The role of the apoplastic transport barriers for radial water and ion uptake in rice (*Oryza sativa* L.) and corn (*Zea mays* L.) roots

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Fakultät Biologie, Chemie und Geowissenschaften
der Universität Bayreuth

von
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Bayreuth, im Juni 2005

Die vorliegende Arbeit wurde am Lehrstuhl für Pflanzenökologie der Universität Bayreuth unter der Leitung von Prof. Dr. E. Steudle durchgeführt und entstand im Zeitraum von Januar 2002 bis Mai 2005. Gefördert mit finanzieller Unterstützung der Deutschen Forschungsgemeinschaft im Rahmen des DFG-Schwerpunktprogramme „Apoplast“ und BMZ (project No. 2000.7860.0-001.00) “Trait and Gene Discovery to Stabilize Rice Yields in Drought Prone Environments”

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Tag der Einreichung: 13.06.2005

Tag der mündlichen Prüfung: 19.09.2005

Prüfungsausschuss:

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Vollständiger Abdruck der von der Fakultät für Biologie, Chemie und Geowissenschaften der Universität Bayreuth genehmigten Dissertation zur Erlangung des Grades eines Doktors der Naturwissenschaften (Dr. rer. Nat.)

Dedicated to my beloved parents

This dissertation is submitted as a "Cumulative Thesis" that covers six (6) publications; four (4) printed articles, two (2) articles are in press. In order to clarify the publications, they are listed below.

Printed articles:

I **Ranathunge K., Steudle E. and Lafitte R.** 2003 Control of water uptake by rice (*Oryza sativa* L.): role of the outer part of the root. **Planta** 217, 193-205 (Chapter 2).

II **Ranathunge K., Kotula L., Steudle E. and Lafitte R.** 2004 Water permeability and reflection coefficient of the outer part of young rice roots are differently affected by closure of water channels (aquaporins) or blockage of apoplastic pores. **Journal of Experimental Botany** 55, 433-447 (Chapter 3).

III **Ranathunge K., Steudle E. and Lafitte R.** 2005 Blockage of apoplastic bypass-flow of water in rice roots by insoluble salt precipitates analogous to a Pfeffer cell. **Plant, Cell and Environment** 28, 121-133 (Chapter 4).

IV **Schreiber L., Franke R., Hartmann K., Ranathunge K. and Steudle E.** 2005 The chemical composition of suberin in apoplastic barriers affects radial hydraulic conductivity differently in the roots of rice (*Oryza sativa* L. cv. IR64) and corn (*Zea mays* L. cv. Helix). **Journal of Experimental Botany** 56, 1427-1436 (Chapter 5).

Articles in press:

V **Ranathunge K., Steudle E. and Lafitte R.** A new precipitation technique provides evidence for the permeability of Casparian bands to ions in young roots of corn (*Zea mays* L.) and rice (*Oryza sativa* L.). 2005 **Plant, Cell and Environment** (online: doi:10.1111/j.1365-3040.2005.01391.x) (Chapter 6).

VI **Steudle E. and Ranathunge K.** Apoplastic water transport in roots. 2005 **Kluwer Academic Publishers, The Netherlands** (in press) (Chapter 7).

Declaration of the self-contribution of research articles

The thesis is compiled with several research articles (7), which included different research work. Most of the research work in the thesis was carried out by myself independently at the Department of Plant Ecology, University of Bayreuth under the supervision of Prof. Dr. E. Steudle.

In Chapters 2, 4 and 6 my contribution was about 80% where I made all experiments and drafted manuscripts, which I completed after discussing with the co-authors. In Chapter 3, most of the research experiments were done by myself in addition to writing the manuscript. My contribution in this chapter was about 70%. Mr. Lukasz Kotula who is the second author of that article assisted to do some experiments while he was in Bayreuth as an ERASMUS student from Katowice, Poland. He especially focused on learning laboratory techniques as well as carried out different experiments, which focused on oxygen transport across rice roots, a topic different from that dealt with in the present work. My contribution of Chapters 5 and 7 were 50%. In Chapter 5, root anatomical studies for rice and corn as well as all hydraulic measurements of rice roots were carried out by myself at Bayreuth under the supervision of Prof. Steudle. The chemical analyses of both rice and corn roots were carried out by members of Prof. Dr. Lukas Schreiber's group at the University of Bonn. I have contributed the parts dealing with anatomy and water transport. The Chapter 7 is a review for a book summarizing work done within the DFG-Schwerpunkt No. 322717 "Apoplast" ("Der Apoplast der höheren Pflanze: Speicher-, Transport- und Reaktionsraum"). I have contributed to the experimental data presented there and wrote the parts dealing with anatomy (see figures) and transport across the rice root.

The co-author Dr. Renee Lafitte (Chapters 2-4, and 6) is the collaborative partner from the International Rice Research Institute (IRRI), Manila, Philippines. Parts of the thesis research were financially supported by the BMZ *via* the IRRI (project no. 2000.7860.0-001.00: "Trait and Gene Discovery to Stabilize Rice Yields in Drought Prone Environments").

All published articles can be downloaded from the worldwide web: http://www.uni-bayreuth.de/departments/planta/research/steudle/index.html.

Acknowledgements

First and foremost, it is with a deep sense of respect and heartfelt gratitude that I extend my unreserved indebtedness to my supervisor, **Prof. Dr. Ernst Steudle**, Department of Plant Ecology, University of Bayreuth, Germany. His expert guidance, advice and genuine assistant in all aspects of my studies have been very helpful to my course of study. His office door has always been open to me, for all kinds of scientific discussions, questions, and dialogues, while I was immersed in plant biophysics, he kept me from getting lost. He stands in my mind as a great icon in the scientific community.

Secondly, I am very grateful to Dr. Renee Lafitte, **International Rice Research Institute (IRR)[1], Manila, Philippines** for her contribution to the rice project and fruitful discussions.

Thirdly, I would like to thank Prof. Dr. Lukas Schreiber, University of Bonn, Germany for discussing the results from my thesis during mutual visits. His expert comments on the chemical composition of apoplastic barriers in relation to root hydraulic properties are very much acknowledged.

My enormous thanks go to Professor Carol Peterson, University of Waterloo, Ontario, Canada, who introduced me into detailed root anatomy giving her valuable technical tips during several stays in the lab as a guest Professor. Many thanks are also going to Professor David Clarkson, University of Bristol, UK, for his advise in root physiology and good English writing. Last but not least, I thank Dr. Tim Colmer, University of Western Australia, Perth for their thoughtful and fruitful comments, suggestions, discussions, and especially during the editing of my manuscripts.

I extend my thanks, gratitude and appreciation to Mr. Burkhard Stumpf for his excellent technical support through all aspects of my studies as well as providing me a friendly, wonderful atmosphere throughout my stay in Bayreuth.

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[1] IRRI is a nonprofit agricultural research and training center established to improve the well-being present and future generations of rice farmers and consumers, particularly those with low incomes. IRRI receives its financial support from donor governments (including Germany), agencies and foundations. More information: http://www.irri.org

My heartfelt gratitude and enormous thanks go to Dr. (Mrs.) Arunika Gunawardena who introduced me to Prof. Steudle in order to get this position in Bayreuth. Her great guidance and encouragements throughout the study are highly appreciated.

I would like to thank Dr. Hilde Zimmermann for her expert help and teaching me the experimental techniques during the early stages of my research. Furthermore, my thanks go to Mr. Lukasz Kotula who helped me conduct research during his stay in Bayreuth as a master's student and for his great friendship. Also, Dr. Seong Hee Lee whose great help on cell pressure probe measurements has been highly appreciated.

My heartfelt gratitude and thanks go to my loved ones; my parents and Thakki, who are beside me, not leaving me alone providing me the moral support during my course of study, encouraging me to succeed.

Last, but not least, I would like to thank my all friends in Bayreuth especially; Alexandra, Anurag, Bogus, Chris, Cristine, Dennis K, Dennis O, Jana, Katja, Marita, Mat, Nabi, Nik, Otilija, Özgün, Petruschka, Qing, Phillip, Sebastian, Thomas, Tobias, Yangmin and there are others, who shared many important and memorable moments with me during my stay in Bayreuth.

Deutsche Forschungsgemeinschaft (**DFG**) and Bundesministerium für wirtschaftliche Zusammenarbeit und Entwicklung (**BMZ/IRRI**) are gratefully acknowledged for the financial support. Without their support these projects would not have been possible.

Content

I

Detailed Summary

1 General introduction

Water is the most abundant constituent in flora and fauna and essential for the existence of life. It is the vehicle for convective transport of solutes, i.e. mineral nutrients, assimilates in plant cells, organs, tissues and even in ecosystems. Plant roots absorb water from the root medium to its xylem across the root cylinder and transport to the shoot along the xylem vessels. Different from nutrient ions, water flow in plants, including the flow across roots involves no direct active pumping or do not use energy (ATP). Both, across the root cylinder and in xylem vessels along the root, water flow is down-hill (passive) following gradients in free energy (water potential) or pressure (Chapter 1.1). Nutrient ions may be dragged with water to reach the plasmalemma ("solvent drag"), or their movement is diffusional in nature in the absence of a drag.

Water uptake by plant roots can be described by simple force/flow relations analogous to Ohm's law and is characterized by hydraulic conductances or resistances or, when referred to unit cross-section, hydraulic conductivities or resistivities (Chapter 1.2). These parameters are known to be highly variable (1.3). This affects the water status of plants. At a given rate of transpiration, the water supply by roots determines the water status of the shoot and its ability to assimilate carbondioxide (Fig. 1). According to the water demand from the shoot, roots can adapt or even regulate water flow changing the pathways (apoplastic *vs.* cell-to-cell) or by regulating water channel (aquaporin) activity. Since the pioneering work of Peter Agre and co-workers, there has been much effort expended in identifying the molecular structure of water channels and their significance to water transport across cell membranes (Murata et al. 2000; Ren et al. 2001). The regulation of water input by roots is as important as that of the output (transpirational loss from stomata). Evidences collected over the past decade show that the phenomenon of variable root hydraulics is not only related to the permeability of root cell membranes to water (as it is largely for nutrient ions), but also depends on some variability along the apoplastic passage. The presence of apoplastic barriers is important (Casparian bands and suberin lamellae in the endo- and exodermis). The anatomical complexity of the root dictates that the flow of water through it will also be complex. The water flow in roots can be described by a composite transport model,

which allows for differences in movement through membranes of individual cells and along the apoplast, as well as through various tissues (Chapter 1.4)**.**

In the following, recent findings are summarized which relate to apoplastic water and ion flow in rice and corn roots. Results have been obtained using root pressure probes, pressure chambers and different types pressure perfusion techniques. Because of differences in the structure of roots of wetland plants (rice) from typical herbaceous plants (corn), they do allow a more detailed view on root hydraulics and tests of current models.

Background

1.1 Concept of water potential

Water potential (ψ) is the key parameter in water relations of plants. It is a quantitative measure of water status of the plant and the driving force that moves water within plants and across plant boundaries to the soil and atmosphere.
Water potential may be split up into four different components, written as the following:

$$\psi = P - \pi - \tau + \psi_g \,, \qquad (1)$$

where,

P - turgor pressure

π – osmotic pressure

τ – matric potential

ψ_g – gravitation potential (= $\rho_w \cdot g \cdot h$; ρ_w= density of water, g = gavity, h = height)

For the sake of simplicity, the matric and gravitation potential can be omitted in the context of this thesis. They refer to effects of surface energy (surface tension) and potential energy, respectively. The matric potential represents the interaction between water and a solid matrix. The gravitational term must be considered when water moves in tall trees and work against gravitation is required. It can be neglected at the organ or cell level. Eventually, we get:

$$\psi = P - \pi \,. \qquad (2)$$

Here, we only consider the pressure and concentration dependence of the water potential. Water potential can be directly derived from the chemical potential of water, which is a quantitative expression of free energy associated with water. Considering the pressure and concentration dependence of the chemical potential of water, we get:

$$\mu_w = \mu_w^* + \bar{V}_w P + RT \ln a_w \,, \tag{3}$$

μ_w is the chemical potential of water in a solution (J mol^{-1}) and μ_w* is the chemical potential of pure liquid-water at the given temperature. The term $\bar{V}_w P$ (J mol^{-1}) represents the pressure dependence of the chemical potential (volume work), where $\bar{V}_w$ is the partial molal volume of water ($\bar{V}_w$ = 1.8 × 10^{-5} m^3 mol^{-1}) and P is the hydrostatic pressure (Pa = J m^{-3} = N m^{-2}). a_w denotes the activity of water or its molar fraction ("Molenbruch" in German), which is a measure of water concentration (a_w = 1 for pure water and a_w < 1 for solutions). According to Eq. (3), the presence of solutes in an aqueous solution tends to decrease the activity of water (a_w). In other words, the molar fraction of water becomes lower than that of pure water when solutes added. The term $RT\ ln\ a_w$ describes the contribution of the osmotic activity of water (osmotic work term), which is usually expressed in terms of the osmotic pressure. It is valid that:

$$RT \ln a_w = -\bar{V}_w \pi \quad . \tag{4}$$

Chemical activity of the solute is related to the concentration. The presence of solutes can lead to develop an osmotic pressure (π) in a solution. When increase the solute concentration in a solution, it raises the osmotic pressure, indicating that π and a_w change to opposite directions (Eq. 4). When osmotic pressure increases, the chemical potential of water tends to decrease.

Hence, the formula for the chemical potential of water can be re-written as:

$$\mu_w = \mu_w^* + \bar{V}_w P - \bar{V}_w \pi \,. \tag{5}$$

Water potential is proportional to the difference between chemical potential in the solution and that of pure water (μ_w - μ_w*) and is defined as:

$$\psi = (\mu_w - \mu_w^*) / \bar{V}_w \quad , \tag{6}$$

or simply:

$$\psi = P - \pi \quad . \tag{7}$$

The advantage of using water potential is that from μ_w, it has a straightforward plausible meaning. The unit is that of pressure (J mol^{-1} / m^3 mol^{-1} = Pa), but it relates to the free energy or Gibbs/Helmholtz free energy, which is a measure of the maximal work, which can be done by the water in a given process. The components of water potential, P and π, are directly measurable. It is possible to predict the behavior of water flow on the basis of two easily measured components (P and π) as well as driving force of water flow as water moves along the water potential gradient (down-hill movement or from a region of high water potential to a region of low water potential). In plants, water movement within the plant body and/or water uptake by roots from the soil is completely governed by the water potential gradient.

1.2 Water transport

In 1948, van den Honert introduced the idea that water movement in plants is analogous to electricity flow. According to basic laws of electricity (Ohm's and Kirchhoff's laws), when components of a circuit are arranged in series, their resistances are additive, and when components are arranged in parallel, their conductances (the inverse of their resistances) are additive. Applying Ohm's law to plants, water flow within a plant is usually related the difference in water potential ($\Delta\psi$):

$$\underbrace{water\ flow}_{\text{flow}} = conductance \times \underbrace{\Delta\psi}_{\text{force}} \quad . \tag{8}$$

According to fundamental principles of irreversible thermodynamics, all flows in a system are governed by all forces, and those forces are linearly related to the flows. In

case of just two forces, a gradient in concentration and pressure, water flow is driven by the gradient of hydrostatic and/or osmotic pressure difference. Solute flow is driven by solute concentration difference, but there is also a component related to the pressure difference. Hence, there are couplings between flows such as, water, solute, and ion (current) as well as they interact with forces. These couplings are systematically described by the thermodynamics of irreversible processes (Kedem and Katchalsky, 1958).

1.2.1 Cell water relations

For the description of plant water and its interactions with solutes, the theory of irreversible thermodynamics is especially useful. This is so because water flow equilibrium (osmotic equilibrium) is well defined and most of the flows and forces can be measured directly. In the following, the thermodynamic theory is applied to a single cell in a medium to work out cell water relations and interactions between water and solute flows. The cell interior (superscript 'i') and the medium (superscript 'o') are treated as two-compartment system and it assumes that cell is surrounded by a homogeneous membrane (Fig. 1). All forces acting on the flows are embraced, a mathematical description for the water (J_v) and solute (J_s) flows will be (Kedem and Katchalsky, 1958):

$$J_v = -\frac{1}{A}\frac{dV}{dt} = \underbrace{Lp \cdot P}_{\text{hydrostatic water flow}} - \underbrace{Lp \cdot \left[RT \cdot \left(c^i - c^o\right) + \sigma_s \cdot RT \cdot \left(c_s^i - c_s^o\right)\right]}_{\text{osmotic water flow}} , \qquad (9)$$

$$J_s = -\frac{1}{A}\frac{dn_s}{dt} = \underbrace{P_s \cdot \left(c_s^i - c_s^o\right)}_{\text{diffusional flow}} + \underbrace{\left(1-\sigma_s\right) \cdot \bar{c}_s \cdot J_v}_{\text{solvent-drag}} + \underbrace{J_s^*}_{\text{active transport}} , \qquad (10)$$

where:

J_v	$[m \cdot s^{-1}]$	volume flow ≈ water flow
J_s	$[mol \cdot m^{-2} \cdot s^{-1}]$	solute flow
J_s^*	$[mol \cdot m^{-2} \cdot s^{-1}]$	active solute flow

A	[m^2]	cell surface area
V	[m^3]	cell volume
Lp	[$m \cdot s^{-1} \cdot MPa^{-1}$]	hydraulic conductivity
P	[MPa]	cell turgor
n_s	[mol]	content of permeating solutes `s` in the cell
$\Delta\pi$	[MPa]	difference in osmotic pressure
σ_s	[1]	reflection coefficient of the solute
P_s	[$m \cdot s^{-1}$]	permeability coefficient for the solute `s`
C	[$mol \cdot m^{-3}$]	concentration of non-permeating solute
C_s	[$mol \cdot m^{-3}$]	concentration of permeating solute
$\overline{C_s}$	[$mol \cdot m^{-3}$]	mean concentration of `s` in the membrane [$(C^o_s + C^i_s)/ 2$]
t	[s]	time
R	[$J \cdot mol^{-1} \cdot K^{-1}$]	universal gas constant (≈ 8.314)
T	[K]	absolute temperature

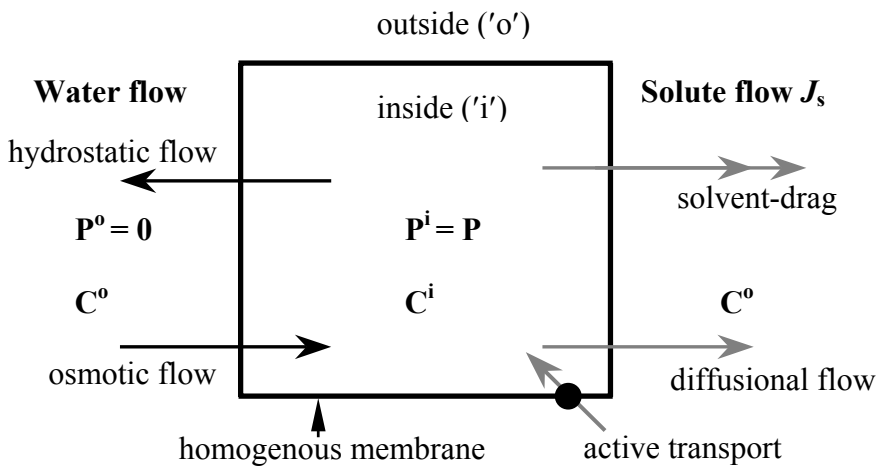

Fig. 1 Two-compartment model of osmosis of a cell. In the model, it is assumed that the inside and outside are separated by a homogeneous membrane which possesses the same permeability characters in each place. The outside atmospheric pressure is considered as zero and used as the reference. Since inside of the system contain over pressure (P), or it is greater than in the outside, it develops hydrostatic water flow (J_v) from internal to external medium. Osmotic water flow is driven by the difference in osmotic pressure. Since $C^i - C^o > 0$, the direction of osmotic water flow is opposite to the hydrostatic water flow. Solute flow (J_s) has three components. Diffusional flow occurs along the concentration gradient from internal to external medium. Water flow couples with solute flow and drag them with water (solvent-drag). The active component of solute flow (J_s*), i.e., the component which relates the transport of solutes to a metabolic reaction, e.g. the splitting of ATP and ATPase.

By convention, Eqs (1) and (2) define flows out of the cell as positive and flows into the cell as negative. Water flow (J_v) has two components, a hydraulic flow driven by gradient in hydrostatic pressure ($Lp \cdot P$; ambient atmospheric pressure is taken as a reference) and an osmotic water flow driven by the difference in osmotic pressure ($Lp \cdot \Delta\pi + Lp \cdot \sigma_s \cdot \Delta\pi$). The osmotic force is opposite to the hydrostatic (minus sign), which build up in the cell as a consequence of the accumulation of solutes ($C_s^i > C_s^o$). Here, both non-permeating and permeating solutes are considered. Total volume flow or water flow (J_v) is resulted by both driving forces called as the hydraulic conductivity (Lp), which is the water permeability of the membrane. The driving force represents a modified water potential gradient ($\Delta\psi = P - \Delta\pi$; $\pi = RT \cdot C$ = osmotic pressure). Since the membrane is considered to be permeable to some solutes, the osmotic component has to be modified by the reflection coefficient (σ_s), which denotes the passive selectivity of the membrane and is usually between zero and unity. It can be also interpreted as a measure of the interaction between water and solutes as they cross the membrane. When $\sigma_s = 0$, the membrane does not distinguish between the solute and water, and solute can readily cross the membrane. If $\sigma_s = 1$, the membrane is not permeable to the solute ($P_s = 0$) and driving force will be equal to the water potential difference ($J_v = Lp\,(P - RT\,\Delta C) = Lp \cdot \Delta\psi$).

Solute flow (J_s) has three components. The first term, the diffusional solute flow [$P_s \cdot (C^i_s - C^o_s)$], which is driven by concentration difference according to Fick's first law. The coefficient relating force and flow in this case is called 'solute permeability' (P_s). The solute permeability coefficient is the passive component of flow of a given solute `s` across the membrane. The second component, $[(1 - \sigma_s) \cdot \overline{C}_s \cdot J_v]$ describes the interaction between water and solutes as they cross the membrane. This is called solvent-drag (the amount of solute dragged along with the water flow in permeable membrane; $\sigma_s < 1$). For a semipermeable membrane ($\sigma_s = 1$; $P_s = 0$), the solvent drag will vanish. On the other hand, for structures, which are not selective at all ($\sigma_s = 0$), the term will be identical with the amount of solutes transported by 'convection' in a water stream. The last term is the active component of solute flow (J_s^*). It represents the transport of a solute as a result of metabolic reaction. In contrast, the active component is missing for the water flow, because no evidence for an active water flow (water pumps driven by metabolic energy). For the solutes usually present in the cell, the

plasma membrane represents a nearly perfect barrier, i.e. σ_s is close to unity and P_s close to zero. On the other hand when the passive selectivity of the cell wall is low ($\sigma_s \approx 0$) and P_s is high.

1.2.2 Water transport in roots

Water relations and water transport across tissues are more complicated than that of an individual cell, where water crosses only the plasma membrane. At the tissue level, there are three parallel pathways involve for the water flow – apoplast, symplast and transcellular path, as well as the tissue cells arrange in series (Fig. 2).

1.2.2.1 Transport pathways in roots

The constitutes outside the plasma membrane of the living cells is termed "apoplast" (Münch 1930). It includes cell walls, intercellular spaces, and the lumena of tracheary elements. The symplast, on the other hand, is the continuum of cytoplasm interconnected by plasmodesmata and excluding the vacuoles. Hence, the terms 'apoplastic' and 'symplastic' transport refer to movements within the two compartments just defined. This may be a reasonable and largely sufficient description for ions, but it definitely does not hold for water (Steudle and Peterson 1998). The simple reason is that water moves across membranes by several orders of magnitude more rapidly than ions. So, a third pathway for water flow must be considered, i.e. the one in which water crosses membranes as well as the short distance of wall space between adjacent cells, which is usually not rate limiting. Hence, there would be three main pathways for water flow in the root cylinder (Fig. 2).

(i) apoplastic path - around protoplasts
(ii) symplastic path - through plasmodesmata
(iii) transcellular or vacuolar path - crossing membranes

There could be, of course, combinations of pathways in that water may travel within the symplast for some distance and may then cross the plasma membrane move within the cell wall etc. (Steudle 2000b). Switching between pathways is important, because roots can adjust their hydraulic conductivity according to the water demand from the shoot.

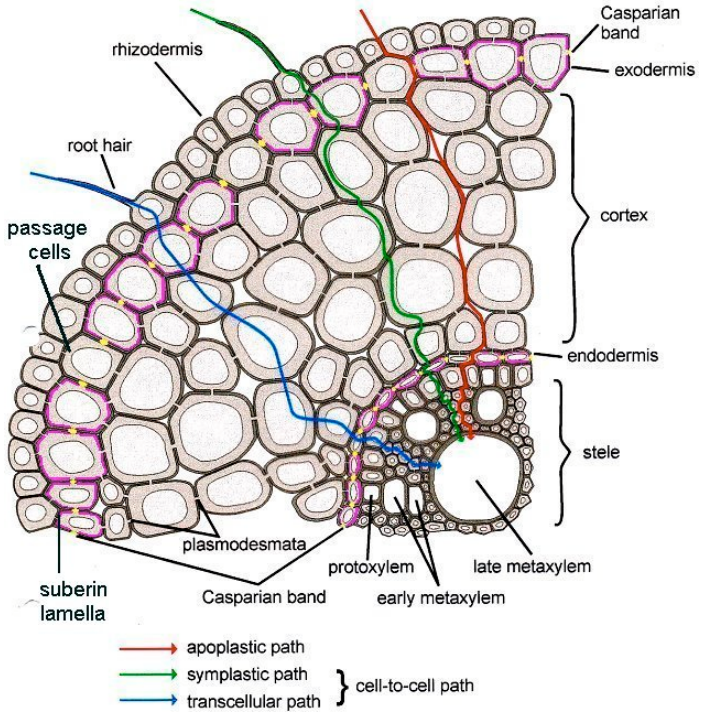

Fig. 2 Pathways for the movement of water and solutes in roots. The apoplast provides a porous path to water, solutes and even for nutrient ions but may be interrupted by Casparian bands in the endo- and exodermis. But it is indicated here, that there may be some passage of water and solutes across the Casparian bands. The symplastic path is through plasmodesmata and the cytosol of cells. Along the transcellular path, water and solutes have to cross many membranes (two per cell layer). It is thought that suberin lamellae in the endo- and exodermis may interrupt the water and solute flow through this path. This path is especially important for water but is for minor important for solutes. Experimentally, the symplastic and transcellular pathways cannot be separated. They are summarized as a cell-to-cell path (modified from Steudle 2000a).

1.2.2.2 Steady-state water flow in roots

In tissues, as in isolated cells, water and solute interactions have to be considered. In addition, active and passive components of solute flow should be distinguish. In the apoplast, only passive (diffusional or convective) solute flow is possible. Both compartments (apoplast and protoplasts) contribute quite differently to the overall tissue volume. Usually, the apoplast contributes a few percent and the protoplasts more than 90% to the total volume. When a gradient of pressure is applied across a tissue, water can use three different pathways as shown in Fig. 3.

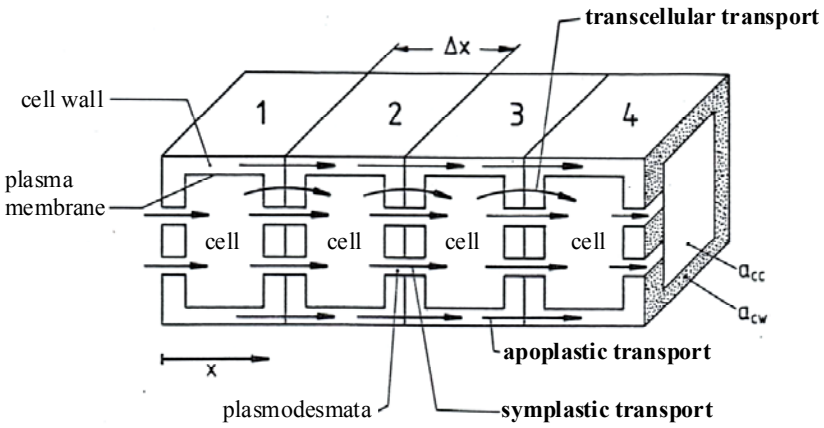

Fig. 3 Schematic representation of the transport pathways across a tissue in one dimension (x). Only four cells are shown. There is an apoplastic (cell wall), a symplastic (*via* plasmodesmata), and a transcellular (vacuolar crossing membranes) pathway. The transcellular and symplastic path is summarized as cell-to-cell path. a_{cc} and a_{cw} are the mean cross-sectional areas for the cell-to-cell and apoplastic paths, respectively. Δx is the thickness of a cell in direction x (modified from Molz and Ferrier, 1982).

With respect to the stationary hydraulic properties of tissues, when considering only two parallel pathways (cell-to-cell and apoplastic) and their flows contribute to the overall flow according to their hydraulic conductivities and cross-sectional areas. The overall hydraulic conductivity of a tissue (Lp_r) in one direction in m s^{-1} MPa^{-1} would be:

$$Lp_r = \left[\underbrace{\gamma_{cc} \cdot \frac{Lp}{2}}_{\text{cell-to-cell component}} + \underbrace{\gamma_{cw} \cdot \frac{Lp_{cw}}{\Delta x}}_{\text{cell wall component}} \right] \cdot \frac{\Delta x}{d} \quad , \tag{11}$$

where Δx = cell thickness in x direction, d = tissue thickness, Lp and Lp_r = hydraulic conductivity of cell-to-cell path and of cell wall material in $m \cdot s^{-1} \cdot MPa^{-1}$, respectively. γ_{cc} and γ_{cw} are the fractional cross-sectional areas of the cell-to-cell and apoplastic path, respectively. Here, hydraulic conductivity of the two parallel pathways are additive. The hydraulic conductivity of the wall path is referred to both unit cross section and path length, whereas Lp refers to unit cell surface area only. A factor of two in the cell-to-cell component is employed, because two membranes would have to be crossed per cell layer. But this theory holds only for hydrostatic gradients, which occur when tensions are created in the xylem during transpiration. Osmotic water flow has not been added

here, since osmotic gradients will only cause small effective driving forces along the apoplastic path. This is so because the reflection coefficient of cell wall material is virtually zero, and nearly no selectivity in the apoplast (see 1.4). By contrast, during the exchange of water between apoplast and protoplast, osmotic forces will be fully exerted.

1.2.2.3 Dynamic water and solute relations of tissues

During dynamic responses such as a change in water potential in a tissue, both the storage properties and hydraulic resistances play an important role. Considering parallel pathways of cell-to-cell and apoplast, and assuming a rapid equilibration between protoplast and the adjacent apoplast, a diffusion type of process for the dynamics of tissue water relation can be obtained. The 'diffusivity' (propagation of water potential through a tissue) of tissue, D_t being (Molz and Ikenberry 1974):

$$D_t = \frac{\Delta x \cdot \left(Lp_{cw} \cdot a_{cw} + {Lp}/{2} \cdot a_{cc} \cdot \Delta x \right)}{C_c + C_{cw}} \quad , \qquad (12)$$

where Lp_{cw} and Lp are the hydraulic conductivities of the wall and the membrane respectively, and a_{cw} and a_{cc} denote the cross-sectional areas. Δx is the thickness of cells in the direction of the propagation of the change. C_{cw} and C_c are the storage capacities of the pathways per cell. It can be seen that the conductances of the pathways are additive and increase D_t, whereas increasing capacities damp the propagation in the tissue. Hence, the physical meaning of D_t is straightforward. It should be stressed that D_t is not a measure of a "diffusional mass flow" of water in the tissue, but rather describes the rate of at which changes in water potential (free energy), cell volume and turgor propagate following a change in water potential. The term "dffusivity" just denotes the fact that the kinetics of the change is of a diffusion type as also observed during ordinary diffusion driven by the thermal motion of molecules.

1.3 Variability of root hydraulics

Water uptake by roots has been shown to be variable for several reasons. The variability of radial hydraulic conductivity is closely related to its complex structure. This

phenomenon has been known for a long time (Brewig 1937; Brouwer 1954; Fiscus 1975; Kramer and Boyer 1995; Steudle 1989; 1994; Steudle and Frensch 1996; Steudle and Peterson 1998). In the longer term (days, weeks), the capacity for water uptake is related to root growth (i.e. increases in root-to-shoot ratio), development and aging or to changes in root morphology and structure (e.g., suberization of roots). Suberization of roots passes through different stages of development of the endo- and exodermis. During state I, Casparian bands (CBs) are forming in transverse and radial walls of the endo- and exodermis. They are primary cell wall modifications, encrusted with lignin as a major component and, to a lesser extent with suberin, the latter assumed to provide most of the resistance towards the movement of polar substances (Schreiber 1996; Zeier and Schreiber 1998; Schreiber et al. 1999; Zimmermann et al. 2000). However, it is usually assumed that CBs are perfect barriers to water and ion movement through the apoplast (Robards and Robb 1972; Singh and Jacobson 1977; Peterson 1987; Peterson 1988; Enstone et al. 2003). During state II, suberin lamellae are laid down in both anticlinal and tangential walls. It is thought that suberin lamellae of roots mainly affects or limits the water flow across the cell-to-cell path. Eventually, cell walls are thickened during state III, which results in the well-known U-shaped cell walls in the endodermis (Steudle 2000b; Steudle and Peterson 1998).

In the shorter term, water uptake may be regulated by mechanisms which alter the physical properties of roots, such as the switching between cellular and apoplastic pathways (composite-transport model of root; Steudle 2001) or by a gating of water channels (aquaporins) of root cell membranes (Azaizeh et al. 1992; Frensch et al. 1996; Henzler et al. 1999; Steudle et al. 1987; Tyerman et al. 1999; Zhu and Steudle 1991). There could be combinations of pathways in that water may travel within the symplast for some distance and may then cross the plasma membrane and move into the cell wall etc. (Steudle 2000b). Switching between pathways helps plant tissues, such as roots to adjust their hydraulic conductivity according to the water demand from the shoot.

1.4 The composite transport model of the root

Switching between water pathways may depend on both the forces that drive flows and on the water permeability (hydraulic conductivity) of components of the pathway.

Because of the porous nature of the apoplast (no selectivity for solutes or $\sigma_s \approx 0$), it does not provide a significant barrier for either water or solutes. It is well known that water flow through the apoplast is hydraulic in nature, driven by gradients in hydrostatic pressure (Steudle 2000a; 2000b; Steudle and Peterson 1998). There are also osmotic water flows in the root cylinder, driven by gradients in osmotic pressure. Even though osmotic gradients are important to drive water flow across membranes, it has less impact or negligible effect on water flow through the apoplast, because this structure does not select or distinguish between water and solutes (as opposed to cell membranes, cell walls have no selective properties). An important feature of the model is that there are two parallel pathways present which exhibit a quite different 'passive selectivity' as expressed by their reflection coefficients (σ_s). To a first approximation, the cell-to-cell (protoplastic) path is semipermeable, i.e. it exhibits a σ_s^{cc} of close to unity. The apoplastic path, on the other hand having a reflection coefficient of virtually zero ($\sigma_s^{cw} \approx 0$). The two pathways interact each other, and the interaction results in phenomena such as a circulation flow of water and a low overall reflection coefficient of the root (σ_{sr}) (Steudle and Frensch 1996; Steudle 1997; 2000a). This mean that root σ_{sr} is smaller than unity. The overall reflection coefficient of the root (σ_{sr}) can be expressed as the following (Steudle 1993):

$$\sigma_{sr} = \sigma_s^{cc} \cdot \frac{\gamma^{cc} \cdot Lp^{cc}}{Lp_r} + \sigma_s^{cw} \cdot \frac{\gamma^{cw} \cdot Lp^{cw}}{Lp_r} \quad , \qquad (13)$$

here:

σ_s^{cc}, σ_s^{cw} = reflection coefficient of cell-to-cell and apoplastic path, respectively

γ^{cc}, γ^{cw} = fractional contribution of pathways to overall cross sectional area ($\gamma^{cc} + \gamma^{cw} = 1$)

Lp^{cc} and Lp^{cw} = hydraulic conductivity of respective pathways

Lp_r = hydraulic conductivity of the root ($Lp_r = \gamma^{cc} \cdot Lp^{cc} + \gamma^{cw} \cdot Lp^{cw}$)

Hence, the overall σ_{sr} in roots locates in between zero and unity as found for different herbaceous and woody plants (Steudle et al. 1987; Peterson et al. 1993; Melchior and Steudle 1993; Rüdinger et al. 1994; Steudle and Heydt 1997; Miyamoto et al. 2001; Ranathunge et al. 2003). According to the model, at zero transpiration, water uptake will be driven by osmotic forces (osmotic pressure difference or $\Delta\pi$ between xylem and

soil solution) across the cell-to-cell path due to the active uptake of solutes by the root. It will cause a high pressure in the xylem and results for some back flow of solution along the non-selective apoplastic path (Fig. 4). The presence of apoplastic barriers, such as CBs in the endo- and exodermis, may reduce backflow of water from the root to the soil solution providing relatively high resistance.

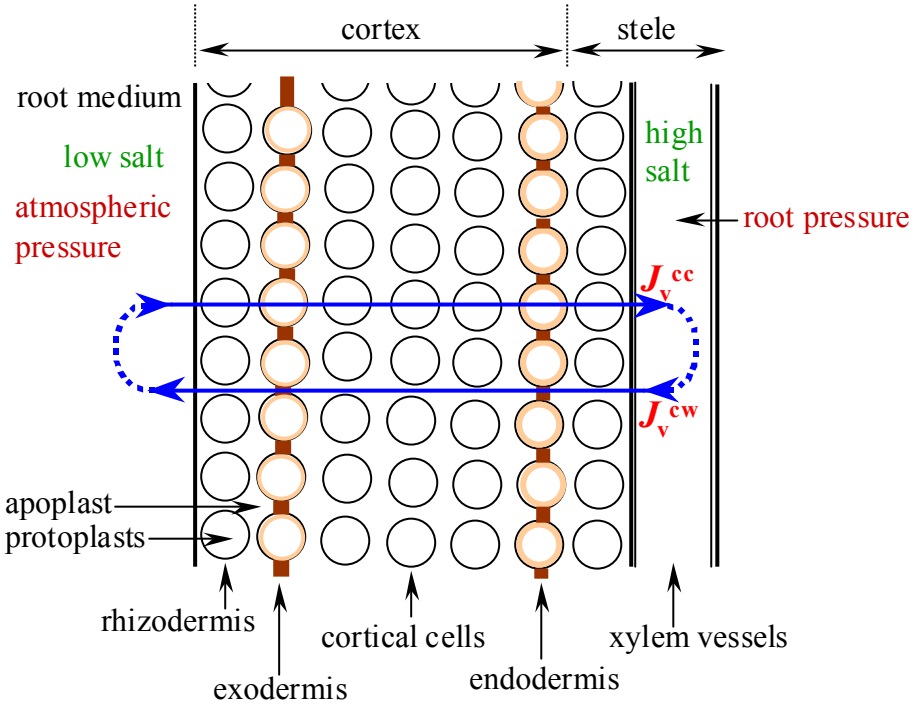

Fig. 4 Composite transport model of root (schematical). The root osmotic barrier is composed of cells (protoplasts) and the apoplast. The apoplastic path may be interrupted by Casparian bands in the endo- and exodermis. Water and solutes move along two parallel pathways (cell-to-cell and apoplastic routes, which are denoted by superscripts 'cc' and 'cw', respectively). The cell-to-cell path has a high selectivity (reflection coefficient, $\sigma_{sr}^{cc} \approx 1$), and the apoplastic path has a very low selectivity for solutes ($\sigma_{sr}^{cc} \approx 0$). At low rates of transpiration, this results in a circulation of water in the root (denoted by J_v) and in a low overall root σ_{sr}. The model explains variable root hydraulic conductivity which depends, in part, on the nature of the driving force.

The hydraulic conductivity of roots depend on the force (osmotic or hydrostatic pressure gradients) which drives water across roots. In the presence of both, osmotic or hydrostatic forces, both pathways (apoplastic and cell-to-cell path) will be used with different intensities. In the presence of a hydrostatic pressure gradient, e.g., generated during transpiration, both pathways will be used. In this case, the hydraulic conductivity

of the root is high. In the presence of an osmotic gradient, cell-to-cell transport will dominate. The hydraulic conductivity of roots should differ depending on the conditions. The physiological consequence of the composite transport is that water uptake by root is adjusted according to the water demand from the shoot.

1.5 Problem in rice plants

Rice (*Oryza sativa* L.) is the most important, staple food crop in Asia, where it provides 35-80% of total calorie uptake. It has been estimated that half the world's population subsists wholly or partially on rice. Rice is the only major cereal crop that is primarily consumed by human directly as harvested (IRRI 1997). About 60% of the rice area is lowland or irrigated and accounts for 75% of total production. However, it has been observed water shortage in rice plants showing leaf rolling and wilting symptoms during day time (midday wilting), even they grow in lowland paddy fields, where water supply from the wet soil should be no problem. This may cause to reduce the productivity or rice yield (Hirasawa et al. 1992; 1996; Ishihara and Sato 1987; Jiang et al. 1988). On the other hand, drought-affected lands with the shortage of irrigation water should be used to grow rice in order to increase the production for rising Asian population. For that reason, it is important to breed rice cultivars with greater water uptake rates. Hence, it is important to find rice cultivars with higher root hydraulic conductivities (Lp_r).

1.5.1 Composite transport in rice roots

To address the rice-water-shortage problem, much effort has been put into research about the regulation of water losses *via* stomata and how external and internal factors contribute to the regulation of the "output function", but, only few research have been done to study or investigate the "input function". Usually, it is thought that the water balance of plant shoots is maintained largely by the regulation of transpiration. However, there is increasing evidence that the water balance can be also regulated at the input, i.e. by a variation the capacity of roots to take up water (Brouwer 1954; Weatherley 1982; Kramer and Boyer 1995; Steudle 2000a; 2000b). This allows for some flexibility in the response of plants to water shortage according to the needs of shoots. In rice, limitation of water uptake by roots may be due to the lack of ability to

adjust the hydraulic conductivity according to the demand from the shoot. As a consequence of insufficient water supply, tensions may be created in the xylem, which result in cavitation and in an interruption of the connection between root and shoot. Measured root hydraulic conductivity (Lp_r) of rice with a root pressure probe located lower than other field crops, such as maize (Miyamoto et al. 2001). Also, rice roots develop apoplastic barriers in the endo- and exodermis and a sclerenchyma layer, which may impede the apoplastic component of water flow across the root cylinder (Miyamoto et al. 2001).

1.5.2 Aerenchyma and the outer part of rice roots

Wetland plants such as paddy rice commonly exposed to hypoxia and anoxia, the partial and complete depletion of environmental oxygen. To survive plants in such oxygen depleted root medium or habitat, oxygen should diffuse from shoots to root tips, which require substantial amount of oxygen because of higher metabolic activities than that of other plant parts. For this reason, rice plants develop a specialized tissue, aerenchyma, abundant, large air spaces throughout the plant body, including in roots (Fig. 5). Aerenchyma in rice roots is a constitutional character (always form during development). The general pattern of cell death and collapse (lysigeny) during aerenchyma formation in rice roots is consistent (Justin and Armstrong 1991). Development of huge aerenchyma in the mid cortex of rice roots caused to separate stele from the outer part of roots (OPR), which comprises only four cell layers; outermost rhyzodermis, an exodermis, sclerenchyma fibre cells, and an innermost unmodified cortical cell layer (Ranathunge et al. 2003; 2004) (Fig. 5).

When diffusing oxygen from basal parts of roots to the tip, it is at risk to lose oxygen from root to oxygen depleted soil across the OPR. It has been observed that the OPR of rice roots contain well developed barriers such as CBs and suberin lamellae to prevent or minimize oxygen loss. This may cause problems for the water and ion uptake, when the apoplastic passage is blocked by CBs and the cell-to-cell passage is affected by suberin lamellae.

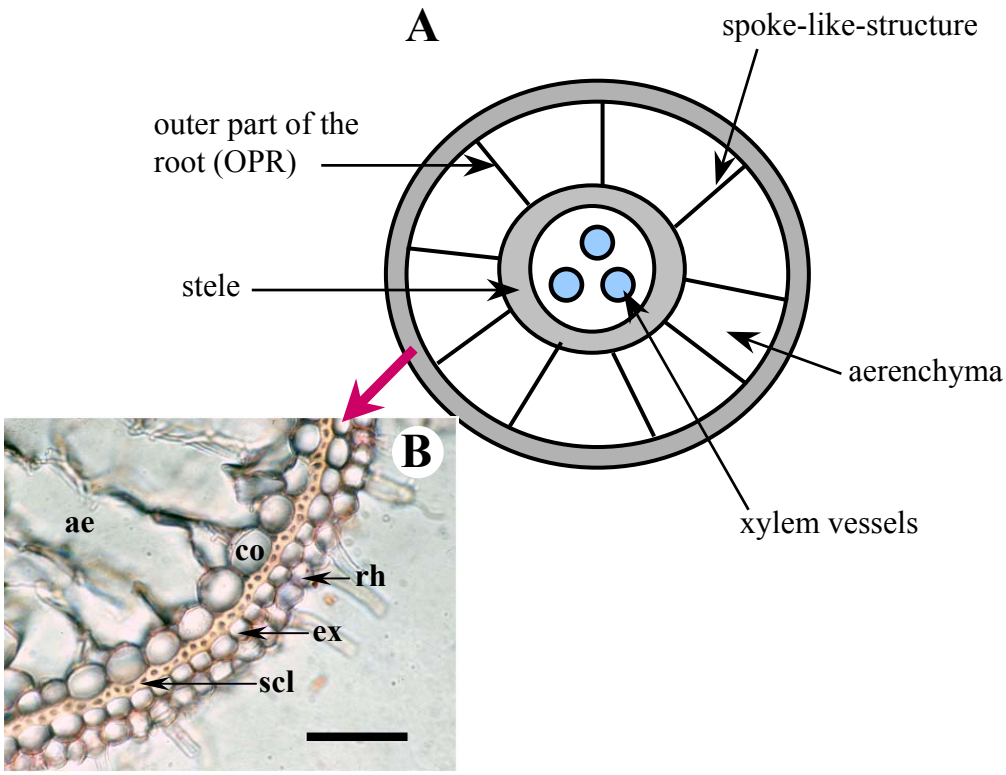

Fig. 5 (A) Schematic diagram of a rice root cross-section. Huge air spaces or aerenchyma separates the stele from the OPR. Spoke-like-structures which are made of remaining cortical cells or cell walls connect the stele to the OPR. (B) The OPR comprises four cell layers: the outermost rhzodermis (rh), an exodermis (ex), a fibre or sclerenchyma cells (scl), and an innermost unmodified cortical cell layer (co). ae = aerenchyma. Bar is 100 µm.

1.6 Aims of the research

Previous research studies of rice roots with two different cultivars (cv. IR64 - lowland and cv. Azucena - upland), which grow in two different conditions showed that their hydraulic conductivities (Lp_r) or radial water permeabilities were similar but smaller than that of other cereal crops, i.e. corn (Miyamoto et al. 2001).

(1) To find out the locations of major apoplastic barriers in rice roots and their relative resistances to the overall root water uptake (hydraulic conductivity; Lp_r).

Although Miyamoto et al. (2001) suggested that the endodermis of rice roots probably represents the major resistance/barrier in the system, they could not experimentally prove it. In this study, we used same rice cultivars (IR64 - lowland, and Azucena -

upland) to find out relative contribution of barriers [endodermis (internal barrier), exodermis (external barrier), aerenchyma] to the overall radial water uptake rates. Since the outer part of the root (OPR) is a well-defined structure, which comprises only four cell layers in series, i.e. rhizodermis, exodermis, sclerenchyma and unmodified cortical cell layer, it could be used for experimental purposes, such as to quantify water relation parameters (hydraulic conductivity, diffusive water permeability, reflection coefficient etc.) using a new pressure perfusion technique as well as to test ion permeability across the barriers using a simple gravitational perfusion apparatus.

(2) To quantify the relative contribution of apoplastic and cell-to-cell paths to the overall radial water flow across the outer part of the root (OPR)

Blocking the apoplastic pores either by China ink particles or by copper ferrocyanide precipitates and closing the water channels in the cell-to-cell path by $HgCl_2$, the relative contribution of above paths to the overall radial water flow as well as the effectiveness of the exodermal CBs as a barrier to the apoplastic water flow could be estimated.

(3) To check the chemical composition of suberin in apoplastic barriers of rice and corn roots and their effectiveness to limit radial water uptake.

Suberin (mainly hydrophobic aliphatic suberin) is one of the major chemical compound in roots that may act as an apoplastic barrier to water and ions. To confirm this idea, total amounts of suberin were determined in rice and corn, and compared with their radial hydraulic conductivities. **Corn was used as a standard to compare with rice**.

(4) To test the permeability of endodermal Casparian bands (CBs) for ions in rice and corn roots.

It is usually assumed and well documented that exo- and endodermal CBs are perfect apoplastic barriers and their permeability to water and nutrient ions is "nil" (Robards and Robb 1972; Singh and Jacobson 1977; Peterson 1987). The validity of this assumption was experimentally checked for above two rice cultivars as well as for corn, which is grown completely in different conditions and holds a different anatomical structure.

This research work can be divided into following sub-sections to investigate above hypotheses:

I Control of water uptake by rice (*Oryza sativa* L.): role of the outer part of the root (Ranathunge et al. 2003).

II Water permeability and reflection coefficient of the outer part of young rice roots are differently affected by closure of water channels (aquaporins) or blockage of apoplastic pores (Ranathunge et al. 2004).

III Blockage of apoplastic bypass-flow of water in rice roots by insoluble salt precipitates analogous to a Pfeffer cell (Ranathunge et al. 2005).

IV The chemical composition of suberin in apoplastic barriers affects radial hydraulic conductivity differently in the roots of rice (*Oryza sativa* L. cv. IR64) and corn (*Zea mays* L. cv. Helix) (Schreiber et al. 2005).

V A new precipitation technique provides evidence for the permeability of Casparian bands to ions in young roots of corn (*Zea mays* L.) and rice (*Oryza sativa* L.) (Ranathunge et al. 2005).

VI Apoplastic water transport in roots (Steudle and Ranathunge 2005).

1.7 Materials and methods

Different methods have been employed for different kind of measurements as shown below. In addition, in order to combine physiological data with root anatomy or their modifications, several histochemical studies had been conducted using different staining techniques.

1.7.1 Pressure chamber and root pressure probe

Two different rice cultivars were used for the experiments (upland cv. Azucena and lowland cv. IR64). Hydraulic conductivity of whole root systems and excised roots were measured with a pressure chamber (Fig. 6A) and a root pressure probe (Fig. 6B), respectively.

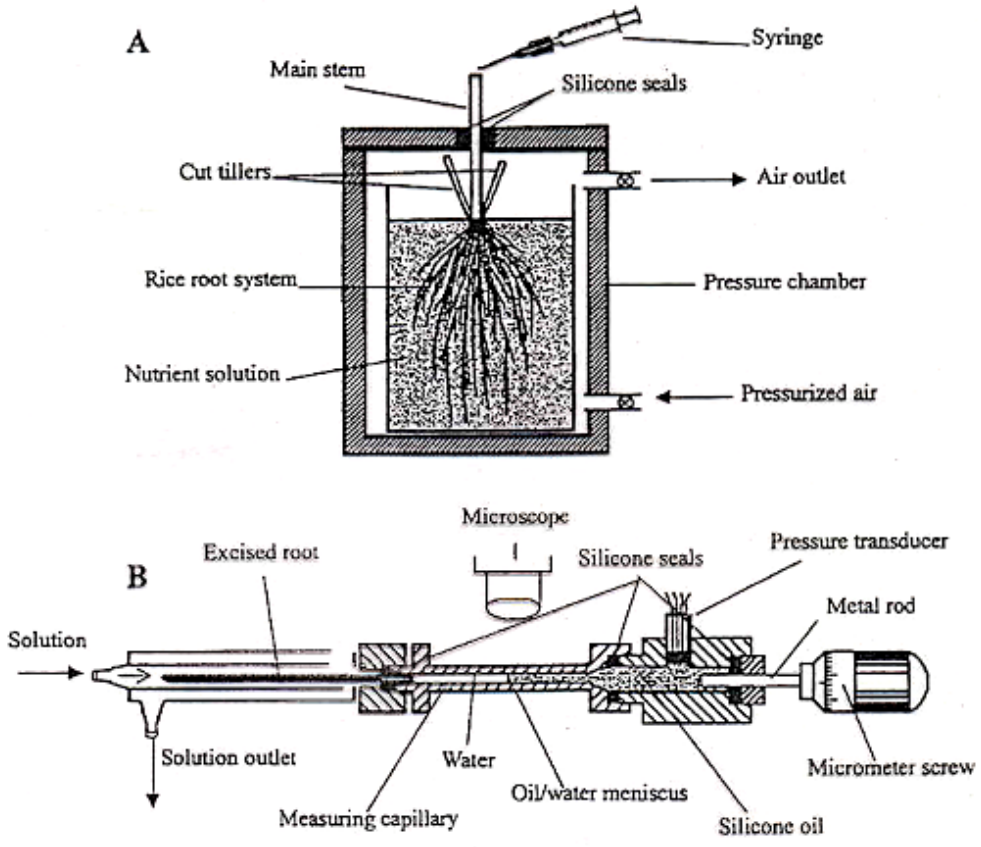

Fig. 6 Pressure chamber (A) and root pressure probe (B) for measuring water flow across root systems and individual roots of young rice plants. (A) The pressure chamber provided the steady-state water flow across the roots by applying pneumatic pressure to the medium. By using silicone seals, the base of the main tiller was tightly sealed to the pressure chamber. Cut ends of the remaining tillers were clamped and

kept in the chamber. With the aid of a syringe, exuded xylem sap was collected in Eppendorf tubes and weighted. (B) Excised roots were connected to a root pressure probe. After steady root pressure had been built up in the system, water flow was induced by either changing the pressure in the probe with the aid of a metal rod or by changing the osmotic pressure of the medium. During measurements oil/water menisci in the measuring capillary of the root pressure probe served as points of reference. From the pressure/time curves obtained, parameters of water and solute flow were calculated.

1.7.1.1 Pressure chamber measurements

Measurement of xylem sap exudation from root systems in the absence of hydrostatic pressure gradients (osmotic exudation)

In the absence of hydrostatic pressure gradients, differences in osmotic pressure ($\Delta\pi$ in MPa) between the medium ($RT \cdot C^o$) and xylem sap ($RT \cdot C^i$) drove the water uptake per unit area by the root (J_{Vr} in $m^3\ m^{-2}\ s^{-1}$), i.e.:

$$Jv_r = Lp_r \times \sigma_{sr} \cdot \Delta\pi = Lp_r \times \sigma_{sr} \cdot RT\left(C^i - C^o\right) \quad . \tag{14}$$

Lp_r and σ_{sr} represent the root's hydraulic conductivity and reflection coefficient, respectively. To calculate Lp_r, a value of $\sigma_{sr} = 0.4$ was used for the reflection coefficient of nutrient salts in xylem and medium (Ranathunge et al. 2003). More details are given in Chapter 2.

Measurement of xylem sap exudation from root systems in the presence of hydrostatic pressure gradients

Pressures in the root chamber were raised in steps of 0.03–0.05 MPa to up to 0.35 MPa above atmospheric. Exuded xylem sap was collected and weighed. For a given applied gas pressure (P_{gas} in MPa), volume exuded from the root system (V in m^3) was plotted against time. Slopes of these relations were calculated and referred to unit root surface area. In hydrostatic experiments, hydraulic conductivity of root systems (Lp_r in $m\ s^{-1}\ MPa^{-1}$) was calculated from the slopes of J_{Vr} plotted against the overall driving forces ($P_{gas} + \sigma_{sr}\Delta\pi$) according ot the following relation:

$$Jv_r = Lp_r\ (P_{gas} + \sigma_{sr} \cdot \Delta\pi) = Lp_r\left[P_{gas} + \sigma_{sr} \cdot RT\ (C^i - C^o)\right] . \tag{15}$$

1.7.1.2 Root pressure probe measurements

Hydrostatic and osmotic relaxations were performed by either changing the xylem pressure (moving the metal rod in the probe) or the osmotic pressure of the medium. Transient responses in pressure were followed which allowed Lp_r to be calculated from rate constants, k_{rw}, or half-times of pressure relaxations ($T_{1/2}^{w}$) according to Steudle et al. (1987):

$$k_{rw} = \ln(2) \Big/ T_{1/2}^{w} = A_r \cdot \Delta P_r / \Delta V_s \cdot Lp_r \quad , \tag{16}$$

where $\Delta P_r/\Delta V_s$ (in MPa · m^{-3}) is the elastic coefficient of the measuring system; V_s denotes the water volume of the system, and A_r is surface area of the root. The ratio of $\Delta P_r/\Delta V_s$ was measured by inducing step changes in the volume and recording the resulting changes in root pressure (ΔP_r). Test solutions used in osmotic experiments were prepared by adding either NaCl or ethanol to the root medium. Responses in root pressure to changes of osmotic pressure of the medium were biphasic. Reflection coefficients (σ_{sr}) of the root for these solutes were calculated using solute phase of the curve. For more details, see Chapter 2.

1.7.2 Steady-state perfusion of root segments by pressure perfusion technique

Using a pressure perfusion technique, hydraulic and osmotic properties (hydraulic resistance or conductivity, reflection coefficients, etc.) of the outer part of rice roots (OPR) or peripheral layers were separated from that of the whole root (Fig. 7). Perfusion of aerenchyma was conducted with root segments (root tip not intact), excised at two difeerent zones from the root tip (20-50 mm and 50-100 mm). At a given pump rate, nutrient solution was pumped into the root segment and the pressure of the set up increased gradually until a stationary positive pressure was established, where the volume flow produced by the pump equalled the radial volume flow across the OPR (Fig. 7B). In a typical steady-state experiment, flow rate was varied step-wise with the aid of the perfusion pump and resulting stationary pressures were measured. Increasing

the pump rate linearly increased steady-state pressure. When plotting pump rate Q_V in $m^3\ s^{-1}$ *vs.* steady-state pressure (MPa), a straight line was obtained.

