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RESEARCH PAPER

Differential responses of maize *MIP* genes to salt stress and ABA

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Abstract

Salt stress is known to reduce root hydraulic conductivity and growth. To examine a concomitant regulation of aquaporins, the expression of the maize *MIP* gene family in response to NaCl was analysed by DNA array hybridization. Plants responded differentially to 100 versus 200 mM NaCl treatments. Leaf water content was reduced rapidly and persistently after the application of 200 mM NaCl in contrast to 100 mM NaCl. Endogenous ABA strongly accumulated in roots after 2 h; it remained at a highly elevated level for 48 h after the addition of 200 mM NaCl, but rapidly declined in plants treated with 100 mM NaCl, indicating an early recovery from water deficit. Interestingly, 2 h after the addition of 100 mM NaCl, when maize regained the osmotic potential allowing water uptake, three highly expressed, specific isoforms *ZmPIP1;1*, *ZmPIP1;5*, and *ZmPIP2;4* were transiently induced. They were preferentially transcribed in the outer root tissue suggesting a role in cellular water transport. None of the *ZmTIP* genes was altered. By contrast, after the addition of 200 mM NaCl these responses were missing. Instead, multiple *ZmPIP* and *ZmTIP* genes were repressed by 200 mM NaCl after 24 h. After 48 h, deregulations were overridden in both cases indicating homeostasis. ABA (1 μM) exogenously applied to the roots transiently induced *ZmPIP2;4* similar to 100 mM NaCl as well as *ZmPIP1;2*. Thus, the early induction of *ZmPIP2;4* by NaCl may be mediated by ABA. Previously, an increase in root hydraulic conductivity had been observed upon ABA application. By contrast, 100 μM ABA led to a complete, possibly non-specific repression of all detected *ZmPIP* and *ZmTIP* genes after 24 h.

Key words: ABA, aquaporins, expression analyses, major intrinsic proteins, water stress, *Zea mays*.

Introduction

Plant water relations are disturbed by drought or high salinity due to the lowered water potential in the environment hampering water uptake or favouring loss of water from plants. Plants have evolved several mechanisms to cope with these stresses. Closing of stomata reduces transpirational losses of water from leaves. The uptake and the biosynthesis of compatible solutes and the regulation of membrane permeability and transport are further measures that either hold back water within the plant or facilitate further uptake of water in the roots (Zhu, 2001; Sharp *et al*., 2004). According to the composite water flux model, water passes plant organs by three pathways, the apoplastic, symplastic, and transcellular pathways. The latter two routes cannot be experimentally distinguished and are combined as the cell-to-cell pathway. However, since both require transport of water across cellular membranes, integral membrane proteins of the major intrinsic protein (MIP) family are supposed to play an important role. Several MIPs have been shown to act as aquaporins facilitating the permeation of water across membranes driven by differences in water potential (Johansson *et al*., 2000; Baiges *et al*., 2002; Maurel *et al*., 2002; Tyerman *et al*., 2002). Structural investigation of the mammalian AQP1 prototype uncovered elements which are involved in forming the water channel and determining its specificity (Sui *et al*., 2001; de Groot *et al*., 2003). In addition to water, individual MIP members may mediate the movement of small non-charged molecules, such as glycerol, urea, CO_2, or formaldehyde (Tyerman

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et al., 2002; Gaspar *et al*., 2003; Uehlein *et al.*, 2003). Furthermore, a role as osmotic or turgor sensors has been suggested for MIPs (Hill *et al*., 2004).

Plant MIPs are classified into four groups, PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), NIPs (nodulin-26 like intrinsic proteins), and SIPs (small and basic intrinsic proteins) (Johanson *et al*., 2001). PIPs are further separated into two, highly homologous subgroups, PIP1 and PIP2 (Schäffner, 1998). Genome projects have revealed 35 MIPs in *Arabidopsis thaliana* and at least 32 members in maize. So far, 13 PIPs split into six PIP1 and seven PIP2, 12 TIPs, four NIPs, and three SIPs were identified in *Zea mays* (Chaumont *et al*., 2001; cDNA accession no. AF057183). Eight maize isoforms have been examined for water channel activity by heterologous expression in *Xenopus* oocytes. ZmTIP1;1, ZmTIP2;3 (Chaumont *et al*., 1998; Lopez *et al.*, 2004) as well as ZmPIP2;1, ZmPIP2;4, and ZmPIP2;5 (Chaumont *et al*., 2000; Fetter *et al*., 2004) have high activity, whereas ZmPIP1;1, ZmPIP1;2, and ZmPIP1;5 had no or very low activity, at least in this system (Chaumont *et al*., 2000; Gaspar *et al*., 2003). However, Fetter *et al*. (2004) could show that co-expression of ZmPIP1 and ZmPIP2 members may synergistically enhance water permeability in *Xenopus* oocytes.

The involvement of MIPs in water relations *in planta* has been approached by antisense strategies targeting *PIP1* and *PIP2* members as well as a single isoform, *AtPIP2;2*, by analysing a knock-out mutant (Martre *et al*., 2002; Javot *et al*., 2003; see below). On the transcriptional level, various reports demonstrated the responsiveness of plant *MIP*s to drought, low temperature, high salinity, light, pathogens, or hormonal stimuli. Thus, aquaporin genes that are up- or down-regulated by drought and salt stress have been pinpointed in *Arabidopsis*, rice, sunflower, cauliflower, wheat, barley, and maize (Kawasaki *et al*., 2001; Seki *et al.*, 2002*a*; Maathuis *et al*., 2003; Martinez-Ballesta *et al*., 2003; Wang *et al.*, 2003; Jang *et al.*, 2004). Suppression of MIPs may be interpreted as a measure to reduce loss of water from cells, to reduce water uptake into cells, and/or to decrease water fluxes through tissues. Reports on the transgenic manipulation of different *PIP1* isoforms in *Arabidopsis* or tobacco were, in part, in agreement with these roles, although seemingly similar genetic alterations lead to contrasting results (Martre *et al.*, 2002; Siefritz *et al.*, 2002; Aharon *et al*., 2003). These findings underscore that plant responses to water stress require complex, differential, and specific regulations of water permeability and, importantly, the consideration of isoform-specific roles (Luu and Maurel, 2005).

