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Effect of overexpression of radish plasma membrane aquaporins on water-use efficiency, photosynthesis and growth of *Eucalyptus* trees

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Summary *Eucalyptus* is a diverse genus of flowering trees with more than 700 genotypic species which are mostly native to Australia. We selected 19 wild provenances of *Eucalyptus camaldulensis* grown in Australia, compared their growth rate and drought tolerance and determined the protein levels of plasma membrane aquaporins (PIPs). There was a positive relationship between the drought tolerance and PIP content. PIPs are divided into two subgroups, PIP1 and PIP2. Most members of the PIP2 subgroup, but not PIP1 subgroup, exhibit water channel activity. We introduced two radish (*Raphanus sativus* L.) PIPs, *RsPIP1;1* and *RsPIP2;1*, into a hybrid clone of *Eucalyptus grandis* and *Eucalyptus urophylla* to examine the effect of their overexpression. Expression of these genes was confirmed by real-time polymerase chain reaction (PCR) and the protein accumulation of RsPIP2;1 by immunoblotting. Drought tolerance was not enhanced in transgenic lines of either gene. However, one transgenic line expressing RsPIP2;1 showed high photosynthesis activity and growth rate under normal growth conditions. For *RsPIP1;1*-transformed lines, the RsPIP1;1 protein did not accumulate, and the abundance of endogenous PIP1 and PIP2 was decreased. The endogenous *PIP1* and *PIP2* genes were suppressed in these lines. Therefore, the decreased levels of PIP1 and PIP2 protein may be due to co-suppression of the *PIP* genes and/or high turnover of PIP proteins. *RsPIP1;1*-expressing lines gave low values of photosynthesis and growth compared with the control. These results suggest that down-regulation of *PIP1* and *PIP2* causes serious damage and that up-regulation of *PIP2* improves the photosynthetic activity and growth of *Eucalyptus* trees.

Keywords: carbon dioxide, leaf, PIP.

Introduction

Recent research on aquaporins has provided new information on their multifunctional roles in various organisms (Agre 2004, King et al. 2004, Maurel et al. 2008). Aquaporin is the most efficient facilitator of transmembrane water movement. In addition to water molecules, other substrates, such as glycerol, urea, ammonia, carbon dioxide, hydrogen peroxide, silicon, boron and arsenate have been reported to be transported by aquaporin members in several plant species (Hanba et al. 2004, Flexas et al. 2006, Ma et al. 2006, Takano et al. 2006, Kamiya et al. 2009). Plant aquaporins are divided into four subfamilies: plasma membrane intrinsic protein (PIP), tonoplast (vacuolar membrane) intrinsic protein (TIP), nodulin 26-like intrinsic protein (NIP) and small basic intrinsic protein (SIP) (reviewed in Hachez et al. 2006, Kaldenhoff and Fischer 2006, Katsuhara et al. 2008, Maurel et al. 2008). Recently, a new subfamily of aquaporins has been reported as the uncategorized X intrinsic proteins, XIPs, which were found in several plant species (Danielson and Johanson 2008). Their intracellular localization and substrates vary between members. The PIP members are thought to be essential for understanding the plant–water relationship.

Forest trees are important as resources of industrial materials and as members of the global environment. Recently, draft genome sequences of the poplar, a model forest tree, have been determined. The genome project identified more than 45,000 putative protein-coding genes in the poplar *Populus trichocarpa* (Tuskan et al. 2006). In addition to PIP, TIP, SIP and NIP, the project identified a new subfamily of aquaporins with five putative members not found in *Arabidopsis thaliana*. At least 45 aquaporin genes were identified in *P. trichocarpa* by computer analysis (Bansal and Sankararamakrishman 2007). Individual aquaporins in forest trees have

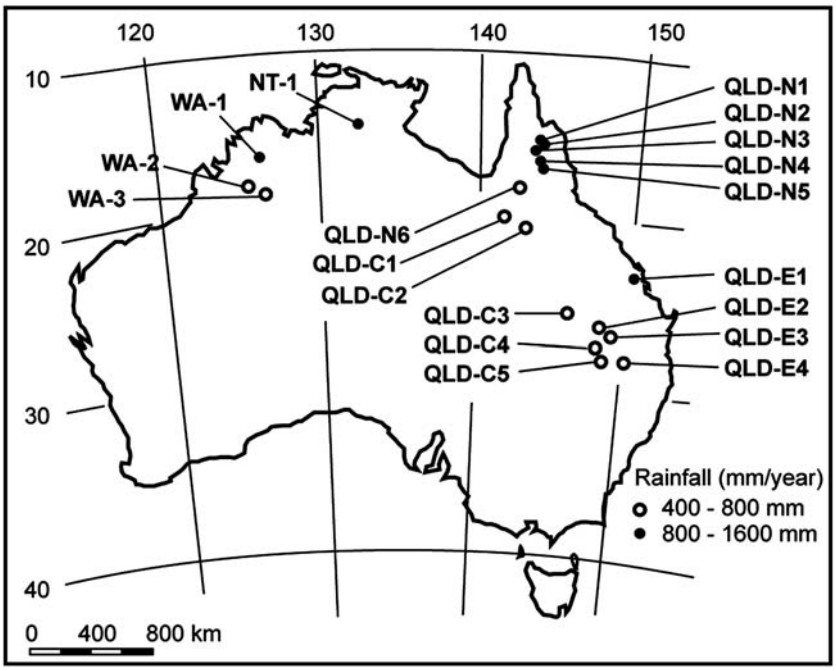

Figure 1. Distribution and drought tolerance of wild provenances of *E. camaldulensis* grown in Australia. Local points where the seeds of *E. camaldulensis* were collected are mapped in the map. Code numbers of provenances such as QLD-N1 and their place names are described in 'Materials and methods'. Letters of N, C and E mean north, central and east areas of Australia. Annual averages of rainfall at each place are marked by open (400–800 mm year^{-1}) and closed circles (800–1,600 mm year^{-1}). Information on the rainfall was obtained from the public site of the Bureau of Meteorology of Commonwealth of Australia (http://www.bom.gov.au/jsp/ncc/climate_averages/rainfall/index.jsp).

not been characterized. There is little information on their physiological roles except for a recent report by Cochard et al. (2007). Their study on walnut (*Juglans regia*) leaves revealed that light illumination stimulates leaf hydraulic conductance, which is tightly correlated with the extensive transcription of PIP2 members (*JrPIP2;1* and *JrPIP2;2*). These diurnal and light-induced variations in the level of PIP transcripts were also reported for rice (Sakurai et al. 2005), *Zea mays* (Lopez et al. 2003) and other plants (Maurel et al. 2008).

In contrast to the model forest tree *P. trichocarpa*, the *Eucalyptus* genome project has not been completed. *Eucalyptus* trees are used as raw materials for the pulp industry because of their good properties of paper making and high growth rate. Forest trees are of increasing importance for the paper industry, building materials, bio-ethanol production, water reserves in forests, oxygen generation and fixation of carbon dioxide in the atmosphere. Although aquaporins play important physiological and environmental roles, little information is available on tree aquaporins.

In this study, we focused on the relationship between aquaporins and the growth of *Eucalyptus*, which is a diverse genus of flowering trees with more than 700 genotypic species which are mostly native to Australia. We collected a number of *Eucalyptus camaldulensis* species native to Australia, examined their growth under drought stresses and quantified the content of aquaporins in these leaves immunochemically. Then, we investigated the relationship between drought tolerance and the content of aquaporins, especially PIPs. A positive relationship was found between drought tolerance and the PIP protein content in leaves. The PIP subfamily is composed of 13 members of *A. thaliana* which are subdivided into two groups, PIP1 and PIP2. The PIP2 members function as a water channel in various plant species (Suga et al. 2002, Suga and Maeshima 2004, Maurel et al. 2008, Sakurai et al. 2008). However, the actual substrate(s) for the PIP1 members is not known except for carbon dioxide for a few members (Flexas et al. 2006, Katsuhara et al. 2008).

Here, we transformed callus cells of *Eucalyptus* with radish *PIP1* and *PIP2* genes and regenerated the transformed trees to examine whether a change in the aquaporin content affects growth. Transformants of the *PIP2* gene were produced and were confirmed to accumulate PIP2 protein. In cells transformed with the *PIP1* gene, its transcript, but not exogenous PIP1 protein, was detected. Furthermore, the level of endogenous PIP1 protein was decreased in *PIP1*-transformant leaves. Therefore, we obtained both *PIP2*-overexpressing transformants and, accidentally, *PIP1*-suppressing transformants. Photosynthetic activity, water-use efficiency and the growth rate of the transformants were determined. The physiological importance of the PIPs in *Eucalyptus* trees is discussed.