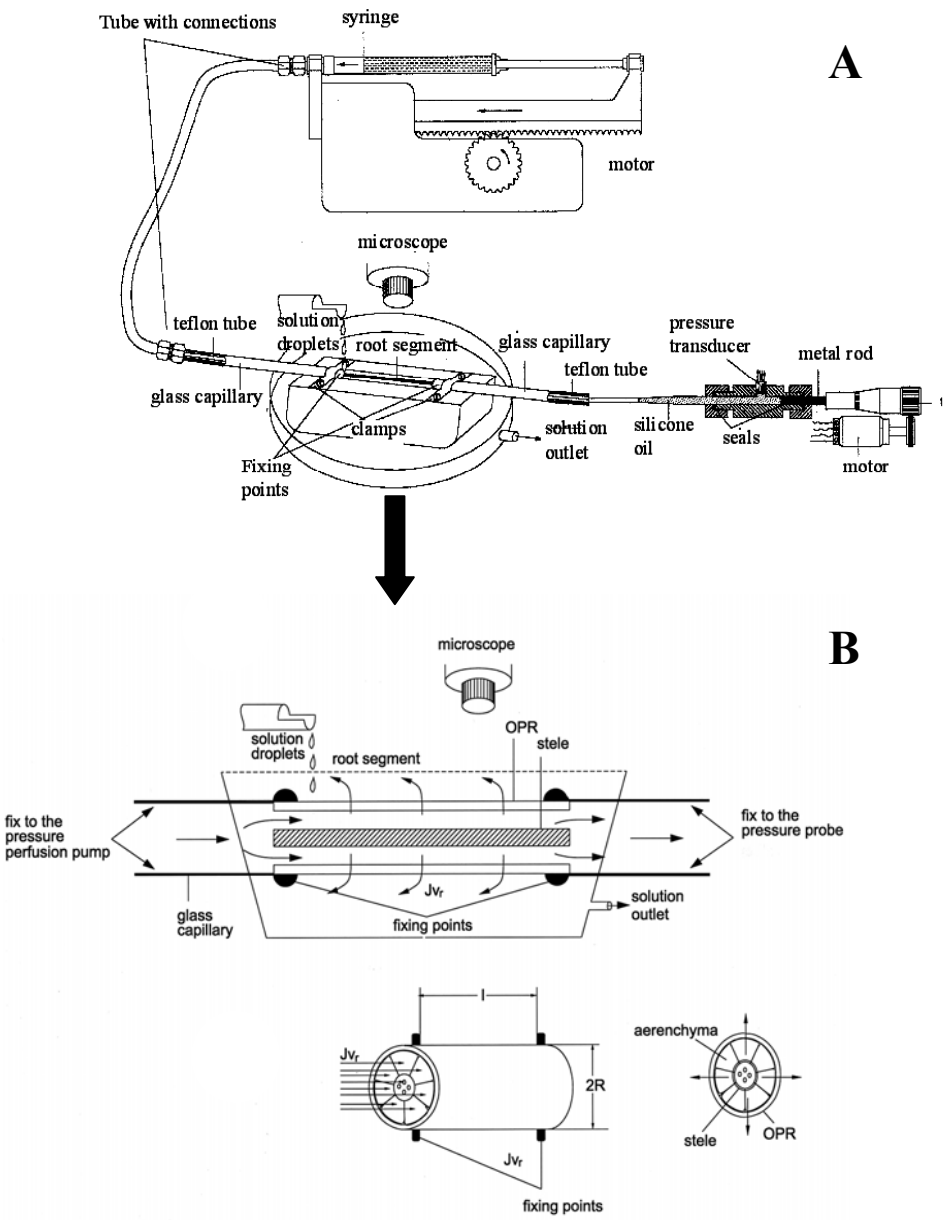

Fig. 7 (A) Pump perfusion setup: A syringe was mounted on a 12 step Braun-Melsungen pump that produced pump rates between 1.7×10^{-9} and 1.1×10^{-7} $mm^3 \cdot s^{-1}$. One end of the root segment was used an inlet. This was fixed to the syringe by a narrow and rigid Teflon tube. The other end was connected to a pressure probe to measure resulting steady state pressures. (B) Schematic diagram with higher magnification to show a root segment with its fixing points. At a given pump rate, stationary pressure was established where the volume flow provided by the pump equalled the radial water/volume flow across the outer part of the root (OPR).

Since the length and diameter of root segments were known, the hydraulic conductivity of the outer part (Lp_{OPR}) was calculated:

$$Q_v = Lp_{OPR} \times P \times A_r \quad . \tag{17}$$

Here, Q_V is the pump rate, P the steady state pressure (reference: atmospheric pressure), and A_r is the surface area of the root segment. Reflection coefficient of the outer part of rice roots (σ_{sOPR}) were estimated adding NaCl (electrolyte) or mannitol (non-electrolyte) to the external medium, and using the resulted pressure drop in the system. More details are given in Chapter 2.

1.7.3 Diffusional water permeability across the OPR measured with HDO

Aerenchyma of root segments were perfused by 3 M solution of HDO displacing air. The root segment was fixed to the pressure perfusion apparatus and held vertically to allow perfusion of the solution by gravity (Fig 8). Radial water movement from the root to the medium was near-isobaric (diffusive) to a good approximation and governed by lateral diffusion of HDO across the OPR. A small pump was employed to mix external solution to equalise distribution of HDO in external medium and minimise the thickness of unstirred layers. For more details, see Chapter 3.

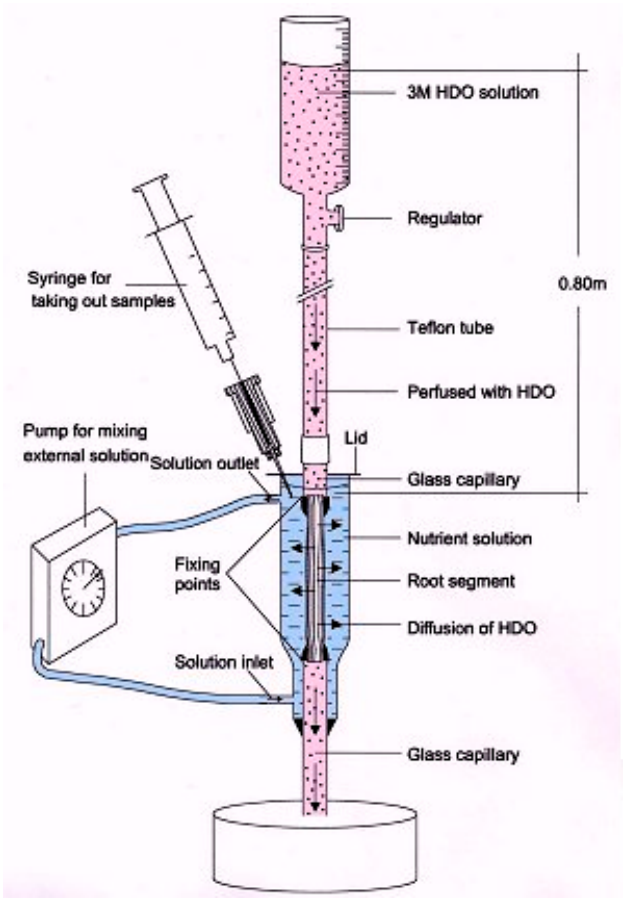

Fig. 8 Experimental setup to measure the diffusional water permeability of the outer part of rice roots. Open ends of root segments were fixed to glass capillaries. Aerenchyma within segments was rapidly perfused with 3M heavy water (HDO). At different time intervals, 50 µl of external solution was taken out using a syringe and concentration of diffused HDO into the outer medium was measured with a freezing point osmometer.

The amount of the solute HDO that diffused to the outer medium was plotted against time. Solute flow across the OPR (J_{sOPR} in moles s^{-1} m^{-2}) was obtained directly from the slope of this curve divided by the surface area of the root segment. Since external (diffused to outer medium) and internal (perfused through aerenchyma) HDO concentrations were known, the driving force or concentration difference between inner and outer compartments (ΔCs in moles s^{-1}) could be evaluated. The diffusional water permeability of the OPR (P_{dOPR} in m s^{-1}) was obtained according to:

$$P_{dOPR} = J_{sOPR} / \Delta C_s \quad . \tag{18}$$

1.7.4 Blockage of apoplastic pores and/or water channels (aquaporins) in the OPR of rice roots

The apoplastic pores (intermicrofibrillar spaces) in the OPR of rice roots were either partially blocked by China ink particles (see Chapter 3) or clogged by brown copper ferrocyanide precipitates {$Cu[CuFe(CN)_6]$} analogous to Pfeffer cell (see chapter 4). Root segments (two different root zones from the tip) were fixed to the pump perfusion set-up, and aerenchyma was perfused with diluted China ink solution with rather high flow rates to block the apoplastic pores in the OPR. In precipitation technique, potassium ferrocyanide was offered on one side of the OPR and copper sulfate on the other. Salts diffused across the barrier and formed a dense precipitates of copper ferrocynide in the apoplast. In order to close water channels in the peripheral layers of rice roots, 50 µM $HgCl_2$ was added to the external medium of the pump perfusion system for 30 min (see Chapter 3). Following these treatments, water relation parameters of the OPR, i.e. hydraulic conductivity, reflection coefficient, and diffusive water permeability were re-measured and compared with the control.

1.7.5 Analyses of chemical composition of apoplastic barriers in rice and corn roots

Cell walls of rice and corn roots were digested incubating in enzymatic solutions and separated stele from the peripheral cell layers or outer part of roots under a binocular microscope using forceps in order to isolate apoplastic barriers, i.e. endodermis and

exodermis. The amounts of suberin were estimated quantitatively by gas chromatography and mass spectrometry following the procedures of tranesterification and depolymerization of cell walls. Amounts of aliphatic and aromatic suberin as well as their substance classes were quantify in the endodermis and exodermis and referred either to the dry weight or to the surface area of the root. The amounts of these apoplastic chemical compounds in roots of rice and corn were compared with their respective radial water uptake rates or hydraulic conductivities (For more details, see Chapter 5).

1.7.6 Permeability of Casparian bands (CBs) in corn and rice to ions

Ion permeability across the CBs of corn and rice roots were tested using a precipitation technique. The test was based on suction of either 100 μM $CuSO_4$ or 200 μM $K_4[Fe(CN)_6]$ into the root from its medium using a pump (excised roots) or transpirational stream (intact seedlings), and subsequent perfusion of xylem of those root segments with the opposite salt, which resulted in precipitation of insoluble Hatchett's brown crystals of copper ferrocyanide ($Cu_2[Fe(CN)_6]$). In order to check the rate of permeabilities of positively charged Cu^{2+} and negatively charged $[Fe(CN)_6]^{4-}$ ions through the negatively charged cell walls, giant *Chara* cell wall preparations were used. More details are given in Chapter 6.

1.7.7 Root anatomical studies

Root anatomical studies were done using different staining techniques i.e., Sudan red 7B and fluorol yellow 088 for suberin lamellae, berberine-aniline blue for Casparian bands, phloroglucinol for lignin etc. Vitality of root cells were checked with Evan's blue and fluorescent dye uranin. For more details, see Chapters 2, 3, 4 and 6.

1.8 Results and Discussion

1.8.1 Root anatomy: development of aerenchyma, Casparian bands (CBs) and suberin lamellae in rice roots

There were no visible differences in the anatomy and development between the two varieties used (upland rice variety, Azucena, and lowland rice variety, IR64). At a distance of 10-15 mm from the root tip, cortical cells started to collapse and aerenchyma gradually developed along roots. Fully developed aerenchyma was observed at a distance of about 100 mm. At distances as short as 20 mm from the tip, suberin lamellae were observed in the endodermis. At a distance of about 50 mm, depositions of secondary suberin were laid down on the inner walls of endodermal cells. Thickness of suberin lamellae in the endodermis increased along roots towards the root base. At a distance of 100 mm from the tip, fully developed, u-shaped wall thickenings were detected in the inner walls of endodermis. Even though suberin lamellae in the exodermis were not as strongly developed as in the endodermis, exodermal development started fairly close to the tip (30 mm). It matured at about 50 mm. As typical for most roots, the endodermis started to develop CBs at distances closer to the tip than the exodermis. In the endodermis, CBs were observed at distances of about 20 mm. Well developed endodermal CBs were found in mature part of roots (100 mm from tip). No exodermal CBs were detectable at about 20 mm from the tip. Maturation of exodermis started at a distances of about 30 mm. Well-developed bands were found at about 50 mm. Highly lignified sclerenchyma was found below the exodermis at about 100 mm. Even at distances of 100 mm from the tip, no suberin could be detected in sclerenchyma tissue by staining with Sudan Red 7B. Initiation of lateral roots was observed at around 50-70 mm and 70-90 mm from the tip for IR64 and Azucena, respectively (see Chapter 2, page 53).

1.8.2 Comparison of hydrostatic and osmotic Lp_r for single roots and root systems

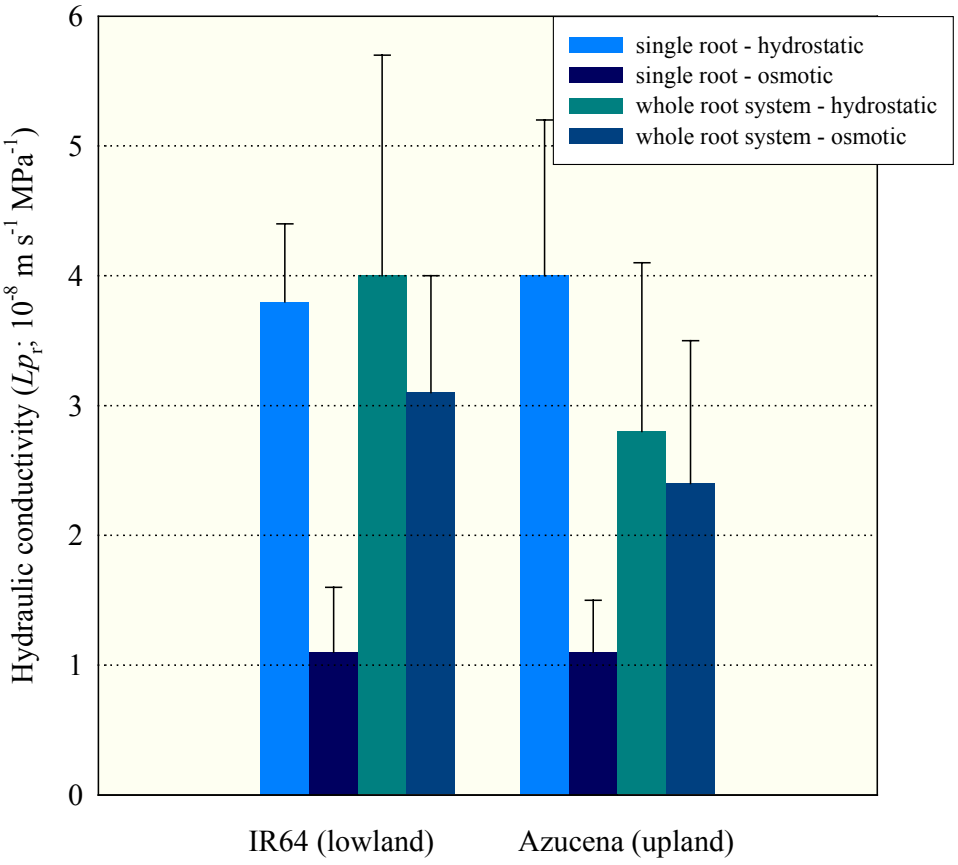

Fig. 9 Hydrostatic and osmotic Lp_r for single roots (measured with a root pressure probe) and whole root systems (measured with a pressure chamber) in two cultivars used. No significant differences were observed between IR64 and Azucena (P = 0.05 level).

In rice (at least for the cultivars used in these experiments), the overall radial hydraulic conductivity was lower than those of other cereal roots (Miyamoto et al. 2001). This has been interpreted as a major limitation of rice roots to supply water to transpiring leaves. The present study confirms this conclusion.

1.8.3 Reflection coefficients of rice roots

Average reflection coefficients for electrolyte, NaCl were σ_{sr} = 0.18 and 0.16 for IR64 and Azucena, respectively. They were significantly greater than those of ethanol, which should rapidly permeate through root cell membranes (σ_{sr} = 0.04 and 0.08,

respectively). Those values were significantly smaller than that of other herbaceous or field crops, i.e. corn, wheat, barley, onion, *Phaseolus* spp. (Barrowclough et al. 2000; Steudle et al. 1987; Steudle and Brinckmann 1989; Steudle and Frensch 1989) indicating a substantial apoplastic bypass flow across the rice root cylinder. More details are given in Chapter 2.

1.8.4 Hydraulic conductivity of the outer part of rice roots (OPR)

Hydraulic conductivity of the OPR (comprises only four cell layers, including an exodermis with well developed Casparian bands and suberin lamellae) was measured for two different root zones from the root tip, i.e. 20-50 mm (immature zone) and 50-100 (mature zone, where apoplastic barriers have already well developed) using a pressure perfusion experiment. No significant differences were observed between two root zones used (P = 0.05 level). By contrast, hydraulic conductivity of the OPR of rice roots was larger by a factor of 30 than that of the whole root or the endodermis/stele. As long as flow across the OPR is hydraulic in nature, this means that OPR would not rate limit water uptake (Ranathunge et al. 2003; see Chapter 2).

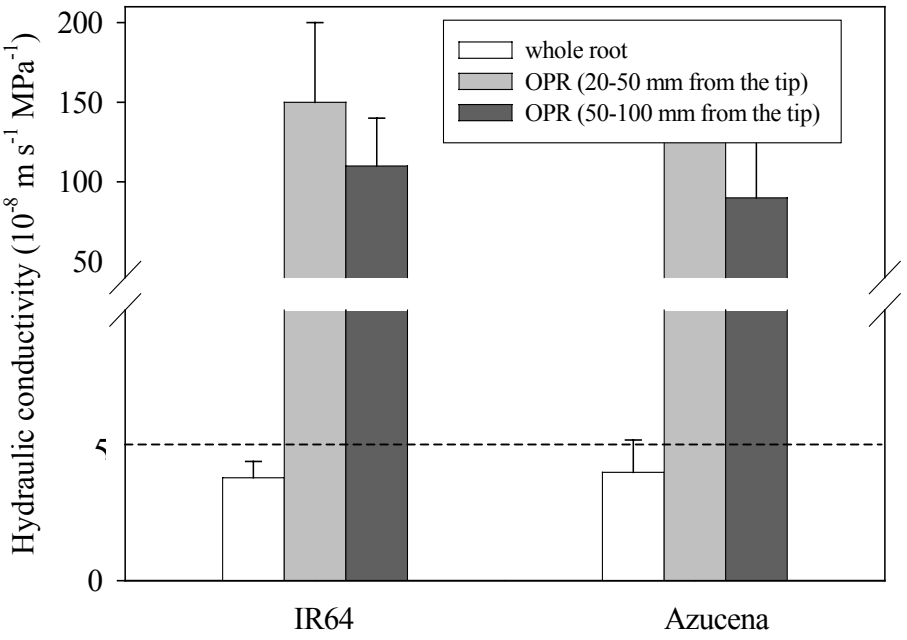

Fig. 10 Hydraulic conductivity of the whole root, and the OPR of two different root zones (20-50 and 50-100 mm from the root tip) in two different rice cultivars. Hydraulic resistance of the OPR was 30-fold smaller than that of the entire root.

1.8.5 Diffusive water permeability across the OPR (P_{dOPR}) of rice roots

Vertical perfusion of aerenchyma by near-isobaric heavy water (HDO) was performed with excised rice root segments at 20-50 or 50-100 mm from the root apex. The diffusional water permeability of the OPR (P_{dOPR}) significantly decreased along the root axis from apex to base. The P_{dOPR} was larger by a factor of two to three in immature (20-50 mm) compared to mature (50-100 mm) root segments (Table 1). Comparison of bulk and diffusional permeabilities showed that the hydraulic/bulk water permeability of the OPR (Lp_{OPR} or P_{fOPR}) was 600 times larger than the diffusional water permeability (P_{dOPR}) at 20-50 mm from the apex and 1200-1400 larger at 50-100 mm from the apex (Table 1). Such big P_f/P_d ratios are expected if the pathway involved a rather long porous path, i.e. apoplast; this would offer a high diffusional resistance for HDO, but should be highly permeable in case of a bulk (hydraulic) water flow (see Chapter 3).

Table 1 Diffusional water permeability (P_d) of the outer part of rice roots, measured by rapidly perfusing aerenchyma with isobaric water (containing HDO). Measurements were performed for two different cultivars, IR64 and Azucena and two different distances from the root apex. Values are means ± SD with the number of measured roots in parenthesis. For both cultivars, P_{dOPR} significantly decreased along the root from the apex (double-sided, unpaired *t*-test, $P = 0.05$). Immature root segments (20–50 mm from root apex) showed significantly higher P_{dOPR} values than mature segments (50–100 mm from the apex). There were no significant differences observed for hydraulic/bulk water permeability of the OPR (Lp_{OPR} or P_{fOPR}) for either distance from the root apex (double-sided, unpaired *t*-test, $P = 0.05$). Hydraulic water permeability of the OPR (Lp_{OPR} or P_{fOPR}) was two to three orders of magnitude higher than diffusional water permeability (P_{dOPR}).

Rice cultivar	Diffusive water permeability of the OPR (P_{dOPR}) × 10^{-7} m·s^{-1}	Hydraulic water permeability of the OPR (P_{fOPR}) × 10^{-7} m·s^{-1}	P_{fOPR} / P_{dOPR} Ratio
IR64			
20-50 mm	3.5 ± 0.5 (7)[a]	2170 ± 683 (10)	620
50-100 mm	1.4 ± 0.8 (6)[b]	1680 ± 255 (10)	1200
Azucena			
20-50 mm	3.0 ± 1.6 (7)[a]	1808 ± 703 (10)	603
50-100 mm	1.0 ± 0.7 (6)[b]	1445 ± 417 (10)	1445

Different superscript letters indicate significant differences at $P = 0.05$ level

1.8.6 Blockage of the apoplastic path and/or cell-to-cell path of the OPR

In order to quantify the relative contribution of the radial pathways (apoplastic *vs*. cell-to-cell) to the overall water flow across the OPR of rice roots (Lp_{OPR}), three types of experiments were conducted. Apoplastic pores of the OPR were either partially blocked with China ink particles (see Chapter 3) or clogged with copper ferrocanide precipitates (see Chapter 4). Whereas, aquaporins or water channels of the OPR were closed with water channel blocker $HgCl_2$. Relative reduction of the Lp_{OPR} in response to these treatments is shown in figure 11.

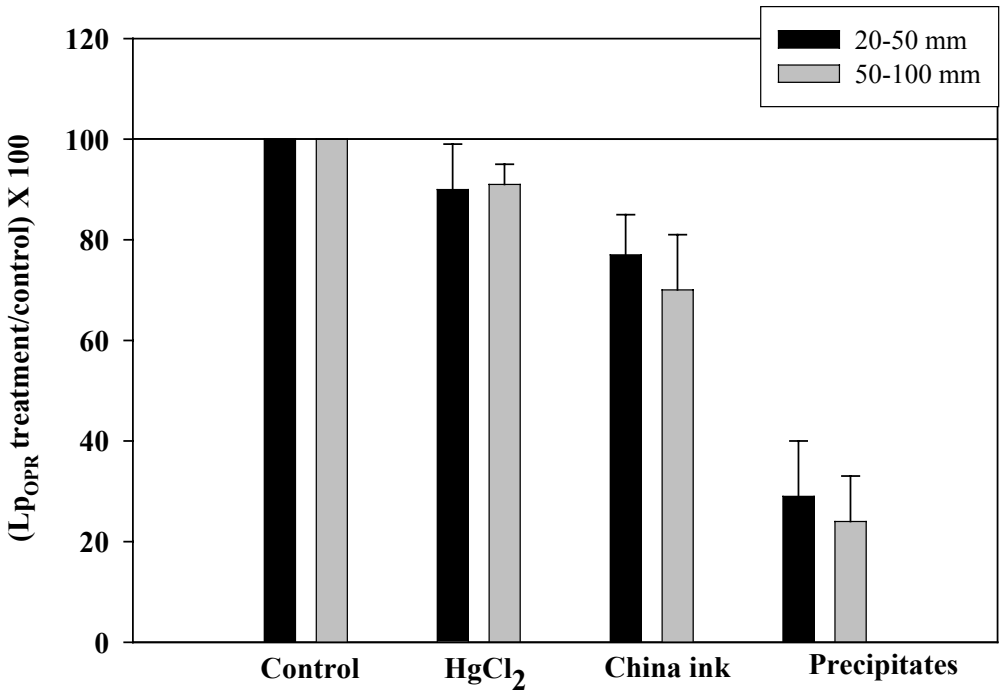

Fig. 11 Reduction of Lp_{OPR} caused by different treatments (relative). Closure of water channels in the OPR with 50 µM $HgCl_2$ resulted in a ≈ 10% reduction of Lp_{OPR}, whereas, blockage of apoplastic pores with China ink particles (partial) and copper ferrocyanide precipitates resulted in substantially greater reductions, ≈ 30% and ≈ 70%, respectively.

These results suggested proportionately greater apoplastic water flow across the OPR (on average 66% - 75% of water used extraprotoplastic pathway) compared to cell-to-cell water flow despite the existence of apoplastic barriers such as Casparian bands, suberin lamellae in the exodermis, and lignified walls of sclerenchyma or fibre cells. This was further supported by increment of σ_{sOPR} after treatments with apoplastic blockers. Blockage of apoplastic pores either with China ink particles or with copper

ferrocyanide precipitates increased σ_{sOPR} by 3-fold. These findings agree with earlier observations of rice roots, which indicated a substantial apoplastic component for NaCl and PTS (Yadev et al. 1996; Yeo et al. 1987). Nevertheless, it has been shown that the endodermis represents the major hydraulic barrier, which is due to its strong suberization (Miyamoto et al. 2001; Ranathunge et al. 2003). Most of the water channels might be concentrated in the endodermis or the stele, as found for other species (Schäffner 1998).

1.8.7 Amounts of suberin in apoplastic barriers in rice and corn roots; relates to their radial water uptake rates

Aliphatic suberin is hydrophobic in nature and thought to be responsible for limiting water uptake in roots (Schreiber 1996; Schreiber et al. 1999). As a comparison, the amounts of aliphatic suberin in rice roots (in the outer part of roots as well as in the central cylinder) was substantially greater than that of maize (in the rhizo-hypodermal cell walls as well as endodermal cell walls). When comparing zones I (immature) and II (mature) of rice, there was no pronounced trend of an increase in aliphatic suberin (Fig. 12). In corn, greater amounts of aliphatic suberin were observed in zone II (mature) than in zone I (immature). For more details, see Chapter 5.

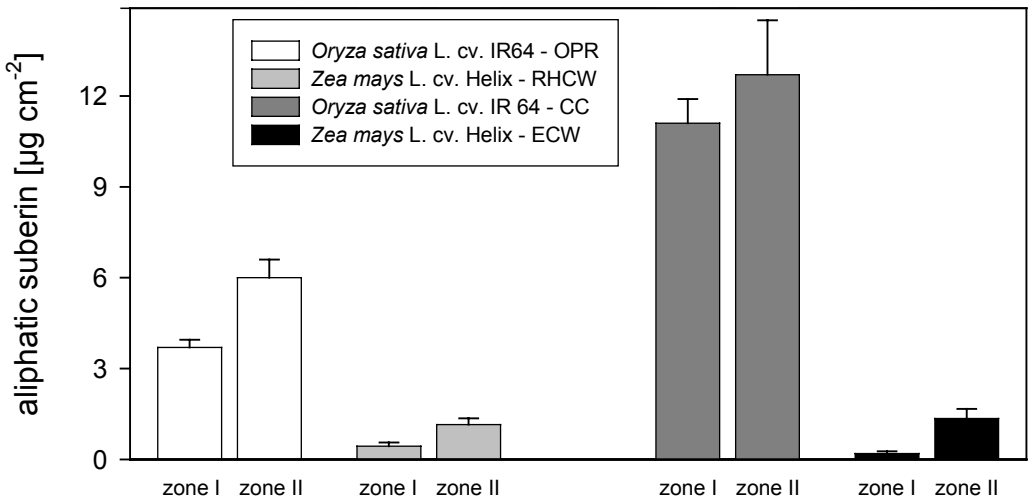

Fig. 12 Total amounts of aliphatic suberin released from both root zones and isolated cell wall samples of rice [*Oryza sativa* L. cv. IR64; the outer part of roots (OPR) and central cylinders (CC)], and corn roots [*Zea mays* L. cv. Helix; rhizodermal and hypodermal (RHCW) and endodermal cell walls (ECW)] related to surface areas of the analyzed cell wall samples [µg cm^{-2}]. Means ± SD (n = 3 replicates).

Nevertheless, this pronounced difference in total amounts of aliphatic suberin in apoplastic transport barriers in roots could help to explain why hydrostatic Lp_r of rice was significantly lower than that of corn, whereas osmotic Lp_r was not significantly different between both species (Fig. 13).

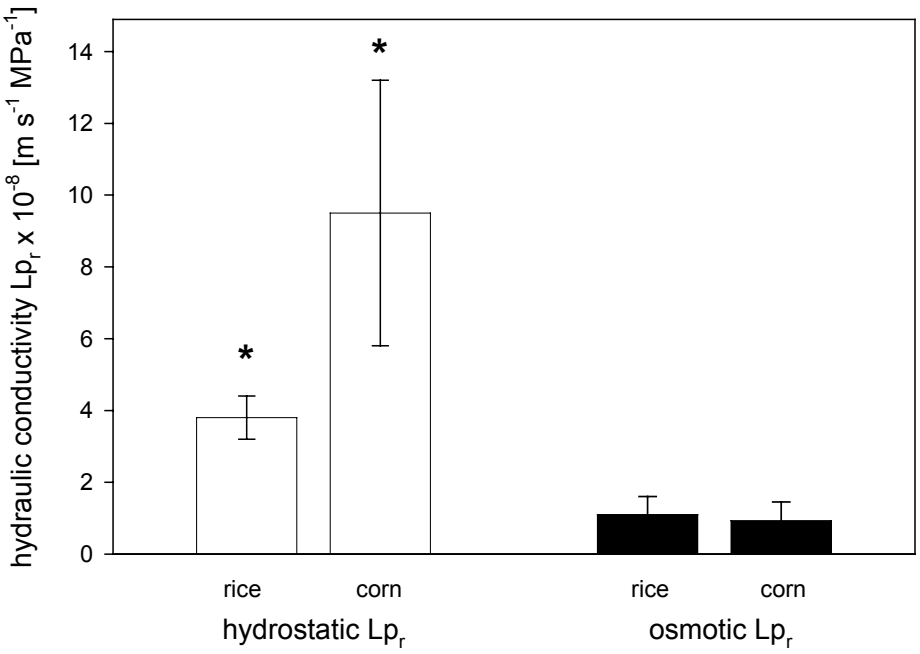

Fig. 13 Hydraulic conductivities (Lp_r) measured by using hydrostatic (hydrostatic Lp_r) and osmotic (osmotic Lp_r) pressure gradients with the end-segments of rice (*Oryza sativa* L. cv. IR64) and corn roots (*Zea mays* L. cv. Helix). In hydrostatic experiments, hydraulic conductivity was measured by changing the root turgor pressure with the aid of the root pressure probe. In osmotic experiments, relaxations were induced by changing the osmotic pressure of the external medium. Means ± SD (n = 6 roots). Bars marked with asterisks indicate a statistically significant difference at 95% confident level (*t*-test).

But the simple conclusion that water permeability would be reduced as the amount of suberin is increasing, is hard to justify according to recent results of water permeability across the outer part of rice roots (Lp_{OPR}). The OPR of rice contains a large amounts of suberin relative to that of corn but is, nevertheless, highly permeable to water. Obviously analyses of total amounts of suberin deposited in apoplastic barriers and of their detailed chemical structure are necessary but not the complete pre-requisite to explain the observed changes in water permeability. The precise molecular and topographical deposition of suberin in root cell walls has to be known as well. The latter determines the reduction of porosity and permeability of roots. In order to make barriers

really watertight, suberin should fill all wall pores (intermicrofibrillar spaces), i.e. it has to impregnate the wall material (such as filling a sponge with water). In fact, hydrophobic aliphatic suberin may have problems to fill pores made of rather hydrophilic material such as cellulose. More details are given in Chapter 5.

1.8.8 Permeability of endodermal Casparian bands (CBs) in corn and rice to ions

Using an insoluble inorganic salt precipitation technique (analogous to Wilhelm Pfeffer's copper ferrocyanide artificial semipermeable membrane technique), the permeability of cell walls and especially of endodermal CBs for ions was tested in young roots of corn (*Zea mays*) and rice (*Oryza sativa*). The test was based on suction of either 0.1 mM $CuSO_4$ or 0.2 mM $K_4[Fe(CN)_6]$ into the root from its medium using a pump (excised roots) or transpirational flow (intact seedlings), and subsequent perfusion of xylem of those root segments with the opposite salt, which resulted in precipitation of insoluble brown crystals of copper ferrocyanide. Under suction, Cu^{2+} could cross the endodermis through the apoplast in both plant species (even though at small rates) developing brown salt precipitates in cell walls of early metaxylem and on the passage between CBs and early metaxylem (see pictures in Chapter 6). Hence, at least Cu^{2+} did pass the endodermis with the water dragged across it. The results suggested that CBs were no perfect barrier to apoplastic fluxes, at least for copper ions. In rice, this is in line with earlier findings of apoplastic passage of ions (Na^+) and tracer dye PTS (Yadev et al. 1996; Yeo et al. 1987). Present findings are further supported by earlier experimental findings of substantial endodermal apoplastic bypass of Ca^{2+} in rye (White et al. 1992), Cl^- in citrus (Storey and Walker 1999), as well as of the stress hormone ABA in corn roots (Freundl et al. 1998; Hose et al. 2000; Schraut et al. 2004). On the contrary, ferrocyanide ions failed to cross the mature endodermis of both corn and rice at detectable amounts with the technique (concentration limit: 0.8 μM of apoplastic copper concentration at a perfusion with 0.2 mM $K_4[Fe(CN)_6]$).

At places where lateral roots emerge from primary roots, the continuity of the endodermis is lost (Peterson et al. 1981). It is, hence, expected that this allows some leakage of water and solutes such as apoplastic dyes. In this study, dense brown precipitates were observed around lateral root emergence points for corn and rice, which

is in line with the earlier observations. These places may act as “open doors” not only for water and apoplastic tracer dyes but also for ions to move freely though the apoplast into the xylem (Clarkson 1993; Ranathunge et al. 2005).

Asymmetric development of precipitates (brown precipitates were developed at the side where ferrocyanide was applied) suggested that the cation, Cu^{2+}, moved faster than the anion, $[Fe(CN)_6]^{4-}$, through cell walls including CBs. Using *Chara* cell wall preparations (“ghost”) as a model system, it was shown that more ferrocyanide ions retained inside wall-tubes than that of Cu^{2+}, which had a higher permeation rate through cell walls (see Chapter 6). The results show that the permeability of CBs to ions is fairly low though not vanishing. CBs represent no perfect barrier for ions, as is usually thought. The permeability of CBs may vary depending on growth conditions, which are known to affect the intensity of formation of bands. More details are given in Chapter 6.

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1.10 Short Summary

For rice, the overall radial hydraulic conductivity (Lp_r) was lower than those of other cereal roots (Miyamoto et al. 2001; Ranathunge et al. 2003). This, together with the limited extension of root systems, is interpreted as a major limitation of rice roots to supply water to transpiring leaves. The stele/endodermis, aerenchyma, and the outer part of roots (OPR) arrange in series and their resistances to the overall radial water flow are additive. However, **the hydraulic resistance of the OPR was smaller by a factor of 30 than the overall values of root Lp_r. Hence, the endodermis rather than the OPR limits water uptake.** It appears that the OPR is constructed to provide a substantial barrier for oxygen rather than for water. The latter is transported down to root tips limiting root extension, which resulted to develop small rice root systems. Both cultivars used here (IR64 and Azucena) developed strong barriers to radial oxygen loss (ROL). The rates of ROL dramatically decreased along the root and reached values close to zero in basal parts (Kotula and Steudle unpublished data). If the radial permeability of oxygen were too high, this would even more limit the root size. The data from this thesis show for the first time that radial uptake of water by rice roots is not limited by the OPR. **Theoretical estimations suggested that the endodermis limits the rate of radial water flow and the resistance of the aerenchyma is in between that of the endodermis and the OPR.**

High values of the Lp_{OPR} could be either brought about by a large apoplastic component of water transport or by a high permeability of membranes of the living cells in the OPR or by both together. If there were a high apoplastic component, this would mean that Casparian bands (CBs) in the exodermis were unusually permeable to water. In order to quantify the relative contribution of the apoplastic *vs* cell-to-cell paths to the overall Lp_{OPR}, apoplastic pores of the OPR were either partially blocked by China ink particles (50 nm in diameter) or clogged with copper ferrocyanide precipitates. In another experiment, water channels (aquaporins) of the OPR were blocked with water channel blocker $HgCl_2$. Resulted Lp_{OPR} values after the treatments suggested that **proportionately greater apoplastic water flow across the OPR compared to cell-to-cell water flow. On average, 66-75% of water used extraprotoplastic path.** This

finding was further supported by substantial increases of the reflection coefficient of the OPR (σ_{sOPR}) after treatments with apoplastic blockers.

Strongest evidence in favour of a predominant apoplastic water transport came from the comparison between diffusional (P_{dOPR}, measured with heavy water, HDO) and osmotic water permeability (P_{fOPR}) or hydraulic conductivity (Lp_{OPR}). **The P_{fOPR} was larger by a factor of 600-1400 than P_{dOPR}. To obtain such huge values of P_f/P_d ratios are expected if the pathway involved a rather long porous path, i.e. a passage along the apoplast;** this would offer a high diffusional resistance for HDO, but should be highly permeable in case of a bulk (hydraulic) water flow. Blockage of apoplastic pores with copper ferrocyanide precipitates significantly affected the bulk rather than the diffusive water flow and caused a 3-5-fold reduction of the P_{fOPR}/P_{dOPR} ratios. These findings suggested a prominent apoplastic bypass flow across the OPR of rice.

Copper ferrocyanide precipitation technique with roots of rice and corn showed that CBs of the exo- and endodermis were not completely impermeable to Cu^{2+} ions. When offering Cu^{2+} and $Fe(CN)_6^{4-}$ on different sides, brown copper ferrocyanide crystals developed on the side where ferrocyanide was applied. This indicated that positively charged copper ions was moving through the barrier and cell walls, much faster than ferrocyanide with its four negative charges. There was a patchiness in the formation of precipitates, which correlated with the maturation of the exodermis in rice roots. **Dense brown precipitates were observed around lateral root emergence points. These places may act as "open doors" for water and apoplastic tracer dyes. To some extent and depending on conditions and developmental state of roots, also ions may move through the apoplast into the xylem and may lead to increase the apoplastic bypass flow in roots.**

Hydrophobic aliphatic suberin is one of the major chemical compound in plant roots that may act as an apoplastic barrier to water. To confirm this idea, total amounts of suberin were determined in corn and rice, and compared with their radial hydraulic conductivities. **On average, exodermal cell walls of rice contained 6-fold greater aliphatic suberin than in corn hypodermis. In endodermal cell walls, amounts were 34-fold greater in rice than that of corn. Substantially higher amounts of suberin**

detected in apoplastic barriers of rice corresponded with substantially lower hydrostatic Lp_r compared to corn. As the OPR of rice is highly porous and fairly permeable to water, it may argue that this holds true only for the endodermis. The results imply that some caution is required when discussing the role of suberin in terms of an efficient transport barrier for water. The simple view that just the amounts of suberin play the important role may not hold. A more detailed consideration of both the chemical nature of suberins and of the microstructure of deposits is required, i.e. how suberin impregnate wall pores. For CBs, the work of Schreiber et al. (1999) indicated that they contain substantial amounts of lignin (besides suberin), which may allow a passage of polar solutes and water, at least to some extent and depending developmental state.

II

Publications

2 Control of water uptake by rice (*Oryza sativa* L.): role of the outer part of root

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Received: 8 October 2002 / Accepted: 27 December 2002 / Published online: 5 March 2003

Planta (2003) 217: 193-205
DOI 10.1007/s00425-003-0984-9

Abstract

A new pressure-perfusion technique was used to measure hydraulic and osmotic properties of outer part of roots (OPR) of 30-d-old rice plants (lowland variety: IR64, and upland variety: Azucena). The OPR comprised rhizodermis, exodermis, sclerenchyma and one cortical cell layer. The technique involved perfusion of aerenchyma of segments from two different root zones (20-50 mm and 50-100 mm from the tip) at precise rates using aerated nutrient solution. The hydraulic conductivity of the OPR ($Lp_{OPR} = 1.2 \times 10^{-6}$ m s^{-1} MPa^{-1}) was larger by a factor of 30 than the overall hydraulic conductivity ($Lp_r = 4 \times 10^{-8}$ m s^{-1} MPa^{-1}) as measured by pressure chamber and root pressure probe. Low reflection coefficients were obtained for mannitol and NaCl for the OPR (σ_{sOPR} = 0.14 and 0.09, respectively). The diffusional water permeability (P_{dOPR}) estimated from isobaric flow of heavy water was smaller by three orders of magnitude than the hydraulic (Lp_{OPR}/P_{fOPR}). Although detailed root anatomy showed well-defined Casparian bands and suberin lamellae in the exodermis, the findings strongly indicate a predominantly apoplastic water flow in the OPR. The Lp_{OPR} of heat-killed root segments increased by a factor of only two, which is in line with the conclusion of a dominating apoplastic water flow. The hydraulic resistance of the OPR was not limiting the passage of water across the root cylinder. Estimations of the hydraulic properties of aerenchyma suggested that the endodermis was rate-limiting the water flow, although the aerenchyma may contribute to the overall resistance. The resistance of the aerenchyma was relatively low, because mono-layered cortical septa crossing the aerenchyma (‘spokes’) short-circuited the air space between the stele and the OPR. Spokes form hydraulic bridges that act like wicks. Low diffusional water permeabilities of the OPR suggest that radial oxygen losses from aerenchyma to medium are also low. It is concluded that in rice roots, water uptake and oxygen retention are optimized in a way that hydraulic water flow can be kept high in the presence of a low efflux of oxygen which is diffusional in nature.

Keywords Aerenchyma · Apoplastic transport · Exodermis · Hydraulic conductivity · Rice roots · Water transport

Introduction

Usually, it is thought that the water balance of plant shoots is maintained largely by the regulation of transpiration. Much is known about the regulation of water losses *via* stomata and how external and internal factors contribute to the regulation of the "output function". Negative effects on water status lead to a closure of stomata. Among other things, this involves the action of the stress hormone abscisic acid (ABA). However, there is increasing evidence that the water balance can be also regulated at the input, i.e. by a variation of the capacity of roots to take up water (Brouwer 1954; Weatherley 1982; Kramer and Boyer 1995; Steudle 2000a, 2000b). In fact, the "input function" may be as important as the output. Water uptake by roots has been shown to be variable for several reasons. In the longer term (days, weeks), the capacity for water uptake is related to root growth (i.e. increases in root-to-shoot ratio), or to changes in root morphology and structure (e.g. suberization of roots). In the shorter term (< day), water uptake may be regulated by mechanisms which alter the physical properties of roots, such as the switching between cellular and apoplastic pathways (composite-transport model of root; Steudle 2001) or by a gating of water channels (aquaporins) of root cells which may change in a diurnal rhythm (Henzler et al. 1999; Tyerman et al. 1999). Switching of water pathways may depend on both the forces that drive flows and on the water permeability (hydraulic conductivity) of components of the pathway. This allows for some flexibility in the response of plants to water shortage according to the needs of shoots.

For rice, water shortage may occur even when plants are growing in paddy fields (Hirasawa et al. 1992). This may be due to a limitation on water uptake by rice roots, which lack the ability to adjust the hydraulic conductivity according to the demand from the shoot (Miyamoto et al. 2001). This is plausible because root systems of rice, growing in the field are usually small. Also, rice roots develop apoplastic barriers in the endo- and exodermis and a sclerenchyma layer which may impede the apoplastic component of water flow across the root cylinder (Clark and Harris 1981; Miyamoto et al. 2001). The aerenchyma may represent an additional barrier. As a consequence of insufficient water supply, tensions may be created in the xylem which result in cavitation and in an interruption of the connection between root and shoot. Miyamoto et

al. (2001) showed that the hydraulic conductivity (Lp_r) of rice roots is rather low in comparison to other species (e.g. maize). They explained this in terms of a composite transport model, which was also employed to interpret the lack of variability in root Lp_r. According to Miyamoto et al. (2001), the reason for the lack of flexibility of rice roots to adjust to demands from the shoot was the fact that there was no or only a small ability to switch between pathways, compared to other plants. Miyamoto et al. (2001) measured overall root hydraulic conductivities, but could not quantify the contribution of different parts or tissues of roots to the overall radial water flow (Lp_r). Although they concluded that the endodermis probably represented the most important resistance in the system, direct evidence was lacking because they were unable to measure cell *Lp* to get an idea about the trans-membrane component of water flow.

The present paper extends the work of Miyamoto et al. (2001) making use of a perfusion technique to separate the hydraulic and osmotic properties (hydraulic resistance or conductivity, reflection coefficients, etc.) of the OPR from that of the whole root. We conclude that the OPR represents a surprisingly low resistance to hydraulic (viscous) water flow. Under these conditions, water flow across the OPR must have a strong apoplastic component despite the presence of a suberized exodermis and a thick layer of sclerenchyma cells. It appears that the resistance of the endodermis/stele is the largest component in the system, that of the aerenchyma is intermediate and that of the OPR the least. The OPR was found to have a very low diffusional water permeability. This is interesting as well. Because the flow of oxygen across the OPR is diffusional in nature, it may suggest that the permeability of the OPR to oxygen is also low. Overall, this may point to an ability of roots to retain oxygen in the presence of a high capacity to take up water. This is advantageous to the plant because oxygen has to be transported along the root without excessive losses to the medium.

Materials and methods

Plant material and growth conditions

Rice seedlings (*Oryza sativa* L. cv. Azucena and IR64 from the International Rice Research Institute, Manila, Philippines) were grown in climatic chambers as detailed previously (Miyamoto et al. 2001). The aerated hydroponic nutrient solution contained 0.09 mM $(NH_4)_2SO_4$, 0.05 mM KH_2PO_4, 0.05 mM KNO_3, 0.03 mM K_2SO_4, 0.06 mM $Ca(NO_3)_2$, 0.07 mM $MgSO_4$, 0.11 mM Fe-EDTA, 4.6 μM H_3BO_3, 1.8 μM $MnSO_4$, 0.3 μM $ZnSO_4$, 0.3 μM $CuSO_4$. The osmotic concentration was 3 mM, which is equivalent to an osmotic pressure of 0.0075 MPa, and the pH was 5.5-6.0. Plants used in experiments were grown for 31–40 d. Overall, the upland variety Azucena grew somewhat faster in hydroponics than the lowland variety, IR64. When used, roots of IR64 from hydroponic culture were 290-360 mm in length. Overall shoot length was 330-350 mm (8th to11th leaf emergence). Azucena developed 500-555 mm roots and shoot height was 480-515 mm (8th - 11th leaf emergence). Diameters of adventitious roots for IR64 and Azucena were up to 0.9 mm and 1.2 mm, respectively. Diameters of the stele for IR64 and Azucena were up to 290 μm and 370 μm, respectively.

Root anatomy and surface area of root systems

Freehand cross–sections were prepared from adventitious roots. Cross sections were taken at the following distances from the root tip: 10, 20, 30, 50, 80, 100, 150, 200, and 250 mm. Sections were stained for 1.5 h with Sudan Red 7B at room temperature (Brundrett et al. 1991). Sections were viewed using an optical microscope (DIALUX 22 EB, Leitz, Germany). For photographs, Kodak Elite 64 ASA film was used. To check for Casparian bands, sections were stained for 1 h with 0.1% berberine hemisulfate and for another hour with 0.5% aniline blue (w/v, Brundrett et al. 1988). Sections were viewed under an epifluorescence microscope using an ultraviolet filter set (excitation filter BP 365, dichroitic mirror FT 395, barrier filter LP 397; Zeiss, Oberkochen, Germany). For photographs, Kodak Elite 200 ASA film was used. Surface areas of root systems were determined using an image–analyzing system based on a video camera

and software (image analysis; Skye Instruments, Llandrindod Wells, UK) as detailed previously (Miyamoto et al. 2001). Surface areas of root systems for IR64 and Azucena ranged between 1.8 and 3.0 × 10^{-2} m^2 and 2.3 and 6.8 × 10^{-2} m^2, respectively.

Pressure chamber measurements

Measurement of xylem sap exudation from root systems in the absence of hydrostatic pressure gradients (osmotic exudation)

The procedures used in these experiments have been described in detail in the previous paper (Miyamoto et al. 2001). Before starting these measurements, shoots were cut off using a razor blade at distances of 40–70 mm from the base. All tillers except the main stem were closed using clamps. Xylem sap exuding from the main stem was collected, transferred to Eppendorf tubes, and weighed. In the absence of hydrostatic pressure gradients, differences in osmotic pressure ($\Delta\pi$ in MPa) between the medium ($RT \cdot C^o$) and xylem sap ($RT \cdot C^i$) drove the water uptake per unit area by the root (J_{Vr} in m^3 m^{-2} s^{-1}), i.e.:

$$Jv_r = Lp_r \times \sigma_{sr} \cdot \Delta\pi = Lp_r \times \sigma_{sr} \cdot RT\left(C^i - C^o\right). \quad (1)$$

Lp_r and σ_{sr} represent the root's hydraulic conductivity and reflection coefficient, respectively. To calculate Lp_r, a value of $\sigma_{sr} = 0.4$ was used for the reflection coefficient of nutrient salts in xylem and medium (Miyamoto et al. 2001). Osmotic concentrations of the medium and of the xylem sap were measured using a freezing–point depression osmometer (Osmomat 030; Gonotec, Berlin, Germany).

Measurement of xylem sap exudation from root systems in the presence of hydrostatic pressure gradients

Plants used for measuring osmotic water flow were also employed to measure hydraulic conductivity of root systems in the presence of hydrostatic pressure gradients (Miyamoto et al. 2001). In order to induce water flows, pressures in the root chamber were raised in steps of 0.03–0.05 MPa to up to 0.35 MPa above atmospheric. Exuded

xylem sap was collected and weighed. For a given applied gas pressure (P_{gas} in MPa), volume exuded from the root system (V in m^3) was plotted against time (not shown). Slopes of these relations were calculated and referred to unit root surface area. Hydraulic conductivity of root systems (Lp_r in m s^{-1} MPa^{-1}) was calculated from the slopes of J_{Vr} plotted against the overall driving forces ($P_{gas} + \sigma_{sr} \Delta\pi$). Alternatively, Lp_r was determined from plots of J_{Vr} against P_{gas} in the range of high pressures, where the contribution of the osmotic component of the driving force was small due to dilution effects. Slopes of the plots were non-linear in the range of low pressures, i.e. root Lp_r depended on the magnitude of water flow as found for other species (Fiscus, 1975; Rüdinger et al. 1994).

Root pressure probe measurements

Root pressure probe measurements were carried out as described previously (e.g. Steudle et al. 1987; Steudle and Frensch 1989). Using cylindrical seals prepared from liquid silicone material (Xantopren; Bayer, Leverkusen, Germany), excised end segments of individual roots (i.e. tips intact) were tightly connected to a root pressure probe. Segments had lengths of 150-200 mm and diameters of 0.8–1.2 mm for Azucena or 0.6–1.0 mm for IR64. Usually, it took 5–12 h to establish stable root pressures. Hydrostatic and osmotic relaxations were performed by either changing the xylem pressure (moving the metal rod in the probe) or the osmotic pressure of the medium. Transient responses in pressure were followed which allowed Lp_r to be calculated from rate constants, k_{rw}, or half-times of pressure relaxations ($T_{1/2}^w$) according to Steudle et al. (1987):

$$k_{rw} = \ln(2) / T_{1/2}^{w} = A_r \cdot \Delta P_r / \Delta V_s \cdot Lp_r \,, \qquad (2)$$

where $\Delta P_r/\Delta V_s$ (in MPa m^{-3}) is the elastic coefficient of the measuring system; V_s denotes the water volume of the system, and A_r is surface area of the root. The ratio of $\Delta P_r/\Delta V_s$ was measured by inducing step changes in the volume and recording the resulting changes in root pressure (ΔP_r). Surface areas were calculated from the lengths and diameters of roots. Test solutions used in osmotic experiments were prepared by

adding either NaCl or ethanol to the root medium. Osmotic pressures of added solutes were 0.13 MPa (50 mOsmol·kg^{-1}) and 0.38 MPa (150 mOsmol kg^{-1}), respectively. Responses in root pressure to changes of osmotic pressure of the medium were biphasic. There was a rapid water phase (water efflux or influx) followed by a slower solute phase (water dragged by solute diffusion). From the solute phase, the permeability coefficient for the given solute (P_{sr} in m s^{-1}) was calculated according to Steudle et al. (1987):

$$k_{sr} = \ln(2) \Big/ T_{1/2}^{s} = A_r \cdot P_{sr} \Big/ V_x , \quad (3)$$

where k_{sr} is the rate constant of permeation of a given solute (NaCl, ethanol) and V_x the volume of xylem. V_x was 1% of root volume (Miyamoto et al. 2001). Root reflection coefficients (σ_{sr}) were calculated using the following equation (Steudle et al. 1987):

$$\sigma_{sr} = \left(P_{ro} - P_{r\min}\right) \Big/ \Delta\pi \times \exp\left(k_{sr} \times t_{\min}\right). \quad (4)$$

Here, P_{ro} is the original steady-state root pressure at the time when osmotic pressure was changed and P_{rmin}, the minimum root pressure according to water efflux. t_{min} is the time required to reach the minimum (P_{rmin}). Cutting experiments were conducted to validate the readings of the root pressure probe at the end of each root pressure probe experiment.

Steady state of perfusion of the outer part of root segments (OPR)

Perfusion of the OPR of segments (root tip not intact), including rhizodermis, exodermis, sclerenchyma and another cortical cell layer (Fig. 2), was conducted with root segments excised at two distances from the root tip (20-50 and 50-100 mm). At 20-50 mm, aerenchyma was not fully developed, but at 50-100 mm it was. Aerated nutrient solution was perfused through the aerenchyma, thus replacing the air with nutrient solution. Then one end of the section was used as an inlet while the other end was connected to a pressure probe, so that solution within the root was under pressure and, therefore flowed outwards across the OPR. A syringe was mounted on a high precision

12-step Braun-Melsungen pump that produced defined pumping rates between 1.7×10^{-9} and 1.1×10^{-7} mm^3 s^{-1}. In the set-up shown in Fig. 1a, perfusion was performed from the inlet side of the root segment, which was fixed to the syringe by a narrow, rigid Teflon tube (inner diameter: 1.5 mm). The resulting steady-state pressure was measured using a pressure probe as a manometer, which was fixed to the other end (outlet) of the root segment. Nutrient solution was pumped into the root segment at a given pump rate and the pressure increased gradually until a stationary pressure was established where the volume flow produced by the pump equalled the radial volume flow across the OPR (Fig. 1b).

Measurements required that root segments be tightly connected to the glass capillaries (inner diameter: 1.3 mm). A polyacrylamide glue (UHU, Bühl, Germany) was used which allowed connection of even wet tissue to glass. To make the seal mechanically rigid, the glue was superposed with a molten mixture of beeswax-collophony (1:3; w/w; Zimmermann and Steudle 1975). To test the tightness of the seal, two types of experiments were performed. In the first type, aerenchyma was perfused with nutrient solution to which the apoplastic fluorescent dye trisodium, 3-hydroxy-5,8,10-pyrene trisulfonate (PTS: Bayer AG, Leverkusen, Germany) was added at a concentration of 0.02% (w/v). Under the microscope, leakages could be detected with UV light, looking for fluorescence that occurred at leaks. In the second type of experiment, small leaks were created by puncturing holes into the OPR of some root segments using a cell pressure probe (tip diameter: 5 μm), and following the decrease in steady-state pressures. Knowing the diameter of the tip of the needle, the size of the leak could be estimated, thus, estimating an upper limit of the overall leakage which could have been still present and relating this to the overall hydraulic conductance of OPR (see Discussion).

In a typical steady-state experiment, flow rate was varied step-wise with the aid of the perfusion pump and resulting stationary pressures were measured. Since the internal volume of the system (water volume of syringe, tube, and aerenchyma) was quite large (high damping), adjustment in steady-state (stationary) pressure required rather long time intervals of about 1-2 h, although the hydraulic conductance was low. Increasing the pump rate linearly increased the steady-state pressure. When plotting pump rate Q_V

in m^3/s *vs.* steady-state pressure (MPa), a straight line was obtained. Since the inner part of the root (stele) did not contribute to the results, the slope was related to the conductance of the outer part of the root (see Fig. 3). Since the length and diameter of root segments were known as well, the hydraulic conductivity of the outer part (Lp_{OPR}) was calculated:

$$Q_v = Lp_{OPR} \times P \times A_r \, . \tag{5}$$

Here, Q_V is the pump rate, P the steady state pressure (reference: atmospheric pressure), and A_r is the surface area of the root segment. To avoid anaerobic conditions, aerated nutrient solution was used for perfusion. Also, root segments were placed in a small chamber and air-saturated nutrient solution was continuously circulated around to allow saturation with oxygen throughout the experiment.

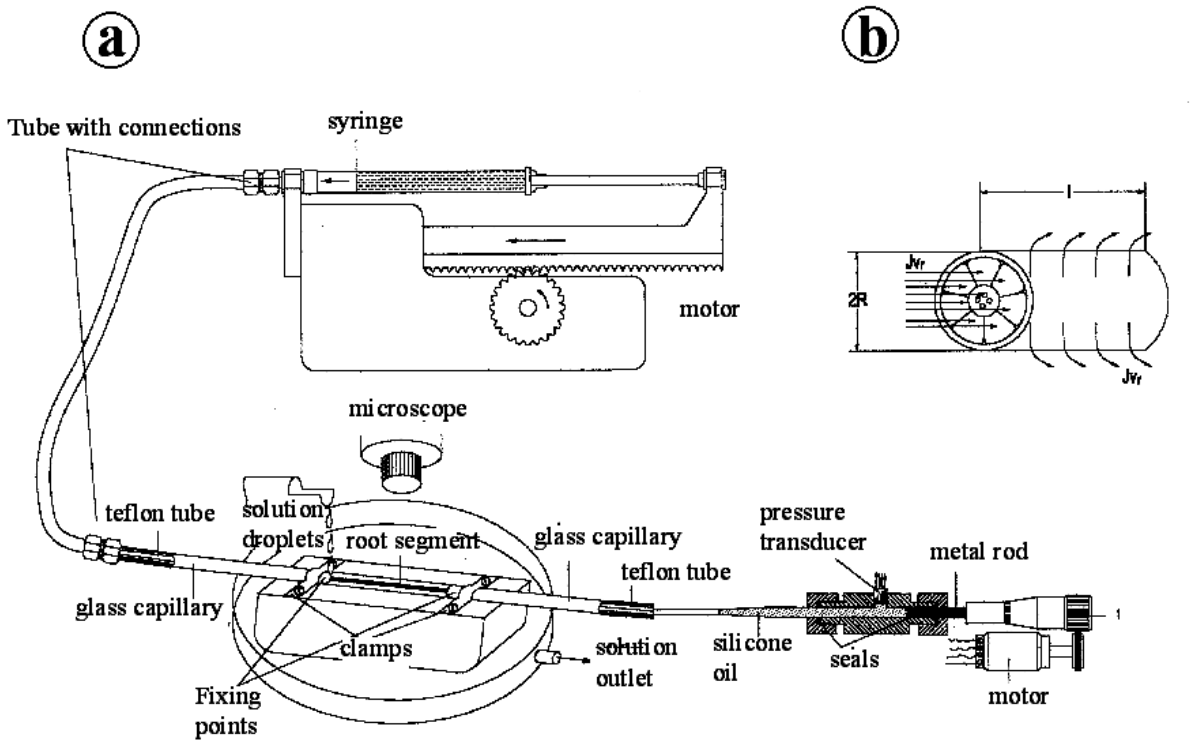

Fig. 1 a Pump perfusion set-up: A syringe was mounted on a 12 step Braun-Melsungen pump that produced pumping rates between 1.7×10^{-9} and 1.1×10^{-7} $mm^3\ s^{-1}$. Perfusion was performed from one side of the root segment, which was fixed to the syringe by a narrow and rigid Teflon tube. The other end was connected to a pressure probe to measure resulting steady-state pressures. **b** Schematic diagram to show radial water flows across the outer part of root segment during pressure perfusion.

