exodermis and endodermis.

A


Epidermis
Exodermis
Suberin lamella
Casparian band
Short cell
Long cell
Vacuole
Cortex
Endodermis
Suberin lamella
Casparian band
Pericycle
Xylem parenchyma
Protoxylem
Xylem
B
Early metaxylem

passage cells. The endodermis is lined with pericycle and encloses the stele. The stele contains parenchyma cells associated with xylem elements involved in the long distance transport of ions. In this study, the pathway and mechanism(s) of Ca^{2+} transport from the soil solution (A) to the lumena of mature xylem elements (B) were explored (Fig 1.2).

Chapter 2

Is there a major apoplastic bypass in onion roots?

2.1 Abstract

Calcium is taken up by onion roots and transported through the xylem to the leaves. It is possible that a combination of pathways for ion transport exists in young onion roots – apoplastic i.e., bypassing the Casparian bands in the endodermis in the root tip area, and symplastic i.e., through cytoplasm and plasmodesmata from the endodermis to the xylem parenchyma. The apoplastic pathway allows solute passage through pores in the cell walls. To assess the extent of a major apoplastic pathway for ions in onion, six to nine day old roots were treated with the non-toxic, highly mobile, fluorescent tracer 8-hydroxy-1,3,6-pyrenetrisulphonate (PTS). After 24 h, leaves were harvested and assayed for the presence of this tracer. No PTS was detected in aqueous extracts of the leaves, indicating that it was not transported with mass flow of water during transpiration. This result shows that the apoplastic pathway in onion roots was ineffective for PTS, i.e. there is no major apoplastic bypass. Observation of whole roots treated with PTS confirmed that the mature exodermis blocked the apoplastic continuum. When roots were irradiated with UV light, a strong green fluorescence was evident in the cortex of the root regions with an immature exodermis which was not seen in older regions of the root. This observation was correlated with the absence or presence of the exodermal Casparian bands, respectivelly. Therefore, incubation of an intact root with PTS can serve as a new, non-destructive technique to detect the presence of a mature exodermis. PTS entered the cortical cells in the young region of the root. The presence of PTS-loaded vesicles was observed with laser scanning confocal microscopy. The first order

kinetics of uptake and insensivity to external pH as well as to the inhibitor probenecid indicated that PTS entered the cell by fluid phase endocytosis.

2.2 Introduction

This investigation was preliminary to a direct study of Ca^{2+} transport in onion roots. The movement of Ca^{2+} across the root is enigmatic. Because of its low concentration in the cytosol, the symplastic pathway for Ca^{2+} transport may not be sufficient to meet the needs of the developing shoot. On the other hand, transport of ions in the apoplast is generally assumed to be terminated at the endodermis by its Casparian bands. There may be, however, some "leaky" zones in the endodermal barrier; one near the apical meristem, where the differentiation of various cell types is not yet complete, and another in mature zones, where developing lateral roots disturb the Casparian bands in the endodermis and create an apoplastic pathway to the stele (Peterson and Emanuel, 1980). The relative significance of this apoplastic pathway is very important for Ca^{2+} transport because of its limited apparent mobility in the symplast (Clarkson, 1974). If there is a substantial apoplastic pathway for solute transport in onion roots that would allow a relatively large tracer molecule to pass, this pathway may be sufficient to supply all the Ca^{2+} required by the shoot. On the other hand, if there is no apoplastic pathway for the tracer, the question how Ca^{2+} is delivered to xylem remains open.

PTS (8-hydroxy-1,3,6- pyrenetrisulphonate) is widely used as an apoplastic tracer. It is a non-toxic, highly mobile, green-fluorescing probe that does not bind to cell walls (Skinner and Radin, 1994). Jachetta et al. (1986) used PTS to measure concentrations of solutes in apoplastic and membrane-filtered fractions in sunflower leaves. PTS was fed to these leaves by the transpiration stream and was extracted from the leaves' apoplast with subsequently applied pressure. With increased pressure the PTS concentration decreased in the leaf extract. This led Jachetta et al. (1986) to conclude that PTS does not cross the plasma membrane (PM). Indeed, numerous studies have been conducted using PTS to detect apoplastic bypasses in roots of various species (Hanson et al. 1985; Yeo et al. 1987; Zimmermann and Steudle, 1998). It has also been used as a symplastic tracer. For example, Wright and Oparka

(1996) facilitated PTS diffusion through the plasma membrane (PM) by using of an acetate derivative and loaded this probe into the cells. In the cytoplasm, the intracellular esterases cleaved the acetate from the PTS which was the trapped in the cytosol. This PTS was used for a detailed study of symplastic unloading of phloem (Wright and Oparka, 1996). The established membrane impermeability of PTS is due to the presence of three sulphonate groups on the molecule that are negatively charged at physiological pH, making this probe an excellent apoplastic tracer (Wright and Oparka, 1996).

The apoplastic pathway in roots is modified by the deposition of hydrophobic materials in the cell walls of the endo- and exodermis. The maturation of the exodermis, i.e. deposition of its Casparian bands, is not readily predictable. It depends on the root growth rate, and can be affected by environmental factors and applied stress. To date, the distribution of exodermal Casparian bands within whole root systems has not been studied (Peterson, 1997). In the past, Casparian bands have been visualized with a variety of methods. Two sensitive fluorescence techniques have been used to stain Casparian bands directly. These stains are an alkaloid extract from *Cheliodonium majus* roots (Peterson et al., 1982), and berberine (Brundrett et al., 1988). Using the former stain, a large number of angiosperm species have been demonstrated to develop a Casparian band in their exodermis (Peterson and Perumalla, 1990; Perumalla et al., 1990). The impermeability of Casparian bands can be visualized by a precipitation technique (Enstone and Peterson, 1992a), where localization of berberine thiocyanate crystals can be detected in the cell walls and correlated with maturation of exodermal Casparian bands. Similarly, incubation of intact onion roots with Cellufluor resulted in staining only the cell walls of the epidermis and outer tangential walls of exodermis because Casparian bands blocked further ingress of the dye (Moon et al., 1986). For all these techniques, it is necessary to section roots and observe them with a fluorescence microscope; the fluorochromes from *C. majus* root extract, berberine and, in the precipitation technique, berberine thiocyanate crystals coat the root surface. Cellufluor binds to cellulose and stains epidermal cell walls throughout the root axis. It was not possible, therefore, to predict maturation of exodermal Casparian bands from external observations. Methods involving sectioning the plant material do not allow for physiological and anatomical studies to be carried out on the same root. A

non-destructive test is needed because determination of the presence of exodermal Casparian bands is essential in studies of transport mechanisms in roots.

In this study, a new technique is reported to detect Casparian bands in the exodermis without sectioning of the roots. In the PTS-labeled segments, this tracer was present in the cell protoplasts. The mechanism of entry of PTS into the cell was investigated and preliminary results indicated that it was taken up by endocytosis.

In plant cells, endocytosis was long considered impossible because of their high turgor pressures (Cram, 1980); however, the evidence for endocytosis in plant cells is now substantial (reviewed by Robinson and Himler, 1990; Battey et al., 1999). In plants, fluid phase endocytosis (pinocytosis) involves a non-selective uptake of the extracellular solution by invagination of the plasma membrane (PM). The resulting vesicles may fuse with intracellular compartments, the vacuole being the major final repository organelle. Steinman et al. (1983) defined fluid-phase endocytotic vesicles as those which become labeled after a brief exposure to an impermeant molecule that does not bind to the outer surface of the PM. A linear kinetics of uptake with respect to increasing substrate concentration (i.e., no saturation over a large external solute concentration) is evidence for fluid-phase endocytosis (Steinman et al., 1983).

Several markers have been used to study endocytotic mechanisms in plant cells. Only relatively small molecules can be used, as the pore size in the cell walls permits diffusion of macromolecules less than 60 kDa in diameter (Carpita et al., 1979). Endocytosis has been demonstrated by the use of fluorescent Lucifer Yellow (Hillmer et al., 1989), fluorescent-tagged dextrans (Cole et al., 1990), heavy metals such as lead (Hübner et al., 1985), nutrients such as biotin (Horn et al., 1990), and elicitors (Horn et al., 1989). To date, no suitable marker that will allow endocytosis in plants to be followed and quantified has been found (Galway et al., 1993). This study reports the use of PTS as a suitable probe for a qualitative and quantitative study of endocytosis in onion root cortical cells. The mechanism of internalization of PTS into the root cells may be relevant of the process of ion transfer to plant cell vacuoles.

2.3 Materials and methods

2.3.1 Plant material

Adventitious roots of onion (*Allium cepa* L. cv. Ebeneezer) were sprouted from bulbs. Prior to planting, the dry outer scales were removed and bulbs were subsequently planted to half of their depth in 20 cm deep pots in vermiculite and grown in a greenhouse (regulated photoperiod 16 h day/8 h night, temperature 25°C day/20°C night). Pots were saturated with tap water daily. Seven to 14 day old plants were used for experiments; at this time the roots were 10 to 20 cm long and lacked lateral roots.

2.3.2 Experimental procedure for testing apoplastic permeability of the roots

Pots with growing onions were watered with 1.9 mM PTS dissolved in tap water. Excess PTS was discarded, and each pot was immersed for 10 min in 1.9 mM PTS, such that PTS saturated the vermiculite completely. The pots were drained and transferred into a container that allowed tight sealing of the surface while exposing the leaves to the outside. There were 2 to 4 cm of PTS solution at the bottom of the container, as liquid continued to drain from the saturated vermiculite during the treatment. Water evaporation from the surface of the pot was prevented by sealing it tightly with aluminum foil and masking tape. The foliage of the shoot was exposed to incandescent light (100 W) in the laboratory at 25°C. The sealed container was weighed throughout the experiment to measure water loss due to transpiration. In some cases, PTS was introduced into the xylem by cutting the roots at an oblique angle under water at a distance of 5 cm from the root tip, followed by incubation in 1.9 mM PTS. Sealing of the containers and measurements of the transpiration were carried out for such onions as well. All experiments were initiated in the morning to assure the same conditions for all replicates. During the experiment it was expected that the stomata would be open, at least initially, and would allow leaves to transpire. Onions were harvested after 3 to 24 h treatments with PTS.

2.3.3 PTS extraction and quantification

For preliminary observations, intact onion leaves were irradiated with a hand-held UV (Sylvania Mercury) lamp to detect PTS. Leaves of PTS-treated and control onions were crushed with a Kimwipe tissue to expose the xylem sap and contents of the cells to UV light.

After the treatment of onion roots with PTS and determination of the water loss due to transpiration, leaves were harvested and weighed. PTS was extracted from the leaves using a modified method of Chung et al. (1992). The tissue was cut into the small pieces with a razor blade. For every 10 g fresh weight of tissue, 50 ml of 80 % methanol was added and this mixture was homogenized in an Omni-Mixer (Sorvall, Norwalk, CONN, USA) on speed 9 for 5 min. The homogenate was filtered through four layers of cheesecloth. For each experiment, a duplicate sample of 5 ml of filtrate was taken for analysis. This was mixed with 10 ml chloroform, incubated for 20 min with occasional shaking, followed by addition of 5 ml of distilled water. Such extracted samples were centrifuged at 2800 g for 10 min. Following centrifugation, the water-soluble fraction which contained PTS was separated from the chloroform-soluble organic fraction which contained the leaf pigments. This partial purification of the extract allowed very precise PTS detection by spectrofluorometry. The amount of PTS was determined using a spectrofluorometer (Photon Technology Inc., South Brunswick, NJ), λ settings for excitation and emission at 405 nm and at 510 nm, respectively. The excitation at 405 nm allows pH independent measurements of the fluorescence intensity (Haugland, 1996). Fluorescence data were processed using Felix software (Photon Technology Inc.).

2.3.4 Determination of PTS accumulation in the roots

To measure the rate of PTS accumulation, root segments 2.5 cm long from young zones (1 to 4 cm from the root tips) were excised with a razor blade and their ends were sealed with sticky wax (Kerr Canda Inc. Toronto). Sealing ensured that PTS entered the root only through the epidermis, and segments were incubated with 1.9 mM PTS. A sample of 3 segments was taken from the PTS solution every 10 min over a total of 120 min. Segments were rinsed with

several changes of tap water for 2 h to remove PTS from the cell walls. After rinsing, the segments were blotted with absorbent (Kimwipe) tissue, the sealed ends cut off with razor blade, and the segments weighed. PTS was extracted from the tissue by adding 3 ml of water and heating at 90°C for 10 min. The root extract was alkalinized with a drop of 0.1 N KOH, and the amount of PTS was determined using spectrofluorometry as described above.

The relationship between PTS accumulation in the roots and external concentration was investigated by incubating sealed segments for 2 h with PTS at concentrations ranging from 0.019 to 1.9 mM. The influence of external pH on PTS (pKa 7.3; Haugland, 1996) accumulation was assessed by incubation of root segments for 2 h in 1.9 mM PTS dissolved in 0.5, 1. 5 and 25 mM HEPS buffers adjusted to pH 8 or 0.5, 5 and 25 mM MES buffer adjusted to pH 5.7. Inhibitors of non-specific inorganic acid transporters (probenecid) and endocytosis (cytochalasin B) were applied for 15 min prior to incubation of the segments in 1.9 mM PTS (±inhibitor) for 2 h. Probenecid (Sigma) was dissolved in 0.1 N NaOH and buffered to pH 5.8 with 0.1 M sodium phosphate buffer. The stock concentration was 100 mM, from which dilutions were performed in PTS-containing solution. Cytochalasin B (Sigma) was dissolved in 80% ethanol to make a 20 mM stock solution and was further dissolved as above. PTS was extracted from the segments as described above.

2.3.5 Detection of Casparian bands

After 24 h of incubation in 1.9 mM PTS, onion plants with intact root systems were removed from vermiculite and rinsed in running tap water. Intact root systems were observed under a hand-held UV lamp. The zones where PTS was present were noted. Cross sections of the roots were taken at the tip, in a young zone, (which exhibited green fluorescence due to the presence of PTS), and in the mature zone, (where fluorescence was absent). All sections were rinsed in water and mounted in water on slides for observation. After determining the presence or absence of PTS in the cells, sections were stained for Casparian bands following the procedure of Brundrett at al. (1988). All observations were made using a Zeiss Axiophot microscope (Carl Zeiss Canada, Don Mills, Ont.) equipped with an Osram HBO 100W mercury lamp and epifluorescence optics. The ultraviolet filter set consisted of an exciter

filter (365 nm), a chromatic beam splitter (395 nm), and a barrier filter (420 nm). Experimental results were recorded on Kodak Ektachrome daylight slide film, ISO 200.

2.3.6 Confocal Laser Scanning Microscopy (CLSM)

CLSM was carried out in the Swedish University of Agricultural Sciences, Dept. of Forest Genetics and Plant Physiology, Umeå, Sweden, with a Zeiss confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with a krypton-argon laser. The standard FITC filter combination was used (excitation at 458 nm, dichroic filter set 510 nm, and barrier filter 570 nm). Thick cross sections from the young zone of the root were incubated in 1.9 mM PTS for 10 min, rinsed, and observed with CLSM. After the first confocal scan was obtained, sections were rescanned within 30 min to detect changes in the location of PTS-filled vesicles.

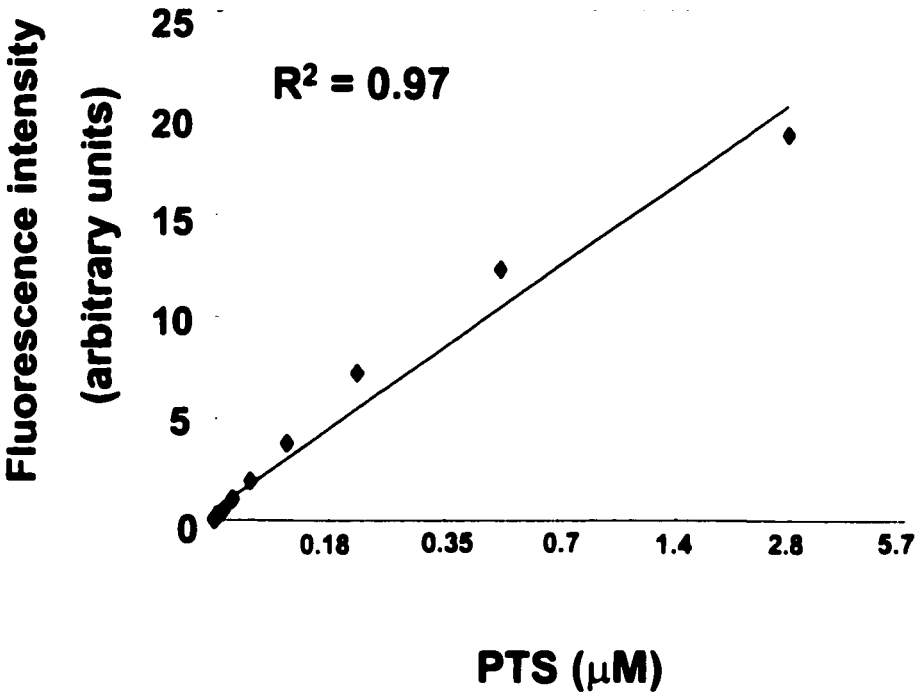
2.4 Results

2.4.1 PTS in leaves after application to the roots

Preliminary observations with the portable UV lamp showed no green fluorescence in either intact or crushed leaves after 24 h of root incubation with 1.9 mM PTS. In contrast, PTS was present in the leaves when cut roots were incubated in PTS, and exhibited green fluorescence which was visible upon irradiation with the UV lamp.

PTS concentrations in the leaves were measured with the spectrofluorometer. For each replicate, a separate calibration curve was obtained to account for the manual adjustment of the shutter opening to obtain maximal sensitivity of the instrument. A typical curve (Fig. 2.1) was generated using 12 data points, with a range of 2.32 nM to 4.77 μM PTS. The linear relationship between fluorescence intensity and PTS concentration ($R^2 = 0.97$) demonstrates that there is no fluorescence quenching at these PTS levels (Fig. 2.1).

Figure 2.1 Calibration curve for PTS measurements. This curve was generated from 12 data points, in a range from 2.32 nM to 4.77 μM PTS.


Fluorescence intensity
(arbitrary units)
25
20
15
10
5
0
R² = 0.97
0.18
0.35
0.7
1.4
2.8
5.7
PTS (μM)

In all leaf extracts measured, no PTS was detected. Emission scans were flat lines with no characteristic emission peak at 510 nm. The sensitivity of the instrument was determined by adding standard concentrations of PTS to the leaf extract. Concentrations above 2 nM PTS could be detected. The least detected concentrations of PTS were corrected for the total volume of the leaf extracts (Table 2. 1).

A control experiment, with onion roots cut open (at an oblique angle with a razor blade) to allow unrestricted PTS access to the xylem elements, was performed to verify tracer mobility in the transpiration stream. In this case, measurable amounts of PTS were present in the leaves (Table 2.1). The concentration of PTS detected in leaf extract was never greater than 50 μM.

2.4.2 Localization of PTS

Control roots (not exposed to PTS) observed under UV light exhibited a blue autofluorescence (Fig. 2.2, A). In roots which had been incubated in PTS for 2 h, some regions fluoresced green. PTS was located in the segments starting 2 mm from the root meristem; no green fluorescence was observed in the root cap or in the meristematic zone (Fig. 2.2, B). PTS was present in the region between 2 mm and 5-6 cm from the root tip (Fig. 2.2, A, B, C). Proximal to that zone, only the blue autofluorescence of the root was observed (Fig. 2. 2, A).

Roots previously stained with PTS were sectioned along their axes and monitored for exodermal Casparian bands. The onset of the deposition of the exodermal Casparian bands was variable. Although the root system of 7 days onions grown in uniform, very moist vermiculite-filled pots, the roots had different lengths. The longest root was 11 cm, with majority between 8 to 6 cm, and many root primordia, less than 1 cm. Different length of roots may reflect the differences in initiation of their growth, as longer roots started to grow

Table 2.1 Amount of PTS in leaves.

Spectrofluorometrical analysis of the aqueous extracts of the leaves of intact onions after roots have been incubated in 1.9 mM PTS. For each experiment, 3 onions were used, ± standard deviation.

Experiment	Time of root exposure to 0.1% PTS (h)	Total transpiration during the experiment (g)	PTS In leaf extract
Intact onions			
1	3	1 ± 0.5	<3.2 ± 1.4 nM
2	6	5 ± 3.0	<7.5 ± 3.1 nM
3	24	31 ± 8	<3.8 ± 1.5 nM
Cut roots			
1	3	3 ± 3	31.1 ± 13.2 μM
2	6	6 ± 2	29.5 ± 8.35 μM
3	24	12 ± 6	36.4 ± 12.3 μM

Figure 2.2 Onion roots incubated in PTS.

A. Left-control root exhibiting blue autofluorescence.

Right- root incubated in 0.1% PTS for 2 h, followed by rinsing. Arrow indicates the point at which exodermal Casparian bands have developed in the exodermis.

B. Root tip incubated in PTS for 2 h. Arrow indicates apical zone exhibiting blue autofluorescence only. Bar = 5 mm

C. Entire root system incubated with PTS, followed by rinsing. Note fluorescent young zones of the roots without exodermal Casparian bands.

D. Median longitudinal section of root tip. Sections were incubated in PTS. Only one cell layer near the surface accumulated PTS. Bar = 1.5 mm

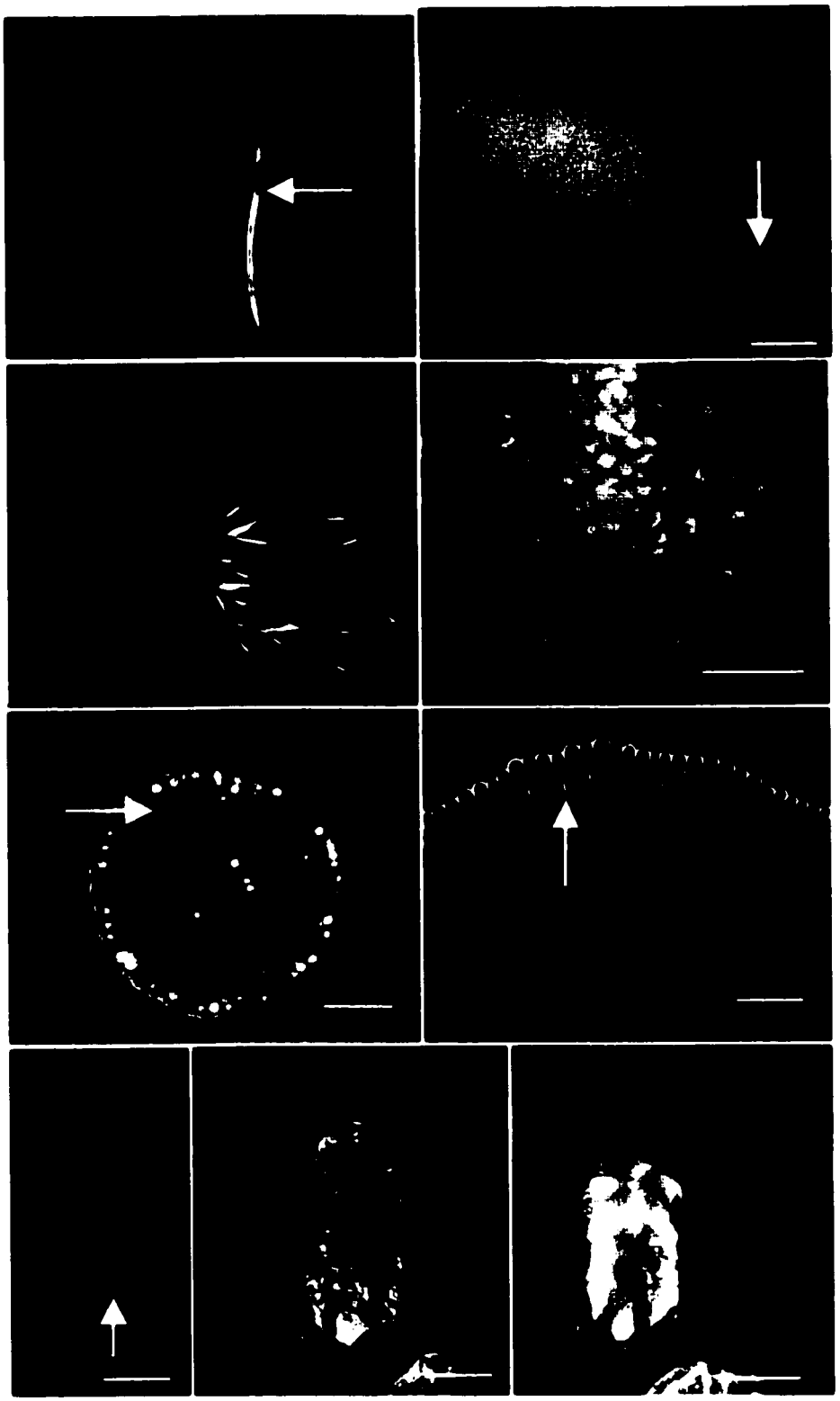
E. Cross section of young zone after entire root was incubated in PTS. Arrow points to one of the brightly fluorescing cortical cell containing PTS. Bar = 3.5 mm.

F. Cross section of mature zone of the root after entire root was incubated in PTS. Stained (berberine/aniline blue) Casparian bands (arrow) prevented entry of PTS into cortical cells. Bar = 0.85 mm

G. Optical section (LCSM) through a cortical parenchyma cell of onion root incubated with PTS for 10 min. Arrow points to a bright vesicle (pseudocolour) on cell periphery. Bar = 0.02 mm

H. Compound picture of optical scans obtained with CLSM of short cell of the exodermis (section taken 2 cm behind root tip). Sections were incubated with PTS for 10 min, followed by 3 min rinse in water. Note peripheral localization of PTS-filled vesicles. Bar = 0.02 mm

I. The same as H, scanned 30 min after the first observation (H). Note disappearance of peripheral vesicles and diffuse fluorescence in the vacuole. Bar = 0.02 mm



earlier than shorter ones. In each case, the transition in PTS staining between the young and old zones predicted exodermal Casparian band development (Fig. 2.2, C).

PTS was not present in the root tips (Fig. 2.2, B). Even the relatively long time of incubation of roots (24 h) did not result in PTS entry into the meristematic zone. In longitudinally bisected root tips incubated in PTS, only the outermost layer of the intact cells was fluorescent (Fig 2 D). Thus, PTS accumulated only in the meristematic cells that were in contact with the medium containing PTS. Neither media containing PTS in MES buffer at pH 5.7 nor the presence of probenecid prevented PTS entry into the meristematic cells in cut root tips (data not shown).

2.4.3 Mechanism of PTS entry into cortical cells

Observations of well-rinsed cross sections of the green fluorescent part of the roots (2 mm to 6 cm behind root tip) revealed the presence of the PTS in the cells (Fig. 2.2, E, G, H, I). When the roots were incubated in 1.9 mM PTS dissolved in MES buffer, pH 5.7 (0.5 to 25 mM) or in HEPS buffer, pH 8 (0.5 to 25 mM) the pattern of PTS uptake into the cells appeared unchanged (data not shown).