Materials and methods

Plant materials

Seeds of *E. camaldulensis* were purchased from the Australian Tree Seed Centre (http://www.csiro.au/places/ATSC.html). These seeds were collected from 19 local provenances

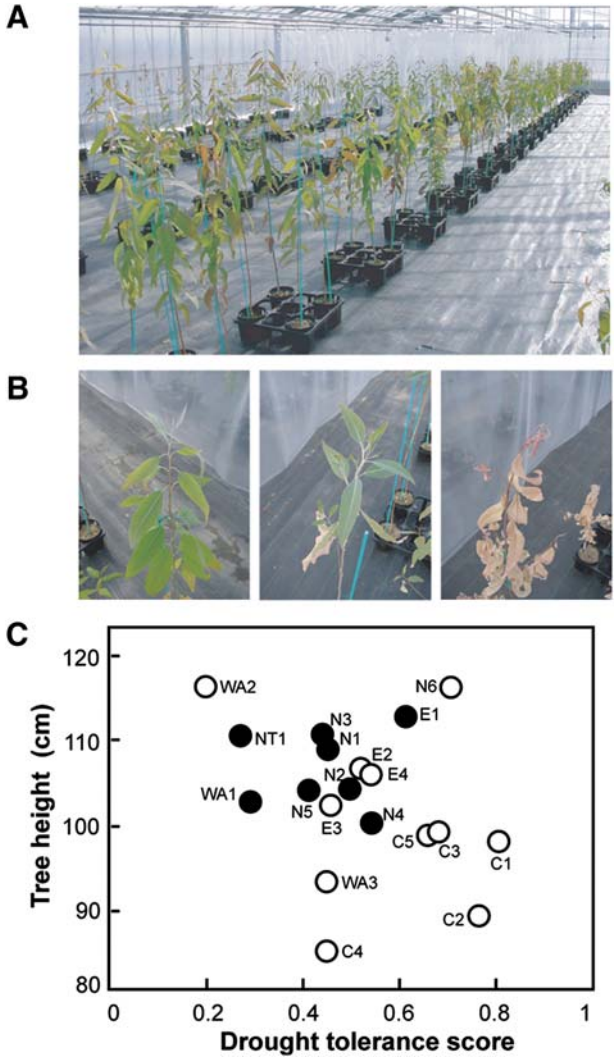

Figure 2. Evaluation of drought tolerance of *Eucalyptus* trees. (A) *Eucalyptus* trees of various provenances grown in a greenhouse of the Forestry Research Institute of Oji Paper Co. Sets of four trees were prepared for drought tolerance test. (B) Typical trees with green leaves (left), intact and withered leaves (middle) and completely withered leaves (right). (C) Comparison of growth rate and drought tolerance of the wild provenances. Names of provenances were abbreviated as listed in the map in Figure 1. Trees grown for 8 months under normal conditions were used for drought tolerance assay. More than four plants for each provenance were grown in the greenhouse. All leaves of plants watered every day were green. Drought tolerance score was determined as described in 'Materials and methods'. Height of trees grown in greenhouse was measured at the stage of 8 months old.

in Australia: Gibb River (No. WA-1), N Fitzroy crossing (WA-2), Mary R Crossing (WA-3), Katharine River (NT-1), Kennedy River (QLD-N1), Laura River (QLD-N2), Wrotham Park (QLD-N3), Springmount (QLD-N4), Gilbert River (QLD-N5), Petford (QLD-N6), Bullock Creek (QLD-C1), N. of Maxwelton (QLD-C2), Alice River (QLD-C3), Lagoons-Par (QLD-C4), SF328 Yuleba CPT116 (QLD-C5), Fitzroy River (QLD-E1), Parish of Hollymount (QLD-E2), Ballon, (QLD-E3) and SF81 Beeboo (QLD-E4) (see Figure 1).

A hybrid tree generated from *Eucalyptus grandis* and *Eucalyptus urophylla* was named GUT5 and used as a host plant. Trees were grown in a greenhouse of the Forestry Research Institute of the Oji Paper Co. (Kameyama, Japan) and the Center for Bioresource Field Science, Kyoto Institute of Technology (Kyoto, Japan).

Transformation of a hybrid line of E. grandis and E. urophylla

cDNA for RsPIP1;1 (previous name, PAQ1) and RsPIP2;1 (PAQ2) was cloned from radish (*Raphanus sativus* L. cv. Tokinashi-daikon) as described previously (Suga et al. 2001). The sequences of *RsPIP1;1* and *RsPIP2;1* of the *Eco*RI-*Xho*I fragments were treated with *Xba*I and then ligated into an *Xba*I-*Sma*I site of the binary vector p35S-nos/Hm (a modified vector of pBI101). The obtained construct was introduced into *Agrobacterium tumefaciens* strain EHA101 and used for the stable transformation of multiple shoots of the hybrid line GUT5 by a patented method (Kawazu et al. 2003). Whole plants were regenerated from the selected transgenic calli and were propagated by cutting of the primary trees.

Plant growth and measurement of drought tolerance

To determine the abilities of drought tolerance, seedlings of the provenances of *E. camaldulensis* were grown in 1.5-l plastic pots filled with 44% loamy soil, 44% culture soil (Fertilizer Business Association in Mie Prefecture, Japan) and 12% perlite (Fuyo Perlite, Tokyo, Japan), which were supplemented with 31.5 g delayed-release fertilizer (085-N180, Chisso Co., Tokyo, Japan), 0.7 g diazinon (Nissan Chemical Industries, Tokyo, Japan) and 0.7 g acephate (Arysta Life Science, Tokyo, Japan) for 8 months in a greenhouse by daily watering. Liquid fertilizer Hyponex (Hyponex Japan, Osaka, Japan) was supplied at a concentration of 1/1,000 biweekly. Daily minimum temperatures ranged from 15 to 25 °C and daily maximum temperatures from 25 to 36 °C. Plants were further grown in a greenhouse for 30 days under different watering conditions: watered 200 ml per pot every day, every 3 days or every 6 days by the shower irrigation or not watered (see Figure 2). The number of intact leaves in four trees grown under each condition was counted after the treatment. All of the leaves were withered after 30 days without watering. Drought tolerance was evaluated from the following equation,

$$\text{Drought tolerance score} = \frac{\text{Intact leaf number (3)} + \text{Intact leaf number (6)}}{\text{Intact leaf number (every day)} \times 2}$$

where (3) and (6) represent watering every 3 and 6 days, respectively.

Leaf gas exchange measurements

To determine the leaf carbon isotope ratio, we grew cuttings of transgenic *Eucalyptus* in a greenhouse under controlled conditions at the Forestry Research Institute of the Oji Paper

Co. (Kameyama, Japan). The temperature in the greenhouse (width, length and height: 9.0, 6.0 and 3.5–4.7 m, respectively) was maintained at 25 to 30 °C in summer and at 20 to 25 °C in winter. Relative humidity was above 50%, and the light was natural sunlight under control using a 50% shading sheet. Plants were individually grown in 4.5-l plastic pots containing the same soil as the experiment of drought tolerance. Plants were grown under different watering conditions: watered 650 ml per pot every day, every 3 days or every 6 days by the flood and drain irrigation system. The cuttings were grown for 8 months, and then fully expanded mature leaves ($n = 4$) were collected from the internode between the third and fourth leaf from the top of the shoot of individual trees in November 2006.

To analyze gas exchange and growth, we transferred the cuttings from 5- to 9-month-old trees to an uncontrolled greenhouse of the Center for Bioresource Field Science, Kyoto Institute of Technology (Kyoto, Japan) in August 2007. The light was natural sunlight with a 50% shading sheet. Plants were grown in 2.9-l pots containing the same amount of soils, delayed-release fertilizer and pesticides as described above. Plants were watered 200 ml per pot daily using an automatic irrigation system (EY4200H, Panasonic, Osaka, Japan) and fertilized weekly with full-strength Hoagland solution (Hewitt and Smith, 1975).

Measurements of the leaf carbon isotope ratio

Fully expanded mature leaves were dried at 60 °C for 48 h and then finely ground. The leaf carbon isotope ratio of ^{13}C to ^{12}C was measured for subsamples of 0.1–0.2 mg with a combined system of an elemental analyzer (EA1108, Carlo-Erba, Italy) and a stable isotope ratio mass spectrometer (Delta S, Finnigan MAT, Bremen, Germany). The mean reproducibility for the isotope measurements was ±0.08‰. The carbon isotope ratio ($\delta^{13}C$) was calculated using the following equation:

$$\delta^{13}C\ (\%) = (R_{sample}/R_{standard} - 1) \times 1000$$

where R_{sample} is the ratio of ^{13}C to ^{12}C in the sample and $R_{standard}$ is the ratio of ^{13}C to ^{12}C in the standard substance (Pee Dee belemnite).