The phytohormone abscisic acid (ABA) is an important signal molecule regulating physiological processes and, in particular, responses to environmental stresses such as drought and salinity (Hose *et al.*, 2002; Sharp *et al*., 2004). ABA accumulates in plants suffering from water deficit and triggers the expression of several ABA-responsive genes (Jia *et al.*, 2002; Himmelbach *et al*., 2003). It stimulates and maintains root development and growth under stress, increases root hydraulic conductivity, and affects stomatal opening. ABA may also regulate the expression of individual *MIP*s in *Arabidopsis*, barley, radish, tomato, *Brassica napus*, and *Craterostigma plantagineum* (Fray *et al.*, 1994; Mariaux *et al*., 1998; Gao *et al*., 1999; Hoth *et al*., 2002; Seki *et al*., 2002*b*; Suga *et al*., 2002). Hose *et al.* (2000) have also shown that ABA transiently induced the water conductivity of roots and the water permeability of cortex cells in maize. Wan *et al.* (2004) measured the root hydraulic conductivity of maize root cortical cells using the cell-pressure probe and concluded that ABA stabilizes aquaporins during high rates of water flow through the pores, which results in increased conductivity.

To assess the contribution of the complete set of maize *MIP* genes in response to salt stress and ABA and to identify isoform-specific roles, the transcriptional regulation of the complete maize MIP family was approached using gene-specific target sequences that eliminate the problem of cross-hybridization when using larger cDNA fragments.

Materials and methods

Plant material and treatment

Seeds of maize (*Zea mays* L. cv. Helix, Kleinwanzlebener Saatzucht AG, Einbeck, Germany) were immersed for 2 h in distilled water and germinated on wetted paper for 4 d at 24 °C. Seedlings were transferred to continuously aerated hydroponic medium (Gibeaut *et al*., 1997) and grown for 7 d under 16 h light at photosynthetically active radiation of 180–230 μmol m^{-2} s^{-1}, temperature was controlled between 22 °C and 25 °C and relative humidity ranged from 45–60%. ABA or high-salinity stress treatments were carried out. For ABA treatments, 1 μM or 100 μM ABA was added to the hydroponic medium. Sampling times were 1, 6, and 24 h. For high-salinity stress, 100 mM or 200 mM NaCl was applied to the hydroponic solution, which resulted in an increase of the osmolality from 14 mOsm to 200 mOsmol kg^{-1} and 373 mOsmol kg^{-1}, respectively. Accordingly, the osmotic pressure changed from 0.04 MPa to 0.50 MPa and 0.92 MPa. After 2, 9, 24, and 48 h of treatment probes were harvested and frozen in liquid nitrogen for further analyses. The osmotic potential of the cell sap of maize roots was determined cryoscopically using the Osmomat 030 osmometer (Gonotec, Berlin, Germany).

Cortex and stele tissues from 10–50 mm zone of primary roots were separated with the help of two pairs of tweezers. One pair of tweezers was used to hold the central, stelar tissue, whereas the second one allowed the outer, cortical tissue to be torn off (Hose, 2000). Control via a microscope showed that the separation occurred right at the endodermis, which was attached to the cortical layers.

Leaf water status was assayed by measuring the fresh weight and dry weight of leaves of NaCl-treated (100 and 200 mM) and control seedlings (treated seedling by 100 mM and 200 mM NaCl and control). Dry weight was obtained after drying the leaves at 104 °C for 3 h.

Nucleic acids

Total RNA was prepared using the TRIZOL reagent (Invitrogen, Karlsruhe, Germany) and then purified using the RNA-Teasy kit

(Qiagen, Hilden, Germany) according to the manufacturer's protocol. After an initial photometric determination of total RNA concentration, ethidium bromide fluorescence of ribosomal bands on the electrophoresis-gel was used for the control of total RNA integrity and the adjustment of equal RNA amounts. Isolation of genomic DNA was performed according to Fabri and Schäffner (1994).

DNA array design and production

To produce gene-specific probes 3′-UTR sequences were selected from *ZmMIP* cDNA sequences (Chaumont *et al*., 2001; cDNA accession AF057183) and *Rab17* as a known ABA-responsive control gene (Kizis and Pagès, 2002) and amplified by PCR from maize genomic DNA as a template. All PCR fragments were cloned into pGEM-Teasy plasmid vector (Promega, Madison, USA) and verified by sequencing. The specificity of *MIP* gene targets was checked by BLAST analyses at http://www.ncbi.nlm.nih.gov/BLAST/ (see below; Supplementary Table 1 can be found at JXB online). Three human cDNAs SP1, SP3, and SP4 (TGF-Beta, accession M60316; HLA class I histocompatibility antigen, M11886; Brachyury, AJ001699) were used as negative, non-hybridizing control genes. For normalization, their target sequences were spotted as well (see below).

The inserts were amplified by PCR using plasmid DNA as templates and oligonucleotides directed to flanking vector sequences. Fragments were concentrated by filtration using filter plates (Millipore, Eschborn, Germany), controlled by gel electrophoresis, and subsequently spotted onto Hybond N^+ nylon membranes (Pharmacia Amersham, Freiburg, Germany) in duplicate (MicroGrid II, BioRobotics, Cambridge, UK). Each array was hybridized with ^{33}P-labelled oligonucleotide 5′-TAATACGACTCACTATAGGG recognizing the flanking vector sequences of the PCR products. These signals served as loading controls and were used for normalization according to Thimm *et al*. (2001) (see below).

