Differences in Aquaporin Levels among Cell Types of Radish and Measurement of Osmotic Water Permeability of Individual Protoplasts

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We investigated tissue- and cell-specific accumulation of radish aquaporin isoforms by immunocytochemical analysis. In taproots, the plasma membrane aquaporins (RsPIP1 and RsPIP2) were accumulated at high levels in the cambium, while the tonoplast aquaporin (RsTIP) was distributed in all tissues. The three isoforms were highly accumulated in the central cylinder of seedling roots and hypocotyls, and rich in the vascular tissue of the petiole of mature plants. The results suggest that RsPIP1 and RsPIP2 are abundant in the cells surrounding the sieve tube of the radish plant. The swelling rate of protoplasts in a hypotonic solution was determined individually by a newly established method to compare the osmotic water permeability of different cell types. All cells of the cortex and endodermis in seedlings showed a high water permeability of more than 300 μm s⁻¹. There was no marked difference in the values between the root endodermis and cortex protoplasts, although the RsPIP level was lower in the cortex than in the endodermis. This inconsistency suggests two possibilities: (1) a low level of aquaporin is enough for high water permeability and (2) the water channel activity of aquaporin in the tissues is regulated individually. The uneven distribution of aquaporins in tissues is discussed along with their physiological roles.

Keywords: Aquaporin — Cell specificity — Osmotic water permeability — Plasma membrane — Protoplast assay — Radish.

Abbreviations: DTT, dithiothreitol; PBS, phosphate-buffered saline; PM, plasma membrane; P₀, osmotic water permeability; PIP, plasma membrane intrinsic protein; RsPIP, radish plasma membrane aquaporin; RsTIP, radish vacuolar membrane aquaporin; TIP, tonoplast intrinsic protein.

Introduction

Plants retain a large quantity of water in their cells for metabolic activities and turgor pressure, whilst retaining water flow in their bodies. Water stream in the bodies is essential for the supply of nutrients, oxygen, hormones and other signal molecules to cells. Also, CO₂ and carbonic ions dissolved in water need to be removed from the non-photosynthetic tissues. The water stream depends on the influx and efflux of water to the cells through plasma membranes (PM).

Aquaporins facilitate the osmotic water transport across PM and tonoplast (Maurel 1997, Chrispeels et al. 1999, Johannson et al. 2000, Teyman et al. 2002). Although water molecules can be transported across membranes by simple diffusion mechanisms and through ion channels, its rate is limited (Haines 1994). This means that water transport, both intercellular and intracellular, depends on the activity and quantity of aquaporins. The distribution of aquaporins in organs and the water channel activity may be one of the factors determining the pathway and capacity of water flow in plant bodies.

The structural diversity, intracellular localization and physiological responses of plant aquaporins have been investigated in detail (Jauh et al. 1999, Jiang et al. 2001, Katsuhara et al. 2000). Remarkable progress has been made into the research on higher-order structures of aquaporins (Daniels et al. 1999, de Groot and Grubmuller 2001, Sui et al. 2001). Furthermore, tissue-specific gene expression has been demonstrated for the tonoplast aquaporins (Barrieu et al. 1998a, Barrieu et al. 1998b, Chaumont et al. 1998, Karlsson et al. 2000, Sarda et al. 1997) and PM aquaporins (Dixit et al. 2001, Otto and Kaldenhoff 2000, Yamada et al. 1995, Yamada et al. 1997) by the promoter assay, in situ hybridization and Northern hybridization. Recently, quantification of aquaporin proteins has been tried in different cell types of Mimosa pudica (Fleurat-Lessard et al. 1997), soybean nodule (Serraj et al. 1998), Brassica oleracea (Marin-Oliver et al. 2000), Mesembryanthemum crystallinum (Kirch et al. 2000), Nicotiana tabacum (Otto and Kaldenhoff 2000) and Brassica napus (Frangne et al. 2001).

The present study was conducted to investigate the distribution of PM aquaporins, RsPIP1 and RsPIP2 (previous names PAQ1 and PAQ2, respectively) and tonoplast aquaporins, RsTIP (previous name VM23) among radish tissues and cells. Their mRNA and protein levels have already been investigated in several organs at different growth stages by Northern hybridization and immunoblot analysis (Suga et al. 2001, Suga et al. 2002). The protein levels of RsPIP1 and RsTIP on a membrane protein basis were relatively constant, whereas the...
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RsPIP2 level varied with tissue and stage. However, immunoblots of the membrane preparations provide information on the average content of aquaporins in tissues composed of several cell types. To analyze the distribution of aquaporins, we applied tissue print immunoblotting and immunocytochemical methods. We found characteristic distributions of aquaporin proteins in different tissues.