Determination of other physiological parameters of transgenic Eucalyptus trees

Plants were watered every day and fertilized with Hoagland solution every week. Intact leaves were collected from three independent trees of each transgenic line and assayed for assimilation of CO_2, transpiration and stomatal CO_2 conductance from 30 October to 2 November 2007. Water-use efficiency was calculated from CO_2 assimilation and transpiration rates. Leaf gas exchange was measured at 740 μmol m^{-2} s^{-1} of photosynthetic photon flux density, 30 Pa of ambient CO_2, 26.4 ± 0.8 °C of leaf temperature and 1,060 ± 170 Pa of vapor pressure difference using experimental equipment described by Hanba et al. (1999). Shoot lengths at similar positions were measured twice at 64-day intervals (20 November 2007 to 23 January 2008), and the numbers of leaves were counted twice at 37-day intervals (20 November to 27 December 2007) for the three shoots for each tree. The mean tree height of GUT5 was 55 cm at the initial stage of analysis.

RNA preparation from leaves

Young leaves were collected from *Eucalyptus* trees, frozen in liquid nitrogen, ground with a mortar and pestle and then homogenized with a TissueLyser (Qiagen, Valencia, CA, USA) for 1 min at 30 Hz. RNA was extracted from the frozen tissue powder using a Plant RNA reagent kit (Invitrogen, Carlsbad, CA, USA) and further purified by sequential precipitation with sodium acetate and lithium chloride.

Analysis of transcript levels of PIP1s and PIP2s in Eucalyptus trees

First-strand cDNA was synthesized from 5 μg total RNA in a 100-μl reaction medium by reverse transcription using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative polymerase chain reaction (PCR) analysis was conducted on a Thermal Cycler Dice Real-Time System (Takara Bio, Otsu, Japan) using a SYBR Premix Ex *Taq* kit (Takara Bio) for 40 cycles with denaturation at 95 °C for 5 s and annealing at 60 °C for 30 s. The primer sets used for real-time PCR (RT-PCR) were as follows: 5′-ATCAAGTCCTGTGGTTTCTGGT-3′ (forward) and 5′-ATTGATTGCCTTAAGCAAAGGAG-3′ (reverse) for *RsPIP1;1*, 5′-TCTCGGATCTTTCAGAAGTGC-3′ (forward) and 5′-AACACAAACAAGAAGATAACAAACTGG-3′ (reverse) for *RsPIP2;1*, 5′-CATCTTTTGCTTCTCTCCTTTCAC-3′ (forward) and 5′-GAAGCATTCACACACTCGAAGTA-3′ (reverse) for *PIP1* of the GUT5 line, 5′-TACTACCAGTTCGTCCTCAGAGC-3′ (forward) and 5′-CTGGTTTCCCCTCTTCATCAT-3′ (reverse) for *PIP2* of *E. camaldulensis* and 5′-CTTGCTCAAGATGAGAGAAATCC-3′ (forward) and 5′-TCGCTTCATTGTAGTACACGTTC-3′ (reverse) for *E. grandis* β-tubulin (TUB-1; gene locus, EF534219). The specificity of these primers was confirmed by PCR. Standard plasmids that contained sequences of *RsPIP1;1* and *RsPIP2;1* were prepared and used for quantitative analysis with adequate primer sets. Copy numbers of the products were calculated from threshold cycles of triplicate real-time PCR assays using the standard curves. Values are expressed as means ± SD calculated for three assays.

Determination of nucleotide sequences of Eucalyptus PIP1 and PIP2

An expressed sequence tag (EST) sequence that covers the full length of *PIP2* (*EcPIP2*) was obtained from the EST database (*E. camaldulensis*; location: Petford, Australia). However, there was no EST that covers the full length of *Eucalyptus*

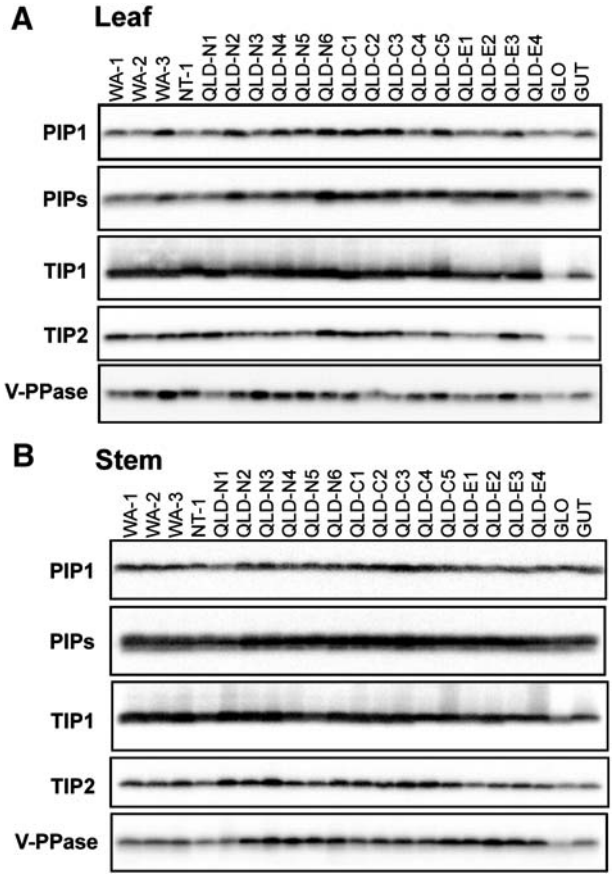

Figure 3. Levels of aquaporins in 19 wild provenances of *E. camaldulensis*. Crude membranes were prepared from leaves (A) and stems (B) of 3-month-old trees. Aliquots (8 μg) of the membrane preparations were subjected to SDS-PAGE and subsequent immunoblotting with antibodies to vacuolar H^+-pyrophosphatase (V-PPase), RsPIP1;1, RsPIPs, radish vacuolar membrane aquaporins (TIP1 and TIP2). GLO, *Eucalyptus globulus*; GUT, a hybrid clone of *E. grandis* and *E. urophylla*.

PIP1. We determined the internal nucleotide sequence that was not in the EST database. A cDNA library was synthesized from the total RNA fraction prepared from the *Eucalyptus* GUT5 line. The cDNA template was amplified to obtain a DNA of *PIP1* of GUT5 line (*EgPIP1*) by PCR using the following two primer sets: 5′-GGCAAAGAAGAGGACGTGAG-3′ (forward) and 5′-GCAAAGTCCTTGTCGAAGATG-3′ (reverse) and 5′-GGCAAAGAAGAGGACGTGAG-3′ (forward) and 5′-GTCTCTGGCATTCCTCTTGG-3′ (reverse). The obtained plasmids were amplified in *Escherichia coli* strain DH5α and then sequenced.

Crude membrane preparation

Young leaves were collected from *Eucalyptus* trees and immediately chilled on ice. In some cases, crude membranes were prepared from the stems of young trees. Tissues were homogenized in a 10-fold volume of medium containing 50 mM Tris-acetate (pH 7.5), 250 mM sorbitol, 1 mM Na-EGTA, 2 mM dithiothreitol, 1% (*w*/*v*) polyvinylpyrrolidone and 20 μM *p*-(amidinophenyl) methanesulfonyl fluoride hydrochloride (APMSF). The homogenate was filtered through four layers of cheesecloth and centrifuged at 10,000*g* for 10 min. After centrifugation of the supernatant at 100,000*g* for 30 min, the pellet was suspended in 20 mM Tris-acetate (pH 7.5), 250 mM sorbitol, 1 mM EGTA, 2 mM $MgCl_2$ and 2 mM DTT and was used as a crude membrane fraction.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting

The crude membranes were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% gel) and subsequent immunoblotting. The blots on an Immobilon-P membrane (Millipore, Bedford, MA, USA) were visualized with horseradish peroxidase-coupled protein A and enhanced chemiluminescence (ECL) western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA). The protein amount was determined using a protein assay reagent kit (Bio-Rad, Hercules, CA, USA). Antibodies specific to RsPIP1;1 (antigen peptide sequence, GKEEDVRVGANKFPERQPIGTSA-Cys) and RsPIP2;1 (MAKDVEAVSGEGFQTRDYQDP-Cys) were prepared previously (Suga et al. 2001). Polyclonal antibodies with broad specificity to plasma membrane aquaporins were prepared previously and used as anti-PIPs (KDYNEPPPAPLFEPGELSSWS-Cys) (Ohshima et al. 2001). Antibodies to vacuolar membrane H^+-pyrophosphatase (V-PPase) (Takasu et al. 1997) and radish vacuolar membrane aquaporins, RsTIP1 (Maeshima 1992) and RsTIP2 (MTSEHVPLASEF) (Suga and Maeshima 2004), were prepared previously.