Similarly, pretreatment with probenecid did not inhibit PTS accumulation in young root segments (Table 2.2). However, incubation of young segments with 1.9 mM PTS in the presence of 0.1 mM Cytochalasin B resulted in a 53% inhibition of PTS accumulation. Lower concentrations of Cytochalasin B did not affect PTS accumulation significantly (Fig. 2.3).

A linear correlation between PTS accumulation and its concentration in the incubation medium was observed (Fig. 2.4). With increasing time of incubation of the roots with 1.9 mM PTS there was a linear increase in PTS accumulation (Fig. 2.5).

Optical sections of cells incubated for 10 min in 1.9 mM PTS were obtained by CSLM. Green-fluorescent vesicles were present at the cell periphery (Fig. 2.2, G, H). The cytoplasm

Figure 2.3 Accumulation of PTS in onion root segments treated with cytochalasin B. Empty bar – control, solid bars – treated, amount of PTS accumulated is not different from control. Hatched bar - 53% inhibition of PTS accumulation in the presence of 0.1 mM cytochalasin B, n = 3, bars represent standard deviation.

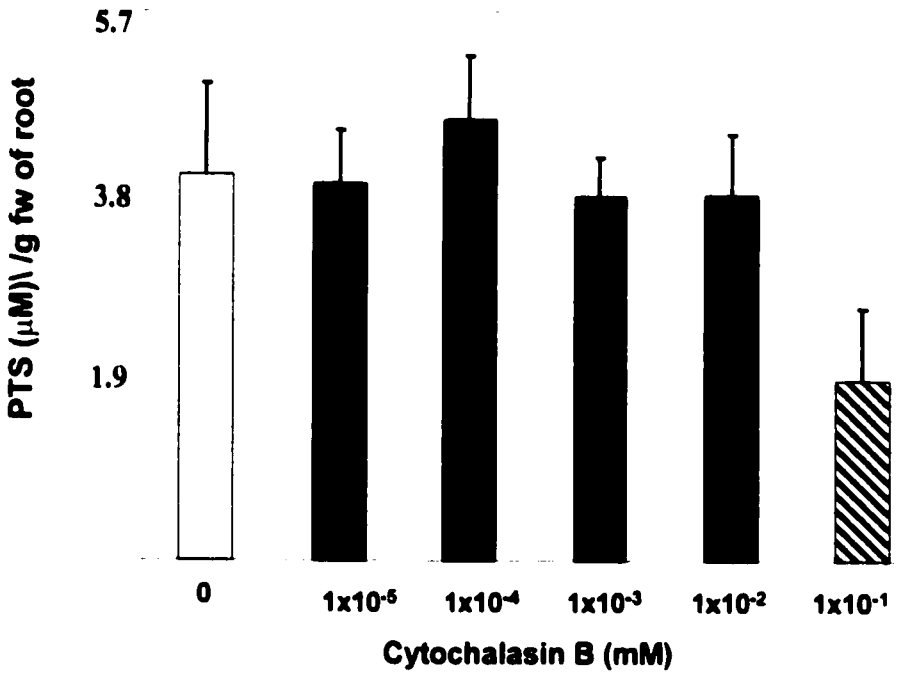

5.7
3.8
1.9
PTS (μM)\ /g fw of root
0
1x10-5
1x10-4
1x10-3
1x10-2
1x10-1
Cytochalasin B (mM)

Figure 2.4 Effect of PTS concentration on its accumulation onion roots. Root segments were incubated in a range of PTS concentration from 0.019 to 1.9 mM for 2 h, n=3, bars represent standard deviation.

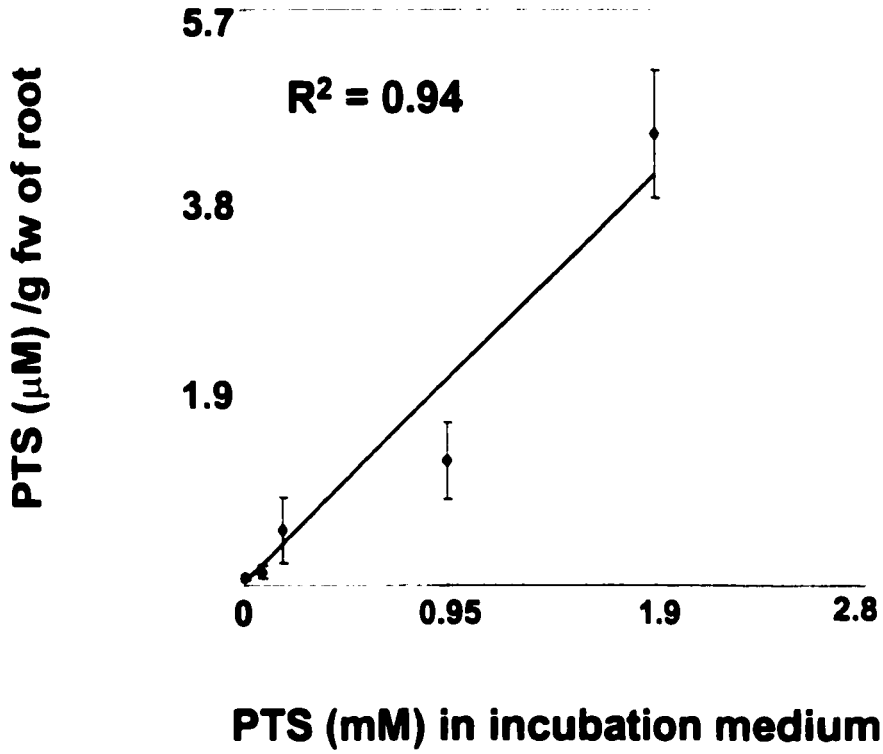

5.7
3.8
1.9
$R^2 = 0.94$
PTS (μM) /g fw of root
0
0.95
1.9
2.8
PTS (mM) in incubation medium

Figure 2.5 Accumulation of PTS in onion root vs. time.

Root segments were incubated in 1.9 mM PTS, n = 3, bars represent standard deviation

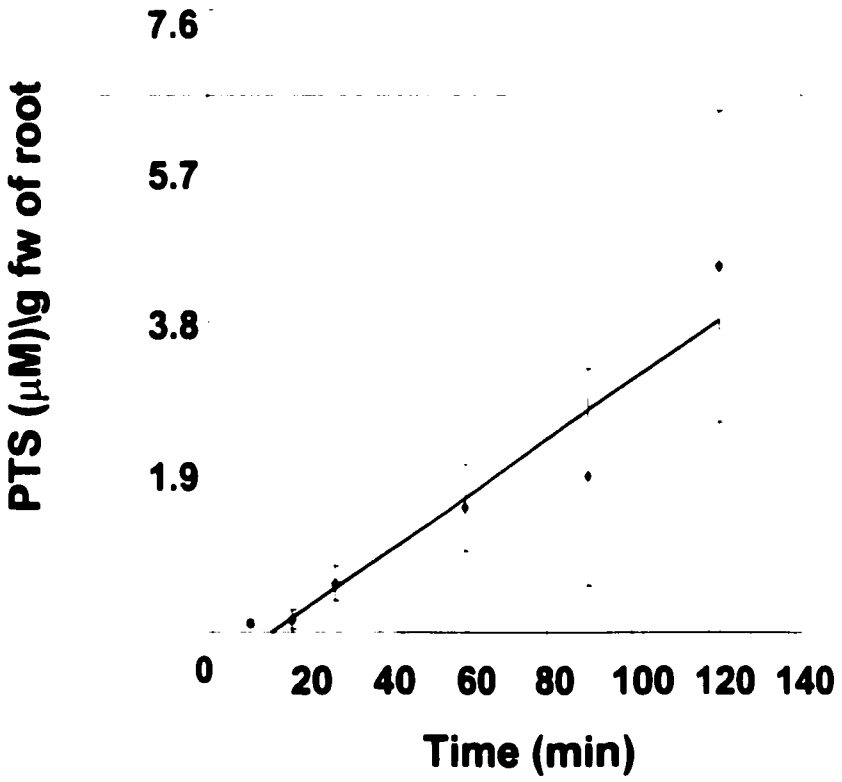

7.6
5.7
3.8
1.9
PTS (μM)\g fw of root
0
20
40
60
80
100
120
140
Time (min)

Table 2.2. Accumulation of PTS in roots treated with probenecid.

Sealed root segments from young zone were incubated increasing concentration of PTS for 2 h in the presence of 0.01, 0.1, and 1 mM probenecid, n=3, ± standard deviation.

PTS (μM)	Control	Probenecid (mM)		
		0.01	0.1	1
19	0.67 ± 0.24	0.85 ± 0.33	0.73 ± 0. 28	1.26 ± 4.2
190	1.96 ± 0. 65	2.85 ± 1.23	6.1 ± 2.1	1.05 ± 24
1900	4.57 ± 1.6	4.75 ± 1.5	3.914 ± 1.3	3.78 ± 1.7

and the vacuole remained PTS-free. Within 30 min, the vacuole started to accumulate PTS, which suggests that the vesicles discharged their contents into this compartment (Fig 2.2, I).

2.5 Discussion

The molecular properties of PTS make it a valuable tracer to detect major apoplastic bypasses in roots. The molecule has an estimated diameter of 1.1 nm for the non-hydrated molecule (Hanson, 1983), and its association with water would increase its diameter. Nevertheless, PTS is mobile within unmodified walls, as their pore sizes range from 3.5 to 5.2 nm (Carpita et al., 1979). The net negative charge on the molecule prevents binding to the numerous negative charges in cell walls. Its properties *in vivo* – high solubility in water, non-toxicity, membrane impermeablity, and retention within live cells - make it ideal for studying fluid-phase endocytosis. Finally, its spectral properties: high fluorescence intensity, little or no photobleaching (observed in this study), and emission maximum at long wavelengths, enable its detection with the light sources available for fluorescence microscopy. In addition, PTS has a wide separation between excitation (405 nm) and emission maxima (510 nm). This property allows a wide range of concentrations PTS to be quantified spectrofluorometrically (Fig. 1). At high concentrations up to 4.77 μM, no fluorescence quenching was detected, and the calibration curve remained linear.

The use of PTS as a tracer for apoplastic bypasses has been reported in several studies (Jachetta et al., 1986; Skinner and Radin, 1994; Yadav et al., 1996; Wang et al., 1997). The recent study of Zimmermann and Steudle (1998) emphasizes that PTS cannot be used as a tracer for water. They conclude that the use of dyes to trace apoplastic water flow should be abandoned, as they found a large discrepancy between water and PTS flow (Zimmermann and Steudle, 1998). Their results are not surprising, given that water is a small, neutral molecule (0.4 nm radius) and can move across cell membranes (reviewed by Steudle and Peterson, 1998). Due to its molecular properties, the larger, charged PTS cannot follow the pathway of water within roots. It can, however, reflect unrestricted apoplastic bypasses present in those

organs (Jachetta et al., 1986; Gracia et al., 1997; Yeo et al., 1987; North and Nobel, 1996). If there was a considerable apoplastic pathway that would allow entry of PTS into the vascular cylinder, such a pathway could easily be utilized by water and ions.

Results of this study show that in young, growing, undisturbed onion roots an apoplastic pathway that would allow the unrestricted entry of PTS to the xylem does not exist with the limits of detection of the instrument. This result confirmed an earlier study, where incubation of onion roots with PTS resulted in absence of this tracer in the leaves (Peterson and Edgington, 1975). By separating PTS from the plant pigment and using a spectrofluorometer in the present study, it was demonstrated that the PTS concentration in the onion leaf extracts is below 2.32 nM (Table 2.1). This indicates that there is no major apoplastic bypass for solute flow in onion roots. However, this result does not completely eliminate the apoplast as a pathway for Ca^{2+} transport, as the barriers that did not permit entry of PTS to the stele may be permeable to smaller cations. Further experiments that establish the apoplastic impermeability of onion roots to Ca^{2+} are presented in subsequent chapters. Apoplastic bypasses were detected using PTS in other studies. For example, Zimmermann and Steudle (1998) found that 0.3% PTS (% of external concentration) was transported in maize. The difference between their results obtained with maize and results of this study may reflect the presence of emerging lateral roots in corn that can cause a temporary discontinuity in the Casparian bands of the endodermis and creation of an unrestricted apoplastic path. The same reason may apply for the higher amount of PTS present in rice (0.3 to 1% of external concentration, Yeo et al., 1987). In contrast, onion roots used in this study had no lateral roots and their endo- and exodermal layers were intact.

It was unexpected to find that injured roots with their conductive xylem elements exposed to external solution were able to filter PTS to some extent, since the cutting open the conducting xylem elements should facilitate a large volume of water flow through the open end. However, the results obtained in this study indicate that some water is transported radially through the remaining part of the root, filtering PTS. Perhaps of some relevance to this finding is that a mercury-sensitive mechanism of water transport in mature zones of onion roots has been demonstrated (Barrowclough and Peterson, 2000). Other workers found a

rapid decline of PTS concentration after its application to cut roots. Twenty four hours after being cut, rice roots filtered the majority of PTS, allowing less than 0.00002% of it to be transported in bypass-flow. Such changes over time may be associated with wound healing processes. In the present study, some PTS was sequestered in the vacuoles of the living cells and this may have contributed (to a minor extent) to the low amount of PTS transported to the leaves

A unique correlation between the presence of PTS in young zones of roots and maturation of the exodermis was observed in this study. Mature zones of roots, where the Casparian bands were deposited in the exodermis, exhibited only blue autofluorescence. In adjacent, younger regions with an immature exodermis, PTS was visible in the root cortex (Fig. 2.2, A). PTS can be used, therefore, as a suitable marker for Casparian band function *in vivo*. Previous anatomical and permeability methods for detection of the exodermal Casparian bands in roots did not allow their detection by external observation (see Introduction), as it was necessary to observe cross sections with a microscope. This is the first report that outlines a technique for detection of exodermal Casparian bands *in vivo*. Preliminary observations indicate that exodermal Casparian bands can also be detected with this method in maize and garlic (unpublished results). This technique has many advantages. It does not kill the roots; thus, it can be used in conjunction with other experiments. It does not require special equipment, as only a simple UV lamp is needed and can be used, therefore, in the field or in any laboratory.

PTS did not enter the meristematic zone and did not accumulate in cells when applied to intact roots. It has been proposed that cell walls in the meristematic zone have smaller pore sizes (Enstone and Peterson, 1992b) than those reported by Carpita et al. (1979) for mature parts of the plant. The results of this study support this interpretation. When the root tips were cut in half and incubated with PTS, its green fluorescence was detected only in the first cell layer exposed by cutting. The presence of PTS in meristematic cells demonstrates that they have an ability to accumulate this tracer, presumably by an endocytotic pathway, as in older cells. The low permeability of the cell walls in apical meristems seems to be a general feature of roots. A somewhat similar pattern of accumulation of fluorescein was observed in pea roots (Dorhout and Kollöffel, 1992; Fig 5). In pea roots, fluorescein accumulated in the cells of the

root cap, differentiation and enlongation zones while it was absent from the meristematic zone of the root. Fluorescein is readily taken up by cells by an ion-trap mechanism, and could reflect the differences in pH along the root. It is also used as a viability test in plant cells, as only living cells can retain this probe in their cytoplasm. Other techniques that allow for a more precise determination of localization of apoplastic tracers also show impermeability of the root meristems. These include the demonstration of exclusion of radioactive calcium and sulphate in maize (Lüttge and Weigl, 1962), lead in onion (Wierzbicka, 1986), aluminium in maize (Rasmussen, 1968), and berberine in onion (Enstone and Peterson, 1992b). Therefore, the findings of this investigation fully support the results of many earlier studies.

This is the second study reporting the presence of PTS in cells, the first being the study by Wright and Oparka (1994), who demonstrated that PTS is accumulated in 5-d-old protoplasts by a probenecid-sensitive mechanism. Three general mechanisms responsible for the uptake and compartmentation of fluorescent probes in plant cells were considered: ion trap, anion transporter, and endocytosis (Oparka, 1991). It is possible that PTS accumulates in the cell cytoplasm by an ion-trap mechanism. This is a well-known process by which neutral molecules are able to diffuse through the PM. The entry of an acidic dye into the cytoplasm is achieved by incubating cells in a medium of pH below the pKa of the dye. Such dyes can be used to visualize localized lowering of pH in the cell walls (Dorhout and Kollöffel, 1992). The apparent pKa of PTS in aqueous buffers is around 7.3 (Haugland, 1996). Thus, incubating roots in the MES buffer at pH 5.7 should facilitate some ion-trap entry of PTS, whereas at pH 8, the majority of PTS should be present in its charged, acidic form. Neither treatment affected PTS accumulation in young zones of onion roots; the presence of PTS in the cells was observed in all conditions. These results exclude the possibility of an ion trap mechanism of PTS accumulation in onion cells.

A second possible mechanism of PTS uptake may involve the transport through a putative, non-specific, organic anion transporter (Wright et al., 1992). Probenecid is a competitive inhibitor of organic acid transport in mammalian cells (Cunningham et al., 1981). In plants, the probenecid-sensitive transport of fluorescent probes occurs mainly in the tonoplast. For example, it has been reported that probenecid inhibited sequestration of fluorescein

isothiocyanate (FITC) into the vacuoles in carrot cells (Cole et al., 1990), Lucifer Yellow in carrot and maize (Cole et al., 1990), and in onion (Oparka et al., 1991). On the other hand, O'Driscoll et al. (1991) demonstrated that in cultured cells of *Morinda citrifolia*, a probenecid-sensitive transport mechanism was present on the PM, not on the tonoplast. More interestingly, Wright and Oparka (1994) reported that probenecid-sensitive transport of PTS developed in 5 d old cultured oat aleurone protoplasts. Young protoplasts up to 5 days did not accumulate PTS. It was suspected, therefore, that a similar transport mechanism for PTS may be present in onion roots. However, treatment with probenecid (in a range 0.01 to 1 mM) did not affect PTS accumulation. Therefore, the possibility that PTS was transported into the cells *via* a non-specific organic acid transporter was eliminated.

A third, and more promising, explanation of PTS uptake involves endocytosis. CLSM observations of sections taken from a young zone and incubated in PTS for only 10 min revealed the presence of fluorescent vesicles at the periphery of the cells. A transmission electron microscope study of onion roots (Chapter 5) revealed that there are different sizes of vesicles, sometimes grouped together as multivesicular bodies. No protein complexes were observed associated with periphery of these vesicles which indicates that they are part of fluid-phase endocytosis, and not receptor-mediated endocytosis (which involves clathrin-coats). Another piece of evidence for fluid phase endocytosis is linear uptake kinetics with respect to the external PTS concentration. It would be desirable to test the endocytotic hypothesis with an inhibitor specific for the process, although such a probe does not exist. Several drugs inhibited endocytosis of Lucifer yellow in *Arabidopsis thaliana* cells (Roszak and Rambur, 1997). The most effective of these, Cytochalasin B, was used in the present study. Cytochalasin B inhibits the polymerization of actin monomers which are involved in the process of endocytosis. At concentrations higher than 0.01 mM, cytochalasin B reduces the growth rate of *A. thalaina* cells to zero, but their viability is retained (Roszak and Rambur, 1997); the drug also inhibits endocytosis. In onion root cells, treatment with 0.1 mM Cytochalasin B resulted in 53% inhibition of PTS accumulation, but lower concentrations were not effective (Fig. 3.3). The effect of the Cytochalasin B on the vitality of onion roots and their ability to perform endocytosis requires further study.

The presence of the endocytotic pathway in the young zone of onion roots may have implications for ion transport. Results that suggested a vesicle-mediated salt accumulation were obtained in a study using *Nitella translucens* as a model system (MacRobbie, 1999). Analyzing the kinetics of uptake of Cl^- and Br^- it was observed that transport of these ions into the vacuoles had two phases: a fast phase important at short times, and a slow phase whose contribution increases with time. The fast phase was attributed to vesicle-trafficking into the vacuole, since there was an equal amount of Cl^- and Br^- transported, despite a strong preference in favour of Cl^- at the PM (MacRobbie, 1999). The discrimination between ions at the PM may arise from the involvement of ion-specific transporters, whereas the lack of discrimination is consistent with the non-specific solute uptake during endocytosis, assuming that PM-derived vesicles discharge their contents into the vacuole. It is of great importance to study the contribution of endocytotic pathway to total ion accumulation in plant roots. Involvement of endocytosis and exocytosis may be beneficial to roots and may allow transport of Ca^{2+} through the cytosol without altering its concentration and activation of the signal transduction pathway.

Chapter 3

Where is the most important and efficient site for Ca^{2+} uptake and translocation in onion roots?

3.1 Abstract

The sites of Ca^{2+} uptake and its subsequent translocation were investigated along the axis of onion root. Three developmentally different zones were exposed to $^{45}Ca^{2+}$ as a tracer. They were: (1) the root tip where Casparian bands matured 5 to 10 mm from the apical meristem and all xylem vessels were immature; (2) the young zone between 15 and 30 mm from the root tip, where the Casparian band was present in the endodermis only, and early metaxylem vessels were functioning; (3) the mature zone, above 100 mm from the root tip where, in addition to the endodermal Casparian bands, there were Casparian bands and suberin lamellae in exodermis, and where early metaxylem vessels were mature. $^{45}Ca^{2+}$ applied to the root tip was not translocated from this zone; radioactivity was detected exclusively in the exposed segment. Application of $^{45}Ca^{2+}$ to the young zone resulted in the highest translocation, as 34 % of the absorbed $^{45}Ca^{2+}$ was detected beyond the zone of application, i.e. in the non-exposed, proximal part of the root and in the leaves. Translocation from mature segments was reduced, as 14 % of the total absorbed $^{45}Ca^{2+}$ was recovered from non-exposed parts of the roots and

leaves. Translocation of $^{45}Ca^{2+}$ occurred only in one direction – towards the base of the roots, and subsequently to the leaves. Results of this study illustrate that xylem-designated $^{45}Ca^{2+}$ is not by-passing the endodermal Casparian band around the root tip in its route to the stele. Furthermore, assuming that the Casparian band is blocking the apoplastic pathway, the evidence of translocation of $^{45}Ca^{2+}$ from young and mature zones suggests that $^{45}Ca^{2+}$ enters the root symplast on its radial pathway to the xylem. In the mature zone, the symplast of both the exodermis and endodermis would be involved.

3.2 Introduction

At the beginning of the last century, the German botanist Haberlandt wrote, "Absorption is not carried out over the whole surface of a subterranean root system. It can, on the contrary, be easily shown that the functional absorbing tissue only occurs in the youngest rootlets and that it is, moreover, confined to a defined zone" (Haberlandt, 1914). Since then, modern techniques have enabled analysis of the structure and function of root systems, as well as definition of the most effective sites of ion uptake.

Growing roots vary both anatomically and physiologically along their longitudinal axes. The region of the root tip is a site of cell division and intensive protein synthesis. This is followed by cell differentiation, starting with vacuolation 5 mm behind the meristem (Russell, 1977). Further back, elongation of the cells occurs as well as maturation of the first conducting tissues in the stele. As protoxylem maturation proceeds, there is a deposition of Casparian bands in the endodermis (Danilova, 1981; Peterson and Permualla, 1984), changing the permeability of the apoplast. Mature zones of the roots may develop a second set of Casparian bands in their exodermis, as was documented for many angiosperm species (Permualla et al. 1990; Peterson and Permualla, 1990). It is now well established that there are differences in ion absorption associated with root age, i.e., the distance from the root tip (Clarkson, 1968).

The ions present in the solution outside of the roots have to be transported across the root tissues and delivered to the xylem for long-distance transport to the aerial parts of the plants.

Ions moving across the root can follow two paths, symplastic, where ions migrate from protoplast to protoplast through plasmodesmata, and apoplastic, in which protoplasts are by-passed and ions move through pores in the cell walls. The general pathway for radial transport of mineral nutrients from outside of the root to the stele is believed to be through the symplastic continuum (Marschner, 1986). For example, the uptake and translocation of phosphate and potassium take place over entire root systems of plants that are several weeks old (Russell, 1977). In contrast, the transport of Ca^{2+} is still described as occurring along the "apoplastic pathway" as the peaks of its absorption have been detected in young zones of the roots (Clarkson, 1991).

There is evidence that Ca^{2+} moves preferentially, if not exclusively, in the apoplastic pathway in its passage across the root (Robards et al., 1973). In barley, the Casparian bands in the endodermis do not develop until 5 to 7 mm from the root tip, which suggests that there is a zone up to 7 mm in length that lacks a barrier for radial apoplastic transport. This fact, taken together with the observation that protoxylem elements were devoid of their cytoplasmic content (which means that they were mature), lead to the conclusion that there is a substantial apoplastic pathway for unrestricted ion transport to the stele (Robards and Jackson, 1976). Russell and Clarkson (1976) have studied Ca^{2+} uptake and translocation in the roots of barley (*Hordeum vulgare*) and melon (*Cucurbita pepo*), and found that Ca^{2+} was rapidly accumulated in the stele when applied near the root tip, but not when applied to the older segments (Russell and Clarkson, 1976). It was further shown that the ability of the root to translocate absorbed Ca^{2+} diminished with increased suberization and thickening of the endodermal cell walls (Robards et al., 1973). Collectively, these reports favour a passive movement of Ca^{2+} within the root with negligible or slow movement in the symplastic pathway.

Since the continuity of the apoplastic free space does change with root growth and development in onion, this study was undertaken to determine the zone from which Ca^{2+} is most efficiently translocated to the aerial parts. Using onion roots as a model system has many advantages, as outlined in Chapter 1, but the principal one for this set of experiments is

the fact that young, growing roots do not develop laterals, the presence of which could influence the pathway of Ca^{2+} transport.

3.3 Materials and methods

3.3.1 Plant material

Onion roots were sprouted from bulbs grown in vermiculite-filled pots in the greenhouse as described in Chapter 2. Plants used in this experiment were 12 days old, with the longest roots being 160 mm. Roots were actively growing and did not reach the bottoms of the pots, and no lateral roots were present in entire root systems used in each experiment.