In some experiments, steady-state pressure was first increased in steps and then decreased again to test for reproducibility and for hysteresis effects due to changes in root segments (data not shown). To characterize root anatomy, especially the anatomy and developmental state of the OPR, a few segments were sectioned following a pressure-perfusion experiment, stained (see above) and viewed under a microscope. Since perfusion experiments lasted for 10-15 h, it was necessary to test for the viability of root cells; Evans blue stain was used (Fischer et al. 1985).

Osmotic experiments with perfused root segments

The experimental set-up allowed estimation of reflection coefficients of the OPR by measuring the changes in steady–state pressure caused by the addition of osmotic solutes to the outer medium at a concentration of about 20 mM (0.05 MPa of osmotic pressure). A non-electrolyte (mannitol) and an electrolyte (NaCl), which have a σ_{sOPR} of virtually unity for plant cell membranes, were used as osmotica. The procedure assumed that Lp_{OPR} values for the osmotic and hydrostatic type of experiments were equal.

Steam treatment

At the end of the pump perfusion experiment, root segments taken at either 20-50 mm, or 50-100 mm from the root tip were exposed to steam for 20-30 s to kill the cells (rhizodermis and cortical cells) at the OPR of the segments. New steady state pressures were measured with respect to flow rates of the perfusion pump. The hydraulic conductivity of the outer part of the root (Lp_{OPR}) was calculated following the steam treatment. Changes in steady-state pressure were also measured by adding the non-electrolyte mannitol at a concentration of 20 mM. Hence, reflection coefficients of the OPR of steamed root segments (σ_{sOPR}) were estimated.

Results

Root anatomy: development of aerenchyma, Casparian bands (CBs) and suberin lamellae

There were no visible differences in the anatomy and development between the two cultivars used (upland rice, Azucena, and lowland rice, IR64). Therefore, photographs taken from cross-sections of adventitious roots are shown only for IR64. At a distance of 10-15 mm from the root tip, cortical cells started to collapse and aerenchyma gradually developed along roots. Fully developed aerenchyma was observed at a distance of about 100 mm (Fig. 2a-c). Aerenchyma was located in the central cortex separating the inner part of the root from the outer (OPR). Well-defined OPR contained only four cell layers (Fig. 2d-f). Rhizodermis was the outermost layer, which surrounded an exodermis (hypodermis with CBs). A single layer of dead sclerenchyma fibre tissue was laid down below the exodermis (Figs. 2e, f; Clark and Harris 1981). An innermost unmodified cortical cell layer was adjacent to large air lacunae in the root. These were separated from each other by radial, monolayered walls, which appeared as spokes in cross-sections (Fig. 2c). At distances as short as 20 mm from the tip, suberin lamellae were observed in the endodermis. At a distance of about 50 mm, depositions of secondary suberin were laid down on the inner walls of endodermal cells. The thickness of suberin lamellae in the endodermis increased along roots towards the root base (not shown). At a distance of 100 mm from the tip, fully developed, u-shaped wall thickenings were detected in the inner walls of endodermis (Fig. 2c). Even though suberin lamellae in the exodermis were not as strongly developed as in the endodermis, exodermal development started fairly close to the tip (30 mm). Slightly developed, exodermal suberin lamellae were observed somewhat later than in the endodermis (at about 30 mm), and these matured at about 50 mm (Sudan Red 7B staining; Fig. 2e). As typical for most roots, the endodermis started to develop CBs at distances closer to the tip than the exodermis. In the endodermis, CBs were observed at distances of about 20 mm (not shown). Well-developed endodermal CBs were found in the mature part of roots (100 mm from tip). No exodermal CBs were detectable at about 20 mm from the tip (berberine aniline blue; Fig. 2g). Maturation of exodermis started at a distances of about 30 mm (Fig. 2h). Well-developed bands were found at about 50 mm (Fig. 2i).

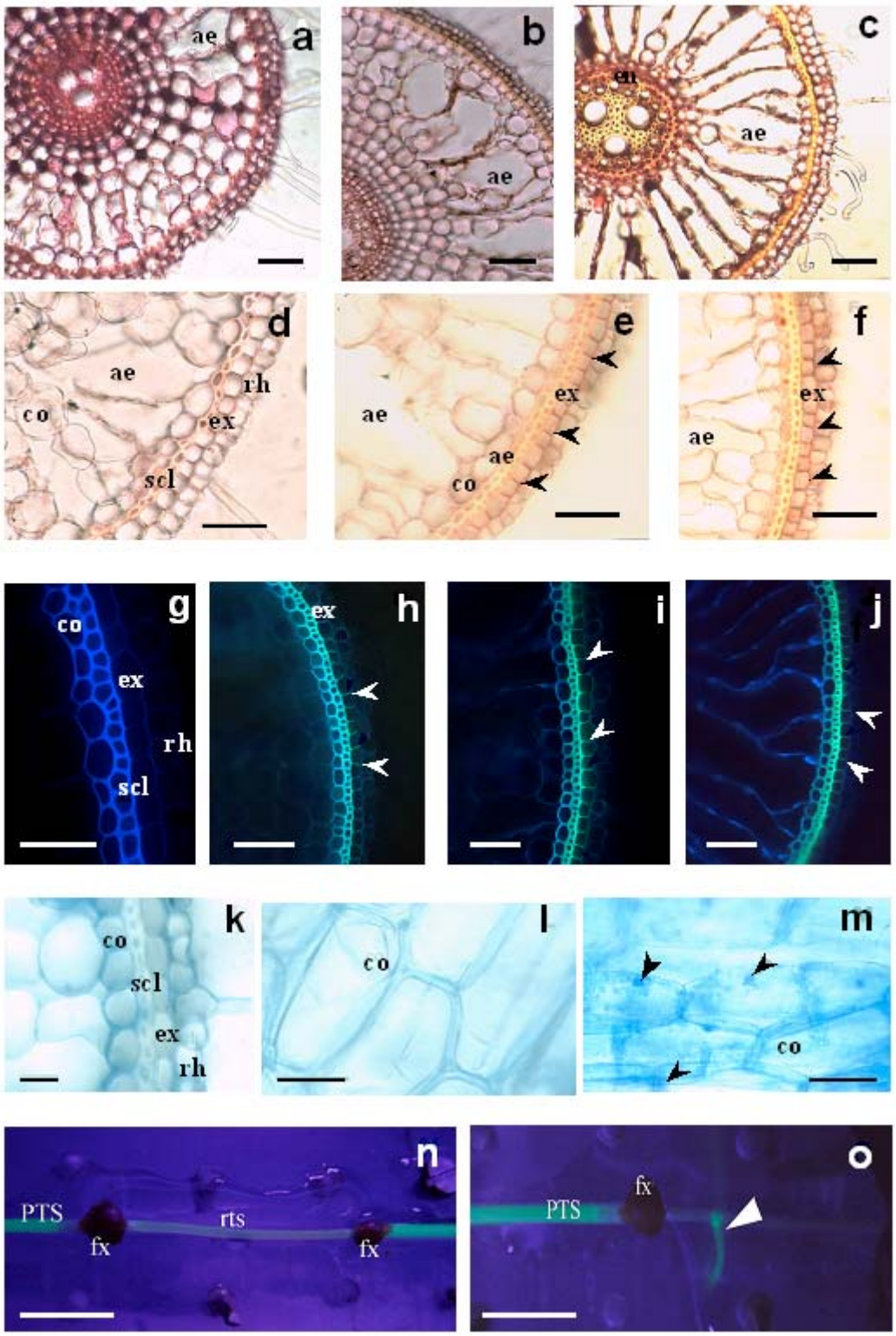

Fig. 2a–c Sections of roots of 30-day-old rice (*Oryza sativa*) plants. Development of aerenchyma at 20 (**a**), 50 (**b**) and 100 mm (**c**) from the tip. Freehand cross-sections were stained with Sudan Red 7B. **d–f** The outer part of roots (OPR) contained four cell layers (rhizodermis, exodermis, sclerenchyma and one cortical cell layer). Freehand cross-sections taken at 20 (**d**), 50 (**e**) and 100 mm (**f**) from the root tip were stained with Sudan Red 7B. *Arrowheads* show suberin lamellae in exodermis. **g-j** Freehand cross-section taken at 20 (**g**), 30 (**h**), 50 (**i**) and 100 mm (**j**) from the root tip were stained with berberine aniline blue

(fluorescent dye). *Arrowheads* show Casparian bands in exodermis. **k-m** Freehand cross-sections (**k**) and longitudinal sections (**l, m**), taken 50 mm from the root tip, stained with Evans Blue to check for the viability of cells of the root segments after finishing the pump perfusion experiment. In killed cells, nuclei were stained blue. **n, o** An experiment using the apoplastic fluorescent dye, trisodium, 3-hydroxy-5,8,10-pyrene trisulfonate (PTS) to check for leaks at the points where root segments were fixed to glass capillaries. Segments were viewed under UV light. *Arrowhead* shows PTS flux coming out after puncturing the root with a 5-µm-diameter micro capillary. *ae* Aerenchyma, *co* cortical cells, *ex* exodermis, *fx* fixing points of the root segment, *rh* rhizodermis, *scl* sclerenchyma fibre tissue. Bars = 90 µm (**a-j**), 40 µm (**k-m**), 20 mm (**n, o**).

Highly lignified sclerenchyma was found below the exodermis at about 100 mm. Even at a distance of 100 mm from the tip, no suberin could be detected in sclerenchyma tissue by staining with Sudan Red 7B. Initiation of lateral roots was observed at around 50-70 mm and 70-90 mm from the tip for IR64 and Azucena, respectively. Lateral roots were fairly rare for those root segments. More root hairs were observed in the mature (50-100 mm) than in the immature part (20–50 mm).

Steady-state hydraulic and osmotic exudation experiments with root systems

At a given air pressure applied to the external nutrient solution (P_{gas}), exuded volumes of xylem sap (V) increased linearly with time, t. Water flows, $J_{V\mathrm{r}}$ (volume per unit time and surface area) obtained from these graphs (not shown) were then plotted against both the gas pressure applied to the root system (P_{gas}) and the overall driving force, $P = P_{gas} + \sigma_{sr}.\ \Delta\pi$, whereby the reflection coefficient of nutrient salts was assumed to be 0.4 (also not shown; Miyamoto et al. 2001). Hydraulic conductivities of whole root systems were obtained as slopes of pressure/flow curves. Mean Lp_r values (±SD) of IR64 and Azucena were $(4.0 \pm 1.7) \times 10^{-8}$ (n = 5 roots) and $(2.8 \pm 1.3) \times 10^{-8}$ m s^{-1} MPa^{-1} (n = 5 roots), respectively (Table 1). There were no significant differences between varieties (t-test; P = 0.05). Osmotic exudation rate and osmotic concentration of xylem sap were measured at an external concentration of 0.0075 MPa. Osmotic Lp_r was calculated as the osmotic exudation rate per unit surface area of root system (A_r) and driving force (difference in osmotic pressure between xylem sap and medium times reflection coefficient of solutes). Mean osmotic Lp_r values (±SD) of IR64 and Azucena were $(3.1 \pm 0.9) \times 10^{-8}$ (n = 5 roots) and $(2.4 \pm 1.1) \times 10^{-8}$ m s^{-1} MPa^{-1} (n = 5 roots), respectively

(Table 1). As for the hydrostatic Lp_r, there were also no significant differences in osmotic Lp_r values, between the cultivars (*t*-test; $P = 0.05$). Osmotic Lp_r and hydrostatic Lp_r values were in the same range.

Table 1 Hydraulic conductivity (Lp_r) of individual rice (*Oryza sativa*) roots and whole root systems, which were grown in hydroponics with bubbled air for 31–40 days, as measured by the root pressure probe (single roots) and pressure chamber techniques (root systems). With both techniques, root Lp_r was measured in response to changes in root or chamber pressure or to changes in the osmotic pressure of the outer medium (osmoticum: 50 mOsmol/kg NaCl; see Material and Methods). Mean values of hydrostatic and osmotic Lp_r were calculated for individual roots and root systems. Number of roots measured are given in parentheses. There were no significant differences of hydrostatic Lp_r values for individual roots and whole root systems. Lp_r values of the cultivars Azucena and IR64 were in the same range, but the means of hydrostatic Lp_r of Azucena and IR64 for single roots were significantly higher than that of osmotic Lp_r (*t*-test, $P = 0.05$) [ratio: Lp_r(hydrostatic)/Lp_r(osmotic) = 3.5]. Overall, values obtained in this paper were similar to those of Miyamoto et al. (2001), except osmotic Lp_r for individual roots.

Rice variety	Hydraulic conductivity of individual roots $Lp_r \times \mathbf{10^{-8}}$ [m . s^{-1} . MPa^{-1}]		Hydraulic conductivity of whole root system $Lp_r \times \mathbf{10^{-8}}$ [m . s^{-1} . MPa^{-1}]		Reference
	Hydrostatic	Osmotic/NaCl	Hydrostatic	Osmotic	
IR64 (lowland)	3.8 ± 0.6 (7)a	1.1 ± 0.5 (6)b	4.0 ± 1.7 (5)	3.1 ± 0.9 (5)	This paper
Azucena (upland)	4.0 ± 1.2 (7)a	1.1 ± 0.4 (6)b	2.8 ± 1.3 (5)	2.4 ± 1.1 (5)	
IR64	5.0 ± 2.5 (8)	9.2 ± 3.0 (6)	5.6 ± 2.7 (18)	4.2 ± 2.5 (18)	Miyamoto
Azucena	4.7 ± 1.0 (10)	4.0 ± 2.5 (6)	6.3 ± 3.1 (14)	5.5 ± 3.7 (14)	et al. 2001

Different letters indicate significant differences at $P = 0.05$ level

Root pressure probe measurements with single roots: transient water flow

Hydrostatic Lp_r for single roots as obtained with the root pressure probe were (3.8 ± 0.6) × 10^{-8} (n = 7 roots) and (4.0 ± 1.2) × 10^{-8} m s^{-1} MPa^{-1} (n = 7 roots) for IR64 and Azucena, respectively. These values were similar to those obtained in steady-state experiments using the pressure chamber. From the water phase of biphasic osmotic relaxations, osmotic Lp_r was calculated. Osmotic Lp_r of IR64 and Azucena were (1.1 ± 0.5) × 10^{-8} and (1.1 ± 0.4) × 10^{-8} m s^{-1} MPa^{-1} (n = 6 roots for each; Table 1). Hence, hydrostatic Lp_r was bigger than the osmotic Lp_r by a factor of 3.6 (significant; *t*-test; P = 0.05). Even though this ratio was bigger than unity, it is significantly lower than that of

corn (Zimmermann and Steudle 1998). The osmotic pressure of the medium was changed by adding solutes like NaCl and ethanol. Average reflection coefficients for NaCl were σ_{sr} = 0.18 and 0.16 for IR64 and Azucena, respectively. They were significantly greater than those of ethanol, which should rapidly permeate root cell membranes (σ_{sr} = 0.04 and 0.08, respectively; see Table 4).

Pressure perfusion of the outer part of root segments (OPR)

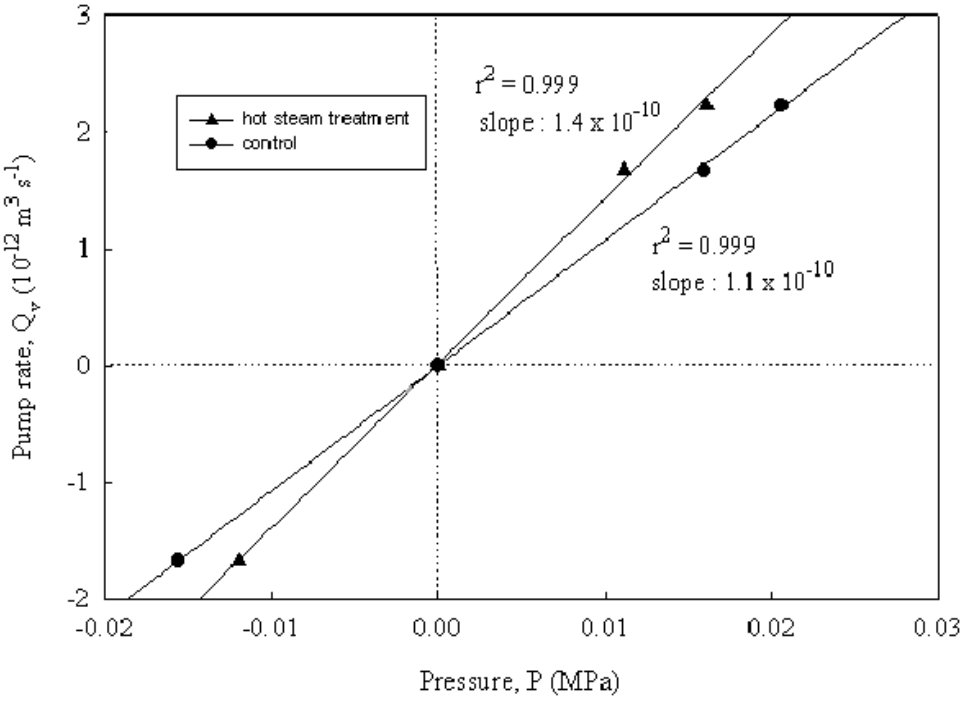

Fig. 3 Effect of heat-killing on the hydraulic conductivity of the OPR (cultivar IR64, 50–100 mm from the tip) of rice. Pump rates (Q_V) plotted against steady-state pressures (P) of the root segment (control and heat-killed). The slope increased after killing the root with steam for 20–30 s. Hydraulic conductivities of the OPR were obtained from slopes of plots, dividing by the surface area of the root segment (A_r = 1.1 × 10^{-4} m^2). In the example shown, Lp_{OPR} increased by 32% during heat-killing. This increase was much smaller than would be expected if membranes (i.e. the cell-to-cell passage across the OPR) were rate-limiting.

Root segments that were fixed to the pressure perfusion set up (Fig. 1a) took at least 2-3 h to build up stable pressure. Increasing the pump rate linearly increased steady-state pressure. Plots of pump rates (Q_V in m^3/s) *vs.* steady-state pressure (P in MPa) yielded a straight line (Fig. 3).

Table 2 Hydraulic conductivity of the outer part of rice roots (Lp_{OPR}), grown in hydroponic culture with bubbled air for 31–40 days, measured by the pump perfusion technique (Fig. 1a). Lp_{OPR} values are given for two different distances from the root tip for IR64 and Azucena separately. Lp_{OPR} values are also given for excised root segments, treated with hot steam for 20-30 s to kill some of the living cells in the OPR. Values are given for individual root segments, which were then averaged (means ± SD, *n* = number of segments). There were no significant differences for Lp_{OPR} values along the root for either cultivar. Killing the segments with hot steam affected Lp_{OPR} of immature part (20-50 mm) more significantly than the mature part (50-100 mm) (*t*-test, $P = 0.05$). Ratios between the Lp_{OPR} of heat-killed roots and that of the control were 2.6 ± 0.7 and 1.8 ± 0.3 for the immature part (20-50 mm) for IR64 and Azucena, respectively, and 1.5 ± 0.4 and 1.4 ± 0.1 for the mature part (50-100 mm). Both ratios were significantly different from unity and from each other (*t*-test, $P = 0.05$).

Root number	Hydraulic conductivity of the OPR Lp_{OPR} (10^{-6} m s^{-1} MPa^{-1})				Lp_{OPR} heat-killed/ Lp_{OPR} control	
	Control roots		Heat-killed roots			
	20-50 mm	50-100 mm	20-50 mm	50-100mm	20-50 mm	50-100 mm
IR64 (lowland)						
1	1.6	1.2	2.9	2.4	1.8	2.0
2	1.4	1.1	3.5	1.4	2.5	1.3
3	1.0	1.2	2.8	1.4	2.8	1.2
4	0.8	0.8	2.9	1.5	3.6	1.9
5	0.8	0.7	1.9	0.9	2.4	1.3
6	1.9	1.3	-	-	-	-
7	1.8	1.5	-	-	-	-
8	2.1	1.3	-	-	-	-
9	2.0	1.2	-	-	-	-
10	1.3	1.2	-	-	-	-
Mean	**1.5**a	**1.1**a	**2.8**b	**1.6**a	**2.6**	**1.5**
SD	0.5	0.3	0.6	0.4	0.7	0.4
n	10	10	5	5	5	5
Azucena (upland)						
1	1.4	1.1	-	1.5	-	1.4
2	0.7	0.6	1.6	-	2.3	-
3	0.9	0.8	-	-	-	-
4	2.0	1.5	3.0	2.1	1.5	1.4
5	1.9	1.0	3.4	-	1.8	-
6	0.7	0.3	1.3	0.4	1.9	1.3
7	1.9	1.4	2.9	1.9	1.5	1.4
8	0.8	0.4	-	-	-	-
9	1.0	0.7	-	1.0	-	1.4
10	1.2	0.8	-	1.3	-	1.6
Mean	**1.3**a	**0.9**a	**2.5**b	**1.4**a	**1.8**	**1.4**
SD	0.5	0.4	1.0	0.6	0.3	0.1
n	10	10	5	6	5	6

Different letters indicate significant differences at $P = 0.05$ level.

When stable pressures were attained after each step change of Q_V, radial volume flow across the OPR equalled the volume flow from the perfusion pump. No hysteresis was observed in $Q_V(P)$ curves, i.e. when pressures (pump rates) were increased, the same results were obtained as during decreases, and Lp_{OPR} remained constant for the rather long time intervals required for an experiment with a given root segment (data not shown).

On average, Lp_{OPR} was 30 times larger than the overall radial hydraulic conductivity of rice roots. With neither cultivar was there any significant difference in Lp_{OPR} along the root axis over the first 100 mm (Table 2). Mean Lp_{OPR} values for root segments 20-50 mm from the tip for IR64 and Azucena were $(1.5 \pm 0.5) \times 10^{-6}$ m s^{-1} MPa^{-1} and $(1.3 \pm 0.5) \times 10^{-6}$ m s^{-1} MPa^{-1}, respectively (n = 10 roots each). Values were $(1.1 \pm 0.3) \times 10^{-6}$ m s^{-1} MPa^{-1} and $(0.9 \pm 0.4) \times 10^{-6}$ m s^{-1} MPa^{-1} for root segments 50-100 mm from the tip for IR64 and Azucena, respectively (n = 10 roots each).

When root cell membranes were damaged by treatment with steam, Lp_{OPR} values increased significantly in both rice varieties and for both distances behind the tip, except for Azucena at 50-100 mm (Table 2). However, in the latter case, there was also a tendency for an increase of Lp_{OPR}. Mean Lp_{OPR} for heat-killed root segments 20-50 mm from root tip for IR64 and Azucena were $(2.8 \pm 0.6) \times 10^{-6}$ m s^{-1} MPa^{-1} and $(2.5 \pm 1.0) \times 10^{-6}$ m s^{-1} MPa$^{-1,}$ respectively (n = 6 and 5 roots each). Values of Lp_{OPR} for root segments taken 50-100 mm behind the tip were $(1.6 \pm 0.4) \times 10^{-6}$ m s^{-1} MPa^{-1} and $(1.4 \pm 0.6) \times 10^{-6}$ m s^{-1} MPa^{-1}, respectively (n = 5 and 6 roots each). Heat-killed segments with immature parts (20-50 mm) showed an increase of 87 and 92% of Lp_{OPR} for the two varieties. It was around 45 (IR 64) and 55% (Azucena) for the mature part (50-100 mm). For both varieties, means of ratios of individual segments (heat-killed/control) were significantly higher for immature segments (20-50 mm) than for mature (50-100 mm). Ratios for root segments 20-50 mm from the tip were 2.6 ± 0.7 and 1.8 ± 0.3 for IR64 and Azucena, respectively. Root segments 50-100 mm behind the tip showed lower ratios for heat-killed/control of 1.5 ± 0.4 and 1.4 ± 0.1 for IR64 and Azucena, respectively. The difference in the change may be interpreted by a larger contribution of trans-membrane water flow and/or changes in the apoplastic component in younger parts.

Osmotic experiments with the OPR

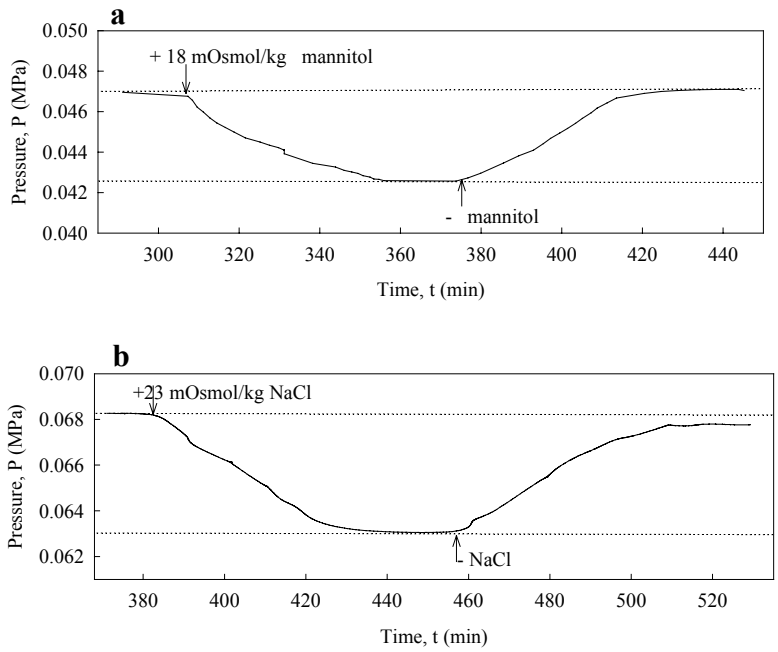

Fig. 4a, b Determination of reflection coefficients of the OPR. Steady-state pressures in segments, perfused at a given rates (Fig. 1) decreased in response to changes in osmotic pressure of the outer medium resulting from mannitol (**a**) and NaCl (**b**). Reflection coefficients of the OPR (σ_{sOPR}) were calculated from pressure differences. They indicated reflection coefficients of as low as 0.1 and 0.09 for mannitol and NaCl, respectively. Segments taken from IR64, 50–100 mm from tip.

Osmotic water flow was induced by changing the osmotic pressure of the outer medium by adding either mannitol or NaCl. Efflux of water from the root caused the stationary root pressure to decline. Maximum drops in pressure were used to calculate the reflection coefficient of the OPR (σ_{sOPR}) for mannitol and NaCl (Fig. 4). After removing the solutes, either the non-electrolyte mannitol or the electrolyte NaCl, from the outer medium, excised root segments re-attained the original stationary pressure.

There were no significant differences in σ_{sOPR} values of mannitol and NaCl in either cultivar (Table 3), and values were almost identical for mature (50-100 mm) and immature parts (20-50 mm) of roots. Mean σ_{sOPR} for mannitol were 0.13 ± 0.04 and 0.15 ± 0.05 for immature root segments (20-50 mm) of IR64 and Azucena, respectively (n = 6 and 8 roots each). Those for mature parts (50-100 mm) were 0.13 ± 0.04 and 0.14 ± 0.10, respectively (n = 6 and 7 roots each).

Table 3 Reflection coefficients of the outer part of rice roots (σ_{sOPR}), which were grown in hydroponics with bubbled air for 31-40 days, as measured by the pump perfusion technique in the presence of an outward water flow (Fig. 1b). Osmotic water flow was induced by increasing the osmotic pressure of the medium by adding either 20 mOsmol/kg mannitol or 20 mOsmol/kg NaCl (=0.05 MPa of osmotic pressure) to the external medium. Values shown are mean values ± SD with the number of measured roots in parentheses. Reflection coefficients (σ_{sOPR}) for mannitol and NaCl are in similar range for both cultivars, IR64 and Azucena, even at the two different distances from root tip. Heat-killing of the OPR resulted in a significant reduction of σ_{sOPR} for mannitol at both distances from root tip (*t*-test, $P = 0.05$). The reflection coefficient for NaCl was lower in the OPR than that of whole individual roots (see Table 4) measured with the root pressure probe.

Rice cultivar	Reflection coefficient (σ_{sOPR}) of the outer part of the root segments		
	Control		Heat–killed roots
	Mannitol	NaCl	Mannitol
IR64			
20 – 50 mm	0.13 ± 0.04 (6)a	0.09 ± 0.02 (5)	0.04 ± 0.01 (5)b
50 – 100mm	0.13 ± 0.04 (6)a	0.11 ± 0.03 (5)	0.06 ± 0.01 (5)b
Azucena			
20 – 50mm	0.15 ± 0.05 (8)a	0.08 ± 0.02 (5)	0.04 ± 0.01 (5)b
50 –100mm	0.14 ± 0.10 (7)a	0.09 ± 0.01 (5)	0.03 ± 0.02 (5)b

Different letters indicate significant differences at $P = 0.05$ level

Heat-killed roots showed significant reductions of σ_{sOPR} values for mannitol for both rice cultivars for both root segments at two different distances from the tip, so that mean σ_{sOPR} values for root segments 20-50 mm from the root tip, which had been killed with steam, were 0.04 ± 0.01 and 0.04 ± 0.01 for IR64 and Azucena, respectively ($n = 5$ roots each). Values were 0.06 ± 0.01 and 0.03 ± 0.02 for the mature part of the roots (50-100 mm; $n = 5$ roots each). The reflection coefficient (σ_s) for NaCl was smaller in the OPR than that of whole rice roots (Table 4). Mean σ_s values in the OPR for NaCl were 0.09 ± 0.02 and 0.08 ± 0.02 for IR64 and Azucena at 20-50 mm from the tip, respectively ($n = 5$ roots each). At a distance of 50-100 mm, reflection coefficients were 0.11 ± 0.03 and 0.09 ± 0.01 for IR64 and Azucena, respectively ($n = 5$ roots each).

Table 4 Reflection coefficients of single adventitious roots of rice (σ_{sr}), which were grown in hydroponics with bubbled air for 31-40 days, as measured with the root pressure probe. Osmotic water flow was induced by increasing the osmotic pressure of the medium by adding either 150 mOsmol/kg ethanol or 50 mOsmol/kg NaCl. Values shown are mean values ± SD with the number of measured roots in parentheses.

Rice variety	Reflection coefficient (σ_{sr}) of excised roots		Reference
	Ethanol	NaCl	
IR 64	0.04 ± 0.02(6)	0.18 ± 0.06(6)	This paper
Azucena	0.08 ± 0.03(6)	0.16 ± 0.05(6)	
IR64	0.09 ± 0.01(6)	0.28 ± 0.11(6)	Miyamoto et al. 2001
Azucena	0.13 ± 0.07(7)	0.28 ± 0.17(6)	Miyamoto et al. 2001

Since the water flows across the OPR was small during the osmotic experiment ($J_{Vr} \approx 10^{-8}$ m s^{-1}), effects of unstirred layers caused by a sweep-away effect (Dainty, 1963) were also rather small. The actual change in concentration immediately at the outer surface of the OPR was slightly smaller than the bulk concentration (factor of $\exp(J_{Vr} \cdot \delta / D_s)$; $J_{Vr} \approx 10^{-8}$ m s^{-1}, unstirred layer, δ = 100 µm and diffusion coefficient of solutes used, $D_s = 10^{-10}$ m^2 s^{-1}).

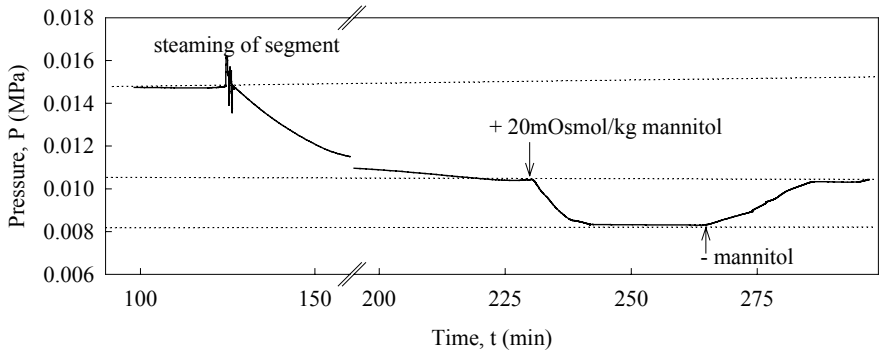

Fig. 5 Reduction of steady-state pressure, after killing with hot steam for 20–30 s (IR64: 50–100 mm from tip). The reflection coefficient (σ_{sOPR} = 0.05) obtained for mannitol for heat-killed root segments was smaller than that of intact segments (Fig. 4).

Tests for leaks and viability of cells of the OPR

At the end of the experiment, possible leaks at fixing points and in other part of the roots were checked using PTS (Fig. 2n). Results from the root segments that showed even small, microscopic leaks were discarded. At the end of the experiment, small leaks were created by puncturing holes in the OPR using a cell pressure probe (tip diameter: 5 μm), and a stream of PTS coming out from the root was observed under UV light (Fig. 2o). The reduction in steady-state pressure was around 15% in the presence of a tiny hole of 5 μm (Fig. 6). No leaks could be observed with PTS at the fixing points (see Discussion).

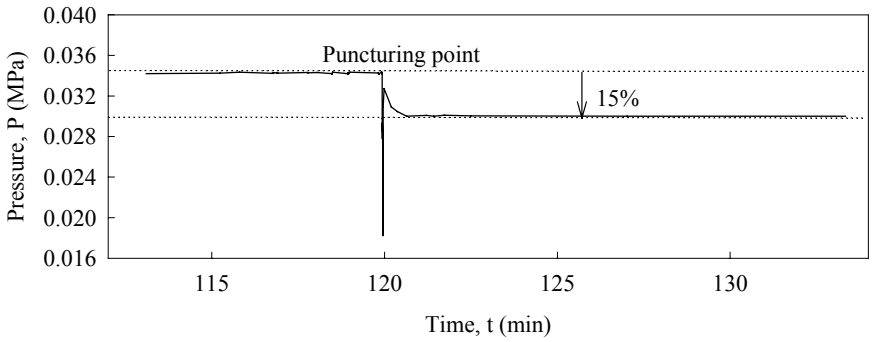

Fig. 6 Effect of puncturing of the OPR of rice roots (IR64: 20–50 mm from root tip). A small leak was created by puncturing a hole in the outer part of the root segment using a cell pressure probe (tip diameter: 5 μm). Pressure reduction was 15%. Reduction was less than calculated from Poiseuille's law (see Discussion). The experiment indicates that any leaks at the points of fixation of root segments to glass capillaries were small. When segments were properly fixed, no visible efflux of dye could be seen in these areas

Since the experiments lasted for 10-15 h, the viability of the cells in the root segments was checked using Evans blue. This stain cannot penetrate through the membrane in living cells and did not stain the nuclei and cytoplasm dark blue (Fig. 2l). The nuclei and cytoplasm of cells in heat-killed root segments were stained dark blue (Fig. 2m).

Discussion

The data show that the OPR offers only a small resistance to the radial passage of water, even when the exodermis is fully developed. It appears that rice roots differ from those of other plants where the exodermis contributes substantially to the overall root Lp_r. In young corn roots, formation of an exodermis decreased root Lp_r 4-fold (Zimmermann and Steudle 1998). In onion, development of an exodermis during root maturation substantially reduced the hydraulic conductivity during root development (Melchior and Steudle 1993). There are to date no data for Lp_{OPR} of aerenchymatous roots of hygrophytes other than rice. However, the diffusional water permeability (P_d) of the OPR of sand sedge (*Carex arenaria*) was surprisingly small at (1-2) × 10^{-8} m s^{-1}, which is equivalent to only (7-15) × 10^{-11} m s^{-1} MPa^{-1}. The values of P_d for perfused corn roots having an aerenchyma was also small, as was that for sleeves prepared from young corn roots (6-13) × 10^{-7} m s^{-1}, equivalent to (4-8) × 10^{-9} m s^{-1} MPa^{-1} (Robards et al. 1979; Clarkson et al. 1987).

For rice, the overall radial hydraulic conductivity was lower than those of other cereal roots (Miyamoto et al. 2001). This has been interpreted as a major limitation of rice roots to supply water to transpiring leaves. The present study confirms this conclusion. However, because the hydraulic resistance of the OPR of rice roots was quite small (present study), it should have little or no influence on overall water uptake. Hence, the overall radial hydraulic conductivity must be limited by some other parts of the pathway. The present study on Lp_{OPR} was possible due to the presence of aerenchyma in roots of rice providing an opportunity to clearly separate resistances without removing the stele from the roots prior to measurements. A tissue of defined structure such as the OPR (four cell layers in series), possessing structural features that have been described as apoplastic barriers in other species, can be analyzed in great detail to test existing models of water transport such as the composite transport model (Steudle 2000a). However, before the data obtained by the new pressure perfusion technique can be considered as real, a few possible sources of error have to be considered.

Leaks in the areas where isolated segments were glued to capillaries could have led to misleadingly high values of Lp_{OPR}. To demonstrate that leaks did not occur, two types

of tests were performed. In one type, segments were perfused with PTS solution to detect leakages under UV light (Fig. 2n). When properly glued to capillaries, no visible leakage was detected in the areas where the sections were fixed. In a second type of experiment, perfused sections were punctured using tips of pressure probes with a diameter of as small as 5 μm; significant leaks of PTS were detected. However, these leaks, even though they were easily detectable by dye leakage, caused a reduction of stationary pressure of only 15% (Fig. 6). This was less than expected for a hole of 5 μm in diameter (Poiseuille's law; thickness of the OPR = 85 μm), which may be explained by a contraction of the hole when the tip was removed. We may conclude that leaks at the points of attachment to capillaries were small, if any. Macroscopic leaks in other parts of sections caused by handling the segments during fixation and the like were immediately evident during dye perfusion. Data collected from these segments were discarded. We could find no leaks in the areas where secondary roots emerged from the roots, even though PTS clearly stained the narrow xylem in these initials (not shown). Although the ability of the OPR to retain the apoplastic dye PTS was rather high, this does not mean that the apoplastic path was completely impermeable to it. In elution experiments with roots preloaded with PTS, Yeo et al. (1987) showed that there was an apoplastic bypass of this solute in rice. However, this general permeability of the dye was relatively small compared with leaks caused by inappropriate fixation or handling. The lack of hysteresis effects and the presence of viable cells not staining with Evans blue strongly suggest that the observed large values of Lp_{OPR} were not due to tissue damage. As the perfusion solution (nutrient solution saturated with air) had an oxygen activity similar to that of air, there should have been no shortage of oxygen within root segments caused by the fact that the air in aerenchyma was replaced by nutrient solution. The external medium was saturated with air as well and rapidly circulated around sections.

Since the measurements were sound, the high hydraulic conductivity of the OPR can be considered real. High Lp_{OPR} could be brought about by a large apoplastic component of water transport or by a high permeability of membranes of the living cells in the rhizodermis, exodermis, and internal cortical cell layer of the OPR. If there were a high apoplastic component, this would mean that Casparian bands in the exodermis (Fig. 2j) were quite permeable to water. In both cases (dominating cell-to-cell or apoplastic

transport), the sclerenchymatous layer consisting of dead cells (Harris and Clark 1981), should have been fairly permeable to water as well. To date, there are, for technical reasons, no data of the *Lp* of membranes of cells of the OPR in rice roots (Miyamoto et al. 2001). Treatment with the water channel blocker mercuric chloride ($HgCl_2$) did not significantly affect Lp_{OPR}. It is astonishing that differences in Lp_{OPR} between younger (20-50 mm from the tip) and older (50-100mm) sections were rather small. In younger sections, suberin lamellae were not mature, but Casparian bands were already fully developed. Detailed chemical analysis of the changes and composition of apoplastic barriers with respect to water transport (suberin lamellae and Casparian bands) as done for corn roots in relation to hydraulic properties of roots (Zimmermann et al. 2000) is needed for rice. Recent experiments, however, in which the apoplastic path was blocked off, indicate that this passage rather than the cell-to-cell component is the dominant path for water flow in the OPR of rice (data not shown).

The hydraulic resistance of the OPR was smaller by a factor of 30 than the overall values of root Lp_r as measured by Miyamoto et al. 2001 and verified in this paper. This may indicate that water uptake may be limited at the endodermis/stele rather than at the OPR. However, caution is needed since this conclusion assumes that water flow across the OPR is hydraulic and not diffusive in nature (see below). Depending on the resistances, tensions (gradients in water potential created by transpiration) in root vessels, which drive water flow, would drop across the endodermis/stele (Miyamoto et al. 2001). However, the additional resistances across the aerenchyma and OPR may also play a role.

The resistance to water vapor in the aerenchyma is diffusional in nature. It can be calculated using the theory developed by Nobel and Cui (1992) for air gaps between roots and dry soil. The theory assumes a steady state flow of water vapor across the gaps according to Fick's first law. This flow is driven by a difference in water vapor concentration, which can be recalculated into a difference in water potential to give hydraulic conductivities in the usual units of m s^{-1} MPa^{-1} (as used here) rather than permeabilities in units of m s^{-1}. For a typical rice root, the thickness of the aerenchyma would be 225 μm, the radius of the stele 190 μm, and the thickness of the OPR 85 μm (overall root diameter:1 mm). Considering the cylindrical geometry of the system, this

results in a hydraulic conductivity of aerenchyma of Lp_{AER} = 1.5 × 10^{-8} m s^{-1} MPa^{-1}. By a factor of 2.7, this value is smaller than that of entire roots (about 4 × 10^{-8} m s^{-1} MPa^{-1}; Table 1) and could be limiting. However, Lp_{AER} represents a lower limit of the resistance of the entire aerenchyma, because there should be a flow of liquid water bypassing air-filled lacunae along the spokes in the cortex of rice roots. Spokes consist of monolayers of cortical cells that were not removed during the formation of lacunae. Most of the cells in spokes appear to be alive. As in a wick, liquid water should be dragged along the spokes from the OPR to the stele together with nutrient ions. At the OPR, the fractional area occupied by spokes was between 15 and 25% (length: 225 μm). There are no data available for the hydraulic conductivity of spokes, but we could either use the same value as that of the OPR (mean of 1.2·10^{-6} × 85·10^{-6} = 1 × 10^{-10} m^2 s^{-1} MPa^{-1}) or a typical value of the hydraulic conductivity of cell wall material (Steudle 1989). Both approaches result in a hydraulic conductivity of 1 × 10^{-10} m^2 s^{-1} MPa^{-1}, which is referred to both unit cross sectional area and unit length. Using a hydraulic conductivity of spokes of 1 × 10^{-10} m^2 s^{-1} MPa^{-1}, we get Lp_{SPK} = 7.4 × 10^{-8} m s^{-1} MPa^{-1}. By a factor of 5, this is larger than Lp_{AER}. The overall Lp value for the aerenchyma (spokes and gas transport = Lp_{AER} + Lp_{SPK}) will be 8.9 × 10^{-8} m s^{-1} MPa^{-1}. This is larger by a factor of only two than the value given for the overall transport (4 × 10^{-8} m s^{-1} MPa^{-1}). However, the measured value of Lp_{OPR} was larger than the overall value for the aerenchyma by a factor of 15. It should hold that the overall hydraulic resistivity ($1/Lp_r$) is given by the sum of the three resistivities arranged in series, i.e. $1/Lp_r = 1/Lp_{END} + 1/(Lp_{SPK} + Lp_{AER}) + 1/Lp_{OPR}$. Using the values given above, this yields an Lp_{END} = 7.2 × 10^{-8} m s^{-1} MPa^{-1}, which is similar to that of the overall value of the aerenchyma. So, the result is that the overall hydraulic resistance of the rice root may be similarly referred to that of the endodermis/stele and aerenchyma (spokes). By far, the smallest of the three resistors in series is at the periphery (OPR).

The calculation suggests that the spokes play an important role in the overall hydraulic conductivity and in the water supply of the plant, as they definitely do in the supply with nutrient ions. They bridge or short-circuit the high resistance to water of the aerenchyma. However, some caution is necessary because the absolute value of the hydraulic conductivity of spokes is not known for sure. The values used to calculate water transport along spokes may be a lower limit because of some film transport along

the spokes outside the walls. Hence, the contribution of this component to the overall resistance or resistivity could be smaller than calculated, although not negligible. If so, calculations of the previous paragraph would be in agreement with the result that the Lp_r measured by steady-state technique (pressure chamber) were similar to those measured by root pressure probe. The former should have included all layers in series, whereas the latter tended to involve just the endodermis/stele (Miyamoto et al. 2001).

In the calculations of Lp_{AER}, we assumed that flow in the vapour phase was diffusional in nature, which is reasonable. In the liquid phase, we assumed a hydraulic mechanism, which should be also reasonable. Across the OPR, differences in water potential may be small, which may be compensated for by a high Lp_{OPR}. In order to compare the Lp_{OPR} of 1.2×10^{-6} m s^{-1} MPa^{-1} with water permeabilities in units of m s^{-1} (such as diffusional water permeabilities of plant cell membranes), we may give it as an 'osmotic permeability coefficient', P_f ($P_f = Lp \cdot RT/V_w$; V_w = molar volume of liquid water). Re-calculation of Lp_{OPR} yields a $P_{fOPR} = 1.6 \times 10^{-4}$ m s^{-1}. This figure is larger than that of P_f of cell membranes (Maurel 1997; Hertel and Steudle 1997) or tissues (House 1974) by one to two orders of magnitude. It is much larger than diffusional water permeabilities (P_d) of cells and tissues as given in the same sources. Ratios of $P_f/P_d > 1$ have been interpreted as indicative of a transport across pores such as in the apoplast of tissues. Provisional data on the P_{dOPR} of rice roots obtained from isotopic water flow [isobaric perfusion with heavy water (HDO)] gave values of P_{dOPR} that were smaller by three orders of magnitude [$P_d \approx 2.5 \times 10^{-7}$ and 5.7×10^{-8} m s^{-1} for immature (20-50 mm from tip) and mature (50-100 mm from tip) root zones, respectively; Ranathunge, Kotula, Steudle in preparation]. The huge difference between P_{fOPR} and P_{dOPR} is a strong indication of a water flow dominated by a porous rather than by a membrane-bound passage in the OPR. This means that either the Casparian bands in the exodermis are fairly permeable to water (Hose et al. 2001), or that this structure develops in a somewhat patchy way, i.e. with arrays lacking Casparian bands and suberin lamellae. However, there is, to date, no anatomical evidence for the latter.

The present values of P_{dOPR} for rice roots were similar to those of the OPR of *Carex arenaria* measured with tritiated water by Robards et al. (1979; $P_{dTHO} = 2 \times 10^{-8}$ m s^{-1}). Both, rice and *Carex* had values smaller than those of aerenchymatous corn roots grown

at low oxygen or of sleeves prepared from basal young corn roots immersed in culture solution (P_{dTHO} = 10^{-7} to 10^{-6} m s^{-1} depending on the position; Clarkson et al. 1987). When exposed to moist air, P_{dTHO} was much smaller (≈ 2 × 10^{-8} m s^{-1}). Under these conditions, roots developed several layers of sclerenchyma underlying the hypodermis. It is known that exposure of corn roots to moist air induces an exodermis as well (hypodermis with Casparian bands; Zimmermann et al. 2000). The early results of Robards et al. (1979) and of Clarkson et al. (1987) indicate that *Carex arenaria* expressing an aerenchyma constitutively (as rice), has a somewhat lower diffusional permeability to water and solutes than aerenchymatous corn roots or sleeves prepared from corn.

Rice roots often grow in anaerobic soils. Oxygen diffuses from shoots to root tips within the aerenchyma, and radial losses of oxygen from roots to the anaerobic root medium are relatively small. Hence, the OPR should have relatively low permeability for oxygen than water. On the other hand, there should be a sufficient hydraulic permeability to take up water. Obviously the problem is solved in rice in that water uptake is hydraulic in nature and oxygen losses are diffusive. Data describing flows of oxygen across roots of intact rice plants are available (Colmer et al. 1998). Recently, it was found that some rice cultivars allow to diffuse oxygen from aerenchyma to the outer surface of roots in order to keep the rhizosphere aerobic under flooded conditions. This is important for the population of ammonia-oxidizing bacteria that convert ammonium forms to nitrate, which is highly available for rice plants (Briones et al. 2002). However, there are no data yet available on the permeability coefficient of the OPR for oxygen and how this would change with growing conditions (e.g. oxic *vs.* anoxic/hypoxic conditions) in parallel with the permeability to water (Lp_{OPR} and P_{dOPR}). Quantitative values are required to understand how Lp_{OPR} and P_{dOPR} would change under different conditions. Provisional data show that the overall root Lp_r did not change much when roots were grown in anoxic conditions (data not shown). However, there are anatomical responses caused by changing conditions which may alter permeabilities, namely for oxygen (data not shown).

The picture of a dominating apoplastic rather than cell-to-cell transport of water within the OPR is in line with the values of reflection coefficients found in this paper for a

non-electrolyte (mannitol) and an electrolyte (NaCl). They were as low as 0.1 or even lower. For both solutes, plant cell membranes would exhibit a $\sigma_s \approx 1$. Measured values of 0.14 and 0.09, respectively, indicate an apoplastic component with a $\sigma_s \approx 0$. According to the composite transport model, this is evident because the parallel pathways should contribute to the overall value according to their hydraulic conductances. Reflection coefficients of the OPR were somewhat smaller than those of the entire roots, which also comprise the endodermis and stele. This is also understandable in terms of composite transport. There is evidence that there is a significant apoplastic bypass for solutes such as NaCl and PTS in rice roots (Yeo et al. 1987; Miyamoto et al. 2001).

In conclusion, the transport data presented indicate that the hydraulic conductivity of the OPR of rice roots is larger by a factor of 30 than that of the endodermis/stele. As long as flow across the OPR is hydraulic in nature, this means that the OPR would not rate-limit water uptake. The fact that the diffusional water permeability (P_{dOPR}) was smaller by a factor of 700 (20-50 mm from tip) and 1,300 (50-100 mm from tip) than the hydraulic conductivity (Lp_{OPR}/P_{fOPR}) points to a dominating apoplastic water flow in the OPR, as do the low reflection coefficients. However, under conditions where water flow is diffusional in nature, the OPR may limit water uptake. It appears that, in rice, water uptake and oxygen retention are optimized in a way that hydraulic water flow can be kept high in the presence of a low efflux of oxygen which is diffusional in nature. The structural basis for this is not yet known. Namely, it is not known how high rates of apoplastic transport can be brought about in the presence of well-developed Casparian bands in the exodermis. To further work out limitations to water and oxygen flow in rice roots, present work in the lab aims at combined measures of the water (P_{dOPR} and Lp_{OPR}, P_{fOPR}) and oxygen permeability [measurement of radial oxygen loss (ROL) and of forces driving O_2 movement within the OPR] along developing roots that have been grown under hypoxic and oxic conditions which affect ROL and root anatomy. This work, using cell pressure probes, should also provide measures of water permeability (hydraulic conductivity) of cells and of the role of water channels (aquaporins) in the OPR.

Acknowledgements

The authors thank Prof. D.T. Clarkson (University of Bristol, UK), Dr. T.D. Colmer (University of Western Australia, Perth), and Prof. C.A. Peterson (University of Waterloo, Ontario, Canada) for carefully reading and discussing the manuscript. The excellent technical support of Burkhard Stumpf (Lehrstuhl Pflanzenökologie, University of Bayreuth) is gratefully acknowledged. This work was supported by a grant from the Deutsche Forschungsgemeinschaft, Schwerpunktprogramm ‘Apoplast‘ to E.S., and by BMZ project No. 2000.7860.0-001.00 “Trait and Gene Discovery to Stabilize Rice Yields in Drought Prone Environments” (R.L.).

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3 Water permeability and reflection coefficient of the outer part of young rice roots are differently affected by closure of water channels (aquaporins) or blockage of apoplastic pores

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Received: 6 June 2003; Accepted: 17 October 2003

Journal of Experimental Botany (2004) 55: 433-447
DOI 10.1093/jxb/erh041

Abstract

The relative contribution of the apoplastic and cell-to-cell paths to the overall hydraulic conductivity of the outer part of rice roots (Lp_{OPR}) was estimated using a pressure perfusion technique for 30-day-old rice plants (lowland cultivar: IR64 and upland cultivar: Azucena). The technique was based on the perfusion of aerenchyma of root segments from two different zones (20-50 and 50-100 mm from the root apex) with aerated nutrient solution using precise pump rates. The outer part of roots (OPR) comprised outermost rhizodermis, exodermis, sclerenchyma fibre cells and the innermost unmodified cortical cell layer. No root anatomical differences were observed for the two cultivars used. Development of apoplastic barriers such as Casparian bands and suberin lamellae in the exodermis were highly variable. On average, mature apoplastic barriers were observed at around 50-70 mm from the root apex. Lignification of the exodermis was completed earlier than that of sclerenchyma cells. Radial water flow across the OPR was impeded either by partially blocking off the porous apoplast with China ink particles (diameter: 50 nm) or by closing water channels (aquaporins) in cell membranes with 50 µM $HgCl_2$. Reduction of Lp_{OPR} was relatively larger in the presence of an apoplastic blockage with ink (≈30%) than in the presence of the water channel blocker (≈10%) suggesting a relatively larger apoplastic water flow. The reflection coefficient of the OPR (σ_{sOPR}) for mannitol significantly increased during both treatments. It was larger when pores of the apoplast were closed, but absolute values were low (overall range of σ_{sOPR} = 0.1 to 0.4), which also suggested a large contribution of the non-selective, apoplastic path to overall water flow. The strongest evidence in favour of a predominantly apoplastic water transport came from the comparison between diffusional (P_{dOPR}, measured with heavy water, HDO) and osmotic water permeability (P_{fOPR}) or hydraulic conductivity (Lp_{OPR}). P_{fOPR} was larger by a factor of 600-1400 compared with P_{dOPR}. The development of OPR along roots resulted in a decrease of P_{dOPR} by a factor of three (segments taken at 20-50 and 50-100 mm from root apex, respectively). Heat-killing of living cells resulted in an increase of P_{dOPR} for both immature (20-50 mm) and mature (50-100 mm) root segments by a factor of two. Even though both pathways (apoplast and cell-to-cell path) contributed to the overall water flow, the findings indicate predominantly apoplastic water flow across the OPR, even in the presence of apoplastic barriers. Low diffusional water permeabilities

may suggest a low rate of oxygen diffusion across the OPR from aerenchyma to the outer anaerobic soil medium (low P_{O2OPR}). Provisional data of radial oxygen losses (ROL) across the OPR suggest that, unlike water, rice roots efficiently retain oxygen within the aerenchyma. This ability strongly increases as roots/OPR develop.

Key words: aerenchyma, apoplastic transport, bulk flow, diffusional water permeabilty, exodermis, hydraulic conductivity, rice root, water channels.

Introduction

Water transport within plants can be divided into several discrete steps, one of which is the radial water flow from soil solution to root xylem vessels. It is known that radial water flow across roots is highly variable. This is due to a considerable variability in root hydraulic conductivity, which differs between plant species and in response to environmental conditions (Brouwer, 1954; Kramer and Boyer, 1995; Steudle, 2000a, 2000b, 2001; Weatherley, 1982). Growth conditions strongly influence the architecture of roots including their anatomy and morphology (Steudle, 2000a; Steudle and Peterson, 1998). Physical and physiological processes can regulate water uptake by roots as well (Steudle, 2000b; Steudle, 2001). Over the long term (days, weeks), the capacity to take up water can be related to root growth or structural, morphological, and anatomical changes of roots (i.e. development of apoplastic barriers). In the short term (<day), root hydraulics are adjusted or are even regulated by physical properties, such as switching between cell-to-cell and apoplastic pathways. Cell-to-cell water flow is regulated by gating of water channels (aquaporins) in the plasma membrane (Henzler and Steudle, 1995; Javot and Maurel, 2002; Steudle, 2001; Tyerman *et al*., 1999). The well-established composite transport model has been used to explain the variable permeability of roots to water (Steudle, 2000a, 2000b; 2001; Steudle and Frensch, 1996; Steudle and Peterson, 1998). The switching of water pathways may depend on both the driving forces and the water permeability of components of the pathway. This may allow some flexibility in the response of plants to water shortage according to the demand from the shoot.

Even for rice crops in paddy fields, water shortage may occur during the day with the appearance of wilting symptoms (Hirasawa *et al*., 1992, 1996). The hydraulic

conductivity of rice roots is somewhat lower than other crop species because of a lack of flexibility in adjusting to demand from the shoot (Miyamoto *et al*., 2001; Ranathunge *et al*., 2003). Apoplastic barriers in rice roots such as well developed exo- and endodermis with Casparian bands and lignified sclerenchyma cells may restrict the water movement through cell walls (Clark and Harris, 1981; Miyamoto *et al*., 2001; Ranathunge *et al*., 2003). The aerenchyma may represent an additional barrier. Comparison of detailed measurements of the overall hydraulic conductivity of rice roots (Lp_r) with that of the outer part of roots (Lp_{OPR}) showed that the contribution of the endodermis/stele to the hydraulic resistance of rice roots was largest, followed by that of the aerenchyma (Ranathunge *et al*., 2003). Despite presence of the exodermis and sclerenchyma cells, the outer part of roots (OPR) had a hydraulic conductivity that was larger by factor of 30 than that of whole roots or root systems (Ranathunge *et al*., 2003). Hence, water flow across the OPR must have either a strong apoplastic component or a prominent membrane bound passage of water or both in parallel. Low reflection coefficients of the OPR (σ_{sOPR}) to non-permeating solutes like mannitol pointed to a dominant apoplastic flow and a fairly porous apoplastic path rather than to a large transmembrane component of water flow across the OPR. These observations are in line with earlier findings of a substantial apoplastic transport of NaCl and the apoplastic dye trisodium, 3-hydroxy-5, 8,10-pyrene trisulfonate (PTS) across the entire root cylinder of rice. (Yeo *et al*., 1987; Yadav *et al*., 1996).