Results

Growth and drought tolerance of Eucalyptus provenances

To compare the growth rate and drought tolerance of wild trees, we collected seeds of 19 provenances of *E. camaldulensis* from various points in Australia (Figure 1). Trees were raised from seeds and grown under normal conditions for 8 months in a greenhouse (Figure 2A).

Drought is one of the major abiotic stresses that adversely affect forest tree growth. In this experiment, we focused on the drought tolerance of these *Eucalyptus* provenances. In the greenhouse, each set of four trees from 19 provenances was watered under different conditions: every day, every 3 days, every 6 days, or no watering. Some typical physiological conditions of the trees, especially the leaves, are shown in Figure 2B. There were no intact leaves in the trees grown without watering. We evaluated drought tolerance by counting intact leaves in trees grown under drought conditions (watering every 3 and 6 days). Under the control conditions, namely everyday watering, all leaves of the trees examined were intact after 30 days. Therefore, the drought tolerance score of these trees was calculated to be 1.0. The drought tolerance of trees

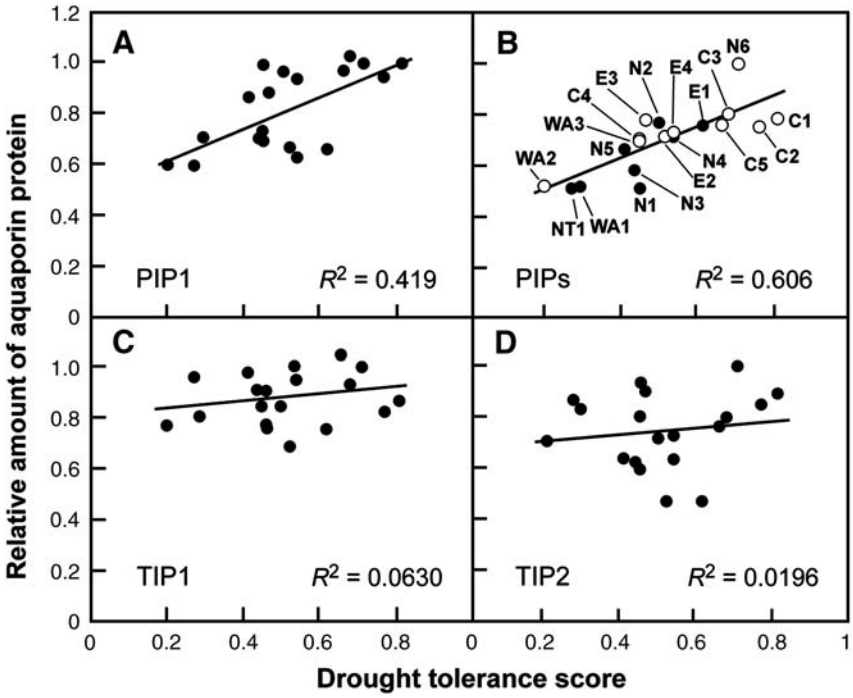

Figure 4. Relationship of the aquaporin level to drought tolerance. Each provenance of *E. camaldulensis* was grown under normal conditions for 80 days and then under drought stress or nonstressed conditions for 30 days. Crude membranes were prepared from leaves and subjected to SDS-PAGE. The protein levels of PIPs and TIPs were determined by immunoblotting with antibodies to RsPIP1 (A), RsPIPs (B), RsTIP1 (C) and RsTIP2 (D) and expressed as a ratio to the value of QLD-N6. In panel B, the provenances names were indicated. Drought tolerance score was determined as described in 'Materials and methods'. Open and closed circles show the annual average rainfall as shown in Figure 1. The values in each panel show the correlation coefficients (R^2). Linear regression was calculated and shown in each panel.

varied with the tree provenance with scores from 0.2 to 0.8 (Figure 2C). No correlation was observed between growth (tree height) and drought tolerance. This suggests that drought tolerance of *Eucalyptus* varies considerably with the local provenance.

Aquaporin content in wild trees of Eucalyptus

To examine the relationship between the aquaporin content and drought tolerance of wild trees of *Eucalyptus*, we prepared crude membrane fractions from young leaves for immunoblotting. Since information on the primary sequence of aquaporin in *Eucalyptus* is not available, we used antibodies prepared for radish PIPs and TIPs. Anti-PIP1 reacts with the PIP1 members of radish and *A. thaliana* (Suga et al. 2001) and anti-PIPs with both members of PIP1 and PIP2 (Ohshima et al. 2001). Anti-TIP1 (Maeshima 1992) and anti-TIP2 (Suga and Maeshima 2004) react with the corresponding members.

Anti-PIP1 and anti-PIPs gave clear bands at 30 kDa in all *Eucalyptus* provenances (Figure 3). The size of the immunostained bands was consistent with that of PIPs of radish. The immunostaining intensity, which reflects the antigen content, varied by provenances. The crude membrane fractions contained vacuolar membranes and gave a 23-kDa band in immunoblots with anti-TIP1 and anti-TIP2. Vacuolar membrane H^+-pyrophosphatase (V-PPase) has been demonstrated to be rich in young tissues (Nakanishi and Maeshima 1998, Maeshima 2000). Anti-V-PPase gave clear bands at 72 kDa in all provenances examined, indicating that the crude membranes were of young leaves and stems.

The aquaporin content was estimated by immunoblotting and plotted against the drought tolerance score for each provenance to evaluate their correlation (Figure 4). There was no correlation between drought tolerance and TIP1 or TIP2. The PIP1 content showed a weak positive correlation (Figure 4A), and the content of PIP proteins detected by anti-PIPs showed a good positive correlation with drought tolerance (Figure 4B). Anti-PIPs reacts with both PIP1 and PIP2 but not with other aquaporins. Therefore, we assume that the PIP2 content is positively related to the drought tolerance in wild *Eucalyptus* trees.

Growth of transformants under drought conditions

The obtained result suggested a positive correlation of the aquaporin content with drought tolerance. Therefore, we transformed *Eucalyptus* trees with aquaporin genes. We selected two types of radish PIPs: RsPIP1;1 and RsPIP2;1. RsPIP2;1 has been demonstrated to have high water channel activity and RsPIP1;1 to have no water channel activity (Suga et al. 2002). Radish aquaporin genes were transformed into a hybrid GUT5 line, which was generated by crossing *E. grandis* and *E. urophylla*. The transformant trees were regenerated from the selected transgenic callus and propagated by

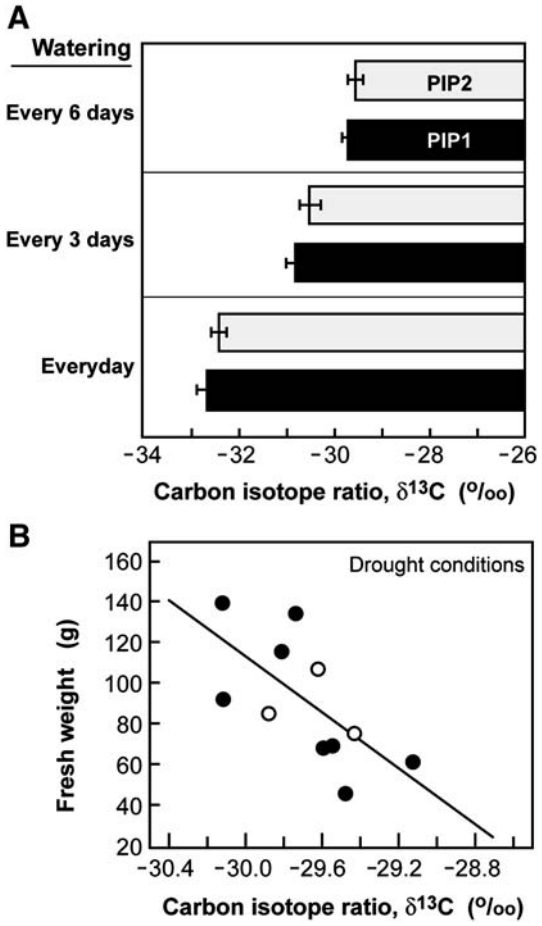

Figure 5. Carbon isotope ratio of transgenic trees. *Eucalyptus* trees transformed with *RsPIP1;1* or *RsPIP2;1* were grown under different watering conditions: every day, every 3 days, or every 6 days at the Forestry Research Institute of Oji Paper Co. Carbon isotope ratio $\delta^{13}C$ (‰) of leaves was determined as described under 'Materials and methods'. (A) Twelve plants of *RsPIP1;1* transformant and six plants of *RsPIP2;1* transformant of *Eucalyptus* were examined, and the mean values of the carbon isotope ratio are expressed. (B) Transgenic plants were grown under drought conditions (watering, every 6 days) for 3 months. Fresh weight of aboveground tissues was determined and plotted against the carbon isotope ratio $\delta^{13}C$ (‰). Open and closed circles means the *RsPIP1;1*- and *RsPIP2;1*-transformed lines, respectively. Linear regression was calculated and shown.