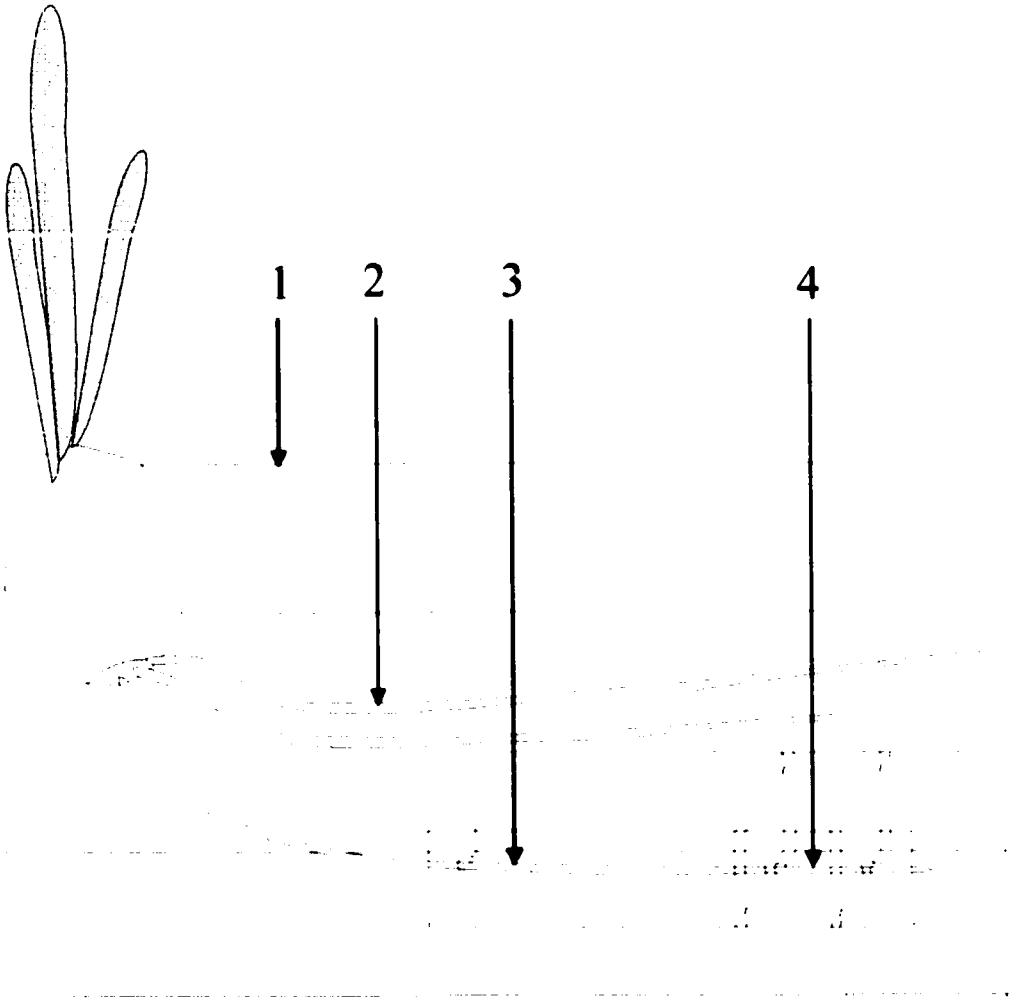
3.3.2 Treatment of various root zones

Radioactive Ca^{2+} was applied to specific zones of the root by means of a particular set of chambers (Fig. 3.1). The uptake assembly included a tray with a lid, and an incubation chamber with double partitions. An entire onion with its intact root system was placed in the tray, which was lined with wet, absorbent paper. Individual roots were placed in the incubation chamber, where partitions were sealed around the zone of interest (Fig 3.1). The sealant consisted of 90% lanolin and 10% parowax (Cronos Co.), heated and mixed together (Barrowclough and Peterson, 2000). Such seals around the roots effectively prevented leakage during the duration of the experiment (24 h).

The sealed incubation chamber held 30 ml of 1 mM $CaSO_4$, labeled with $^{45}CaCl_2$ (3.7×10^{-4} Bq ml^{-1}). At the beginning of the experiment, 100 µl of the labeled solution was removed to measure initial radioactivity in the medium, then the entire assembly was covered with the lid lined with wet absorbent paper in such a way that only the onion leaves were exposed to the atmosphere and allowed to transpire. Leaves were exposed to continuous light supplied from a bulb at an intensity of 100 µmoles/s/cm^2. The tray was placed on a balance to measure

Figure 3.1 Diagram of the apparatus for studying Ca^{2+} uptake along an onion root axis.

1- Plexiglass container holding an assembly. It was covered with a lid so that only the leaves were exposed to the atmosphere during experiments. 2 - one of the control roots not exposed to $^{45}Ca^{2+}$, covered with vermiculite during the experiment. 3 - experimental root exposed to $^{45}Ca^{2+}$ placed within the container with removable partitions. Openings in partitions were occluded with a sealant. 4 - segment of the root exposed to $^{45}Ca^{2+}$. Radioactivity was measured in the incubation solution, the exposed segment, apical zone, basal zone, and in the bulb and leaves. Control roots were used for determination of the maturation of Casparianthe bands, maturation of the xylem elements, as well as being assayed for $^{45}Ca^{2+}$. In addition, the solutions between the double partitions were assayed for the presence of $^{45}Ca^{2+}$ to determine whether any leakage occurred during experiment. In cases of leakage, the experimental data were discarded. The openings in the partitions to accommodate the root were slightly nabove the base of the container.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weight loss due to transpiration. The remaining, non-exposed roots of the same onion were sprayed with a non-labeled solution of 1 mM $CaSO_4$ and covered with wet vermiculite.

Experiments were run for 24 h. At the conclusion of each experiment, radioactive solution was removed, and the exposed segment washed with 3 changes of 1 mM $CaSO_4$ for 10 min. The solution on each side of the partition was probed for radioactivity to detect any leakage from the treated chamber. Any data from experiments with leakage were discarded.

To measure the actual length of the exposed segment, the incubation chamber was filled with Toluidine blue O (Fisher Inc.) and incubated for 5 min. After removal of dye, the root was measured and divided into 3 parts, which included: the treated segment, the basal part of the root, and the apical part of the root. The bulb and the leaves were separated and the remaining, non-exposed roots were cut from the bulb and one of them was used as a control for radioactivity counting. Other roots of the same length were used for determination of their anatomy and development. All parts of the onion were ashed at 500°C overnight, cooled, made up to 5 ml with 0.2 N HCl and counted in liquid scintillation counter set on channel 8 for 10 min (Searle Analytic Inc., Model Mark III) upon addition of scintillation liquid (Ecolite, ICN) to a total of 20 ml.

3.3.3 Anatomical observations and determination of xylem maturation

Casparian bands were detected as described previously (Chapter 2) using the procedure of Brundrett et al. (1988). Suberin lamellae were detected by staining sections with Fat red (Sigma, St. Louis, Mo) and observing them under white light with a microscope. Xylem maturation was determined with the method of Peterson and Steudle (1993). This involved cutting the root at an oblique angle 3 cm above the root tip. The cut segment was immobilized on a slide by a smear of vaseline. The cut part of the root was quickly immersed in 0.001% Cellufluor (Polysciences Inc. Worrington, PA) and the entire assembly placed in a humid chamber for 2 h. Root segments were subsequently removed, and cut ends rinsed. Whole roots were flattened between two slides, and blue-white fluorescence in the conducting

xylem was observed with epifluorescence optics in a Zeiss microscope (Fig. 3.2, A). Results were recorded on Ektachrome ISO 200 colour slide film. Filter sets used for observations were the same as described in Chapter 2.

3.4 Results

3.4.1 Developmental stages of the root segments used in experiments

The root tip zone (5 mm) included the root cap, apical meristem and differentiating, but immature xylem elements in the stele (Fig. 3.2, B, C, D). Casparian bands in the endodermis were detected, on average, 8 mm behind the apical meristem (Fig. 3.2, D). This treatment ensured that externally applied $^{45}Ca^{2+}$ had an access to the part of the root tip where Casparian bands were not yet deposited.

The young zone of the root (1.5 to 3 cm behind the tip) had Casparian bands present in the endodermis (Fig. 3.2, D). Functioning xylem elements were also present, as treatment of the roots with Cellufluor allowed visualization of the conducting vessels, which matured, on average, 1.3 cm behind root tip (Fig. 3.2, A). This zone, therefore, was characterized by the presence of both functional xylem and an endodermal Casparian band. No modification of the cell walls in the exodermis was detected. Therefore, externally applied $^{45}Ca^{2+}$ had unrestricted access to the cortical free space in the apoplast up to the endodermis.

The mature zone (10 cm above the root tip) had two sets of Casparian bands (Fig. 3.2, E). The outermost one was present in the exodermis. In addition to the Casparian bands, long cells of the exodermis developed suberin lamellae. Short cells of the exodermis were without suberin lamellae (Fig. 3.2, E). The second set of Casparian bands was present in the endodermis. Suberin lamellae were observed in the cells opposite the phloem poles, whereas in the endodermal cells opposite the xylem poles suberin lamellae were not detected (Fig. 3.2,

Figure 3.2 Onion root - anatomical characterization of various zones used in present study.

A. Onion root tip exhibiting a blue-white fluorescence of Cellufluor white introduced to the xylem. Arrow indicates the point of maturation of early metaxylem vessels which were conductive and stained with Cellufluor. Bar = 2 mm.

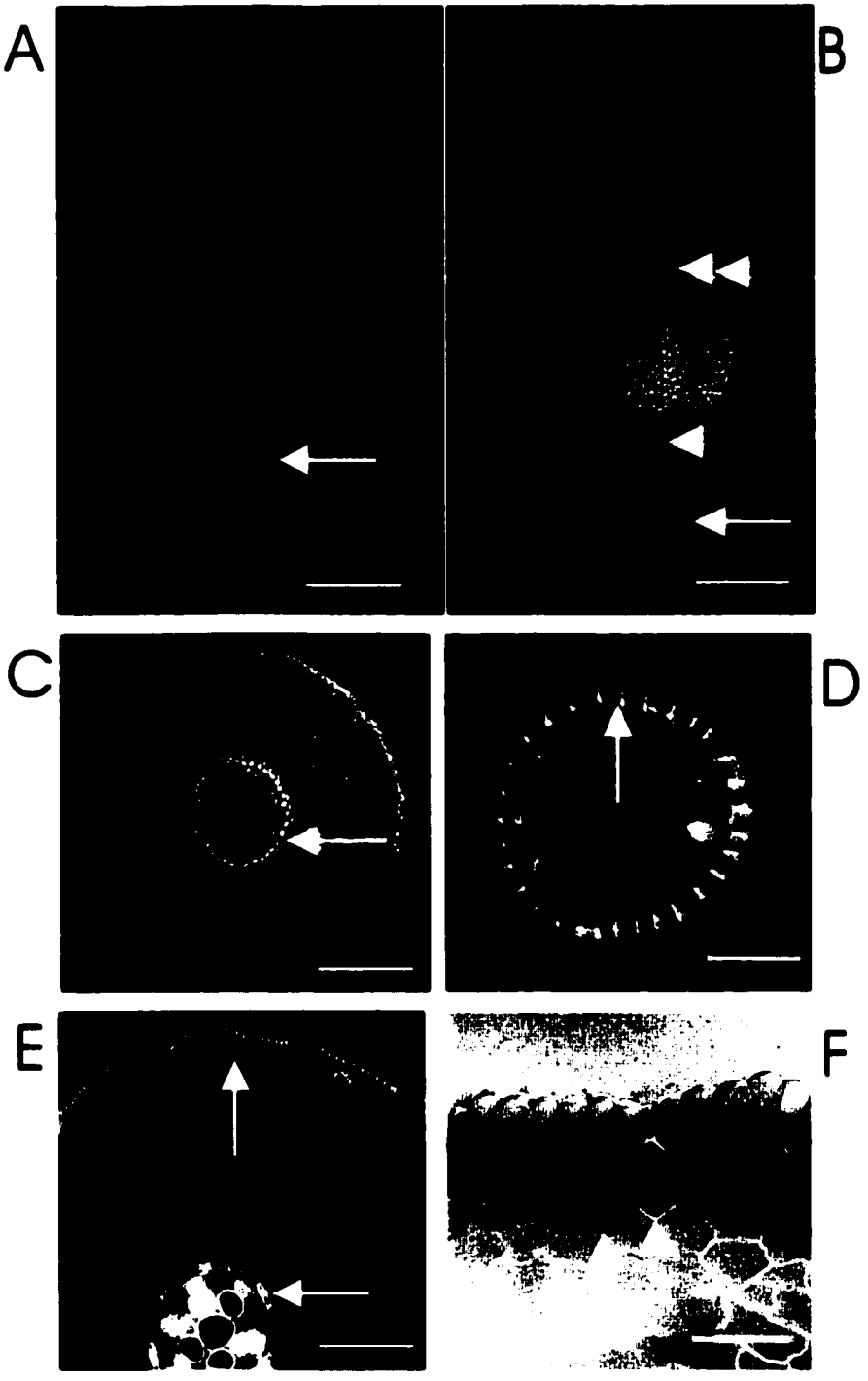
B. Median longitudinal section through root tip; stained with berberine. Arrow points to a root cap, arrowhead indicates apical meristem, double arrowheads are pointing to the differentiating stele. Bar = 5 mm

C. Cross section of the root tip, 3-4 mm behind meristem; stained with berberine. Arrow points to the differentiated stele (no Casparian bands were observed). Bar = 3 mm.

D. Casparian bands in endodermis, section taken 8 mm behind root tip. Note that xylem vessels in the stele are not yet lignified. Bar = 1 mm

E. Casparian bands in the exodermis of mature zone of the root that blocked root permeability to PTS. Some of the endodermal cells have deposited suberin lamella while others remain without suberin lamellae (i.e., are passage cells). Bar = 2 mm.

F. Mature zone as in E. Section stained with Fat Red to visualize suberin lamellae in the exodermis (arrow). Cell to the right of the arrow is a passage cell without a suberin lamella (arrowhead). Bar = 0.5 mm.


A
B
C
D
E
F

E). It was assumed that at this distance from the root tip, the early metaxylem vessels were fully functional. No lateral root development was observed along the entire axes of the roots used in experiments.

3.4.2 Determination of the amount of Ca^{2+} in the tissues

The amounts of radioactivity detected from control, non-exposed roots were at the level of the control, unlabeled incubation solution (data not shown). This result demonstrates that no Ca^{2+} was redistributed from the treated root to other roots on the same bulb. Having established lack of redistribution in the preliminary experiments, only one control root was ashed and its radioactivity counted as an assurance of the lack of leakage or contamination with radioactive label during the course of the experiments.

3.4.2.1 Apical zone

Upon application of the $^{45}Ca^{2+}$ to the apical zone of the root, radioactivity was detected in the labeled segment only. The remaining part of the root, as well as the bulb and the leaves, were free of label (their DPM were not different from control, non-exposed onions). The observed accumulation of Ca^{2+} which was, on average, 38 nmoles of Ca^{2+}/root tip, was independent of the amount of water transpired during the experiments (Table 3.1). The differences in amounts of water transpired can be attributed to the size of the leaves of onions used in experiments. (The number of leaves depended on the bulb size used for sprouting adventitious roots.) During the 24 h incubation period, root tips grew an average of 3 mm, which give a total of 8 mm of exposed apical zone. This increase in length did not induce significant developmental changes, as no mature xylem elements were present in this zone. (They matured, on average, 1.3 cm from the tip.)

3.4.2.2 Young zone

Application of $^{45}Ca^{2+}$-labeled solution to the root segment in the young zone (1.5 to 3 cm behind the root tip) resulted in 35% translocation of the label from the site of application (Table 3.2). The total uptake of Ca^{2+} was 99 nmoles/1.3 cm segment, from which treated

Table 3.1 Accumulation of Ca^{2+} in the tips of onion roots.

Amount of Ca^{2+}in the tissue wa calculated based on radioactivity recovered from the tissue after 24 h exposure to 45 Ca^{2+} -labbeled 1 mM $CaSO_4$.

Replicates	Water loss due to transpiration (g)	Ca^{2+} (nmoles per root tip)
1	21	46.3
2	15	20.1
3	8	35.7
4	10	52.3
Average	13.5	38.6
±SD	5.8	14.1

Table 3.2 Uptake and translocation of $^{45}Ca^{2+}$ from young zone of the root.

Replicate	Water loss due to transpiration (g)	Ca^{2+} (nmoles /1.3 cm root segment)			Translocation (%)
		Treated segment	Translocated	Total	
1	10	61	28	89	31
2	8	52	27	79	34
3	8	60	36	96	38
4	9	88	48	135	35
Average	8.7	65	34.7	99.7	34.5
±SD	0.95	15.6	9.7	24.8	2.7

segments accumulated 65 nmoles Ca^{2+} and 34 nmoles were translocated (i.e., recovered as radioactivity in the remaining part of the root, bulb and leaves). In this zone, the maximum translocation of Ca^{2+} occurred; it was twice as much as the translocation from mature zone (Table 3.3). No $^{45}Ca^{2+}$ was detected distal to the root tips. Therefore, Ca^{2+} was translocated in one direction only: towards the root base, and into the aerial parts of the onion. The uptake and translocation of Ca^{2+} was not correlated with the amount of water transpired.

3.4.2.3 Mature zone

Application of $^{45}Ca^{2+}$-labeled solution to the root segment in the mature zone (10 cm or more proximal to the root tip) resulted in 14% translocation of Ca^{2+}(Table 3.3). Total uptake was 80 nmoles Ca^{2+}/1.3 cm segment, from which treated segments accumulated 69 nmoles Ca^{2+}, and 11 nmoles were recovered as radioactivity in the remaining proximal part of the root, bulb and leaves. As in the young zone, the Ca^{2+} was translocated in one direction only – towards the root base, and into the aerial parts of the onion. No radioactivity was detected in the distal part of the root. In this zone, the translocation of Ca^{2+} was half of the amount of that from young zone (Table 3.2). Statistical analysis showed a significant difference in the amount of translocated Ca^{2+} from young and mature zones, as Student T test revealed $P = 0.005$ at $\alpha = 0.5$.

3.5 Discussion

The entry of an ion in one part of the root system can vary greatly depending on its absorption in other parts, as well as by other root members (Russell, 1977). Thus, the inherent ability of different parts of a root system to absorb and translocate nutrients can be shown unequivocally only if all parts of the root system are exposed to the same external ion concentration (Clarkson, 1974). This requirement was met in the present study, as the entire root system was exposed to the same $CaSO_4$ concentration (1 mM), and the $^{45}Ca^{2+}$ was added only to one zone of the root, from whence its translocation was measured. Therefore, there was no difference in the roots' environment which might have caused variations in the Ca^{2+}

Table 3.3 Uptake and translocation of Ca^{2+} from mature zone of the root

Replicate	Water loss due to transpiration (g)	Ca^{2+}/ (nmoles/1.3 cm root segment)			Translocation (%)
		Treated segment	Translocated	Total	
1	20	53	10	63	5.7
2	18	56	10	66	15
3	22	69	7	76	8.7
4	12	97	18	115	16
Average	18	69	11	80	13.75
±SD	4.32	20	4.9	23.99	3.48

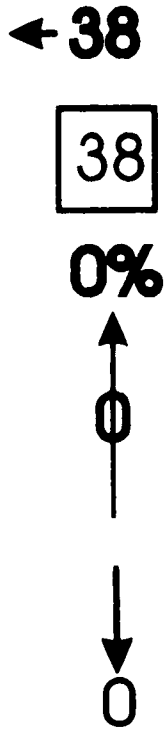
uptake. However, this approach may be responsible for low counts of radioactivity and overall small percent of translocation of the total Ca^{2+} absorbed in the labeled segment. A different approach was used to study K^+ transport in barley roots. The single root used to trace the uptake rate was continuously exposed to 1 mM K^+, and its ability to transport K^+ to the xylem was enhanced by depriving the other roots of their K^+ supply (Drew et al., 1990). Such an approach would be unsuitable for a study of Ca^{2+} uptake, as roots require a continuous supply of this essential element (Marschner, 1986). However, in the field, the distribution of Ca^{2+} and its concentration in the soil solution may not be uniform. Therefore, roots would have to acquire Ca^{2+} in the zones where Ca^{2+} is present.

The only apoplastic pathway that exists in young, growing onion roots is at the root tip, where the endodermal Casparian bands are not yet deposited. In this study no translocation of Ca^{2+} occurred from such an apical zone. Since development of the Casparian bands in the walls of the endodermis occurred 5 to 10 mm behind the root apical meristem, there was a potential apoplastic pathway for Ca^{2+} to be transported to the stele. If such transport occurred, this Ca^{2+} must have been incorporated into the rapidly dividing and growing cells in the apical region, and not translocated. All of the absorbed Ca^{2+} remained in the tip (Fig. 3.3). This finding eliminates the possibility that Ca^{2+} may be loaded into the xylem *via* an apoplastic pathway, by-passing the endodermal Casparian bands around the root tip. Due to the absence of mature xylem elements in the apical zone (Fig. 3.2, A, C, D), it can be assumed that long distance transport of any solute from this zone to the aerial parts of the onion is virtually impossible. The research on water transport in roots led Steudle and Peterson (1998) to call tips of maize roots a "hydraulically isolated zone", as no long distance transport of water occurred from this region, where early metaxylem vessels were not mature (Steudle and Frensch, 1996; Azaizeh and Steudle, 1991).

In young zones of onion roots (1.5 to 3 cm behind root tip), externally applied Ca^{2+} had access to the all cell layers of the root up to the anticlinal walls of the endodermis, where the Casparian bands were deposited. The maximal translocation of Ca^{2+} from this zone (Fig. 3.3) may reflect the fact that externally applied $^{45}Ca^{2+}$ had access to the large surface area of the plasma membrane (PM) of cortical parenchyma cells, as well as PM in the outer tangential

Fig 3.3 Sumary of calcium uptake and translocation along the root axes in intact onion.

Boxes indicate treated zones.

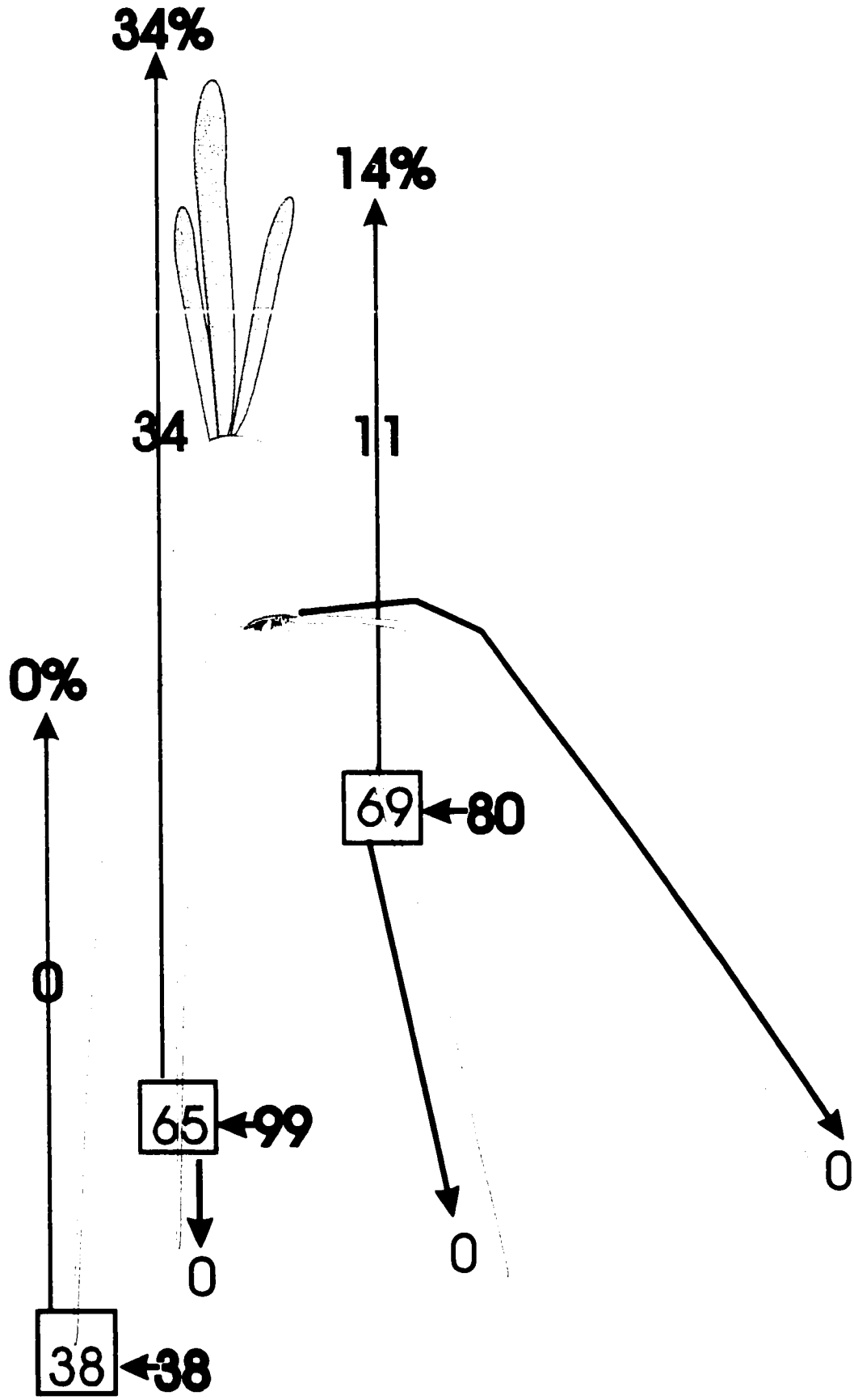


Total Ca^{2+} uptake in apical zone. (Young and mature zones are indicated on subsequent roots).

Ca^{2+} retained in apical zone. (Young and mature zones are indicated on subsequent roots).

Green number: amount of Ca^{2+} (nmoles) translocated from the treated zones.
Red number: Ca^{2+} translocated (percent of total taken up).

Ca^{2+} translocated towards the root tip from treated segment. or relocation from treated to non-treated root on the same bulb.


34%
14%
34
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0%
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69
80
65
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38
38

surface of the endodermis. Since in this region there is no deposition of suberin lamellae in endodermal cells, all of them may contribute equally to Ca^{2+} transport to the stele. The early metaxylem elements were mature and conductive in this zone, facilitating a low-resistance pathway to the shoot. Similarly, the net Ca^{2+} influx in the young zone (7 to 40 mm from the root tip) of maize roots was directly demonstrated by use of a microelectrode (Shabala et al., 1997). This influx may reflect the Ca^{2+} uptake into the root cells. However, microelectrode measurement revealed also the presence of Ca^{2+} efflux from the cells (Shabala et al., 1997). This efflux may be caused by active Ca^{2+} extrusion from the cells, as a means of regulating its level in their cytosols during steady state conditions.

In the mature zone of the root (above 10 cm from root tip), in addition to the partial deposition of suberin lamellae in endodermis, a Casparian band was developed in the exodermis (Fig. 3.2, E). The long cells of exodermis had deposited suberin lamellae (Fig. 3.2, F). Thus, the externally applied Ca^{2+} had access only to the cells of epidermis and non-suberized short cells of the exodermis. The observed translocation of Ca^{2+} from this zone (Fig. 3.3), as in the younger zone, indicates that externally applied Ca^{2+} was transported symplastically in its radial pathway to the stele, where the xylem elements were mature and conductive.