In this study, we extend the previous work on the hydraulic and osmotic properties of the OPR of rice roots has been extended in order to get an estimation of the contribution of pathways (apoplastic *versus* cell-to-cell components). Two different cultivars, a lowland (IR64) and an upland (Azucena) have been used again which may differ in their ability to take up water (root Lp_r). Even though both pathways in roots contribute to the overall high water permeability across the OPR, it was found that the contribution of the apoplast was larger than that of the transmembrane passage of water flow favouring the presence of significant apoplastic bypasses. This was also suggested from experiments in which either the apoplastic passage was partially blocked using a suspension of China ink particles of an average diameter of 50 nm, or the membrane permeability was reduced using the water channel blocker $HgCl_2$. Treatments created a significant reduction in Lp_{OPR}, but the reduction caused by blockage of the apoplast by

ink particles was larger by a factor of 3 than that following $HgCl_2$ treatment. Blockage caused a much bigger increase in the reflection coefficient of the OPR, when the apoplast was blocked. As expected in the presence of a fairly porous apoplastic bypass, there were huge differences between osmotic (bulk) and diffusional water permeabilities.

Materials and methods

Plant materials and growth conditions

Rice seedlings [*Oryza sativa* L. cv. Azucena (upland) and IR64 (lowland) from the International Rice Research Institute, Manila, Philippines] were grown from seeds in climatic chambers using aerated hydroponics as detailed previously (Ranathunge *et al*., 2003). Plants used in experiments were grown for 31-40 d including the time for germination.

Plant morphology and root anatomy

Height of young rice plants used in experiments was 330-350 mm and 480-515 mm (eighth to eleventh leaf emergence) for IR64 and Azucena, respectively. Root lengths were typically 290-360 mm (IR64) and 500-550 mm (Azucena). Freehand cross-sections were taken at distances of 20, 50 and 100 mm from the root apex and stained with Sudan Red 7B at room temperature for 1.5 h (Brundrett *et al*., 1991) to observe development of aerenchyma along the roots. Sections were viewed using an optical microscope (DIALUX 22 EB, Leitz, Germany) and photographed using Kodak Elite 64 ASA film. To confirm the presence of suberin lamellae in the exodermis, freehand cross-sections were stained for 1 h with Fluorol Yellow 088 (Brundrett *et al*., 1991) and viewed under an epifluorescence microscope using an ultraviolet filter set (excitation filter BP 365, dichroitic mirror FT 395, barrier filter LP 397; Zeiss, Oberkochen, Germany). To detect lignin in cell walls of the OPR, freehand cross-sections were stained for several minutes with phloroglucinol/hydrochloride at room temperature (Jensen, 1962). Lignin stained as a bright red layer within the cell walls.

Measurement of hydraulic conductivity of the outer part of roots (OPR) by pressure- perfusion

Root segments were excised 20-50 mm or 50-100 mm from the root apex. Aerenchyma was not fully developed at 20-50 mm from the apex, but it was at 50-100 mm. Both ends of each segment were fixed to glass capillaries with an inner diameter of 1.3 mm (Fig. 1A).

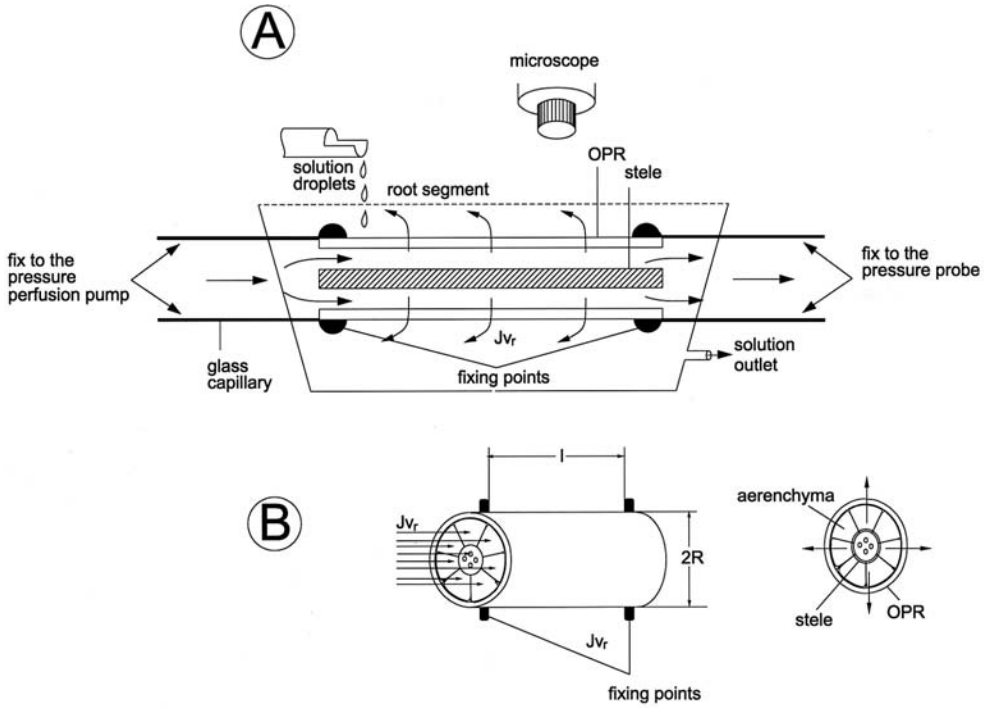


Fig. 1 (A) Pump perfusion setup: a syringe was mounted on a Braun-Melsungen pump that created pump rates between 1.7×10^{-9} and 1.1×10^{-7} mm^3 s^{-1}. One end of the root segment was used as an inlet. This was fixed to the syringe by a narrow and rigid Teflon tube. The other end was connected to a pressure probe to measure resulting steady state pressures. (B) Schematic diagram to show radial water flow across the outer part of the root segment during pressure perfusion. At a given pump rate, stationary pressure was established where the volume flow provided by the pump equalled the radial volume flow across the outer part of the root (OPR).

One of the glass capillaries (inlet side) was connected to a syringe while the other (outlet side) was connected to a pressure probe by a narrow, rigid Teflon tube (inner diameter: 1.5 mm). The syringe was mounted on a 12-step Braun-Melsungen pump that produced defined pumping rates (water flows; Qv) between 1.7 x 10^{-9} and 1.1 x 10^{-7} $mm^3 \cdot s^{-1}$. Perfusion was commenced from the inlet side of root segment. Aerated nutrient solution was perfused through the aerenchyma, displacing air. At a given pump rate,

nutrient solution was pumped into the root segment and the pressure increased gradually until a stationary pressure was established where the volume flow produced by the pump equalled the radial volume flow across the OPR (Fig. 1B). The resulting steady-state pressures were measured using a pressure probe as a manometer. Stationary pressures were measured with respect to the flow rates. The hydraulic conductivity of the outer part of the root (Lp_{OPR} in m s^{-1} MPa^{-1}) was calculated according to Eq. 1:

$$Qv = Lp_{OPR} \times P \times A_r \,. \qquad (1)$$

Here, Q_V is the pump rate in $m^3 \cdot s^{-1}$, P is the steady-state pressure in MPa (reference: atmospheric pressure), and A_r is the surface area of the root segment (m^2). To avoid anaerobic conditions, root segments were placed in a small chamber and air-saturated nutrient solution was continuously circulated around the roots. A poly-acrylamide glue (UHU, Bühl, Germany) was used to fix root segments to glass capillaries, which allowed successful connection of tissues to glass, even when wet. To make the seal mechanically rigid, the glue was superimposed with a molten mixture of beeswax/collophony (1:3; w/w Zimmermann and Steudle, 1975). The tightness of the seal was tested at the end of the experiments by perfusion with nutrient solution which contained the apoplastic fluorescence dye 0.02% PTS (trisodium, 3-hydroxy-5,8,10-pyrene trisulfonate) as described previously (Ranathunge *et al*., 2003). In cases where root segments were not sealed properly to glass capillaries, the pressure-perfusion data were discarded. Given that the pressure-perfusion experiments lasted for 10-15 h, root segments were randomly selected for treatment with Evan's Blue stain in order to test for the viability of the OPR cells (Fisher *et al*., 1985).

Osmotic experiments with perfused root segments

Reflection coefficients of the OPR (σ_{sOPR}) were estimated by measuring changes in steady–state pressures caused by adding 14 mOsmol/kg mannitol (equivalent to 0.035 MPa of osmotic pressure) to the external medium, which did not change the hydraulic conductivity of the OPR (Lp_{OPR}). Hence, comparing the osmotic pressure applied with the change in hydraulic pressure gives the reflection coefficient ($\sigma_{sOPR} \equiv -Lp_{DOPR}/Lp_{OPR}$; Lp_{DOPR} = osmotic coefficient of the OPR). Mannitol was used as the test osmoticum

since it has a σ of unity for plant cell membranes. The nutrient solution in the external medium was replaced with mannitol solution and the decline in steady-state pressures was observed. Maximum drops in pressures were used to calculate σ_{sOPR}. Original steady-state pressures were obtained following the removal of mannitol from the external medium.

Blockage of cell-to-cell path of the OPR with water channel blocker $HgCl_2$

50 μM $HgCl_2$ was used as the blocking agent for cells of the OPR. After estimating the original steady-state pressures and reflection coefficients of the OPR (control), $HgCl_2$ was added to the external medium of the root segments for 30 min, during which the direction of pumping was reversed (pump rate of 1.7×10^{-12} $m^3 \cdot s^{-1}$). This established a slightly negative pressure gradient across the OPR (- 0.02 MPa relative to atmospheric pressure) drawing $HgCl_2$ deeper into the tissue. Mercuric chloride could be also added to the perfusion medium. However, it was then difficult to get rid of it after the treatment. Excess $HgCl_2$ was flushed from the system before new steady-state pressures and reflection coefficients were measured using the same flow rates as during the control. Following measurements, Hg^{2+} bound to the cell membranes of the OPR was scavenged by 4 mM 2-mercaptoethanol (Carvajal *et al*., 1996; Henzler and Steudle, 1995).

Blockage of porous apoplast by China ink particles

China ink particles (Rotring-Werke Riepe KG, Hamburg, Germany) were used to block apoplastic pores in the OPR in order to investigate the contribution of the apoplastic path to overall water movement across the OPR. Prior to use, commercial China ink was diluted 1:1 with nutrient solution and cleared of small molecular weight compounds by dialysis against the nutrient solution. The osmotic concentration of the purified ink suspension was the same as that of nutrient solution. The diameter of ink particles was 51 ± 22 nm as measured using a Particle Sizing System (PSS Nicomp, Santa Barbara, California, USA) by courtesy of Professor S.D. Tyerman, University of Adelaide, Australia. Root segments (20-50 mm or 50-100 mm from the root apex), previously used to measure the original steady-state pressures and reflection coefficients, were

perfused with the diluted, purified China ink suspension for at least 1 h at relatively high flow rates directed from the inside to the outside, as described above, to block off pores in the apoplast (0.1 MPa pressure difference between aerenchyma and medium). Following ink treatment, root segments were perfused with nutrient solution to sweep away excessive ink particles trapped inside the aerenchyma. As for the control, the resulting new steady-state pressures and reflection coefficients of the OPR were measured. The diluted, purified ink suspension was not toxic to cells of the OPR.

Diffusional water permeability across the OPR with heavy water (HDO)

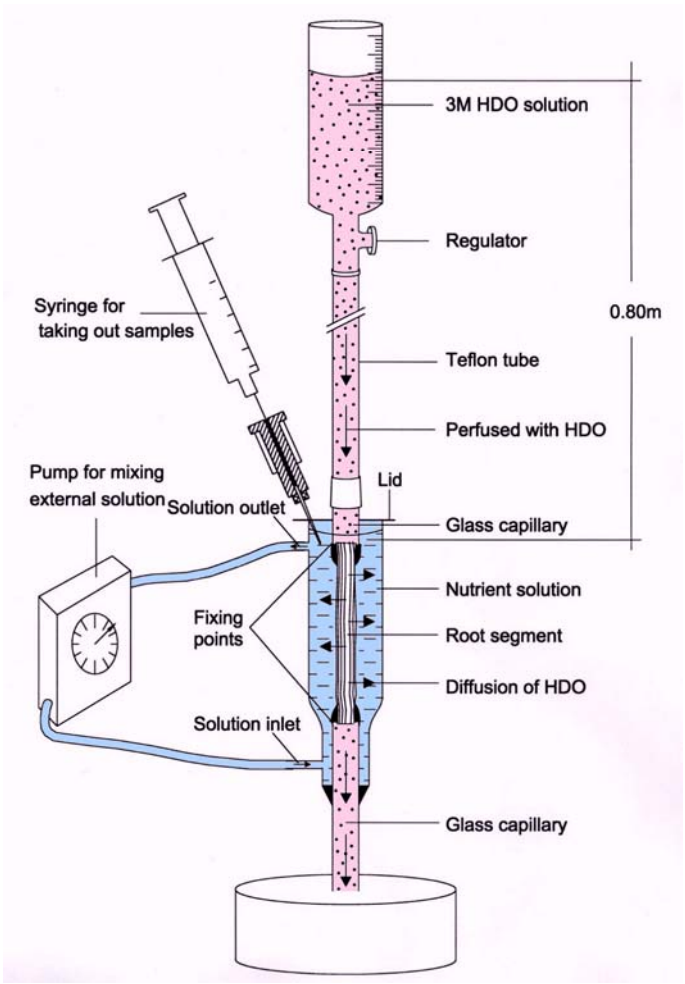


Fig. 2 Experimental set-up to measure the diffusional water permeability of the outer part of rice roots. Open ends of root segments were fixed to glass capillaries. Aerenchyma within segments was rapidly perfused with 3M heavy water (HDO). At different time intervals, concentration of HDO diffusing into the outer medium was measured with a freezing point osmometer. The external solution was stirred vigorously throughout the experiment, using a small pump.

Steady-state perfusion using heavy water (HDO) was performed with root segments excised at either 20-50 or 50-100 mm from the root apex.

A 3 M solution of HDO was perfused through the aerenchyma of root segments, displacing air with solution. The root segment was held vertically to allow perfusion of the solution by gravity (Fig 2). The upper, open end of the segment (used as an inlet) was connected to a syringe by a Teflon tube filled with 3 M HDO. The other end of the root segment remained open as an outlet. As shown in Fig. 2, the syringe was placed 0.8 m above the root segment providing a gravitational force of 0.008 MPa, significantly less than in the previous pump perfusion experiment (0.04-0.05 MPa; see above). Hence, water movement was near-isobaric (diffusive) to a good approximation and governed by the lateral diffusion of HDO across the OPR. Root segments were bathed in aerated nutrient solution of known volume (5 ml). At different time intervals, 50 µl of the outer medium was taken out by a syringe and the HDO concentration of each sample was measured by a freezing point osmometer. The successive reduction of volume of the outer medium was accounted for. Since HDO and H_2O form mixed crystals, the freezing point of samples containing HDO increased above that of distilled water in proportion to concentration, as verified by a calibration curve (freezing points of distilled water: 0°C; pure D_2O: +4°C). Measurements of diffusional water flow did not require that root segments to be so tightly connected to the glass capillaries (inner diameter 1.3 mm) as during steady-state perfusion. Poly-acrylamide glue was sufficient to connect wet tissue to glass. A small pump was employed to mix external solution in order to equalize the distribution of HDO in the external medium and to minimize the thickness of unstirred layers (Fig. 2).

In typical diffusional experiments, heavy water was perfused through the aerenchyma and diffused across the OPR to the external medium. The amount of the solute HDO that diffused to the outer medium was plotted against time. Solute flow across the OPR (J_{sOPR} in moles s^{-1} m^{-2}) was obtained directly from the slope of this curve divided by the surface area of the root segment. Since the external (diffused to outer medium) and internal (perfused through aerenchyma) HDO concentrations were known, the driving force or concentration difference between the inner and outer compartments (ΔCs in

moles s^{-1}) could be evaluated. The diffusional water permeability of the OPR (P_{dOPR} in m s^{-1}) was obtained according to Eq. 2:

$$P_{dOPR} = \frac{J_{sOPR}}{\Delta C_s}. \quad (2)$$

The external concentration was usually much smaller than the internal, i.e. the back flow of HDO could be neglected. In order to compare the bulk/hydraulic water permeability (P_{fOPR}) across the OPR with diffusional water permeability (P_{dOPR}), the hydraulic conductivity of the OPR (Lp_{OPR}) was converted to P_{fOPR} (House, 1974):

$$P_{fOPR} = \frac{Lp_{OPR} \times RT}{\overline{V_w}}. \quad (3)$$

Here, V_w, was molar volume of liquid water. Since the units of the P_{fOPR} were m s^{-1}, the P_{fOPR}:P_{dOPR} ratio was calculated directly. Following the diffusional permeability experiments, root segments were taken from the chamber and exposed to steam for 20-30 s to kill part of the living cells of the OPR. Then the diffusion experiment was repeated with steam-treated root segments.

Results

Root anatomy

No visible anatomical or developmental differences were observed in the adventitious roots of the rice cultivars IR64 (lowland) or Azucena (upland). Therefore, Fig. 3 only refers to cross-sections of the lowland variety, IR64. At 20 mm from the root apex, cortical cells had partially collapsed to form large, gas-filled spaces, commonly called aerenchyma (Fig. 3A). At 100 mm from the apex, development of aerenchyma was complete (Fig. 3B). The initiation of cortical cell collapse was observed 5-6 cell layers from the innermost cortical layer. The OPR was separated from the stele by the large gas-filled spaces of aerenchyma. The OPR comprised rhizodermis, exodermis, sclerenchyma (fibre cells), and one unmodified cortical cell layer. The dense packing of sclerenchyma cells with thick cell walls indicated some physical strength of the OPR of rice roots. The cytoplasm of sclerenchyma cells was rarely observed closer to the root base, suggesting that they were definitely dead. Diamond-shaped air spaces were visible

between the exodermis and the rhizodermis of the OPR where they loosely connected to each other. The OPR was connected to the stele by 40 to 50 spokes, i.e. monolayers of cells, which separated the different voids of the aerenchyma.

Weakly developed suberin lamellae were observed in the exodermis at a distance of 20 mm from the root apex (Fig. 3C), however, lamellae were fully mature at 50 mm from the root apex (Fig. 3D). No suberin was detected in sclerenchyma cell walls, even in mature zones close to the root base. Sections taken at 50-100 mm from the apex showed weak deposits of lignin in the external tangential cell walls of sclerenchyma tissue (Fig. 3E). At a given distance from the root apex, more lignin was observed in the exodermis than in the sclerenchyma.

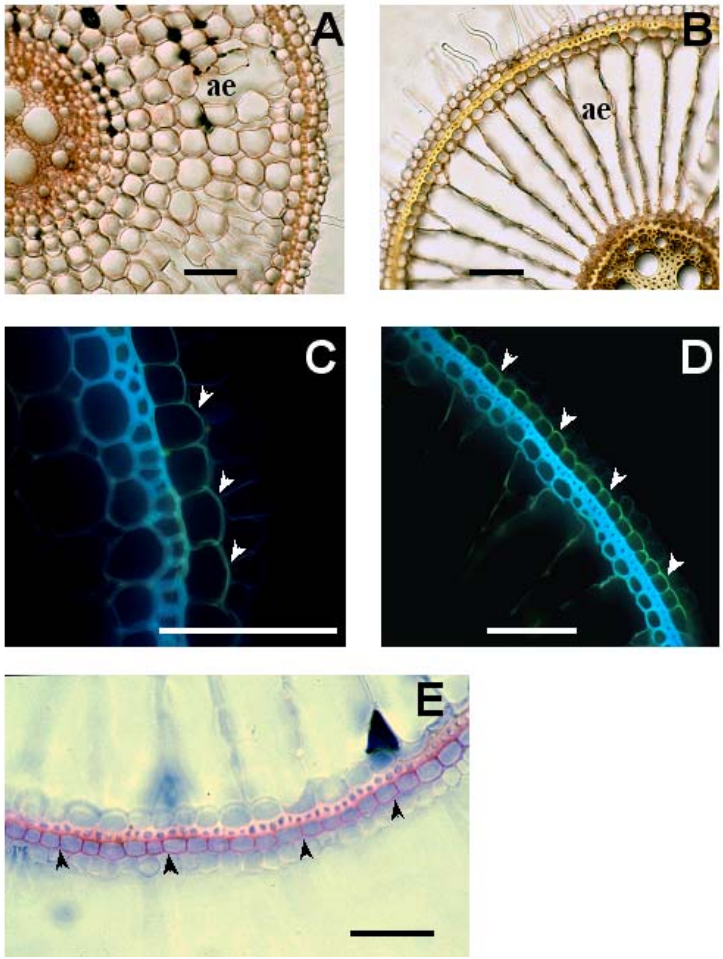


Fig. 3 (A-D) Cross-sections of roots of 30-d-old rice (*Oryza sativa*); cultivar IR64 plants. (A-B) Development of aerenchyma at 20 (A) and 100 mm (B) from the root apex. Freehand cross-sections were stained with Sudan Red 7B. (C-E) Freehand cross-sections taken at 20 (C), 50 (D), and 100 mm (E) from the root apex stained with Fluorol Yellow 088 (C, D) and phloroglucinol/hydrochloride (E), respectively. *Arrowheads* show suberin lamellae in the exodermis (C, D) and lignin in cell walls of sclerenchyma and exodermis (E), respectively. (ae = aerenchyma); bar = 100 µm.

Pressure-perfusion experiments with rice root segments

Two to three hours after fixing root segments to the pressure-perfusion pump, stable pressures were established. These long time intervals were caused by the large volume (as compared to the conducting area of root segments) of the system, including the syringe and tubing). Increasing pump rates linearly increased steady-state pressures. Stepwise increasing the pump rate (Q_V in $m^3\ s^{-1}$) and then decreasing it again resulted in the same pressure/flow curves and Lp_{OPR}. There was no hysteresis in the $Q_V(P)$ curves. Neither IR64 (lowland) nor Azucena (upland) showed significant differences in Lp_{OPR} measured over the first 100 mm from the root apex (Table 1). The OPR was quite permeable to bulk water. At 20-50 mm from the root apex, Lp_{OPR} values were $(0.98 \pm 0.30) \times 10^{-6}$ and $(0.99 \pm 0.20) \times 10^{-6}$ m s^{-1} MPa^{-1} for IR64 and Azucena, respectively (n = 9 root segments each). Similar values were found for root segments at 50-100 mm from the apex (Table 1).

Table 1 Hydraulic conductivity of the outer part of rice roots (Lp_{OPR}) treated with either 50 µM $HgCl_2$ or diluted China ink. Plants were grown for 31-40 d in aerated hydroponic culture. Measurements were performed using pump perfusion technique. Lp_{OPR} given for two different distances from the root apex and two cultivars, IR64 and Azucena. Values are means ± SD (n = 9 root segments). Using ratios of treatment / control, both treatments significantly reduced Lp_{OPR} compared with the control (double-sided, unpaired t-test, P = 0.05). While Lp_{OPR} of segments perfused with China ink was significantly lower compared to segments treated with 50 µM $HgCl_2$ treatment (double-sided, unpaired t-test, P = 0.05).

Rice cultivar	Hydraulic conductivity of the outer part of the root, Lp_{OPR} (10^{-6} m s^{-1} MPa^{-1})				Lp_{OPR} treatment / Lp_{OPR} control	
	20-50 mm		50-100 mm		20-50 mm	50-100 mm
	control	$HgCl_2$ treated	control	$HgCl_2$ treated		
IR64	0.98 ± 0.30	0.87 ± 0.27	0.81 ± 0.21	0.74 ± 0.18	0.90 ± 0.10a	0.92 ± 0.03a
Azucena	0.99 ± 0.20	0.92 ± 0.23	0.77 ± 0.20	0.70 ± 0.18	0.91 ± 0.08a	0.91 ± 0.04a
	control	ink treated	control	ink treated		
IR64	0.98 ± 0.50	0.75 ± 0.42	0.74 ± 0.33	0.44 ± 0.17	0.75 ± 0.09b	0.67 ± 0.13b
Azucena	0.90 ± 0.33	0.73 ± 0.28	0.80 ± 0.18	0.59 ± 0.19	0.81 ± 0.06b	0.73 ± 0.10b
IR64	1.50 ± 0.50		1.10 ± 0.30			
Azucena	1.30 ± 0.50		0.90 ± 0.40		Ranathunge et al. 2003	

Different letters indicate significant differences at P = 0.05 level

When all data were pooled and compared, the addition of 50 µM $HgCl_2$ to the external medium did not significantly affect Lp_{OPR} (t-test; P = 0.05) because of a large variation

between root segments. However, when the results were presented as ratios of treatment/control Lp_{OPR}, the addition of $HgCl_2$ reduced Lp_{OPR} significantly, by 10% in both cultivars and at both distances from the root apex (Table 1; *t*-test, $P = 0.05$).

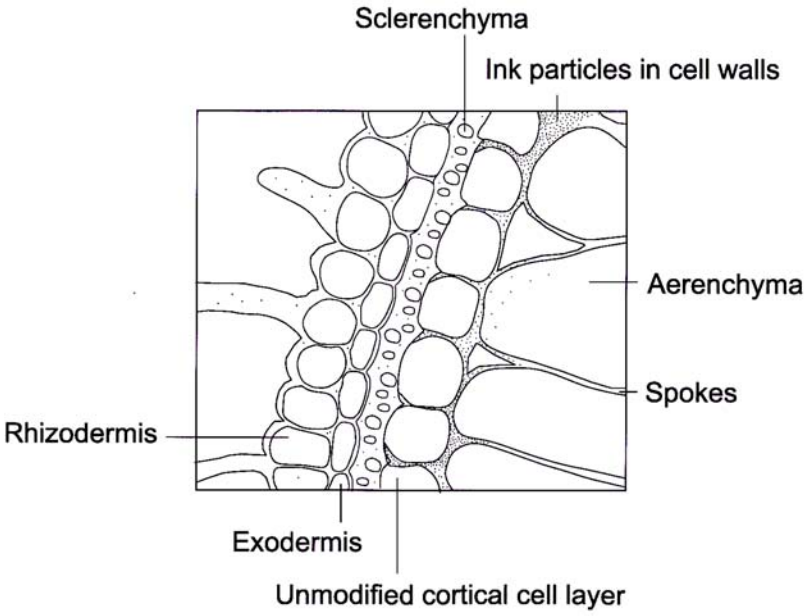


Fig. 4 Schematic diagram to show blockage of apoplastic (cell wall) pores in the outer part of rice roots (OPR) with China ink particles (particle diameter: 51 nm) after perfusing root segments with ink for 1 h.

Root segments that were perfused with China ink for 1 h, to block the porous apoplast of the OPR (Fig. 4), had visibly darker root surfaces than controls (Fig. 5). Treatment with China ink decreased radial water flow across the OPR by 25% at 20-50 mm from the root apex, giving rates of $(0.75 \pm 0.42) \times 10^{-6}$ and $(0.73 \pm 0.28) \times 10^{-6}$ m s^{-1} MPa^{-1} for IR64 and Azucena, respectively (Table 1; $n = 9$ root segments). The reduction was 30% at 50-100 mm from the apex, giving rates of $(0.44 \pm 0.28) \times 10^{-6}$ and $(0.59 \pm 0.19) \times 10^{-6}$ m s^{-1} MPa^{-1} ($n = 9$ root segments). Means of the treatment/control ratios of Lp_{OPR} of individual roots were significantly smaller than, unity (*t*-test; $P = 0.05$).

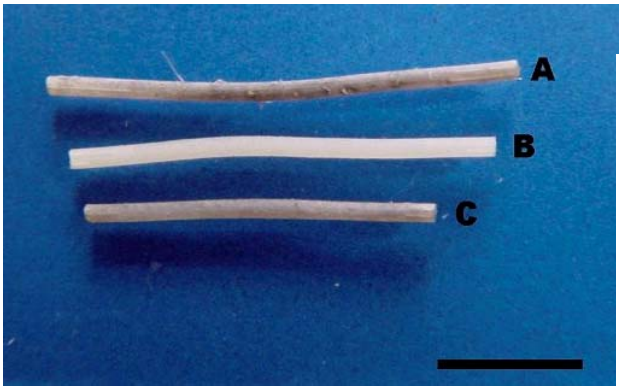


Fig. 5 Outer appearance of rice root segments (cv. IR64) after perfusion with either China ink (A,C) or nutrient solution (B) for 1 h. (A) 50-100 mm from the root apex, (B, C) 20-50 mm from the root apex. Bar = 10 mm.

Overall, the treatment/control ratios of Lp_{OPR} obtained from the China ink treatment were significantly smaller (the effect of China ink treatment was bigger) than the ratios obtained from $HgCl_2$ treatment. This was true for both rice cultivars at 20-50 mm and 50-100 mm from the root apex (*t*-test; $P = 0.05$; Fig. 6). It may suggest that more water passed through the apoplast rather than crossing the transmembrane passage.

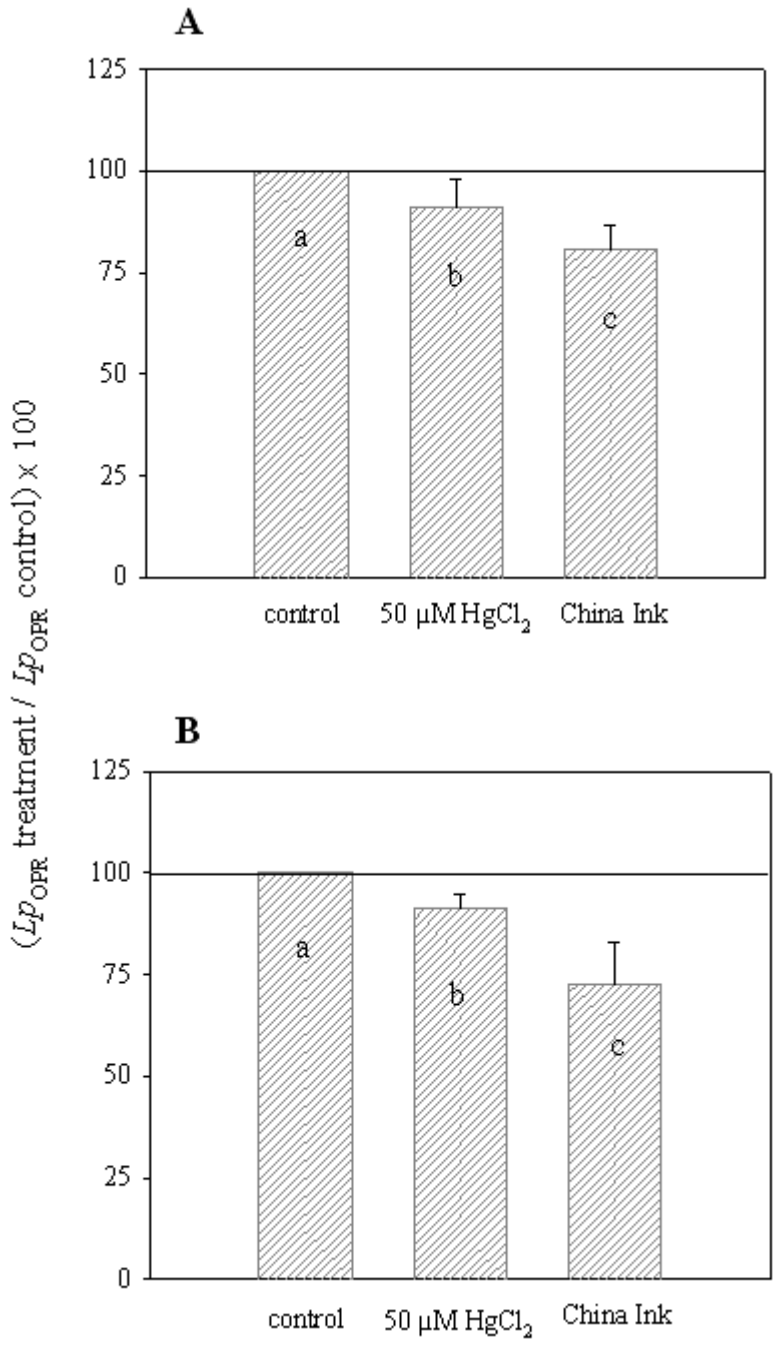


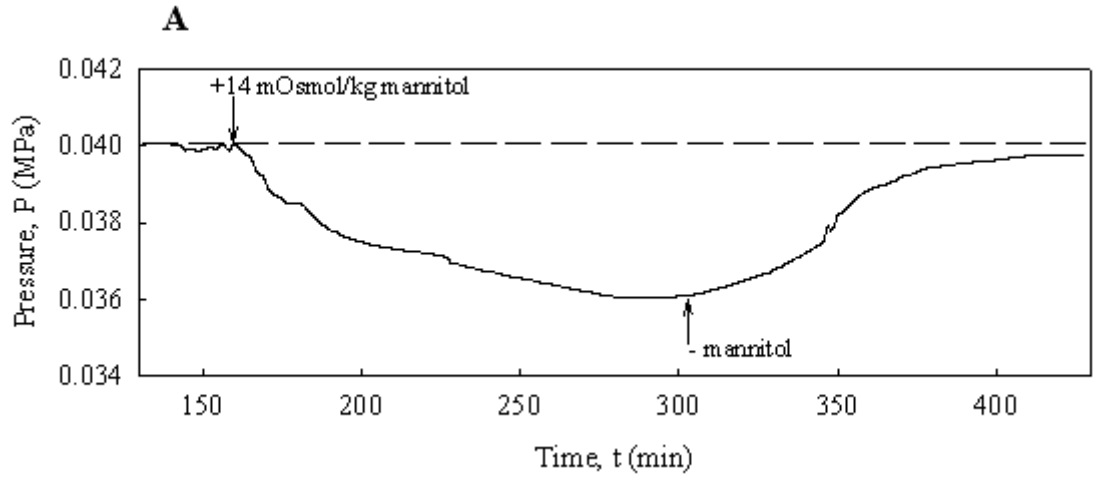
Fig. 6 Effect of treatments with 50 µM $HgCl_2$ and China ink on hydraulic conductivity of outer part of rice roots (Lp_{OPR}; cv. IR64). (A) 20–50 mm from the root apex; (B) 50-100 mm from the root apex, respectively. Treatment/control ratios were calculated to remove substantial variations between roots

(Table 1). According to the ratios, both treatments showed significant reductions of Lp_{OPR} (double-sided, unpaired *t*-test, $P = 0.05$). Reduction of Lp_{OPR} with China ink was significantly higher than that of 50 µM $HgCl_2$ treatment (double-sided, unpaired *t*-test, $P = 0.05$).

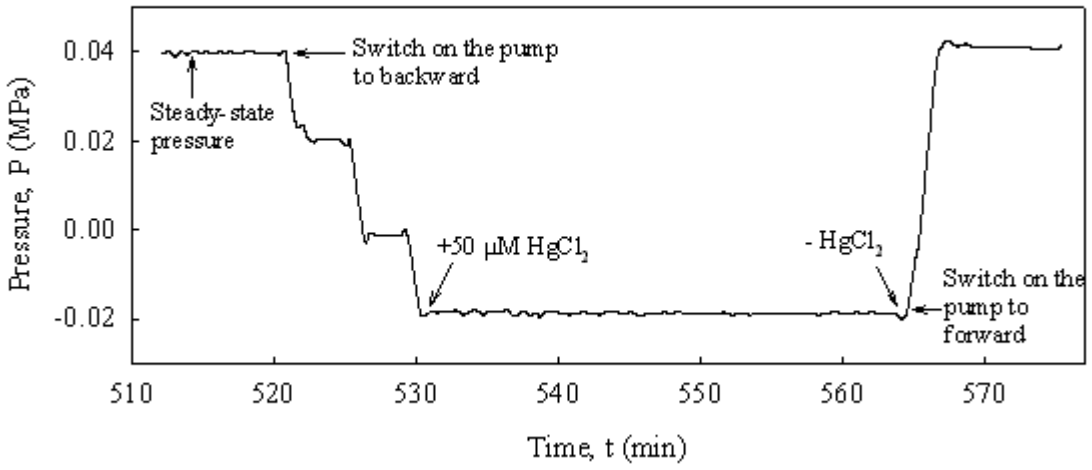
Osmotic experiments with the outer part of rice roots

Efflux of water from the root segments was induced by adding 14 mOsmol/kg mannitol. The resulting outward water flow caused a decline in stationary pressures of root segments at constant Lp_{OPR} (see materials and methods; Fig. 7A, D as controls). The original steady-state pressures were restored on removal of mannitol from the external medium.

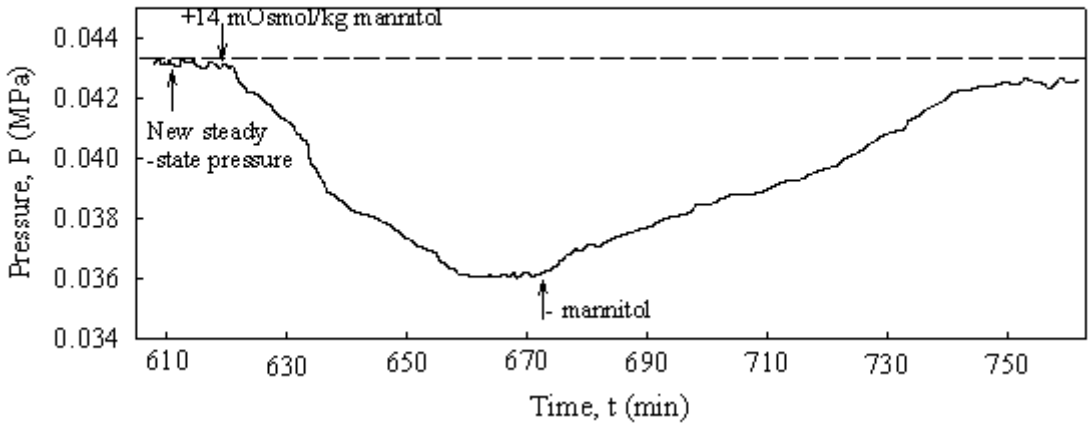
Before adding 50µM $HgCl_2$, steady-state pressures of root segments were reduced to sub-atmospheric levels (slightly negative ≈ -0.02 MPa with reference to atmospheric pressure) as shown in Fig. 7B. This resulted in drawing $HgCl_2$ deeper into the tissues. Higher steady-state pressures were obtained following the $HgCl_2$ treatment than the control at the same pump rate (Fig. 7C). Root segments treated with $HgCl_2$, showed larger drops in steady-state pressures upon the addition of mannitol than controls (Fig. 7A, C). In root segments 20-50 mm from the root apex, the $HgCl_2$ treatment increased the σ_{sOPR} from 0.12 ± 0.04 to 0.23 ± 0.05 in IR64 and 0.10 ± 0.02 to 0.20 ± 0.04 in Azucena (*t*-test; $P = 0.05$; $n = 6$ root segments). There was a similar increase in σ_{sOPR} for root segments 50-100 mm behind the root apex (Table 2). On average for both root segments, the addition of $HgCl_2$ increased the σ_{sOPR} by a factor of about two.



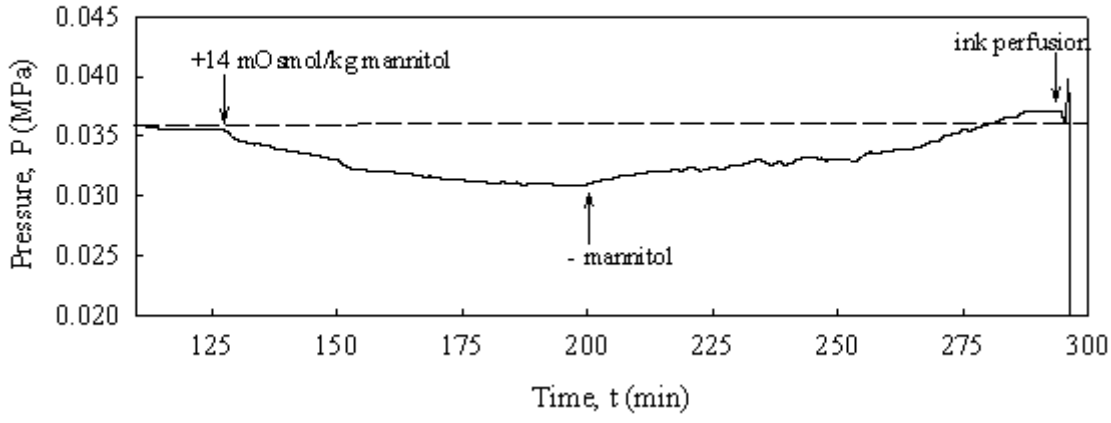
B


0.04
0.02
0.00
-0.02
Pressure, P (MPa)
Steady- state pressure
Switch on the pump to backward
+50 μM HgCl2
- HgCl2
Switch on the pump to forward
510
520
530
540
550
560
570
Time, t (min)

C


+14 mOsmol/kg mannitol
0.044
0.042
0.040
0.038
0.036
0.034
Pressure, P (MPa)
New steady -state pressure
- mannitol
610
630
650
670
690
710
730
750
Time, t (min)

D


0.045
0.040
0.035
0.030
0.025
0.020
Pressure, P (MPa)
+14 mOsmol/kg mannitol
ink perfusion
- mannitol
125
150
175
200
225
250
275
300
Time, t (min)

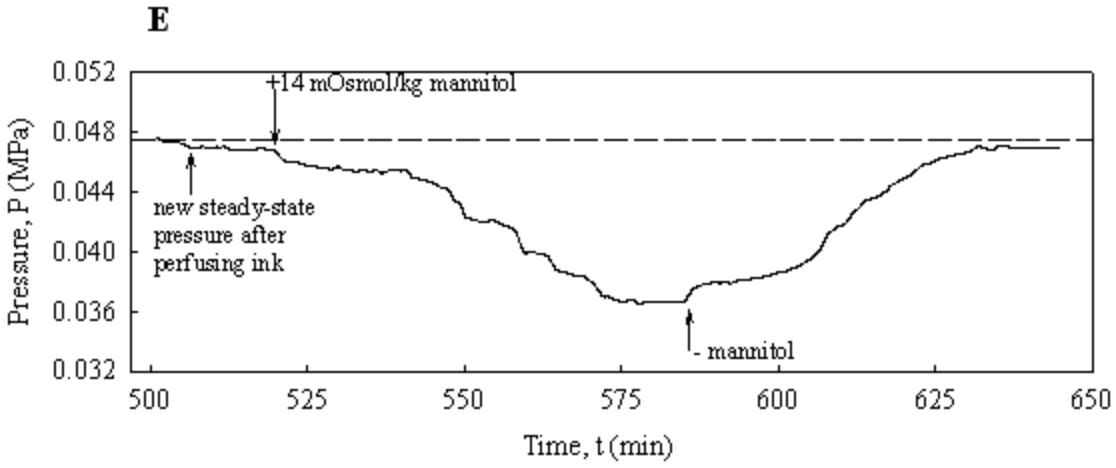


Fig. 7 (A, C-E) Typical experiments showing drop of steady-state pressures in response to changes in osmotic pressure through the addition of mannitol to the outer medium for control root segments (A, D) or segments treated with either 50 µM $HgCl_2$ (C) or China ink particles (E) (cv. IR64, 20-50 mm from the root apex). Blockage of cell-to-cell path with $HgCl_2$ increased the reflection coefficient (σ_{sOPR}) from 0.11 (A) to 0.21 (C). Reversed usage of pump resulted for a slightly negative pressure gradients (reference to atmospheric pressure) in root segments (B). Partial blockage of apoplast with ink particles caused an increase in stationary pressure by 37% from 0.035 to 0.048 MPa, and caused σ_{sOPR} to rise from 0.11 (D) to 0.34 (E).

Root segments perfused with diluted China ink for 1 h, developed higher stationary pressures than controls. The decline in stationary pressures upon the addition of mannitol was larger than that of controls (Fig. 7E). At a distance of 20-50 mm from the root apex, σ_{sOPR} increased from 0.13 ± 0.04 to 0.40 ± 0.09 in IR64 and 0.11 ± 0.03 to 0.27 ± 0.04 in Azucena (Table 2; n = 6 root segments). Similar results were obtained for root segments taken 50-100 mm from the root apex for both cultivars. Overall, China ink perfusion of root segments increased the σ_{sOPR} by a factor of about three. The increase in σ_{sOPR} due to the China ink treatment was significantly greater than that due to the $HgCl_2$ treatment (t-test; P = 0.05).

Table 2 Reflection coefficient of the outer part of rice roots (σ_{sOPR}) for mannitol for root segments treated either with 50 µM $HgCl_2$ or perfused with China ink. Plants were grown for 31-40 d in aerated hydroponic culture. The σ_{sOPR} given for two different distances from the root apex and two cultivars, IR64 and Azucena. Values are means ± SD (n = 6 root segments). The reflection coefficient (σ_{sOPR}) for mannitol did not differ significantly between cultivars or over different root segments (20-50 mm or 50-100 mm from the root apex). Root segments treated with either $HgCl_2$ or perfused with China ink showed a significant increase in σ_{sOPR} for mannitol over control values (double sided, unpaired t-test, P = 0.05). Root segments perfused with China ink had significantly higher σ_{sOPR} values than those treated with $HgCl_2$ (double sided, unpaired t-test, P = 0.05).

Rice cultivar	Reflection coefficient of the outer part of rice roots (σ_{sOPR}) for mannitol			
	control	50µM $HgCl_2$ treated	control	ink perfused
IR64				
20-50 mm	0.12 ± 0.04a	0.23 ± 0.05b	0.13 ± 0.04a	0.40 ± 0.09c
50-100 mm	0.14 ± 0.04a	0.28 ± 0.07b	0.11 ± 0.03a	0.35 ± 0.06c
Azucena				
20-50 mm	0.10 ± 0.02a	0.20 ± 0.04b	0.11 ± 0.03a	0.27 ± 0.04c
50-100 mm	0.13 ± 0.03a	0.21 ± 0.03b	0.12 ± 0.06a	0.30 ± 0.05c

	Mannitol	NaCl	Reference
IR64			
20-50 mm	0.13 ± 0.04 (n = 6)	0.09 ± 0.02 (n = 5)	Ranathunge et al. 2003
50-100 mm	0.13 ± 0.04 (n = 6)	0.11 ± 0.03 (n = 5)	
Azucena			Ranathunge et al. 2003
20-50 mm	0.15 ± 0.05 (n = 8)	0.08 ± 0.02 (n = 5)	
50-100 mm	0.14 ± 0.10 (n = 7)	0.09 ± 0.01 (n = 5)	

Different letters indicate significant differences at P = 0.05 level

Diffusional water permeability of the OPR with heavy water

Vertical perfusion of aerenchyma by near-isobaric heavy water (HDO) was performed with rice root segments 20-50 mm or 50-100 mm from the root apex. The amount of HDO diffused into the external medium increased with time (Fig. 8). At any time, the external concentration of HDO was substantially smaller than that of HDO perfused through the aerenchyma, which was constant. Killing the roots by exposing them to steam for 30 s, doubled the radial diffusion of HDO across the OPR into the external medium for both immature (20-50 mm) and mature (50-100 mm) root segments for both cultivars used (t-test; P = 0.05).

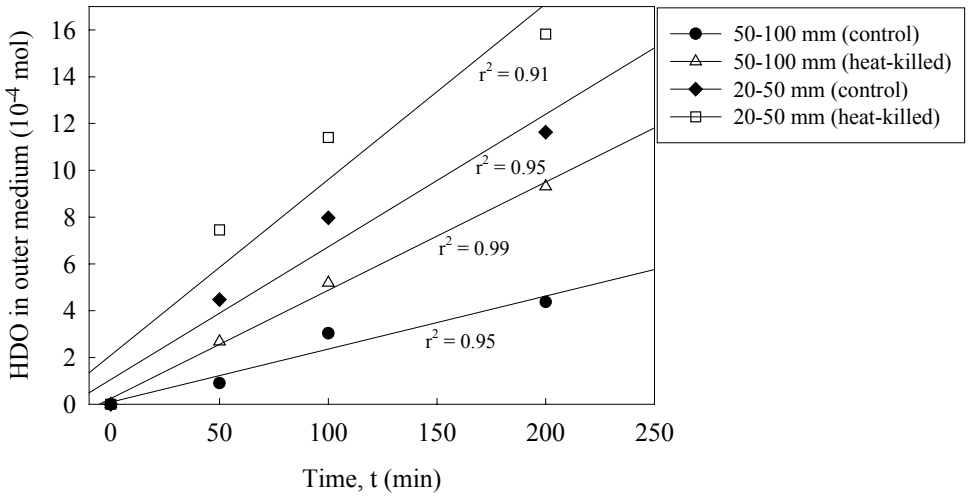


Fig. 8 Increases of external HDO concentration with time for root segments 20-50 mm or 50-100 mm from the root apex (cv. IR64). The amount of HDO, diffused into the outer medium was significantly higher for immature segments (20-50 mm from the apex) than mature (50-100 mm from the apex) (double-sided, unpaired *t*-test, $P = 0.05$). By a factor of two, heat-killing of root segments increased the difusional water permeability (P_{dOPR}) at both distances from the root apex.

The diffusional permeability of the OPR (P_{dOPR}) was obtained as defined by equation 2. Since the concentration of HDO in the aerenchyma was much larger than that of the external medium, this concentration was used as the driving force in equation 2. The diffusional water permeability of the OPR significantly decreased along the root axis from apex to base (*t*-test; $P = 0.05$; n = 6-7 roots). P_{dOPR} was larger by a factor of two to three immature (20-50 mm) compared with mature (50-100 mm) root segments. The P_{dOPR} of root segments 20-50 from the root apex were 3.5 ± 0.5 and $3.0 \pm 1.6 \times 10^{-7}$ m s^{-1} in IR64 and Azucena, respectively. At a distance of 50-100 mm from the apex, values were 1.4 ± 0.8 and $1.0 \pm 1.6 \times 10^{-7}$ m s^{-1} in IR64 and Azucena, respectively. Steam treatment of root segments increased P_{dOPR} by a factor of about two for both rice cultivars at both distances from the root apex. Comparison of bulk and diffusional permeabilities showed that the hydraulic/bulk water permeability of the OPR (P_{fOPR}) was 600 times larger than the diffusional water permeability (P_{dOPR}) at 20-50 mm from the apex and 1200-1400 larger at 50-100 mm from the apex (Table 3).

Table 3 Diffusional water permeability (P_d) of the outer part of rice roots, measured by rapidly perfusing aerenchyma with isobaric water (containing HDO). Plants were grown for 31-40 d in aerated hydroponics. Measurements were performed for two different cultivars, IR64 and Azucena and two different distances from the root apex. Values are means ± SD with the number of measured roots in parenthesis. P_{dOPR} values are given for control (living) and steam-treated root segments. For both cultivars, P_{dOPR} significantly decreased along the root from the apex (double-sided, unpaired *t*-test, P = 0.05). Immature root segments (20–50 mm from root apex) showed significantly higher P_{dOPR} values than mature segments (50–100 mm from the apex). Steam-treated root segments increased the P_{dOPR} by a factor of two on average for both cultivars and over both distances from the root apex (double-sided, unpaired *t*-test, P = 0.05). There were no significant differences observed for hydraulic/bulk water permeability of the OPR (P_{fOPR}) for either distance from the root apex (double-sided, unpaired *t*-test, P = 0.05). Hydraulic water permeability of the OPR (P_{fOPR}) was two to three orders of magnitude higher than diffusional water permeability (P_{dOPR}).

Rice cultivar	Diffusional water permeability of the OPR (P_{dOPR}) × 10^{-7} m s^{-1}		Hydraulic water permeability of the OPR (P_{fOPR}) × 10^{-7} m s^{-1}	P_{fOPR} / P_{dOPR} Ratio
	Control roots	Steam-treated roots		
IR64				
20-50 mm	3.5 ± 0.5 (7)a	5.1 ± 0.7 (6)	2170 ± 683 (10)	620
50-100 mm	1.4 ± 0.8 (6)b	2.7 ± 2.2 (5)	1680 ± 255 (10)	1200
Azucena				
20-50 mm	3.0 ± 1.6 (7)a	5.8 ± 2.8 (5)	1808 ± 703 (10)	603
50-100 mm	2.0 ± 0.7 (6)b	2.0 ± 1.0 (5)	1445 ± 417 (10)	1445

Different letters indicate significant differences at P = 0.05 level

Discussion

The present study provides further evidence of the passage of water through both the apoplast and the cell-to-cell path of the OPR of young rice roots. The passage across the two parallel pathways has been partially inhibited by either affecting water channel activity with $HgCl_2$ or by closing pores in the apoplast with ink particles. Blocking off the apoplast is not easy. To the authors' knowledge, the technique used here is unique. The results suggest that both pathways contribute to the overall water flow. The contribution of the apoplast appeared to be bigger, although roots develop apoplastic barriers as revealed by anatomical studies (e.g. suberin lamellae, Casparian bands). Because it comprises just four cells layers, the OPR of rice is a useful and well-defined structure for studying the tissue transport of water in the presence of apoplastic barriers. The anatomy of the OPR can be easily characterized by observing the development of an exodermis and sclerenchyma and the deposition of apoplastic barriers in parallel with changes in transport. Current models of tissue (root) water transport may be applied (Steudle, 2000a, 2001; Steudle and Frensch, 1996; Steudle and Peterson, 1998). Ranathunge *et al*. (2003) have shown that the apoplast contributes to most of the radial permeability for water of the Lp_{OPR}. This is supported by the current results.

The absolute figure of Lp_{OPR} was larger by a factor of 30 than the overall Lp_r value of rice roots. It seems that at least these two rice cultivars differ from other crop plants where the contribution of the hydraulic resistance of the exodermis is much bigger. Maturation of the exodermis substantially reduced the radial water flow in onion (Melchior and Steudle, 1993). In young corn plants, development of the exodermis caused in a 4-fold reduction of root Lp_r (Zimmermann and Steudle, 1998).

Even for the same cultivar grown under similar conditions, detailed anatomical studies of the OPR of rice roots confirmed that exodermis maturation was highly variable, as previously shown by Perumalla and Peterson (1986) for onion and corn roots. The specialized type of hypodermis (exodermis) observed in the rice cultivars allowed a rather free flow of bulk water across the periphery of roots. Even though fully matured exodermis with Casparian bands was found beyond 60–80 mm from the root apex, this did not significantly reduce radial water flow across the OPR. This may be due to local

disruption of the exodermis when developing lateral roots from the pericycle allowing high apoplastic bypasses through these cracks (Peterson *et al*., 1981). In roots lacking an exodermis, water and ions can potentially move apoplastically through the cell walls of the epidermis and cortex as far as the endodermis (Peterson, 1988). Despite having the exodermis, the OPR was reasonably permeable for water. Preliminary results showed that charged ions could also pass through premature Casparian bands in the rice exodermis, at least to some extent (data not shown). This is an agreement with earlier findings of Flowers and co-workers of an apoplastic bypass flow of sodium and the apoplastic tracer PTS (Yadav *et al*., 1996; Yeo *et al*., 1987).

It may be argued that the exodermis is permeable because of the patchiness of Casparian bands or because of the presence of passage cells lacking suberin lamellae. It is known that rice roots contain passage cells in the exodermis (Clark and Harris, 1981). However, in these histochemical studies it was not possible to confirm large number of passage cells in the exodermis for the rice cultivars used here. There was no evidence for patchiness of the exodermis. On the contrary, maturation of Casparian bands and suberization of the hypodermis was fairly uniform along the roots for both cultivars. It was fully completed at a distance of 50-60 mm from the apex. It was known that the presence of suberin lamellae in hypodermal cell walls of corn sleeves did not necessarily indicate a low permeability to water or solutes (Clarkson *et al*., 1987). This is an agreement with the present data.

The permeability properties of the apoplastic barriers for water or solutes are related to the amount and chemical composition of aliphatic and aromatic suberin and lignin (Hose *et al*., 2001; Schreiber *et al*., 1999; Zimmermann *et al*., 2000). Neither uniseriate sclerenchymatous layer formed of short fibres (Clark and Harris, 1981) nor suberized and lignified mature exodermis greatly impeded the radial bulk flow of water across the OPR much. Histochemical studies with rice roots showed that lignification of the exodermis started as close as 50 mm from the root apex. No lignin was detected in sclerenchyma fibre cells at the same distance. According to Clark and Harris (1981), lignified sclerenchyma fibre cells without cytoplasm were observed as far as 150 mm from the root apex. Unusually low amounts of lignification of sclerenchyma fibre cells

at distances of up to 100 mm may indicate a rather high permeability of this structure, which is largely composed of cellulose.

Basically, radial water flow across the roots could use either the apoplastic or cell-to-cell pathways or both. There is strong evidence that water channels (aquaporins) play a central role in plant water relations (Chrispeels and Maurel, 1994; Henzler and Steudle, 1995; Javot and Maurel, 2002; Maurel, 1997; Steudle and Peterson, 1998; Steudle, 2001; Tyerman *et al*., 1999, 2002). Water channel activity can be affected by different parameters such as high salinity, nutrient deprivation, drought, diurnal rhythms, and heavy metals (Azaizeh and Steudle, 1991; Carvajal *et al*., 1996, 1999, 2000; Henzler *et al*., 1999; Henzler and Steudle, 1995; North and Nobel, 2000). The exact mechanisms of the gating of channels are poorly understood (Steudle, 2000a, 2000b, 2001; Tyerman *et al*., 1999, 2002; Ye *et al*., 2003; Wan *et al*., 2003). A tentative indicator of water channel involvement is the observation that heavy metals like Hg^{2+} can reversibly reduce the hydraulic conductivity of roots by binding to –SH groups of water channels (Barrowclough *et al*., 2000; Carvajal *et al*., 1996; Henzler and Steudle, 1995; Maggio and Joly, 1995; North and Nobel, 2000; Wan and Zwiazek, 1999). According to authors' best knowledge, this is the first study in which water channels were blocked off with $HgCl_2$ only for a part of roots (just its outer part or periphery).

For *Agave deserti*, the reduction of radial hydraulic conductivity was 60% in the presence of 50 μM $HgCl_2$ under wetted conditions (Martre *et al*., 2001). It was 4-fold in the basal root zones of onion (Barrowclough *et al*., 2000). These figures differ from the OPR of rice roots which showed only 10% reduction. The main reason for that might be that apoplastic barriers like Casparian bands, suberin lamellae or lignin restricted the penetration of even non-dissociated $HgCl_2$ into OPR and to plasma membranes. Perhaps, the reduction of the radial water flow across the OPR resulted of only a closure of water channels in the rhizodermis and external membranes of the exodermis. Alternatively, the water channels in the OPR may not contain many –SH groups or these groups are difficult to access with $HgCl_2$ because of the suberization. Hence, roots were not particularly sensitive to $HgCl_2$. Since the major barrier to radial water flow in rice roots was the endodermis (Miyamoto *et al*., 2001; Ranathunge *et al*., 2003), most of

the water channels might be located around the endodermis as found for other species (Schäffner, 1998).