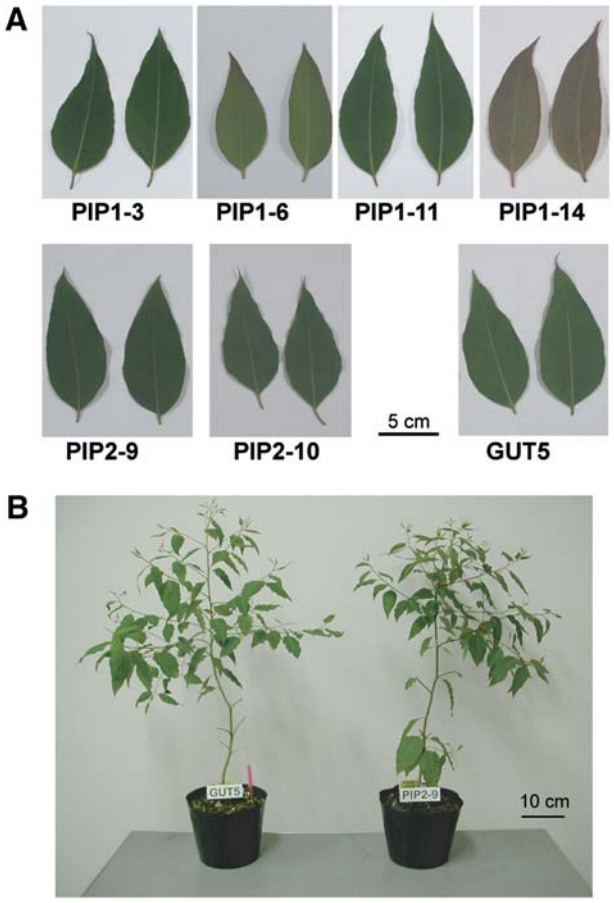

Figure 6. *Eucalyptus* trees transformed with *RsPIP1;1* and *RsPIP2;1*. Trees were grown in a glasshouse at Kyoto Institute of Technology. (A) Leaves from each line of the transgenic trees transformed with *RsPIP1;1* or *RsPIP2;1*. (B) GUT5 (left) and PIP2-9 (right) lines at 22-month-old stage were photographed.

cuttings of the primary trees. Hereafter, we refer to the transformants expressing *RsPIP1;1* and *RsPIP2;1* as the PIP1 and PIP2 lines, respectively.

To examine the water-use efficiency, which is related to drought tolerance, we grew the transgenic plants under three conditions of watering: every day, every 3 days and every 6 days. Then we determined the carbon isotope ratio ($\delta^{13}C$). The carbon isotope ratio of leaf dry matter reflects the water-use efficiency (Farquhar et al. 1989). Both PIP1 and PIP2 lines gave $\delta^{13}C$ values of approximately −32.5‰ when grown under well-watered conditions (Figure 5A). In both lines, the values were increased to −30‰ under drought conditions (watering every 6 days), indicating that the leaf water-use efficiency was enhanced under drought conditions. We observed no significant difference in the $\delta^{13}C$ values between the PIP1 and PIP2 lines. The $\delta^{13}C$ values and growth (fresh weight) varied by line (Figure 5B).

Therefore, we grew the transgenic lines under normal conditions and determined the transcription and translation levels of transferred DNA in each transformant. Plural lines were generated successfully and grown in a greenhouse (Figure 6B). The PIP2-9 and PIP2-10 lines grew well. Leaves of the PIP2-10 were thicker than those of the PIP2-9 or GUT5 line. Leaf mass per area of the GUT, PIP2-9 and PIP2-10 lines was determined to be 3.33 ± 0.28, 3.35 ± 0.31 and 4.25 ± 0.51 mg cm^{-2} (each, $n = 9$), respectively. Furthermore, PIP1-6 and PIP1-14 lines had reddish, hard leaves, although the leaves of PIP1-3 and PIP1-11 were as normal as those of GUT5 (Figure 6A). The red color might be due to the accumulation of anthocyanin in the leaves. The reddish leaf color of the PIP1-6 and PIP1-14 lines indicates that the transgenic plants suffered some stress even though the plants were

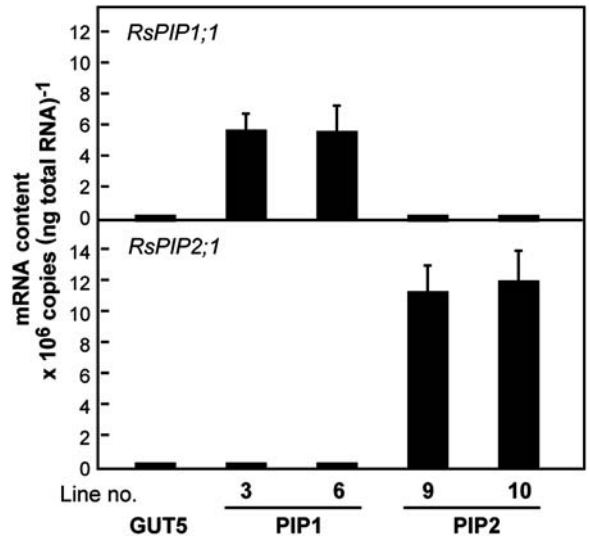

Figure 7. Transcript levels of *RsPIP1;1* and *RsPIP2;1* in transgenic trees. Total RNA was prepared from leaves of each line (single plant from PIP1-3 and PIP1-6 lines; three plants from each line of PIP2-9, PIP2-10 and GUT5). The transcript levels were determined by quantitative PCR using primer sets specific to *RsPIP1;1* or *RsPIP2;1* as described under 'Materials and methods'. Values shown represent mean ± SE of three replicates.

grown under normal conditions. The PIP1-14 line did not grow well for half a year and had harder leaves than the other lines.

RsPIP1;1 and RsPIP2;1 were transcribed in transgenic trees

We introduced *RsPIP1;1* and *RsPIP2;1* genes into *Eucalyptus* plants under the control of the CaMV 35S promoter. We used GUT5 as the host, which is one of the most cultivated *Eucalyptus* trees for the paper industry. Genes with the 35S promoter are typically expressed in most tissues at detectable levels. We determined the transcripts of exogenous *RsPIP1;1* and *RsPIP2;1* in transgenic trees. The *RsPIP1;1* gene was transcribed in the PIP1-3 and PIP1-6 lines but not in the PIP2 lines (Figure 7). The *RsPIP2;1* gene was also equally transcribed in the PIP2-9 and PIP2-10 lines at a relatively high level. The control plant, GUT5, expressed neither *RsPIP1;1* nor *RsPIP2;1*. We used these lines as *RsPIP1;1* and *RsPIP2;1* transformants for further characterization.

RsPIP2 protein, but not RsPIP1 protein, was accumulated in transgenic trees

Protein levels of RsPIP1 and RsPIP2 in the transgenic trees were quantified by immunoblotting with antibodies to radish PIPs. Transformation with *RsPIP1;1* did not increase the PIP1 abundance in the *RsPIP1;1* transformants (Figure 8A and C). The crude membrane fractions prepared from GUT5 and PIP2 lines showed a clear band at 30 kDa. These bands might correspond to *Eucalyptus* endogenous PIP1, not exogenous radish PIP1. The membrane preparations from the PIP1 line gave only a weak band at the same position. The