The contribution of each zone to the total Ca^{2+} translocated can be estimated based upon the distances from the root tip where the early metaxylem and endodermis matured. The average length of the roots used in this experiment was 15 cm. The apical part of the root, up to 1.3 cm behind the root tip did not contribute to Ca^{2+} translocation. The Casparian bands and suberin lamellae in the long cells of the exodermis developed simultaneously at a distance of 4 to 4.5 cm behind root tip. Therefore, the young zone (with conductive xylem elements and no deposition of Casparian bands in the exodermis) can be estimated as 3 cm long. Based on amount of Ca^{2+} translocated by a 1.3 cm long segment (Table 3.2) this zone could translocate a total of 78.4 nmoles of Ca^{2+}.

The mature zone, which was 10 cm long in roots used for the experiments, could translocate 86.4 nmoles Ca^{2+} (estimated from 11.24 nmoles translocated from a 1.3 cm segment, Table

3.3). It can be concluded that the young and mature zone would contribute approximately equal amounts of translocated Ca^{2+} in growing onion roots.

The results of this study do not agree with the findings of Russell and Clarkson (1976), where a fluctuation in Ca^{2+} absorption and translocation was detected along barley roots. They demonstrated that the maximal absorption (around 0.9 nmoles/mm^3/day) and translocation of Ca^{2+} occurred from the zone 0.2 cm from root apex. About 76% of the total absorbed Ca^{2+} was translocated. In the next zone, 1 cm behind root tip, a decrease in the amount of absorbed Ca^{2+} was observed (0.26 nmoles/mm^3/day), of which 72% was translocated. A relative increase was observed in the zone 5 cm from the root tip (absorbed 0.4 nmoles/mm^3/day, from which 59% was translocated). In the zones above 15 cm from the root tip, a decrease of uptake to 0.13 nmoles/mm^3/day was observed, from which only a small fraction of Ca^{2+} was translocated. In a developmental study of barley roots, Danilova (1981) reported that in the apical zone (3 to 5 mm behind root tip) there were no developed Casparian bands in the endodermis; furthermore, protoxylem tracheary elements were not mature. Maturation of these elements occured 6 to 9 mm from root tip and was correlated with completion of the formation of the Casparian bands in the endodermis (Danilova, 1981). Unless the growing conditions changed the development of barley roots in Russell and Clarkson's (1976) study and/or there is a different mechanism for Ca^{2+} transport in barley than in onion, it is difficult to explain how 72% of absorbed Ca^{2+} was translocated from the apical zone of barley roots in the absence of mature xylem vessels.

No such fluctuation was reported for *Cucurbita pepo* roots, where Ca^{2+} absorption and translocation was measured along the root starting at 2 cm from the tip (Harrison-Murray and Clarkson, 1973). At 2 and 3 cm from root tip, the maximum uptake (11 to 13 nmoles/mm^3/day) and subsequent 50% translocation of Ca^{2+} was detected. Older parts of the root, above 10 cm from the root tip, were absorbing between 8 and 10 nmoles/mm^3/day and translocated about 20% of it. A similar absorption was observed even at a distance of 50 cm from the root tip, but only a trace of it was translocated (Harrison-Murray and Clarkson, 1973). The findings of present study are similar to those reported for *C. pepo*, as the maximum of Ca^{2+} translocation was found in the young zone of onion roots.

Interesting results were reported in the study of Marschner and Richter (1973). They applied $^{45}Ca^{2+}$ to 3 cm zones at distances of 0-3, 6-9, and 12-15 cm from the root tip of maize. They found a uniform translocation in all these zones of 2.2 to 2.4 microequivalents per 12 plants in 24 h. However, total Ca^{2+} absorption decreased with the root age from 6.5 to 2.8 microequivalents per 12 plants in 24 h, in the youngest and oldest zones, respectively (Marschner and Richter, 1973). These results indicate that in maize, as in onion (this study), there is a translocation from the mature parts of the root, probably *via* a symplasic pathway.

The studies of Ca^{2+} fluxes in wheat using microelectrodes demonstrated that there is a 4-fold greater Ca^{2+} influx at 1 and 2 mm back from the root apex than at 5 mm or more from the root tip (Huang et al., 1992a). Furthermore, a subsequent study (Huang et al., 1993) demonstrated that the 5 mm apical zone of wheat root translocated twice the amount of Ca^{2+} than a more mature zone. These results are in agreement work of Russell and Clarkson (1976). Huang et al. (1993) reported, however, that the treated apical segment increased in length by 8 to 10 mm during the duration of the experiment (24 h) to a total length of 14 mm. It is possible, therefore, that maturation of xylem occurred and facilitated Ca^{2+} translocation from the apical segment to the basal part of the root in their experiments.

In the present study, the peak of Ca^{2+} translocation was detected in the young segment of the onion roots (1.5 to 3 cm from the root tip). This result is in agreement with findings of Robards et al. (1973). In their work, application of $^{45}Ca^{2+}$ to restricted portions of intact roots of barley resulted in determination of the maximum $^{45}Ca^{2+}$ translocation site, which occurred between 3 to 6 cm behind root tip, in seminal and nodal roots. In lateral roots, this maximum was observed between 1.75 and 3.75 cm behind root tip. Accordingly, the translocation mechanism of this particular site of the root may be associated with the maturation of the stelar conducting xylem elements. In addition to the detected translocation peak (3 to 6 cm behind root tip) in barley roots, a considerable amount of Ca^{2+} was translocated from the distance of 2 cm behind root tip, as well as from the mature zone above 10 cm from the root tip (Robards et al., 1973).

The difference in translocational efficiency depending on the distance from the apex suggests that the specificity and activity of the root cells for Ca^{2+} uptake undergo changes during cell growth and development. The observed higher translocation of Ca^{2+} from young zones of the roots can be explained as a consequence of two processes. Firstly, there is an increased accessibility of the absorbing surface of the PM to ions in this zone (Kamula et al., 1994), as ions can penetrate in the continuous free space of the cell walls up to the endodermis. This point of view is supported by most researchers and represented in almost all textbooks on ion transport (Clarkson, 1974; Epstein, 1972; Sutcliffe and Baker, 1974) and on plant physiology (Salisbury and Ross, 1978). Secondly, it is possible that young cortical cells have an increased capacity for ion uptake. The results of the study of Van Iren and Boers-van der Sluijs (1980) demonstrated that young cortex cells in barley roots possessed a primary absorption capacity, which was comparable to that of the epidermis of the mature zone of the root. Furthermore, from the results of autoradiography of the distibution of ^{86}Rb, they concluded that this special feature of young cortical cells is lost during cell differentiation, as in the young zone, all cell layers were labeled, but in the mature region, only the epidermis was labeled, even after root plasmolysis (Van Iren and Boers-van der Sluijs, 1980).

It is likely that both explanations are correct, and the increased absorption and translocation of Ca^{2+} in the young zone is due to the cooperative action of the above-mentioned processes. Further purification of Ca^{2+} transporters followed by their *in situ* localization may elucidate which mechanism is predominant.

In barley roots, simultaneous application of $^{45}Ca^{2+}$ and $^{28}Mg^{2+}$ along the axis of the root resulted in similar translocation of these ions (Ferguson and Clarkson, 1976). Application of these radioisotopes to the zone 1 cm from the root tip yielded 45 and 56% translocation of Ca^{2+} and Mg^{2+}, respectively. The most effective segment for uptake was 6 cm behind the root tip, where translocation was over 70% for both ions. When applied to the base of the root, 30 cm from the root tip, translocation was negligible (Ferguson and Clarkson, 1976). This study did not correlate the anatomical development of the roots with the sites of uptake; nevertheless, it can be assumed that at a distance of 6 cm from the root tip, there were mature Casparian bands in the endodermis. The negligible translocation of ions from the root bases

(30 cm behind root tip) was assumed to reflect deposition of suberin lamellae, which restricted the apoplastic pathway. In the experiments reported by Ferguson and Clarkson (1976) barley roots were grown hydroponically, and the studied basal segments adjacent to the shoot might be exposed at times to the humid air above the nutrient solution. Such exposure induces the rapid development of an exodermis, and deposition of suberin lamellae in its cells (Enstone and Peterson, 1998). Thus, the lack of ion translocation in this zone could well be due to the modification of the outer cell layers in the roots caused by the exposure of the segment. Yet, the similar pattern of Ca^{2+} and Mg^{2+} translocation indicates that both ions are moving radially *via* similar pathways, despite the common assumption that Mg^{2+} is more mobile within plant tissue and can be readily translocated *via* the symplast while Ca^{2+} is not.

In the present study, the mature zone (above 10 cm from the root tip) translocated 14% of the total absorbed Ca^{2+}. In order to be translocated, Ca^{2+} must move through both the exodermis and endodermis. At this distance from the root tip, both had developed suberin lamella in addition to the Casparian bands. The ability of onion root to translocate Ca^{2+} from this zone strongly suggests that Ca^{2+} is moving in the symplast on its radial pathway to the stele. Translocation of Ca^{2+} from a mature zone of the root is not unique to onion, as similar results were found for maize roots, where application of $^{45}Ca^{2+}$ to the zone 12-15 cm behind root tip resulted in 14% of its translocation (Marschner and Richer, 1973). The symplastic origin of Ca^{2+} in the xylem exudate was demonstrated in the study of Jarvis and House (1970). During a 24 h experiment, the level of Ca^{2+} in the xylem exudate was the same whether roots were maintained in a solution of $CaCl_2$ or in water vapour, despite the fact that the volume of exudate in water vapour was only a third of that in the nutrient solution (Jarvis and House, 1970).

When applied to the segments proximal to the root tip, no translocation of Ca^{2+} towards the root tip was observed in this study. This result supports the general view that Ca^{2+} is immobile in the phloem and is not circulated within plants (Marschner, 1986). With the uniform supply of Ca^{2+} along the root, it seems logical that developing cells in the apical zone will absorb a sufficient amount of Ca^{2+} from the external medium, and there would be no need

for an additional Ca^{2+} supply from older regions. Results of the present study are in agreement with earlier reports, where only basipetal translocation of Ca^{2+} was observed in excised onion roots (Maclon, 1975; Maclon and Sim, 1981). The same pattern of basipetal-only Ca^{2+} translocation was reported for the seminal root of maize (Marschner and Richer, 1973). Occasionally, there are reports of acropetal transport of Ca^{2+}. For example, Huang et al. (1993b) demonstrated that in wheat, 5% of the total translocated Ca^{2+} was transported toward the root tip. There was no redistribution of $^{45}Ca^{2+}$ from the labeled root to other roots of onion during the experiments. The findings of this study are contrary to the report of Huang et al. (1993b). Additionally, in 3-day-old wheat seedlings, a significant amount of $^{45}Ca^{2+}$ redistributed from one labeled seminal root to other seminal, non-labeled, roots of the same seedling (Huang et al., 1993b). No such redistribution was found in onion (Fig. 3.3).

The measured water loss due to transpiration in onions had no correlation with the amount of translocated Ca^{2+} in this study. This result cannot indicate any correlation between water and Ca^{2+} transport, as onion with its numerous roots cannot be used for such detailed study ($^{45}Ca^{2+}$ was applied to one root only, while the remaining roots could freely contribute to water transport). However, in a number of other studies on Ca^{2+} uptake and translocation, a positive correlation between water uptake and Ca^{2+} transport was found, e.g: in pea (Hylmö, 1953), in bean (Bell and Biddulph, 1963). In other studies, an increase in transpiration resulted in an increased translocation of Ca^{2+}, only when the Ca^{2+} concentration of the nutrient solution was high (for example, Drew and Biddulph, 1971). On the contrary, Ca^{2+} translocation was independent of transpiration in cucumber (Bengtsson, 1982) and in maize (Jarvis and House, 1970). Clearly, more studies are needed to determine whether or not Ca^{2+} translocation depends upon the rate of water transport.

The evidence obtained in this study demonstrates that the zone 1.5 to 3 cm behind the root tip possesses optimal Ca^{2+} translocation capacity in onions. This zone could potentially contribute about 50% of the total translocated Ca^{2+} in 15 cm long roots. The mature zone of the root is also capable of translocating Ca^{2+}, although with smaller efficiency. The absorbed Ca^{2+} at the root tip is not translocated from it. This result eliminates the hypothesis that Ca^{2+} is

transported apoplastically and enters the xylem elements in the stele bypassing the endodermis around the root tip, where there are no Casparian bands.

Chapter 4

Exodermal Casparian bands of onion roots are impermeable to calcium: evidence from compartmental elution

4.1 Abstract

Calcium is an essential macronutrient, and its constant supply in the range of 1 to 10 mM is required to sustain plant growth. This ion may be taken up by roots through a combination of apoplastic and symplastic pathways but there is a controversy concerning which pathway predominates. The symplastic pathway may be insufficient to meet the plant's demand, since Ca^{2+} concentrations in the cytosol are low, in the nM range. On the other hand, the apoplastic pathway may be blocked by Casparian bands in the endodermis, and in many species, in the exodermis. The permeability of the exodermal Casparian bands of onion roots to Ca^{2+} was tested by means of compartmental elution. Root segments with mature exodermal bands were incubated in $^{45}CaSO_4$ solution. The amount of $^{45}Ca^{2+}$ eluted from the cell walls of intact segments with sealed ends (where $^{45}Ca^{2+}$ had access only to epidermal cell walls) was compared to that of dissected segments of the roots, (longitudinally split with steles removed). In later $^{45}Ca^{2+}$ had access to the cortical cell walls in addition to the walls of epidermis. Analysis of elution curves revealed that $^{45}Ca^{2+}$ was eluted from three compartments in both

conditions: cell wall, cytosol, and vacuole. The amount of radioactivity eluted from the wall compartment in bisected segments was larger than in intact segments, and the difference correlated with the expected volumes of the cell walls. This result indicates that exodermal Casparian bands are not freely permeable to Ca^{2+}. Therefore, the apoplastic pathway of Ca^{2+} transport would be restricted in mature parts of the onion roots. In addition, it was observed that there was a substantial accumulation of Ca^{2+} in intact segments. Comparison of the amount of Ca^{2+} accumulated by intact and bisected segments taken from 2 to 4 cm behind root tip showed that accumulated Ca^{2+} was present in the stelar apoplast. Hence, the Casparian bands in the anticlinal walls of both the endodermis and the exodermis are believed to block the movement of Ca^{2+}.

4.2 Introduction

This study primarily concerns the permeability of exodermal Casparian bands to Ca^{2+}. Results presented in Chapter 3 demonstrated that externally applied Ca^{2+} was transported across the root, loaded into the xylem, and transported to the aerial parts of the onion. The transport from mature zone of the root (Chapter 3, Table 3.3) accounted for 50% of the total Ca^{2+} translocated in growing onion roots used for experiments (Chapter 3, discussion). In this zone, Casparian bands were deposited in the walls of the exodermis. It is not known how Ca^{2+} entered the root and whether or not exodermal Casparian bands pose a barrier for its apoplastic radial transport. Determination of the permeability of these Casparian bands to Ca^{2+} is important, since the majority of angiosperm species develop Casparian bands next to the epidermis. It has been demonstrated that 89% of 207 angiosperm species from 52 families had a modification in the cell walls of the exodermis in the form of Casparian bands (Peterson and Perumalla, 1990; Permualla et al., 1990). Under typical field conditions, the Casparian band in the exodermis may be present in the major part of the root, as its maturation has been demonstrated within 5 mm of the tip (Peterson and Perumalla, 1984). If the exodermal Casparian bands were impermeable to solutes, as is believed for endodermal ones, the radial apoplastic pathway for Ca^{2+} would be blocked in the zones of the roots where they are deposited.

The composition of Casparian bands, especially the demonstration that they contain the hydrophobic polymer suberin (Zeier and Schreiber, 1997) indicates that, they may block the entry of the solutes into the root cortex. The permeability of the exodermal Casparian bands has been addressed experimentally with conflicting results. With the aid of fluorescent dyes, the blocking role of Casparian bands has been visualized (Moon et al., 1986). Results of other studies (Marshner, 1995; Clarkson, 1996) indicate that exodermal Casparian bands are impermeable to potassium ion as well. Other studies suggest that they may be permeable to water and ions (Kochian and Lucas, 1982; Zimmerman and Steudle, 1998). Partial permeability was demonstrated using Energy Dispersive X-ray Analysis by applying Rb^{+} as a K^{+} tracer to barley roots where it was concluded that "restriction of apoplastic ion flow by the outermost cortex cell layers is rather effective but not complete" (Gierth et al., 1999). Furthermore, Sanderson (1983b) suggested that there is some perceptible transfer of Ca^{2+} in the root zones where the endodermis is depositing layers of suberin lamellae and tertiary walls, which represents residual permeability of the endodermal walls. Since this transfer of Ca^{2+} was dependent on transpiration, it was pointed out that the destruction of the attachment of the plasmalemma to the Casparian band during secondary and tertiary wall deposition opens up a small apoplastic channel (Sanderson, 1983a).

Recent analyses of the chemical composition of exodermal cell walls revealed that lignin is the major polymer present in the Casparian band, while suberin-derived polymers accounted only for 1% of the total detected polymers (Zeier and Schreiber, 1997). Since lignin is less hydrophobic than suberin , these results may indicate that the exodermal Casparian band may not completely block the apoplastic pathway for water and ions. Previous dye tracer studies which demonstrated impermeability of Casparian band were questioned, as the tracer molecules are much larger than ions and the diffusivity of large uncharged dyes may be quite different from those of hydrated inorganic ions (Byliss et al., 1996). The only study that addressed this question and used a divalent anion to probe the permeablility of exodermal Casparian bands was that of Peterson (1987), who demonstrated that Casparian bands present in the onion root exodermis are impermeable to sulphate ions.

Compartmental elution is the established method for characterizing exchange properties of multicomponent systems. This procedure was initially developed to study the kinetics of ion movement in giant algal cells (reviewed by MacRobbie, 1971). Many such studies have been conducted for a variety of ions using both excised root segments (Cram, 1968; Balke and Price, 1988; Peterson, 1987; Macklon et al., 1990) and intact root systems (Jeschke and Jambor, 1981; Jeschke, 1982; Macklon and Sim, 1987; 1990; Rauser, 1987). Despite the widespread use of this technique, the assignment of particular kinetically defined phases to their corresponding subcellular compartments has not always been unequivocal. Many different compartments and half-times have been obtained (Table 4.1). These discrepancies may reflect the variety of strategies used to analyze the efflux data, as well as the different parameters used to assign the linear phases in semilogarithmic efflux plots to specific subcellular compartments.

In this study, the permeability of the exodermal Casparian band to Ca^{2+} was investigated by measuring the amount of Ca^{2+} eluted from the wall compartment. For this purpose, Ca^{2+} eluted from intact, sealed, segments was compared to that from bisected segments with removed steles. In the former, the treatment solution had access to the root through the epidermal and exodermal cell walls only, and in the latter, both the epidermis and cortex were exposed. If Casparian bands were not restricting apoplastic diffusion, there should be no difference in the amount of Ca^{2+} eluted from the cell walls in intact and bisected segments. If, however, Casparian bands do restrict the apoplastic pathway, the cell wall compartment in the bisected segments should be substantially larger than in the intact segments.

The volume of the apoplast accessible to solutes is classically divided between water free space (WFS) and Donnan free space (DFS). WFS is defined as the volume of the free space in the cell walls in which the electrical interactions of the free ions and fixed charges is negligible, and both Ca^{2+} and anions are freely diffusible. DFS corresponds to the space in the cell walls where free ions are in the range of the electrostatic field of the ionized, fixed charges. The distinction between WFS and DFS may be possible under the assumption of an ideal Donnan model, where the charges present in the cell walls are immobilized on structural polymers. This condition is probably satisfied only under low ionic strength of the external

Table 4.1 Summary of the half times of elution obtained during compartmental elution experiments.

References: a- Deviennne et al.,1994; b – Rauser, 1987; c – Maclon, 1975; d - Kronzucker et al., 1995a; e - Kronzucker et al., 1995b; f – Rygiewicz et al., 1984; g - DiTomaso et al., 1993; h - White et al., 1992; i – Peterson, 1987; j - Drew and Biddulph, 1971.

racer and plant species	Half-times of elution from compartments						
	Loading time	Superficial film	Cell wall		Cytoplasm	Vacuole	Ref.
			Water free space	Donnan free space			
$^{15}NO_3$, *Triticum cereale*, intact wheat roots.			14 s		4.6 min	9.4 h	a
^{109}Cd, *Agrostis gigantea*	23 h		2.7 to10.3 min		40.2 to 73.7 min	52.5 h to 133 h	b
^{109}Cd, *Zea mays*			3.5 to 8.1 min		59 to 88 min	79 h to 213 h	b
^{45}Ca, *Allium cepa*, root segments	17 h	12.6 s	1.5 min	18 min	54 min	29.6 h	c
$^{13}NH_4$, *Picea glanca*, intact roots	60 min	2 s	30 s		14 min		d
$^{13}NO_3$, *Picea glanca*, intact roots	35 min		2.5 s	20 s	7 min		e
^{86}Rb, *Pseudotsnogas manzesii*, intact roots	18 h		1.5 to 9.2 min		17.8 to 60 min	8.2 to8.7 h	f
$^{13}NO_3$, *Hordeum vulgare*, intact roots	18 h		1.2 to 3.7 min		39 to 64 min	25 to 31 h	f
[^{14}C]paraquat, *Zea mays*	24 h		16.6 min		58.8 min	7.3 h	g
^{45}Ca, *Secale cereale* intact roots	14 days		2 min		6.7 to 25 min	28.5 h	h
$^{35}SO_4$ *Allium cepa*, root segments	16 h	>15 s	1 min		12.4 min	4.9 days	i
^{45}Ca, *Phaseolus vulgaris*, intact roots	24h		2.1 min		13 min	8.3 h	j
^{45}Ca, *Allium cepa*, root segments Mature Intact	17 h		57 s		17 min	3.6 h	This study
Mature bisected, stele removed	17 h		55 s		10 min	3.5 h	
Young Intact	17 h		45 s		9.3 min	1.9 h	This study
Young bisected	17 h		35 s		6.7 min	2.1 h	

medium, or in lignified materials (Wolterbeek, 1987). The DFS changes when there are electrostatic interactions between the charged polymers and osmotic gradient between the medium and the cell walls (Ritcher and Dainty, 1989; Ritchie and Larkum, 1982).

Cell walls are composed of matrices of complexed polysaccharides. There are four major components: cellulose microfibrils, hemicellulosic polysaccharides, pectic substances, and glycoproteins. Association of Ca^{2+} with the plant cell walls occurs through Donnan effects, mainly the ionic bonding of free carboxyl groups on uronic acid polymers (pectic substances). The proposed "egg box" model of Ca^{2+} binding to polygalacturonic acid involves positive cooperativity (Grant et al., 1973). It has been proposed that in the egg-box model there are "high affinity internal sites" i.e., urinates facing each other inside dimerized pectic chains, and "low affinity external sites" i.e., galacturonates on the outside faces of the assembly that bind cations less strongly (Messien et al., 1997). Some scientists envisage a single class of binding sites (Sentenac and Grigon, 1981; Bush and McColl, 1987) whereas Baydoun and Brett (1984) interpreted their analysis as evidence for high and low affinity binding sites. Clearly, more research needs to be performed to understand the mechanism and chemistry of Ca^{2+} binding to plant cell walls.

4.3 Materials and methods

4.3.1 Plant material and segment preparation

Onion (*Allium cepa* L.) roots grown from bulbs in vermiculite in the greenhouse for 12 d as described in Chapter 2 were used for experiments. Roots were 15 cm long on average. Uniform roots from one bulb were used for one replicate of each experiment. Segments were cut from the part of the roots above 10 cm from the root tip and used for determination of exodermal Casparian band permeability to Ca^{2+}. One set of 10 segments was prepared for

each experimental condition: for one, a set 2.5-cm-long intact segments had their ends sealed with sticky wax (Kerr Dental Specialties, Inc.), so that only 2 cm were exposed to the external solution. For the second set, 2-cm-long segments were dissected with the aid of fine forceps and dissecting microscope, their steles were removed. To determine the accuracy of the removal of the steles, the diameters of cross-sections of the segments were compared to the diameters of the steles and this ratio was compared to the weights of intact and dissected ones.

Segments cut from a young zone of the root, 2 to 4 cm behind root tip, were used for experiments to determine the site of Ca^{2+}accumulation. For elution experiments, two sets of 10 segments were prepared. Intact segments were 2.5 cm long with sealed ends (as described above), and 2 cm segments were bisected, but their steles were not removed.

4.3.2 Anatomical and permeability analyses

The maturity of the exodermis in the segments was checked by examining cross sections from the remaining roots at both proximal and distal ends of the segments. Sections were stained following the procedure of Brundrett at al. (1988) which facilitated visualization of the Casparian bands.

The permeability of the walls and the effectiveness of the wax seal were determined by incubation of selected segments in 0.001% Cellufluor white. Stained segments were rinsed, sectioned and observed with epifluorescence optics, with UV illumination using excitation filter G 365 (365 nm emission peak), chromatic beam splitter FT 395 (395 nm) and barrier filter LP 420 (>420 nm bandpass). Unemerged lateral root primordia were detected by the clearing method of Hackett and Steward 1969.

4.3.3 Labeling and elution

Labeling and elutions were performed following the method of Peterson (1987) for sulphate ions. Segments, both dissected and with sealed ends, were incubated overnight (17 h, which met the requirement of the loading time to be 4 to 5 times longer than half-time of elution) in 25 ml aerated solution of 1 mM $CaSO_4$, labeled with ^{45}Ca (final specific activity, 3.7 x 10^{-4} Bq ml^{-1}). At the end of the loading period, tissue was removed from the bathing solution and blotted with absorbent tissue.

Elution was performed by transferring the segments into vials containing 10 ml unlabelled, aerated, unbuffered 1 mM $CaSO_4$ dissolved in distilled water, adjusted to pH 5.5. Vials were constructed from segments of 5 ml pipette tips by sealing plastic micromesh at one end of the tip (to prevent root segment loss during elution and transferring) and securing an aeration tube to assure constant mixing. Ten segments were placed in each vial. Between each change, vials were lifted just above the solution, and the tissue was rinsed further with 1 ml of unlabelled 1 mM $CaSO_4$ solution to minimize error due to ullage. The transfers to fresh washout solution were carried out at following time intervals in 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, wax caps were removed from sealed segments, and intact and dissected or bisected segments were weighed. ^{45}Ca was extracted from the tissue by soaking in 1 ml of 0.2 N HCl for 20 min, diluted with 5 ml of water for 10 min, and scintillation liquid (Ecolite, ICN, Cat. No.882475) was added to reach 20 ml total volume. From each of the washout solutions, a 1 ml aliquot was taken and mixed with scintillation fluid to a total volume of 6 ml. Samples were counted for β-emissions in a scintillation counter (Searle Analytic Inc. Model Mark III). Each reading was corrected for the background present in the control tissue, which had not been exposed to $^{45}Ca^{2+}$.