Blockage of the apoplastic pathway by ink treatment reduced Lp_{OPR} by 30% which was significantly larger than the 10% inhibition caused by $HgCl_2$. Perhaps, the ink suspension used could not effectively close all pores in the cell walls because of the relatively large particle sizes. The mean was 51 ± 22 nm which is bigger than the diameter of interfibrillar pores (interstices) which are about 5-30 nm (Nobel, 1999). Partial blockage of the apoplast in the OPR reduced radial water flow by more than the water channel blocker $HgCl_2$, suggesting relatively large apoplastic bypasses. There is a need for better apoplastic blockers. Tests are underway with suspensions of particles of smaller mean diameter, which should result in a larger reduction of Lp_{OPR} of, say, by a factor of 5-10. Completeness of blockage may be tested by measuring the reflection coefficient, which should increase when blockage of the apoplast is complete or nearly so.

The picture of a dominating apoplastic rather than cell-to-cell path for radial water flow within the OPR is in line with the low overall reflection coefficients ($\sigma_{sOPR} \approx 0.1$; Table 2; Ranathunge *et al*., 2003) as well as the doubling of σ_{sOPR} upon partial pore closure by ink particles. The solute mannitol used to measure effects on σ_{sOPR} does not permeate plant cell membranes and should have a $\sigma_s^{cc} \approx 1$ along the cell-to-cell path (nearly semipermeable membranes). Unlike membranes, however, apoplast should have $\sigma_s^{cw} \approx 0$ with virtually no selectivity expected (Steudle, 2000a, 2000b). Roots, having complex structures with the two pathways arranged in parallel as well as in series, then overall σ_s usually locates in between 0 and 1, which can be calculated using a relation derived from basic irreversible thermodynamics (Kedem and Katchalsky, 1963a*,* 1963b). Partial closure of apoplastic pores with ink resulted in a decrease of the movement of solutes (mannitol) through the apoplast leading to a higher σ_{sOPR} than the treatment with the water channel blocker $HgCl_2$.

According to the composite transport model, which may be applied for complex structures such as roots, the overall reflection coefficient (σ_s) for a parallel arrangement of membranes should decrease after closing water channels with $HgCl_2$ (assuming that

$\sigma_s^{cc} \approx 1$ and $\sigma_s^{cw} \approx 0$). Data presented in this paper showed the opposite trend: σ_s increased after $HgCl_2$ treatment. The OPR of rice roots contain 4 cell layers in series, indicating that both parallel and serial membrane models contribute to the overall reflection coefficient. It is not clear why this deviation occured but it may arise because the OPR comprises both parallel (apoplast *versus* cell-to-cell) and four different series layers of cells, which may differ in their transport properties. For example, the Kedem-Katchalsky (1963b) treatment of patchy membrane systems predicts that, in a series array, the overall reflection coefficient would be the weighed sum of individual arrays, whereby the series elements contribute according to their solute (mannitol) permeability. It cannot be excluded that the permeability of mannitol of different layers is affected by $HgCl_2$ treatment, although direct evidence is missing.

In order to compare the Lp_{OPR} (units: m s^{-1} MPa^{-1}) with the osmotic water permeability, P_f in units of m s^{-1}, equation (3) was used. P_f rather than Lp is usually given in animal physiology (e.g. Table 5.6 in House, 1974). The diffusional permeability of the OPR of rice roots (P_{dOPR}) was much lower than the osmotic water permeability (P_{fOPR}). Absolute values of P_{dOPR} were bigger than those of sleeves of the aerenchymatous species *Carex arenaria* ($\approx 10^{-8}$ m s^{-1}; Robards *et al*., 1979), but smaller than the P_{dOPR} of sleeves obtained from aerenchymatous corn roots ($\approx 10^{-6}$ to 10^{-7} m s^{-1}, depending on the position from the root apex; Clarkson *et al*., 1987). By a factor of as large as 600-1400, the osmotic water permeability (P_{fOPR}) was greater than that of diffusional water permeability (P_{dOPR}). These values were larger than the P_f/P_d ratio of artificial membranes (P_f/P_d = 1-730; Table 4.4 in House, 1974) and various animal tissues (P_f/P_d = 1-300; Table 9.5 in; House, 1974). It should be noted that, in the present experiments, diffusional water flows were measured under near-isobaric but not completely isobaric conditions. Hence, P_d may be overestimated, and the big ratios represent a lower limit. Such large P_f/P_d ratios are expected if the pathway involved a rather long porous path; this would offer a high diffusional resistance for HDO, but should be highly permeable in case of a bulk (hydraulic) water flow. In single-file pores such as water channels, ratios of $P_f/P_d>1$ are a measure of the number of water molecules aligned within the pore (Levitt, 1974). It is well documented that P_f/P_d ratios may be overestimated in the presence of unstirred layers, which affect P_d rather than P_f. However, during the measurements in this study, the solutions in both compartments (aerenchyma and

external solution) were well-stirred, tending to reduce the effect. Hence, large ratios were not due to effects of unstirred layers. In the context of the other findings of (i) effects of blocking experiments and (ii) low reflection coefficients, the huge P_f/P_d ratios provide the strongest evidence for a major passage of water along the apoplast, even in the presence of apoplastic barriers.

Rice roots often grow under water-logged conditions in hypoxic soil environments. At first sight, a low diffusional permeability (as found for water) may also refer to oxygen, which diffuses from the shoot to root tips through the aerenchyma under hypoxic conditions (Armstrong, 1979; Colmer *et al*., 1998). To reach root tips, it is required that there are no excessive losses to the soil, i.e. P_{O2} should be low. The differences between diffusional (HDO) and bulk water (*Lp*) permeabilities indicate that this could be achieved by differences in the transport mechanism (diffusional *versus* bulk flow; Ranathunge *et al*., 2003). Hence, rice roots could have rather high bulk water permeability in the presence of a low permeability to oxygen, which reduces radial oxygen losses (ROL). This would be favourable to the plant. The present data show that the diffusional water permeability was reduced by only a factor of 3 as roots developed. At the same time, radial oxygen loss (ROL) drastically decreased along the roots and ends up with rates of close to zero at a distance of 50 mm from the root apex for the cultivars used (Kotula L, Ranathunge K, Steudle E, Lafitte R, unpublished data). Apparently, the diffusion of oxygen from aerenchyma to the outer medium is strongly restricted by the existence of apoplastic barriers, which retain oxygen more effectively than water. This may point to differences in the transport path for the two compounds. However, there are, to date, no data of the permeability coefficients of oxygen across the OPR to compare with permeabilities of water and how this would change during root development. These values are badly needed.

The data show that apoplastic water flow contributes much more to the overall water flow across the OPR of rice roots than the transmembrane component. The findings suggest that exodermal apoplastic barriers such as Casparian bands and suberin lamellae are fairly permeable to water. Partial blockage of the porous apoplast with ink particles proportionately reduced the radial water flow across the OPR more than $HgCl_2$ did along the cell-to-cell path, suggesting that there were prominent apoplastic bypasses.

This was in line with substantial relative increases of σ_{sOPR} in response to blockage of the apoplast with ink rather than the cell-to-cell path. However, absolute values of reflection coefficients remained rather low. The diffusional water permeability (P_{dOPR}) was smaller by two (for immature root segments) or three (for mature root segments) orders of magnitude than the osmotic (P_{fOPR}). This strongly supported the view that there was substantial apoplastic transport of water across the OPR of rice, even in the presence of Casparian bands and suberin lamellae. Diffusional and bulk water permeabilities did not decrease much during root development. Hence, the small effect of root development on the diffusional permeability of water differed from that found for oxygen (Colmer *et al*., 1998). This suggested that the two diffusants use different pathways within the OPR.

Acknowledgements

We thank Ms. Alaina Garthwaite, University of Western Australia, Perth for carefully reading and correcting the manuscript. We wish to thank two anonymous refrees for helpful comments and suggestions. The excellent technical assistance of Burkhard Stumpf (Lehrstuhl Pflanzenökologie, University of Bayreuth) is gratefully acknowledged. We are also grateful to the Deutsche Forschungsgemeinschaft: Ste319/3-3 (E.S.), and BMZ (project No. 2000.7860.0-001.00) “Trait and Gene Discovery to Stabilize Rice Yields in Drought Prone Environments“ (R.L.) for financial support. LK thanks for a grant within the ERASMUS/SOCRATES programme of the EU.

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4 Blockage of apoplastic bypass-flow of water in rice roots by insoluble salt precipitates analogous to a Pfeffer cell

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Plant, Cell and Environment (2005) 28: 121-133

Abstract

Precipitates of insoluble inorganic salts were used to clog apoplastic pores in cell walls of the outer part of rice roots (OPR) in two rice cultivars (lowland cv. IR64 and upland cv. Azucena). Aerenchyma of two different root zones (20-50 and 50-100 mm from the apex) was perfused with 1 mM potassium ferrocyanide ($K_4[Fe(CN)_6]$) while the whole root segments were bathed in 0.5 mM copper sulfate medium ($CuSO_4$). In another experiment, salts were applied on opposite sides of the OPR. The copper-ferrocyanide precipitation technique resembles the famous osmotic experiments of the German botanist Wilhelm Pfeffer, in which he used them with clay diaphragms. Precipitates were observed on the side where ferrocyanide was applied, suggesting that Cu^{2+} and SO_4^{2-} were passing the barrier including the Casparian bands (CBs) of the exodermis much faster than ferrocyanide. There was a patchiness in the formation of precipitates, correlated with the maturation of the exodermis. The intensity of copper ferrocyanide staining decreased along developing rice roots. No precipitates were observed in mature parts beyond 70-80 mm from the root apex, except for sites around the emergence of secondary roots, which were fairly leaky to both water and ions. Blockage of the apoplastic pores with precipitates caused a massive three- to four-fold reduction of hydraulic conductivity of the OPR (Lp_{OPR}). The reflection coefficient of the OPR (σ_{sOPR}) increased in response to the blockage with precipitates. The osmotic *versus* diffusive water permeability ratios of the OPR (P_{fOPR}/P_{dOPR}) were around 600 for immature and 1200 for mature root segments. Treatment significantly affected the bulk rather than the diffusive water flow and caused a three- to five-fold reduction of the P_{fOPR}/P_{dOPR} ratios. Results indicated that despite the existence of an exodermis with Casparian bands, most of the water moved around cells rather than using the cell-to-cell passage.

Key words: aerenchyma; apoplast; Casparian bands; exodermis; hydraulic conductivity; rice roots; wall precipitates.

Introduction

In the past, the phenomenon of variable hydraulics of roots has been explained in terms of a composite transport model. The complex 'composite anatomical structure' of roots results in 'composite transport' of both water and solutes (Steudle and Peterson 1998). The parallel arrangement of the apoplastic and cell-to-cell paths and the switching between pathways are important features of this model. The apoplastic (extraprotoplastic/cell wall) component of water flow may be restricted by the existence of barriers such as Casparian bands. Along the cell-to-cell path (transmembrane and symplastic component *via* plasmodesmata), aquaporins, plasmodesmata, and suberin lamellae may regulate the intensity of water flow (Oparka and Prior 1992; Peterson and Cholewa 1998; Tyerman et al. 1999; Javot and Maurel 2002; Tyerman et al. 2002). By switching between apoplastic and cell-to-cell paths, the composite transport model allows for an adjustment or even a regulation of water uptake according to the demand from the shoot. The model may also be applied to rice roots, which have aerenchyma that separates the stele from the outer part of the root (OPR). The OPR comprises an innermost unmodified cortical layer, sclerenchyma cells, an exodermis, and outermost rhizodermis (Ranathunge et al. 2003).

Even though rice is grown in paddy fields, symptoms of water shortage may occur during daytime, resulting in leaf rolling (Hirasawa et al. 1992; Hirasawa et al. 1996). This has been attributed to the hydraulic resistance of rice roots being higher than that of other crop plants due to a lack of flexibility in switching between pathways and adjusting to demand from the shoot (Miyamoto et al. 2001; Ranathunge et al. 2003). Even though there are apoplastic barriers in rice roots in both the exo- and endodermis (Clark and Harris 1981; Ranathunge et al. 2003), there are substantial apoplastic bypass-flows of polar solutes such as NaCl across the entire root cylinder (Yeo et al. 1987; see Discussion). For water, it has been shown that the main resistance in rice roots is located in the endodermis (Miyamoto et al. 2001; Ranathunge et al. 2003). Despite the presence of a suberized exodermis with Casparian bands (CBs) and an additional layer of lignified fibre cells, the OPR had the lowest resistance of any part of the radial path; the resistance of aerenchyma was calculated to be intermediate (Ranathunge et al. 2003). The low resistance of the OPR might be due to the fact that

lignin rather than suberin is the major chemical component of rice CBs as found for other plant species (Schreiber 1996; Zeier and Schreiber 1998).

Researchers are interested in distinguishing relative contributions of apoplastic and cell-to-cell paths for the overall radial water flow across roots. For rice, Ranathunge et al. (2004) presented evidence that the relative contribution of the apoplastic path was much larger than that of cell-to-cell path for overall radial water flow. This suggested that the CBs of the OPR was not a major barrier to water flow. Using a perfusion technique, the authors showed huge ratios between the bulk and diffusional permeabilities of the OPR, which are indicative of a dominating porous path. Ranathunge et al. (2004) used ink particles of a mean diameter of 50 nm to clog apoplastic pores and decreased hydraulic conductivity of the OPR (Lp_{OPR}) by 30%. The effect was larger than that of the water channel blocker $HgCl_2$ but the technique, perhaps, was not sufficient enough to block most of the apoplastic pores.

In the present study, the technique of blocking the apoplastic path was improved using insoluble salt precipitates. We perfused the aerenchyma and added one component (salt) to the perfusion medium and the other to the outside. Salts diffused across the apoplast of the OPR and formed a coloured precipitate where they met. Copper sulfate and potassium ferrocyanide were used in millimolar concentrations. These formed reddish brown precipitates of copper ferrocyanide (Hatchett's brown) that were visible from the outside of the root. The technique was adapted from the famous experiments of Wilhelm Pfeffer (1921), who used a precipitation technique to produce semipermeable precipitation membranes in clay diaphragms. For the first time, Pfeffer's technique was used in a living plant tissue. Upon blockage of the apoplast we found a several-fold decrease in the hydraulic conductivity, consistent with the former idea of a dominating apoplastic water flow.

Materials and methods

Plant material

Rice (*Oryza sativa* L.) seeds (International Rice Research Institute, Manila, Philippines) of IR64 (lowland cultivar) and Azucena (upland cultivar), the same cultivars used in previous studies (Ranathunge et al. 2003, 2004), were germinated on moistened filter paper placed within a Petri dish under light conditions (500 µmol m^{-2} s^{-1} of PAR). Seedlings with 15 mm long roots and shoots were transferred to aerated hydroponic culture and grown in climatic chambers as previously described by Miyamoto et al. (2001). After 31-40 d growth, roots were excised at the basal node of the stem and used in experiments.

Plant morphology and root anatomy

The shoot heights of young rice plants used in the experiments were 340 ± 10 mm and 500 ± 15 mm for IR64 and Azucena, respectively. Roots were 320 ± 40 mm (IR64) and 530 ± 25 mm (Azucena) long. Two different root zones (20-50 and 50-100 mm from the root apex) were employed for measurements. The aerenchyma was not fully developed at 20-50 mm from the root apex, as it was at 50-100 mm. Freehand cross-sections were made approximately 70 mm from the root apex and stained with 0.05% Toluidine blue O at room temperature for 2 min (O'Brien et al. 1964) to observe the emergence of secondary roots from the primary root. Sections were examined using a light microscope and photographed using Kodak Elite 64 ASA film.

Blocking the apoplast of the OPR by precipitates

Selection of salts

Insoluble salts were used to block apoplastic pores in the OPR. The reaction between $CuSO_4$ and $K_4[Fe(CN)_6]$ gave rusty brown, insoluble crystals (precipitates) of $Cu_2[Fe(CN)_6]$ or $Cu[CuFe(CN)_6]$. Equivalent precipitates using $FeSO_4$ and $K_4[Fe(CN)_6]$ resulted in a bluish colour of $K[Fe^{III}Fe^{II}(CN)_6]$ due to contamination by Fe^{III} (Holleman

and Wiberg 1995). As $CuSO_4$ permeated the OPR faster than the ferrocyanide, precipitates largely occurred at the side where the ferrocyanide was offered. Different combinations of $CuSO_4$ and $K_4[Fe(CN)_6]$ were tested to find the combination that yielded the most intense precipitates. This occurred when 0.5 mM $CuSO_4$ was offered outside and 1.0 mM $K_4[Fe(CN)_6]$ offered inside the aerenchyma of root segments. This combination effectively clogged the apoplastic pores in the OPR. $FeSO_4$ and $K_4[Fe(CN)_6]$ were tested in the same way. However, in some experiments 1 mM of both solutions were used, or the sides of application were reversed.

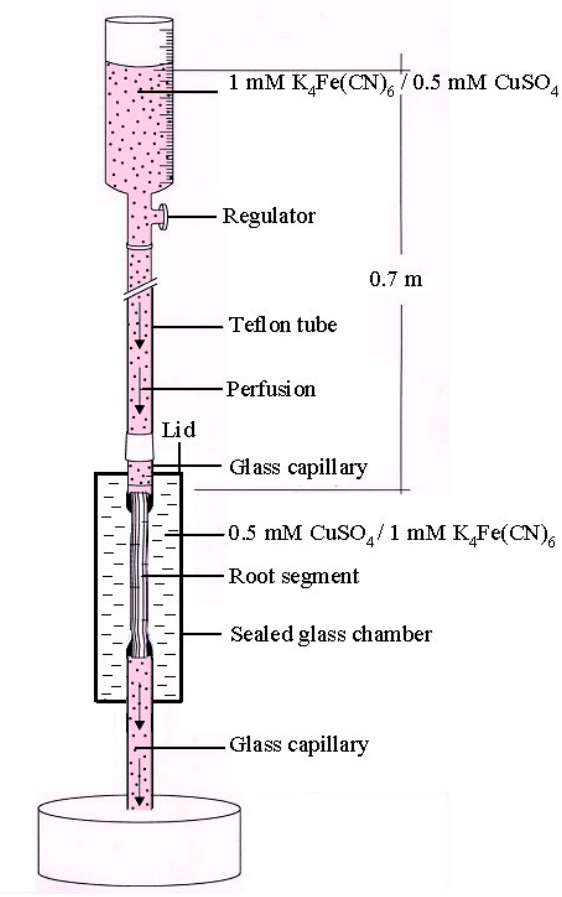

Fig. 1 Diagram of a root perfusion set-up with an attached root segment, bathed in 0.5 mM $CuSO_4$ or 1 mM $K_4[Fe(CN)_6]$. Open ends of root segments were fixed to glass capillaries (inner diameter: 1.3 mm). Aerenchyma of root segments was rapidly perfused either by 1 mM $K_4[Fe(CN)_6]$ or 0.5 mM $CuSO_4$ by gravity to develop precipitates of copper ferrocyanide in the apoplast of the outer part of the root (OPR; see Figs. 4b-d).

Root segments excised either at 20-50 mm or 50-100 mm from the root apex were fixed to glass capillaries (inner diameter: 1.3 mm), using a polyacrylamide glue (UHU, Bühl, Germany), and then connected to the simple perfusion apparatus shown in Fig. 1. The upper, open end of the root segment (used as an inlet) was connected to a syringe by a

Teflon tube filled with either $CuSO_4$ or $K_4[Fe(CN)_6]$ solution. The other end of the root segment was connected to a glass capillary, and remained open as an outlet. The syringe was placed 0.7 m above the root segment providing a gravitational force of 0.007 MPa, and the root segment was held vertically to allow perfusion of the solution by gravity. $K_4[Fe(CN)_6]$ solution was perfused through the aerenchyma of the root segment, displacing air, while the whole root segment was bathed in $CuSO_4$ solution. Trials were carried out for the combinations given above.

Selection of optimum time period for perfusion

Perfusion of inorganic salt solutions was conducted for four different time periods (2, 3, 4, 5 h) to determine the optimum time for the development of insoluble crystals to clog apoplastic pores in the cell walls of the OPR. Freehand cross-sections were prepared and coloured crystals in the cell walls were viewed using an optical microscope (DIALUX 22EB, Leitz, Nürnberg, Germany) after each period of perfusion. A period of 3 h was established to be the optimum time period for perfusion.

Measurement of radial water flow across the outer part of roots (OPR) by steady-state of perfusion

Measurements were done for two different root zones (root tip not intact) of 20-50 mm and 50-100 mm from the apex. Control (pre-perfused with water instead of salt solutions) or treated (apoplastic pores in the cell walls of the OPR blocked off by precipitates) root segments were attached to the pump perfusion apparatus as previously detailed by Ranathunge et al. (2003). A syringe filled with aerated nutrient solution was mounted on a pump (high-precision, 12-step) and connected to the inlet side of the root segment, while a pressure device (pressure probe) was connected to the outlet side. Aerated nutrient solution was perfused through the aerenchyma of rice root segments at a rate of 1.7×10^{-12} m^3 s^{-1} and resulting steady-state pressures (P in MPa above atmospheric) were measured. The hydraulic conductivity of the OPR (Lp_{OPR} in m s^{-1} MPa^{-1}) was calculated using equation 1:

$$Qv = Lp_{OPR} \times P \times A_r \,. \qquad (1)$$

where Q_v is the pump rate (in m^3 s^{-1}), and A_r is the surface area of the root segment (m^2).

To avoid anaerobic conditions, root segments were placed in a small, open chamber and nutrient solution was continuously circulated around them. The effectiveness of the seals (places where the open ends of root segments attached to glass capillaries by using a polyacrylamide glue and a molten mixture of bee's-wax:colophony) were tested before and after experiments by passing a fluorescent dye, 0.01% trisodium,3-hydroxy-5,8,10-pyrene trisulfonate (PTS: Bayer AG, Leverkusen, Germany) through the root segments and checking for leakages into the outer medium. At the end of the experiment, only segments without leaks were used to calculate the Lp_{OPR}.

Determination of reflection coefficient of the OPR (σ_{sOPR}) for perfused root segments

The reflection coefficient of the OPR (σ_{sOPR}: passive selectivity of cell membrane for a given solute) was determined from the change in steady-state pressure in response to adding osmotic solutes to the external medium. Mannitol (20 mOsmol kg^{-1}, equivalent to 0.05 MPa of osmotic pressure) was used as the test solute, as it has a σ of unity (ideal semi-permeable) for plant cell membranes (Steudle and Tyerman 1983). Replacing the external nutrient solution with mannitol brought about a decline of steady-state pressure. Its maximum drop was used to calculate σ_{sOPR}. The original steady-state pressure was obtained following the removal of mannitol from the external medium. Measurements were carried out for control and treated root segments (apoplastic pores in the cell walls of the OPR clogged with salt precipitates), separately. At the end of the experiment, root segments were randomly selected; freehand longitudinal sections were made and stained with Evans blue for 20 min in order to check the viability of cells in the OPR (Fischer et al. 1985). Longitudinal sections were soaked in 10 mM ethylenediamine tetra-acetic acid (EDTA) for 30 min and washed with distilled water to dissolve precipitated salt crystals in cell walls, prior to staining with Evans blue.

Diffusional water permeability across the OPR (P_{dOPR}) with heavy water (HDO)

Control and treated (apoplastic pores in the OPR blocked off with precipitates) rice root segments were attached to a perfusion apparatus similar to that illustrated in Fig. 1. The

technique was based on a steady-state perfusion of 3 M HDO through aerenchyma of root segments, displacing air. The upper, open end of the root segment (used as an inlet) was attached to a small reservoir by a Teflon tube filled with 3 M HDO. The other end was kept open as an outlet. The root segment was placed 0.8 m (gravitational force = 0.008 MPa) below the small reservoir, and held vertically to allow perfusion of the solution by gravity. Such small forces allowed the measurement of diffusion of HDO across the OPR, i.e. the lateral water movement from aerenchyma to external well was nearly isobaric. The root segment was placed in a small chamber with known volume (5 ml) of nutrient solution and stirred continuously using a small pump to minimize the thickness of unstirred layers. At different time intervals, 50 µl of the external medium was taken out using a syringe, and the HDO concentration of each sample was measured by a freezing point osmometer (Ranathunge et al. 2004). The amount of HDO diffusing to the outer chamber was plotted against time. The solute/HDO flow across the OPR (J_{sOPR} in mol s^{-1} m^{-2}) was obtained, dividing the slope of the curve by the surface area of the root segment. ΔC_s (mol s^{-1}) was evaluated using the HDO concentration difference between the internal (perfused through aerenchyma) and external (diffused into the external chamber) media. The diffusional water permeability of the OPR (P_{dOPR}) was obtained according to Eq. 2:

$$P_{dOPR} = \frac{J_{sOPR}}{\Delta C_s}. \qquad (2)$$

Further details are given in Ranathunge et al. (2004). At the end of the experiment, root segments were randomly selected and stained with Evans blue to confirm the viability of cells in the OPR.

Measurement of the P_{dOPR} for $HgCl_2$ treated root segments

Prior to treatment with mercurials, P_{dOPR} was measured for control root segments using perfusion of aerenchyma with HDO as described above. In order to close the water channels (aquaporins) in cell membranes of the OPR, 50 µM $HgCl_2$ was added to the outer surface of rice root segments for 20 min (Carvajal et al. 1996; Henzler and Steudle 1995). Excess $HgCl_2$ was flushed from the outer surface of root segments with the aid of flowing distilled water. New P_{dOPR} values were obtained for root segments by

perfusing 3 M HDO through aerenchyma that had been treated with 50 μM $HgCl_2$. The toxicity of 50 μM $HgCl_2$ applied for 20 min to rice root segments was investigated by staining them with Evans blue as described above.

Photography

All photographs were taken either by using slide film, white-light illumination with Kodak Elite 64 ASA or using a digital camera (Sony-DSC-F505V, Sony Corporation, Tokyo, Japan).

Comparison of bulk/hydraulic water permeability with diffusional water permeability across the OPR

In order to compare the bulk/hydraulic water permeability (P_{fOPR}) across the OPR with diffusional water permeability (P_{dOPR}), the hydraulic conductivity of the OPR (Lp_{OPR}) was converted to P_{fOPR}, according to House (1974):

$$P_{fOPR} = \frac{Lp_{OPR} \times RT}{\overline{V_w}} . \qquad (3)$$

Here, $\overline{V_w}$ is the partial molar volume of liquid water. The P_{fOPR}/P_{dOPR} ratio was obtained directly; hence the units were m s^{-1} for both the P_{fOPR} and P_{dOPR}.

Results

Anatomy

Since there were no visible anatomical or developmental differences between cultivars (upland cv. Azucena and lowland cv. IR64), all pictures presented in this paper are only for cv. Azucena. Immature regions of rice roots (around 10 mm from the apex) consisted of closely packed flattened cells (Clark and Harris 1981; Ranathunge et al. 2004). However, it changed dramatically along the root, from apex to base, modifying its structure. As close as 20 mm from the root apex, cortical cells had partially collapsed to form large, air-filled spaces (aerenchyma) as a result of lysigeny (Evans 2003; Ranathunge et al. 2004). In both cultivars, aerenchyma formed as cells in the mid cortex died, which caused separation of the stele from the OPR. OPR consisted of four discrete layers. The outermost rhizodermis, hypodermis with Casparian bands, a layer of sclerenchyma and the innermost unmodified cortical cell layer. Casparian strips in the hypodermis started to develop approximately 30 mm from the root apex. Fluorol Yellow 088 stained complete suberin lamellae in the hypodermal cell walls at a distance of 50 mm from the root apex in both cultivars (Ranathunge et al. 2003; 2004).

Identification of precipitated salt crystals along the root axis

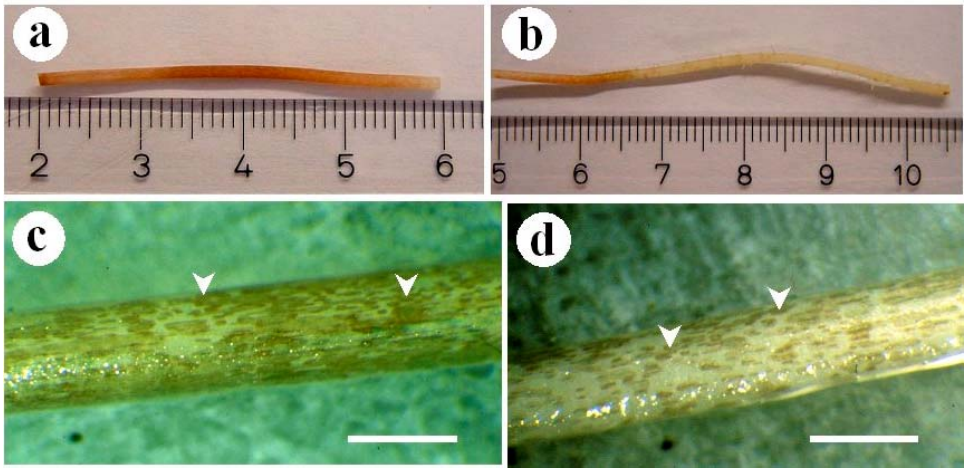

Fig. 2 Naked eyed (a,b) and stereo microscopic (c,d) views of the outer surface of rice root segments (cv. Azucena) after perfusion of aerenchyma with 1 mM $K_4[Fe(CN)_6]$ for 3 h. 0.5 mM $CuSO_4$ was used as the external medium. In all cases, copper ferrocyanide precipitates formed in cell walls of the inner unmodified cortical layer of the OPR (see Figs 4b,c), but were visible from outside. (a) Uniformly

distributed brown precipitates were observed throughout the immature (20-50 mm from the apex) root segments, (b) no precipitates were observed beyond 70-80 mm from the root apex in mature (50-100 mm) root segments. Stereo-microscopic observations showed brown precipitate patches on root surfaces, where the density was higher in immature (approximately 35 mm from the apex; c) and lower in mature (approximately 65 mm from the apex; d). Arrowheads show brown precipitate patches on the root surface. Bar = 1 mm.

Uniformly distributed brown precipitated crystals of $Cu_2[Fe(CN)_6]$ (= $Cu[CuFe(CN)_6]$; Holleman and Wiberg 1995) were observed on the surface of immature root segments (20-50 mm from the apex; Fig. 2a) after perfusing aerenchyma with 1 mM $K_4[Fe(CN)_6]$ for 3 h, while it was placed in a chamber filled with 0.5 mM $CuSO_4$. The brown colour gradually faded along the mature part of the root segment (between 50-80 mm from the apex) and it was not visible approximately 70-80 mm from the apex (Fig. 2b). In contrast, stereo microscopic observations showed irregular brown patches on the root surface, where the intensity and density were higher in immature (Fig. 2c) and lower in mature (Fig. 2d) root segments. Brown, precipitated salt crystals were observed as rings around the points where secondary roots emerged from the primary root (Fig. 3a), even when treated (perfusing aerenchyma with 1 mM $K_4[Fe(CN)_6]$ while keeping 0.5 mM $CuSO_4$ as the external medium) for a time as short as 1 h. Development of secondary roots from the pericycle (Fig. 3b) created regions of discontinuity in the endo- and hypodermal Casparian bands and regions of intense precipitates.

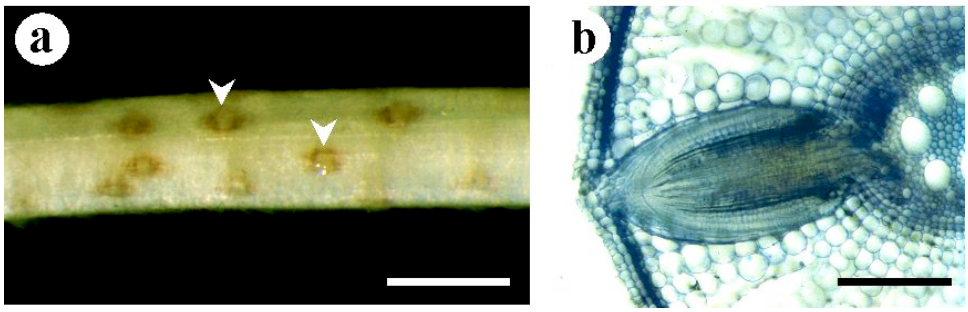

Fig. 3 Emergence of secondary roots from a primary rice root. (a) Brown copper ferrocyanide precipitates were observed as rings around the points where secondary roots emerge from the primary root (arrowheads). Here, aerenchyma was perfused by 1 mM $K_4[Fe(CN)_6]$ and 0.5 mM $CuSO_4$ was applied as the external medium for 1 h. Bar = 1 mm. (b) Emergence of a secondary root from a primary root as it breaks the OPR. Free-hand cross-section taken approximately 70 mm from the root apex, stained with toluidine blue O. Bar = 200 μm.

Precipitated salt crystals in cell walls of the OPR

Blue salt crystals of $K[Fe^{III}Fe^{II}(CN)_6]$ were observed in the walls of the inner unmodified cortical cells of the OPR (Fig. 4a) after perfusing the aerenchyma with $K_4[Fe(CN)_6]$ while the whole root segment was bathed in $FeSO_4$ medium. The bluish colour resulted from the fact that part of the Fe^{II} offered in the medium was oxidized to Fe^{III} thus causing the precipitation of "Berliner Blau" (Holleman and Wiberg 1995). But majority of precipitate was white coloured $Fe_2[Fe(CN)_6]$. Apparently, the diffusion of Fe^{II}/Fe^{III} into the aerenchyma *via* apoplastic pores (intermicrofibrillar spaces) was much faster than that of $Fe(CN)_6^{4-}$ (from the aerenchyma to the external chamber) because crystals were deposited in cell walls of the inner cortical layer of the OPR. Dense, rusty brown deposits characteristic of iron(III) hydroxide were seen in the walls and lumens of the rhizodermal cells (Fig. 4a). Because of the obvious iron(III) toxicity to the rhizodermal cells, such root segments were discarded without using for further permeability measurements.

Perfusion of root segments with $K_4[Fe(CN)_6]$ while they were bathing in $CuSO_4$ medium resulted in the development of brown, insoluble crystals of $Cu[CuFe(CN)_6]$ in cell walls (especially inner tangential) of the cortical layer (Fig. 4b,c). Dense, brown crystals were observed in the walls of cortical files of cells around the secondary roots. These cortical cell chains did not collapse but continued to expand in a radial direction (Fig. 4d). Cells of newly emerged secondary roots from the primary root were completely covered with brown precipitate (Fig. 4d). When salts were applied on opposite sides of the OPR, brown crystals were observed in cell walls of the rhizodermis and especially in outer tangential walls of the exodermis (Fig. 4e). Even though Cu^{2+} could pass through the immature hypodermal Casparian bands, this structure appeared to be rather impermeable to $Fe(CN)_6^{4-}$.

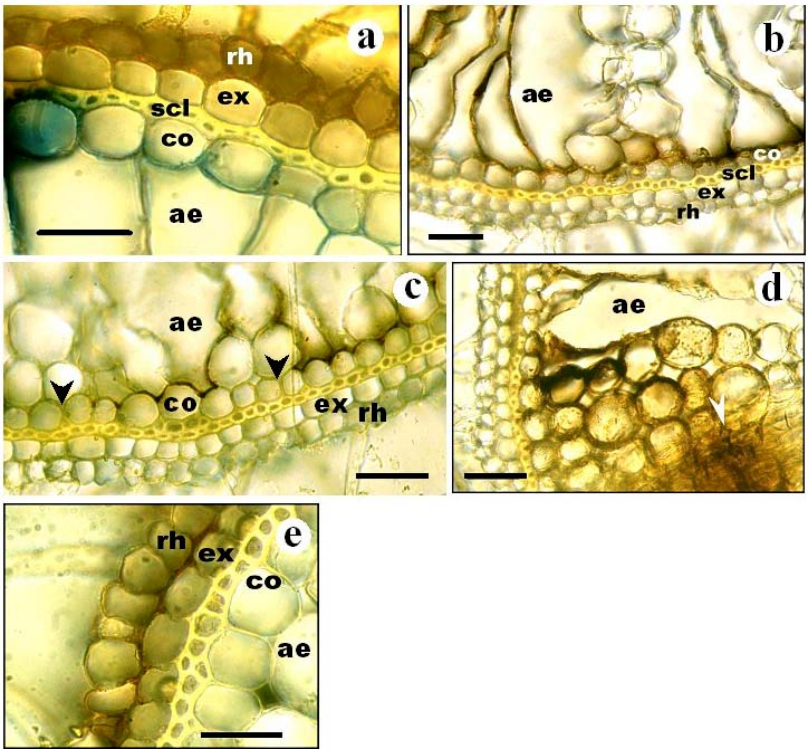

Fig. 4 (a-e) Free-hand cross-sections of rice root segments (cv. Azucena), made after blocking off the apoplast of the OPR with precipitated salt crystals. (a) Blue salt crystals of $K[Fe^{III}Fe^{II}(CN)_6]$ deposited in the walls of inner unmodified cortical cells as a result of perfusion of aerenchyma by 1 mM $K_4[Fe(CN)_6]$ and simultaneous application of Fe^{III} contaminated 1 mM $FeSO_4$ to the external medium for 3 h. Cross-section was made approximately 60 mm from the root apex. Heavy depositions of rusty brown oxidised iron (Fe^{III}) in the walls and cytoplasm of the outermost rhizodermis. (b-d) Brown precipitated salt crystals of $Cu_2[Fe(CN)_6]$ deposited in cell walls inner to the exodermal ring. Here, 1 mM $K_4[Fe(CN)_6]$ was perfused trough the aerenchyma and 0.5 mM $CuSO_4$ was applied to the outer medium for 3 h. Continuous brown precipitates in cell walls of the inner unmodified cortical cells of the OPR in immature root zones (b), but it was discontinuous in mature (c). Cross-sections were taken at 35 mm and 65 mm from the root apex, respectively. Arrowheads show places without precipitates. (d) Dense, brown precipitated crystals in cell walls where the secondary roots emerge breaking the outer surface of the primary root. Arrowhead shows the lateral root. (e) Brown precipitated copper ferrocyanide crystals in the apoplast (cell walls) of the rhizodermis as a result of simultaneous application of 0.5 mM $CuSO_4$ inside aerenchyma and 1 mM $K_4[Fe(CN)_6]$ into the external medium for 3 h. Cross-section was made approximately 40 mm from the root apex. Bar = 50 µm. rh = rhizodermis, scl = sclerenchyma, ex = exodermis, co = cortical cell layer, ae = aerenchyma.

Hydraulic/bulk water flow across the OPR

After fixing root segments to the pressure-perfusion pump, about 1.5-2 h of perfusion was required to obtain steady-state pressures. This is due to the large internal volume of

the system (compared to conducting area of the root segment), which dampens pressure changes. There was a linear relationship between pump rate (Q_v) and steady-state pressure (P). Further details are given in Ranathunge et al. (2003). Since water permeability across the OPR was temperature sensitive, measurements were done at constant temperature (25°C). Compared to whole roots or root systems, the OPR was quite permeable to water (Ranathunge et al. 2003). In controls, hydraulic/bulk water flow across the OPR (Lp_{OPR}) was similar for both cultivars (t-test, $P > 0.05$; Table 1). Even though there was a 20-25% reduction of the Lp_{OPR} in mature versus immature segments, it did not differ significantly along the root axis over the first 100 mm from the apex (Table 1; t-test, $P > 0.05$). This small reduction of the Lp_{OPR} did not correlate with maturation of Casparian bands or increased suberin deposits in the hypodermis either for IR64 or Azucena. In precipitation treatments, Lp_{OPR} measurements were only done for root segments containing salt crystals in walls of the inner cell layers (Fig. 4b,c). When crystals were only present in walls of the rhizodermis (Fig. 4e), segments were not employed for Lp_{OPR} measurements. In these roots, during perfusion under pressure, the bulk flow of water from the aerenchyma to the external medium tended to wash out precipitated crystals from intermicrofibrillar spaces of the cell walls.

Table 1 Hydraulic conductivity of the outer part of young rice roots (Lp_{OPR}) for control and treatment (blockage of the apoplastic pores by precipitates of copper ferrocyanide). Plants were grown for 30-40 d in aerated hydroponic culture. Measurements were performed using a pump perfusion technique. Means ± SD are given for two different root zones and two different cultivars, IR64 and Azucena. Number of measured roots is given in parenthesis.

Treatment	Hydraulic conductivity of the outer part of the root, Lp_{OPR} (10^{-7} m s^{-1} MPa^{-1})			
	IR64		Azucena	
	20–50 mm	50-100 mm	20-50 mm	50-100 mm
Control roots	14.8 ± 3.8 (15)a	11.4 ± 1.9 (15)a	12.5 ± 4.1 (15)a	9.0 ± 2.8 (15)a
Roots with precipitates	4.2 ± 1.3 (07)b	3.6 ± 1.1 (06)b	3.6 ± 1.0 (09)b	2.2 ± 0.9 (10)c
Average factor of reduction	**3.6 ± 1.2**	**3.2 ± 0.8**	**3.5 ± 1.3**	**4.1 ± 1.6**

Different letters indicate significant differences at $P = 0.05$ level. Statistical analysis was based on t-test (hence, lack of homogeneity of variances in samples, data were transformed to logarithmic scale, before running the t-test).

Compared with the control, blockage of apoplastic pores in cell walls of the OPR by insoluble crystals caused a 3- to 4-fold (66-75%) reduction of Lp_{OPR} in both cultivars and both root zones (Table 1). At a distance of 20-50 mm from the root apex, Lp_{OPR} decreased from (14.8 ± 3.8) to (4.2 ± 1.3) × 10^{-7} m s^{-1} MPa^{-1} in IR64 and (12.5 ± 4.1) to (3.6 ± 1.0) × 10^{-7} m s^{-1} MPa^{-1} in Azucena. Similar reductions were obtained for root segments taken 50-100 mm from the apex (Table 1).

Reflection coefficients of modified OPR

Reflection coefficients were measured with two different root zones (20-50 and 50-100 mm from the apex) for control and treated root segments. This involved altering water flow by changing the osmotic pressure of the outer medium by adding 20 mOsmol kg^{-1} (equivalent to 0.05 MPa of osmotic pressure) mannitol. Efflux of water (exosmosis) from root segments caused stationary pressures to decline at constant Lp_{OPR}. Comparison of the osmotic pressure applied with the change in hydraulic pressure gives the reflection coefficient ($\sigma_{sOPR} \equiv -Lp_{DOPR} / Lp_{OPR}$; Lp_{DOPR} = osmotic coefficient of the OPR).

Table 2 Reflection coefficient of the outer part of young rice roots (σ_{sOPR}) for the permeating solutes NaCl and mannitol. Mean values ± SD are given for two different root zones and two different cultivars, IR64 and Azucena for control and the treatment (blocking off the apoplastic pores in cell walls of the OPR with precipitates). Number of measured roots is in parenthesis. Plants were grown in aerated hydroponics for 30-40 d.

Rice cultivar	Reflection coefficient of the outer part of rice roots (σ_{sOPR})		
	Control roots*		Roots with precipitates
	NaCl	Mannitol	Mannitol
IR64			
20-50 mm	0.09 ± 0.02 (5)	0.13 ± 0.04 (6)a	0.24 ± 0.03 (5)b
50-100 mm	0.11 ± 0.03 (5)	0.13 ± 0.04 (6)a	0.26 ± 0.05 (5)b
Azucena			
20-50 mm	0.08 ± 0.02 (5)	0.15 ± 0.05 (8)a	0.26 ± 0.06 (5)b
50-100 mm	0.09 ± 0.01 (5)	0.14 ± 0.10 (7)a	0.28 ± 0.04 (5)b

Different letters indicate significant differences at $P = 0.05$ level. Statistical analysis was based on *t*-test (data distributed normally). *Ranathunge et al. 2003.

Table 2 summarizes reflection coefficients of the OPR of young rice roots. As shown by Ranathunge et al. (2003), the σ_{sOPR} did not change along the root axis (*t*-test; $P > 0.05$).

Clogging apoplastic pores in the OPR caused an increase in the σ_{sOPR} for mannitol by a factor of about 2 in both immature and mature root zones. As observed in the control, the σ_{sOPR} for mannitol did not change along the root axis (*t*-test; $P = 0.05$).

Investigation of viability of cells in the OPR

Since the experiments were time consuming (approximately 10-12 h), it was essential to check the viability of the cells, namely in the presence Cu^{2+} which may be toxic to plant cells at mM concentrations (Murphy et al. 1999). Evans blue is a non-penetrating dye in living cells; it cannot cross an intact membrane (Taylor and West 1980). In living cells, the cytoplasm and nuclei did not stain (Fig. 5a). If cells were dying or dead, the plasma membrane should be leaky allowing the dye to diffuse into the cell. Nuclei and cytoplasm of dead cells were stained dark blue (Fig. 5b). Results confirmed that the cells in the root segments used for permeability measurements were alive.

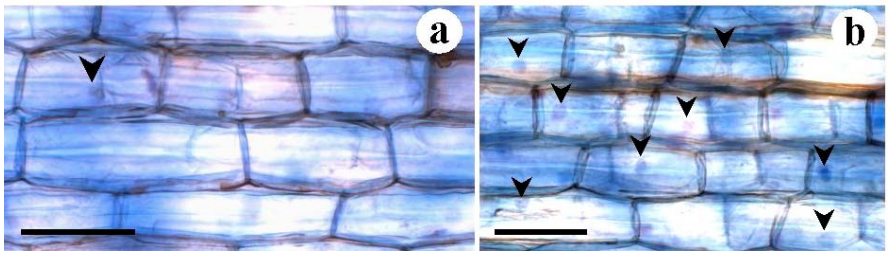

Fig. 5 Free-hand longitudinal sections of rice roots (cv. Azucena), taken approximately 60 mm from the root apex, stained with Evans blue to check the viability of cells in the outer part of the root at the end of the pump perfusion experiment. In dead cells, nuclei were stained blue (arrowheads). (a) A few dead cells (<5%) were observed at the end of the pump perfusion experiment (8-10 h after excising from the intact plant). (b) Number of dead cells was more than 70%, 36 h after excising from the intact plant. Bar = 50 µm.

Diffusional water permeability of the OPR (P_{dOPR})

Vertical perfusion of root segments (aerenchyma) by heavy water (HDO) was performed for root zones at 20-50 and 50-100 mm from the apex for the control and two different treatments, i.e. either by blocking off apoplastic pores in the OPR with salt crystals or closing the membrane water channels with 50 µM $HgCl_2$. Amounts of HDO that diffused into the external chamber increased linearly with time (Fig. 6). Both treatments (precipitates and $HgCl_2$) reduced the diffusion of HDO into the external

medium compared with that of the control. At any time and up to 200-250 min from the start, the external HDO concentration was substantially smaller than the concentration of perfused HDO in the aerenchyma. Hence, the concentration of HDO perfused through the aerenchyma was used as the driving force in equation 2.

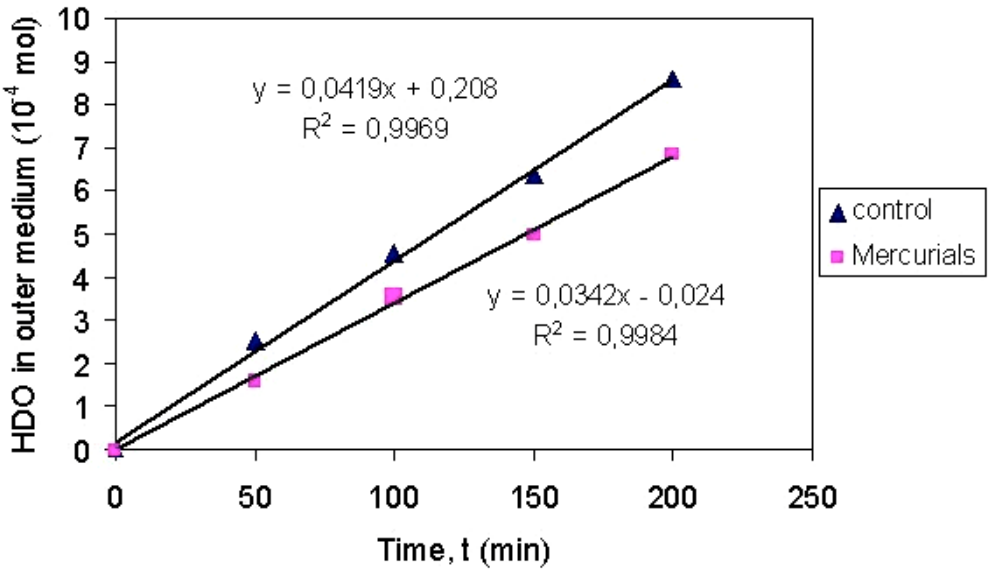

Fig. 6 Increases of HDO concentration in the external medium with time for root segments of control and treatment (addition of 50 μM $HgCl_2$ to the outer surface of the root segment for 20 min, aiming to close the water channels in membranes of the OPR). Root segment was taken at 20-50 mm from the root apex (cv. Azucena). The amount of HDO diffused into the outer medium was reduced by about 20-30% after the treatment.

The diffusive water permeability of immature root segments, which had been excised at 20-50 mm from the root apex, had significantly higher values than that of mature (50-100 mm) root zones as shown in Table 3 (*t*-test; $P < 0.05$). Those values were same for both cultivars. Even though blockage of apoplastic pores with insoluble salt crystals reduced the P_{dOPR} compared to the control, treatments did not cause significant changes at $P = 0.05$ level. However, there was a large variation between root segments. When results were presented as ratios, blockage of apoplastic path reduced the P_{dOPR} by about 20% in Azucena for both root zones. For IR64, reductions were 12% at 20-50 mm and 23% at 50-100 mm, respectively (Table 3).

Table 3 Diffusional water permeability of the outer part of rice roots (P_{dOPR}), measured by rapidly perfusing aerenchyma of root segments with near-isobaric water (HDO) in control and after blockage of the apoplastic pores of the OPR with precipitates. Plants were grown in aerated hydroponics for 30-40 d. P_{dOPR} is given for two different distances from the root apex and two cultivars, IR64 and Azucena. P_{dOPR} significantly decreased along the root from the apex at $P = 0.05$ level. The blockage of apoplastic pores of the OPR with precipitates did not significantly decrease P_{dOPR} at $P = 0.05$. Values are means ± SD with the number of measured roots in parenthesis.

Type of the treatment	Diffusional water permeability of the OPR (P_{dOPR}); 10^{-7} m s^{-1}			
	IR64		Azucena	
	20–50 mm	50-100 mm	20-50 mm	50-100 mm
Control (A)	3.6 ± 0.6 (7)a	1.5 ± 0.4 (6)b	3.4 ± 0.7 (7)a	1.3 ± 0.4 (6)b
Roots with precipitates (B)	2.9 ± 0.7 (6)a	1.2 ± 0.5 (6)b	3.0 ± 0.9 (6)a	1.0 ± 0.5 (6)b
(B)/(A) ratio	**0.81 ± 0.24**	**0.80 ± 0.39**	**0.88 ± 0.32**	**0.77 ± 0.44**

Different letters indicate significant differences at $P = 0.05$ level. Statistical analysis was based on *t*-test (data distributed normally).

For both cultivars and root zones, closure of water channels in cell membranes of OPR with 50 µM $HgCl_2$ caused approximately 30% reduction of P_{dOPR} (Table 4). Toxicity of $HgCl_2$ was tested with Evans blue stain. Cells of the central cortex and the rhizodermis in the OPR were alive even when treated with 50 µM $HgCl_2$ for 20 min (data not shown).

Table 4 Diffusional water permeability of the outer part of rice roots (P_{dOPR}) measured with near-isobaric water (HDO) for control and after treating with 50 µM $HgCl_2$ for 20 min. P_{dOPR} is given for two different root zones and two cultivars, IR64 and Azucena. Values are means ± SD and number of measured roots (*n*) is six. Treatment (50 µM $HgCl_2$)/control ratios were calculated to remove variabilities between roots and the reduction of P_{dOPR} is given as a percentage.

Rice cultivar	Diffusional water permeability of the OPR (P_{dOPR}); 10^{-7} m s^{-1}		50 µM $HgCl_2$ / control ratio	Reduction as a %
	Control	50 µM $HgCl_2$		
IR64				
20-50 mm	3.2 ± 0.9	2.3 ± 0.5	0.71 ± 0.07	**29 ± 07**
50-100 mm	1.5 ± 0.5	1.2 ± 0.4	0.74 ± 0.15	**26 ± 15**
Azucena				
20-50 mm	3.4 ± 0.6	2.4 ± 0.8	0.68 ± 0.15	**32 ± 15**
50-100 mm	1.6 ± 1.0	1.3 ± 0.9	0.74 ± 0.11	**26 ± 15**

Bulk/Hydraulic *versus* diffusive water permeability of the OPR

Using equation 3, Lp_{OPR} values obtained by pressure perfusion were converted to osmotic/hydraulic water permeability (P_{fOPR}) to compare bulk and diffusive (P_{dOPR}) water permeabilities in the same units. There were huge differences between P_{fOPR} and P_{dOPR} of roots from both cultivars (Table 5). The P_{fOPR} was about 600 times greater than the P_{dOPR} in immature root zones (20-50 mm). It was larger by a factor of 1200 in mature root zones (50-100 mm). Blockage of apoplastic pores with salt crystals caused a three- to five-fold reduction of P_{fOPR}/P_{dOPR} ratio in both rice cultivars and both root zones indicating that the treatment with salt crystals did reduce $Lp_{OPR}(P_{fOPR})$ substantially more than P_{dOPR}.

Table 5 Ratios between hydraulic/osmotic water permeability (P_{fOPR}) and diffusional water permeability of the OPR (P_{dOPR}) for control and the treatment (roots with precipitates). Plants were grown in aerated hydroponics for 30-40 d. Prior to calculating the ratios, hydraulic conductivity of the OPR (Lp_{OPR}; m s^{-1} MPa^{-1}), was converted to hydraulic/osmotic water permeability (P_{fOPR}), which has the same units (m s^{-1}) as diffusional water permeability (P_{dOPR}). P_{fOPR} values are means ± SD with the number of measured roots in parenthesis. P_{fOPR}/P_{dOPR} ratios are given for two different root zones and two rice cultivars, IR64 and Azucena. Blocking off the apoplastic pores of the OPR with precipitates reduced the P_{fOPR}/P_{dOPR} ratio by a factor of three to five relative to the control.

Rice cultivar	Hydraulic water permeability of the OPR (P_{fOPR}) (10^{-7} m s^{-1})		P_{fOPR} / P_{dOPR} Ratios	
	Control	Roots with ppt.	Control	Roots with ppt.
IR64				
20-50 mm	2124 ± 498 (7)a	573 ± 113 (6)b	607 ± 163	212 ± 68
50-100 mm	1681 ± 179 (7)a	483 ± 145 (6)b	1200 ± 360	402 ± 180
Azucena				
20-50 mm	1782 ± 545 (7)a	491 ± 142 (9)b	594 ± 236	144 ± 56
50-100 mm	1265 ± 355 (7)a	296 ± 69 (8)c	1265 ± 407	227 ± 45

Different letters indicate significant differences at $P = 0.05$ level. Statistical analysis was based on the *t*-test (hence, lack of homogeneity of variances in samples, data were transformed to logarithmic scale before running the *t*-test).

Discussion

According to authors' best knowledge, this is the first study in which inorganic salt precipitates have been successfully used to block off intermicrofibrillar spaces in the apoplast of living tissue and to measure responses in water transport. In a different approach, Enstone and Peterson (1992) introduced berberine hemisulphate and potassium thiocyanate sequentially into the xylem to check the permeability of endodermal Casparian bands of onion, corn and broad bean roots. The present technique was based on Wilhelm Pfeffer's successful development of artificial semi-permeable precipitation membranes using cells with clay diaphragms to verify van't Hoff's theory of osmosis (van't Hoff 1887; Pfeffer 1921). Pfeffer used precipitation membranes (invented by Traube, 1867) of insoluble copper ferrocyanide, which clogged the pores within the diaphragms. In Pfeffer's experiments, diluted solutions of $CuSO_4$ and $K_4[Fe(CN)_6]$ were placed on either side of the diaphragms. When we used the technique with living tissue, great care was given to check the toxicity of the chemical salts (in millimolar concentrations) to living cells. The $FeSO_4/K_4[Fe(CN)_6]$ treatment was discarded because of the toxicity of iron to living cells, even when used in millimolar concentrations (Dobermann and Fairhurst 2000). In contrast, the reaction between 0.5 mM $CuSO_4$ and 1 mM $K_4[Fe(CN)_6]$ produced small, insoluble salt crystals of $Cu_2[Fe(CN)_6]$ or $Cu[CuFe(CN)_6]$ which could successfully clog pores with average diameters of 5-30 nm (Nobel 1999) in cell walls of the OPR. The solubility product of $Cu_2[Fe(CN)_6]$ at 25°C is 1.3×10^{-16} M^3 (Hill and Petrucci 1999). So, the concentration of Cu^{2+} and $[Fe(CN)_6]^{4-}$ ions in a solution of pure water containing a precipitation of $Cu_2[Fe(CN)_6]$ would be 8.04 µM and 4.02 µM, respectively. Thus, the concentrations of applied salts during the experiment should have been sufficient even to produce precipitates in the apparent free spaces in the apoplast, because we offered much higher concentrations than required (0.5 mM and 1 mM for Cu^{2+} and $[Fe(CN)_6]^{4-}$, respectively). It was shown that salts and crystals did not affect the viability of root cells during treatments.

Blockage of the apoplast by insoluble salt crystals (precipitates) reduced L_{OPR} by a factor of three to four. However, blockage may have not been complete. It cannot be ignored that a small amount of water moved radially along the continuum of middle

lamellae where the access of Cu^{2+} and $[Fe(CN)^6]^{4-}$ may have been limited. Groh et al. (2002) presented evidence that 2-4% of water could move along the middle lamellae of isolated phellems of trees. The present results imply that at least two-thirds to three-quarters of water is crossing the OPR apoplastically. Although segments taken from a distance of 50-100 mm from the tip were less stained than those from 20-50 mm, the percent-reduction in Lp_{OPR} was similar indicating that the contribution of apoplastic flow was less in more mature parts where the exodermis was mature. It would be interesting to see changes at older regions of the root; these have not yet been measured. In the range of up to 100 mm, the relative contribution of the apoplast is larger than the cell-to-cell path despite the existence of apoplastic barriers in the OPR such as Casparian bands and lignified walls of sclerenchyma (Ranathunge et al. 2003).