DNA array expression analyses

For hybridizations, 10 μg total RNA was spiked with a single batch of *in vitro* transcribed RNA from human genes SP1 (100 pg), SP3 (50 pg), SP4 (50 pg) (NASC Arabidopsis Stock Centre, UK), and labelled with 2.1 MBq [α-^{33}P]-dATP (42.9 kBq pmol^{-1}) by oligo-dT primed reverse transcription according to the manufacturer (Strip-EZ™ RT, Ambion, Huntingham, UK). Heat-denatured purified probes were hybridized for at least 18 h in 5× SSC, 5× Denhardt's buffer, 0.5% SDS, and 100 μg ml^{-1} ssDNA at 65 °C. Washing involved a final step at 0.2× SSC/0.1% SDS at 68 °C for 30 min. Primary data were obtained by exposure of imaging plates and reading using a phosphoimager (Fuji FLA3000); software for evaluation was ArrayVision 5.1 (InterFocus, Mering, Germany).

For further processing of data, the local background measured within each 3×3 subgrid was subtracted from the value of each individual signal. These values were divided by the mean of the signal measured for three internal standard transcripts (SP1, SP3, and SP4) for a first normalization regarding labelling by reverse transcription and exposure. These values were divided by individual reference hybridization values (T7 oligonucleotide see above) in order to compensate for differences in the amount of spotted DNA probes (Thimm *et al*., 2001). Next, the mean for each gene was calculated from the duplicate spots. Final 'gene activities' were determined as the mean of three biological replicates. In order to compare two different scenarios, the gene activities of individual experiments from treated versus untreated samples were divided to result in a relative gene activity or 'expression ratio'. Means and standard deviations were calculated from three independent biological replicates. The result was subjected to *t*-test; *P*-values were calculated to assess the statistical relevance of changes (Precht and Kraft, 1993). Changes were classified as inductions with varying extent: ratio ⩾2.0, $P \leqslant 0.05$; ratio=1.67–2.0, $P \leqslant 0.05$; ratio ⩾2.0, $0.05 < P \leqslant 0.1$. Reductions are indicated by: ratio <0.5, $P \leqslant 0.05$; ratio <0.5, $0.05 < P \leqslant 0.10$; ratio=0.50–0.60, $P \leqslant 0.05$.

Quantitative RT-PCR

For real-time RT-PCR, total RNA was incubated with RNase-free DNase at 37 °C for 15 min, purified by phenol-chloroform, precipitated by ethanol and dissolved in DEPC-treated water. The concentration and integrity was controlled as described above.

For cDNA synthesis 2 μg total RNA, 10^6 copies pAW109 (GeneAmp PCR system, Applied Biosystems, Darmstadt, Germany), 0.5 μg oligo $(dT)_{15}$ were incubated in 12 μl at 70 °C for 10 min and chilled on ice. Then, an 8 μl cocktail was added, resulting in final concentrations of 1× first strand buffer, 500 μM dNTP Mix (Roche, Mannheim, Germany), 0.01 M DTT, and 25 U RNase inhibitor and 200 U Superscript II enzyme (Gibco, Karlsruhe, Germany). The mixture was incubated at 42 °C for 60 min followed by 15 min at 70 °C. The cDNA solution was stored at −20 °C or directly used for quantitative PCR. For each RNA sample a reaction without reverse transcription was performed to control contamination by genomic DNA. For real-time PCR analysis 4 μl of 1:50 diluted cDNA samples were used in 25 μl reactions containing 0.5 μM gene-specific primers (Supplementary Table 1 can be found at JXB online) and 12.5 μl Absolute QPCR SYBR Green ROX Mix (ABgene, Surrey, UK) in an Applied Biosystems PE-7700 (Applied Biosystems, Darmstadt, Germany). The program was as follows: 15 min at 95°C for DNA polymerase activation, 40 cycles of 20 s at 95 °C, 30 s at 55 °C, and 45 s at 72 °C. Data collection was performed at 72 °C for *ZmPIP1;1* and *ZmPIP1;2* or at 81 °C for *ZmPIP1;5*, *ZmPIP2;4*, and pAW109 according to the melting curves of the respective products. A control experiment without cDNA was included. The program for analysis of melting curves consisted of 10 s at 95 °C, 30 s at 60 °C, heating to 90 °C at a rate of 0.2 °C s^{-1}, and data were continuously collected. The data were analysed according to the $2^{-\Delta\Delta Ct}$ method (Pfaffl *et al*., 2002). Externally added pAW109 was used for normalization.

Determination of ABA content

Freeze-dried tissue samples were homogenized and extracted in 80% methanol. Extracts were passed through a Sep Pak C_{18}-cartridge. Methanol was removed under reduced pressure and the aqueous residue partitioned three times against ethyl acetate at pH 3.0. The ethyl acetate of the combined organic fractions was evaporated under reduced pressure. The residue was taken up in TBS-buffer (TRIS-buffered saline; 150 mM NaCl, 1 mM $MgCl_2$, 50 mM TRIS; pH 7.8) and subjected to an immunological ABA assay (ELISA) as described earlier (Peuke *et al*., 1994). The accuracy of the ELISA was verified for maize in earlier investigations (Hartung *et al*., 1994).

Results

Gene-specific DNA targets for maize MIP *gene expression analysis*

In order to analyse the expression profile of *MIP* genes in maize, a DNA array was established that harbours probes for 11 *ZmPIP*, 12 *ZmTIP*, three *ZmSIP*, and four *ZmNIP* genes (see Materials and methods). In particular, among subfamily members, there are high DNA sequence similarities. Within the coding regions of *ZmPIP*, *ZmTIP*, *ZmSIP*, and *ZmNIP* subfamilies, the sequence similarity varies from 59.2% to 99.3%, 56.6% to 99.6%, 47.7% to 81.4%, and 51.7% to

92.6%, respectively. Therefore, 110–350 bp fragments were PCR-amplified as targets for hybridization originating from the more divergent 3′-UTR sequences compared with maize cDNA sequences by BLAST analyses (see Materials and methods). Nineteen target sequences did not detect any other homologous maize genes. Ten additional target sequences exhibited considerable homologies to other *ZmMIP* members, however, encompassing only short stretches of up to 67 nt at 88% homology (Supplementary Table 1 can be found at JXB online). This level of homology does not hamper specific detection since cross-hybridizations was not detectable under the stringency conditions used (Supplementary Fig. 1 can be found at JXB online), which is in accordance with other reports (Xu *et al*., 2001). The target sequences of *ZmTIP3;1* and *ZmTIP3;2* showing homologies of 84% over 98 nt might weakly cross-hybridize. Finally, two pairs of highly homologous genes, *ZmTIP2;3*/*ZmTIP2;4* and *ZmPIP1;3*/*ZmPIP1;4*[†], were not discernible; therefore a single target detecting both was designed (Supplementary Table 1 can be found at JXB online; see Materials and methods).