The radioisotope content in each wash solution was plotted against the time of elution. Since the elution is a sum of several exponential processes, the logarithm of the radioactivity

remaining in the tissue had to be plotted. Examination of the kinetics reveals that the plot of the total radioactivity during the experiment can be resolved into distinct components which have logarithmic rates of elution. When the radioactivity reaching the external solution was derived from more than one compartment, the relationship was curved. The linear part of the curve indicates that a single compartment determines this rate of elution. Identification of compartments was performed by "curve-peeling". To avoid an error of subjective selection of the number of data points to be included in regressions of each phase, a maximization of R^2 (correlation coefficient) for linear regression (Rygiewicz et al., 1984) was used as the criterion. The sum of radioactivity of all of the elution solutions plus the residue in the segments gave the total radioactivity at the beginning of the elution, t_o. The amounts removed at subsequent sampling periods are subtracted, stepwise, from the initial quantity, giving the amount remaining in the tissue at any given time. The slowest-eluting compartment was assumed to be the vacuole. The half times for the exchange were then calculated by extrapolating the linear part of the curve to the origin (t_o). The intercept of the linear portion of the curve with the origin gives an approximation of the total quantity of the ion in the analyzed compartment. More rapidly eluted compartments and their half times were determined in a similar manner.

To verify the identity of the cell wall compartment, an elution experiment was performed on ice. The elution solution containing 1 mM $CaSO_4$ was cooled down to 4°C. The temperature of the solution in the vials was measured throughout the experiments and was constant at 4°C during 360 min of experiment. Intact and dissected segments from the mature zone of the root were incubated in 1 m M $CaSO_4$ (^{45}Ca-labelled, 3.7 x 10^{-4} Bq ml^{-1}) at 22±1°C for 17 h. At the end of this labeling period, segments were removed from the last eluting solution, blotted with absorbent tissue, and eluted (as described earlier) at 4°C. The half time of elution from wall compartment should not be sensitive to reduced temperature, whereas the half-times of elution from the membrane-bound compartments (including the cytoplasm) should be increased.

4.4 Results

4.4.1 Developmental stage of the root segments used in experiments

The presence of exodermal Casparian bands in root segments used for experiments was confirmed by observations of sections stained with berberine/aniline blue. Casparian bands were present in all sections taken both proximal and distal to experimental segments. The Casparian bands were visible as a strong, yellow fluorescence in the radial walls of the exodermis. These Casparian bands restricted the movement of Cellufluor, as incubation of the segments with it resulted in staining of the epidermal and outer tangential walls of the exodermis only.

Sealing of the cut ends with wax proved effective, as Cellufluor was not observed in the walls of the segments under the seal, while exposed epidermal and outer tangential walls of the exodermis were stained, and when illuminated with UV light gave a strong, bluish-white fluorescence. Those results fully confirmed the previous findings of Peterson (1987).

The segments taken from young zones (1.5 to 4 cm) of the roots had Casparian bands in the endodermis, but they were not developed in the exodermis, as staining with berberine/aniline blue failed to detect them. Such young segments were used for investigation of the sites of accumulation of Ca^{2+}.

4.4.2 Accuracy of the removal of the steles from dissected segments

Segments cut in half longitudinally had their steles removed (dissected) and discarded to avoid complications in estimating the amount eluted from cell walls, as the stelar apoplast and vessel lumena may add to the amount of eluted Ca^{2+} from cell walls in an unpredictable way. By comparing the ratios of the root and stelar areas to the ratio of fresh weight of intact and bisected segments, it was calculated that steles were removed with 90% accuracy. This result may reflect the fact that the dissected segments underwent more manipulation than intact ones and, during removal of the steles, some of the air present in the extracellular spaces was replaced with water.

4.4.3 Analysis of efflux curves

Efflux of Ca^{2+} was determined by plotting the log of Ca^{2+} remaining in the root segments versus time. The resulting data yielded typical efflux curves (Fig 4.1). These data adequately fitted a triple exponential function indicating contribution from three compartments, i. e., cell walls, cytoplasm, and vacuole (although the identity of the last compartment is not known with any certainty). The intercept with the origin of the line drawn through a linear phase of eluted amounts yielded an estimate of the amount of Ca^{2+} accumulated in the slowest-exchangeable compartment at the end of the loading period (Table 4.2), and was subsequently used to determine the amount of Ca^{2+} present in the faster compartments. Subtraction of this line from the total efflux data yielded a curve that had a linear phase as well, and was interpreted as the time course of the efflux from the second compartment, the cytoplasm. The cell wall compartment was eluted most rapidly. The superficial film compartment reported in some studies (Table 1, Peterson, 1987; Maclon, 1975; Kronzucker, 1995a,b) was not detected.

The cell wall compartment was eluted with a mean half time of 57 s for intact segments and 55 s for dissected (steles removed) segments. Similar rates of elution for intact and bisected

Figure 4.1 Typical result of an elution experiment

Elution curve was resolved into 3 compartments: A – vacuole, B – cytoplasm, and C – cell wall.

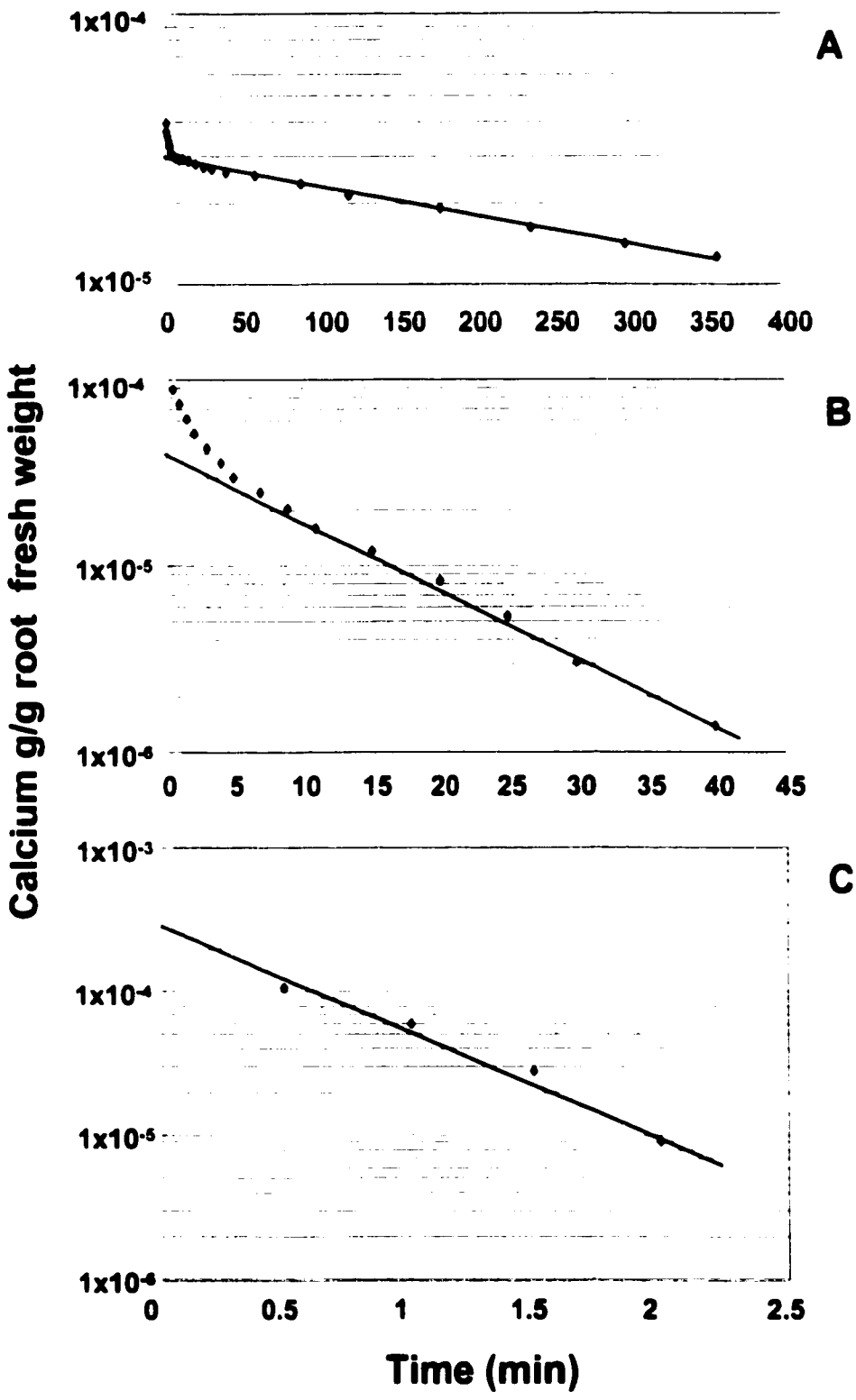

A
B
C
1x10-4
1x10-5
0
50
100
150
200
250
300
350
400
1x10-4
1x10-5
1x10-6
0
5
10
15
20
25
30
35
40
45
1x10-3
1x10-4
1x10-5
1x10-6
0
0.5
1
1.5
2
2.5
Calcium g/g root fresh weight
Time (min)

Table 4. 2 Summary of elution experiments.

Reported results are for whole and dissected segments taken from the mature zone of roots.

Compartments	Whole segments		Dissected segments	
	µmoles Ca/g fw	Half-times	µmoles Ca/g fw	Half-times
wall	4.78	30 s	9.10	30 s
	12.45	90 s	10.73	90 s
	6.39	120 s	85.32	60 s
	8.48	45 s	16.52	60 s
	17.84	30 s	69.42	60 s
	5.90	30 s	76.76	30 s
Average	**9.31**	**57.5 s**	**44.64**	**55 s**
±SD	**4.98**	**38.4**	**36.07**	**22.6**
cytoplasm	1.91	15 min	8.86	9 min
	6.23	30 min	11.9	12 min
	5.11	30 min	4.27	8 min
	0.71	8 min	8.59	8 min
	1.34	12 min	6.94	15 min
	3.67	9 min	5.31	8 min
Average	**3.16**	**17.33 min**	**7.32**	**10**
±SD	**2.21**	**10.11**	**2.70**	**2.9**
vacuole	2.58	350 min	1.52	300 min
	2.86	250 min	0.48	200 min
	0.20	200 min	0.24	180 min
	0.61	200 min	0.77	200 min
	1.74	200 min	0.40	220 min
	2.24	130 min	0.83	170 min
Average	**1.71**	**221.7 min**	**0.71**	**211.7 min**
±SD	**1.08**	**73.6**	**0.46**	**46.65**

segments indicated that Ca^{2+} was eluted from the same compartment. However, the amount of eluted Ca^{2+} was 4.8-fold larger in bisected segments compared to intact ones (Table 4.2). Statistical analysis of log transformed data revealed a significant difference in amount of Ca^{2+} eluted from cell wall compartment in intact and bisected segments, as $P = 0.037$ at $\alpha = 0.5$ (Student T test, 2 tailed, 1 type).

The second compartment (cytoplasm) was eluted with half-times of 17 and 10 min from intact and dissected segments, respectively (Table 4.2). The amount of eluted Ca^{2+} was 2.3 times larger from dissected segments than from intact ones (Table 4.2.). The external elution solution was in contact with cortical cells in dissected segments during elution so that all Ca^{2+} pumped out of the cytosol of the cells was recovered. In sealed segments, this Ca^{2+} was trapped by Casparian bands in the apoplast of intact segments.

The elution from intracellular membrane-bound compartments (possibly vacuole and other organelles such as endoplasmic reticulum, plastids and mitochondria) had a mean half-time of 211 min for mature segments (Table 4.2). It has been reported that the half-times of ion exchange from vacuoles are very long (Table 4.1), and probably do not reflect a complete equilibrium that can be reached.

Half-times of elution from cell wall compartments from young segments was on average 45 and 35 s, from intact and bisected segments, respectively (Table 4.3). Those half times are somewhat faster than the ones obtained for mature segments (55 and 57 s, Table 4.2), which may reflect differences in their cell wall composition. Half times of elution from cytosol of young segments (9.33 min and 6.67 min, Table 4.2) were somewhat faster than from mature segments. The amount of eluted Ca^{2+} from intact young segments was larger than from cut ones. However, the difference in the amount of Ca^{2+} eluted from intact and bisected segments was not statistically significant, as Student T test revealed $P = 0.285$ at $\alpha = 0.5$ (log transformed data, 2 tailed, 1 type). .

The amount of Ca^{2+} recovered from the segments at the end of elution was over 2 times larger in intact segments than in dissected or bisected ones. While the amount of Ca^{2+} reflects its

Table 4.3 Summary of elution experiment from young zone.

Amounts of Ca^{2+} in the compartments and half times of the efflux of the elution experiments of the intact and bisected root segments collected from young zone (2 to 4 cm) of the root. Amount of Ca^{2+} is represented in μmoles/g fresh weight, n = 3.

Compartments	Whole segments		Bisected segments	
	µmoles Ca/g fw	Half-times	µmoles Ca/g fw	Half-times
wall	15.0	45 s	14.88	45 s
	20.5	60 s	29.50	30 s
	14.08	30 s	47.75	30 s
Average	**16.55**	**45 s**	**30.71**	**35 s**
±SD	**3.51**	**15 s**	**16.47**	**8.6 s**
cytoplasm	3.80	9 min	0.94	6 min
	1.62	12 min	1.25	9 min
	3.58	7 min	1.79	5 min
Average	**3.00**	**9.33**	**1.33**	**6.67**
±SD	**1.20**	**2.5**	**0.43**	**2.08**
vacuole	4.45	120 min	2.44	80 min
	2.39	90 min	0.39	200 min
	3.58	140 min	1.79	100 min
Average	**3.47**	**116.6** min	**1.54**	**126.6** min
±SDEV	**1.04**	**25.16**	**1.05**	**64.29**

accumulation in the intercellular stores in dissected and bisected segments, it represents a deposition of Ca^{2+} in the stelar apoplast in intact ones (see results below).

The elution of the segments on ice (4°C) resulted in elimination of the second (cytoplasm) compartment. The half-time of the elution from the fastest (wall) compartment was comparable to that eluted at 21°C, and averaged 60 s (Fig. 4.2).

4.4.4 Determination of the site of accumulation in intact root segments

To determine a site of accumulation of Ca^{2+} in intact root segments, the total radioactivity remaining in the intact segments was compared to that of those cut in half. For this purpose, the segments from the young zone of the root (between 2 and 4 cm from the root tip) without exodermal Casparian bands were used for the experiments (Table 4.3). It was assumed that endodermal Casparian bands block the apoplastic Ca^{2+} movement. If the sites of Ca^{2+} accumulation were in the intracellular compartments, the amount of Ca^{2+} retained in bisected segments should be larger than in the whole segments, since in the former the external labeled solution had an access to all cells. In fact, the opposite result was obtained, as the intact segments from young zones of the roots with sealed ends accumulated 80 % more Ca^{2+} than bisected ones (Table 4.4). These results suggest that the majority of Ca^{2+} absorbed in the root had its destiny in the stelar apoplast for long distance transport to the leaves. The retention of accumulated Ca^{2+} during elution supports the generally accepted hypothesis that the endodermis blocks the apoplastic leakage of accumulated ions from the stele. Accumulation of Ca^{2+} was also observed in intact root segments cut from the roots above 10 cm from root tip. When compared to the dissected segments, 93% more Ca^{2+} was recovered from intact segments (Table 4.4). Since in dissected segments the steles were removed, it can be estimated that the average difference of 1.16 μmoles Ca^{2+}/g fw between intact and dissected segments is due to Ca^{2+} accumulation in the stele.

Fig. 4.2 Typical result of elution of onion roots at 4 °C.

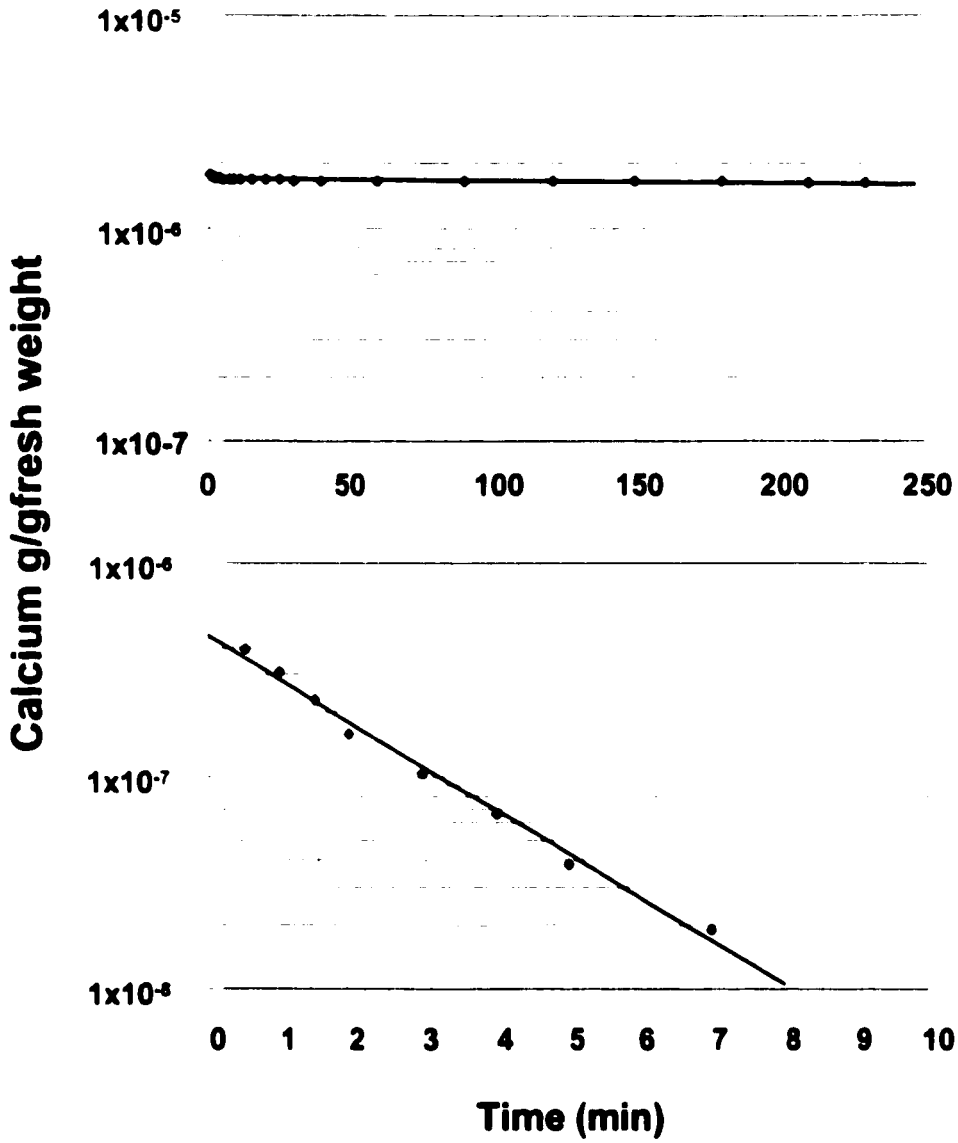

1x10-5
1x10-6
1x10-7
0
50
100
150
200
250
1x10-6
1x10-7
1x10-8
0
1
2
3
4
5
6
7
8
9
10
Calcium g/gfresh weight
Time (min)

Table 4.4 Accumulation of $^{45}Ca^{2+}$ in intact root segments.

The intact segments with sealed ends were compared to bisected ones. Segments were taken from young zone (1.5 to 4 cm from root tip). These data were generated by using counts of the total radioactivity present in the segments after elution series had been completed (360 min). The differences in fw between replicates were accounted for, as the data is presented in g $^{45}Ca^{2+}g^{-1}$ fw. For each replicate of the experiment, 10 intact and 20 halves of the root segments from one bulb were used.

Exp.	Intact segments µmoles Ca/g fw	Bisected µmoles Ca/g fw	Difference µmoles Ca/g fw	% accumulation
1	0.025	0.0049	0.0199	80.11
2	0.052	0.0115	0.0400	77.63
3	0.071	0.0118	0.0595	83.40
Average	0.049	0.0094	0.0398	80.38
±SD	0.023	0.0038	0.0198	2.98

4.5 Discussion

4.5.1 Estimation of the rate of Ca^{2+}accumulation in the stele

The average difference in concentration of Ca^{2+} accumulated in intact vs. cut segments during the 17 h loading period is 0.0398 μmoles Ca^{2+} /g fw. This difference can be attributed to Ca^{2+} accumulation in the stele. Assuming a constant rate of Ca^{2+} accumulation during the loading period, it can be calculated that Ca^{2+} entered the stele at a rate of 2.3 nmoles Ca^{2+} g^{-1} h^{-1}. The estimated volume contributed by the stele to the volume of 1 ml of root is 0.19 ml in the samples used in this experiments (ratio of measured diameters from cross-sections). Therefore, the concentration of Ca^{2+} taken up in the stele was estimated as 0.2 mM. The actual concentration of Ca^{2+} in the lumena of xylem vessels may be higher, since the above calculations do not take into account the volume occupied by living cells in the stele. This result reflects a high capacity of the root to accumulate Ca^{2+}. The calculated concentration of Ca^{2+} in the stele may be overestimated for physiological conditions, as there was no translocation of Ca^{2+} from the loaded segment, as would probably occur if the roots were intact and attached to the bulb.

4.5.2 Benefits of using onion as a model system

Onion roots have been used as a model system in many physiological studies for several reasons. It is relatively easy to obtain large number of roots from one bulb, which ensures uniform samples for each experiment. Especially for this study, onion roots are ideal for the estimation of amounts of Ca^{2+} present in the cell walls, as the results do not develop hairs when well watered. The presence of root hairs could alter the interpretation of the results, as they are easily damaged during handling of the segments. The amount of Ca^{2+} eluted from damaged root hairs could made the analyses of the result impossible. In addition, onion roots

can be manipulated without problems and there is no sign of the vitality loss after 48 hour of excision (Peterson, 1987).

4.5.3 Anatomical features of onion

The stage of root development was determined for each replicate of the experiment. As anticipated, segments taken between 2 and 4 cm from root tip did not have exodermal Casparian bands, whereas those in the endodermis were clearly visible upon berberine/aniline blue staining. Segments collected from the mature (>10 cm from root tip) zone of the root had both endo- and exodermal Casparian bands. The permeability of the mature root is affected by the development of lateral root primordia (Dumbroff and Peirson, 1971; Peterson and Humphreys, 1981). The root segments used in this study did not possess laterals, as the method of Hackett and Steward (1969) using acridine orange staining failed to detect them. The use of uniform segments of defined developmental stage allowed a precise comparison of permeability of the cell walls in onion roots.

4.5.4 Compartmental elution technique

For single celled organisms, it was possible to correlate the kinetics of ion efflux with distinct compartments (reviewed by McRobbie, 1971). In subsequent studies, efflux analyses have been used to investigate transport processes in higher plant organs with the assumption that their compartments are also arranged in a series, i.e., vacuole, cytoplasm, cell wall and surface film (Table 4.1, and references therein). Since the transport processes may be differentially regulated in these compartments, the use of the compartmental analysis technique to study transport of ions in plants has been questioned. In addition, it has been argued that labeled ions may be slowly released from cell wall binding sites (Jorgenson, 1966), chemically bound in the cytosol (Walker and Pitman 1976) or compartmentalized in organelles such as plastids (Cheeseman, 1986). Under these conditions, analysis of the data from elution experiments may lead to an overestimation of the accumulation of the ion in the slow-releasing

compartments such as the cytoplasm or vacuole and the simple, four-compartment model would not apply. Theoretical, computerized analysis of simulated, error-free four compartment data using nonlinear regression techniques led Cheeseman (1986) to the conclusion that efflux analysis alone lacks the power to provide valuable information about multicompartment systems. Despite this criticism, efflux analysis is the only technique that allows for semi-quantitative comparison of ion pools present in given compartments in the roots. Since the compartment of interest in the present study was the apoplast, which is easily identified, efflux analysis allowed a comparison of the amount of Ca^{2+} present there.

4.5.5 Problems associated with use of a divalent cation for elution

Using a divalent cation for efflux analysis can be problematic due to the significant amount of the ion binding to the negative sites in the cell walls. From reported studies, it can be concluded that the cation binding properties of the cell walls are different for different species as well as for different cations. Compartmental efflux analysis could not be performed on roots of ryegrass (*Lolium perenne* L.) using Cu^{2+} because it was bound to the cell walls and slowly released from them and, thus, interfered with measurements of Cu^{2+} efflux from the vacuole (Thorton, 1991). No such interference was reported for Ca^{2+} (Maclon, 1975; White et al., 1992), and present study due to the rapid elution of the bound Ca^{2+}, Cd^{2+} (Rauser, 1987), or for the divalent cation herbicide, paraquat (DiTomaso et al., 1993).

4.5.6 Number of compartments identified with elution procedure

The number of compartments identified in various compartmental elution studies ranged from 3 to 5 (Table 4.1). Some studies were able to reveal five compartments for Ca^{2+} which are, in order of decreasing rates of exchange: the superficial film, water free space, Donnan free space, cytoplasm, and vacuole (Maclon, 1975). It is known, however, that a rigid separation of the Donnan free space and water free space may be an oversimplification, since it is not

possible to make any clear spatial differentiation between those two compartments (Platt-Aloia et al., 1980; Starrach and Mayer, 1986).

The presence of residual treatment solution (superficial film) on the surface of onion roots during elution experiments was reported by Peterson (1987) and Maclon (1975). The thickness of the surface film in intact segments of mature onion roots was reported to be 14.8 to 19.8 μm, and 34.6 to 19.5 μm in dissected ones, as estimated during elution experiments with sulphate ion (Peterson, 1987). The present study failed to detect the presence of fast-exchangeable Ca^{2+} in a superficial film. It was possible that the time lapse for blotting (in minutes) between the removal of segments from labeling solution and start of the elution series was adequate to allow Ca^{2+} present in the surface film to interact with the cell walls. If this Ca^{2+} interacted with the charges present at the surface of the cell walls, their elution would not be different from that of the cell wall compartment. If this is the cause of failure to detect a surface film for Ca^{2+}, the difference in the amount of Ca^{2+} eluted from cell wall of dissected and intact segments may be slightly overestimated, as the latter had a 1.6 times thicker film (Peterson, 1987). The possible incorporation of the surface film into the amount of $^{45}Ca^{2+}$ present in the cell walls cannot, however, account for the large differences observed between dissected and intact segments (see discussion below).

For the purpose of this study (to compare free space accessible to Ca^{2+} in the cell walls) the elution time was set at the time intervals of total 360 min, and three compartments were recognized. Longer times of elution (1200 min) experiments did not result in identification of more than three compartments for Ca^{2+} in rye roots (White et al., 1992), or for Cd^{2+} in *Agrostis gigantea* and maize (Rauser, 1987).