The present results agree with our previous findings that blockage of the apoplast with ink particles reduced the overall hydraulic conductivity of the OPR. However, blockage with ink particles resulted in a reduction of only 30%; this was much less than with that by precipitates. The present reduction was much more pronounced than that caused by the water channel blocker $HgCl_2$. Closure of water channels in the membranes of the OPR reduced the Lp_{OPR} by only 10% (Ranathunge et al. 2004). Although the closure of water channels by $HgCl_2$ may have been incomplete, the present results do indicate a dominating apoplastic water flow across the OPR rather than a cell-to-cell flow. This agrees with earlier observations of rice roots, which indicated a substantial apoplastic component for NaCl and PTS (Yadev et al. 1996; Yeo et al. 1987; see Introduction). Root anatomical features support the view of a non-dominant cell-to-cell water flow across the OPR. The symplasmic continuum could be limited by formation of a suberized exodermis (Ranathunge et al. 2004) as well as scarcity of pitting and plasmodesmata in the sclerenchymatous layer (Clark and Harris 1981). Nevertheless, it has been shown that the endodermis represents the major hydraulic barrier, which is due to its strong suberization (Miyamoto et al. 2001; Ranathunge et al. 2003). Most of the water channels might be concentrated in the endodermis or the stele, as found for other species (Schäffner 1998).

There was a striking asymmetry in the development of precipitates. They only occurred on the side where ferrocyanide was added suggesting that Cu^{2+} and SO_4^{2-} rather than

$[Fe(CN)_6]^{4-}$ were passing the barrier including Casparian bands of the hypodermis (when not completely matured). The reason may be that, unlike $CuSO_4$, ferrocyanide with its four negative charges moved very slowly across the barrier. In view of the general attitude that Casparian bands of the hypodermis are completely impermeable for ions, the finding of a considerable permeability of Casparian bands to CuSO4 is unusual. One may argue that the permeation properties of the exodermis of the OPR of rice may be unusual because we used hydroponically grown rather than soil-grown rice. Experiments with the latter and with other cereals (corn) are under way to check the permeability of their exodermis using the precipitation technique. Currently, the technique is also being used for the endodermis of rice and for species lacking aerenchyma.

At a distance of up to 70-80 mm from the apex, apoplastic salt precipitates revealed a somewhat patchy structure of the exodermis (Figs 2c,d). However, our histochemical studies failed to show hypodermal passage cells lacking suberin lamellae. The patchiness might be correlated with the maturation of Casparian bands in the hypodermis, which was not uniform. Some of the hypodermal Casparian bands of hydroponically grown rice stained with fluorescent dye berberine may have been not completely functional and did not completely block off ions and water (Ranathunge et al. 2003). However, from an anatomical point of view, stained bands did look like intact Casparian strips. Further investigations are required to confirm the idea that Casparian bands in the exodermis of rice roots have an unusual high permeability. By using other combinations of salts (ions) which form precipitates (e.g. of $BaSO_4$ or Ca-oxalate), the idea should be followed further. Perhaps, the unusual high permeability of Casparian bands is due to a different chemical composition as compared with bands of other species. For example, when lignin is a major chemical component of bands rather than suberin, the high permeability to polar solutes may be understandable (Schreiber 1996; Zeier and Schreiber 1998). A chemical analysis of bands is required.

It has been postulated that places where secondary roots emerge from primary roots are leaky to water and solutes (Peterson et al. 1981). In this study, dense precipitates have been observed around secondary roots. These places may act as ‘open doors’ for relatively free movement of water and nutrient ions. This is in agreement with Peterson

and co-workers who used the apoplastic tracer PTS (Peterson et al. 1981). Zimmermann and Steudle (1998) observed that the radial PTS flow into the xylem was similar in exodermal and non-exodermal corn roots suggesting a rate limitation at the endodermis. In mature rice root segments, local disruptions of the exodermis when developing secondary roots from the pericycle are most likely to allow for high apoplastic bypasses. When secondary roots mature, we observed that bypasses were healed in more basal parts of the roots. No precipitates were observed around mature secondary roots situated beyond 150 mm from the apex (data not shown).

The picture of a dominating apoplastic rather than cell-to-cell path for bulk water flow across the OPR was consistent with low reflection coefficients ($\sigma_{sOPR} \approx 0.1$; Table 2), which were smaller by a factor of two to three than those of whole roots ($\sigma_{sr} = 0.20$ to 0.30 for NaCl; Ranathunge et al. 2003). Apoplastic blockage by precipitates caused an increase of σ_{sOPR} by a factor of two for mannitol. This is smaller than one would expect according to the composite transport model of the root assuming a $\sigma_s^{cc} \approx 1$ for the cell-to-cell passage (semipermeable membranes; Steudle and Frensch 1996). The small effect is not completely understood, but may be related to the fact that precipitates especially blocked the pores in tangential cell walls of the OPR rather than radial and transverse as observed in cross-sections (Figs 4b,c). Therefore, there was also an effect on the cell-to-cell passage. According to the composite transport model, this may have added up to the overall σ_{sOPR}, which was smaller than one would expect at first glance.

Unlike Lp_{OPR}, the P_{dOPR} significantly decreased along the root axis. Development of exodermal suberin lamellae may impede plasma membrane and restrict HDO diffusion across membranes of the OPR. These are barriers for water and ion movement from the apoplast to the symplast, and vice versa, of individual cells (Peterson and Cholewa 1998). The reduction of P_{dOPR} along the root nicely correlated with the increment of rhizodermal-exodermal suberin along rice roots (data not shown). Unlike $Lp_{OPR,}$ the reduction of P_{dOPR} was in the same range for both treatments used (either blockage of apoplastic pores with precipitates or closure of water channels with 50 μM $HgCl_2$). Development of precipitates was not uniform in cell walls. Dense precipitates were observed in the tangential walls (Figs 4b,c). Less or no precipitates were found in the radial and anticlinal cell walls. So it is clear that precipitates in the cell walls could not

completely block the diffusion of HDO across membranes and allowed a flow of HDO at the direction of the radial and anticlinal cell walls resulting in a lower effect than expected.

The strongest evidence in favour of a predominantly apoplastic water flow came from comparing the ratios between P_{fOPR} and P_{dOPR} ($P_{fOPR} = Lp_{OPR} \times RT/V_w$). For a pore-less membrane, such as an oil film, the P_f/P_d ratio is around 1. In particular, if P_f/P_d is significantly greater than 1, one can surmise that water crosses a porous path (Finkelstein 1987). In *Chara*, >90% of the water moves through water channels, which represent single-file-pores, and the P_f/P_d ratio is around 40 (Henzler et al. 2004). The P_{fOPR}/P_{dOPR} ratios for rice roots were as large as 600–1200, depending on the position of the root (Ranathunge et al. 2004). This provided strong evidence that water moved through a fairly porous path. Blockage of the apoplastic path by precipitates caused a 3-5-fold reduction of P_{fOPR}/P_{dOPR} ratio (Table 5) indicating that the treatment did significantly affect the bulk rather than diffusive water flow. Solutions in both compartments (inside aerenchyma and external medium) were well stirred, so these large ratios could not be obtained due to unstirred layers, which mainly affect P_d rather than P_f. Effects of unstirred layers of solution at the inner and outer surfaces can be corrected according to Finkelstein (1987), i.e. $1/(P_{dOPR})_{obs} = 1/(P_{dOPR})_{cor} + \delta^1/D_w + \delta^2/D_w$, where the resistances are in series. Here, $(P_{dOPR})_{obs}$, $(P_{dOPR})_{cor}$ = observed and corrected permeability coefficients, D_w = self diffusion coefficient of HDO (24×10^{-10} m^2 s^{-1}), δ^1, δ^2 = thickness of inner and outer unstirred layers (pessimistic assumption of 50 μM each used). The observed values underestimated the corrected value by less than 2% in both root zones as well as in both control and treatment. Hence, large ratios were not due to unstirred layers, and did reflect an intrinsic property of the composite barrier.

We show for the first time that salt precipitates in the apoplast of the OPR of rice roots substantially affect the hydraulic conductivity of this tissue. Despite the existence of an exodermis with Casparian bands, the results indicate a preferred flow of water around cells rather than a cell-to-cell passage. At least in the immature parts of the roots, charged ions such as Cu^{2+} or SO_4^{2-} could pass through the exodermis and sclerenchymatous layer. Asymmetrical development of precipitates suggested that Cu^{2+} and SO_4^{2-} moved faster than $Fe(CN)_6^{4-}$ across the exodermis and sclerenchyma layer.

There was a patchiness of the formation of precipitates, which may correlate with the maturation of the exodermis. However, in mature parts, places of emergence of secondary roots were fairly leaky to both water and ions. Blockage of the apoplastic pores with precipitated salt crystals caused a massive three- to four-fold reduction of Lp_{OPR} suggesting that there were prominent apoplastic bypasses. This finding was in line with increments of σ_{sOPR} in response to the blockage of apoplastic path with precipitates. Huge P_{fOPR}/P_{dOPR} ratios suggested a predominant apoplastic water flow across the OPR as well. Blockage of apoplastic path with precipitates caused a three- to five-fold reduction of P_{fOPR}/P_{dOPR} ratios and the treatment significantly affected the bulk water flow rather than the diffusive water flow.

Acknowledgements

The authors thank Prof. C.A. Peterson and Chris Meyer (University of Waterloo, Ontario, Canada), and Prof. D.T. Clarkson (University of Bristol, UK) for their thoughtful comments that helped to improve the manuscript. The excellent technical support of Burkhard Stumpf (Department of Plant Ecology, University of Bayreuth) is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft: Ste319/3-3 (ES), and BMZ (project No. 2000.7860.0-001.00) ″Trait and Gene Discovery to Stabilize Rice Yields in Drought Environments“ (RL)

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5 The chemical composition of suberin in apoplastic barriers affects radial hydraulic conductivity differently in the roots of rice (*Oryza sativa* L. cv. IR64) and corn (*Zea mays* L. cv. Helix)

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Received: 21 October 2004; Accepted: 23 February 2005

Journal of Experimental Botany (2005) 56: 1427-1436
doi:10.1093/jxb/eri144

Abstract

Apoplastic transport barriers of rice (*Oryza sativa* L. cv. IR64) and corn (*Zea mays* L. cv. Helix) roots were isolated enzymatically. Following chemical degradation (monomerization, derivatization), amounts of aliphatic and aromatic suberin monomers were analyzed quantitatively by gas chromatography and mass spectrometry. In corn, suberin was determined for isolated endodermal (ECW) and rhizo-hypodermal (RHCW) cell walls. In rice, the strong lignification of the central cylinder (CC), did not allow isolation of endodermal cell walls. Similarly, exodermal walls could not be separated from the rhizodermal and sclerenchyma cell layers. Suberin analyses of ECW and RHCW of rice, thus, refer to either the entire CC or to the entire outer part of the root (OPR), the latter lacking the inner cortical cell layer. In both species, aromatic suberin was mainly composed of coumaric and ferulic acid. Aliphatic suberin monomers released from rice and corn belonged to five substance classes: primary fatty acids, primary alcohols, diacids, ω-hydroxy fatty acids, and 2-hydroxy fatty acids with ω-hydroxy fatty acids being the most prominent substance class. Qualitative composition of aliphatic suberin of rice was different from that of corn; (i) it was much less diverse, and (ii) besides monomers with chain lengths of C_{16}, a second maximum of C_{28} was evident. In corn, C_{24} monomers represented the most prominent class of chain lengths. When total amounts of suberin were related to surface areas of the respective tissues of interest (hypodermis and/or exodermis and endodermis), exodermal cell walls of rice contained, on average, six-times more aliphatic suberin than in corn. In endodermal cell walls, amounts were 34-fold greater in rice than that of corn. Significantly higher amounts of suberin detected in apoplastic barriers of rice corresponded with substantially lower root hydraulic conductivity (Lp_r) compared to corn, when water flow was driven by hydrostatic pressure gradients across the apoplast. As the OPR of rice is highly porous and permeable to water, it argued that this holds true only for the endodermis. The results imply that some caution is required when discussing the role of suberin in terms of an efficient transport barrier for water. The simple view that just the amounts of suberin play the important role, may not hold. A more detailed consideration of both the chemical nature of suberins and of the microstructure of deposits is required, i.e., how suberins impregnate wall pores.

Key words: Apoplast, Cell wall, Endodermis, Hydraulic conductivity, Subrrin, Water transport.

Abbreviations: AE, aerenchyma; CC, isolated central cylinders; CO, cortical cells; ECW, isolated endodermal cell walls; EN, endodermis; EX, exodermis; HY, hypodermis; Lp_{OPR}, hydraulic conductivity of the outer part of rice roots; Lp_r, hydraulic conductivity of end segments of rice or corn roots; OPR, outer part of rice root; RH, rhyzodermis; RHCW, isolated rhizodermal and hypodermal cell walls; SCL, sclerenchyma cells.

Introduction

Hydraulic properties of roots largely vary between species (Kramer and Boyer 1995). They are strongly affected by environmental conditions, which result in changes in root anatomy and morphology (Steudle and Peterson 1998). To explain variable water uptake, a composite transport model has been set up, which includes physical as well as physiological elements (Steudle, 2000, 2001). According to the model, there are three parallel pathways of water uptake from the soil solution into the central cylinder of the root: (i) the apoplastic pathway around protoplasts, (ii) the symplastic pathway through plasmodesmata, and (iii) the transcellular pathway with the water molecules moving from one living cell to the next by crossing two plasma membranes and part of the apoplast at each cell layer. The symplastic and transcellular components together are usually considered as the cell-to-cell path, since it is, to date, not possible to separate them experimentally. Along the cell-to-cell path, water transport may be regulated in terms of an expression and/or activation of aquaporins and development of suberin lamellae (Peterson and Cholewa 1998; Tyerman *et al*., 1999). The hydraulic conductivity of the apoplastic path may be decreased by the deposition of suberin in Casparian bands in the cell wall (Zimmermann *et al*., 2000).

Chemically, apoplastic barriers of roots are depositions of the biopolymers lignin and suberin within the cell wall matrix, which may occlude wall pores previously filled with water (Schreiber *et al*., 1999). Among the different substances, aliphatic suberin rather than lignin or aromatic suberin has the most pronounced effect on the barrier function of biopolymers (Schreiber *et al*., 1999). In the endodermis of primary roots, the deposition of apoplastic barriers first occurs when Casparian bands form in the primary state of endodermis (for a review see Ma and Peterson 2003). Later, many species form suberin lamellae at the inner tangential surfaces of endodermal cells (with the exception of the passage cells; secondary developmental state of endodermis). Frequently, apoplastic barriers are found in the hypodermis of roots (Hose *et al*., 2001). Either suberin lamellae are deposited at the inner surfaces of hypodermal cell walls, or even Casparian bands are formed prior to the deposition of lamellae. A hypodermis with Casparian bands is called an exodermis (Peterson and Perumalla 1984).

Environmental stresses i.e. drought and salt stress, as well as growth conditions, intensify the formation of apoplastic barriers in roots (North and Nobel 1994; Radin and Matthews 1989; Reinhardt and Rost 1995). For example, cultivation of corn seedlings in aeroponic induced an exodermis in primary roots which is not expressed in hydroponic culture (Zimmermann *et al*., 2000). In turn, water uptake was substantially reduced. Hence, 'root hydraulics' are efficiently controlled or are even regulated by modifying the hydraulic resistance along the apoplastic and cell-to-cell pathways by the deposition of the hydrophobic biopolymer suberin.

When rice is grown in paddy fields, one would expect that its hydraulics would not limit water uptake. However, this is not so. In rapidly transpiring shoots of field-grown rice, a water shortage has been observed, even when roots were exposed to a medium fully saturated with water (Hirasawa *et al*., 1992; 1996). The explanation for this observation is that radial water transport across rice roots is limiting, and that high demands for water from the shoot were not met by the supply from the root. This hypothesis is supported by the fact that there are pronounced apoplastic transport barriers in rice roots, i.e. well developed exodermal and endodermal cell layers in addition to a lignified layer of sclerenchyma cells at the inner side of the exodermis (Ranathunge *et al*., 2003). Not surprisingly, the measured overall radial hydraulic conductivity of rice roots was significantly lower than that of roots of other cereals such as corn (Miyamoto et al. 2001).

In this investigation, we aim to provide more detailed information on the structural reasons for the differences between in the hydraulics of roots of rice and corn. The chemical composition of endodermal and exodermal cell walls of corn roots has been worked out in great detail (Zeier *et al*., 1999). To date, nothing is known about the chemical composition of apoplastic barriers in rice roots. Corn was selected for comparison, because (i) it is a closely related crop species, but cultivated under completely different conditions. (ii) Corn roots also exhibit depositions of suberin in endodermal and hypodermal cell walls despite having a different root anatomy from that of rice (Zeier *et al*., 1999). (iii) It has been shown that environmentally induced changes in the composition of apoplastic barriers influence radial hydraulic conductivity of water in roots (Zimmermann *et al*., 2000). Chemical analyses have been related to

changes in the hydraulic conductivity of both species to answer the question whether or not the lower hydraulic conductivity of rice roots can be explained by differences in the density and/or chemical composition of apoplastic barriers.

Materials and methods

Plant materials and cultivation

Seeds of rice (*Oryza sativa* L. cv. IR 64; International Rice Research Institute, Manila, Philippines) were germinated for 5 days on wet filter paper in the light at 27°C. Cultivation of seedlings was continued for another 35 d in climatic chambers on an aerated hydroponic culture system with 12 h light (500 µM m^{-2} s^{-1} of PAR) and temperatures varying between 27°C during the day and 22°C at night. The nutrient solution was replaced every week. It contained (in mM): 0.09 $(NH_4)_2SO_4$, 0.05 KH_2PO_4, 0.05 KNO_3, 0.03 K_2SO_4, 0.06 $Ca(NO_3)_2$, 0.07$MgSO_4$, 0.11 Fe-EDTA, and the micronutrients (in µM) 4.6 H_3BO_4, 1.8 $MnSO_4$, 0.3 $ZnSO_4$ and 0.3 $CuSO_4$.

Seeds of corn (*Zea mays* L. cv. Helix; Kleinwanzlebener Saatzucht AG, Kleinwanzleben, Germany) were germinated for 5 d on wet filter paper in the dark. Cultivation was continued for another 7 days in climatic chambers on an aerated hydroponic culture system (14 h light; PAR: 500-600 µM m^{-2} s^{-1}) and day/night temperatures of 20/ 17°C. The nutrient solution contained (in mM): 0.7 K_2SO_4, 0.1 KCl, 2 $Ca(NO_3)_2$, 0.5 $MgSO_4$, 0.1 KH_2PO_4, with the micronutrients (in µM) 1 H_3BO_4, 0.5 $MnSO_4$, 0.5 $ZnSO_4$, 0.2 $CuSO_4$, 0.01$(NH_4)_6Mo_7O_{24}$, and 200 Fe-EDTA.

Roots of 12-d-old corn plants had an average length of 0.26 m. Roots of 40-d-old rice plants were 0.45 m long. For chemical analysis, roots of both species were divided into two different zones. In younger root zones (zone I), laterals were not yet (corn) or were not very frequently (rice) emerged. In older root zones (zone II), both species had well developed laterals. Average lengths of zone I of corn and rice roots were 0.11 m and 0.06 m, respectively. Average lengths of zone II of corn and rice roots were 0.15 m and 0.39 m, respectively.

Light microscopy

Freehand cross-sections were made at different distances from the root tip with both species. To examine the developmental stages of rice endodermis, cross sections were taken at 30, 50, 100 and 200 mm from the root tip. Since exodermis of rice mature within short length of the root, to check the exodermis, cross sections were made at 30 and 60 mm from the tip. As did for rice, for the corn endodermis, cross sections were taken at 50, 60, 100 and 150 mm and for the corn hypodermis at 60 and 120 mm. Sections were stained with Sudan Red 7B at room temperature for 1.5 h to visualize the suberin lamellae and to count the passage cells (Brundrett et al. 1991). Sections were examined using a light microscope (DIALUX 22 EB, Leitz, Germany) and photographed using Kodak Elite 64 ASA film.

Cell wall isolation and preparation

About 20-50 g fresh weight (FW) of material from either zones I or II of roots of rice and corn were incubated separately in enzymatic solutions of cellulase (Onozuka R-10, Serva) and pectinase (Macerozyme R-10, Serva) in citric buffer (0.01 M) adjusted to pH 3.0. After several days, cell walls, which had resisted the enzymatic attack, could be sampled under a binocular using a forceps. By pulling, central cylinders of corn roots could be separated from cortical sleeves, and endodermal cell walls (ECW) enclosing xylem vessels could successfully be isolated from the rest of the stele as well (Zeier *et al.*, 1999). Rhizodermal and hypodermal cell walls (RHCW) of corn roots could not be separated from each other and were always isolated and analyzed together. Isolated wall samples were washed in borate buffer (0.01 M, pH 9.2), dried and stored over silica gel.

Cell walls of the central cylinder of rice including the endodermis as the most outer cell layer completely resisted the enzymatic attack. Therefore, suberin analyses of the endodermis of rice roots had to be carried out for entire isolated central cylinders (CC). The central cortex of rice roots was composed of an aerenchyma, which separated the stele from four cell layers at the root periphery: rhizodermis, exodermis, sclerenchyma and one layer of unmodified cortical cells. These four cell layers have been called outer part of roots (OPR: Ranathunge *et al*., 2003). During enzymatic treatment, only walls of

the innermost unmodified cortical layer of the OPR could be digested away. Hence, wall preparations used for analyses still consisted of walls of rhizodermal, exodermal, and sclerenchymatous cells. With the exception of the innermost cortical layer, they were identical with those of the outer part of roots (OPR) as used in transport studies by Ranathunge *et al*. (2003). Cell wall preparations of rice were washed and stored as described above.

Suberin analysis

Prior to transesterification cell wall preparations were thoroughly extracted for 16 h using a mixture (1:1; v:v) of chloroform (Roth, Karlsruhe, Germany) and methanol (p.a.; Roth) at 40°C. For suberin depolymerization, the resulting extracted samples of known dry weight (between 1 to 10 mg) were transesterified as described in detail by Zeier and Schreiber (1998) using a mixture of methanol/borontrifluoride ($MeOH/BF_3$; Fluka), according to the procedure of Kolattukudy and Agrawal (1974). Released suberin monomers were derivatized for 40 min at 70°C using a 1:1 mixture of 10 µl dry pyridine (GC-grade, Merck, Darmstadt, Germany) and 10 µl of BSTFA (*N*,*N*-bis-trimethylsilyltrifluoroacetamide; Machery-Nagel, Düren, Germany). This procedure converted free carboxy- and hydroxyl groups to their trimethylsilyl (TMS) esters and ethers, respectively.

TMS-derivatives were analyzed by means of gas chromatography (GC) and mass spectroscopy (MS). Released monomers were quantified by a gas chromatography and flame ionization detection (GC-FID; HP 5890 Series II, Hewlett-Packard, Palo Alto, California, USA) using the HPChemStation software (Hewlett-Packard) and referring to an internal standard (20 µg dotriacontane). Monomers were identified using gas chromatography connected with a quadrupole mass selective detector (HP 5971A, Hewlett-Packard).

Results of suberin analyses were either expressed on the basis of total dry weight of original isolates used for the depolymerization or to the surface area of layers where apoplastic barriers are located (exodermis, hypodermis, and endodermis, respectively). Surface areas were calculated from root lengths and diameters obtained by light microscopy of root cross sections. For 10 individual root samples, correlations between

sample dry weights and corresponding surface areas have been worked out, whereby weights of wall preparations (ranging between 100 µg and 1 mg; see above) were precisely determined using a microbalance with an accuracy of 1 µg (Sartorius Microwaage, Göttingen, Germany). Using ratios between dry weights of wall preparations and surface areas, suberin amounts obtained by chemical analysis could be recalculated and related to surface areas.

Root-pressure probe experiments

Root pressure probe experiments were performed as described earlier for corn and rice (Miyamoto *et al*., 2001; Zimmermann *et al*., 2000). Excised root segments were tightly connected to a root pressure probe using a cylindrical silicone seal prepared from liquid silicone material (Xantopren from Bayer, Leverkusen, Germany). Segments of corn roots used had a length of between 75-120 mm and diameters between 0.65-1.1 mm. End segments of rice roots were 150-200 mm long (diameter: 0.8-1.2 mm). In corn, stable root pressures were normally observed after 1 to 3 h, whereas in rice it took between 5 to 12 h (Miyamoto *et al*., 2001). Root segments fixed to the probe were bathed in nutrient solution, which circulated along the roots to avoid problems with unstirred layers. Hydrostatic and osmotic relaxations were performed by either changing xylem pressure (moving the metal rod in the probe) or the osmotic pressure of the external medium. Test solutions used in osmotic experiments contained 20-40 mM NaCl (≈ 40-80 mOsmol/kg of osmotic concentration, which is equivalent to osmotic pressures of 0.1-0.2 MPa) in addition to the nutrients of the medium. Transient changes of pressure were followed. Root hydraulic conductivity (Lp_r) was calculated according to eqn 1 using half-time of water exchange ($T^w_{1/2}$) or rate constants k_{rw} (Steudle *et al*., 1987):

$$k_{rw} = \frac{\ln(2)}{T^W_{1/2}} = A_r\left(\frac{\Delta P_r}{\Delta V_s}\right)Lp_r \quad . \tag{1}$$

A_r is the effective surface area of the root investigated and $\Delta P_r/\Delta V_s$ (in MPa m^{-3}) is the elastic coefficient of the measuring system. $\Delta P_r/\Delta V_s$ was measured by inducing step-changes in the volume and recording the resulting changes in root pressure (ΔP_r).

Responses in root pressure to changes in osmotic pressure were biphasic, with a rapid water phase (efflux or influx) followed by a slower solute phase.

At the end of each experiment, the proper functioning of the mounted root was tested by cutting off the root at the seal and checking the decrease of the time constants of pressure relaxations. When root xylem remained open during fixation to the probe, there was a drastic decrease in $T^{w}_{1/2}$ after the cut. If not, the data obtained from the experiment was discarded.

Statistics

Suberin analyses and root pressure probe experiments were done for the same set of plants cultivated together at the same time under identical conditions. Radial hydraulic conductivity of rice and corn roots was determined measuring 6 replicates for each species. Hydraulic conductivities measured with the roots of this set of rice plants have already been communicated in a separate publication (Ranathunge *et al*., 2003), whereas hydraulic conductivities measured with the corn roots for this set of corn plants are presented here for the first time. Suberin composition of isolated cell wall samples of corn (ECW and RHCW) and rice (CC and OPR) were determined analyzing three replicates. Each replicate consisted of roots sampled from at least 20-30 individual plants. Results are given as means ± SD. To check for statistical significance, *t*-tests were conducted between pairs of means.

Results

Root anatomy

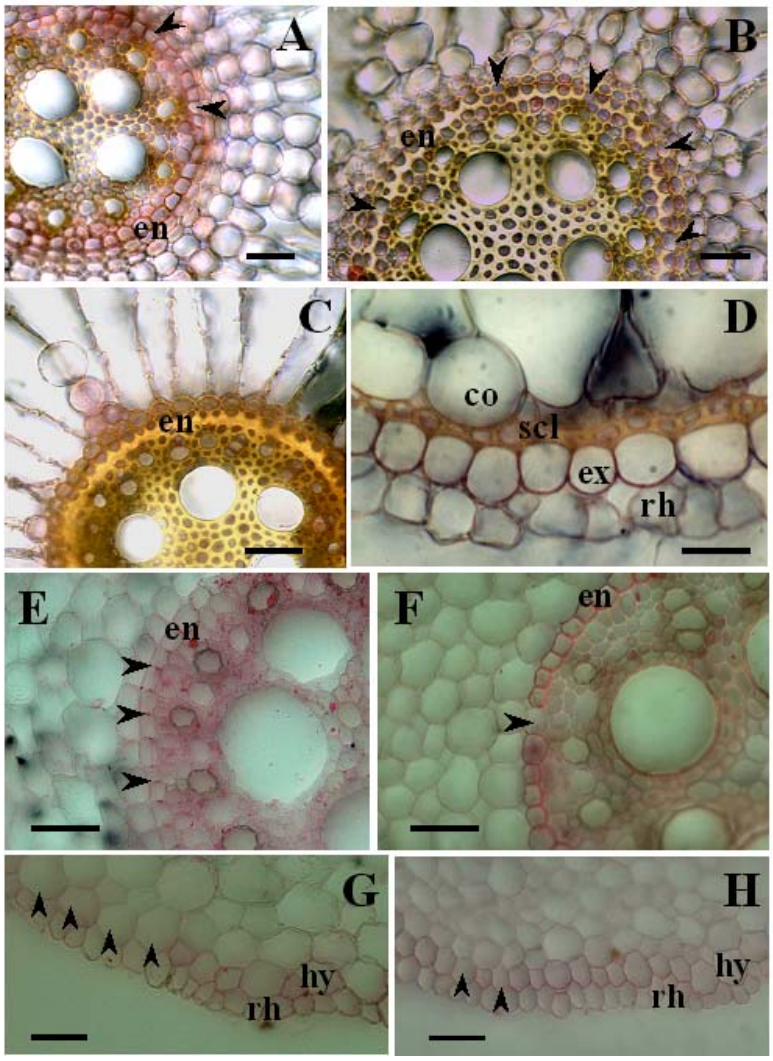

Fig. 1 Light microscopic pictures of Sudan Red 7B stained freehand cross-sections of rice (*Oryza sativa* L. cv. IR64) and corn (*Zea mays* L. cv. Helix) roots. **(A)** Rice endodermis (en) 30 mm behind the root apex with suberin lamellae and passage cells (black arrowheads). **(B)** Rice endodermis (en) 100 mm behind the root apex in its tertiary developmental state with passage cells (black arrowheads). **(C)** Rice endodermis (en) 200 mm behind the root apex in its tertiary developmental state without passage cells. **(D)** Rice exodermis (ex) 60 mm behind the root apex with well-developed suberin lamellae. The outer part of the rice root (OPR) is characterized by 4 cell layers: the rhizodermis (rh), the exodermis (ex), the sclerenchyma (scl) and the layer of unmodified cortical cells (co). **(E)** Corn endodermis (en) 50 mm behind the root apex in its primary developmental state with Casparian bands (black arrowheads). **(F)** Corn endodermis (en) 150 mm behind the root apex in its secondary developmental state with well-developed suberin lamellae and passage cells (black arrowheads). **(G)** Corn hypodermis (hy) and epidermis (ep) 60 mm behind the root apex. The hypodermis is characterized by a patchy suberization

with passage cells (black arrow heads). **(H)** Corn hypodermis (hy) and epidermis (ep) 120 mm behind the root apex. The hypodermis is still characterized by a patchy suberization with passage cells (black arrow heads).

In rice roots, endodermal suberin lamellae appeared 30 mm behind the root tip (Fig. 1A) and all endodermal cells had suberin lamellae at 50 mm with 5-8 passage cells. At 100 mm, endodermal cells were characterized by U-shaped tertiary cell wall depositions, and there were on average, still 5-8 passage cells (Fig. 1B). At 200 mm behind the root tip, no passage cells were observed in the endodermis (Fig. 1C). In rice, exodermal suberin lamellae started to develop 30 mm behind the root tip, and they were fully developed at about 60 mm behind the tip (Fig. 1D). In corn, there were no endodermal suberin lamellae at a distance of 50 mm behind the root tip (Fig. 1E). But it started to appear at 60 mm behind the root tip. They were fully developed at distances of between 100 and 150 mm behind the tip (Fig. 1F). On average, the endodermis had 3-4 passage cells at 100 mm and this number decreased up to 2-3 at 150 mm behind the root tip. Suberin lamellae in the corn root hypodermis appeared at 60 mm behind the root tip (Fig. 1G), but the deposition of suberin lamellae in the hypodermis was not complete even at a distance of 120 mm behind the root tip (Fig. 1H).

Suberin composition

Average lengths of zone I of corn and rice roots were 0.11 m and 0.06 m, respectively. Average lengths of zone II of corn and rice roots were 0.15 m and 0.39 m, respectively. In isolated OPR of zone II of rice, chain lengths distributions of aliphatic suberin monomers belonging to the five detected substance classes ranged from C_{16} to C_{30} (Fig. 2A). Chain lengths of C_{16} and C_{28} were the most abundant in rice suberin, but chain lengths C_{22} and C_{24} could not be detected. As in the OPR of zone II, similar qualitative suberin compositions were also found for the CC of root zone II and for the OPR and CC of root zones I of rice (data not shown). Chain length distribution of the five substance classes in the RHCW in zone II of corn roots ranged from C_{16} to C_{26} with C_{24} being the most abundant chain length (Fig. 2B). A similar qualitative pattern of suberin composition such as that shown in Fig. 2B was found for the ECW of root zone II and for RHCW and ECW of root zones I of corn (data not shown).

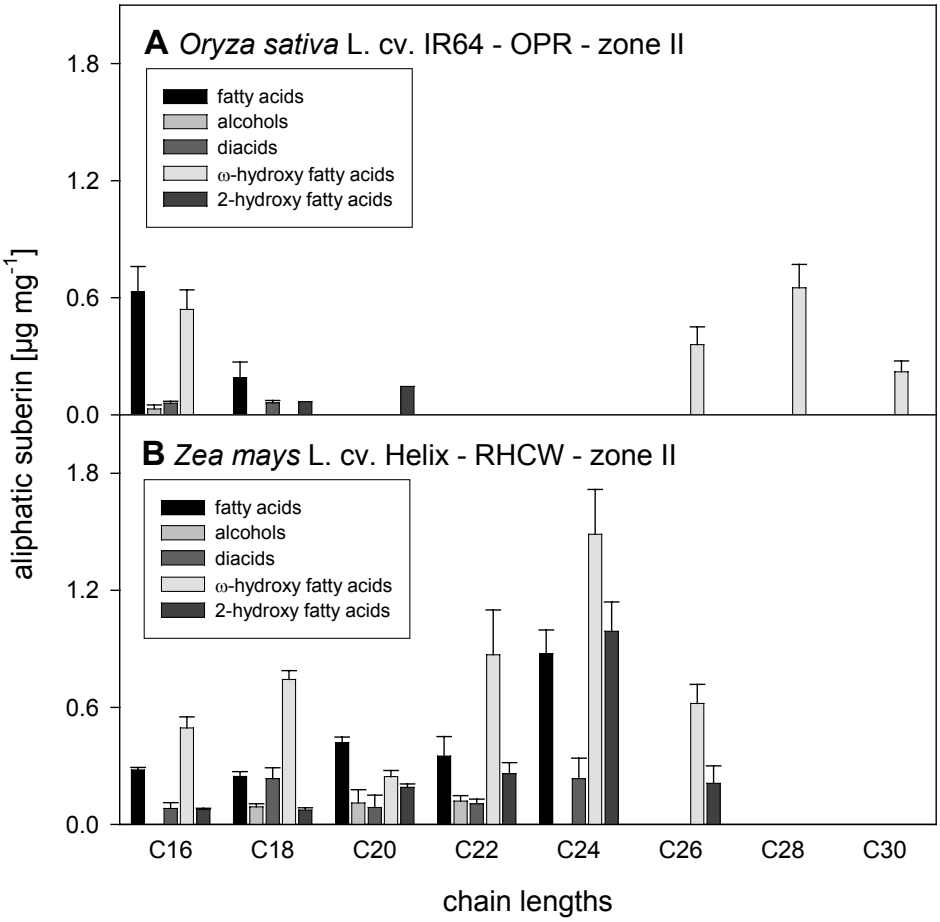

Fig. 2 Chain lengths distribution and substance class composition of (**A**) aliphatic suberin amounts released from the outer part of roots (OPR) isolated from zone II of rice (*Oryza sativa* L. cv. IR64) roots in comparison to suberin amounts released from (**B**) rhizodermal and hypodermal cell walls (RHCW) isolated from zone II of corn roots (*Zea mays* L. cv. Helix). Means ± SD (n = 3 replicates).

When comparing zones I and II of rice, there was no pronounced trend of an increase in aliphatic suberin (Fig. 3A). ω-Hydroxy fatty acids formed the most prominent substance class of rice root suberin (Fig. 3A). In corn, greater amounts of aliphatic suberin were observed in zone II than in zone I (Fig. 3B). Similar to rice, ω-hydroxy fatty acids represented the most prominent class of substances of aliphatic suberin in corn roots (Fig. 3B).

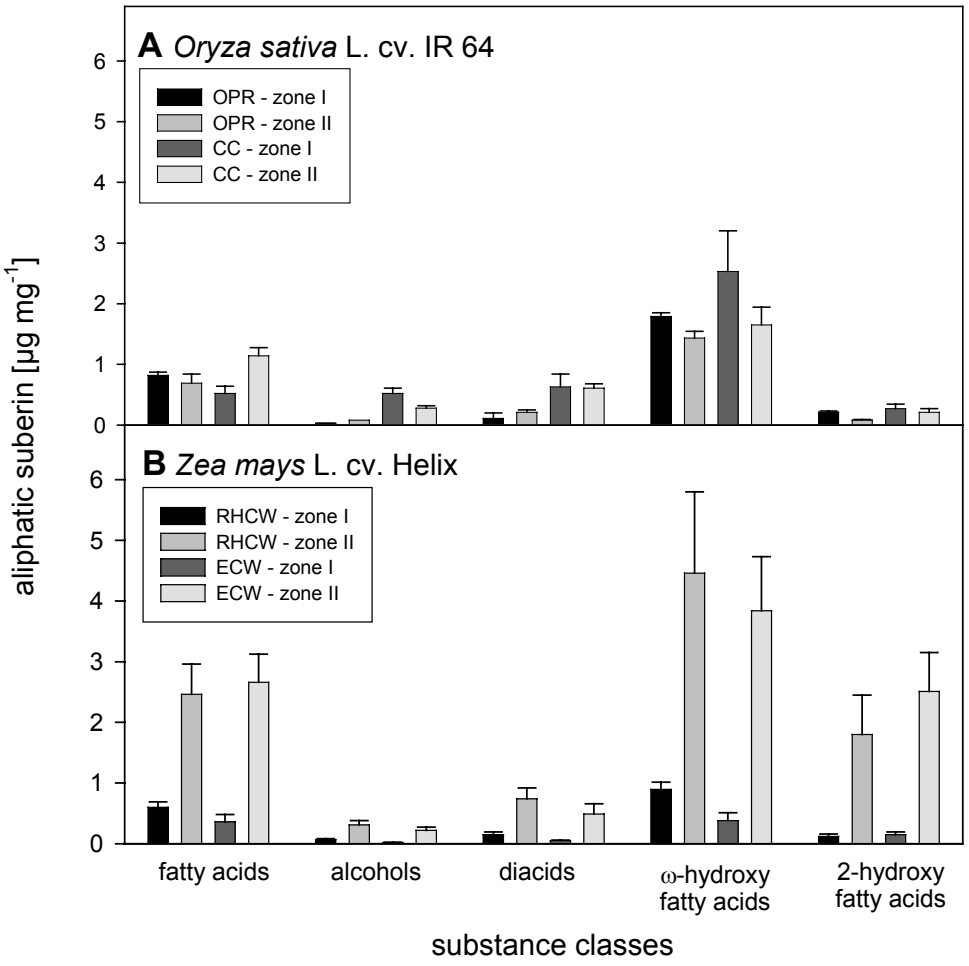

Fig. 3 Substance class composition of (**A**) aliphatic suberin amounts released from the outer part of roots (OPR) and central cylinders (CC) isolated from zones I and II of rice (*Oryza sativa* L. cv. IR64) roots in comparison to aliphatic suberin amounts released from (**B**) rhizodermal and hypodermal (RHCW) and endodermal cell walls (ECW) isolated from zones I and II of corn roots (*Zea mays* L. cv. Helix). Means ± SD (n = 3 replicates).

Total amounts of suberin

When referring the total amounts of aliphatic suberin released from the OPR and CC of both zones of rice roots to the dry weight of the isolated cell wall material, there were no differences between root zones. However, in corn roots there was a pronounced increase of suberin amounts, when comparing zones I and II (Fig. 4A). Compared to suberin amounts of both root zones of rice, suberin amounts in zone I of corn were lower. However, suberin amounts in root zone II of corn were higher (Fig. 4A). Referring suberin amounts to root surface areas, a completely different picture emerged.

By factors of six and 34, aliphatic suberin contents were, on average, larger in both zones of rice roots than in corn (OPR vs. RHCW and CC vs. ECW, respectively; Fig. 4B). In both species, there was a slight trend of increasing aliphatic suberin amounts along the roots from root zone I to II (Fig. 4B).

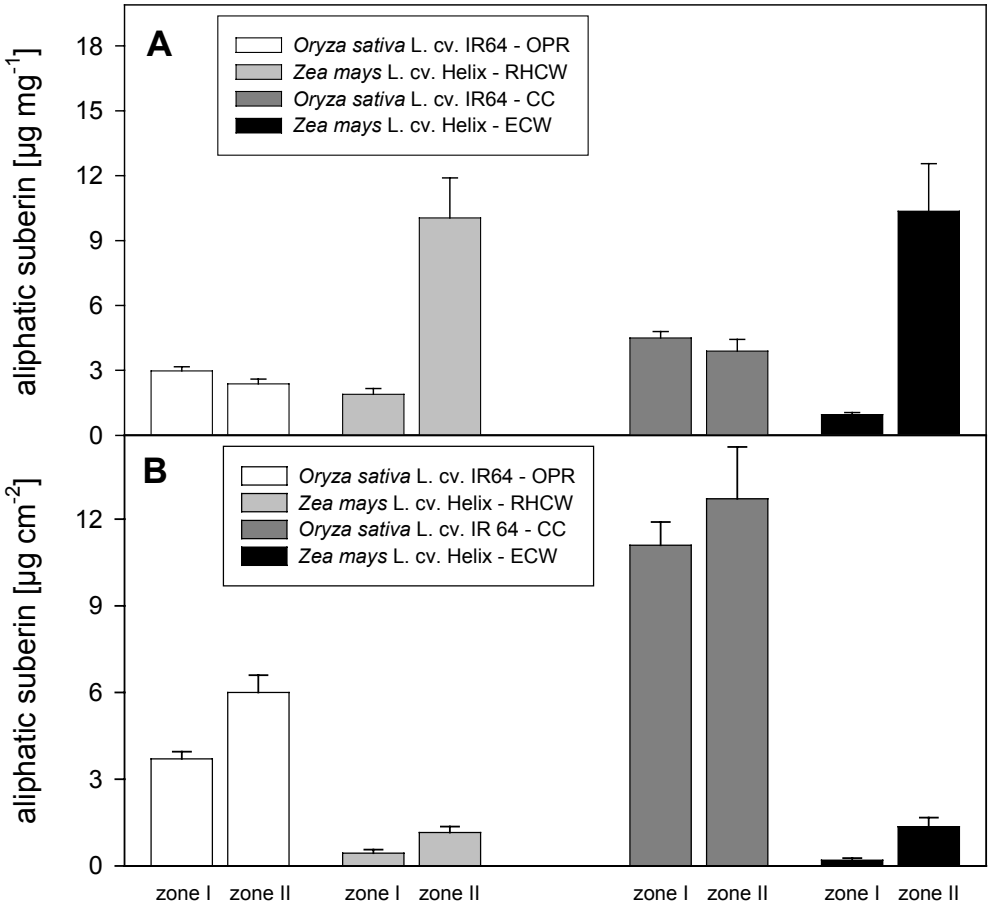

Fig. 4 (**A**) Total amounts of aliphatic suberin released from both root zones and isolated cell wall samples of rice [*Oryza sativa* L. cv. IR64; outer part of roots (OPR) and central cylinders (CC)], and corn roots [*Zea mays* L. cv. Helix; rhizodermal and hypodermal (RHCW) and endodermal cell walls (ECW)] related to dry weights of the analyzed cell wall samples [µg mg^{-1}] in comparison to (**B**) total amounts of aliphatic suberin related to surface areas of the analyzed cell wall samples [µg cm^{-2}]. Means ± SD (n = 3 replicates).

In both species, aromatic suberin was basically composed of coumaric and ferulic acids. When referring to dry weight of the isolated wall material, amounts of aromatic suberin were similar in the OPR and CC of both root zones of rice (Fig. 5A). Compared to rice, total amounts of aromatic suberin of corn were lower by a factor of three to four (Fig.

5A). Referring aromatic suberin to root surface areas, much higher differences emerged between rice and corn. The amount of aromatic suberin in rice OPR was, on average, larger by factor of 80 than that of corn RHCW and it was factor of 50, when comparing rice CC with corn ECW (Fig. 5B). The reason for the changes is that dry weights are larger in rice than in corn, and this vanishes, when referring to surface areas, which were similar. However, reference to surface area is physiologically relevant. In both species, contents of aromatic suberin were greater in root zone II as compared with zone I (Fig. 5B).

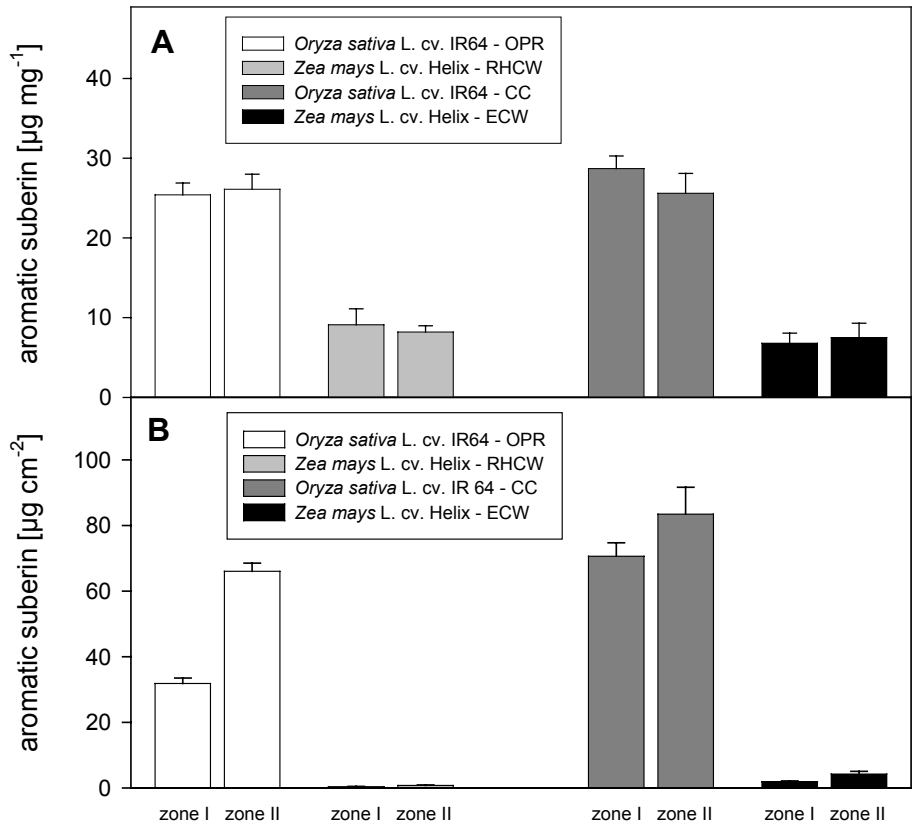

Fig. 5 (**A**) Total amounts of aromatic suberin released from both root zones and isolated cell wall samples of rice [*Oryza sativa* L. cv. IR64; outer part of roots (OPR) and central cylinders (CC)], and corn roots [*Zea mays* L. cv. Helix; rhizodermal and hypodermal (RHCW) and endodermal cell walls (ECW)] related to dry weights of the analyzed cell wall samples [µg mg^{-1}] in comparison to (**B**) total amounts of aromatic suberin related to surface areas of the analyzed cell wall samples [µg cm^{-2}]. Means ± SD (n = 3 replicates).

Hydraulic conductivities

Table 1 Hydraulic conductivities of end segments of 30-day-old rice (*Oryza sativa* L. cv. IR64; **A**), and 12-day-old corn roots (*Zea mays* L. cv. Helix; **B**) measured with the root pressure probe. Plants were cultivated in aerated hydroponics, and hydrostatic pressure gradients between the xylem and the medium were induced by hydrostatic relaxations. Osmotic water flow was induced by adding NaCl to the external nutrient solution.

A

Root (rice)	Surface area of measured rice root segments [mm^2]	Hydraulic conductivity Lp_r, as measured in hydrostatic relaxations [$m\ s^{-1}\ MPa^{-1}\ 10^{-8}$]	Hydraulic conductivity Lp_r, as measured in osmotic relaxations [$m\ s^{-1}\ MPa^{-1}\ 10^{-8}$]
1	438	3.3	0.9
2	535	3.6	0.9
3	273	3.0	0.7
4	326	4.4	1.0
5	374	4.0	2.0
6	531	3.3	1.0
mean	**413**	**3.7**	**1.1**
SD	**108**	**0.6**	**0.5**

B

Root (corn)	Surface area of measured corn root segments [mm^2]	Hydraulic conductivity Lp_r, as measured in hydrostatic relaxations [$m\ s^{-1}\ MPa^{-1}\ 10^{-8}$]	Hydraulic conductivity Lp_r, as measured in osmotic relaxations [$m\ s^{-1}\ MPa^{-1}\ 10^{-8}$]
1	350	9.9	1.1
2	390	4.7	0.27
3	310	8.6	1.4
4	380	6.5	0.31
5	320	15	1.0
6	280	13	1.5
mean	**339**	**9.5**	**0.93**
SD	**42**	**3.7**	**0.52**

By a factor of about 10, radial hydraulic conductivity of the distal segments of corn roots (Lp_r) was higher, when measured in the presence of a hydrostatic pressure gradient compared to an osmotic pressure gradient (Table 1). Compared to corn roots, Lp_r of rice roots measured in the presence of a hydrostatic pressure gradient was lower by a factor of 2.6 (significant at the 95%-level; Fig. 6). In the presence of an osmotic pressure gradient, however, radial hydraulic conductivities of the two species were not significantly different (*t*-test; Fig. 6).

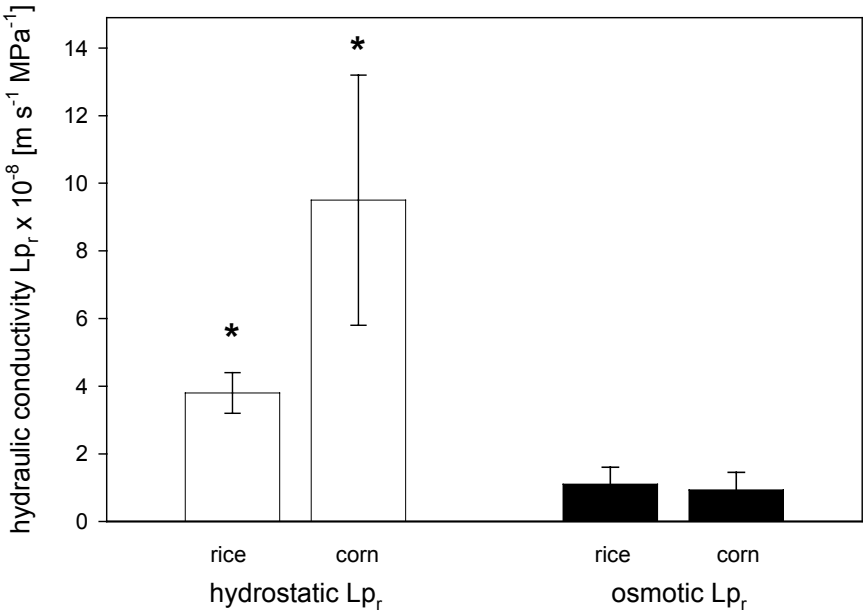

Fig. 6 Hydraulic conductivities (Lp_r) measured by using hydrostatic (hydrostatic Lp_r) and osmotic (osmotic Lp_r) pressure gradients with the end-segments of rice (*Oryza sativa* L. cv. IR64) and corn roots (*Zea mays* L. cv. Helix). Data of rice are from Ranathunge et al. (2003) and data of corn were taken from Table 1. In hydrostatic experiments, hydraulic conductivity was measured by changing the root turgor pressure with the aid of the root pressure probe. In osmotic experiments, relaxations were induced by changing the osmotic pressure of the external medium. Means ± SD (n = 6 roots). Bars marked with asterisks indicate a statistically significant difference at 95% confident level (*t*-test).

Discussion

According to the composite transport model of the root, water transport in the apoplast can be distinguished from water transport along the cell-to-cell path by the type of pressure gradient applied. In the presence of an osmotic pressure gradient, water flow is largely restricted to the cell-to-cell rather than to the apoplastic path around protoplast. In the presence of hydrostatic pressure gradients, there will an apoplastic water flow on top of the cell-to-cell component. There was no difference in the osmotic Lp_r between rice and corn roots, but there was a pronounced difference when comparing hydrostatic Lp_r., Hydrostatic Lp_r was smaller by a factor of 2.6 in rice than in corn (Fig. 6). This suggested differences in the apoplastic component, which affected by apoplastic barriers.

Differences in the formation and/or structure of apoplastic barriers between roots of both species support this view as indicated by light microscopy and histochemistry. Our data show that the exodermis of rice was already fully developed at 30-60 mm from the root tip (including a suberin lamella). In hydroponically grown corn, an exodermis is missing (Zimmermann *et al*., 2000) and in the hypodermis, suberin lamellae started to deposit quite far away from the root tip (at around 60 mm), and it was still patchy at mature root zones (at around 120 mm). Furthermore, apoplastic barriers in the rice endodermis (Casparain bands and a suberin lamella) developed much earlier compared to corn. But rice endodermis retained greater number of passage cells without developing suberin lamellae than that of corn at mature parts of the root.

Roots of the two species showed differences in both the qualitative chemical composition of suberin and in the total amounts of these compounds, which were quantified. It is known that the biopolymer suberin forms most of the barrier against water transport, and that the aliphatic domain should be more efficient than the aromatic (Schönherr, 1982; Vogt *et al*., 1983). Therefore, analyses were restricted to the aliphatic suberin, although it has been shown that apoplastic barriers in roots contain significant amounts of other biopolymers such as lignin, carbohydrates and cell wall proteins (Schreiber *et al*., 1999). Comparing the qualitative composition of aliphatic suberins of rice with corn, it is evident that rice is much less diverse. All 5 substance classes

(primary fatty acids, primary alcohols, diacids, ω-hydroxy fatty acids and 2-hydroxy fatty acids) could only be detected at lower chain lengths between C_{16} and C_{20}). The chain lengths C_{22} and C_{24} were completely missing and higher chain lengths from C_{26} to C_{30} were only represented by ω-hydroxy fatty acids (Fig. 2A). In corn, chain lengths of the monomers continuously increased from C_{16} to C_{26}. Each chain length was formed by at least two, in most cases three or more substance classes leading to a suberin with a larger variation in terms of detected monomers. Although, there are obvious differences in the qualitative suberin composition between both species, it is difficult to deduce functional differences between both types of suberin in terms of barrier properties. However, based on the lower diversity of monomers and especially on the longer chain lengths formed by only one substance class, we would speculate that rice suberin is more hydrophobic than that of corn.

When proceeding from the younger (zone I) to the older part of the root (zone II), a pronounced developmental gradient was evident in corn hypodermis and endodermis (Zeier *et al*., 1999). In all five substance classes, amounts of monomers significantly increased by factors between 3 to 15 in corn depending on the root zone and the substance class (Fig. 3B). In rice, gradients in suberin development were less (Fig. 3A). Obviously, the amounts of suberin required for the formation of the apoplastic barriers were already fully deposited in zone I of rice roots, and there was no need for further deposition of suberin in zone II (Fig. 3A). We conclude that, unlike corn, there were already well-suberized apoplastic arrays in younger parts of rice roots as it was also evident from the microscopic investigations of the roots. In corn roots, however, there was a pronounced developmental gradient along the root, which became also evident form the microscopic investigations (Fig. 1). For rice, this may represent an adaptation to the flooded habitat, where it is normally growing. Comparing the total amounts of all monomers added, the developmental gradient of aliphatic suberin along corn roots is clear compared to rice. However, this was not the case for the aromatic suberin monomers detected.

From light microscopy and histochemistry it is evident that most of the aliphatic suberin monomers released from the different isolated cell wall samples should be located in the apoplastic barriers of the endo- and hypo- or exodermis of both species. Therefore, it

seems questionable comparing the chemical composition of apoplastic barriers between different species such as rice and corn, by relating detected suberin amounts to the dry weight of the isolated cell wall samples. Only with the corn endodermis, isolated cell wall samples (ECW) really represented the suberized apoplastic barrier of interest. This was not the case with the hypodermis of corn, since isolated cell wall material was composed of hypodermal and rhizodermal cell walls (RHCW), although microscopy and histochemistry clearly showed that the aliphatic suberin was mostly deposited in the hypodermal cell walls (Fig. 1). For rice, this argument becomes even more important, since suberin in the exodermis had to be analyzed using isolated cell wall samples composed of three cell layers (OPR). Finally, the endodermal suberin could only be analyzed using complete central cylinders (CC), due to the fact that lignified walls resisted enzymatic degradation in rice.

Consequently, detected amounts of suberin will necessarily underestimate the actual suberin content in the barrier, when are related to cell wall isolates containing additional cell wall material leading to increased dry weights. Therefore, data of Figures 4A and 5A were re-plotted referring suberin amounts to the surface areas of the respective tissues of interest (exodermis, hypodermis and endodermis). Physiologically, reference to surface area makes more sense when comparing different species.

The pronounced differences between rice and corn became visible, when suberin amounts were related to surface areas. Amounts of aliphatic suberin in rice exodermis were on average 6-times higher compared to corn hypodermis and in rice endodermis, on average, 35-fold more suberin was deposited compared to corn endodermis (Fig. 4B). In both species, there was a slight trend to increase the amounts of aliphatic suberin along the roots from zone I to zone II. When compared the aromatic suberin, even larger differences were obtained re-plotting them and referring to the unit surface area of roots. The amounts of aromatic suberin were, on average, 50 to 80-times greater in apoplastic barriers of rice compared to corn (Fig. 5B). However, in contrast to aliphatic suberin, quantification of aromatic suberin could be overestimated. It is known that aromatics (ferulic and coumaric acids) are also covalently linked to normal non-lignified or suberized cell walls in grasses (Chabbert, 1994). This is also evident from the pronounced autofluorescence of all cell walls in corn and rice roots, indicating the

existence of aromatic compounds in nearly all cell walls. Nevertheless, this pronounced difference in total amounts of aliphatic suberin in apoplastic transport barriers in roots could help to explain why hydrostatic Lp_r of rice was significantly lower than that of corn, whereas osmotic Lp_r was not significantly different between both species.

The simple conclusion that water permeability would be reduced as the amount of suberin is increasing, is hard to justify according to recent results of water permeability across the outer part of rice roots (Lp_{OPR}). The OPR of rice contains a large amounts of suberin relative to that of corn but is, nevertheless, highly permeable to water. It was shown that the Lp_{OPR} was larger by a facror of 30 than the overall Lp_r of intact rice roots, which contained both exo- and endodermis, arranged in series (Ranathunge *et al*., 2003). Clogging off the apoplastic pores of the OPR by small ink particles or and copper-ferrocyanide precipitates substantially decreased the apoplastic water flow (Ranathunge *et al*., 2004, 2005). The effect was even more pronounced when insoluble salts were precipitated in the OPR (Ranathunge *et al*., 2005), These results suggest that wall pores of the OPR were still open despite containing large amounts of suberin. In rice, there is even an apoplastic bypass-flow of ions (Yadev *et al*., 1996; Yeo *et al*., 1987). We conclude that, at least for rice, any conclusion drawn from suberin contents about the water permeability would be premature.