Abundance of MIP *transcripts in unstressed maize roots*

To determine the expression of maize *MIP* members in unstressed roots using the maize *MIP* array, RNA was isolated from 11-d-old roots of hydroponically cultivated plants. Although signals derived from different hybridization targets cannot be compared in a strict, quantitative manner, it was evident that *MIP* genes were transcribed in roots at very different levels. By far, *ZmPIP1;5* and *ZmTIP1;1* showed the highest expression. In addition, *ZmPIP1;1*, *ZmPIP2;1*, *ZmPIP2;4*, *ZmPIP2;5*, *ZmPIP2;6*, *ZmTIP2;1*, *ZmTIP2;2*, and *ZmTIP2;4* were highly transcribed in roots (Fig. 1A). All other MIP members were expressed at low levels or not detected at all (Fig. 1A). Considering the sensitivity of the hybridization (Supplementary Fig. 1D can be found at JXB online) the latter genes are not significantly transcribed in unstressed maize roots or restricted to a specific, numerically minor cell type obviating their detection.

Furthermore, it was interesting to assess whether *ZmMIP*s were differentially expressed in different root cell layers. The zone 10–50 mm from tip of primary roots of 6-d-old seedlings was separated into inner and outer tissue at the endodermis. The outer layers contained epidermis, cortex, and the endodermis, the inner tissue consisted of the stele (Supplementary Fig. 2 can be found at JXB online). Interestingly, DNA array hybridization revealed that several highly expressed *ZmMIP* members had higher abundance in the outer cell layers than in the stele. There was no difference for *ZmPIP1;2*, *ZmPIP1;3*, and *ZmPIP2;*6 (Fig. 1B).

[†] *ZmPIP1;3* and *ZmPIP1;4* may indeed correspond to the same gene (Chaumont *et al*., 2001).

Effect of salt stress on water content of maize leaves and root osmotic potential

Application of NaCl to the root system of maize plants exerts a strong water stress onto the plants. The reactions of 11-d-old, hydroponically grown maize seedlings towards two different salt concentrations were compared by determining the water content of their leaves and assessing the osmotic potential of the root cells.

Application of 100 mM NaCl slightly, but not significantly decreased the water content of leaves in comparison to control plants (Fig. 2A). By contrast, 200 mM NaCl rapidly and significantly reduced the water content of leaves in comparison with both control and 100 mM NaCl treatment (P <0.05) (Fig. 2A). However, after 1 d, leaves appeared turgid in both cases indicating a recovery from stress and regaining water homeostasis. For the severe treatment with 200 mM NaCl, a constant, but lower water content was established.

After 2 h of salt stress, the osmotic potential of the root cells was strongly reduced (Fig. 2B). In the case of 100 mM NaCl, the osmotic potential was already slightly lower than the surrounding medium. After 24 h, a further reduction was observed. Although the reduction of the osmotic potential was even stronger after 2 h of 200 mM NaCl, it did not reach the level of the medium. Yet, after 24 h, the external and internal osmotic potential was also roughly equal in this case.

Effect of NaCl on gene expression of maize MIP *genes*

NaCl strongly influences plant water relations (Hasegawa *et al*., 2000; Zhu, 2001; Munns, 2002; Fig. 2). Therefore, the expression of maize *MIP* genes encoding aquaporins in roots of 11-d-old seedlings was assayed after the administration of 100 mM and 200 mM NaCl to the hydroponic solution. In addition to the differential impact on leaf water content and root osmotic potential the stress- and ABA-responsive maize gene *Rab17* was, at most, weakly induced by 100 mM NaCl at 24 h, but significantly up-regulated by 200 mM NaCl at 9, 24, and 48 h (Table 1).

Interestingly, the transcriptional responses of *ZmPIP* and *ZmTIP* genes were also different for both NaCl concentrations. Two hours after the addition of 100 mM, but not of 200 mM NaCl, a transient induction of the highly expressed PIP isoforms *ZmPIP1;1* and *ZmPIP2;4* was observed; in addition, the most highly expressed maize *PIP* gene in roots, *ZmPIP1;5*, showed a weak, yet significant induction (1.6-fold, P-value 0.004) (Table 1). Quantitative RT-PCR confirmed these results (Fig. 3A-D). Although these inductions were weak, it has to be stressed that the respective *ZmPIP* members were already expressed at high levels in unstressed maize roots. None of the *ZmTIP* genes was significantly altered 2 h after the application of NaCl.

The expression of the *MIP* genes to both NaCl concentrations was not significantly altered after 9 h. However, the situation changed completely 24 h after NaCl addition.