We developed a new technique to determine the osmotic water permeability ($P_{os}$) of protoplasts. There are several methods to measure $P_{os}$ of living cells, such as the plasmometric method and the pressure probe technique (Maurel 1997). These methods are technically difficult to apply to inner or small cells of tissues. Protoplasts were assayed for the water permeability in some plants (Maurel 1997, Moshelion et al. 2002). Ramahaleo et al. (1999) reported a new technique using protoplasts in a transference chamber. We further improved accuracy and simplified the method to measure $P_{os}$ of protoplasts that were isolated directly from target tissues. We determined $P_{os}$ using our refined method to compare cells either rich or poor in aquaporins. Here we discuss the relationship between the $P_{os}$ values and the level of aquaporin proteins.

**Results**

**Levels of aquaporin proteins in specific cells of young taproots**

To analyze the distribution of aquaporins, we examined the taproots and petioles of 1-month-old plants using tissue print immunoblotting. The anti-RsPIP1 and anti-RsPIP2 antibodies recognize the subfamily of RsPIP1 (RsPIP1-1, 1-2, 1-3) and preferentially the RsPIP2-1 isoform, respectively (Suga et al. 2001). It was confirmed that the anti-RsTIP (previous name, anti-VM23) reacts mainly with the RsTIP1 ($\gamma$-VM23) protein using the recombinant RsTIP1 and RsTIP2 proteins (data not shown). As shown in Fig. 1, the distribution of RsTIP was different from that of RsPIPs. The vascular cambium of taproots was stained densely with anti-RsPIP1 and anti-RsPIP2, but not with anti-RsTIP. The rim of the cross-section of the young taproots, which in part are composed of the periderm and sub-periderm (inner cell layer), was clearly stained with the anti-RsTIP (Fig. 1). The region including the xylem was clearly stained with all three antibodies. The tissue print immunoblot for the petiole indicated that the levels of RsPIP1, RsPIP2 and RsTIP were the highest, at least in the vascular bundles, compared with other tissues.

These observations were confirmed by the immunocytochemical analysis (Fig. 2). For the analysis we used a thin, lower part of the taproot from a 1-month-old radish to obtain small tissues, although large upper parts of the taproot were used for the tissue print immunoblotting as shown in Fig. 1. The phloem and the vascular cambium of the taproot were clearly stained brown with anti-RsPIP1 (Fig. 2a) and anti-RsPIP2 (Fig. 2d, f), anti-RsTIP (Fig. 2g–i) and the control antibody (Fig. 2k).

**Accumulation of aquaporins in the central cylinder of seedlings**

Fig. 3 shows diagrams of the cross-sections from seedling roots and young taproots. Active cell proliferation occurs in the cambium of the central cylinder of seedling roots and hypocotyls, with the central cylinder growing into a taproot. On the other hand, the cortex and epidermis of roots and hypocotyls peel away during the growth of the taproots.

Fig. 4 shows the distribution patterns of RsPIP1, RsPIP2 and RsTIP in seedling roots and hypocotyls. A number of root hairs distinguish roots from hypocotyls. The central cylinders in both organs were stained dark brown. The levels of RsPIP1, RsPIP2 and RsTIP were high in the cambium of the central cylinder and the endodermis of seedling roots (Fig. 4a–f). It is clear in an enlarged photograph of the central cylinder (Fig. 4i). On the other hand, aquaporin levels were found to be lower in the pericycle or cells around the metaxylem. The hypocotyls and seedling roots showed a similar pattern. It should be noted that the levels of three aquaporins were higher in the root endodermis than in the hypocotyl (Fig. 4a, c, e). The other feature of seedling roots is that the epidermis contained relatively high levels of RsTIP (Fig. 4e).
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The high levels of aquaporins in specific cells raise a question: Does the osmotic water permeability of the cells reflect the aquaporin level? To answer this question, we determined $P_{\text{os}}$ of protoplasts individually. Fig. 5 shows typical protoplasts from the cortex and endodermis of seedling roots. The radius of protoplasts isolated from the cortex and endodermis were 23–45 μm and 18–25 μm.