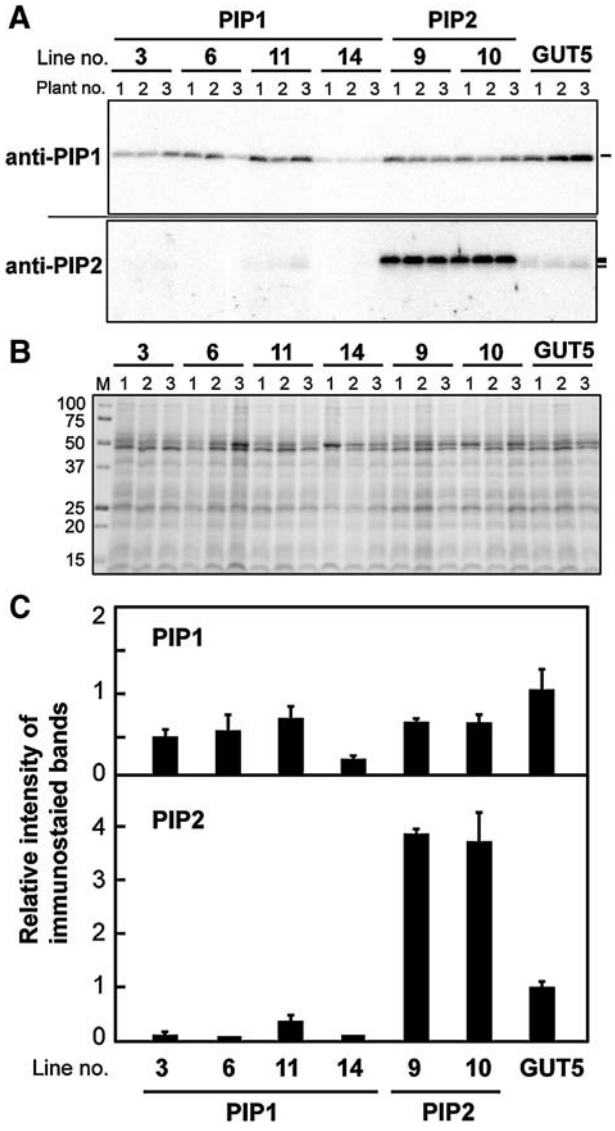

Figure 8. Protein levels of PIP1 and PIP2 in transgenic trees. Five leaves were collected from individual lines of *Eucalyptus* tree transformed with *RsPIP1;1* or *RsPIP2;1* and used for crude membrane preparation. Aliquots of the membrane fraction (8 μg of protein) were subjected to SDS-PAGE. (A) Immunoblots with anti-RsPIP1 (upper panel) or anti-RsPIP2 (lower panel). (B) Coomassie-brilliant blue stained gel. Lane M, molecular size marker with indicated values (kDa). (C) Relative contents of PIP1 and PIP2 determined by immunoblotting. The intensities of the immunostained bands were densitometrically quantified and are expressed relative to that of GUT5.

immunostained intensity of these faint bands was markedly low compared with that of GUT5. The difference is not due to the difference in the protein amount applied. The protein samples had similar protein profiles (Figure 8B). This result indicated that exogenous RsPIP1;1 was not accumulated and that endogenous PIP1 was decreased in the *RsPIP1;1* transformants.

Anti-RsPIP2 clearly reacted with a 30-kDa antigen in the crude membranes from *RsPIP2;1* transformants (Figure 8A and C). The result confirmed the overexpression of *RsPIP2;1*

Table 1. Physiological properties of *Eucalyptus* trees transformed with *RsPIP1;1*, *RsPIP2;1* or vacant vector. Transgenic trees were grown for approximately 12 months in 50% shaded light conditions in 2.9-l pots. Plants were watered every day and fertilized with Hoagland solution every week. Intact leaves were collected from three independent trees of each transgenic line and assayed for assimilation of CO_2, transpiration and stomatal CO_2 conductance. Water-use efficiency was calculated from CO_2 assimilation and transpiration rates. The details are described in 'Materials and methods'. Shoot lengths at similar positions were measured twice at 64-day intervals, and numbers of the leaves were counted twice at 37-day intervals for the three shoots for each tree. The mean tree height of GUT5 was 55 cm at the initial stage of analysis. Data were means ± SD.

Line	CO_2 assimilation (μmol m^{-2} s^{-1})	Transpiration (mmol m^{-2} s^{-1})	Stomatal CO_2 conductance (mol m^{-2} s^{-1})	Water-use efficiency (mmol mol^{-1})	Shoot growth (cm)	Increase in leaf number
GUT5	25.5 ± 2.2	3.04 ± 1.04	0.160 ± 0.027	8.88 ± 2.15	7.8 ± 1.3	13 ± 8
PIP1-3	26.3 ± 5.4	2.60 ± 0.14	0.152 ± 0.028	10.1 ± 1.9	4.8 ± 2.9	30 ± 4*
PIP1-6	13.1 ± 0.5*	2.95 ± 0.10	0.197 ± 0.019	4.43 ± 0.29*	1.7 ± 3.2*	3 ± 0
PIP1-11	24.5 ± 4.5	2.95 ± 0.53	0.140 ± 0.031	8.28 ± 0.18	3.8 ± 1.8	19 ± 9
PIP1-14	12.9 ± 1.7*	3.13 ± 0.16	0.194 ± 0.018	4.16 ± 0.78*	1.7 ± 1.5*	3 ± 1
PIP2-9	23.2 + 0.5	2.65 ± 0.24	0.171 ± 0.015	8.81 ± 1.01	8.4 ± 2.6	20 ± 13
PIP2-10	34.1 ± 0.3*	2.57 ± 0.22	0.161 ± 0.028	13.3 ± 1.3*	11.2 ± 0.8	36 ± 17*

*$P < 0.05$ (Student's t tests), the values were statistically different from those of GUT5.

in *Eucalyptus* trees at the protein level. A faint band was detected in the membranes from the GUT5 line and the *RsPIP1;1* transformants (PIP1-3 and PIP1-11 lines) at a slightly lower position (29.8 kDa). The immunospecificity of anti-RsPIP2 was confirmed previously (Suga et al. 2001). Therefore, this faint band might be that of the *Eucalyptus* endogenous PIP2. It was hard to detect PIP2 in the PIP1-6 and PIP1-14 lines.

Physiological properties of transformants

To determine physiological properties of the transgenic *Eucalyptus*, we determined the CO_2 assimilation rate, stomatal CO_2 conductance, transpiration and water-use efficiency in leaves from each line by the established methods, respectively. The PIP1-6 and PIP1-14 lines had CO_2 assimilation rates approximately half of that of the control GUT5 line (25.5 μmol m^{-2} s^{-1}) (Table 1). On the other hand, the rate in the PIP2-10 line was 33% higher than the control. The transpiration rate in the four PIP1 lines (2.91 mmol m^{-2} s^{-1}) was similar to that in GUT5. The PIP2-9 and PIP2-10 lines showed lower values. Also, the drought tolerance of these transgenic lines was examined under different watering conditions (every day, every 4 days or every 7 days). Under drought conditions, the transgenic plants lost more than 60% of leaves after 3 weeks. No transgenic lines showed improved drought tolerance with a statistical significance (data not shown).

The water-use efficiency, which is the ratio of the amount of CO_2 accumulated in leaves to the amount of H_2O evaporated from leaves, was calculated from the CO_2 assimilation and transpiration rates. The water-use efficiency in GUT5 was 8.38 mmol mol^{-1}. The PIP1-6 and PIP1-14 lines had water-use efficiency approximately 50% of that of GUT5. The PIP2-10 line (13.3 mmol mol^{-1}) had 50% higher water-use efficiency than the control line, and the PIP2-9 line also had a higher value than the PIP1 lines.

The stomatal CO_2 conductance was 0.16 mol m^{-2} s^{-1} in GUT5, and the transgenic lines showed comparable values with a variation of 20%. The content of chlorophyll ($a + b$) in leaves was also determined in the same trees. The values were 422 (GUT5 line), 345 (PIP1-3), 356 (PIP1-6), 404 (PIP2-9) and 446 μmol m^{-2} (PIP2-10). Therefore, the chlorophyll ($a + b$) content in leaves of the PIP1 lines was remarkably low, and that of the RsPIP2 transformants was comparable to or slightly higher than that of GUT5.

The growth of transgenic trees was compared with shoot growth under normal conditions (Table 1). Shoots of the GUT5 line grew 7.8 cm for 64 days, and shoots of the PIP2-9 and PIP2-10 lines grew 8.4 and 11.2 cm, respectively. However, the shoots of the PIP1 lines grew slowly. The PIP1-6 and PIP1-11 lines grew only 1.7 cm. The GUT5 line had 21 leaves, and the PIP2-10 lines had 45 leaves. The PIP1-6 and PIP1-11 lines had 14 and 26 leaves, respectively; however, their leaves were reddish (Figure 6). The average diameter of the trunk of trees at a height of 5 cm from the soil was 8.8, 8.5 and 10.7 mm in GUT5, PIP1 and PIP2 lines, respectively. These results indicate that the PIP2 lines had relatively high water-use efficiency and growth rates. However, the transformation of *RsPIP1* resulted in the suppression of photosynthetic activity and tree growth.