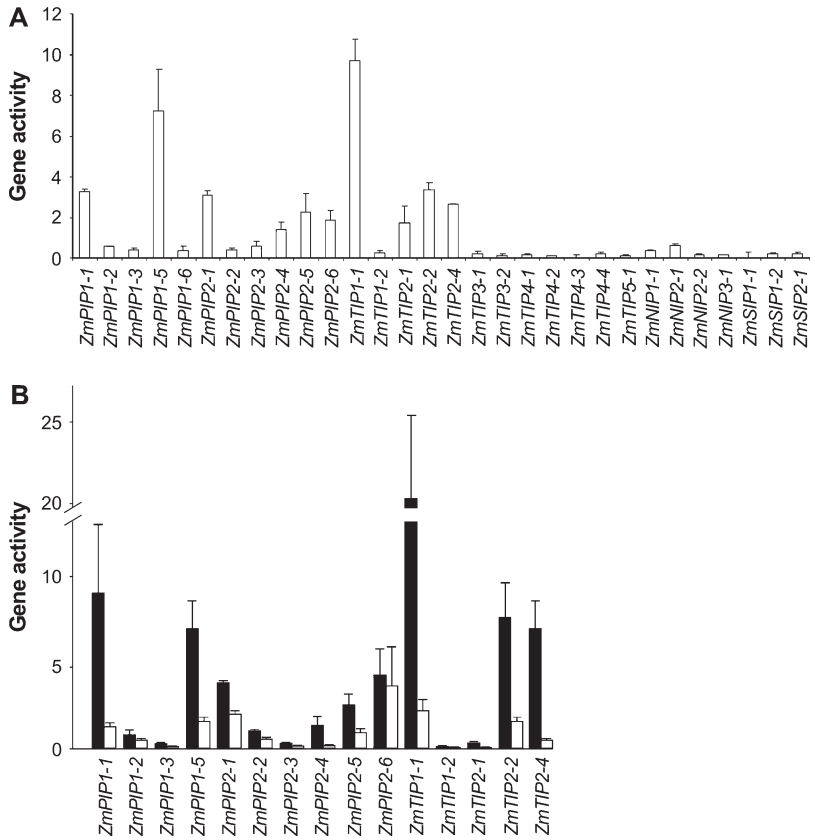


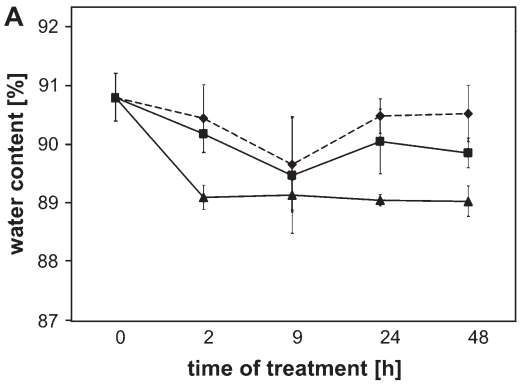
Fig. 1. Abundance of *ZmMIP* transcripts in maize roots. (A) Roots were harvested from 11-d-old seedlings cultured in hydroponic solution. Total RNA (10 μg) extracted from roots was assayed by maize MIP DNA array for gene activities. Relative gene activities (arbitrary units) above 0.20 were significantly detected 2-fold above background values. Original hydridization signals were normalized by internal standards (see Materials and methods). (B) Distribution of *ZmPIP* transcripts in the outer cell layers and stele. Total RNA was isolated from separated outer cell layers (black) and stele (white) tissues of 6-d-old primary roots (see Materials and methods) and gene activities of *ZmPIP*s were assayed by DNA array hybridization. The mean values and standard deviation of three independent experiments are shown.

With 100 mM NaCl added, no significant inductions or suppressions were detected, except a weak up-regulation of *ZmTIP2;4*. However, multiple *ZmPIP* and *ZmTIP* transcripts were reduced by 200 mM NaCl after 24 h (Table 1). The repressed genes included both less highly and more highly transcribed members of the *PIP1* and *PIP2* subfamilies, *ZmPIP1;2*, *ZmPIP1;5, ZmPIP1;6*, *ZmPIP2;4*, *ZmPIP2;5*, and *ZmPIP2;6*, as well as most *ZmTIP* genes. Interestingly, the highly expressed *ZmPIP1;1* and *ZmPIP2;1* were not affected. After 48 h, transcriptional deregulation of all *ZmPIP* and *ZmTIP* genes was basically abolished at both NaCl concentrations, indicating recovery and homeostasis in both cases. Nevertheless, the ABA-responsive marker *Rab17* continued to be enhanced in the 200 mM NaCl treatment. The differential responses of *ZmPIP1;1*, *ZmPIP1;2*, *ZmPIP1;5* and *ZmPIP2;4* to 200 mM NaCl after 24 h were confirmed by quantitative RT-PCR (Fig. 3A–D).

Data for *ZmSIP* and *ZmNIP* were collected as well (Supplementary Table 2 can be found at JXB online), but they are not discussed here because of their low expression, which renders expression ratios less certain.

Effect of salt stress on ABA concentration

ABA is known to accumulate in response to drought and salt stress. Since differential reactions to two NaCl concentrations were observed for leaf water content, root osmotic potential, and *ZmMIP* transcription in roots, ABA levels in roots were determined in parallel (see Material and methods). Indeed, salt stress significantly induced the amount of ABA in maize roots (Fig. 4). After 2 h, ABA accumulated to identical levels both in 100 mM and in 200 mM NaCl experiments. However, at later time points there was a clear difference between both treatments. ABA levels were strongly reduced to values 2–3-fold above controls in roots treated with 100 mM NaCl, whereas they remained at very high levels, up to 17-fold, throughout the whole period examined after the application of 200 mM NaCl; nevertheless, a clear decline was also observed after 48 h (Fig. 4).



B

Osmotic potential [MPa]

		Medium	Root
Control		-0.035	-0.47 +/- 0.007
100mM NaCl	2 h	-0.50	-0.55 +/- 0.007
	24 h		-0.67 +/- 0.007
200 mM NaCl	2 h	-0.92	-0.75 +/- 0.027
	24 h		-0.90 +/- 0.010

Fig. 2. Water content of maize leaves and root osmotic potential after the addition of NaCl to roots. (A) Water content was assayed after treatment of 11-d-old seedlings by 0 mM (filled diamonds), 100 mM (filled squares), and 200 mM (filled triangles) NaCl for the times indicated. The experiments were started at 10.00 h. Data are means ±SD for n=3. (B) Measurement of osmotic potentials. The osmotic potential of the hydroponic medium was compared with the osmotic potential of the root cell sap of 11-d-old maize plants after 2 h and 24 h. Data are means ±SD for n=4 (see Materials and methods).