We developed a procedure for the selective isolation of a target protoplast and a new assay method for $P_{\text{os}}$ of protoplasts. Discs of roots and hypocotyls were pretreated with a maceration medium, and were rinsed in 0.45 M sorbitol (Fig. 6A). We then selected a particular part in the disc and isolated it using a capillary. The small cell cluster was subsequently dispersed and washed in 0.45 M sorbitol. A few protoplasts were transferred to a 0.45-M sorbitol droplet in a culture dish filled with paraffin oil. A target protoplast in the droplet was selected under a microscope, placed into a 0.3-M sorbitol droplet using a micromanipulator, and monitored under an inverted microscope (Fig. 6B, C). The protoplasts were stable for 2 h in 0.45 M sorbitol without any additional reagents such as Ca$^{2+}$. This is a simple method to determine the swelling rate of the protoplast of interest.

Time-course changes of the volume of protoplasts prepared from the cortex and endodermis were compared (Fig. 7). Cells from the same tissue were reproducibly swollen in the hypotonic medium at the same rate. The volume gradually increased to 150% in the hypotonic 0.30 M sorbitol solution within 30 s. The steady-state volume in the hypotonic medium is comparable to the theoretical value 150% calculated from the osmolarities of 0.45 and 0.30 M sorbitol solutions.

**Fig. 2** High level of RsPIPs and RsTIP proteins in specific cells of radish taproot and petiole. Thin cross-sections of 1-month-old taproots (3–4 mm in diameter) and petioles were immunostained with anti-RsPIP1 (a, b, c), anti-RsPIP2 (d, e, f), anti-RsTIP (g, h, i) and control antibody (j, k, l). High magnifications of vascular bundles of petioles (b, e, h, k) are shown in the right panels (c, f, i, l). Roman numerals in panel (a) indicate phloem (1), vascular cambium (2), secondary xylem (3) and the central part (4).
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High $P_{os}$ values of radish protoplasts

The $P_{os}$ values were calculated from the rates of volume increase under consideration of changes in the cell volume and the internal solute concentration as described in Materials and Methods. Fig. 8 shows the three theoretical lines of cell volume increase, which were calculated for a protoplast with a radius of 26.3 $\mu$m under the assumption that $P_{os}$ is 180, 280 and 380 $\mu$m s$^{-1}$. The experimental results, shown as open circles, fitted well with the theoretical line of 280 $\mu$m s$^{-1}$. This value was determined as $P_{os}$ of the protoplast. The $P_{os}$ values of 20 protoplasts from the endodermis and cortex of the roots were calculated to be 323±90 and 365±90 $\mu$m s$^{-1}$, respectively (Fig. 9). The $P_{os}$ values of the endodermis and cortex cells gave a single peak on this histogram. A mathematical $t$-test showed no difference between the $P_{os}$ values of the endodermis and cortex cells ($p = 0.1721$, df = 38).

This novel method for determining $P_{os}$ can be applied to small protoplasts. We tried to determine $P_{os}$ of cambium cells in the central cylinder but it was hard to select individual protoplasts among several cell types, because the central cylinder consists of the pericycle, cambium, metaxylem, protoxylem and xylem parenchyma cells (Fig. 3). Also, epidermis cells containing thicker cell walls proved difficult for protoplast preparation. The method for protoplast preparation and isolation of these special tissues remains to be improved.

Discussion

Aquaporins are highly accumulated in the central cylinder and vascular tissue

In the present study, we applied methods of tissue print immunoblotting and immunocytochemistry to examine the distribution of RsPIPs and RsTIP proteins. Immunological quantification provided essential information on the uneven distribution of aquaporins in tissues. The first point is that RsPIPs are highly accumulated in the cambium of taproots, the vascular tissue of petioles, and the central cylinder. The RsPIP level in the cambium was estimated to be several times higher than that of the pericycle and the inner parenchyma cells. High signal intensity in the vascular cambium and the central cylinder was not found to be due to differences in cell size. The levels of RsPIP1 and RsPIP2, but not RsTIP, were high in the vascular cambium of the young taproot (Fig. 2). The cell-specific immunostaining was also clearly observed in the central cylinder and endodermis (Fig. 4i).