Identification of endogenous PIPs and their expression in transgenic lines

Reduction in the amounts of endogenous PIP proteins in the transgenic trees led us to examine the transcription levels of endogenous *PIP1* and *PIP2* genes. At present, genome information for *Eucalyptus* is not available. A full-length sequence of a PIP2 member was obtained from the EST database. A single cDNA of PIP1 could be isolated from the cDNA library of *Eucalyptus* using a partial sequence obtained from the EST. As shown in Figure 9, the amino acid sequences of *Eucalyptus* PIP1 (EgPIP1, originating from the *Eucalyptus* GUT5 line)

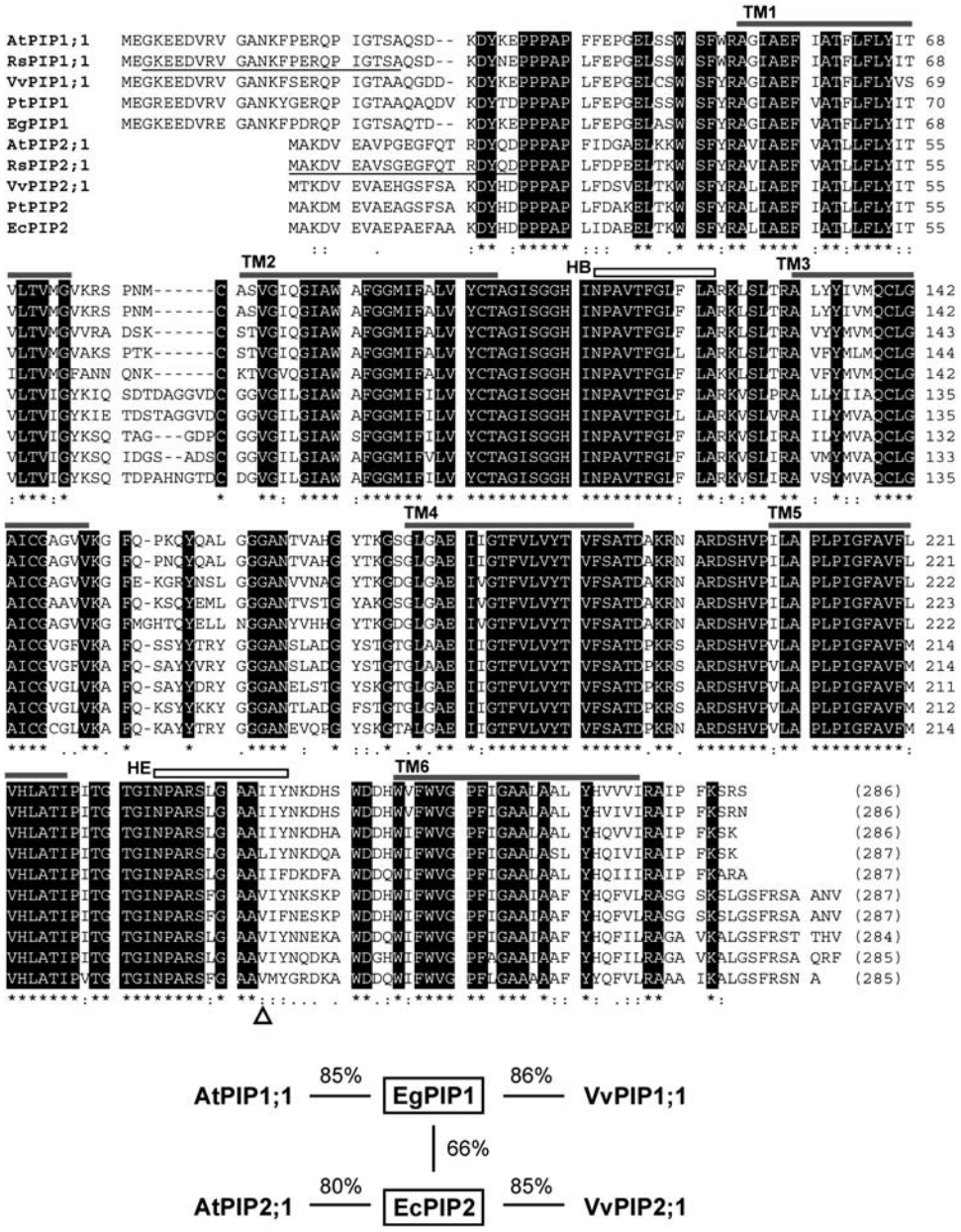

Figure 9. Amino acid sequence alignment of endogenous *Eucalyptus* PIP1 and PIP2 with related proteins from various organisms: AtPIP1;1 (*A. thaliana*; gene name, At3g61430), RsPIP1;1 (*R. sativus*; accession number, AB012044), VvPIP1;1 (*V. vinifera*; EF364432), PtPIP1 (*P. trichocarpa*; National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/; XM_002323529), EgPIP1 (*Eucalyptus*), AtPIP2;1 (At3g53420) RsPIP2;1 (AB0120045), VvPIP2;1 (AY823263), PtPIP2 (XM_002316331) and EcPIP2 (*Eucalyptus*). Identical residues among these proteins are highlighted and marked by asterisks. Six putative transmembrane domains (TM1 to TM6) predicted from hydropathy profiles are marked by thick lines. Open boxes indicate half α-helices containing a NPA motif (HB and HE). The sequences used for preparation of antibodies are marked by dotted lines. Triangle indicates position of Ile-245 (EgPIP1) and Val-237 (EcPIP2). Amino acid sequence identity between *Eucalyptus* PIPs and related PIPs of *A. thaliana* and *V. vinifera* is shown at the bottom.

and PIP2 (EcPIP2, based on the EST database of *E. camaldulensis*) are similar to those of *A. thaliana*, *R. sativus* (radish), *Vitis vinifera* (grape) and *P. trichocarpa.* Both *Eucalyptus* PIP1 and PIP2 share a relatively high sequence identity of 66%. EgPIP1 has an extensive hydrophilic region of 14 residues at the N terminus and a short tail at the C terminus compared with PIP2s. In addition to these characteristics, EgPIP1 and EcPIP2 contain some subgroup-specific residues. For example, the valine residue in the half-helix loop HE has been shown to be essential for the water channel activity of RsPIP2 (Suga et al. 2002). This residue is substituted with Ile in RsPIP1s, which have little water channel activity. These PIP1- and PIP2-type specific functional residues are conserved in EgPIP1 (Ile-245) and EcPIP2 (Val-237), respectively.

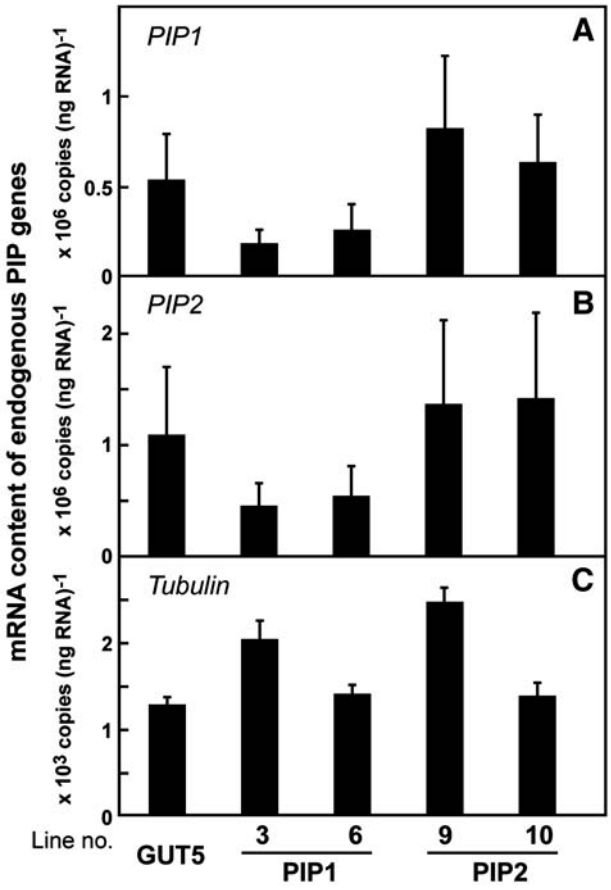

Figure 10. Expression of endogenous *Eucalyptus PIP1* and *PIP2* in transgenic trees. Five leaves were collected from each plant. The total RNA fraction was prepared from young leaves and the mRNA contents of endogenous *EgPIP1*, *EgPIP2* and tubulin-1 were determined individually by quantitative real-time PCR.

We then determined the mRNA levels of endogenous *EgPIP1* and *EgPIP2* genes in the control and transgenic lines by real-time PCR with primer sets for endogenous *Eucalyptus* PIP genes. Transformation with radish *PIP1* caused down-regulation of endogenous *PIP1* as shown in Figure 10. The transcript level of *EgPIP2* was also decreased in the PIP1-3 and PIP1-6 lines compared with that of GUT5. Expression of *EgPIP1* or *EgPIP2* was not extensively changed in the PIP2-10 lines.

Discussion

Correlation of aquaporin content to drought tolerance of Eucalyptus

Aquaporin members facilitate membrane transport of water molecules and other small compounds such as glycerol and contribute to diverse physiological phenomena including growth, fruit development, flower opening, drought tolerance and low-temperature sensitivity (Hachez et al. 2006, Kaldenhoff and Fischer 2006, Azad et al. 2008, Katsuhara et al. 2008, Maurel et al. 2008, Murai-Hatano et al. 2008). Among the five subfamilies (PIP, TIP, NIP, SIP and XIP) of plant aquaporins, the NIP members have been demonstrated to facilitate the transport of various substrate compounds (Ma et al. 2006, Choi and Roberts 2007, Kamiya et al. 2009). Despite these elaborations of higher aquaporin function, little is known about the physiological roles of aquaporin members in trees. We focused on the plasma membrane aquaporins (PIPs) in *Eucalyptus* trees because the PIP members predominantly function as water channels. We assumed that the PIP members among plant aquaporins are tightly related to water physiology in trees.