Effect of ABA on the expression of MIP *genes*

Since NaCl increased the ABA concentration in roots, whether the transcriptional reaction of *ZmMIP* genes to NaCl was mediated by ABA would be interesting. Therefore, 11-d-old, hydroponically grown maize seedlings were subjected to 1 μM ABA for 1, 6, and 24 h. Previous and parallel analyses had shown that internal ABA concentrations rapidly increased by a factor of 3–8 after the external addition of 1 μM ABA, which is in the range observed here after NaCl treatment (Fig. 4). Furthermore, considering a possible delay of ABA biosynthesis after NaCl application compared with the direct effect of exogenously added ABA, the first two time points in the ABA experiments were chosen earlier than in the NaCl experiments. Transcription was analysed using the *ZmMIP* DNA array.

After 1 h treatment with 1 μM ABA only *ZmPIP1;2* and *ZmPIP2;4* were specifically induced (P <0.05) (Table 1). In addition, *ZmPIP1;1* showed a weak, but less significant enhancement of transcription (1.68 fold; P=0.052). Quantitative RT-PCR corroborated the induction of *ZmPIP1;2* and *ZmPIP2;4* (Fig. 3E–H). For *ZmPIP2;4* this reaction was reminiscent of the early response to NaCl (Table 1). After 6 h and 24 h treatment by 1 μM ABA, only a weak enhancement of *ZmPIP1;2* was persistent (Table 1), whereas no other *ZmMIP* transcripts significantly deviated from the control levels. Importantly, none of the *ZmTIP* genes showed a significant alteration of gene expression during the whole treatment.

Instead of 1 μM ABA, several studies reported by other laboratories used up to 100 μM ABA in order to provoke more drastic changes in gene expression, although such

Table 1. ZmPIP *and* ZmTIP *expression ratio (stressed versus unstressed control) in response to salt stress and ABA*

NaCl treatment refers to 100 mM and 200 mM NaCl added, ABA treatment refers to 1 μM and 100 μM. Inductions are highlighted in red (ratio ⩾2.0, P ⩽0.05), orange (ratio=1.67–2.0, P ⩽0.05), or light red (less significant, ratio ⩾2.0, 0.05 < P ⩽0.1), or yellow (see text). Reductions are indicated in aquamarine (ratio <0.5, P ⩽ 0.05), green (ratio ⩽0.5, 0.05 <P ⩽ 0.10), or light green (ratio=0.50–0.60, P ⩽0.05) (see Materials and methods). The mean expression ratios of three independent replicates are given (see Materials and methods). Non-responsive or non-detectable *ZmMIP* genes are not displayed; for a complete set of data including standard deviations and P-values refer to Supplementary Tables 2 and 3 that can be found at JXB online. Gene activities in unstressed roots are indicated for comparison, higher expression levels (>1.0 arbitrary units) are printed in bold; nd, not detected.

Gene	Gene activity	NaCl treatment								ABA treatment			
		2 h 100	9 h 100	24 h 100	48 h 100	2 h 200	9 h 200	24 h 200	48 h 200	1 h 1	6 h 1	24 h 1	24 h 100
ZmPIP1-1	**3.3**	**1.87**	0.74	1.32	1.49	1.31	1.02	0.91	**1.70**	1.68	1.59	1.63	0.45
ZmPIP1-2	0.57	1.18	nd	1.04	1.38	1.32	nd	0.58	1.47	**1.87**	**1.99**	**1.90**	0.20
ZmPIP1-3	0.41	1.51	nd	1.01	1.20	1.10	nd	0.72	1.14	1.41	1.44	1.63	0.23
ZmPIP1-5	**7.2**	**1.56**	1.13	1.26	1.71	0.87	0.92	0.50	1.14	1.62	1.35	1.10	0.12
ZmPIP1-6	0.37	1.25	nd	0.97	1.07	0.93	nd	0.31	0.83	1.59	nd	1.05	0.16
ZmPIP2-1	**3.1**	1.40	0.95	1.20	1.14	0.96	0.74	1.01	0.99	1.46	1.55	1.43	0.31
ZmPIP2-2	0.42	0.84	nd	1.89	1.19	0.86	nd	1.20	1.60	1.42	0.95	0.90	0.14
ZmPIP2-4	**1.4**	**1.97**	1.17	1.03	0.95	1.08	1.00	0.32	1.00	**2.35**	1.31	1.65	0.19
ZmPIP2-5	**2.3**	1.10	1.04	0.94	1.10	0.92	0.64	0.31	0.68	1.48	1.52	1.32	0.11
ZmPIP2-6	**1.9**	1.92	0.73	0.81	0.83	1.08	1.10	0.53	0.71	1.45	1.31	1.55	0.11
ZmTIP1-1	**9.7**	1.29	0.68	0.81	1.02	0.76	0.92	0.55	0.77	1.43	1.48	1.34	0.39
ZmTIP1-2	0.25	1.52	nd	0.94	nd	1.11	nd	0.52	1.15	nd	nd	1.29	0.20
ZmTIP2-1	**1.7**	1.10	0.88	1.00	0.93	0.92	0.69	0.35	0.67	1.59	1.02	1.04	0.21
ZmTIP2-2	**3.4**	0.91	0.85	1.62	1.07	0.80	0.33	0.12	0.56	1.24	0.81	0.76	0.06
ZmTIP2-4	**2.6**	1.36	1.01	**1.81**	**1.69**	0.96	1.07	0.59	1.14	1.19	1.07	0.90	0.15
RAB17	0.09	1.41	nd	**2.50**	nd	1.37	**26.15**	**39.41**	**42.07**	nd	2.28	**2.86**	**50.51**