We thought that a high amount of RsPIPs in the cambium supports the rapid flow of water from the phloems, which are located in the central cylinder, toward the pericycle. The high levels of RsPIPs may facilitate water circulation between the xylem and phloem in both the taproot and central cylinder. PM aquaporins are essential to facilitate the intercellular water flow from the cells surrounding the fine-structured sieve tubes to the relatively large parenchyma cells. Rapid cell growth in this region requires a prompt water supply. The high levels of RsPIPs in the cambium seemed to be regulated at the transcriptional step since in situ hybridization revealed a high steady-state mRNA level of RsPIP1 and RsPIP2 in the root cambium (data not shown). The active transcription and translation of RsPIPs may supply a large amount of aquaporin proteins to growing cambium cells.

The second point is that RsPIP1, RsPIP2 and RsTIP were highly accumulated in the central cylinder, especially in the cambium and the phloem (Fig. 4). There was a marked difference in stained intensity between the cambium and the metaxylem-surrounding cells, although they were similar in cell size. The cambium in the central cylinder is active in cell proliferation and growth. The xylem and phloem in the central cylinder are the main waterways. A dense distribution of RsPIPs in the central cylinder may provide rapid dispersion of water from the phloem to the cortex. It should be noted that the endodermis of seedling roots, but not of hypocotyls, were strongly stained with antibodies to RsTIP, RsPIP1 and RsPIP2. This may be related to the presence (roots) or absence (hypocotyls) of the Casparian strip that intercepts the apoplastic water stream. The endodermis of seedling roots contains the Casparian strip, in which the membrane of the large central vacuoles is a bar-

Fig. 3 Schematic diagrams of tissues of seedling root and growing young taproot of radish. The inside part within the endodermis (central cylinder) grows into a taproot.
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This is because water molecules cross the cells through plasmodesmata, as discussed for the basal endosperm transfer cells (Barrieu et al. 1998b). Thus, the high level of RsTIP provides endodermis cells for rapid transport of water. The abundance in the periderm and sub-periderm is a characteristic of RsTIP. It is clear that the sub-peridermal cells may directly sustain the epidermis through high turgor pressure. These growing sub-peridermal cells may be required for quick water absorption for their growth. Rapid cell growth occurs by rapid expansion of the vacuole. A large quantity of RsTIP may support a quick water transport across the tonoplast in these cells.

The results are consistent in part with the previous results of *Mesembryanthemum crystallinum* PM aquaporins (Yamada...
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et al. 1995, Yamada et al. 1997). MipA was highly expressed in the developing root xylem cells and the leaf vascular tissue, whereas MipB was expressed in the central cylinder. The MipB promoter has been reported to be active in cells surrounding vascular tissues as well as xylem parenchyma cells and the sub-epidermal cells. Barrieu et al. (1998b) reported the high expression of ZmTIP1 in the subepidermis and endodermis of young roots of maize.

**Improvement of selection of protoplast and $P_{os}$ assay method**

We developed a method for selection of specific protoplasts and measurement of $P_{os}$. A target portion was selected and isolated using a capillary from a tissue segment that was digested in the maceration medium. Thereafter proper protoplasts could be selected from the protoplast suspension using microscopy.

The present assay method for $P_{os}$ has some advantages as compared with other methods. Recently, Ramahaleo et al. (1999) developed a new method for the $P_{os}$ measurement of an isolated protoplast that was held by a micropipette in a transfer-chamber. The $P_{os}$ values were calculated from the initial swelling rate during the first 20% increase in the hypotonic medium. They discussed the systematic errors of underestima-

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**Fig. 5** A cross-section (A) of young root which was treated with the maceration solution and protoplasts of the cortex (B) and the endodermis (C) of the root of 4-day-old radish seedling.