We investigated the aquaporin content and its relationship to growth and drought tolerance of local provenances of *Eucalyptus* trees (Figures 1, 2 and 3). The analysis of native provenances grown in Australia revealed a positive correlation between the content of PIPs, especially PIP2 and drought tolerance (Figure 4). Water absorption, water supply to cells in tissues, transpiration from leaves and photosynthesis may be tightly related to the content and function of aquaporins. Aquaporins, especially PIPs, are a key factor that determines the amount, rate and direction of water flow in plant tissues. The water flow in the tissues affects the circulation of nutrients, carbon dioxide and signal compounds. Therefore, we assume that a relatively high amount of PIPs contributes to efficient water absorption and water flow in tissues under drought conditions.

Positive effect of overexpression of radish PIP2 in Eucalyptus

The transgenic approach is a useful method to evaluate gene products in plants. We introduced radish *PIP1* and *PIP2* genes into *Eucalyptus* callus and regenerated the transformants. The *PIP2* transformants expressed exogenous *RsPIP2* and accumulated the translation product. The PIP2 lines showed good assimilation of CO_2 and strong shoot growth and produced many leaves with a low transpiration rate (Table 1). As a result, the water-use efficiency, shoot growth and leaf numbers were all better than those of the control. Radish PIP2;1 has been demonstrated to function as an efficient water channel as well as PIP2;2 and PIP2;3, but radish PIP1;1 and PIP1;2 show no water channel activity when examined in a yeast heterologous expression system (Suga et al. 2002). Also, it has been reported that an increase in PIP2 transcripts is tightly related to high hydraulic conductance in leaves of walnut trees (*J. regia*) (Cochard et al. 2007). Therefore, we concluded that additional RsPIP2;1 functioned as an efficient water channel together with endogenous PIP2s in *Eucalyptus* trees and provided a positive effect on water-use efficiency and growth under normal conditions.

It should be noted that the overexpression of PIP2 did not enhance drought tolerance, even though the transpiration rate of the transformants decreased by 10% compared with GUT5. It is possible that the increase in the number of leaves,

which was more than 150% of that of the control trees, resulted in an increase in the total amount of water evaporated. It has been reported that a T-DNA insertion knockout mutant of *PIP2* results in a reduction in water permeability of the roots in *A. thaliana* (Javot et al. 2003). Therefore, the present study also indicates the importance of PIP2 in forest trees *in planta*.

We should note the difference in physiological properties between PIP2-9 and PIP2-10 (Table 1). Plants of both lines had similar levels of radish PIP2 mRNA and protein (Figures 7 and 8). The physiological variation between PIP2-9 and PIP2-10 lines might be a reflection of the DNA insertion site in the *Eucalyptus* genome and intracellular localization of the PIP2 protein, which was exogenously expressed. It is possible that a small part of RsPIP2;1 translated in the rough endoplasmic reticulum (ER) membranes was retained in the ER, Golgi, or small vesicles in cells of PIP2-9 trees before final localization. Furthermore, the increment of number and thickness of leaves in the PIP2-10 line may totally increase the photosynthetic capacity. For these reasons, the physiological parameters may be improved. Indeed, there is a report that overexpression of PIP in rice caused thick leaf morphology and increased CO_2 assimilation (Hanba et al. 2004).

We obtained another PIP2 line, PIP2-54, which grew very well as a PIP2-10 line. The properties of PIP2-54 are not described here because the season of regeneration and propagation by cutting was different from that of the other lines. The assimilation rate of CO_2 and leaf mass per area of PIP2-54 leaves was 24 and 10% greater than those of GUT5, respectively, indicating high photosynthetic activity in relatively thick leaves. Thick leaf morphology is consistent with a previous observation for rice transformed with barley PIP2;1 (Hanba et al. 2004), although we cannot explain the reason why the leaves became thick in the *PIP2* line. In conclusion, the present study suggests that transformation with *PIP2* is one possible method to improve the growth of *Eucalyptus* trees. In this study, radish *PIP2* was expressed in *Eucalyptus* trees using the CaMV 35S promoter. Therefore, expression using other promoters, such as root- or leaf-specific promoters, should be examined.

Knockdown transformants obtained by overexpression of radish PIP1

In contrast to PIP2 lines, PIP1 lines did not accumulate protein of radish PIP1. Also, the abundance of endogenous PIP2 seemed to be decreased as discussed later. The transcript of *RsPIP1;1* was confirmed by RT-PCR. However, the exogenous radish PIP1 did not accumulate, and the endogenous PIP1 protein was markedly decreased in these lines (Figure 8). It should be noted that the marked decrease in the level of PIP1 protein resulted in a low rate of CO_2 assimilation (Table 1). A tobacco PIP1 member (NtAQP1) has been reported to be involved in mesophyll conductance to CO_2 (Flexas et al. 2006). Therefore, photosynthetic activity may be partially affected by the decreased amount of PIP1.

We found that the reduction in endogenous PIP1 abundance was mainly caused by a reduction of the transcript level. Indeed, the mRNA level of endogenous *EgPIP1* was remarkably suppressed (Figure 10). These phenomena are well known as co-suppression or post-transcriptional gene silencing (Linder and Owttrim 2009). The transcript of *RsPIP1;1* probably accumulated in the transformants beyond a critical threshold level that led to the co-suppression of the endogenous gene and transformed gene. The mRNA level of endogenous *PIP2* was also partially decreased in the PIP1 lines. There are two possible explanations for the cross-suppression by *RsPIP1;1*. The expression of the endogenous *EgPIP1* gene probably controls the expression of *EcPIP2* by an unknown cross-talk mechanism. Therefore, the co-suppression of *EgPIP1* by exogenous *RsPIP1;1* causes low expression of *EcPIP2*. Second, transformation with *RsPIP1;1* may cause the co-suppression of *EgPIP1* and *EcPIP2* because the nucleotide sequence of *RsPIP1;1* has high identity with that of *EcPIP2* (61%). The detailed mechanism remains to be resolved.

In the present study, we accidentally obtained knockdown mutant lines of PIP1 in which protein contents and mRNA levels of endogenous PIP1 and PIP2 were markedly decreased (Figures 8 and 10). The results clearly indicate that suppression of PIP proteins was critical to growth. Growth was markedly suppressed in two (PIP1-6 and PIP1-14) out of four PIP1 lines. These two lines gave low water-use efficiency, with half values of that of the GUT5 line (Table 1). Therefore, we should note that PIPs play an essential role for normal growth and that a low accumulation of the PIP proteins severely suppresses growth of *Eucalyptus* trees. This result is consistent with previous observations for PIP1 in addition to reports for PIP2. Aharon et al. (2003) reported that overexpression of AtPIP1;2 in tobacco plants significantly increased plant growth rate, transpiration rate and photosynthetic efficiency but decreased tolerance to salt and drought stresses. Another group reported that tobacco plants impaired in NtAQP1 expression showed reduced root hydraulic conductivity and lower water stress (Siefritz et al. 2002).

Limited information was available on PIPs in forest trees. A full-length cDNA for EgPIP1 was determined in this study, and a cDNA for EcPIP2 was picked up from an EST database. *Eucalyptus* PIP1 and PIP2 share high identity with corresponding PIP members in other plants including their N- and C-terminal regions (Figure 9). EcPIP2 shares identity of more than 80% with other PIP2 members, which have been demonstrated to be water channels. EcPIP2 also has common motifs among aquaporins including two NPA motifs and residues (Phe-87, Asn-107 and Arg-231 in EcPIP2) for an aromatic/Arg selectivity region (for review; Maurel et al. 2008). A valine residue in the half-helix loop HE has been reported to be essential for water channel activity of radish PIP2s when compared with PIP1s (Suga et al. 2002). It has been reported that substitution of valine residue of PIP2 with isoleucine, which is found in the

corresponding position of PIP1 members, results in the loss of water channel activity. In contrast, PIP1 showed significant water channel activity when the isoleucine residue in the half-helix loop HE was substituted with valine. This key residue is conserved in EcPIP2 (Val-237) but not in EgPIP1 (Ile-245). Therefore, EcPIP2 may function as a water channel in *Eucalyptus* trees.

In conclusion, this study showed the occurrence of cosuppression of *PIP1* and *PIP2* genes caused by the overexpression of *PIP1* and revealed the physiological importance of abundance of PIP1 and PIP2 members in *Eucalyptus* trees. Further studies are needed to determine endogenous *PIP* genes of *Eucalyptus* and the actual substrate(s) for PIP1.

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