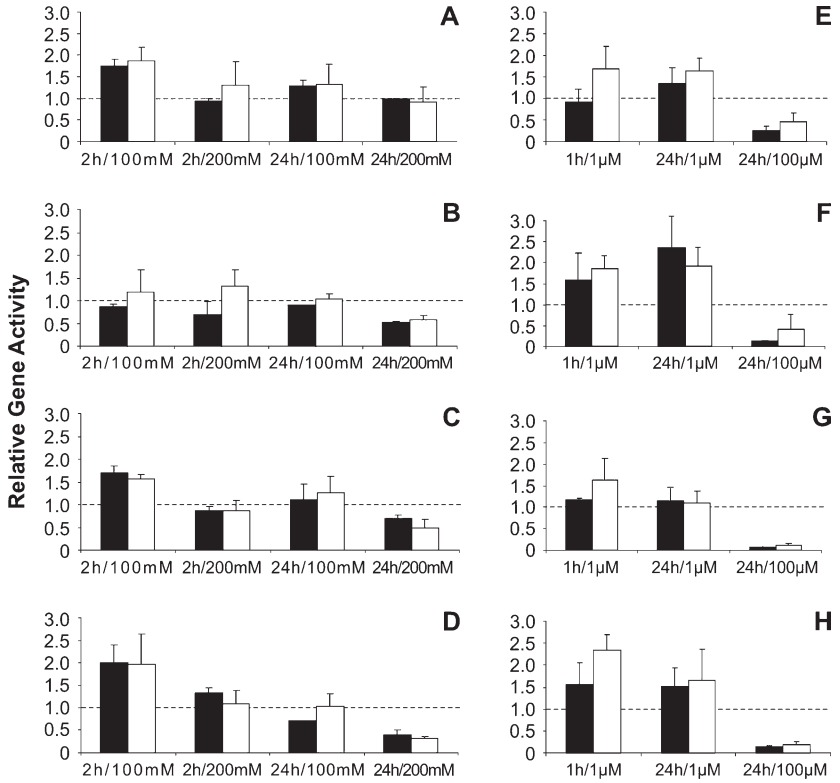


Fig. 3. Independent assessment of NaCl and ABA induced transcriptional changes by DNA array hybridization (white) and quantitative RT-PCR (black). *ZmPIP1;1* (A, E), *ZmPIP1;2* (B, F), *ZmPIP1;5* (C, G), and *ZmPIP2;4* (D, H) were analysed in RNA samples from roots of maize seedlings subjected to treatment with 0, 100, or 200 mM NaCl and to treatment with 0, 1, or 100 μM ABA for the indicated times. The transcript levels of each gene were plotted as the expression relative to the untreated control (0 mM NaCl or 0 μM ABA) plants.

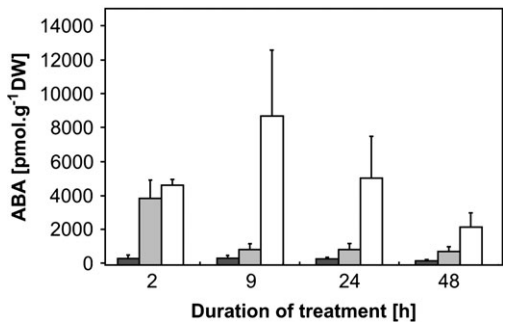


Fig. 4. ABA concentration in maize roots subjected to salt stress. ABA was determined by an immunoassay from lyophilized samples taken in parallel to the expression analyses and referred to dry weight (DW) (see Materials and methods). Controls (black), 100 mM NaCl (grey), 200 mM NaCl (white). Data are means ±SD of three independent experiments.

a high concentration may also lead to non-hormonal effects. Indeed, when 100 μM ABA was applied, a completely altered situation was apparent, indicated by the total repression of all *ZmPIP* and *ZmTIP* genes after 24 h (Table 1; Fig. 3E–H). At earlier time points, no consistent changes attributable to ABA could be detected (data not shown).

Semi-quantitative RT-PCR analyses of the known ABA-responsive genes *Rab17* and *Dbf1* served as controls, showing that the plants investigated responded to both ABA concentrations showing similar kinetics and ABA-sensitivity as described previously (Kizis and Pagès, 2002; Supplementary Fig. 3 can be found at JXB online).

Discussion

Expression of MIP *in unstressed maize roots*

A DNA array harbouring gene-specific target sequences has been used to assess the *ZmMIP* members that are expressed in maize roots. Six *PIP* and four *TIP* members were highly transcribed in roots indicating that these isoforms will be most important in roots. Nevertheless, other members of the *MIP* complement, including *NIP* and *SIP* isoforms that were detected only at low levels, could fulfil roles in specialized cell types or under certain conditions. This result was in accordance with the analyses of expressed sequence tags that revealed higher numbers for *ZmPIP1;1*, *ZmPIP1;5*, *ZmPIP2;1*, and *ZmPIP2;4* among the *PIP* members (Chaumont *et al.*, 2001).

Furthermore, it was interesting to find that several of the highly expressed *ZmMIP* members were not evenly expressed across the root, but prevailed in the outer cell layers. The highly expressed *ZmPIP2;6* was expressed at

the same level in both the outer layers and the stele. The higher expression of major plasma membrane and tonoplast aquaporins could indicate an important role in transcellular water transport across the epidermis, cortex, and endodermis, or alternative functions in these cells (see below). Nevertheless, these findings might be at variance with other studies that indicated high(er) expression in the vascular tissue. However, it has to be noted that the endodermis, which may harbour vascular-expressed *MIP* members, was included in the outer layers after tissue separation (Supplementary Fig. 2 can be found at JXB online). Furthermore, several studies on maize *PIP* or *TIP* genes comply with these results. In particular, Barrieu *et al.* (1998) showed that *ZmTIP1;1* is highly expressed in root epidermis and endodermis as well as in xylem parenchyma and the pericycle; Gaspar *et al.* (2003) found the highly transcribed *ZmPIP1;5* in all root cells with strong expression in the epidermis. In addition, *in situ* visualization may underestimate the expression in the larger cells of the cortex layer. Similar observations and considerations apply to *ZmPIP2;5* (Fetter *et al*., 2004).