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**Fig. 6** Protoplast assay system for osmotic water permeability. (A) A schematic diagram of the procedure. Tissue discs were prepared from young roots and hypocotyls of 4-day-old seedlings. The disc was treated with a maceration medium, and then rinsed to remove enzymes. A cluster of protoplasts was picked out from the disc and suspended in 0.45 M sorbitol and 1 mM DTT. The protoplasts obtained were placed in a droplet of 0.45 M sorbitol in a plastic culture dish filled with paraffin oil. A target protoplast in the droplet was picked using a capillary. The capillary was immersed into a droplet of 0.30 M sorbitol and the protoplast was released under an inverted microscope. The swelling of the protoplast in the hypotonic solution was monitored by the microscope and recorded through a CCD video camera. (B) Photograph of a culture dish containing several droplets in paraffin oil. The dish was set on the stage of the inverted microscope. (C) Overall view of apparatus used for the protoplast assay. A micro-manipulator, cell injector and CCD video camera were attached to a microscope.
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The underestimation comes from the assumption that the cell surface and the solute concentration gradient were kept at their initial values. Actually, the protoplast surface increases and the solute concentration in protoplast decreases during swelling. To avoid underestimation, we considered the changes as shown in equations (4) and (11). When the $P_{\text{os}}$ values were determined from the data in Fig. 8, using the method of Ramahaleo et al. (1999), the value of a protoplast with a radius of 26.3 $\mu$m was calculated to be 202 $\mu$m s$^{-1}$ (25% increase in the volume in the first 4 s), which was smaller than the present result (280 $\mu$m s$^{-1}$). The experimental results of $P_{\text{os}}$ fitted well with the theoretical curve for the whole assay period. Thus, we concluded that this curve-fitting method is appropriate for the total process of protoplast swelling.

The improved method has other advantages. Calculation of volume changes of protoplasts is easy using the present method, since a free protoplast is used. A glass pipette with a diameter of 25 $\mu$m was used to trap a protoplast (Ramahaleo et al. 1999). In the case of small protoplasts but not of large protoplasts, the surface area that is attached with a pipette is not negligible. Furthermore, the surface area may change during swelling and shrinking of the protoplast. Therefore the investigator should consider this factor. The present method does not
require a complex transference chamber for the protoplast assay and is applicable to most laboratories.

Cortex and endodermis protoplasts have high osmotic water permeability

The protoplasts from the cortex and endodermis gave significantly high \( P_{os} \) of more than 300 \( \mu m \) s\(^{-1}\). Such high \( P_{os} \) strongly supports the aquaporin-mediated water transport across PM. The values are comparable to those of whole red blood cells, which exhibit high \( P_{os} \) among living cells (530 \( \mu m \) s\(^{-1}\)) (Tsai et al. 1991). Typical values for membranes with aquaporins are 100–200 \( \mu m \) s\(^{-1}\) (Zeidel et al. 1994). Ramahaleo et al. (1999) reported permeabilities of approximately 10, 400 and 330 \( \mu m \) s\(^{-1}\) for protoplasts of onion leaf, rape hypocotyl and rape root. The \( P_{os} \) of protoplasts from Samanea motor cells has been reported to be regulated diurnally with a range from 3.1 to 5.0 \( \mu m \) s\(^{-1}\) (Mosshelen et al. 2002). Simple diffusion of water molecules across lipid bilayers gave lower values less than 50 \( \mu m \) s\(^{-1}\) (Haines 1994), and was dependent on the phase transition temperature of the membrane. Plants need to maintain the water flow in their organs, especially in the cambium and cells surrounding the sieve tube, even at low temperatures. Thus, the PM and tonoplast must contain a large quantity of aquaporins in plants.

In the radish, the tonoplast may not limit \( P_{os} \) of protoplasts, since \( P_{os} \) of tonoplast, determined by the stopped-flow spectrophotometrical method, was significantly higher than that of PM (Ohshima et al. 2001). Thus, the \( P_{os} \) values of protoplasts may be closely related to the water channel activity through RsPIPs. From this point of view, there are two explanations why \( P_{os} \) do not reflect the aquaporin level. First, quick water transport in protoplasts occurs even at low RsPIP levels in the cortex. Indeed, red blood cells show high \( P_{os} \) (530 \( \mu m \) s\(^{-1}\)) (Tsai et al. 1991) even though the red cell membranes of humans and rats contain only 2.4% and 3.7%, respectively (Nielsen et al. 1993). In the radish, RsPIPs and RsTIP occupy 10% and 40% of the total protein in PM and tonoplast, respectively (Higuchi et al. 1998, Maeshima 2001, Ohshima et al. 2001). Therefore, the RsPIP levels in radish are estimated to be at least a few percentages of the membrane proteins. One can estimate that the low content is enough for expression of high \( P_{os} \).