Schreiber *et al*. (2004) described a comparable situation for suberized periderm of potato. Wound periderm of potato, having about 60% of the suberin amounts detected in native periderm, had water permeabilities which were about 100-times higher. This is difficult to explain in terms of suberin content as well. In wound periderm, the major function of suberin obviously is related to pathogen defence rather than in the protection of living tissue from desiccation. In a similar way it could be argued that strong suberin depositions in rice OPR have to protect rice roots grown in paddy fields from pathogen attack. However, it is more likely that suberin deposition prevents losses of oxygen from the rice aerenchyma as indicated by measurement of changes of the permeability coefficient of oxygen along developing rice roots (Kotula and Steudle, in preparation). Obviously analyses of total amounts of suberin deposited in apoplastic barriers and of their detailed chemical structure are a necessary but not the complete pre-requisite to explain the observed changes in water permeability. The precise molecular and

topographical deposition of suberin in root cell walls has to be known as well. The latter determines the reduction of porosity and permeability of roots. In order to make barriers really water-tight, suberin should fill all wall pores (intermicrofibrillar spaces), i.e. it has to impregnate the wall material (such as filling a sponge with water). In fact, hydrophobic aliphatic suberin may have problems to fill pores made of rather hydrophilic material such as cellulose. To date, virtually nothing is known about the microstructure of apoplastic barriers. Histochemistry does not help much here. Techniques are required to resolve, with high spatial resolution, porosity in arrays containing apoplastic suberin depositions such as the precipitation techniques mentioned above or molecular techniques (i.e. suberin-specific antibodies).

The results show that the deposition of suberins in root cell walls do not straightforwardly allow conclusions about the degree of inhibition of water and ion transport. Comparison between roots of rice and corn indicates that the main hydraulic resistances were located at the endodermis having 35-times more suberin in rice than in corn. Future work relating the radial hydraulic conductivity of roots to the existence of apoplastic transport barriers badly requires to answer the following questions: (i) To what extent does the hypodermis of corn roots contribute to the overall resistance of the radial water flow and is it similar or completely different from rice? (ii) What is the exact local deposition of suberin in apoplastic barriers and are there techniques available offering the necessary spatial resolution? (iii) To what extent are additional structural biopolymers such as lignin or cell wall proteins import constituents of apoplastic barriers in rice roots? This information is required for a better understanding of the structure and the function of suberized apoplastic barriers in roots.

Acknowledgements

We thank Prof. Carol A. Peterson, University of Waterloo, Canada for reading and discussing the manuscript. The authors gratefully acknowledge financial support by the Deutsche Forschungsgemeinschaft (DFG) within the Schwerpunktprogramm “Apoplast” to L.S. and E.S. (SCHR 506/5-2 and Ste 319/3-3).

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6 A new precipitation technique provides evidence for the permeability of Casparian bands to ions in young roots of corn (*Zea mays* L.) and rice (*Oryza sativa* L.)

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Plant, Cell and Environment (online publication)
doi:10.1111/j.1365-3040.2005.01391.x

Abstract

Using an insoluble inorganic salt precipitation technique, the permeability of cell walls and especially of endodermal Casparian bands (CBs) for ions was tested in young roots of corn (*Zea mays*) and rice (*Oryza sativa*). The test was based on suction of either 100 μM $CuSO_4$ or 200 μM $K_4[Fe(CN)_6]$ into the root from its medium using a pump (excised roots) or transpirational stream (intact seedlings), and subsequent perfusion of xylem of those root segments with the opposite salt component, which resulted in precipitation of insoluble brown crystals of copper ferrocyanide. Under suction, Cu^{2+} could cross the endodermis apoplastically in both plant species (though at low rates) developing brown salt precipitates in cell walls of early metaxylem and in the region between CBs and functioning metaxylem vessels. Hence, at least Cu^{2+} did cross the endodermis dragged along with the water. The results suggested that CBs were not perfect barriers to apoplastic ion fluxes, at least for copper. In contrast, ferrocyanide ions failed to cross the mature endodermis of both corn and rice at detectable amounts. The concentration limit of apoplastic copper was 0.8 μM at a perfusion with 200 μM $K_4[Fe(CN)_6]$. Asymmetric development of precipitates suggested that the cation, Cu^{2+}, moved faster than the anion, $[Fe(CN)_6]^{4-}$, through cell walls including CBs. Using *Chara* cell wall preparations ("ghosts") as a model system, it was observed that, different from Cu^{2+}, ferrocyanide ions remained inside wall-tubes suggesting a substantially lower permeability of the latter which agreed with the finding of an asymmetric development of precipitates. In both, corn and rice roots, there was a significant apoplastic flux of ions in regions where laterals penetrated the endodermis. Overall, the results show that the permeability of CBs to ions is fairly low though not zero. CBs do not represent perfect barrier for ions, as is usually thought. The permeability of CBs may vary depending on growth conditions, which are known to affect the intensity of formation of bands.

Key-words: apoplast; Casparian band; corn root; endodermis; ion permeability; rice root; salt precipitates.

Introduction

In the plant body, the apoplast denotes the extraprotoplastic compartment. It comprises cell walls, gas- or water-filled intercellular spaces, and the xylem. Chemically, cell walls are highly complex, i.e. they contain cellulose and matrix materials such as hemicellulose, pectic substances and structural proteins (Peterson & Cholewa 1998). The pectic substances are made of galacturonic acids entities with –COOH groups (carboxyl groups), which are responsible for the overall negative fixed charge of the cell wall. Cell walls provide mechanical strength to the plant, as well as functioning as a porous network involved in a diverse range of passive transport processes (gas, water, nutrient ions, assimilates). The porous network consists of intermicrofibrillar and intermicellar spaces that range in size from 3.5 to 30 nm (Shepherd & Gootwin 1989; Chesson, Gardner & Wood 1997; Nobel 1999), and therefore it does not represent a major barrier for both water and nutrient flows, even when considering relatively large molecules.

Usually, flows of water across cell walls or the apoplast are driven by hydrostatic pressure gradients and are viscous in nature. Nutrient ions may be dragged with the water to reach the plasmalemma ("solvent drag"), or may move by diffusion in the absence of a drag (Nobel 1999). The velocity of water and nutrient movement may be hampered by friction and tortuosity along the porous path (Sattelmacher 2001). It may be reduced by adsorption or fixation to negatively charged cell wall matrix (in case of cations) or by repulsion (in case of anions; Clarkson 1991; Marschner 1995). Moreover, in roots, water and ion movement through the apoplast may be hampered by the presence of Casparian bands (CBs) in radial and transverse walls of the endo- and exodermis, and there may be suberin lamellae as well.

The Casparian band is a primary wall modification, encrusted with lignin as a major component and, to a lesser extent with suberin, the latter assumed to provide most of the resistance towards the movement of polar substances (Schreiber 1996; Zeier & Schreiber 1998; Schreiber *et al*. 1999; Zimmermann *et al*. 2000). It is usually assumed that CBs are perfect barriers to water and ion movement through the apoplast (Robards & Robb 1972; Singh & Jacobson 1977; Peterson 1987; Enstone, Peterson & Ma 2003).

However, results from recent studies suggested that CBs are imperfect barriers to apoplastic fluxes of water, dissolved solutes and ions, i.e. Ca^{2+}, Zn^{2+}, Cd^{2+}, and apoplastic tracer dyes, as well as for the stress hormone ABA (Sanderson 1983; Yeo, Yeo & Flowers 1987; White, Banfield & Diaz 1992; Steudle, Murrmann & Peterson 1993; Yadav, Flowers & Yeo 1996; Freundl, Steudle & Hartung 1998; Steudle & Peterson 1998; Schreiber *et al*. 1999; Hose *et al*. 2001; White 2001; White *et al*. 2002; Ranathunge, Steudle & Lafitte 2003; 2005; Lux *et al.* 2004). In rice roots, the permeability of CBs in the exodermis for water and different ions (copper and ferrocyanide) has been intensively studied by Ranathunge *et al*. (2003; 2005). The results suggested a substantial apoplastic bypass flow of water across the mature exodermis and, surprisingly, even divalent Cu^{2+} ions were able to cross the barrier, at least to some extent. Since findings were contrary to general assumption that the permeability of CBs to water and nutrient ions is nil, it is important to test the validity of such statements verifying by direct experimentation.

In the present study, we extended our previous research on permeability of CBs of the rice endodermis (with its specialized root anatomy) incorporating 'normal' (less modified) roots of corn as another test object. We applied 100 µM $CuSO_4$ to the root medium, and subsequently perfused xylem vessels with 200 µM $K_4[Fe(CN)_6]$. Those salts moved through the apoplast and readily precipitated as Hatchett's brown $Cu_2[Fe(CN)_6]$, where they met. Suction of $CuSO_4$ from the root medium, either by using a pump (excised roots) or the transpirational stream (intact seedlings), dragged Cu^{2+} ions into the stele with the water flow through the apoplast crossing the endodermis, and developed brown precipitates just opposite to CBs and in the passage between CBs and early metaxylem. The observation was evident even in some regions of basal, mature root zones of both plant species suggesting that CBs are "imperfect barriers" to nutrient salts, at least for Cu^{2+} and SO_4^{2-} ions. A striking asymmetry in the development of precipitates (on the side where ferrocyanide was applied) proposed that movement of copper ions through cell walls was faster (less hindered) than that of ferrocyanide. This hypothesis was tested using cell wall preparations of *Chara* internodes as a model system. It was shown that at points where lateral roots emerged from the primary root were leaky for both, Cu^{2+} and $[Fe(CN)_6]^{4-}$ ions.

Materials and methods

Plant materials

Seeds of maize (*Zea mays* L. cv. Helix; Kws Saat AG, Einbeck, Germany) were germinated for 4 d on wet filter paper in the dark. Seedlings were raised hydroponically under well-aerated conditions in a solution containing (a) macronutrients (mM) 0.7 K_2SO_4, 0.1 KCl, 2.0 $Ca(NO_3)_2$, 0.5 $MgSO_4$, 0.1 KH_2PO_4 and (b) micronutrients (μM) 1 H_3BO_3, 0.5 $MnSO_4$, 0.5 $ZnSO_4$, 0.2 $CuSO_4$, 0.01 $(NH_4)_6Mo_7O_{24}$, 200 Fe-EDTA, at a pH of 6.0 (Steudle & Frensch 1989). Roots from 7- to 10-d-old plants were used. They were 160-220 mm long and 0.8 to 1.2 mm in diameter.

Seeds of rice [*Oryza sativa* L. cvs. Azucena (upland) and IR64 (lowland)] were germinated for 5-6 d in the light in a climate chamber on tissue soaked with tap water. Seedlings were transferred to a hydroponic culture system which containing (a) macronutrients (in mM) 0.09 $(NH_4)_2SO_4$, 0.05 KH_2PO_4, 0.05 KNO_3, 0.03 K_2SO_4, 0.06 $Ca(NO_3)_2$, 0.07 $MgSO_4$, 0.11 Fe-EDTA and (b) micronutrients (in μM) 4.6 H_3BO_3, 1.8 $MnSO_4$, 0.3 $ZnSO_4$ and 0.3 $CuSO_4$, with pH of 5.5-6.0. Boxes of nutrient solution (10 L) accommodated 12 seedlings as described by Miyamoto *et al*. (2001). Roots from 30- to 40-d-old plants (including the time for germination) were used for experiments. The lengths of root systems of Azucena and IR64 were 350-550 mm and 250-450 mm, respectively. Average diameters of adventitious roots of Azucena and IR64 were 1.2 and 0.9 mm, respectively.

Test of apoplastic permeability (including the endodermis) of $CuSO_4$ and $K_4[Fe(CN)_6]$ in corn roots

Four different types of experiments were employed to check for the permeability of cell walls including Casparian bands (CBs) in the endodermis for the above salts. The reaction between $CuSO_4$ and $K_4[Fe(CN)_6]$ developed rusty-brown, insoluble crystals (precipitates) of $Cu_2[Fe(CN)_6]$ or $Cu[CuFe(CN)_6]$, which were easy to detect in cross sections (for details, see Ranathunge *et al*. 2005). Low concentrations of copper sulfate

and potassium ferrocyanide were used to treat roots to minimize adverse effects to living tissues.

In experiment one, intact seedlings of 10-d-old corn were transferred to a beaker and roots were covered with an aluminium foil, leaving only the shoot exposed to sunlight. 100 µM $CuSO_4$ and 200 µM $K_4[Fe(CN)_6]$ were sequentially applied to the root medium of transpiring corn seedlings and allowed to transpire for 2 h in each salt on a sunny summer day outside the lab with an average light intensity of 700 Wm^{-2} of PAR. In some experiments, the sequence of salt application was reversed.

In experiment two, roots were excised near the kernel under water and fixed to a glass capillary (inner diameter of 1.3 mm) using a polyacrylamide glue (UHU, Bühl, Germany), which was then superposed with a molten mixture of beewax-collophony (1:3 w/w; Zimmermann & Steudle 1975). The other end of the glass capillary was fixed to a vacuum pump (Vacuumbrand GmbH, Wertheim, Germany) through a connector as shown in Fig. 1a. The entire root was dipped in 100 µM $CuSO_4$ solution and a suction of –0.07 MPa applied from the pump for 90 min to drag $CuSO_4$ radially into the root through the apoplast. In some experiments, only the tip part was dipped in $CuSO_4$ and suction was created (20 mm from the tip where vessels were not yet developed). At the end of this period, the tip part of the root was removed (20 mm from the tip) and the remainder was fixed to the perfusion set-up using a silicone seal (Fig. 1b). Proper tightening of the screw cap and rubber silicone seal ensured a flow only through open xylem vessels of the root. The other end of the root segment, which had already been connected to the glass capillary, remained open as an outlet. The syringe was placed 0.7 m above the root (gravitational force of 0.007 MPa) and xylem vessels perfused with 200 µM $K_4[Fe(CN)_6]$ under gravity for 3 h. The external medium of 100 µM $CuSO_4$ was continuously stirred using a pump. In some experiments, the side of salt application was reversed (suction was created applying 200 µM $K_4[Fe(CN)_6]$ to the root medium and xylem vessels were perfused with 100 µM $CuSO_4$).

In the third experiment, 100 µM $CuSO_4$ was applied to the root medium of transpiring corn seedlings for 2 h and subsequently perfused the xylem vessels with 200 µM $K_4[Fe(CN)_6]$ under gravity as described in the experiment two.

In experiment four, to check for the toxicity of chemicals used (especially $CuSO_4$), root exposure time to the salts as well as the concentrations has been increased. Xylem vessels of root segments were perfused with 1000 µM $K_4[Fe(CN)_6]$ for as long as 48 h while they were bathing in 500 µM $CuSO_4$ medium. In some experiments the side of salt application was reversed.

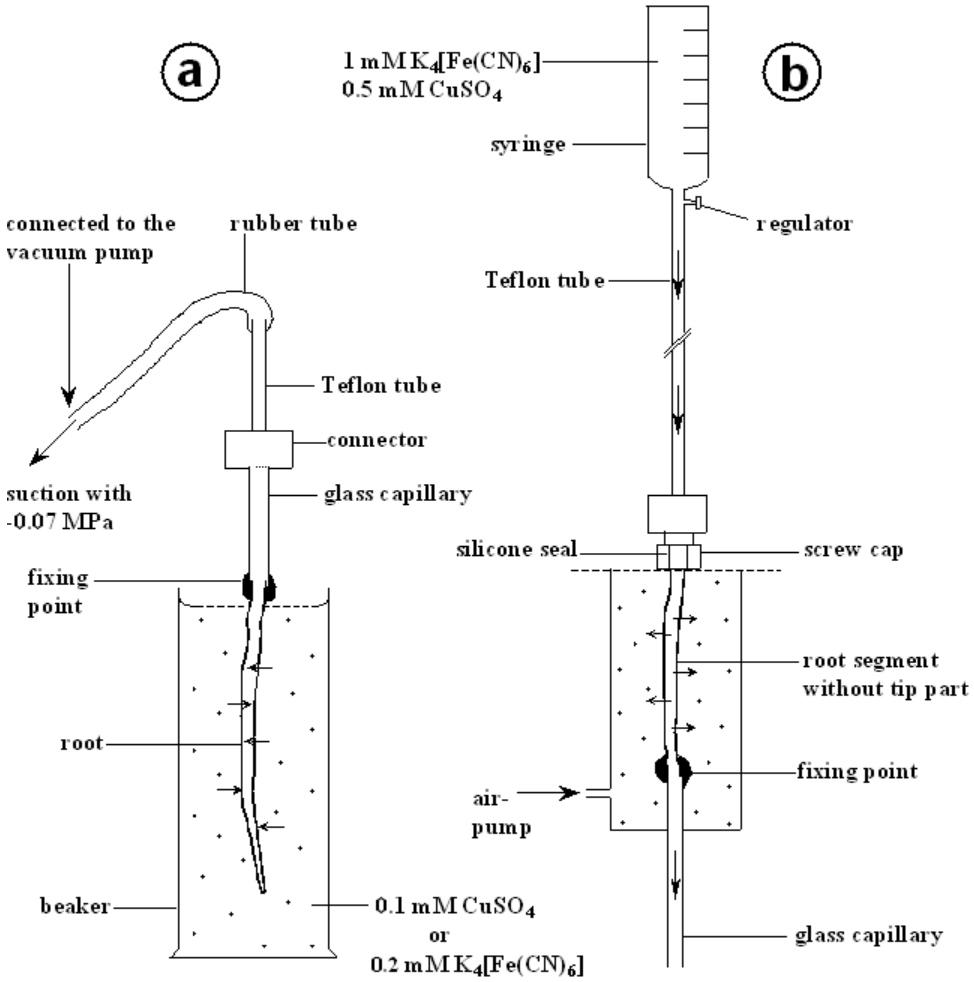

Figure 1 Experimental set-up to check the permeability of the apoplast including Casparian bands of the endodermis in corn and rice roots. (a) Excised roots were connected to a vacuum pump through a connector, and the root medium of either 100 mM $CuSO_4$ or 200 µM $K_4[Fe(CN)_6]$ was sucked into the stele for 90 min. Afterwards, tip parts of roots were removed and segments fixed to the perfusion apparatus (b). Xylem of root segments was perfused with the opposite salt that of in the root medium, under gravity for 3 h.

Test of apoplastic permeability (including the endo- and exodermis) of $CuSO_4$ and $K_4[Fe(CN)_6]$ in rice roots

Two different types of experiments were employed to assess the apoplastic permeability (including the endo- and exodermis) of rice roots for these salts. Here, 100 µM $CuSO_4$ was sucked into the stele from the root medium in excised roots as did in corn, and xylem vessels and aerenchyma of root segments were subsequently perfused with 200 µM $K_4[Fe(CN)_6]$ for 3 h. (**experiment five**). **In experiment six**, above treatment was repeated for roots after removing or damaging the outer part or peripheral layers of roots using a razor blade and fine-tipped forceps under a dissecting microscope (Makroskop M 420, Wild, Heerbrugg, Switzerland). Five roots were tested in each experiment, (n = 5).

Vitality test

Since permeability experiments lasted for several hours (4-5 h) and even low Cu^{2+} concentrations may be toxic to plant cells (Murphy *et al*. 1999), it was essential to check viability of root cells at the end of the experiments. Free-hand longitudinal sections were made and stained either with 0.5% (w/w) Evan's blue for 15 min (Taylor & West 1980) or with fluorescent dye 0.01% (w/v) uranin (disodium fluorescein) for 10 min (Stadelmann & Kinzel 1972) to determine cell vitality.

Photography

At the end of the experiments with corn and rice roots, free-hand cross-sections were made at different distances from the root tip (3, 20, 40, 60, 80, 100 mm) and observed under a light microscope to localize copper ferrocyanide precipitates in the treated roots. Sections stained with uranin were observed under a fluorescent microscope with blue filters (Zeiss, Oberkochen, Germany). Photographs were taken either using a Kodak Elite 64 ASA film (Kodak limited, England, UK) or using a digital camera (Sony-DSC-F505V; Sony Corporation, Tokyo, Japan).

Cell pressure probe measurements

It may be argued that applied copper altered (decreased) the water permeability through plasma membranes tending to increase the relative amount of water that crosses the endodermis through the apoplast. This idea was tested measuring the hydraulic conductivity of individual cortical cells (*Lp*) of corn roots by a cell pressure probe as previously described (Steudle 1993), assuming that endodermal cells would behave similar as other cortical cells. *Lp* of the cells of the outer cortex were measured for control as well as re-measured after sucking 100 µM $CuSO_4$ into the stele from the root medium for 90 min. A total of five cells was measured from five roots ($n = 5$).

Measurements of *Chara* cell wall permeability for copper sulphate ($CuSO_4$) and potassium ferrocyanide ($K_4[Fe(CN)_6]$)

In order to isolate internodal cell walls, the nodes of mature cells of *Chara corallina* were excised and the cellular contents flushed out gently with a syringe. To remove plasmalemma and other residues, the cell wall tubes were perfused with pure ethanol followed by water. Kannula needles from which pointed ends were removed (Braun Melsungen AG, Melsungen, Germany) were glued to open ends of the cell wall tubes using a polyacrylamide glue (UHU, Bühl, Germany) and superposed with a molten mixture of beewax-collophony (Fig. 2).

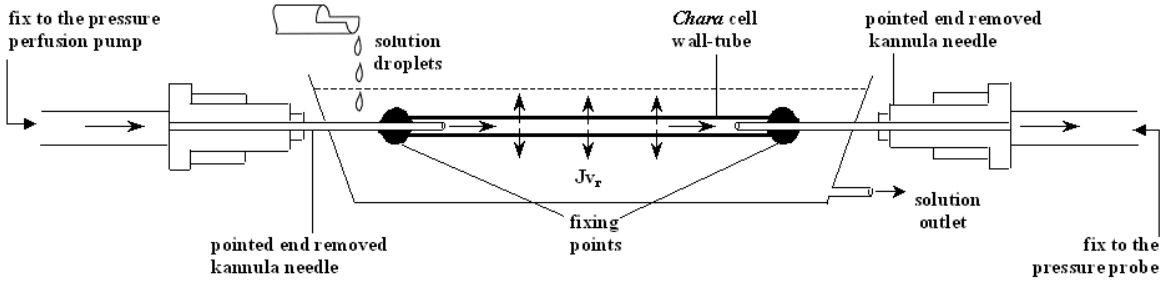

Figure 2 Pump perfusion set-up: Open ends of cleared *Chara* cell wall preparations (untreated walls or ghosts containing copper ferrocyanide precipitates) were fixed to kannula needles (without pointed end), and inlet ends connected to a Braun-Melsungen pump through a Teflon tube. The pump created pump rates of 3 to 6 × 10^{-11} m^3 s^{-1}. The other ends (outlet ends) of preparations were connected to a pressure

probe to measure steady-state pressures resulting in the system. At steady-state, the volume flow provided by the pump equaled the radial volume flow across wall preparations.

One kannula needle (inlet end) was connected to a 12-step Braun-Melsungen pump using a Teflon tube. The outlet end was fixed to a pressure probe to measure steady-state pressures in the system as described by Ranathunge *et al*. (2003). A syringe was filled with 100 mOsmol/kg $CuSO_4$ and mounted on the pump, and the cell wall tube was perfused at a pump rate of either by 3×10^{-11} or 6×10^{-11} m^3 s^{-1}. Pressure in the system increased gradually until a stationary pressure established. A defined volume of distilled water (2 mL) was added to the external chamber as the bathing medium of the cell wall tube. To prevent evaporation, the chamber was covered with a lid. It was stirred throughout the experiment. At different time intervals, 50 µl of the outer medium was taken out by a pipette and the $CuSO_4$ concentration of each sample was measured by a freezing point osmometer. Hence, the amount of $CuSO_4$ that permeated to the external medium was measured and plotted against time. The rate of $CuSO_4$ permeation was directly obtained from the slope of this curve (mOsmol kg^{-1} s^{-1}). In another experiment, cell wall-tubes were perfused with 100 mOsmol/kg $K_4[Fe(CN)_6]$ to measure the rate of permeation for this solute as well.

Results

Apoplastic permeability of $CuSO_4$ and $K_4[Fe(CN)_6]$ in corn roots

Sequential application of 100 µM $CuSO_4$ and 200 µM $K_4[Fe(CN)_6]$ to the root medium in transpiring seedlings resulted in a development of copper ferrocyanide precipitates in the outer layers of corn roots (**experiment one**). Dense brown $Cu_2[Fe(CN)_6]$ precipitates were accumulated between the epidermis and the hypodermis as a result of diffusion of salts into the root close to the tip (3 mm from the tip; Fig. 3a).

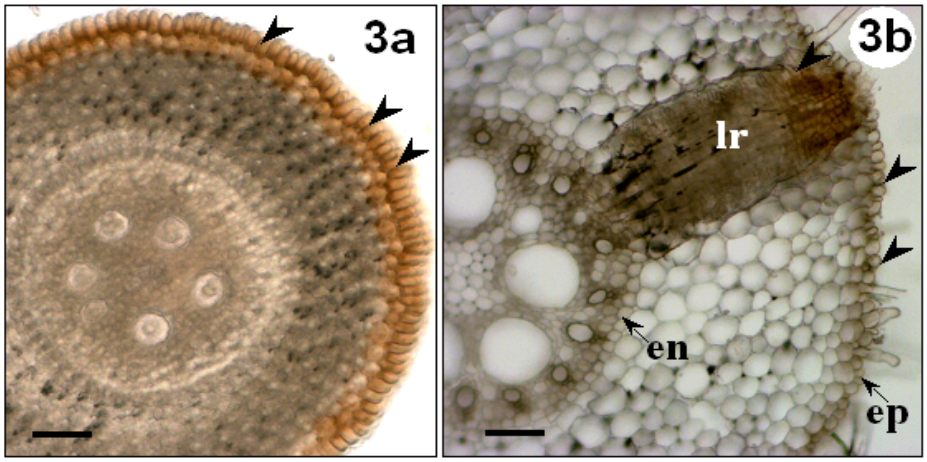

Figure 3 (a, b) Cross-sections of corn roots: treated subsequently by adding 100 µM $CuSO_4$ and 200 µM $K_4[Fe(CN)_6]$ to the root medium of transpiring seedlings. (a) Brown precipitates were accumulated in between the epidermis and the hypodermis at close to the root tip (3 mm from the tip). These two cell layers were stained with brown copper ferrocyanide crystals. In mature parts at 70 mm (b) from the tip, epidermal cell walls were stained with brown precipitates. (b) Lateral root emerging points from the primary root were stained dark brown indicating that these areas provided some kind of an 'open door' for ion intake. Arrowheads show brown precipitates in cell walls. Bars = 50 µm. co, cortical cells; en, endodermis; ep, epidermis; hy, hypodermis; lr, lateral roots.

In addition, these two cell layers were stained with brown crystals. No mature/functional xylem vessels were observed in this region. In mature parts, precipitates were only observed in outer tangential walls of the epidermis (Fig. 3b). Sequential salt applications developed a semipermeable precipitation membrane/barrier in outer walls of the epidermis, preventing further movement or drag of ions apoplastically into the inner tissues with the water. Lateral root emergence points on the primary root were stained dark brown. At these points, salts could move into inner

layers of the cortex. Cells in the lateral roots were covered with dense brown crystals (Fig. 3b).

In experiment two, suction of 100 µM $CuSO_4$ from the root medium into the stele in entire roots and subsequent perfusion of 200 µM $K_4[Fe(CN)_6]$ through xylem vessels under gravity resulted in development of brown crystals in the walls of the stele. Precipitates were especially concentrated around the early metaxylem vessels as well as in cell walls of the passage between the endodermis and early metaxylem (Figs 4a, b).

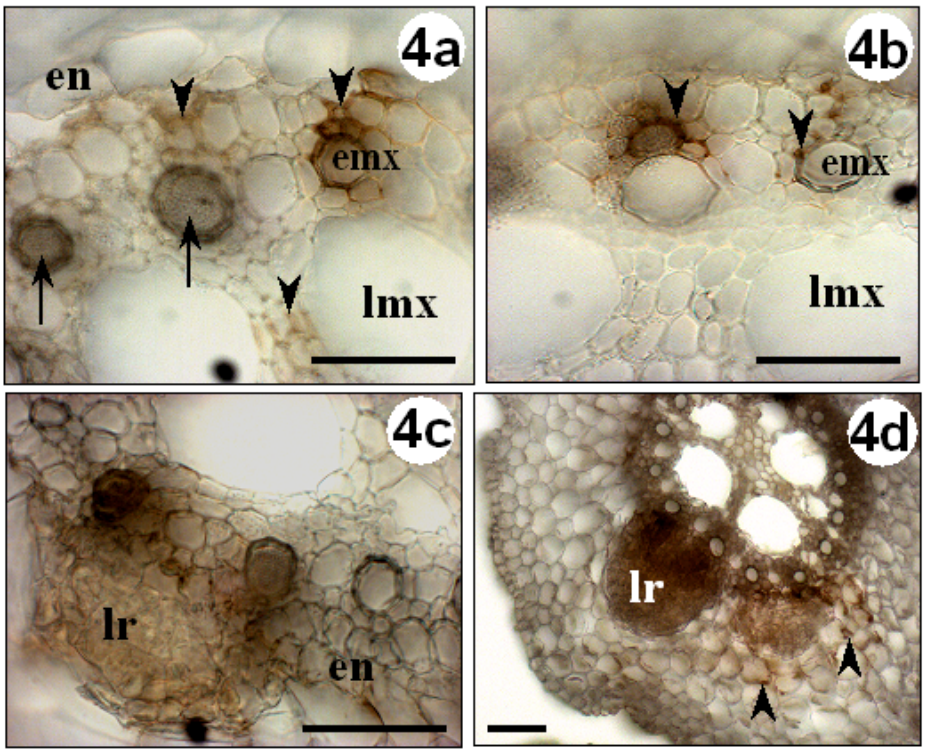

Figure 4. (a)-(d) Free-hand cross-sections of corn roots, made after sucking 100 µM $CuSO_4$ into the stele from the root medium using a vacuum pump, followed by perfusing xylem with 200 µM $K_4[Fe(CN)_6]$. Brown precipitates were found in cell walls of the early metaxylem, and along the passage between CBs of endodermis and xylem vessels at 40 (a) and 70 mm (b) from the root tip. Cell walls of the pith also stained with brown crystals. (c, d) Intense, brown precipitates were found in cell walls at places where laterals penetrated through the endodermis. Arrowheads show precipitated brown crystals in the apoplast (cell walls). Stalked-arrowheads show embolised early metaxylem vessels. Bars = 50 µm. emx, early metaxylem; en, endodermis; lmx, late metaxylem, lr, lateral roots.

Drifting precipitation towards the metaxylem was common (Fig. 4b). Cell walls of 3-5 early metaxylem vessels were stained with brown crystals, which represented 18-35% from the total xylem strands. Observations were similar for middle (40 mm from the tip:

Fig. 4a) and basal parts of the root (70 mm from the tip; Fig. 4b). Brown crystals were noticed in the parenchyma cell walls of the pith (Fig. 4a). To develop drifting precipitates in cell walls towards the xylem vessels, Cu^{2+} ions should have crossed the endodermis apoplastically, where Casparian bands (CBs) do exist in the radial and transverse walls as primary cell wall modifications. Treatment of $CuSO_4$ only for the root tip, instead of the entire root resulted in no brown crystals in the stele. Neither hydroponically-grown corn nor rice developed functional xylem closer to root tips. It started to function approximately 20 mm beyond the root tip, however at this distance, both, corn and rice started to develop CBs in the endodermis (Zimmermann & Steudle 1998; Ranathunge *et al*. 2003; 2004). Emergence of laterals from the pericycle resulted in a discontinuity of the endodermis, allowing a free movement of Cu^{2+} ions into the stele through the apoplast. In such places, intense brown crystals were observed in cell walls throughout the stele. Similarly $[Fe(CN)_6]^{4-}$ could leak out, and brown crystals developed in cell walls of the cortex (Figs 4c, d).

In experiment three, the transpirational stream dragged $CuSO_4$ from the root medium into the stele. Subsequent perfusion of $K_4[Fe(CN)_6]$ through xylem vessels resulted in development of brown crystals in cell walls of the stele, but precipitates were less intense than in the experiment with vacuum suction (experiment two; Fig. 5a).

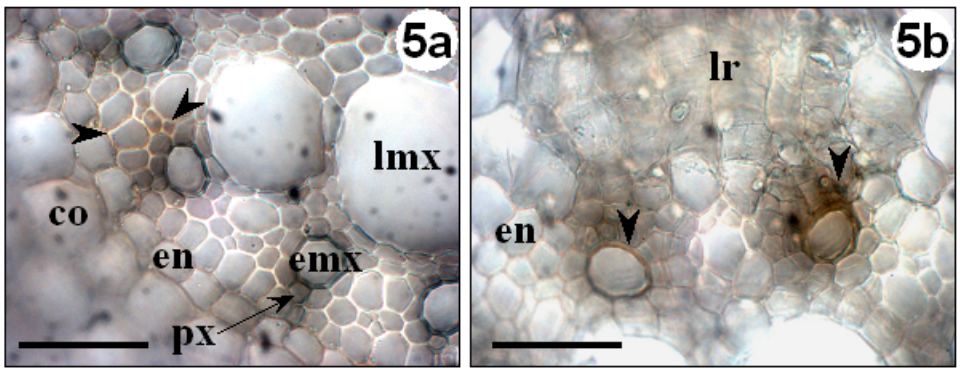

Figure 5 (a, b) Free-hand cross-sections of corn roots (70 mm from the tip), prepared after adding 100 µM $CuSO_4$ to the root medium of transpiring corn seedlings followed by perfusing xylem vessels with 200 µM $K_4[Fe(CN)_6]$. (a) Walls of cells in between the endodermis and metaxylem were stained light brown, but too faint to be visible in print. (b) Dense brown precipitates in the walls of early metaxylem at places laterals emerged. Arrowheads show brown precipitates in cell walls. Bars = 50 µm. co, cortical cells; en, endodermis; emx, early metaxylem; lmx, late metaxylem; lr, lateral roots; px, protoxylem.

Intense brown precipitates were noticed around lateral root emergence points (Fig. 5b). When salt application was reversed, brown crystals were observed neither in the cortex nor in the stele. Possible reasons would be either $[Fe(CN)_6]^{4-}$ could not cross the exodermis at detectable amounts or Cu^{2+} had to move a long way from the stele to the medium under simple diffusion instead of solvent drag mechanism.

Long term treatment of roots with higher salt concentrations (**experiment four**) resulted in cell death and disintegration of the tissue. This led to develop a leaky structure, which allowed free movement of salts from external to inner xylem and *vice versa*. Well-plasmolysed cells were evident in the cortex as well as in the stele (Figs 6a, b). Plasmalemmata were still attached to the radial walls of plasmolysed cells in the endodermis (Fig. 6b). Brown crystals were observed inside the plasmolysed cytoplasm and cell walls.

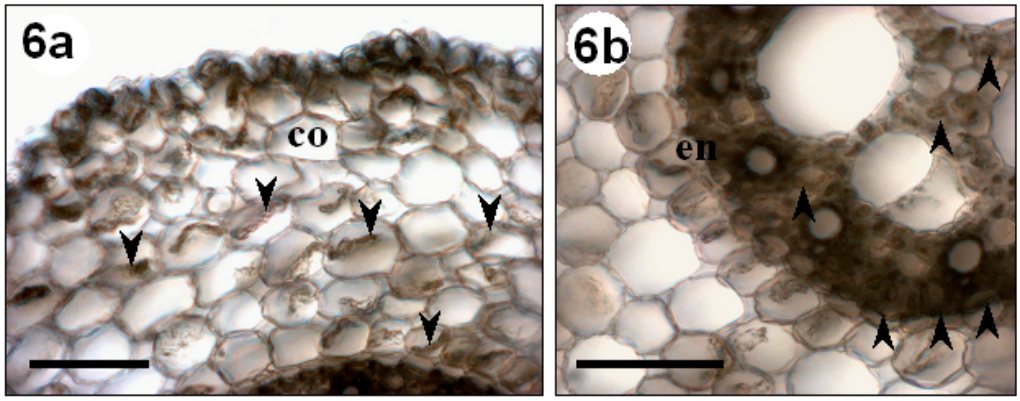

Figure 6 (a, b) Long term treatment (48 h) of corn root segments with 500 µM $CuSO_4$ and 1000 µM $K_4[Fe(CN)_6]$ caused cell death or loss of integrity of plasma membrane. (a, b) Well-plasmolysed cells in the mid cortex and in the stele with localized brown crystals in the cytoplasm. (b) Plasmalammata were attached to the radial walls of plasmolysed cells in the endodermis. Arrowheads show shrank dead cytoplasm with brown crystals. Bar = 50 µm. co, cortical cells; en, endodermis.

Test of apoplastic permeability of $CuSO_4$ and $K_4[Fe(CN)_6]$ in rice roots

In experiment five, water dragged $CuSO_4$ from the root medium into the mid cortex crossing the exodermis and developed brown crystals reacting with ferrocyanide in cell walls of the cortex up to the endodermis (Figs 7a, b). No precipitates were observed in the stele. Since $[Fe(CN)_6]^{4-}$ could not cross the exodermis at sufficient rates (Ranathunge *et al*., 2005), no precipitates were found either in the epidermis or in the

outer tangential walls of the exodermis, but sclerenchyma cell walls were intensively stained brown (Fig. 7a). Once the side of salt application was reversed, brown crystals were only found in the cell walls of the epidermis and in the outer tangential walls of the exodermis bordering epidermis (data not shown, but see Ranathunge *et al*. 2005).

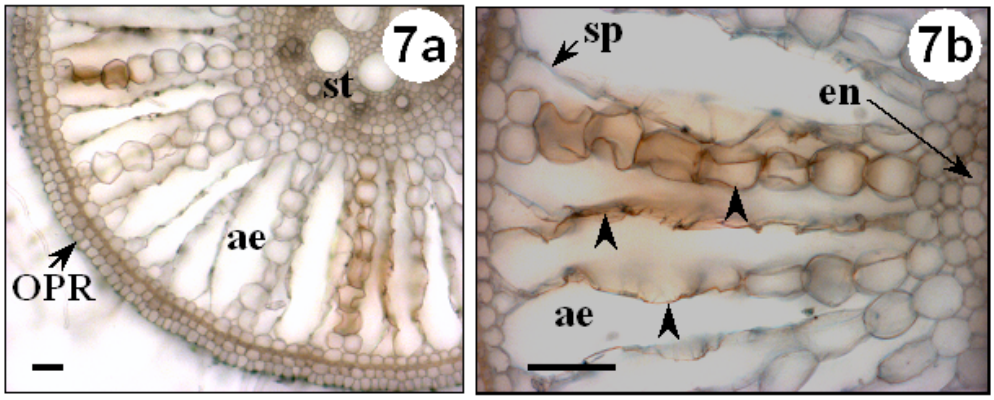

Figure 7 (a, b) Free-hand cross-sections of rice roots (80 mm from the root tip), made after sucking 100 µM $CuSO_4$ into the root from the medium and subsequently perfusing the stele and aerenchyma with 200 µM $K_4[Fe(CN)_6]$. (a, b) Brown crystals were in cortical cell walls and in spoke like structures up to the endodermis. (a) Sclerenchyma cell walls were stained with brown crystals, too. Arrowheads show brown precipitates in cortical cell walls as well as in spoke like structures. Bar = 50 µm. ae, aerenchyma; en, endodermis; OPR, outer part of the root; sp, spoke like structure; st, stele.

When the permeability of CBs in the endodermis of rice roots was investigated, salts were directly applied to the endodermis by perfusion of the aerenchyma (**experiment six**). In immature parts (20 mm from the root tip), suction of 100 µM $CuSO_4$ from the root medium and subsequent perfusion of 200 µM $K_4[Fe(CN)_6]$ through xylem vessels (or *vice versa*) resulted in development of brown precipitates inside the stele as well as in the cortex external to the endodermis (Figs 8a, b). At this distance, CBs of the endodermis have already started to develop but not yet fully matured (Ranathunge *et al*. 2003). At 40 mm from the tip, the development of CBs was complete (Ranathunge *et al*. 2003), but it was evident that Cu^{2+} ions still could cross the endodermis apoplastically, and brown crystals developed and accumulated at the inner side to the CBs bordering to the pericycle (Fig. 8c). Even in mature root zones (at a distance of beyond 60 mm from the tip), intense brown crystals were observed in the walls of some early metaxylem vessels (Figs 8d, e). The number of stained vessels (1-3 out of 12-15) was similar in both at 60 and 100 mm from the root tip (Figs 8d, e).

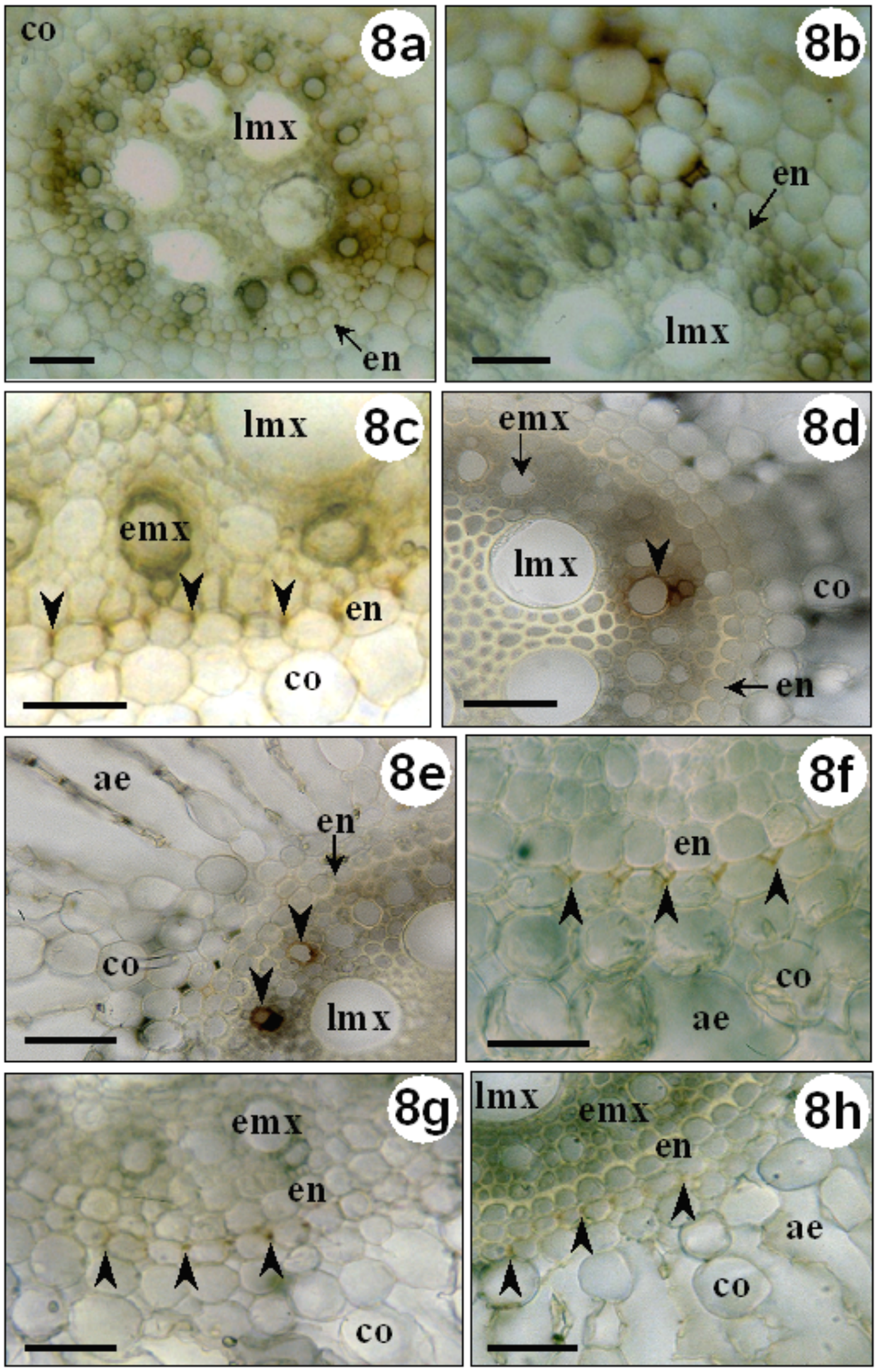

Figure 8 Cross-sections of OPR removed/damaged rice roots, prepared after either sucking 100 µM $CuSO_4$ into the stele from the root medium, followed by perfusion of the xylem with 200 µM $K_4[Fe(CN)_6]$ (a-e) or treating roots with salts in the opposite way (sucking 200 µM $K_4[Fe(CN)_6]$ into the stele, followed by perfusion of the xylem with 100 µM $CuSO_4$; f-h). (a, b) In immature parts (20 mm from the tip), brown crystals deposited in the apoplast of the stele and the cortex. In mature zones, when copper was applied from the outside, brown precipitates deposited at the inner side to Casparian bands bordering to the pericycle (40 mm from the tip; c) as well as in the cell walls of the early metaxylem at 60

(d) and 100 mm (e) from the tip. When ferrocyanide was applied from the outside, light brown precipitates accumulated outside to the Casparian bands bordering cortical cells at 40 (f), 60 (g), and 100 mm (h) from the root tip. Arrowheads show brown crystals in the apoplast or cell walls. Bars = 50 µm. ae, aerenchyma; co, cortical cells; emx, early metaxylem; en, endodermis; lmx, late metaxylem.

Once $CuSO_4$ was applied from the inside, brown precipitates were observed just outside to the CBs of the endodermis bordering the cortical cell layer, but precipitates were less intense (Figs 8f-h). Obviously $[Fe(CN)_6]^{4-}$ ions failed to pass CBs of the mature endodermis in detectable amounts. Movement of Cu^{2+} from the stele to outer cortex crossing the endodermis appeared to be low under gravity (0.007 MPa of pressure), which did not account the solvent drag effect. By contrast, vacuum suction (-0.07 MPa), which is analogous to transpiration, dragged more Cu^{2+} into the stele apoplastically, as already seen for corn. In addition, intense brown precipitates were noticed at the places where laterals penetrated through the endodermis (Figs 9a, b).

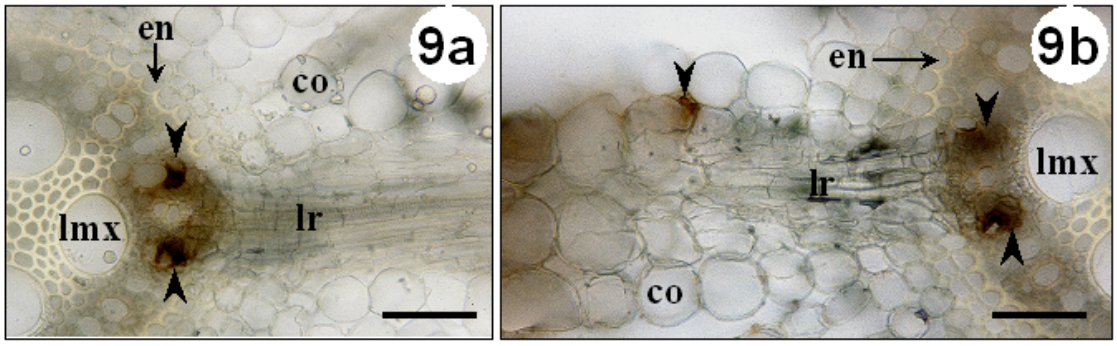

Figure 9 (a, b) Intense brown crystals deposited at the places where laterals emerged from the primary root discontinuing the endodermis. Copper and ferrocyanide ions could cross the barrier moving through these cracks developing brown precipitates at 80 (a) and 100 mm (b) from the root tip. Bar = 50 µm. co, cortical cells; en, endodermis; lr, lateral roots.

Vitality of root cells

The vitality of cells in salt-treated roots (namely in the presence of copper ions) was examined in two ways, i.e. with Evan's blue, a non-permeating dye in living cells, and with the fluorescent dye uranin. Evan's blue cannot pass an intact, healthy plasma membrane (Taylor & West 1980). If cells are dying or dead, the plasma membrane loses integrity becoming leaky for the dye, allowing it to diffuse into the cell. The cytoplasm and nuclei of dead cells are stained blue. In the experiments, salt treated root cells did

not stain blue either in corn (Fig. 10a) or in rice (Fig. 10b) confirming that those roots used for permeability measurements were alive. The tests with the fluorescent dye uranin supported these results. With uranin, the cytoplasm and nuclei of cells were stained green in both treated corn and rice roots indicating that those cells were alive (Figs 10c, d).

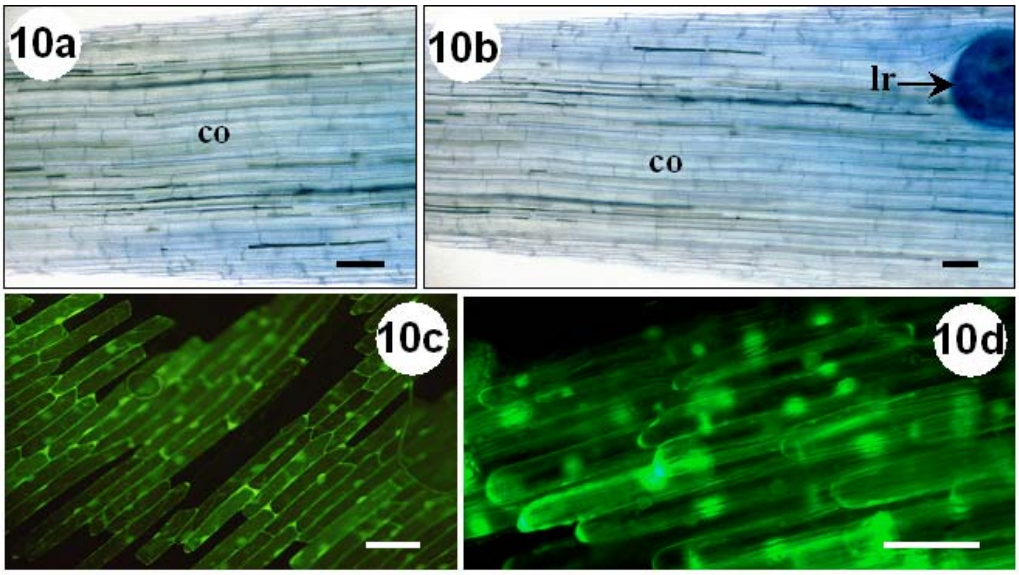

Figure 10 Free-hand longitudinal sections of corn (a, c) and rice (b, d), taken 60 mm from the root tip, stained with either Evan's blue (a, b) or uranin (c, d) to check the viability of cells at the end of the experiments. Generally, in dead cells, cytoplasm and nuclei are stained dark blue with Evan's blue. But, after treatments, in both species, cytoplasm and nuclei were not stained indicating that roots are alive after the treatment. It was further confirmed by stained living cells with uranin. Bar = 50 µm. co ,cortical cells; lr, lateral roots.

Hydraulic conductivity (*Lp*) of individual cortical cells

The *Lp* ($\propto T_{1/2}^{w}$; half-time of water exchange) values of control and Cu^{2+} treated individual cortical cells of outer cortex, measured from water flow generated by a cell pressure probe, did not differ significantly from each other ($T_{1/2}^{w}$ values of control and Cu^{2+} treated cells were 2.3 ± 0.4 and 2.9 ± 0.5 s, respectively.) However, once the control/Cu^{2+} treated ratio was prepared, in order to reduce the variability between cells (Ye, Muhr & Steudle 2005), $CuSO_4$ treatment reduced the membrane water permeability by 30 ± 17%. As in the case of *Lp*, $CuSO_4$ treatment resulted in decline of the cell turgor by 31 ± 9%. The results do show some membrane alteration to ions by the $CuSO_4$ treatment, probably partial inhibition of ion channels leading to lower ion

uptake compared to the control. It may be resulted in a small decline of cell turgor than that of the control (see Discussion).

Radial wall permeability rates of copper sulphate and potassium ferrocyanide in *Chara* cell wall preparations

Chara cell-wall tubes were perfused either with 100 mOsmol/kg $CuSO_4$ or 100 mOsmol/kg $K_4[Fe(CN)_6]$. The amount of salts permeated into the external medium increased with time (Fig. 11). When wall-tubes were perfused with the pump rate of 3×10^{-11} m s^{-1}, higher stationary pressures were generated for ferrocyanide (~ 0.06 MPa) than for copper sulphate (~ 0.03 MPa) indicating that more ferrocyanide ions were retained inside wall preparations. However, in that case, the permeability rate of ferrocyanide was greater than that of copper sulphate. It is correlated with the solvent drag effect (drag of ions through the pores by water, induced by hydrostatic pressure difference), which was bigger for the former than the latter. Once the pump rate was doubled only for $CuSO_4$, similar stationary pressures were obtained for both salts (~ 0.06 MPa). In that case, the hydrostatic pressure gradient through the wall was similar for both salts, and the permeability rate of $CuSO_4$ through *Chara* cell wall-tubes was greater by a factor of ~1.5 than that of $K_4[Fe(CN)_6]$ (Fig. 11). The back flow and dilution of internal perfused solution was negligible (< 5-7%) because of the large internal volume of the experimental set-up. The results showed that movement of copper ions through cell walls was faster (less hindered) than that of ferrocyanide.

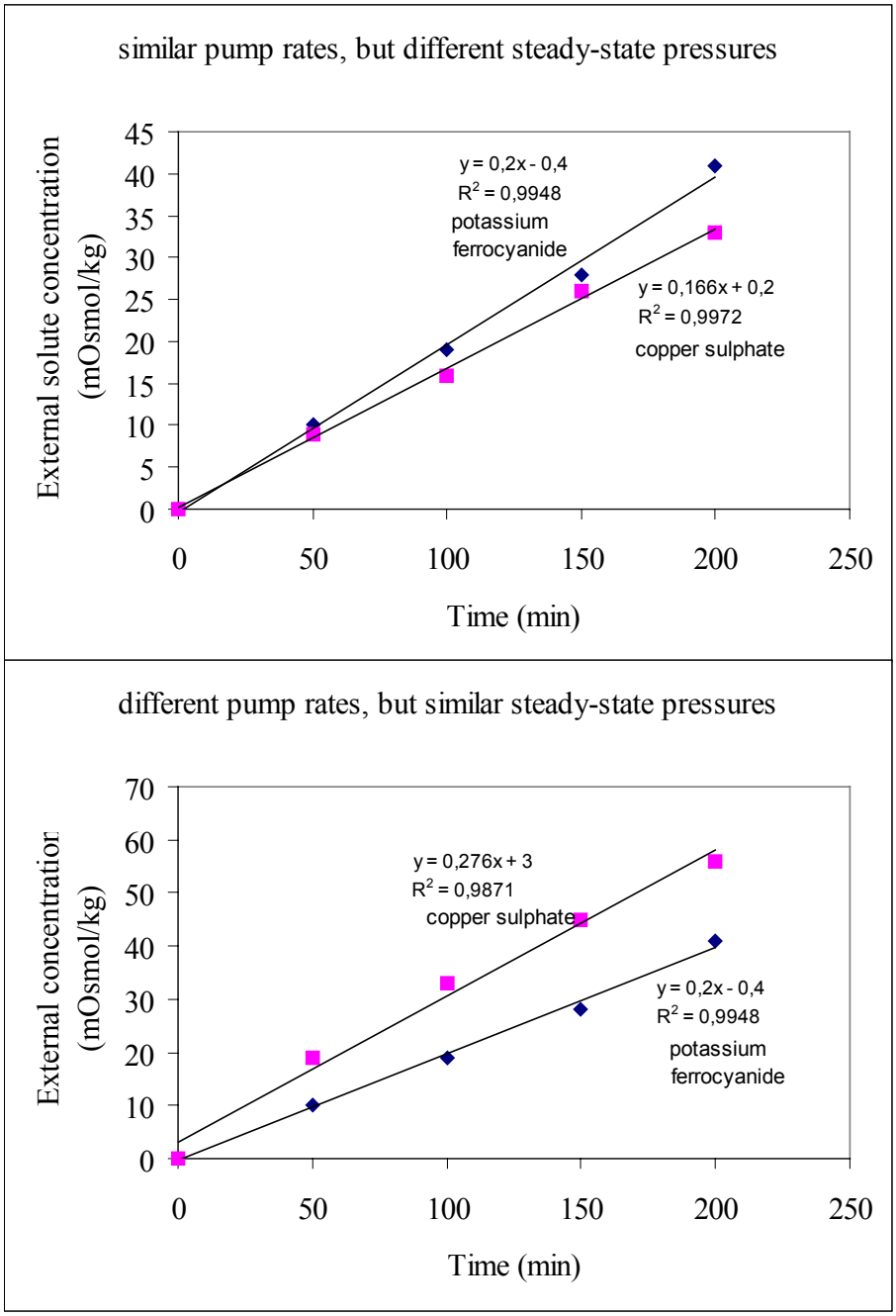

Figure 12 (a, b) Increases of either copper sulphate or potassium ferrocyanide concentration in the external medium with time for untreated *Chara* cell wall preparations. With similar pump rates, cell wall preparations retained more potassium ferrocyanide than that of copper sulphate resulting higher steady-state pressures in the system. In this situation, solvent drag effect for ferrocyanide was greater than that of copper, hence higher permeation rates were observed for ferrocyanide (a). Once similar steady-state pressures (similar pressure gradients from wall tubes to external medium) were obtained for both salts changing pump rates, permeation rate of copper through cell walls was greater than that of ferrocyanide (b).

Discussion

The results provide direct anatomical evidence that Casparian bands (CBs) do allow some passive passage of ions besides water. In the precipitation technique used, 100 µM $CuSO_4$ and 200 µM $K_4[Fe(CN)]_6$ were applied either in the xylem or in the medium of roots. Those salts were dragged into the xylem with the water (solvent drag), either by transpiration (intact plants) or pump suction (excised roots). The presence of precipitates of insoluble copper ferrocyanide at the endodermis close to CBs and drifting precipitation towards the metaxylem vessels indicated a passage of ions across CBs. However, this finding does not mean that CBs do not represent a substantial barrier for ions. It just means that the barrier is not completely impermeable.