The identities of the compartments are often confirmed by experiments where the external concentration of labeled solution is increased (Peterson, 1987). The amount of ion eluted from the free space in cell walls that is in equilibrium with external solution should be proportional to the strength of labeled solution, but will not be proportional if the ion is bound in the compartment. Such an experiment cannot be applied to identify the wall free space for Ca^{2+}. The capacity of pectic substances (mainly polygalacturonic acid) to bind Ca^{2+} and form gels

increases with increased external Ca^{2+} concentration (Willats et al., 1999). Other compounds, such as cell wall proteins, are unlikely to bind large amounts of Ca^{2+}. Structural wall proteins such as extensin and potato tuber agglutinin have a high isoelectric pH (pI between 9.65 to 12. Cooper et al., 1984; Leach et al., 1982). The standard procedure for isolation of cell walls associated enzymes involves treatment with 0.1 to 0.5 M $CaCl_2$ (Scott and O'Neil, 1984) and yields proteins of a low molecular weight (14 to 31 kDa) which are basic in nature (β-glucosidase, α-mannosidase, and acid phosphatase), and have their pI above 7.9 (Nagahashi and Seibles, 1986). It seems that high Ca^{2+} concentrations break the association (covalent and/or ionic) of positive charges present on the proteins with negative charges present on the cell walls. Therefore, a high external Ca^{2+} concentration may result in the formation of new binding sites which have different affinities for the ion. Also, the increase of divalent cation may replace H^+ from binding sites, thus diminishing protonation of the fixed charges in the cell walls. It has been reported that Ca^{2+} efflux from cell walls during elution was smaller when external Ca^{2+} concentration was doubled from 0.75 mM to 1.5 mM. In addition, irregularities (discontinuity) in the efflux curves were reported (Jansen and Kylin, 1980).

The estimated amount of Ca^{2+} in the cytosol (3 and 7 μmoles Ca/g fw for whole and dissected segments, respectively) was higher than the estimated amount in the vacuolar compartment (1.7 and 0.7 μmoles Ca/g fw for whole and dissected segments, respectively). The cytosolic free Ca^{2+} level is maintained in a low (nanomolar) range in plant cells, the presence of larger amount of Ca^{2+} in the cytosolic compartment illustrate its high capacity for Ca^{2+} buffering. Calcium binding proteins such as calmodulin and calcireticulin may be involved in chelating Ca^{2+} in the cytosol. Such chelated Ca^{2+} is readily exchangeable and available for efflux, as half times of elution of cytosolic Ca^{2+} ranged from 10 to 17 min in dissected and intact segments (Table 4.2)

4.5.7 Comparison of DFS in whole and dissected segments from the mature zone

In the present study, the identity of the wall compartment was confirmed by a cold treatment. As predicted, neither the amount of Ca^{2+} in the wall compartment nor its half-time of elution

was influenced by temperature. The fastest compartment, which eluted with a half-time of less than 1 min in this study, was attributed as an exchange from the walls. Comparable half times of elution from cell walls were obtained in earlier studies (Table 4.1). Gierth et al. (1999) calculated (under the assumption that the movement of Rb^+ within the apoplast was due to the free diffusion) that Rb^+ would penetrate 500 μm into the tissue within 2 min. The combined thickness of epidermal and cortical cell layers of onion root segments used in this study was approximately 500 μm. Our finding that $^{45}Ca^{2+}$ elution from cell walls had a half-time of 1 min fully supports the findings of Gierth et al., (1999).

The amount of Ca^{2+} present in the cell wall compartment was larger in dissected segments than in intact ones (Table 4.2). The average ratio of the amount of eluted Ca^{2+} from intact/dissected segments was 1:4.8. This ratio is comparable to the one obtained by Peterson (1987) in onion roots for sulphate. It is clear that in bisected and in dissected segments, $^{45}Ca^{2+}$ had unrestricted contact with the cell walls of the epidermis as well as all cortical cell layers, while in intact ones this contact was restricted to the cell walls of epidermis and outer tangential walls of exodermis. This result illustrates that Casparian bands present in the cell walls of the exodermis restricted apoplastic permeablilty of these walls to Ca^{2+}.

4.5.8 Comparison of DFS and half-times in mature and young zones

In the segments from young regions of the roots, the cell wall compartment was eluted somewhat faster (45 and 35 s, Table 3) than from those from the mature root zone (57 and 55 s, Table 4.2). Faster times of exchange of $^{45}Ca^{2+}$ from young walls may reflect their composition. It is possible that in this region the pectic substances (polygalacturonic acid) still exist in esterified form (Dolan et al., 1997), and there are less carboxylic groups to bind Ca^{2+}, so its exchange is faster. In addition, the amount of Ca^{2+} eluted from the epidermal and cortical walls in the young zone (16. 5 μmoles/g fw, whole segments, Table 4.3) was smaller than the amount eluted from the mature zone (44.6 μmoles/g fw, dissected, Table 4.2). This difference reflects the higher capability of cell walls from mature segments to bind Ca^{2+}.

4.5.9 Accumulation of Ca^{2+} in the stele

During the course of this study it was observed that at the end of the elution series, intact segments accumulated a much larger amount of Ca^{2+} than dissected ones (Tables 4.4 and 4.5). The following experiments were designed to determine the location of the accumulated Ca^{2+}. For this purpose, segments were chosen from a young region of the roots where the exodermal Casparian bands were not yet developed. In intact segments with sealed ends, the external solution had access to the epidermis and all cortical cell layers up to the endodermis. Such segments were compared to similar ones, which were bisected longitudinally. In bisected segments, the external solution was in contact with all cells, including those present in the steles. If the vacuole was the site of Ca^{2+} accumulation, there should be a larger amount of radioactivity recovered from cut segments, as more cells were in contact with the label. However, the opposite results were obtained in this study, as maximal accumulation of Ca^{2+} was detected in sealed segments (0.071 and 0.018 μmoles/g fw intact and bisected, respectively, Table 4.4). This was attributed to trapping of Ca^{2+} in the stelar apoplast, including the lumena of the xylem vessels, as during the long loading period, some of the Ca^{2+} was probably transferred to the stelar apoplast. Since the stele in intact segments was sealed at both ends, and endodermal Casparian bands prevented diffusion of Ca^{2+} to the cortex, Ca^{2+} was trapped, whereas it was eluted from bisected segments. The influx of Ca^{2+} to the stele was estimated to occur at the rate of 2.3 nmoles g^{-1} fw h^{-1}. This value is lower than reported 37 nmoles g^{-1} fw h^{-1} for excised rye roots, where a linear, steady state Ca^{2+} accumulation in the xylem was recorded for 12 hours after excision (White et al., 1992).

Plant cells are known to accumulate Ca^{2+} in their vacuoles. Such accumulation did not occur during the 17 hour loading period in onion roots as the amount present in the bisected segments after elution was completed was small (Tables 4.4, and 4.5). In an earlier study, Maclon (1975) concluded that the accumulation of Ca^{2+} in the vacuoles of onion roots is negligible, as there was no difference in either hot water-exchangeable Ca^{2+} or total Ca^{2+} content of the excised segments preincubated with the tracers for 30 h. Based on findings of this and earlier studies (Maclon, 1975), it can be concluded that cells of onion roots are not accumulating high amounts of Ca^{2+} in their vacuoles and that most of the Ca^{2+} taken up is

Table 4.5 Accumulation of $^{45}Ca^{2+}$ in intact and dissected segments.

Segments were taken from mature zone of the root. After 17 h of incubation, segments were eluted for 360 min. Data was calculated based on radioactivity present in the root tissue after the elution was completed.

Experiment	Intact segments µmoles Ca/g fw	Dissected segments µmoles Ca/g fw	Difference µmoles Ca/g fw	% Accumulation (Intact vs. dissected segments)
1	1.199	0.089	1.11	92.6
2	1.001	0.096	0.91	90.5
3	1.470	0.149	1.32	89.9
4	1.257	0.084	1.17	93.3
5	1.593	0.083	1.51	94.8
6	0.989	0.028	0.96	97.2
Average ±SD	1.253 0.243	0.088 0.038	1.16 0.22	93.0 2.72

transported across the root to the stele. On the contrary, White et al., (1992) demonstrated a steady-state $^{45}Ca^{2+}$ accumulation into the root vacuoles of rye (*Secale cereale*) between 40 min and 12 h after introduction of the radioisotope to the nutrient solution. Their data suggested that the tonoplast has a low permeability to Ca^{2+} and that it was effectively sequestered within the vacuole. It can be concluded, therefore, that onion roots take up Ca^{2+} for specifically transporting it to the stele for long distance transport to the shoot.

4.6 Conclusions

The experimental system developed by Peterson (1987) proved useful in comparing the cell wall space accessible to Ca^{2+} under conditions where exodermal Casparian bands are present on the radial pathway of Ca^{2+} movement from the external medium into the root. This study confirms that exodermal Casparian bands restrict radial ion movement and demonstrates that those impede apoplastic Ca^{2+} transport. In addition, results of this study indicate that the majority of Ca^{2+} absorbed by the root segment is accumulated in the stele. Such accumulation was observed in the segments cut from 2 to 4 cm from the root tip, as well as in the segments cut more than 10 cm from the tip. This accumulation was not obscured by the presence of the Casparian bands in the exodermis in the mature zone. Since elution data indicate that Casparian bands are not permeable to Ca^{2+} ions, it can be predicted that absorbed Ca^{2+} is transported into stele *via* other than cell wall pathways.

Chapter 5
Subcellular localization of calcium in onion roots

5.1 Abstract

In the present study, the patterns of localization of free Ca^{2+} in different zones of onion roots were determined using a Ca^{2+} antimonate precipitation procedure and an improved method for root fixation. In all living cell types, Ca^{2+} antimonate precipitates were predominantly associated with the outer leaflet of the plasma membrane and the inner leaflet of the tonoplast. Additionally, Ca^{2+} antimonate precipitates were present in the periplasmic space, vacuoles, mitochondria, plastids, golgi sacks, endoplasmic reticulum, and multivesicular bodies. The cytosol and cell walls were free of precipitate with the exception of those of the stelar parenchyma, specifically the walls that were in contact with mature metaxylem vessels. Other cell walls in the stele were precipitate free. The presence of free Ca^{2+} in the cell wall adjacent to the metaxylem indicates an intracellular origin of this ion, which may be delivered to the apoplast by two processes: active transport *via* Ca^{2+}-ATPases and/or incorporation of cytoplasmic vesicles filled with Ca^{2+} *via* exocytosis. It may also reflect possible differences in cell wall porosity or chemistry of Ca^{2+} binding sites. The majority of the precipitates were removed during incubation of the sections in EGTA, confirming that Ca^{2+} was the primary ion present in them.

5.2 Introduction

Earlier results from this thesis demonstrated that Ca^{2+} is absorbed and translocated from zones greater than 10 mm from the root tip (Chapter 3) and that the exodermal Casparian bands in the mature zones restrict the apoplastic movement of Ca^{2+} (Chapter 4). The present study was performed to localize free and loosely bound Ca^{2+} within onion roots in an attempt to clarify its pathway of radial transport from the external medium to the stele. Ca^{2+} readily enters the apoplast as a divalent ion and is transported in the xylem in its ionic form as well. The fate and form of Ca^{2+} between its entry into the root and its exit in the xylem are unknown.

The possible pathways of Ca^{2+} transport within the root which have been considered to date are apoplastic and symplastic. The apoplastic pathway involves transport within cell walls, while symplastic pathway requires uptake into the cell cytosol, and transport within the cytoplasmic continuum from cell to cell through plasmodesmata. Since in the root cell cytosol, as in all living cells, the Ca^{2+} level is extremely low (nM range), the symplastic pathway was considered inefficient for Ca^{2+} supply (Hanger, 1979). On the other hand, the apoplastic Ca^{2+} transport pathway is restricted in the major part of the onion root due to the development of exodermal Casparian bands (Chapter 4). In the young part of the root, it is possible that Ca^{2+} is transported apoplastically up to the endodermis, assuming that the endodermal Casparian band is also impermeable to Ca^{2+}. According to a hypothetical model, Ca^{2+} enters the root and moves radially within the cortical apoplast, up to the root endodermis, where it may be shunted across the endodermal cells (Clarkson, 1984). If this is true, there should be an accumulation of Ca^{2+} in the vicinity of endodermis in young zones. Similarly, this could also be the case in the vicinity of the exodermis in older root zones. Any such accumulation could be demonstrated by the antimonate precipitation technique. Special attention was paid in this study to verify the above predictions.

Although no translocation of Ca^{2+} occurred from apical zones of the onion roots (Chapter 3), the radial transport of Ca^{2+} and its accumulation in the immature xylem elements (especially in their vacuoles) cannot be excluded. A recent cryo-analysis of maize roots demonstrated K^+ accumulation in living vessel elements at the root tip (Enns et al., 1998). Localization of Ca^{2+} with antimonate could determine if this ion is also present at this location in onion roots.

A second pathway of transport is possible in onion roots, namely endocytosis, as indicated by a previous study of PTS uptake (Chapter 2). Endocytotic vesicles containing cationized ferritin were observed in soybean protoplasts with transmission electron microscope (TEM) (Tanchak et al., 1988). To the best of my knowledge, there is no study that reports endocytotic vesicles in onion roots at the ultrastructural level. The precipitation technique using antimonate could determine whether such vesicles containing $Ca^{2+}$are present in onion roots.

Several techniques have been developed to study subcellular Ca^{2+} distribution including Ca^{2+} detection with metallochromic dyes, luminescent proteins and fluorescent probes (McCormack and Cobbold, 1991). These techniques are useful for following Ca^{2+} dynamics in single cells, but are not suitable for tissue or organ-level studies. The limited spatial resolution of fluorescent probes renders them unsuitable for a study of the subcellular localization of Ca^{2+}, even when a laser scanning confocal microscope is employed. There is no method that would allow simultaneous monitoring of free Ca^{2+} in the apoplast as well as in the intracellular compartments of the symplast.

The antimonate precipitation method has been widely used to localize Ca^{2+} in both plant and animal tissues (reviewed by Wick and Hepler, 1982). The range of Ca^{2+} concentrations at which a Ca^{2+}-antimonate precipitate is formed is linear between 10^{-8} to 10^{-3} M Ca^{2+} in solution. The addition of potassium ions does not affect the Ca^{2+} antimonate precipitation. In addition, the efficiency of Ca^{2+} precipitation using 28 mM antimonate is greater than 80% (Slocum and Roux, 1982). The validity of the technique has been verified (Wick and Hepler, 1982). It appears that the key to a successful precipitation is the efficient and rapid delivery of antimonate anions to the site of Ca^{2+} within the cell.

Upon the development of an excellent protocol for fixation and embedding of onion roots (Ma and Peterson, 2000), an attempt was undertaken in this present study to monitor the distribution of free Ca^{2+} to illustrate the possible pathway of its uptake, transport through the cortical cell layers, and delivery to the xylem elements for long-distance transport. This approach allows localization of free Ca^{2+} at the ultrastructural level in both young and mature zones. Precipitates were observed on the outer surface of the plasma membrane (PM), inner surface of tonoplast, in the periplasmic space, intracellular organelles, multivesicular bodies, as well as in the cell walls of the stellar parenchyma in association with mature metaxylem vessels. Other cell walls, as well as the cytosol of all cells, were free of precipitates. These results are discussed in a view of radial Ca^{2+} transport and its release to the xylem.

5.3 Materials and methods

5.3.1 Plant material

Adventitious roots of onion (*Allium cepa* L.) were sprouted from bulbs grown in a greenhouse as described in Chapter 1. For experiments, 12 d old onions were used, which had 120 to 150 mm long roots. Entire root systems of transpiring onions were preincubated in distilled water (control), 1 or 10 mM $CaSO_4$ for 10 min or 2 h. Root segments 0.5 to 1 mm long were collected from four zones that varied developmentally: 5, 20, 40, and 100 mm proximal to the root tip (see Fig. 1.1).

5.3.2 Transmission electron microscopy

To avoid Ca^{2+} redistribution during sample collection (cutting), entire roots were immersed in a prefixative containing: 2% gluteraldehyde (Baker Inc. Phillipsburg, NJ), 2% acrolein (Aldrich Ltd. Oakville, ON), and 2% potassium antimonate (Aldrich Ltd. Oakville, ON), in 25 mM sodium-phosphate buffer, pH 7.6. Segments 0.5 to 1 mm long were collected in the

prefixative. Samples were kept at 4°C overnight. The prefixative was then rinsed off by incubation of the samples in buffered 2% antimonate (pH 7.6), 3 × 20 min, followed by a rinse with distilled water adjusted to pH 10. This step was carried out to remove the soluble sodium and potassium antimonate salts while the insoluble Ca^{2+} antimonate remained as precipitates. Samples were postfixed in a mixture containing 2% potassium antimonate and 0.5% OsO_4 (Electron Microscopy Sciences), in the sodium-phosphate buffer, pH 7.6, at 4°C overnight. Rinsing was carried out as following the prefixation. Specimens were further processed for embedding in Spurr's resin (Polysciences Inc., Warrington, PA) using the protocol of Ma and Peterson (2000).

Cross, and tangential longitudinal sections were cut with a glass knife on a Reichert Ultracut E microtome (Leica AG Reichert Division, Austria). Semithin sections were stained with 0.05% toluidine blue O and observed with a light microscope. Ultrathin sections (80 nm) were collected on Formvar-coated 75-mesh copper grids. Some sections were post-stained in uranyl acetate and lead citrate to check the quality of preservation. Unstained sections were examined to determine the presence of Ca^{2+}-antimonate in the tissues. Observation was made using a Philips CM 10 electron microscope operated at 60 kV. The Ca^{2+} antimonate precipitates were quite characteristic and easily recognizable. For clarity, photography was done on unstained sections.

The specificity of the Ca^{2+}-antimonate precipitate was confirmed by dissolving precipitate from already analyzed sections in 200 mM EGTA, pH 8, at 60°C for 1 h. As a control, some sections were treated in distilled water at 60°C for 1 h.

5.4 Results

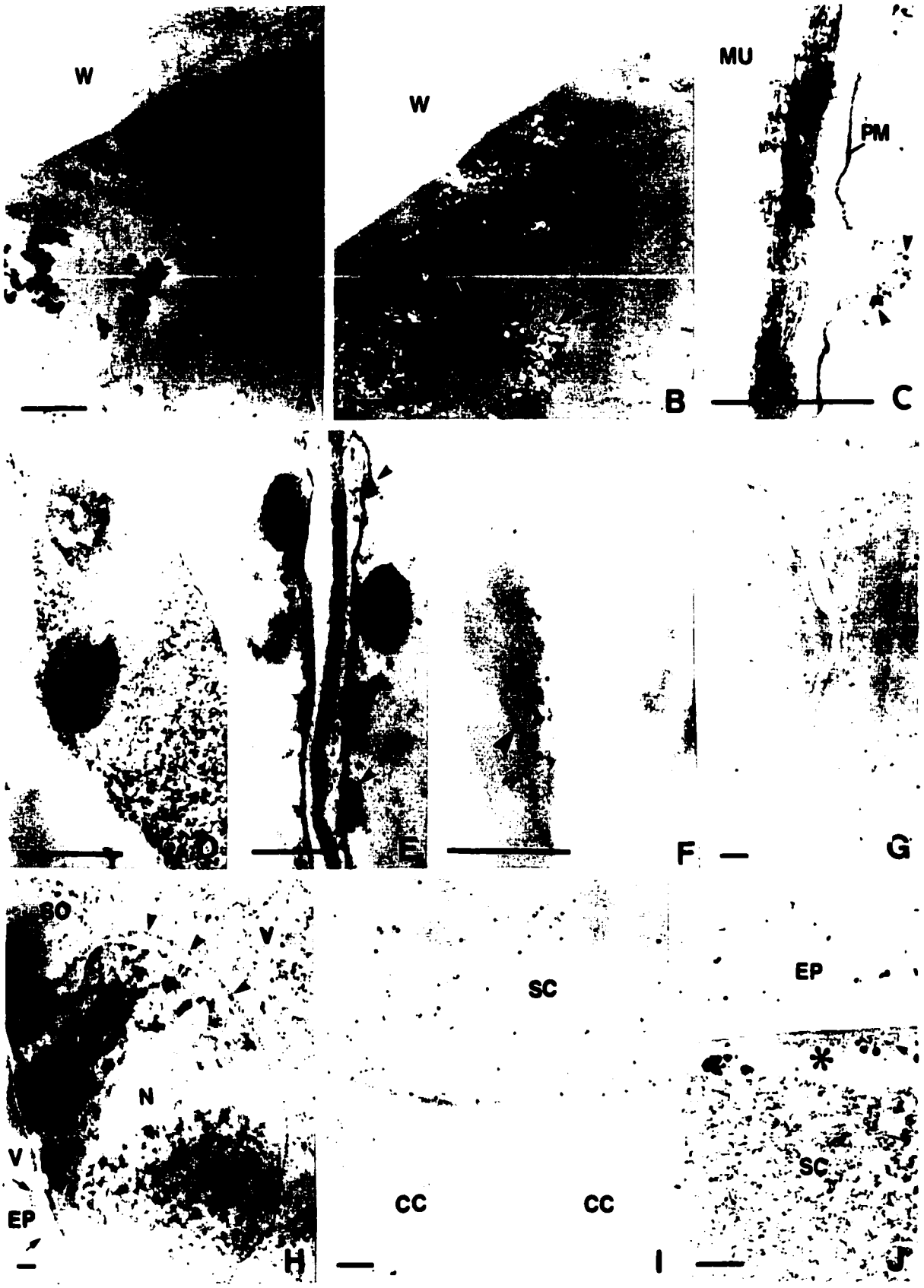
5.4.1 The technique for ultrastructural localization of free Ca^{2+} in onion roots

The TEM protocol of Ma and Peterson (2000) allowed successful preservation of root tissues in all developmental stages. The ultrastructure of the cytosol was readily observed in all post-stained sections. The presence of organized cortical microtubules was used as an indication of the satisfactory quality of tissue fixation and preservation. Gluteraldehyde and acreolin in the prefixative could enhance tissue fixation and facilitate penetration of antimonate to the inside of the root segments. The phosphate buffer was postulated to aid in the precipitation of mobile Ca^{2+} (Coleman and Trepka, 1972) and enhance its specific localization, possibly by decreasing the sensitivity of antimonate for sodium and potassium (Torak and LaValle, 1970).

In onion root sections fixed in the presence of antimonate, electron-dense crystals were observed with the TEM (Fig. 5.1, A). To demonstrate that these were indeed calcium deposits and not osmophilic globules (lipid bodies) or other structures, tissue sections fixed with and without potassium antimonate were compared. Precipitates were only observed in tissues that were fixed in the presence of antimonate. The specificity of the reaction of Ca^{2+} with antimonate was determined by treatment of sections with EGTA, a chelating agent, which is known to bind Ca^{2+}. Treatment of previously observed sections containing precipitates with EGTA, resulted in a disappearance of the precipitate deposits, leaving empty holes in the resin
(Fig. 5.1, B). Occasionally, smaller crystals, embedded in resin (thus, not in contact with the EGTA solution) remained undissolved.

Figure 5.1 Electron micrographs of ultrastructural localization of free Ca^{2+} in onion roots

A. Mature xylem element. Arrowheads point to electron-dense Ca^{2+} antimonate precipitates.
B. The same as A, after treatment with EGTA. Arrows indicate the holes in the resin where Ca^{2+} antimonate precipitates were located.
C. Outer tangential wall of the epidermis in the meristematic zone of the root. MU-mucilage, PM-plasma membrane, arrowheads indicate the Ca^{2+} antimonate within the mucilage in the invagination of the outer surface of PM.
D. Ca^{2+} antimonate present in the plastids of exodermis 5 mm from the root tip.
E. Radial wall of the epidermis of the meristematic zone. Invaginations of the PM along the wall are indicated by arrowheads. Asterisk - periplasmic space containing Ca^{2+} antimonate.
F. Outer tangential wall of the epidermis from mature zone. Ca^{2+} antimonate-filled invagination of PM (Arrowhead).
G. Proendodermis 5 mm from the root tip. Ca^{2+} antimonate in vacuoles.
H. Exodermis 5 mm from the root tip. Note accumulation of Ca^{2+} antimonate along the inner face of the tonoplast of short cell (arrowheads) of short cell (SC). Sparse Ca^{2+} antimonate precipitates are associated with the tonoplast (arrows) of the epidermal cell (EP). N-nucleus, V-vacuole.
I. Young zone of the root (20 mm from the root tip). Precipitate-free wall between exodermal short cell (SC) and cortical cells (CC).
J. Mature zone of the root (100 mm from the root tip). Difference in Ca^{2+} antimonate precipitates density between epidermal cell and short cell of exodermis. EP-epidermis, SC-short cell, asterisk-cytosol of short cell with some precipitates.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5.4.2 Root development

5.4.2.1 5 mm zone

This region was immediately adjacent the meristematic zone, characterized by cells that were expanding. The proendodermis was differentiated; Casparian bands were not present (Fig. 5.1, G). In the stele, mature protophloem sieve elements were observed, while protoxylem elements were not yet mature, i.e., they retained their protoplasts. Stelar parenchyma cells had dense cytoplasm, with extensive rough endoplasmic reticulum and mitochondria. Their walls contained numerous plasmodesmata.

5.4.2.2 20 mm zone

An endodermal Casparian band was recognizable as an electron-dense incrustation located in the radial walls of the endodermis. The plasma membrane was tightly associated to the bands. In the stele, in addition to the mature sieve elements in the phloem, mature protoxylem vessels were observed. The metaxylem vessels had enlarged but still contained cytoplasm.

5.4.2.3 40 mm zone

In some sections, exodermal Casparian bands were initiated; while in others, no exodermal Casparian bands were detected. Protoxylem vessels were mature; metaxylem vessels had a large central vacuole and cytoplasm.

5.4.2.4 100 mm zone

This was the most mature zone of the root sampled. Casparian bands were developed in all exodermal cells. Suberin lamellae were also deposited in the exodermal long cells. Internal to the mature protoxylem vessels, early metaxylem vessels were mature. The cell walls of stelar parenchyma cells were slightly thickened.

5.4.3 Description of the Ca^{2+} antimonate precipitates

5.4.3.1 Epidermis

At 5 mm from the root tip, epidermal cells were characterized by thin walls typical of the meristematic zone. The cells had numerous organelles including mitochondria, golgi apparatus, rough and smooth endoplasmic reticulum, and plastids. Cortical microtubules were observed. On the outside of the epidermis there was a layer of mucilage that contained Ca^{2+} antimonate crystals. The outer tangential walls of epidermal cells were free of Ca^{2+} antimonate precipitates. Precipitate crystals were present in the periplasmic space outside the outer surface of the PM. Invaginations of the PM containing crystals were also present along the radial walls of the epidermis (Fig. 5.1, E). Nevertheless, no precipitates were observed within the radial walls. The PM invaginations at the outer tangential surface may be correlated with the secretory functions of the epidermis (i.e., forming mucilage). Within the cytosol, there were vesicles that contained Ca^{2+} antimonate precipitates. Occasionally some Ca^{2+} antimonate crystals were observed in association with the plasmodesmata between the epidermis and exodermis. Generally, there were more Ca^{2+} antimonate crystals in the vacuoles of epidermal cells than of any other cells.