Secondly, the PM aquaporins are functionally regulated. There is a possibility that the water channel activity of RsPIPs in the endodermis is suppressed under the assay conditions. Several possible factors may regulate the function of aquaporins; namely, the cytoplasmic Ca\(^{2+}\) concentration, phytohormones, protein kinase, protein phosphatase, the external osmotic pressure and light (Chrispeels et al. 1999, Johansson et al. 2000, Maurel 1997). The \( P_{os} \) of protoplasts isolated from different tissues under physiological conditions should be determined in consideration of these factors. Further quantitative studies of the water channel activity and its functional regulation of each aquaporin isomor should provide novel information on the water stream in plants.

Materials and Methods

Plant material

Radish plants (Raphanus sativus L. cv. Tokinashi-daikon) were grown on the Nagoya University farm for 1 month. Radish seedlings were soaked in water for 1 day and germinated on a net floating on 0.3 mM CaSO\(_4\) for 3 or 4 d at 26\(^\circ\)C under long-day conditions (light/dark regime of 16 h/8 h, cool-white lamp).

Antibody preparation, tissue print immunoblot and immunocytochemistry

An antibody to RsTIP (anti-RsTIP) (Maeshima 1992) and antipeptide antibodies to RsPIP1 (anti-RsPIP1) and RsPIP2 (anti-RsPIP2) (Suga et al. 2001) were prepared in advance. The reactivity of antibodies to the aquaporin isoforms was described previously (Suga et al. 2002). The anti-RsPIP2 antibody is specific to the RsPIP2-1 isomor. The anti-RsPIP1 antibody equally reacts with RsPIP1-1, RsPIP1-2 and RsPIP1-3. The anti-RsTIP antibody specifically reacts with RsTIP1 but not with RsTIP2. Tissue print immunoblot analysis was carried out on a polyvinylilene difluoride membrane (Millipore Co., Bedford, MA, U.S.A.) to investigate the distribution of aquaporins following a standard procedure (Cassab and Varner 1987).

Immunocytochemical analysis was carried out after fixation of tissues with glutaraldehyde. Small pieces cut from the roots, hypocotyls, taproots and petioles were fixed with 2% glutaraldehyde in phosphate-buffered saline (PBS) for 4 h at 4\(^\circ\)C. The tissue pieces were embedded in 5% agar, and thereafter thin sections of them were prepared. The thickness was 90 \( \mu m \) for seedling roots, 110 \( \mu m \) for seedling hypocotyls, 75 \( \mu m \) for taproots and 200 \( \mu m \) for petioles. The sections were dehydrated in a series of ethanol (30, 50, 70, 90 and 100% in sequence) and rehydrated by exchanging the ethanol solution (100, 90, 70, 50 and 30%). The sections were treated with 1% deflated milk, 0.05% Tween-20 and 0.1% Na\(_2\)SO\(_4\) in PBS and then treated with the primary antibody. After rinsing with 0.5% deflated milk, 0.05% Tween-20 in PBS, the blotted membranes were treated with horseradish peroxide-linked protein A (Amersham Biosciences Co., Piscataway, NJ, U.S.A.). The blots were visualized in 0.06% (w/v) 3,3’-diaminobenzidine tetrahydrochloride, 0.03% (w/v) CoCl\(_2\) and 0.03% (v/v) \( H_2O_2\) and then photographed.

Protoplast preparation and water permeability measurement of protoplasts

Tissue segments (200–300 \( \mu m \) in thickness) cut from roots (upper portion) and hypocotyls (central portion) from 4-day-old seedlings were incubated in 2 ml of a maceration solution. The maceration solution contained 2% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Ind. Co., Tokyo, Japan), 0.1% (w/v) Macerozyme R-10 (Yakult Pharmaceutical Ind. Co., Tokyo, Japan), 0.03% (w/v) Pectolyase Y23 (Kikkoman Co., Noda, Japan), 20 mM MES-KOH, pH 5.5, 2 mM dithiothreitol (DTT) and 0.39 M sorbitol at 25\(^\circ\)C for 20 to 60 min. The cell wall was partially hydrolyzed by this treatment.