Before the data obtained from the new precipitation technique can be considered as real, a few possible sources of error or artifacts have to be considered. (i) A great care was given when handling roots in the experiments without exposing to physical stresses or bending the roots to prevent structural defects in the CBs or endodermis. Even roots with tiny natural wounds could be identified during the experiments because of heavy brown precipitates accumulated at wounding places within short period of time. Such roots were discarded without using for further experiments. (ii) It was concerned about the possible effects of Cu^{2+} toxicity on the plasma membrane. That could create a leaky structure for ions. Viability tests with sensitive dyes proved that Cu^{2+} treated roots were alive and plasma membranes were intact even at the end of the experiments. Furthermore, if membranes were damaged or leaky, brown precipitates could have been observed everywhere in the roots. However, in these experiments, brown crystals were localized to certain places, and this clearly indicated that Cu^{2+} treated roots were alive. (iii) The idea of the transition metal, Cu^{2+} can reduce water flow through membranes, resulting in a relatively greater apoplastic flow was tested. Copper ions may inhibit water channel (aquaporin) activity similar to mercurials ($HgCl_2$) by attachment to SH groups of cystein residues (Henzler & Steudle 1995; Maurel 1997; Zhang & Tyerman 1999). Hence, it may be argued that Cu^{2+} treatment artificially increased the apoplastic water flow creating a relatively greater solvent drag effect, which led to drag Cu^{2+} across CBs. It was found that, even though, Cu^{2+} treatment reduced the membrane water flow across cortex cells, the effect was as small as 30% and substantially smaller than

that of $HgCl_2$ (as one would expect according to the higher affinity of Hg^{2+} to sulfhydryl groups as compared with Cu^{2+}). For example, in wheat root cells, Hg^{2+} reduced the membrane water flow by 75% (Zhang & Tyerman 1999). Reduction was 9-fold in onion and 7-fold in corn (Barrowclogh, Peterson & Steudle 2000; Wan, Steudle & Hartung 2004). The present results showed that Cu^{2+} did affect the membrane water permeability but not as substantial as Hg^{2+}. This may have increased the pressure gradient across the endodermis, thus leading to a somewhat bigger water flow across CBs. Anyhow, even if there was a relative increase in the apoplastic component, this does not affect the conclusion that CBs are not completely interrupting the apoplastic water and ion flow.

In addition to dye-vitality tests, the pressure-probe experiments have been used as an another indicator to check the vitality of root cells after the $CuSO_4$ treatment. If cells are dead, no turgor pressure as well as no responses to pressure relaxations can be expected. However, measurements with cell pressure probe proved that root cell membranes were intact even after the Cu^{2+} treatment, also turgor was reduced by 31%, perhaps, by inhibiting nutrient uptake besides the water.

There is already some evidence that CBs are permeable to water, at least to some extent (Steudle 1989; Steudle, Murrmann & Peterson 1993; Zimmermann & Steudle 1998; Steudle & Peterson 1998). The results of the present study support and extend this view in that ion movement is incorporated as well. In the past, a substantial contribution of the apoplastic path to both water and solutes has been inferred from comparison between cell Lp and root Lp_r and from measurements of root reflection coefficients (σ_{sr}) and permeability coefficients (P_{sr}) (Steudle & Frensch 1989; Steudle & Peterson 1998; Zimmermann & Steudle 1998). For technical reasons, there are, to date, only a few direct results indicating a permeability of CBs to water and ions. Zimmermann & Steudle (1998) grew corn seedlings under different conditions. In hydroponics, roots developed no exodermis but in aeroponics they did. This corresponded to a substantially lower hydraulic conductivity. However, solute permeability was not much affected. The findings have been interpreted that water flow was not completely interrupted by CBs and solute permeability was limited at the endodermis. In rice, anatomical studies showed the presence of an exodermis, which, however, was permeable to both water

and ions. Ranathunge *et al*. (2003; 2004) experimentally demonstrated that despite the existence of an exodermis with CBs, most of the water moved around cells rather than using a cell-to-cell passage. In copper ferrocyanide precipitation experiments, brown precipitates were noticed at the side where ferrocyanide was applied, suggesting that copper ions were passing the barrier including the exodermal CBs (Ranathunge *et al*. 2005). This and the present evidence are in line with earlier findings of an apoplastic passage of ions (Na^+) and tracer dye PTS in rice roots (Yeo *et al*. 1987; Yadev *et al*. 1996). Present findings are further supported by earlier experimental findings of substantial endodermal apoplastic bypass of Ca^{2+} in rye (White *et al*. 1992), Cl^- in citrus (Storey & Walker 1999), and heavy metals, i.e. Zn^{2+} in *Thlaspi caerulescens* (White *et al*. 2002), Cd^{2+} in *Salix* (Lux *et al*. 2004) as well as of the stress hormone ABA in corn roots (Freundl *et al*. 1998; Hose, Steudle & Hartung 2000; Schraut, Ullrich & Hartung 2004).

It may be argued that movement of Cu^{2+} into the stele through the endodermis is exclusively through the plasma membrane using ion channels or Cu^{2+} transporters. If this was true, the findings could be interpreted as a cellular movement of Cu^{2+} across the endodermis rather than through the apoplast. However, apoplastic Cu^{2+} flow could be substantial because (i) according to the authors' best knowledge, neither Cu^{2+} channels nor Cu^{2+} transporters were yet found in the plasma membrane of root cells, though Zn^{2+} transporters were found in *Arabidopsis thaliana* and heavy metal sensitive *Thalspi caerulescens* (Pence *et al*. 2000; Hussein *et al*. 2004). (ii) Other ion channels transporting divalent cations (such as Ca^{2+} channels) are expected to be sufficiently selective to prevent movement of Cu^{2+} and of other heavy metals to the xylem (White 2001). (iii) Heavy metal cations (i.e. Cu^{2+}, Zn^{2+}) are usually absorbed by roots as chelates after binding to organic acids, amino acids or peptides as a detoxifying mechanism, and move through the apoplast without binding to negatively charged cell walls (Marschner 1995; Hall 2002). (iv) If majority of Cu^{2+} ions move through the membrane, that leads to increase the Cu^{2+} concentration in the cytosol. So, most of cytosolic Cu^{2+} should be pumped into the vacuole (vacuolar compartmentalization) as well as adsorb onto the cell walls as heavy metal tolerance mechanisms (Hall 2002). Hence, intense, brown crystals could be expected in cell walls especially of the endodermis and all over the stele. However, in these experiments, crystals were

localized into certain places. (v) Most important, however, was the finding, in both species, that precipitates were found just opposite to CBs and on the passage between CBs and early metaxylem (drifting precipitation towards the early metaxylem) where they had be swept to the stele with the water flow across CBs. Massive precipitates were also observed at places where secondary root initials immerged through the endodermis. These findings could not be interpreted in terms of an artifact during the preparation of sections. One could argue that during sectioning, the barrier between two compartments separated by the endodermis destroyed and led to brown precipitates. However, in this case brown crystals should have been observed also along the membranes of tangential walls of the endodermis. (v) Moreover, brown copper ferrocyanide precipitates were only noticed in the cell walls rather than in the symplast, suggesting that most of the used ions moved through the apoplast. All these evidences clearly show that there was some movement of Cu^{2+} ions *via* the apoplast with the transpiration stream, i.e. CBs were not completely impermeable.

When considering the structure of the root, one can argue that Cu^{2+} could enter to the stele apoplastically at the root tip, where CBs were nonexistent. This idea could be excluded because no functional or mature xylem vessels were detected closer to the root tip in hydroponically grown corn and rice, and started to develop at around 20 mm from the tip. However at this distance, both, corn and rice started to develop CBs in the endodermis (Zimmermann & Steudle 1998; Ranathunge *et al*. 2003). On the other hand, if Cu^{2+} entered to the stele from the root tip and moved upward, brown crystals could be observed in all xylem poles rather than in few localized precipitates. However, we didn't notice such observations. Even though, corn and rice, both exist passage cells in the endodermis, they do contain CBs in transverse and radial cell walls as primary cell wall modifications (Clark & Harris 1981; Zimmermann & Steudle 1998; Ranathunge *et al*. 2003). Hence, the idea that ions move apoplastically only at the passage cells can be excluded.

The technique used here was very sensitive, hence very low concentrations of salts in the apoplast could be detected. At 25°C, the solubility product of $Cu_2[Fe(CN)_6]$ is 1.3×10^{-16} M^3 (Hill & Petrucci 1999). So, when we perfused the xylem with a $K_4[Fe(CN)_6]$ solution of 200 μM, we may have detected copper ions in the apoplast at a

concentration of as low as 0.8 μM (assuming 200 μM $[Fe(CN)_6]^{4-}$ throughout the apoplast). The absence of brown precipitates in the symplast, neither imply that lack of a flux through the cell-to-cell path (symplastic path through plasmodesmata and transmembrane path) nor all ions moved through the apoplast crossing the endodermal CBs. This may be due to either the insufficient concentration and accumulation of Cu^{2+} and $[Fe(CN)_6]^{4-}$ in the symplast for a precipitate to form or the used method to detect crystals was not sensitive enough for less intense crystals in the symplast. Hence, results can be interpreted in terms of an apoplastic as well as cell-to-cell flow of ion rather than an exclusive membrane bound ion flow.

When salts were provided at different sides of the barrier, an asymmetric development of precipitates were obtained. This suggested that the cation, Cu^{2+}, moved faster than the anion $[Fe(CN)_6]^{4-}$ through cell walls. This is in agreement with the previous finding of Ranathunge *et al*. (2005) for the OPR of rice roots. In order to check this hypothesis, *Chara* cell wall preparations ("ghost") were used as a model system. It has been suggested that the effective diameter of the wall-pores in *Chara* ranged between 2-10 nm besides some pores with diameters of greater than 10 nm (Berestovsky, Ternovsky & Kataev 2001; Dainty & Hope 1959). Steady-state perfusion of salts through cell wall-tubes showed that retention of ferrocyanide inside wall-tubes was greater than that of copper, which had a higher permeation rate through cell walls to the outer medium. Cell wall pectic substances are generally made of galacturonic acid entities with –COOH groups, which are responsible for the overall negative charge of the cell wall. Those negatively charged cell walls might repel and restrict movement of anions through the large intermicrofibrillar spaces (Clarkson 1991).

At places where lateral roots emerge from primary roots, the continuity of the endodermis is lost (Peterson, Emanuel & Humphreys 1981). It is, hence, expected that this allows some leakage of water and solutes such as apoplastic dyes. In this study, dense brown precipitates were observed around lateral root emergence points for corn and rice, which is in line with the earlier observations. These places may act as "open doors" not only for water and apoplastic tracer dyes but also for ions to move freely though the apoplast into the xylem (Clarkson 1993; Ranathunge *et al*. 2005). In mature/basal parts, discontinuity of the endodermis in roots of corn and rice, as well as

local disruptions of the exodermis in rice, when lateral roots develop from the pericycle, are most likely to allow substantial apoplastic bypasses. Those sites may act as a locus for water, dye and ion movement into the xylem, likely to be healed when laterals mature (Peterson *et al*. 1981; Ranathunge *et al*. 2005).

In conclusion, the data show that the application of a new precipitation technique provides evidence of some permeability of endodermal CBs for ions such as Cu^{2+} in roots of corn and rice. Permeability of the endodermal CBs of corn and rice were intensively tested with copper and ferrocyanide ions. Under suction or transpirational flow, Cu^{2+} could cross the endodermis apoplastically in both plant species (even though at small rates) suggesting that CBs are not perfect barriers to apoplastic fluxes, at least for copper ions. The hypodermis of corn and exodermis of rice could not occlude the movement of Cu^{2+} into the mid cortex *via* the apoplast. On the contrary, ferrocyanide ions failed to cross the mature endodermis of both corn and rice as well as the exodermis of rice in detectable amounts. Asymmetric development of precipitates provided evidence that the cation, Cu^{2+} moved faster than the anion, $[Fe(CN)_6]^{4-}$, through cell walls, most likely because of four negative charges of the ferrocyanide ion which are likely to be repelled by negatively charged cell walls. Using *Chara* cell wall preparations ("ghost") as a model system, it was shown that more ferrocyanide ions remained inside wall-tubes than copper ions, which had a higher permeation rate through cell walls. In roots, there was a significant apoplastic flux of ions in regions where laterals penetrated the endodermis and exodermis, and Cu^{2+} and $[Fe(CN)_6]^{4-}$ ions could freely cross the barriers through the apoplast developing brown copper ferrocyanide precipitates. Overall, the conventional assumption that "the permeability of CBs to water, nutrient ions is nil" (Peterson & Cholewa 1998) should be modified, although we agree that permeability of CBs to ions should be fairly low. It may vary depending on growth conditions, which have an impact on the intensity of bands. By using other combinations of salts that form precipitates i.e. calcium oxalate, barium sulphate, this idea can be tested. These types of experiments are under way.

Acknowledgements

We thank Dr. Seong Hee Lee (Department of Plant Ecology, University of Bayreuth) for her expert assistance with the cell pressure probe and Burkhard Stumpf (University of Bayreuth) for outstanding technical help. This work was supported by the Deutsche Forschungsgemeinschaft: Ste319/3-4 (E.S.), and B.M.Z. (project no. 2000.7860.0-001.00) 'Trait and Gene Discovery to Stabilize Rice Yields in Drought Environments' (R.L.).

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7 Apoplastic water transport in roots

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Kluwer Academic Publishers, The Netherlands (in press)

Abstract

Results obtained from combined measurements at the cell and root levels (cell and root pressure probe) indicate an important role of apoplastic water transport in roots, even in the presence of apoplastic barriers (Casparian bands and suberin lamellae in the endo- and exodermis). The composite transport model of the root explains the variable root hydraulic conductivity (Lp_r) and its physiological benefits, as well as low root reflection coefficients and the switching between pathways (apoplastic *vs.* cell-to-cell) depending on demands for water from the shoot. Switching between pathways provides a coarse, and changes in aquaporin activity a fine regulation of root Lp_r. Recent measurements of the hydraulics of rice roots support the composite transport model and the view that apoplastic barriers exhibit water permeabilities of larger than usually assumed.

Introduction

Different from ions, water flow across roots involves no active pumping. Both, across the root cylinder and in xylem vessels along the root, water flow is down-hill following gradients in free energy (water potential) or pressure. Water uptake by plant roots can be described by simple force/flow relations analogous to Ohm's law and is characterized by hydraulic conductances or resistances. The latter parameters are known to be highly variable. This affects the water status of plants. At a given rate of transpiration, the water supply by roots determines the water status of the shoot and its ability to assimilate carbon dioxide. The regulation of water input by roots is as important as that of the output (stomata). For technical reasons, much is known about the regulation of the latter, but little about the regulation of water uptake. Evidence collected over the past decade shows that the phenomenon of variable root hydraulics is not only related to the permeability of root cell membranes for water (as it largely is for nutrient ions), but also depends on its apoplastic passage. The presence of apoplastic barriers is important (Casparian bands and suberin lamellae in the endo- and exodermis). The anatomical complexity of the root dictates that the flow of water through it will also be complex. Flow is best described by a composite transport model which allows for differences in movement through membranes of individual cells and along the apoplast, as well as through various tissues (see reviews: Hose, Clarkson, Steudle, Schreiber and Hartung, 2001; Steudle 2000a, 2000b; 2001; 2002a, 2002b; Steudle and Frensch 1996; Steudle and Heydt 1997; Steudle and Peterson 1998). In the following, recent findings are summarized which relate to apoplastic water flow in roots. Results have been obtained using cell and root pressure probes (Steudle 1993) and different types of pressure chambers and pressure perfusion techniques for herbacous (maize, sunflower, bean, onion, barley) and woody (oak, spruce, beech) plants. Some recent results for rice have been added (Ranathunge et al. 2003; Ranathunge et al. 2004). Because of differences in the structure of roots of wetland plants, they allow a more detailed view on root hydraulics and tests of current models as do others.

Water flow in roots is variable: flows and forces

The numerous factors which affect the capability of roots to take up water may be classified in two groups, i.e. those which affect the hydraulic resistance of roots and those which refer to the different forces that move water. The hydraulic resistance depends on the size of root systems, i.e. on the area available for water uptake. However, it also very much depends on the specific hydraulic conductivity which is a measure of conductance per unit surface area (sometimes also given per unit root length) and per unit driving force given in units of a pressure (Pa or MPa). Hydraulic conductivities (Lp_r in m s^{-1} MPa^{-1}) rather than conductances are used for a quantitative comparison of roots of different species or roots at different developmental states of a given species.

Usually, the radial transport of water across the root cylinder rather than the axial transport within the root xylem dominates the overall water uptake by plant roots (Steudle & Peterson, 1998). When referred to unit area of outer root surface, the radial flow of water across a root (J_{Vr} in $m^3 \cdot m^{-2} \cdot s^{-1}$) is given by:

$$J_{Vr} = Lp_r \, [P_r - \sigma_{sr}(\pi_x - \pi_m)] \; . \qquad (1)$$

The expression in brackets represents the driving force, which comprises the pressure difference between root xylem and soil solution (root xylem pressure P_r referred to reference atmospheric pressure), and the difference in osmotic pressure between the two compartments (xylem, π_x, and root medium, π_m). In case of a transpiring plant, P_r would be negative, but may be also positive in the absence of transpiration (root pressure). It can be seen from Eq. (1), that the osmotic component is modified by the root reflection coefficient, σ_{sr}. This indicates that roots may not behave like an ideal semipermeable osmometer and $\sigma_{sr} < 1$. The reflection coefficient is a measure of the selectivity of roots to solutes such as nutrient ions or others present in xylem sap and soil solution.

Pathways for water in roots

In the root cylinder, water uses the pathway(s) of lowest resistance. Usually, this is the apoplast, i.e. the passage around protoplasts. During root development this may change by the formation of Casparian bands and suberin lamellae in the endo- and exodermis. Casparian bands are formed in one or more layers of the hypodermis (exodermis) or in the endodermis by the deposition of suberin and lignin in anticlinal cell walls. They may largely interrupt water and ion flow and enforce a transmembrane movement of these compounds. Depending on the species, but also on the root's developmental and physiological state, root Lp_r comprises different components. It clearly incorporates the Lp of root cell membranes, which, in turn, has a symplastic (mediated by plasmodesmata) and a transmembrane component. The latter is dominated by aquaporins or water channels, the activity of which is subjected to some "gating" by different internal or external parameters such as drought, high salinity, temperature, nutrient status, or diurnal rhythm. Aquaporins are thought to be the molecular basis of water relations (Clarkson et al. 2000; Henzleret al. 1999; Javot and Maurel 2002; Maurel 1997; Maurel and Chrispeels 2001; Schäffner 1998; Steudle 2000a, 2000b; 2001; Steudle and Henzler 1995; Tyerman et al. 1999; Tyerman et al. 2002; Ye et al. 2003). The symplastic and transmembrane components cannot be separated to date experimentally, and are, hence, summarized as a cell-to-cell component, which is measurable using the cell pressure probe (Steudle 1993). Pressure probes also permit measurement at the root level (individual roots and root systems; Hose et al. 2000). Comparison of data allowed conclusions about pathways and transport models (see reviews).

Driving forces during water uptake and losses: composite transport

According to Eq. (1), there are two distinct driving forces for water. Provided that the reflection coefficient is unity (semipermeable osmotic barrier), they can be summarized by an overall difference in water potential. The hydrostatic driving force (P_r) should dominate in transpiring plants where transpiration from leaf surfaces causes tensions in the root xylem and the xylem osmotic pressure is rather small. On the other hand, there should be positive root pressures in its absence, when osmotic forces dominate. From Eq. (1), one would expect that root Lp_r is the same regardless of a pressure (hydrostatic) or osmotic

driving force (hydrostatic or osmotic water flow). However, this not so. Differences can be as large as a few orders of magnitude in woody and up to a factor of ten in herbaceous species (see reviews). The reason for the deviations is that, in the presence of osmotic forces across the root cylinder, the driving force along the apoplast is small due the low selectivity and low reflection coefficient of the cell wall compartment ($\sigma_{cw} \approx 0$). Along the cell-to-cell passage this is different ($\sigma_{cc} \approx 1$). Overall, the contribution of the apoplast to osmotic water uptake should be small. This is different, when pressure gradients are present which are effective both along the apoplastic and cell-to-cell pathways.

Differences are well understood in terms of the ”composite transport model”. Composite transport explains the contribution of different pathways to overall root Lp_r and the switching between pathways depending on conditions. The term “composite transport” results from irreversible thermodynamics where it has been used to explain the overall permeability of patchy membranes in terms of permeabilities of individual arrays (see reviews). To some extent, the theory implies a non-additive of coefficients. The composite transport of roots explains the findings of (i) variable root Lp_r, (ii) low reflection coefficients, (iii) differences between osmotic and hydrostatic root Lp_r (i.e. the switching), and differences between species (e.g. herbaceous *vs.* woody; Steudle and Frensch 1996; Steudle and Peterson 1998).

When used with roots, the parallel arrangement of apoplastic and cell-to-cell pathways is the most relevant feature. Composite transport is more efficient in explaining variable root Lp_r and the other findings as do other models, which assume a variation in driving force or of root membrane permeability (e.g. Fiscus 1975; Weatherley 1982).

Composite transport in roots of herbaceous plants

Most of the evidence in favour of the composite transport model has been derived from pressure probe work (cell and root level) with excised roots of herbaceous plants such as corn, bean, onion, or sunflower. Some of the evidence is summarized in the following. Early work with the root pressure probe indicated that roots behave like osmometers, though not like ideal ones (low root σ_{sr}; see reviews). Results indicated an apoplastic bypass for both water and small solutes, which also referred to the endo- and exodermal

Casparian bands. Apoplastic barriers, however, showed some flexibility in their selectivity and permeability depending on the species, growth conditions, and developmental state of these structures (Barrowclough et al. 2000; Frensch et al. 1996; Hose et al. 2001). For example, in roots of corn and sunflower the rather big ABA molecule could be swept from the root medium into the root xylem by solvent drag (Freundl et al. 1998; 2000). The fact that the solvent drag was bigger in roots of corn than in those of sunflower may indicate that the amount of "porosity" of Casparian bands is bigger in the former. It turned out that reflection coefficients for ABA (as estimated from solvent drag) depended on pH. At slightly alkaline pH = 8, when ABA was present in anionic form, the reflection coefficient was bigger than at slightly acid (pH = 4.8), when part of the ABA was present in unsdissociated form. As a general rule, uncharged solutes were more permeable than charged nutrient ions.

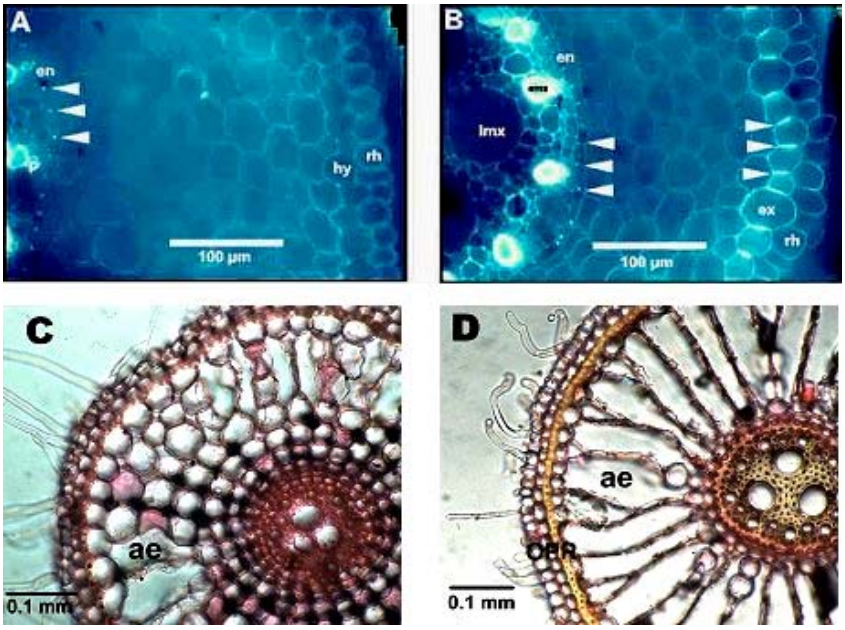

Figure 1 A, B Freehand cross-sections of 8-d-old maize plants stained with berberine-aniline blue and viewed under an epifluorescence microscope. Lignified vessels and Casparian bands appear bright. In **A** (hydroponic culture), the distance from the tip was 80 mm. rh=rhizodermis, hy= hypodermis **without** Casparian bands; en=endodermis (primary state) with Casparian bands (arrowheads); p=mature protoxylem. In **B** (aeroponic culture), the distance from the root tip was 50 mm. ex=mature exodermis, (secondary state) **with** Casparian bands (arrowheads). **C, D** Cross-sections of 30-d-old rice plants were stained with Sudan Red 7B. Development of aerenchyma at 20 (**C**) and 100 mm (**D**) from the root tip .ae=aerenchyma, OPR=outer part of the root.

In corn roots, the effect of a Casparian bands in the exodermis was tested for by growing roots with and without an exodermis. The existence of an exodermis caused a decrease of root hydrostatic root Lp_r by a factor of 3.6 at constant root membrane *Lp* (Zimmermann and Steudle 1998). There was no change in the presence of low osmotic gradients as expected from the composite transport model. Results were correlated with chemical analyses in different root zones, which indicated that aliphatic suberin in Casparian bands of the exodermis caused the decrease in root Lp_r (Zimmermann et al. 2000).

Despite these results, there is still a lack of information of how different growth conditions, stresses and other treatments affect the permeability of apoplastic barriers (see Schreiber et al. this issue). From the work of Schreiber's group in Bonn much is known about the chemical composition of apoplastic barriers (Schreiber et al. 1999). However, there is still considerable uncertainty about the actual porosity and submicroscopic structure of these barriers (Hose et al. 2001; Ma and Peterson 2003) As they form, permeability is reduced, but rarely to zero. Passive selectivity (reflection coefficients) increases as the cell-to-cell path becomes more important for water and solutes. Different from Casparian bands, there is much less quantitative evidence about the role of suberin lamellae.

Physiological benefits of composite transport

Composite transport of water in roots is beneficial for the plant. It provides a mechanism to regulate water uptake according to the needs of the shoot. In the presence of high rates of transpiration, demands for water are high. Under these conditions, water flow across the root is hydraulic in nature and root Lp_r high. Both, the apoplastic and the cell-to-cell pathways are used. When transpiration is switched off, the cell-to-cell passage is left which has a relatively high resistance. This prevents water losses to a dry soil during the night. This type of a physical adjustment due to a switching on or off of the apoplastic passage has been termed a "coarse regulation". It allows rapid changes of root Lp_r according to the needs of the plant. It would explain most of the variability in root Lp_r. Under conditions of water shortage, plants develop roots, which are heavily suberized and have a low root Lp_r because of substantial apoplastic barriers. These barriers prevent water losses to the dry soil, but should have a negative effect on water uptake, when conditions become more

favorable. The disadvantage can be compensated for by the existence of water channels which are under metabolic control and are inhibited/gated by factors such as heavy metals, hypoxia, nutrient deficiency, low temperature, drought and high salinity (see reviews). Water channel activity may provide a "fine regulation" of water uptake and the only way to take up water under harsh conditions.

Apoplastic barriers are much discussed in relation to water uptake. However, they are probably as important as barriers preventing excessive water losses to the dry soil under conditions of water shortage in the absence of a sufficient transpirational force for water uptake (Stavosky and Peterson 1993; Steudle 2000b; Taleisnik et al. 1999). Although it is well documented that roots suberize in response to drought and other stresses, there are hardly measurements of changes in root Lp_r and in root cell Lp. These measurements are badly needed, as is the comparison between cell and root level to work out contributions of pathways (Azaizeh et al. 1992). Besides the interplay between the physical switching between pathways and water channel activity, there are of course other means for plants to regulate water uptake such as by root growth under favorable and root death under adverse conditions. However, these responses are on a much longer time scale compared to those mentioned above.

Composite transport in rice roots

Wetland plants such as paddy rice develop root systems, which grow into hypoxic substrates at the risk that oxygen delivered from the shoot down to root tips would be largely lost by radial diffusion across the outer part of the OPR (= outer parts of roots; Ranathunge et al. 2003). Hence, there should be barriers such as Casparian bands and suberin lamellae to prevent these losses. In Fig. 1D, it is shown that the OPR of rice roots has a well-developed exodermis with Casparian bands and suberin lamellae. This may cause problems for the water uptake, when the passage across Casparian bands is blocked and the cell-to-cell passage affected by suberin lamellae. Accordingly, it has been shown that rice plants, even though rooting in a wet substrate, may suffer from water shortage (Hirasawa et al. 1992).

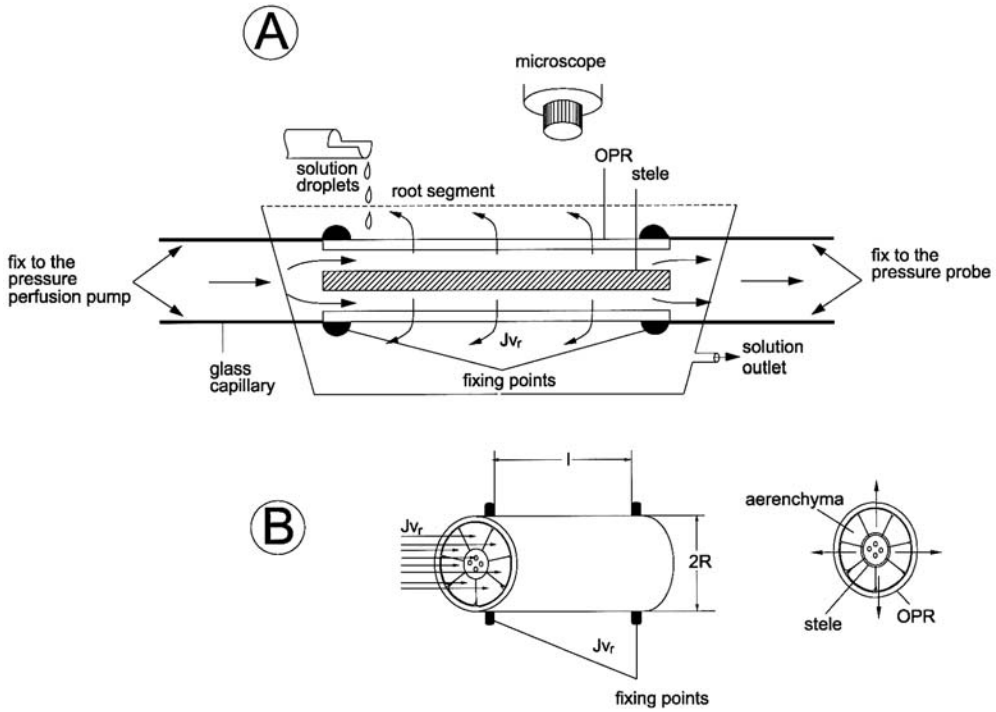

Figure 2 (**A**) Pump perfusion setup: A syringe was mounted on a Braun-Melsungen pump (not shown) that created pump rates between 1.7×10^{-9} and 1.1×10^{-7} mm^3 s^{-1}. One end of the root segment was used as an inlet. This was fixed to the syringe by a narrow and rigid Teflon tube. The other end was connected to a pressure probe to measure resulting steady state pressures. (**B**) Schematic diagram to show radial water flow across the outer part of the root segment during pressure perfusion. At a given pump rate, stationary pressure was established where the volume flow provided by the pump equaled the radial volume flow across the outer part of the root (OPR).

For the first time, we studied the hydraulics of rice roots in some detail using pressure chambers and probes as well as a new perfusion technique (Fig. 2). The results indicated the importance of apoplastic water transport, at least in the OPR. Miyamoto et al. (2001) showed that root Lp_r and σ_{sr} were rather low which was interpreted as a major resistance at the endodermis and some apoplastic bypass of water and solutes despite suberization. When the small size of rice root systems is taken into account, the water supply of the shoot must indeed be quite limited.

Table 1 (**A**) Hydraulic conductivity (Lp_r) of individual rice (*Oryza sativa*, L.) roots, whole root systems and outer part of roots (Lp_{OPR}), which were grown in hydroponics with bubbled air for 31-40 days, as measured by the root pressure probe (single roots), pressure chamber techniques (root systems) and pressure perfusion technique (outer part of roots). (**B**) Calculated treatment / control ratios of Lp_{OPR} after perfusing root segments (two different distances from the root tip) either diluted China ink suspension or treated with 50 µM $HgCl_2$ for 20 minutes. Ratio of bulk (P_{fOPR}) and diffusional (P_{dOPR}) water permeability of the OPR also given for two different distances from the root tip. (**C**) Reflection coefficients of single adventitious roots (σ_{sr}) and outer part of roots (σ_{sOPR}) as measured with root pressure probe or pump perfusion technique. Osmotic water flow was induced by increasing the osmotic pressure of the medium by adding different osmotica.

A

	Hydraulic conductivity (Lp) × 10^{-8} m s^{-1} MPa^{-1}				References
	Whole root systems	Individual roots	Outer part of the root (OPR)		
			20-50 mm	50-100 m	
Hydrostatic	4.0 ± 1.7 5.6 ± 2.7	3.8 ± 0.6 5.0 ± 2.5	150 ± 60 -	110 ± 30 -	Ranathunge et al 2003 Miyamoto et al. 2001
Osmotic (NaCl)	3.1 ± 0.9 4.2 ± 2.5	1.1 ± 0.5 9.2 ± 3.0	- -	- -	Ranathunge et al 2003 Miyamoto et al. 2001

B

Distance from root tip (mm)	Treatment /control ratio of Lp_{OPR}		Ratio of bulk (P_{fOPR}) and diffusional (P_{dOPR}) water permeability of the OPR	References
	$HgCl_2$/control	Ink / control		
20-50 50-100	0.90 ± 0.10 0.92 ± 0.03	0.75 ± 0.09 0.67 ± 0.13	620 1200	Ranathunge et al. 2003

C

	Reflection coefficient (σ_{sr}) of excised roots		References
	Ethanol	NaCl	
Whole root	0.04 ± 0.02 0.09 ± 0.01	0.18 ± 0.06 0.28 ± 0.11	Ranathunge et al. 2003 Miyamoto et al. 2001
Distance from the root tip (mm)	Reflection coefficient (σ_{sOPR}) of outer part of the root		References
	Mannitol	NaCl	
20-50 50-100	0.13 ± 0.04 0.13 ± 0.04	0.09 ± 0.02 0.11 ± 0.03	Ranathunge et al. 2003

In subsequent measurements, a new perfusion technique was used to measure the OPR separately and to get more detailed information as to whether the exo- or the endodermis was limiting water uptake. Despite suberization of the exodermis, the Lp_{OPR} was larger by a factor of 30 than the overall root Lp_r (Table 1A) and σ_{OPR} siginifcantly smaller than the overall σ_{sr} (Table 1C). So, despite suberization there was a considerable bypass of water

and small solutes in the exodermis as proposed for other species (see above). A dominating apoplastic transport component was also indicated by the fact the diffusional water permeability (measured with heavy water) was smaller than the bulk water permeability by a factor of as large as 600-1200 (Table 1B). The partial blockage of the apoplastic pathway by China ink particles and of the transmembrane path with the water channel blocker mercuric chloride indicated that the former treatment was more effective. This was in line with the idea of a dominating apoplastic water flow. The rice system shows that apoplastic transport of water is possible even in the presence of a well-developed exodermis with apoplastic barriers. Anatomical studies did not reveal any kind of patchiness or of other irregularities of the exodermis.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, Schwerpunktprogramm "Apoplast". Publications obtained within this progamme are indicated by an asterix (*).

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8 Summary

Roots of plants growing in waterlogged soils, such as paddy rice, are exposed to an anaerobic and chemically reduced environment. Rice plants usually form a large amount of aerenchyma that facilitates the movement of oxygen from the shoots to the root tips, but at the risk that oxygen would be largely lost by radial diffusion across the peripheral layers or outer part of the root (OPR). **The OPR of rice roots comprises, an outermost rhizodermis (epidermis), one layer of exodermis, sclerenchyma cell layer and an innermost unmodified cortical cell layer. To restrict or minimize radial oxygen loss (ROL) from aerenchyma to the external anaerobic soil substrate, rice roots develop barriers in the outer layers. The OPR of rice roots has a well-developed exodermis with Casparian bands (CBs) and suberin lamellae, in addition to a lignified sclerenchyma cell layer**. This may cause problems for the water uptake, when water flow across the apoplast is blocked by CBs and the cell-to-cell passage affected by suberin lamellae. Accordingly, **it has been shown that rice plants, even though rooting in a wet substrate, may suffer from water shortage showing leaf-rolling and wilting symptoms during the day time (Hirasawa et al. 1992).**

For rice, the overall radial hydraulic conductivity (Lp_r), measured with the pressure chamber and root pressure probe was lower than those of other cereal roots. This has been interpreted as a major limitation of rice roots to supply water to transpiring leaves. The stele/endodermis, aerenchyma, and the outer part of roots (OPR) arrange in series and their resistances to the overall radial water flow are additive. However, **the hydraulic resistance of the OPR, a tissue of defined structure with four cell layers in series including an exodermis (hypodermis with CBs and well developed suberin lamellae) was smaller by a factor of 30 than the overall values of root Lp_r.** As long as flow across the OPR is hydraulic in nature, this means that OPR would not rate limit water uptake. Hence, the overall radial hydraulic conductivity must be limited by some other parts of the pathway. **Estimations of hydraulic properties of aerenchyma suggested that the endodermis was rate limiting water flow, although the aerenchyma may contribute to the overall resistance. The resistance of the aerenchyma was relatively low, and locates in between the endodermis and the OPR.** Mono-layered cortical septae crossing the aerenchyma (‘spokes’) short-circuited

the air space between the stele and the OPR. The spokes form hydraulic bridges, may act like wicks.

High values of the overall hydraulic conductivity of the OPR (Lp_{OPR}) could be brought about by a large apoplastic component of water transport or by a high permeability of membranes of the living cells in the OPR or by both together. If there were a greater apoplastic component, this would mean that CBs in the exodermis were quite permeable to water. **In order to assess the relative contribution of the apoplastic and cell-to-cell paths to the overall Lp_{OPR}, apoplastic pores of the OPR were either partially blocked by China ink particles or clogged with copper ferrocyanide precipitates. In another experiment, water channels (aquaporins) of the OPR were blocked with water channel blocker $HgCl_2$. New Lp_{OPR} measurements after the treatments suggested that proportionately greater apoplastic water flow across the OPR (on average 66-75% of water used extraprotoplastic pathway) compared to cell-to-cell water flow, despite the existence of apoplastic barriers such as CBs, suberin lamellae in the exodermis, and lignified walls of sclerenchyma.** This was further supported by an increment of the reflection coefficient of the OPR (σ_{sOPR}) after treatments with apoplastic blockers. Treatments with China ink particles or copper ferrocyanide precipitates increased the σ_{sOPR} by 3-fold.

Strongest evidence in favour of a predominant apoplastic water transport came from the comparison between diffusional (P_{dOPR}, measured with heavy water, HDO) and osmotic water permeability (P_{fOPR}) or hydraulic conductivity (Lp_{OPR}). **P_{fOPR} was larger by a factor of 600-1400 than P_{dOPR}. Such huge values of P_f/P_d ratios are expected if the pathway involved a rather long porous path, i.e. apoplast;** this would offer a high diffusional resistance for HDO, but should be highly permeable in case of a bulk (hydraulic) water flow. **Blockage of apoplastic pores with copper ferrocyanide precipitates significantly affected the bulk rather than the diffusive water flow and caused a 3-5-fold reduction of the P_{fOPR}/P_{dOPR} ratios.** These findings also indicated a prominent apoplastic bypass flow across the OPR of rice roots indicating that CBs in the exodermis were not perfect barriers to water.

It is usually assumed and well documented that exo- and endodermal CBs are perfect apoplastic barriers and their permeability to water and nutrient ions is "nil" (Robards and Robb 1972; Singh and Jacobson 1977; Peterson 1987). The validity of this assumption was experimentally checked for above two rice cultivars as well as for corn using a new precipitation technique (copper ferrocyanide). Corn was used as a standard, which is grown in completely different conditions and holds a different anatomical structure. The technique used here resembles Pfeffer's famous precipitation technique for producing artificial osmotic cells. Cu^{2+} and $[Fe(CN)_6]^{4-}$ ions were offered on different sides of the barrier (external medium and xylem). **Results showed that Cu^{2+} were passing the barrier including the CBs of the exodermis and endodermis, developing brown copper ferrocyanide crystals on the side where ferrocyanide was applied.** Since the passage across the membranes could be excluded, the result indicated that copper was moving through the barrier and cell walls much faster than ferrocyanide. There was patchiness in the formation of precipitates, correlated with the maturation of the exodermis in rice roots. **Dense brown precipitates were observed around lateral root emergence points. These places may act as passages not only for water and apoplastic tracer dyes but also for ions to move through the apoplast into the xylem and may lead to increase the apoplastic bypass flow in roots**. This does not mean that there was no difference on the permeability of CBs for water and solute ions. What it means is that the barriers were not completely impermeable to polar solutes and to the water, still exhibiting a selectivity (as shown by the fact that the negatively charged ferrocyanide was not passing at measurable amounts). **Overall, the results showed that the permeability of CBs to ions was fairly low, but bands were not perfect barriers to ions or even apoplastic tracer dyes.** This result differs from textbook knowledge (Strasburger et al. 1998).

Suberin (mainly hydrophobic aliphatic suberin) is one of the major chemical compounds, which may act as an apoplastic barrier to water in roots. To confirm this idea, total amounts of suberin were determined in corn and rice, and compared with their radial hydraulic conductivities. **On average, exodermal cell walls of rice contained 6-fold greater aliphatic suberin than in corn hypodermis. In endodermal cell walls, amounts were 34-fold greater in rice than that of corn. Significantly higher amounts of suberin detected in apoplastic barriers of rice corresponded**

with substantially lower root hydraulic conductivity (Lp_r) compared to corn, when water flow was driven by hydrostatic pressure gradients across the apoplast. As the OPR of rice is highly porous and permeable to water, it argued that this holds true only for the endodermis. The results imply that some caution is required when discussing the role of suberin in terms of an efficient transport barrier for water. The simple view that just the amounts of suberin play the important role, may not hold. A more detailed consideration of both the chemical nature of suberins and of the microstructure of deposits is required, i.e. how suberin impregnate wall pores.

In conclusion, rice roots often grow in anaerobic (hypoxic or anoxic) soils. Oxygen diffuses from shoot to root tips within the aerenchyma, and radial oxygen loss (ROL) from roots to the anaerobic root medium are relatively small. Hence, the OPR should have a low permeability for oxygen. On the other hand, there should be a sufficient hydraulic permeability to take up water. It appears that in rice, water uptake and oxygen retention are optimized in a way that hydraulic water flow can be kept high in the presence of a low efflux of oxygen, which is diffusional in nature.

Brief Summary

(1) In rice roots, the main hydraulic barrier locates inside the root, namely the endodermis. The outer part of the root (OPR), which includes the exodermis with well developed Casparian bands (CBs) and sclerenchyma fibre cells is highly permeable for water.

(2) Field-grown rice plants develop small roots systems in order to diffuse oxygen from basal parts of the root to the tip effectively, without losing to the anaerobic soil medium. In addition, the apoplastic barriers of the OPR limit or restrict the radial oxygen loss (ROL) from aerenchyma to the soil medium.

(3) A proportionately greater apoplastic water flow across the OPR (on average 66-75% of water used extraprotoplastic pathway) compared to cell-to-cell water flow, despite the existence of apoplastic barriers such as CBs, suberin lamellae in the exodermis, and lignified walls of sclerenchyma.

(4) Exo- and endodermal CBs of rice and corn roots do allow for a small passage of divalent ions, Cu^{2+}, but severely hinder negatively charged ions, such as $[Fe(CN)_6]^{4-}$. Hence the permeability of CBs to ions is fairly low, but bands are not perfect barriers to ions

(5) The hypothesis of a substantial apoplastic transport of water across the exodermis of rice OPR is supported by the comparison of osmotic (P_f) and diffusive (P_d) water permeabilities (P_f/P_d ratios). This ratio was as large as 600-1400. By a factor of 3-5, blockage of the apoplastic pores of the OPR with copper ferrocyanide precipitates declined this ratio.

(6) The permeability of CBs to the divalent cation Cu^{2+} is directly demonstrated by using a new precipitation technique for both rice and corn. The permeability decreases during the root development. The secondary (lateral) root emergence points from the primary root are leaky for water and ions.

(7) The amounts of aliphatic suberin correlate with the changes of hydraulic conductivities (Lp_r) or radial water permeabilities of roots. But a more detailed consideration of both the chemical nature of suberins and of the microstructure of deposits is more useful, i.e. how suberin impregnate wall pores.

(8) The results do not indicate that CBs allow a free passage to ions through the apoplast. Bands restrict ion movement, but it is not complete. Hence, the conventional assumption that "the permeability of CBs to water and nutrient ions is nil" should be modified.

9 Zusammenfassung

Die Wurzeln von Hygrophyten, die, wie etwa der Reis, in wasserreichen Böden wachsen, sind einer anaeroben und chemisch reduzierenden Umgebung ausgesetzt. Reispflanzen bilden normalerweise ein großes Aerenchym, welches den axialen Transport des Sauerstoffs vom Spross bis in die Wurzelspitzen erleichtert. Sie tun dies jedoch unter der Gefahr, dass große Mengen Sauerstoff durch Radialdiffusion über die äußeren Teile der Wurzel (OPR[1]) verlorengehen. Der OPR der Reiswurzeln besteht, von außen nach innen gesehen, aus der Rhizodermis (Epidermis), einer Schicht Exodermis, einer Schicht Sklerenchym und einer unveränderten inneren Schicht von Cortexzellen. Um den radialen Verlust des Sauerstoffs (ROL[2]) aus dem Aerenchym zum umgebenden anaeroben Bodensubstrat einzuschränken, sollte es Barrieren in den äußeren Schichten der Reiswurzeln geben. Das OPR der Reiswurzeln hat zusätzlich zu der ligninangereicherten sklerenchymatischen Zellschicht eine gut entwickelte Exodermis mit Casparyschen Streifen (CBs[3]) und Suberinlamellen. Dies könnte bei der Wasseraufnahme Probleme verursachen, wenn der apoplastische Fluß durch die Casparychen Streifen blockiert und der Durchgang von Zelle zu Zelle durch das Vorhandensein von Suberinlamellen reduziert ist. Es konnte gezeigt werden, daß Reispflanzen selbst dann, wenn sie in einem feuchten Substrat wurzeln, unter Wassermangel leiden können, was sich durch das Einrollen der Blätter und durch Symptome des Welkens während des Tages bemerkbar macht (Hirasawa et al. 1992). Das Problem der Wasseraufnahme durch die Reiswurzel ist wichtig, vor allem im Hinblick auf die Züchtung von Reissorten, die auf bewässerten Böden oder gar unter Trockenstress gezogen werden. Weltweit sind Züchter daran interessiert. Reissorten zu entwickeln, die mit weniger Wasser auf relativ trockenen Böden auskommen. Deshalb wurden die Untersuchungen in dieser Arbeit in Zusammenarbeit mit dem IRRI (International Rice Research Institute, Manila, Philippinen) durchgeführt, von dem auch die verwendeten Reissorten stammten.

Bei den Reispflanzen war die hydraulische Leitfähigkeit des Wurzelzylinders (Lp_r), die mit die Druckkammer und mit der Wurzeldruckmesssonde gemessen wurde, niedriger als die von anderen Getreidewurzeln. Dies ist als Ursache dafür gedeutet worden, daß

[1] OPR = Outer Part of the Root [2] ROL = Radial Oxygen Loss [3] CBs = Casparian bands

beim Reis die Wasseraufnahme durch die Wurzeln die Versorgung des Sprosses eher begrenzen kann als bei anderen Gräsern. Hinzu kommt die relative geringe Ausdehnung des Wurzelsystems im anaeroben Substrat. Die Stele/Endodermis, das Aerenchym und der äußere Teil der Wurzeln (OPR) sind in einer Schichtenfolge angeordnet, und ihre hydraulischen Widerstände addieren sich zum Gesamtwiderstand. In der vorliegenden Arbeit sollte die relative Bedeutung der verschiedenen Widerstände quantifiziert werden. Der hydraulische Leitfähigkeit des OPR, ein Gewebe mit klar definierter Struktur mit vier aneinandergereihten Zellschichten, welche die Exodermis einschließt (Hypodermis mit Casparycher Streifen und gut entwickelten Suberinlamellen), war um den Faktor 30 höher als das $L\mathrm{p}_\mathrm{r}$ der ganzen Wurzel. Solange der Fluß durch den OPR hydraulisch ist, bedeutet dies, daß der OPR die Wasseraufnahme nicht begrenzt. Folglich ist die hydraulische Radialleitfähigkeit an anderer Stelle eingeschränkt. Theoretische Überlegungen zu den hydraulischen Eigenschaften des Aerenchyms zeigten, daß es die Endodermis ist, die den Wasserfluss begrenzt, obgleich das Aerenchym zum gesamten Widerstand beitragen kann. Der Widerstand des Aerenchyms lag zwischen dem der Endodermis und dem des OPR. Die einschichtigen kortikalen Septae, die das Aerenchym kreuzen („Speichen“), überbrücken den Luftraum zwischen dem Wurzelinneren und dem OPR. Ähnlich einem Docht, verhalten sich die Speichen wie hydraulische Brücken, über die das Wasser von den äußeren Teilen der Wurzel in deren Inneres gesogen wird .

Die hohen Werte der gesamten hydraulischen Leitfähigkeit des OPR (Lp_{OPR}) können entweder auf eine große apoplastische Komponente des Wassertransportes oder durch eine hohe Durchlässigkeit der Membranen der lebenden Zellen im OPR (Aquaporine) oder durch beides zusammen hervorgebracht werden. Gäbe es einen größeren apoplastischen Anteil, hätte dies zur Folge, daß die CBs in der Exodermis ziemlich wasserdurchlässig wären. Um den relativen Beitrag des apoplastischen Weg und des Weges von Zelle zu Zelle zum gesamten Lp_{OPR} zu ermitteln, wurden die apoplastischen Poren des OPR ganz oder teilweise durch Chinatuschepartikel (mittlerer Durchmesser: 50 nm) geschlossen oder mit Fällungen von Kupfer-hexacyanoferrat(II) verstopft. In einem anderen Experiment wurden die Wasserkanäle (Aquaporine) des OPR mit dem Wasserkanal-Blocker $HgCl_2$ geschlossen. Die im Anschluß an diese Behandlungen erfolgten neuerlichen Lp_{OPR}-Messungen zeigten, dass im Vergleich zum Wasserfluß von

Zelle zu Zelle ein erheblicher Anteil von mindestens 66-75% des Wassers ($Cu_2[Fe(CN)_6]$-Präzipitate) durch den Apoplasten floss. Im Vergleich damit war die Inhibierung mit den Tuschepartikeln geringer (30%). Da Inhibierungen des Membrantransportes des Wassers ausgeschlossen werden konnten, zeigt das Ergebnis, dass trotz des Bestehens apoplastischer Barrieren (CBs und Suberinlamellen in der Exodermis, ligninhaltige Wände des Sklerenchyms) ein substantieller apoplastischer Wasserfluß durch den OPR erfolgt. Diese Schlussfolgerung wird gestützt durch die Tatsache, dass nach den Behandlungen mit apoplastischen Blockern die Reflexionskoeffizienten des OPR (σ_{sOPR}) deutlich zunahmen. Behandlungen mit Chinatintepartikeln oder mit Kupfer-hexacyanoferrat(II)-Partikeln erhöhten das σ_{sOPR} um das Dreifache.

Die stärkste Beweiskraft eines überwiegend apoplastisch erfolgenden Wassertransportes lieferte der Vergleich zwischen der diffusiven (P_{dOPR}, gemessen mit schwerem Wasser, HDO) und der osmotischen Wasserdurchlässigkeit (P_{fOPR}) bzw. der hydraulische Leitfähigkeit (Lp_{OPR}). P_{fOPR} war um einen Faktor 600-1400 größer als P_{dOPR}. So hohe Werte im Verhältnisse von P_f zu P_d sind zu erwarten, wenn der Wasserweg eine ziemlich lange poröse Strecke durchläuft, die dem Tracer HDO einen hohen Diffusionswiderstand entgegensetzt. Für den viskosen (hydraulischen) Wasserfluss sollte eine solche Barriere aber in hohem Maße durchlässig sein. Das Blockieren der apoplastischen Poren mit Partikeln von Kupfer-hexacyanoferrat(II)-Partikeln (s.o.) beeinflusste den hydraulischen Wasserfluss konsequenterweise erheblich mehr als den diffusiven. Sie verursachte eine Verringerung der P_{fOPR}/P_{dOPR} Verhältnisse um das Drei- bis Fünffache. Die Befunde deuten auf eine erhebliche apoplastische Komponente des Wasserflusses im OPR der Reiswurzeln hin. Sie zeigen, dass die CBs in der Exodermis des OPR für das Wasser keine unüberwindbaren Barrieren darstellen.

Um die Ionendurchlässigkeit der exodermalen CBs bei Reis sowie der endodermalen CBs von jungen Reis- und von Maispflanzen zu überprüfen, wurde ebenfalls die neue Fällungstechnik verwendet, wobei Cu^{2+} und das Hexacyanoferrat(II)-Anion auf jeweils verschiedenen Seiten der Barriere (Medium bzw. Xylem) angeboten wurde. Die Ergebnisse zeigten, daß Cu^{2+} und SO_4^{2-} für die Barrieren (einschließlich der CBs der Exodermis und der Endodermis) letztlich permeabel war. Im Gegensatz dazu war die

Permeabilität für das Hexacyanoferrat(II)-Anion gering. Infolgedessen bildeten sich die Niederschläge jeweils auf der Seite aus, auf der $[Fe(CN)_6]^{4-}$ appliziert worden war. Dies zeigte, dass das Kupfer-Ion sehr viel schneller die Barrieren und die Zellwände durchläuft als das Hexacyanoferrat(II)-Ion. Die Unterschiede in der Permeabilität wurde der Tatsache zugeschrieben, dass die Zellwände im Apoplasten Kationenaustauscher darstellen, die stark negativ geladene Ionen aufgrund Coulombscher Wechselwirkungen zurückstoßen. Je nach dem Entwicklungszustand der Exodermis der Reiswurzeln fanden sich Unterschiede in deren Permeationseigenschaften für die Ionen. Um Seitenwurzelinitialen herum wurden dichte braune Niederschlag beobachtet. Diese Stellen können als "offene Türen" nicht nur für Wasser und apoplastische Farbindikatoren (tracer dyes) dienen. Im Einklang mit der Literatur sind sie auch für ionische Substanzen relativ leicht passierbar. Die Ursache dafür ist, dass in den frühen Stadien der Seitenwurzelbildung, die Kontinuität von Exo- und Endodermis unterbrochen ist. Insgesamt zeigten die Resultate, dass die CBs für die Ionen doch ziemlich undurchlässig waren, obwohl sie weder für die Ionen noch für die apoplastischen Farbindikatoren nicht wirklich unüberwindliche Barrieren darstellen.

Vor allem das hydrophobe aliphatische Suberin soll die Leitfähigkeit apoplastischer Barrieren in Wurzeln gegenüber dem Wasser und anderen polaren Stoffen reduzieren. Um diese Annahme zu bestätigen, wurden die Gesamtmengen von Suberin in Mais- und Reiswurzeln bestimmt und mit den radialen hydraulischen Leitfähigkeiten korreliert. Im Durchschnitt enthielten die exodermalen Zellwände von Reis um das Sechsfache mehr aliphatisches Suberin als die in der Hypodermis von Mais. In endodermalen Zellwänden waren die Mengen bei Reis sogar um den Faktor 34 höher als bei Mais. Die signifikant höheren Suberinmengen, die in den apoplastischen Barrieren von Reis gefunden wurden, korrelierten mit einer deutlich geringeren hydraulischen Leitfähigkeit der Wurzel (Lp_r) bei Reis im Vergleich mit Mais, wenn der Wasserfluss nach hydrostatischen Drucksteigerungen vorwiegend durch den Apoplasten erfolgte. Da bei Reis der OPR relativ porös und daher wasserdurchlässig ist, ließ dies den Schluß zu, daß dies ausschließlich für die Endodermis zutrifft. Darüberhinaus zeigen die Resultate, daß einige Vorsicht geboten ist, wenn man die Wirksamkeit des Suberin als Transportbarriere für Wasser diskutiert. Die einfache Annahme, dass lediglich die Menge des Suberins wichtig ist, läßt sich möglicherweise nicht aufrechterhalten. Eine

genauere Kenntnis sowohl der chemischen Zusammensetzung der Suberine als auch der Mikrostruktur der porösen Matrix ist notwendig, d.h. eine Untersuchung darüber, wie die Suberine die Wandporen imprägnieren.

Zusammenfassend läßt sich sagen,

(1) dass in der Reiswurzel die hydraulischen Barriere vor allem im Wurzelinneren lokalisiert sind. Der OPR setzt dem Wasser einen relativ geringen Widerstand entgegen. Dies kann beim Sauerstoff anders sein.
(2) Im Freiland ist die geringe Größe des Wurzelsystem offenbar sauerstofflimitiert..
(3) Die hohe hydraulische Leitfähigkeit des OPR ist auf eine relativ hohe apoplastische Komponente zurückzuführen, selbst in Gegenwart Casparyscher Streifen in der Exodermis.
(4) Der Apoplast, einschließlich der Casparyschen Streifen, ist selbst für zweiwertige Kationen passierbar, wenn auch eingeschränkt.. Er ist wenig permeabel für mehrfach negativ geladene Anionen.
(5) Die Hypothese eines substantiellen apoplastischen Transportes des Wassers durch die Exodermis wird durch den Vergleich von osmotischer und diffusiver Wasserpermeabilität gestützt (P_f/P_d.Verhältnis). Verschluss der Casparyschen Streifen durch Salzfällung verringert P_f/P_d erheblich.
(6) Eine Permeabilität des zweiwertigen Kations Cu^{2+} ist Mithilfe einer neuartigen Fällungsmethode direkt nachweisbar, und zwar sowohl beim Reis als auch beim Mais. Die Permeabilität nimmt während der Wurzelentwicklung und der damit einhergehenden Entwicklung der CBs und Suberinlamellen ab. Im Einklang mit der Literatur findet sich an Orten, an denen Initialen von Seitenwurzeln die Endodermis durchbrechen, eine besonders hohe Kationen- und wohl auch Wasserpermeabilität.
(7) Die Menge des aliphatischen Suberins korreliert mit den Änderungen der hydraulischen Leitfähigkeit (Lp_r) oder der radialen Wasserpermeabilität der Wurzeln. Eine detaillierte Betrachtung sowohl der chemischen Struktur als auch der Mikrostruktur der Einlagerung der Suberine ist sinnvoller, z.B. wie die Suberine der Wandporen Imprägnierung.

(8) Die Ergebnisse bedeuten nicht, dass CBs gar kein Rückhaltevermögen für Ionen besitzen. Sie besagen lediglich, das CBs der Endodermis sowohl für Wasser als auch für Ionen eine Permeabilität besitzen, die von Null abweicht. In Lehrbüchern wird oft davon ausgegangen, dass CBs völlig impermeabel sind. Nach den Ergebnissen dieser Arbeit sind diese Aussagen zu modifizieren.

10 Erklärung

Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegeben Quellen und Hilfsmittel benutzt habe.

Ferner erkläre ich, dass ich anderweitig mit oder ohne Erfolg nicht versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden.

Bayreuth, den 13 Juni 2005

(Kosala Manjupriya Ranathunge)