In the zone 20 mm from the root tip, occasional crystals were observed in the cell wall interface between the epidermis and exodermis. These were rare observations as generally cell walls were precipitate-free.

In the mature zone, 100 mm from the root tip, there were no precipitates present in the cell walls of the epidermis. These walls were characterized by thickening at the outer tangential side. Epidermal cells were alive, as cytoplasm with numerous mitochondria, nuclei and ER strands were observed. All organelles were free of precipitates. Some precipitates were in association with the PM. Additionally, fine crystals of Ca^{2+} antimonate were present in the vacuoles. Occasionally, precipitate-filled vesicles were present in association with the outer tangential walls of epidermal cells (Fig. 5.1, F).

5.4.3.2 Exodermis

At 5 mm from the root tip, all exodermal cells contained a lot of organelles, such as golgi, rough ER, mitochondria, and plastids. Cortical microtubules were present parallel to the outer PM and tangential wall. Plasmodesmata were rarely observed in the interface between the epidermis and exodermis, because of their low frequency (Ma and Peterson, 1999).

In sections taken 20 mm from the root tip, differences between cells in the exodermis were observed. Some cells had a high content of Ca^{2+} antimonate precipitates distributed throughout the vacuoles, whereas neighboring cells had precipitates associated only with the inner surface of their tonoplasts. There was also a difference between some cells of the epidermis and exodermis; it appeared that exodermal cells accumulated more Ca^{2+} in their vacuoles as compared with epidermal cells (Fig 5.1, J).

At a distance of 100 mm from the root tip, short cells had cytosols free of precipitates (Fig. 5.1, I). Crystals were either lining the inner side of their tonoplast or dispersed in the vacuole (Fig. 5.1, J). Occasionally, invaginations of PM filled with Ca^{2+} antimonate precipitates were observed. Long cells had developed suberin lamellae and their protoplasts had started to degenerate. Ca^{2+} animonate precipitates were observed in their nuclei. Scattered crystals were present within the cytoplasm (Fig. 5. 2, A).

5.4.3.3 Central cortex

The cortex 5 mm from the root was characterized by having isodiametric, thin walled cells. Ca^{2+}-antimonate precipitate was observed in intercellular spaces, but its distribution varied for different sizes of spaces. Crystals were distributed throughout in the small, triangular spaces, whereas in large spaces, crystals were located only in the corners. This difference may reflect the water status of these spaces. It is suspected that small spaces are entirely filled with solution, whereas bigger ones are largely filled with air, and the solution is only lining the cell

Figure 5.2 Electron micrographs of Ca^{2+} antimonate precipitates in cells of onion root.

A. Mature exodermis. EP-epidermis, LC-long cell of exodermis, SL-suberin lamella. Ca^{2+} antimonite precipitates are located in the vacuole of epidermal cell. Long cell cytosol disintegrates upon deposition of suberin lamella.
B. Central cortex in the young zone (20 mm from root tip) V-vacuoles are lined with Ca^{2+} antimonite (arrowheads). Cell wall is free of precipitate.
C. Mature zone of the root (>100 mm from the root tip). V-vacuoles with the inner surface of tonoplast lined with Ca^{2+} antimonite. Arrowheads are pointing to the precipitate-free plasmodesmata.
D. Fully developed Casparian band (asterix) in the endodermis. Note precipitate-free cell walls in the vicinity of Casparian band V-vacuoles lined with Ca^{2+} antimonite (arrowheads).
E. Vesicles containing Ca^{2+} antimonite in the endoderimis of mature zone. PM-plasma membrane invagination encapsulates vesicles.
F. Invagination of plasma membrane associated with deposition of suberin lamella in the endodermis of mature zone of the root.
G. Post stained section of pericyle. V-vacuole lined with Ca^{2+} antimonite (arrowheads). Note numerous organelles in the cytoplasm.
H. Stelar parenchyma. Membrane-bound vesicles containing Ca^{2+} antimonite are present in the vacuoles.
I. Cell wall of stelar parenchyma (SP) and xylem vessel (XY). Arrowheads point to the Ca^{2+} antimonite present in the cell wall.
J. Mature protoxylem vessel filled with Ca^{2+} antimonite.

Scale bars = 0.5 μm in all micrographs.


EP
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SL
LC
A
V
B
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PM
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D
E
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W
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I

walls and is thicker in the corners. Some precipitates were observed in the plastids. Vesicles loaded with Ca^{2+} antimonate precipitate were present between the cell wall and PM. The cytosols of the central cortical cells, however, were free of Ca^{2+} antimonate precipitates.

At a distance of 20 mm from the root tip, there were no crystals present in the periplasmic space between the PMs and cell walls of cortical cells (Fig. 5.2, B). Association of Ca^{2+} antimonate precipitate with plasmodesmata was observed in the cortical walls. Crystals were usually present at the neck regions of plasmodesmata. It is not known if this is the true location of Ca^{2+} within PD or an artifact due to the presence of crystals lining the outer surface of the PM. Within transvacuolar strands, electron-dense particles were observed associated with inner surface of the tonoplast. These particles were not Ca^{2+} antimonate precipitates, as they were different in shape (round) and were removed by EGTA.

In the sections taken 100 mm from the root tip, a very sharp difference was observed concerning the distribution of Ca^{2+} antimonate within the vacuoles of the central cortex. Unlike in younger regions where the most typical distribution of Ca^{2+} antimonate was observed in association with the inner surface of the tonoplast; in this zone of the root, precipitates were either clustered or evenly dispersed in the vacuole. Precipitate was absent from cell wall, cytosol, periplasmic space, and plasmodesmata (Fig. 5.2, C).

5.4.3.4 Endodermis

At any stage of development, the cell walls of the endodermis were free of precipitate. In the young zone (2 mm from the root tip where Casparian bands were not formed yet), along the radial walls, there were no Ca^{2+} antimonate crystals present in the periplasmic space, despite the fact that the PM was not tightly associated with the wall. In more mature zones of the root, Ca^{2+} antimonate precipitate was present lining the inner side of the tonoplast or scattered in the vacuoles. Occasionally, vesicles containing Ca^{2+} antimonate precipitate were observed associated with the radial walls of the endodermal cells. In some sections, there were differences between the endodermis and pericycle, more crystals being present in the vacuoles of the former than in those of the latter. In the mature zone of the root (100 mm from the root

tip), where endodermal Casparian bands were fully developed, there were no Ca^{2+} antimonate crystals present within the wall region of Casparian bands, or associated in any way with the PM (Fig. 5.2, D). In some endodermal cells, suberin lamella deposition had commenced, where the presence of vesicles was always observed in the vicinity of PM adjacent to the suberin lamellae. These vesicles contained Ca^{2+} antimonate precipitate (Fig. 5.2, E, F).

5.4.3.5 Pericycle

The presence of the dense cytoplasm was characteristic for pericycle cells. Numeorus organelles were observed in the cytoplasm. Very light Ca^{2+} antimonate deposition were observed in vacuoles of the pericycle (Fig. 5.2, G)

5.4.3.6 Stelar parenchyma

At 5 mm from the root tip, Ca^{2+} antimonate crystals were present in the vacuoles. At 20 mm from the root tip, vesicles containing Ca^{2+} antimonate were occasionally observed in the stelar parenchyma. In the mature zone of the root (100 mm from the tip), there were precipitate-filled vesicles in the vacuoles (Fig. 5.2, H). In this zone, there was invariably Ca^{2+} antimonate precipitates in the cell walls of stelar parenchyma adjacent to xylem vessels (Fig. 5.2, I).

5.4.4.7 Xylem vessels

At 5 mm from the root tip, some Ca^{2+} antimonate crystals were present in the vacuoles, but not the walls, of the immature xylem vessel members. The density of precipitates was similar to that in the vacuoles of stelar parenchyma cells. At 20 mm from the root tip, the lumena of the mature protoxylem vessels were scattered with fine precipitates of Ca^{2+} antimonate. On some occasions, vesicles filled with Ca^{2+} antimonate were observed in the stelar parenchyma abutting the protoxylem poles immediately internal to the pericycle. The cell walls of these cells also contained some Ca^{2+} antimonate precipitate similar to these present in mature zone (Fig. 5.2, I). In the mature zone, the lumena of mature early metaxylem vessels were full of

dense Ca^{2+} antimonate crystals (Fig. 5.2, J). Frequently, Ca^{2+} antimonate crystals were present in the thickened parenchyma walls associated with xylem elements (Fig. 5.2, I).

5.4.4.8 Phloem

In the zone 5 mm from the root tip, Ca^{2+} antimonate crystals were present in the vacuoles, along the inner surface of the tonoplast, in the periplasmic space, and, occasionally, in the cell walls. No crystals were present in mature protophloem sieve elements.

5.5 Discussion

5.5.1 Evaluation of the Ca^{2+} antimonate precipitation

The Ca^{2+} antimonate precipitation technique is the only technique available that indicates the position of free Ca^{2+} ions at the ultrastructural level. The validity of this technique has been demonstrated by an experiment in which the distribution of Ca^{2+} in flax hypocotyl was identical whether the samples were cryofixed or prepared by standard fixation in conjunction with the antimonate precipitation technique (Rihouey et al., 1995). Application of other methods can produce misleading results concerning the role of calcium in physiological responses. For example, by imaging *Arabidopsis thaliana* L. roots loaded with the fluorescent, Ca^{2+}-indicator Indo-1, Legue et al. (1997) concluded that there is no asymmetry in cytosolic Ca^{2+} levels in response to gravitropism. Methods like this allow one to monitor only the cytosolic Ca^{2+} levels, while Ca^{2+} in other compartments of the root cells remained undetected. In contrast, the Ca^{2+}-antimonate technique allows simultaneous monitoring of Ca^{2+} in all compartments. By using the latter technique, a higher level of Ca^{2+} was detected in the vacuoles of the cortical cells of the gravistimulated roots than in those of the controls (Dauwalder et al., 1985). In the gravistimulated columella cells of the root cap of maize, asymmetric staining with antimonate was observed in the cell walls adjacent to the distal ends. No other walls of the columella cells contained precipitate in either gravistimulated or control roots (Moore, 1986a). These examples illustrate the possibility of achieving an

integrated overview of free Ca^{2+} distribution in whole roots using the Ca^{2+} antimonite precipitation technique.

The precipitate produced after treatment with antimonate contains predominantly Ca^{2+} as demonstrated by X-ray analysis and application of chelators (Wick and Hepler, 1982; Foissner, 1998). In the present study, the nature of the cations present in the antimonate precipitates was inferred from chelation experiments. The result confirmed earlier findings (Slocum and Roux, 1982). In addition, by using the improved TEM protocol (Ma and Peterson, 2000), both tissue preservation and precipitation were enhanced. Thus, major drawbacks of the precipitation technique were minimized. These include poor preservation of tissues (especially subcellular structures), low permeability of aldehyde-fixed membranes to antimonate, and random distribution of the precipitates (which then cannot be associated with any subcellular structures). Taken together, the results obtained in the present study verified that the location of electron dense precipitates is a reliable indicator of free Ca^{2+} in onion roots.

5.5.2 Apoplast

The almost complete lack of precipitate in the walls is, at first glance, surprising. Ca^{2+} was certainly present in the cell walls, as the roots were treated with 10 mM $CaSO_4$ for 10 min or 2 h before fixation. It was expected that the short treatment with Ca^{2+} would result in the detection of the initial pathway by which Ca^{2+} enters the roots, whereas within 2 h, Ca^{2+} from the external solution would equilibrate with and saturate the apoplastic pool of free Ca^{2+}. However, no difference was observed between the two treatments in the amount or distribution of free Ca^{2+} in the roots, as very little precipitate was present in the epidermal and cortical cell walls. In a study of maize roots, the absence of Ca^{2+} antimonate precipitate in cell walls was reported (Moore, 1986a). These results could be due to the strong buffering capacity of cell walls for free Ca^{2+}. In cell walls, the agents that bind Ca^{2+} are matrix substances, especially pectins, which are rich in galacturonic acid (Fry, 1988). These have been described as a block polymer of homogalacturonan, consisting mainly of contiguous unbranched D-galacturonic acid (pyranose) residues, some of which can contain methyl ester

groups, whereas others are un-esterified. Blocks of about a dozen or more consecutive un-esterified residues can became cross-linked *via* Ca^{2+} bridges, whereas esterified and partially esterified blocks cannot (Fry, 1988). The negatively charged oligogalacturonate blocks within pectic polysaccharidies are believed to permit the lateral association of pectin molecules via Ca^{2+} bridges, leading to the formation of a network of noncovalently linked polymers (Jarvis, 1984). The observation that there is very little ionic Ca^{2+} present in cell walls under normal, physiological conditions may be an indication that the level of free Ca^{2+} is not only regulated in the cell's cytosol, but also in the wall. Willats et al., (1999) investigated the primary wall structure *in vivo* by using antibodies for de-esterified blocks of homogalacturonan. The binding of the antibodies was enhanced by addition of 0.2 mM Ca^{2+}. When the amount of calcium added was higher than 1 mM, a pectate gel was formed and the assay was no longer possible. In the latter case, Ca^{2+} must have changed the structure of the pectic blocks so that the antibody no longer recognized them.

The observed absence of free Ca^{2+} in cell walls cannot be attributed to the pH (7.6) of the fixative. Under physiological conditions, the pH of cell walls in numerous plant species is 4-7, with the majority 5-6.5. The pKa of polygalacturonic acid is 3 (Grignon and Sentenac, 1991). Hence, the carboxylic groups in the pectic substances would be negatively charged at all times. It has been demonstrated that the concentration of polygalacturonic acid in primary cell walls of storage parenchyma is above 100 mol m^3 (Michael and Ehward, 1996). That gives an enormous binding capacity of walls for Ca^{2+}.

It is concluded, therefore, that the antimonate precipitation reflects the free Ca^{2+} status in the onion root cell walls. Furthermore, the presence of antimonate precipitate in the walls of stelar parenchyma cells in this study supports the above conclusion, in that it demonstrates that ionic Ca^{2+} can be detected in cell walls, when present. Other studies showed a dramatic asymmetry of Ca^{2+} localization in columella cell walls when roots were positioned horizontally (Moore, 1986a). The antimonate reacted with Ca^{2+} in a portion of the cell walls adjacent to the plasmalemma. This staining was restricted to half of the cell wall. Also, deposition of Ca^{2+} into cell walls was observed in etiolated oat coleoptile cells irradiated with red light followed by far-red light (Tretyn et al., 1992).

As in the majority of cell walls, no antimonate precipitates were found in the walls of the epidermis in this study. Those walls are known to be highly permeable; the application of fluorescent dyes in all reported experiments led to staining of the epidermal walls (Moon et al., 1986). The precipitation of the other ions (e.g., Cl^- with Ag^+) resulted in a heavy deposition of the precipitate in the epidermal walls (Ma, personal communication). Therefore, epidermal cell walls are permeable and porous enough to allow Ca^{2+} diffusion and antimonate crystal formation.

The fact that there was no Ca^{2+} precipitate in onion root cell walls opens a different perspective on how Ca^{2+} is transported within the apoplast. There are two possibilities: (1) Ca^{2+} is not transported within cell walls or (2) Ca^{2+} is transported within walls as in a cation exchange column, interacting with the fixed negative charges of the pectic substances. The presence of free Ca^{2+} in the cell walls of stelar parenchyma (at the site of xylem loading) supports the first possibility, as free Ca^{2+} would be detected in the cell walls if it is present there. But the absence of free Ca^{2+} in the cell walls of the epidermis supports the second possibility, as it has to pass through this wall to be absorbed by the root. There may be, therefore, a combination of the two processes in radial Ca^{2+} transport through the onion root apoplast. Nevertheless, no accumulation of free Ca^{2+} in the vicinity of the endo- and exodermal Casparian bands was observed, as would be expected if Ca^{2+} was transported as a free ion in the cell walls.

The presence of Ca^{2+} antimonate precipitate in intercellular spaces of the central cortex may reflect their water status. In small spaces, which may be filled with water, precipitate was present in the center, whereas, in larger ones, which may contain air, precipitate was present only in the corners. This reflects the localization of Ca^{2+}-containing solution outside of the cell walls, as in the larger spaces, solution is only lining the cell walls. Another possible explanation of such a localization of precipitates is their correlation with recently observed amorphous matrix materials lining intercellular spaces. Small spaces could be filled with such materials, whereas large spaces have only their edges associated with matrix materials

(Ma, personal communication). The presence of such matrix materials could facilitate holding the precipitates in the intracellular spaces during fixation and embeding.

5.5.3 Plasma membrane

In the present study, association of the Ca^{2+} antimonate precipitate with the outer surface of the PM was consistently observed. Similar observations were reported in etiolated oat coleoptile (Tretyn et al., 1992) and maize root cap (Moore, 1986a), and in bean root tips (Moore, 1986b). It seems that this is a universal phenomenon. This may reflect an active regulation of the Ca^{2+} level in the cytosol by extrusion *via* Ca^{2+}-ATPases. However, it is not clear whether the precipitation technique could detect all Ca^{2+} ions associated with outer surface of PM, as it is possible that part of the PM-associated Ca^{2+} will bind to the head groups of phospholipids. The presence of membrane-bound Ca^{2+} was demonstrated in studies using chlorotetracycline (CTC) as a Ca^{2+} probe (Muhling et al., 1998). CTC is a lipophilic chelator that binds preferentially to divalent cations at the membrane outer surface, especially to the head groups of phospholipids (Caswel and Hutchison, 1971). The presence of Ca^{2+} is necessary for the integrity of the PM. The antimonate-precipitation technique was not able to detect the membrane-bound Ca^{2+}, as precipitates were not observed on the outer surface of the membrane. However, crystals were present in the periplasmic space, which reflected the localization of the free calcium.

5.5.4 Plasmodesmata

In the young zone of the root (20 mm from root tip), Ca^{2+} antimonate precipitates were present in some plasmodesmata in the cortex. It is possible that deposits of Ca^{2+} antimonate within plasmodesmata may reflect Ca^{2+} movement from cell to cell. Due to the heavy deposits that obscured plasmodesmatal structure, the exact position of the precipitates within these fine connections was not determined. It is possible that precipitates formed within the cytolplasmic annulus or the appressed endoplasmic reticulum. There is still a third possibility, namely the formation of precipitates between the PM and cell wall at the neck region of the plasmodesmata. Symplastic transport of Ca^{2+} was proposed in chickpea

trichomes as Ca^{2+} was detected in both the plasmodesmata (upon treatment with antimonate) and the secretion droplets (Lazzaro and Thomson, 1992). In the young (5 mm from the tip) and mature zones of onion roots (100 mm from the tip) the majority of the plasmodesmata were free of precipitate.

5.5.5 Multivesicular bodies

The present study revealed the presence of invaginations of the PM and associated vesicles that contained Ca^{2+}. These structures have been described on numerous occasions in plant tissues under different names such as multivesicular bodies, paramural bodies, plasmatubules, and plasmalemmasomes. The authenticity of these structures has been questioned as an artifact of chemical fixation, but there is growing evidence supporting their actual presence in plant cells. These structures were observed upon cryofixation (Caffey and Harris, 1985). A specific dense labeling in immunocytochemical studies provides convincing evidence of their genuine nature (Wierzbicka, 1998; Herman and Lamb, 1992; Robinson et al., 1996; Olmos and Hellin, 1997). However, little is known about the formation of multivesicular bodies and their physiological importance (Caffey and Harris, 1985). They are often associated in the tissues where high transport fluxes are present, e.g., in the transfer cells of young pea leaves (Harris and Chaffey, 1985). Robinson et al. (1996) demonstrated a high expression of aquaporins in the multivesicular bodies and proposed that their role was to mediate water transport between the apoplast and vacuole. Olmos and Hellin (1997) localized PM H^+-ATPase and apoplastic phosphatase in multivesicular bodies associated with the PM and proposed that these structures are involved in the process of incorporation of H^+-ATPase into the PM and secretion of phosphatase into the apoplast. Results of the study of PTS accumulation in onion roots (Chapter 2) indicated that endocytosis takes place. This study demonstrated the presence of Ca^{2+} in the vesicles. Taken together, these results suggest that the endocytotic pathway may contribute to Ca^{2+} uptake and transport. To date, such a pathway for Ca^{2+} in roots has not been considered. Further studies may elucidate its relevance to ion transport in general.

5.5.7 Xylem

The accumulation of potassium in the vacuoles of the immature xylem vessels in maize roots led McCully et al. (1987) to the conclusion that absorbed minerals are released into the transpiration stream upon the maturity of the vessels. Numerous studies of the site of Ca^{2+} uptake demonstrated the Ca^{2+} influx into the root occurs at the root tip, where endodermal Casparian bands are not developed (reviewed in Chapter 3). Accumulation of Ca^{2+} in the developing vessel members may be speculated. However, our results did not prove this. In fact, the developing vessels resemble cortical and stellar parenchyma cells in the density and distribution pattern of precipitates.

The presence of Ca^{2+} pumps in plant cell membranes is now firmly established (Muchhal et al., 1997; Olbe and Sommarin, 1998; Askerlund, 1997; Hwang et al. 1997). These Ca^{2+}-ATPases are responsible for the active export of Ca^{2+} from the cytosol and maintaining its low, physiological level. The presence of the Ca^{2+} antimonate precipitates along the membranes in onion root cells may reflect the activity of such pumps. If this assumption is correct, the presence of precipitate in the stelar parenchyma associated with the mature metaxylem vessels may indicate that there is also an active export of Ca^{2+} from stelar parenchyma cells. Once in the walls of vessels, ions can freely diffuse to the lumena.

The parenchyma cells near the xylem may be specialized for the transport of ions. In fact, a study by Jahn et al. (1998) demonstrated that the PM H^+-ATPase was highly localized in the cells facing xylem poles, forming "bridges" between the endodermis and metaxylem. Since PM H^+-ATPase maintains the pH gradient across the PM and enables ion transport against their gradients, high expression of H^+-ATPase in parenchyma cells abutting the xylem poles strongly suggests that those cells are playing a primary role in xylem loading. Results of this study verified the presence of free Ca^{2+} in the walls of the stelar parenchyma cells facing the xylem poles. This indicates that there may be a specialized high localization of Ca^{2+} transporters in these cells that are pumping ionic Ca^{2+} into the cell wall. With progress in Ca^{2+}-ATPases characterization and purification, it would be possible to localize these transporters using antibodies in the future.

Chapter 6
Effect of inhibitors on Ca^{2+} accumulation in onion roots

6.1 Abstract

In mature zones of the root, the exodermal Casparian bands are blocking the apoplastic pathway for Ca^{2+}(Chapter 4). However, Ca^{2+} accumulates and it is transported from that zone (Chapter 3). A mechanism of Ca^{2+} uptake and accumulation in onion root segments was investigated by means of inhibitors. Ca^{2+} entry into the root is partially mediated through lanthanum-sensitive Ca^{2+} channels, as treatment with lanthanum resulted in 43% of inhibition of Ca^{2+} accumulation. The exit of Ca^{2+} from the symplast and its loading into the xylem is mediated in part by P-type Ca^{2+} ATPases, which are vanadate-sensitive. Incorporating vanadate into the transpiration stream resulted in 53% inhibition of Ca^{2+} accumulation. A less specific inhibitor, ruthenium red, caused a moderate (19%) inhibition of Ca^{2+} accumulation. The general respiratory inhibitor KCN, and potent Ca^{2+} transport inhibitor, erythrosine B, did not affect the levels of accumulated Ca^{2+} in root segments. Taken together, these results indicate the complexity of Ca^{2+} uptake and its delivery to the xylem.

6.2 Introduction

The uptake and translocation of Ca^{2+} can occur along the onion root, starting approximately 1.3 cm behind the root tip (Chapter 3). In a more mature zone of the root, where exodermal Casparian bands have been developed, these modifications restrict the apoplastic pathway for radial Ca^{2+} movement (Chapter 4). It can be concluded, therefore, that the absorbed and

translocated Ca^{2+} was transported radially *via* the symplastic pathway, at least through the exodermis.

The uptake of Ca^{2+} from the soil solution, its transport across the root, and its delivery to the apoplast of the stele are complex processes. They involve Ca^{2+} entry into the cells, radial transport across the root and release into xylem vessels. The entry of Ca^{2+} into the symplast is governed by the plasma membrane (PM). Ca^{2+} enters the root passively (with is electrochemical gradient) through channels. The presence of Ca^{2+} channels in roots cells is well documented (reviewed by White, 1998). How Ca^{2+} is transported radially across the multiple cell layers in the root is unknown. Nevertheless, Ca^{2+} is delivered to the stele and is loaded into the xylem vessels. This can be achieved by Ca^{2+}-ATPases (Bush, 1993). This study was designed to investigate the mechanism of Ca^{2+} entry into the root, and its subsequent accumulation in the xylem by the use of specific inhibitors.

There is a considerable electrochemical gradient for passive Ca^{2+} entry into the cell through Ca^{2+} channels. Several types of Ca^{2+} channels have been characterized for barley, wheat and maize roots. Some of them are described as non-specific outward-rectified (K^+) channels, having a low unitary conductance for Ca^{2+}, activating at positive voltages, and unlikely being major Ca^{2+} influx sites (White, 1997). More specific Ca^{2+} channels are: the maxi cation channel, which is inhibited by ruthenium red (White, 1993), and the voltage-dependent cation channel two (VDCC2), which is inhibited by lanthanum (White, 1997). In maize root PM vesicles, the class of Ca^{2+} channels that is inhibited by lanthanum mediates 70% of the Ca^{2+} influx. The remaining 30% of Ca^{2+} influx is inhibited by Gd^{3+} but not La^{3+} (Marshall et al., 1994). Another class of Ca^{2+} channels is inhibited by verapamil (White, 1998). It seems that lanthanum-sensitive channels may be exclusive Ca^{2+} transporters in wheat roots (Huang at al., 1994, 1996; Sasaki et al., 1994). In addition to the classes of channels described above, others are present in roots. Stretch-activated Ca^{2+} channels in the tips of root hairs were found to be inhibited by La^{3+} (Felle and Hepler, 1997) or verapamil (Wymer et al., 1997). These channels are responsible for establishing the Ca^{2+} gradient at the tip of root hair which mediates tip growth. A variety of hormonal and external stimuli trigger the opening of Ca^{2+} channels during the signal transduction pathway (reviewed by Bush, 1995). Since these invoke

transient Ca^{2+} increases in the cytosol, leading to the initiation of signal transduction pathway and metabolic responses, they are not discussed here.