These discs were then carefully rinsed in 2 ml of 0.45 M sorbitol and 1 mM DTT to remove the maceration enzymes. DTT was added to prevent oxidation of cell components and the pH of the sorbitol was about 5.8. The target parts such as the cortex were sucked up with a small capillary (100–150 \( \mu m \) in internal diameter). For the endodermis, the central cylinder containing endodermis cells was taken from the disc using a relatively large capillary (400 \( \mu m \) in internal diameter), thereafter the endodermis cells lined around the central cylinder.
were picked and chosen using a small capillary (100 μm) under microscopy. A few protoplasts in the rinse medium were transferred to a droplet (30 μl) of 0.45 M sorbitol in a culture dish (Falcon) filled with paraffin oil (see Fig. 6). The droplets of sorbitol solution were stable on the bottom of the dish. The temperature in the paraffin oil was monitored with a thermometer. A target protoplast in the droplet was picked up using microscopy with a micro-capillary (100 μm in internal diameter) pre-filled with paraffin oil. The tip of the capillary was moved into a droplet (100 μl) of hypotonic solution of 0.30 M sorbitol in the same dish and the microscope was focused on the tip of the capillary. The protoplast was then released into the droplet from the capillary. The micro-capillary attached with a cell injector was handled with a micromanipulator (see Fig. 6C). When a protoplast was released into a droplet of the hypotonic solution, a small volume of 0.45-M sorbitol solution also entered into the droplet. The volume was negligible, however, being only 1–10 nl. All sorbitol solutions contained 1 mM DTT to protect membrane components from oxidation during the experiment.

Protoplasts were viewed using an inverted microscope (Nikon, Diaphot TMD, Tokyo, Japan) equipped with a differential interference contrast (DIC) system and recorded by a charge-coupled device (CCD) camera (Sony, DXC-107A, Tokyo, Japan). The assay was carried out at 25°C and the diameter and volume of the protoplasts were calculated from the images on the video monitor using a NIH/Image program.

Calculation of osmotic water permeability

Osmotic water permeability \( P_{os} \) (m s\(^{-1}\)) of protoplasts was determined by the swelling rate of protoplasts after exposure to hypotonic conditions. The rate of change in protoplast volume \( \nu \) is given by

\[
d\nu/dt = P_{os} S \nu (C - C_0)
\]

where \( t \) is time (s), \( S \) is the surface area of the protoplasm (m\(^2\)), \( C \) and \( C_0 \) are the solute (osmotically active particles) concentration inside and outside the protoplast, respectively (mol m\(^{-3}\)). \( P_{os} \) is assumed to be independent of changes in protoplasm surface area. \( \nu \) is the partial molar volume of water (18×10\(^{-6}\) m\(^3\) mol\(^{-1}\)). If the protoplast is assumed to be a perfect sphere, \( S \) and \( V \) are written as

\[
S = 4\pi r^2,
\]

\[
V = 4/3\pi r^3,
\]

where \( r \) is the radius of the protoplast, and \( \pi \) the circular constant. Assuming that \( C \) decreases inversely proportional with increasing volume of the protoplasm, \( C \) is expressed as

\[
C = (V_0/V) C_0
\]

where

\[
V_0 = 4/3\pi r_0^3,
\]

and \( V_0 \) and \( C_0 \) are initial values of \( V \), \( C \), and \( r \), respectively. Combining equation (4) with equation (1) yields

\[
d\nu/dt = P_{os} SV_0 [(V_0/V) C_0 - C_0].
\]

Next, substituting equations (2), (3) and (5) into equation (6) yields

\[
ddr/dt = 3r^2P_{os} V_0 [(r/r_0)^3 C_0 - C_0].
\]

Since \( dV/dt = 3r^2(dr/dt) \), equation (7) can be rewritten as

\[
[r_0^3(r/r_0^3 + b^3)] (dr/dt) = -C_0 P_{os} V_0 r_0
\]

where:

\[
r_0 = (r/r_0),
\]

\[
b = (4/3 C_0 C_0)^{1/3}.
\]

After integrating equation (8) under the initial condition \( r = r_0 \) for \( t = 0 \) we obtain

\[
(r_0 - 1) - \frac{b}{6} \ln \left[ \frac{r_0 + b}{r_0 + b^3} \right] - \frac{b}{6} \ln \left[ 1 + b \right] = -\frac{C_0 P_{os} V_0}{r_0}
\]

When the values of \( r_0 \), \( C_0 \) and \( C_0 \) have been determined, we can calculate the theoretical time course of changes in \( V \) for any value of \( P_{os} \) from equation (11). The value of \( P_{os} \) can then be determined from the fitted curve (Fig. 8).

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