The exit of Ca^{2+} from the symplast into the cell walls of the pericycle or stelar parenchyma in the stele occurs against its electrochemical gradient and requires an input of metabolic energy. A number of PM Ca^{2+}-ATPases from different plant species has been characterized (reviewed by Bush, 1995). They belong to the P-type ATPase family, i.e. they form a phosphorylated intermediate during the catalytic cycle. Orthovanadate (VO_4^{-3}) inhibits PM ATPases (Sze, 1985; Serrano, 1990), and phosphorylases which form a phosphorylated intermediate (Macara, 1980), and acid phosphatases (Gallagher and Leonard, 1982). In the present study, vanadate was incorporated into the transpiration stream to investigate a role of P-type Ca^{2+}-ATPaes in xylem loading.

Another inhibitor, erythrosine B [3', 6'-dihydroxy-2',4',5',7'tetraiodospiro isobenzofuran-1-(3H),9'-(9H)xanthen]-3-one], is a biological stain used as food colouring (Windholdz, 1976). Although erythrosine B is known to be specific inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, it has been also reported to inhibit PM Ca^{2+}-dependent Mg-ATPase (Rasi-Caldogno et al., 1987; 1989). Because of their potency to inhibit Ca^{2+} pumps, these chemicals were used in this study to investigate the mechanism of Ca^{2+} loading into the xylem. KCN is a general inhibitor of mitochondrial respiration. It reduces ATP synthesis (Voet and Voet, 1991), which is needed for maintaining low levels of Ca^{2+} in the plant cell cytosol. It could potentially interfere with Ca^{2+}transport, especially its loading to the xylem.

6.3 Materials and Methods

Onion roots were obtained from bulbs sprouted in vermiculite-filled pots in a greenhouse as described in Chapter 1. Roots 12-14 cm long from two-week-old plants were used for experiments. For each replicate, 10 segments 2.5-cm-long were excised and the cut ends were sealed with sticky wax. Segments were taken from a mature zone of the root (>10 cm from the root tip) with the exception of those to be treated with lanthanum where in addition to mature segments, additional ones from a young zone (1.5 to 4 cm from the root tip) were

tested. Control segments were incubated in an unbuffered, aerated solution of 1 mM $CaSO_4$, adjusted to pH 5.5. Experimental segments were incubated in 1 mM $CaSO_4$ like the control, but with added inhibitors. Lanthanum chloride or potassium cyanide were added to the incubation solutions to the final concentration of 1 mM 15 min prior to labeling with $^{45}Ca^{2+}$. Lanthanum was used for testing the entry of Ca^{2+} through channels in young and mature zones, whereas cyanide was used to determine the dependence of Ca^{2+} accumulation on metabolic energy.

Testing the exit of Ca^{2+} in the stele and the possible involvement of Ca^{2+}-ATPases was performed by feeding the inhibitor-containing solution into the transpiration stream. Vanadate (1 mM), ruthenium red (1 mM), and erythrosine B (100 μM) were introduced into the xylem by cutting the roots at the distance of 3 cm from the root tip, placing them in the treatment solution, and allowing the plant to transpire. It was assumed that the transpiration stream would pull the solution containing inhibitors into the xylem vessels, from whence they would diffuse throughout the stellar apoplast and have access to all cells in the stele. Control plants were fed the same way with 1 mM $CaSO_4$. After 2 h preincubation with control or inhibitor-containing solutions, roots were cut off the bulb under water at their bases to relieve any xylem tension, which could cause an air embolism in the stele and relocation of the inhibitors. Such treated roots were cut in 2.5 cm root segments, and their ends sealed. For each replicate of the experiment, 10 segments were used. They were incubated in an aerated solution of 1 mM $CaSO_4$, labeled with 10 μl $^{45}CaCl$ (0.001 μCi) for 17 h. The radioactive solution was withdrawn, and the segments were washed 3x10 min in non-labeled 1mM $CaSO_4$. Segments were blotted with absorbent tissue, the wax caps were removed, and segments were weighed. Radioactivity present in the segments was measured as described in Chapter 4.

6.4 Results

The involvement of PM Ca^{2+} channels in Ca^{2+} uptake into the roots became evident upon treatment with lanthanum. An external treatment of segments with 1 mM La^{3+} resulted in an average of 42% inhibition of Ca^{2+} accumulation in the segments from young and mature

zones (Table 6.1). The incorporation of vanadate into the transpiration stream resulted in a 53% inhibition of Ca^{2+} accumulation. Ruthenium red caused a moderate, 19% inhibition of Ca^{2+} accumulation, whereas erythrosine B and KCN did not have any effect (Table 6.1).

6.5 Discussion

The entry of Ca^{2+} into the root involves crossing the PM. Chapters 3 and 4 illustrated that Ca^{2+} can be absorbed and translocated from mature part of the root, where the presence of exodermal Casparian bands is hindering its apoplastic pathway.

Pharmacological evidence presented in this study suggests that Ca^{2+} influx into the root is at least partially mediated by lanthanum-sensitive Ca^{2+}channels. Lanthanum is a well-known antagonist of Ca^{2+} influx, and does not penetrate the PM (DuPont and Leonard, 1977; Lonergan and Williamson, 1988; Perdue et al., 1988). In the present study, treatment with lanthanum resulted in a 42% inhibition of Ca^{2+} accumulation in both, young and mature zones of the root
(Table 6.1), demonstrating some involvement of La^{3+}-sensitive Ca^{2+} channels in Ca^{2+} transport in onion roots. This incomplete inhibition may reflect the complex mechanism of Ca^{2+} transport by roots. It is apparent that other classes of Ca^{2+}-channels could be present in PM. 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 PM as well. Moreover, the root is composed of many different cell types, each specialized to a specific task. It is possible that the Ca^{2+} channel complement of each cell type differs.

To date, the entry of Ca^{2+} into the cell is thought to be mediated almost exclusively through Ca^{2+}-selective channels. However, there may be other pathways for $Ca^{2+}$to enter the root. For example, there is growing evidence that the process of endocytosis occurs in plant cells (reviewed by Battey et al., 1999). The endocytotic pathway could be an efficient way to transport Ca^{2+} through the cytosol without altering its low concentration, since the moved Ca^{2+} would be within membrane-bound vesicles. Preliminary data (Chapter 2) suggest that

Table 6.1 The effect of various inhibitors on the accumulation of Ca^{2+} in onion root segments.

Reported values are means of 3 replicates ± SD. Lanthanum and KCN were applied to sealed segments as an external solution, whereas vanadate, ruthenium red, and erythrosine B were introduced directly into the transpiration stream through a cut in the root.

Inhibitors and zones	Controls µmoles Ca/g fw	Inhibitors µmoles Ca/g fw	Change (%)
Lanthanum	-		% Inhibition
Young zone	0.048 ±0.008	0.027 ±0.007	43 ±17
Mature Zone	1.17 ±0.24	0.69 ±0.12	40 ±7
Vanadate	-		%Inhibition
Mature zone	1.25 ±0.23	0.50 ±0.06	53 ±4
Ruthenium Red	-		%Inhibition
Mature zone	1.94 ±0.26	1.57 ±0.22	19 ±1.7
+KCN	-		No change
Mature zone	0.92 ±0.18	0.98 ±0.35	
Erythrosine B	-		No change
Mature zone	1.25 ±0.27	1.57 ±0.43	

endocytosis is occurring in onion roots. Furthermore, the ultrastructural study (Chapter 6) provided evidence for the presence of Ca^{2+}-filled vesicles in association with the PM. The significance of the endocytotic pathway for Ca^{2+} transport has not been tested quantitatively.

Ca^{2+} exit from the symplast in the stele and its loading into the xylem may involve Ca^{2+}-ATPases, which are present in PM (Muchhal et al., 1997). The observed 53% inhibition of Ca^{2+} accumulation in the present study strongly supports the hypothesis that Ca^{2+} pumps are involved in xylem loading. Another potential inhibitor that was introduced into the xylem of onion root is ruthenium red. Ruthenium red is a polycation, whose inhibitory action was suggested to be by counteracting the association of Ca^{2+} with negatively charged phospholipids (Missiaen et al., 1990). White (1996) demonstrated the presence of ruthenium red-inhibited Ca^{2+} channels in PM-derived vesicles from rye. Ruthenium red also inhibits Ca^{2+}-ATPase present in the endoplasmic reticulum and interferes with the uptake of Ca^{2+} into intracellular organelles (Kreimer et al., 1985; 1988; McComark, 1985; Schumaker and Sze, 1987). In addition, ruthenium red inhibited Ca^{2+} extrusion across the PM (Hinds et al., 1981). It was tested, therefore, whether ruthenium red-sensitive Ca^{2+} transporters are present ion onion roots. Treatment of onion root segments with ruthenium red resulted a 19% decrease in $^{45}Ca^{2+}$accumulation in this study. A somewhat higher inhibition (50%) was reported for sorghum root tips (Wilkinson and Dunkan, 1993). This may be due to the different part of the root used for experiments (tip vs. mature zone) as well as species-related differences. Another inhibitor used in the present study was erythrosine B. Erythrosine B is a potent inhibitor of Ca^{2+}-ATPases and Ca^{2+}-release channels (BCC1) in the mechanosensitive tissue of *Bryonia dioica* (Liss et al., 1998). Similar to the present study, erythrosine B was fed into a tendril of *B. dioica via* the transpiration stream. Touch-induced coiling was reduced by 50% in erythrosine B-treated tendrils (Liss et al., 1998). In roots, erythrosine B decreased absorption of $^{45}Ca^{2+}$ by 35% in sorgum root tips (Wilkinson and Dunkan, 1995). However, in this study, erythrosine B did not reduce Ca^{2+} accumulation. It is possible that erythrosine B is a more specific inhibitor of Ca^{2+} ATPases present on the intracellular membranes than the PM, and only vanadate-sensitive P-type of Ca^{2+} pumps are involved in xylem loading.

The absence of a clear effect of temperature (Drew and Biddulph, 1971), or of a variety of metabolic inhibitors (Clarkson, 1984) on Ca^{2+} absorption in roots supported the conclusion that the transport processes measured with $^{45}Ca^{2+}$ are essentially independent of metabolism. In this study, treatment with KCN did not affect Ca^{2+} accumulation. KCN inhibits mitochondrial respiration by interacting with the cytochrome path of electron transport (Voet and Voet, 1998). Lambers et al. (1991) provided an overview, which points out that in roots, treatment with 0.01 mM KCN inhibits respiration (oxygen uptake). This, in turn, should interfere with the cell's metabolism and reduce the level of the metabolic energy for cellular processes. Metabolic inhibitors can lead to alterations of membrane permeability, allowing leakage of inorganic ions from tissues (Lambers et al., 1991). In the case of Ca^{2+}, metabolic energy is needed to maintain its low level in the cytosol. Therefore, interfering with mitochondrial production of ATP should lead to an increase in cytosolic Ca^{2+} concentrations in the root cells, as the Ca^{2+} extruding ATPases may be affected, and total amount of Ca^{2+} in the tissue may remain high. On the other hand, Lambers et al. (1991) suggested that there are two pathways in the mitochondria of roots, one which partitions electrons through the cytochrome path, and the other alternative path, which is cyanide resistant, but inhibited by salicylhydroxamic acid. If the alternative pathway is supplying metabolic energy in the presence of KCN, Ca^{2+} transport may be not affected. In contrast, a strong inhibition of Ca^{2+} movement by treatment with KCN across gravistimulated root tips of maize was detected (Lee et al., 1983; 1984). But, the same roots were able to transport Ca^{2+} across the elongation zone at the same level in the presence or absence of KCN (Lee et al., 1983). Those conflicting results highlight the differences between the developmental zones in Ca^{2+} transport.

The data presented in this study support a concept of multiple avenues of Ca^{2+} transport through the root. This Ca^{2+} must be transported radially across the root and loaded into the xylem for long distance transport. To the best of my knowledge, this is the first report that demonstrates the involvement of Ca^{2+} channels in the process of Ca^{2+} uptake leading to its long-distance transport by mature roots, as well as providing direct evidence for active Ca^{2+} release into the xylem by a P-type Ca^{2+} ATPase in the stelar tissue.

Chapter 7

General discussion

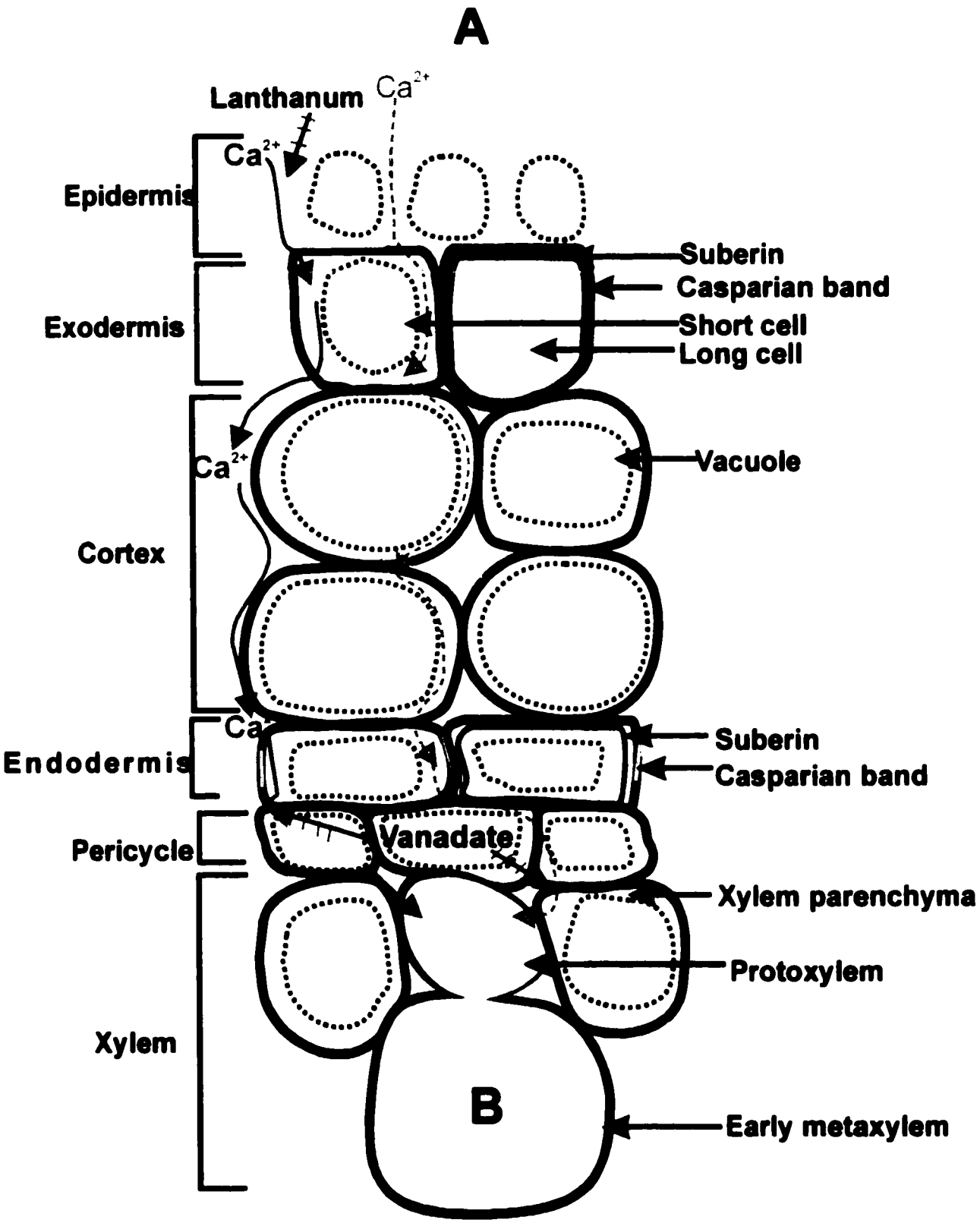
Based on the research reported to date about Ca^{2+} transport in roots, McLaughin and Wimmer (1999) in their recent review announce, "The typical pathway for uptake of mineral element [Ca^{2+}] involves initial movement into the free space of the root apoplast, and subsequent basipetal movement through apoplastic pathways. This pattern of movement through the cell walls avoids trans-membrane passage and the toxicity problems that contact with the cytoplasm would entail. Thus, Ca uptake is confined to the unsuberized fine root tips that have no Casparian strip, which would force symplastic detour". The widely publicized belief that Ca^{2+} uptake and transport within the roots occurs principally by passive movement by diffusion or in the mass flow of water is based on a range of studies that demonstrate a positive correlation between Ca^{2+} transport and the rates of water movement to transpiring organs of the plant (reviewed by Bangerth, 1979). The presence of an "apoplastic" pathway for Ca^{2+} transport seems to be established as a fact and led Yeo et al. (1987) to use $^{45}Ca^{2+}$ as an indicator for the apoplastic bypasses in rice roots.

The results of this study provide evidence that in onions: Ca^{2+} is absorbed at the root tip, but not translocated from it; Ca^{2+} uptake and its subsequent translocation occur in more mature parts of the root, despite the impermeability of the exodermal Casparian bands present there; Ca^{2+} channels are involved in its uptake; Ca^{2+} pumps are involved in its release to xylem. Thus, the presented evidence contradicts thoroughly the generally accepted view of radial Ca^{2+} movement in roots.

The contribution of this study can be fitted into a new model of radial Ca^{2+} movement in roots Fig. (7.1). Ca^{2+} is taken up and translocated in the xylem in the mature zone of the root

Figure 7.1 Pathways of radial transport of Ca^{2+} from soil soution (A) to the lumena of xylem vessels (B).

Red print - contribution of present study
Green print - hypothetical pathway


A
Lanthanum
Ca2+
Ca2+
Epidermis
Exodermis
Cortex
Endodermis
Pericycle
Xylem
Suberin
Casparian band
Short cell
Long cell
Vacuole
Suberin
Casparian band
Vanadate
Xylem parenchyma
Protoxylem
B
Early metaxylem

(Chapter 3). However, the results of compartmental elution analyses (Chapter 4) demonstrate that exodermal Casparian bands hinder the radial apoplastic pathway. Therefore, Ca^{2+} has to enter the symplast near the root periphery. The most probable site of Ca^{2+} entry is the outer tangential surface of the PM of the short cell of the exodermis, as Ca^{2+} can move up to this point within the radial walls of the epidermis. A 43% inhibition of Ca^{2+} uptake was observed upon treatment with lanthanum (Chapter 6). This indicates that lanthanum-sensitive Ca^{2+} channels are at least partially mediating Ca^{2+} entry into the root symplast. It is unknown whether Ca^{2+} is transported apoplastically or symplastically through the cells in the central cortex. However, low cytosolic Ca^{2+} levels in living cells and their ability to extrude Ca^{2+} into their walls suggest that most of the Ca^{2+}would be sequestered into the apoplast. The fast time of exchange of Ca^{2+} from cell walls (half-time 1 min, Chapter 4) shows that Ca^{2+} is loosely bound and can move quicly within the walls by cation exchange reactions with the negative charges present on the walls. Based on the permeability study of exodermal Casparian bands (Chapter 4) as well as literature data it can be assumed that the Casparian bands present in the endodermis are not permeable to Ca^{2+} as well. At this point of its radial pathway, Ca^{2+} would have to enter the symplast, perhaps via Ca^{2+} channels located in the outer tangential surface of the PM of the endodermal cells. Some endodermal cells are depositing suberin lamellae. Those would be inaccessible to Ca^{2+}. But passage cells, located in the proximity to xylem poles remain unsuberized. Those cells could facilitate Ca^{2+} transport to the stele. To enter the stelar apoplast, Ca^{2+} has to be transported against its electrochemical gradient. Inhibition of Ca^{2+} accumulation in the xylem upon treatment with vanadate (53%, Chapter 6) shows that P-type Ca^{2+}-ATPaes are mediating this process. The precise site of Ca^{2+} exit from the symplast is unknown, but it is logical to suggest that the inner surface of the PM of the endodermal cells contain Ca^{2+} pumps. Once in the stelar apoplast, Ca^{2+} has an unrestricted pathway to the lumena of the xylem vessels. The TEM study (Chapter 5) of Ca^{2+} localization in the tissues of the root revealed the presence of free Ca^{2+} in the cell walls of xylem parenchyma. It is possible, therefore, that xylem parenchyma is primarily involved in the loading of the vessels. Taken all together, the contributions of the present study imply that Ca^{2+} is transported, at least in part, via a symplastic pathway.

From a consideration of this model, a number of questions arise.

(1) Is Ca^{2+} moving by a cation exchange reaction along cell walls?

Indirect evidence for Ca^{2+} transport in cation exchange reaction along cell walls has been obtained in a few studies. Van de Geijn and Smeulders (1981) found an enhancement of $^{45}Ca^{2+}$ transport into the apical meristem of excised tomato shoots by darkness and concluded that Ca^{2+} transport to this region and low-transpiring organs is controlled by a cation exchange reaction rather than by root pressure. It is possible that auxin plays an important role in Ca^{2+} transport along cation exchange sites in developing, low-transpiring organs since inhibition of Ca^{2+} transport and reduction in cation exchange capacities by inhibition of auxin transport has been demonstrated (Bangerth, 1976; Kirkby and Pilbeam, 1984;, Banuelos et al., 1988). Further, studies of gravitropism in roots have demonstrated that inhibitors of auxin transport prevent Ca^{2+} relocation in root tips (Lee et al., 1984). Results presented in Chapter 4 of this study demonstrate that onion root cell walls have a large capacity for binding and exchanging Ca^{2+}. The half-time for the release of Ca^{2+} from the cell wall compartment was about 1 min. It is possible, therefore, that Ca^{2+} can be transported very fast within cell walls by a cation exchange process. The amount of Ca^{2+} eluted from the cell wall compartment was larger than the one eluted from cytosol (Chapter 4). It is likely, that more of the Ca^{2+} taken up by the cells at the root periphery is pumped out of their cytosol into the cell walls of the inner layers of the root, where the Ca^{2+} can be transported radially within the apoplast.

(2) Is it possible that there is asymmetric localization of Ca^{2+} transporters in onion roots?

Immunofluorescence microscopy was used to localize P-type H^+-ATPases in maize root apices. It revealed that there is an asymmetric localization of this pump, with the most abundant amount in epidermal and endodermal cells, as well as xylem parenchyma (Jahn et al. 1998). This distribution may reflect the fact that root epidermal cells are a primary site for nutrient uptake from the soil solution, and is consistent with the expected high level of membrane transport activity. Antibodies have been raised to Ca^{2+}-ATPase (LCA1 and BCA1) and these immunoreactive proteins colocalize with both plasma membrane marker enzyme and Ca^{2+}-ATPase activities during membrane marker enzyme studies (Askerlund, 1997). Unfortunately, to date no *in situ* localization of Ca^{2+}-ATPase has been published. Nevertheless, based on localization of H+-ATPase (Jahn et al., 1998) and on observation of

the presence of free Ca^{2+} in the cell walls of xylem parenchyma (Chapter 5), it can be predicted that localized high levels of Ca^{2+}-ATPases are present in the stele.

However, results of the TEM study (Chapter 5) did not reveal any presence of free Ca^{2+} in the stelar parenchyma. It seems that Ca^{2+} may be "stored" in xylem parenchyma and slowly released to the xylem. Modern techniques may provide supplementary evidence for other possible mechanisms by which plants could store Ca^{2+}. For example, Nelson et al. (1997) found significant amounts of calreticulin (a newly discovered major Ca^{2+}-storing protein in plants) in mature seeds of *Arabidopsis thaliana*. The authors of the study describe this discovery as "intriguing" and propose that calcireticulin may have a role in protecting the seed during desiccation and dormancy (Nelson et al., 1997). It is tempting to speculate that this protein may be a source of Ca^{2+} for initial development of the radicle.

(3) Is it possible that Ca^{2+} is bypassing the cytosolic compartment by means of membrane-bound vesicles?

The process of endocytosis and its contribution to solute transport in roots has not been studied. In the present study (Chapter 1) it was demonstrated that endocytosis occurs in onion roots cortical cells. PTS was transported from the cell wall to the vacuole in the membrane-bound vesicles. There are only a few reports which demonstrate the transfer of fluorescent tracers into the vacuole via an endocytotic pathway, e.g. in soybean protoplasts (Tanchak and Fowke, 1987), *Arabidopsis* protoplasts (Roszak and Rambour, 1997), and intact leaves of *Commelina communis* (Himler et al., 1990). For this process to contribute to the radial Ca^{2+} transport through the root, vesicle delivery would need to be made to the PM on the inner tangential side of the cell, or from a vacuole to this location. Whether such a mechanism contributes to the radial transport of Ca^{2+}has yet to be determined. The general physiologist's view of the vacuole is that it is a static organelle with transport proteins embedded in its tonoplast, mediating single ion transfers across that membrane, into and out of the vacuole. There is, however, some evidence that solute transfer from the vacuole to the apoplast can occur. These studies concentrated on the fast responses, such as stomatal closure or movement in *Mimosa* which can be observed and followed over a short time interval. Indirect evidence pointed to vesicles mediating the loss of sucrose from the vacuole to the apoplast in

guard cells (Talbott and Zeiber, 1996). Another example is a rapid turgor loss in *Mimosa* pulvinar motor cells. The movement in *Mimosa* follows mechanical disturbance, which gives rise to a propagated action potential in the phloem and, on its arrival in the primary pulvinus, a longer-lasting action potential is elicited in motor cells, leading to a massive loss of solute from vacuole and cell, loss of turgor and collapse of the pulvinus. Studies have been concentrated on activation of K^+ and Cl^- channels involved in the action potential, but there are observations that stimulation of the action potential in sieve tubes and motor cells involves release of both ions and sucrose to the apoplast (Fromm and Eschrich 1988 a, b, c). There is a microscopy study that reports the appearance of vesicles in the cytoplasm during the dramatic shrinkage of the vacuole associated with excitation (Weintraub, 1951). The author of this study suggested that these vesicles budded off the vacuole, thereby reducing its volume. Unfortunately, microscopical studies are still heavily criticized and often observations of vesicles are attributed to their formation during tissue fixation and preparation for the microscopy. Development of improved methods for *in situ* hybridization together with the use of the membrane-specific antibodies may prove that criticism of microscopical studies lacks its validity. Using a specific antibody and immunogold labeling of aquaporins Robinson et al. (1996) presented an excellent example for the demonstration of the genuine nature of the vesicles derived from PM and their involvement in water transport. So far, studies on vesicle incorporation into the PM have focused on the deposition of the cell wall materials during cell elongation, as in root hair tip growth or pollen tip growth (reviewed by Battey et al., 1999). Future studies may show whether such vesicles are involved in Ca^{2+} transport in roots.

The radial pathway of Ca^{2+} transport from soil solution to the xylem elements for long distance transport is far from being solved. The evidence presented in this study may initiate a reconsideration of the generally accepted apoplastic pathway for Ca^{2+} transport in roots. With the progress in characterization and generation of Ca^{2+} transporters specific antibodies the radial pathway of Ca^{2+} transport in roots could be determined.

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