Differential responses to salt stress

Differential responses of maize seedlings were observed after adding 100 mM or 200 mM NaCl to the medium. These salt concentrations increase the osmotic pressure from 0.014 to 0.200 and 0.373 Osmol kg^{-1}, respectively, leading to a reduction in the external water potential from −0.035 MPa to −0.50 and −0.92 MPa. In the control medium, the water potential of the root cells, which is composed of an osmotic pressure of 0.47 MPa and a turgor in the range of 0.5–0.7 MPa (Fig. 2; Rodriguez *et al.*, 1997), will be close to equilibrium with the external solution.

Thus, in the case of 100 mM NaCl added to the medium, a complete loss of turgor could already account for most of the difference in water potential (0.46 MPa) since the cellular osmotic potential is around −0.47 MPa (Fig. 2B). An immediate consequence of this osmotic effect is the sudden reduction in growth of the roots and leaves (Frensch and Hsiao, 1994; Rodriguez *et al*., 1997; Munns, 2002). In addition to the sole efflux of water, measures to lower the osmotic potential are initated in order to regain turgor and the capability of water uptake. The synthesis of compatible solutes as well as the uptake of Na^+ and Cl^- is involved in the osmoregulation, although both ions are toxic at high concentrations (Azaizeh *et al*., 1992; Frensch and Hsiao, 1994; Rodriguez *et al.*, 1997; Amtmann and Sanders, 1999; Uozumi *et al*., 2000; Munns, 2002; Essah *et al*., 2003; Wang *et al.*, 2003). Within 2 h after the addition of 100 mM NaCl, the root osmotic pressure was increased to 0.55 MPa (Fig. 2), similar to that described by Rodriguez *et al*. (1997).

Coincidently, three highly expressed *ZmPIP* isoforms (*ZmPIP1;1*, *ZmPIP1;5*, *ZmPIP2;4*) were specifically induced at this stage; all three genes showed higher expression in the outer cell layers of unstressed maize roots (Table 1; Fig. 1B). Therefore, the transient induction of these specific *PIP* isoforms indicates that they may be involved in cellular water uptake in the outer cell layers facilitated by the appropriate decrease in cellular osmotic potential. Commencing water homeostasis after 2 h treatment with 100 mM NaCl is also supported by the observation that the water content of the leaves was not different from that of control plants (Fig. 2). Later, a further reduction in the cellular osmotic potential (Fig. 2B; Frensch and Hsiao, 1994; Rodriguez *et al*., 1997) may supersede the requirement for enhanced aquaporin expression.

The observed transient induction of maize *PIP* members was reminiscent of the salt-sensitive rice line IR29 (Kawasaki *et al.*, 2001). Treatment with 150 mM NaCl transiently induced two rice *PIP* members after 3 h. Interestingly, the rice genes aligned to the transiently enhanced maize *ZmPIP1;1* and *ZmPIP2;4* (Supplementary Fig. 4 can be found at JXB online). Thus, they may reflect orthologous genes, having an isoform-specific and conserved function in maize and in the salt-sensitive rice cultivar. This correlation, as well as the specific and focused response of individual *PIP* members, emphasizes the importance of isoform-specific roles of individual *MIP* members as already proposed by Luu and Maurel (2005).

Maize plants responded in a different way after the severe shock with 200 mM NaCl. After 2 h the decrease of the root osmotic potential was not sufficient to allow the cellular uptake of water (intracellular −0.75 versus external −0.92 MPa; Fig. 2). Thus, in this scenario, even further shrinking of cells and plasmolysis is possible until the cellular osmoticum roughly equalled the external concentration after 24 h (Fig. 2; Munns, 2002). In accordance with this situation, under the severe 200 mM NaCl conditions a transient induction of aquaporins at 2 h was not observed.

In contrast to the treatment with 100 mM NaCl, 200 mM NaCl significantly reduced the water content of leaves at 2 h. This lowered water content persisted at a constant level, indicating that stressed plants manifested a new water balance. Furthermore, the reduced water status and continued stress was also reflected by the high level of root ABA, which lasted for at least 24 h, indicating a continued reduction of root cell volume during this period as suggested by Jia *et al.* (2001).

However, 24 h after the addition of 200 mM NaCl most *ZmPIP*s and *ZmTIP*s were repressed in a much less specific manner, which may be correlated with a prolonged retardation of growth and sustained reduction of water flux due to a diminished transpiration. There is previous physiological evidence that recovery of growth of maize roots, at least at a strongly reduced rate, is retarded with increasing osmotic stress. In addition, the root growth zone was reduced with high salt stress (Frensch and Hsiao, 1994, 1995; Neumann *et al*., 1994; Sharp *et al.*, 2004). In

Arabidopsis thaliana, a relatively severe stress of 100 mM NaCl strongly reduced hydraulic conductivity and *AtPIP1* transcription after 24 h (Martínez-Ballesta *et al*., 2003). In contrast to most other *PIP*s, the highly expressed *ZmPIP1;1* and *ZmPIP2;1* were not affected, which again argues for isoform-specific roles of the aquaporins.

Nevertheless, even in the case of 200 mM NaCl, both the stabilized water content of maize leaves and the significant reduction of ABA content in roots after 48 h implies that water homeostasis was established. Accordingly, *ZmMIP* transcription recovered back to control levels.

Wang *et al.* (2003) found that seven *ZmPIP*s and two *ZmTIP*s were repressed after 12 h treatment with 150 mM NaCl in maize roots. Since 150 mM NaCl also constitutes a more severe osmotic stress, with an osmotic potential exceeding the magnitude of the cellular turgor as discussed above, these results were in agreement with their analyses[‡]. Several analyses of salt-induced transcriptional changes have been performed in other plants, in particular with *Arabidopsis*. However, apparently contradictory results have been reported, which might be attributed to different developmental and growth conditions (Pih *et al.*, 1997; Kreps *et al*., 2002; Martinez-Ballesta *et al*., 2003; Jang *et al*., 2004). Nevertheless, there are also consistent results like the transient induction of *AtPIP1;4* (Pih *et al*., 1999; Kreps *et al*., 2002, Jang *et al*., 2004), which are reminisent of the induction of *ZmPIP1;2*.

Another interesting precedent also distinguished the early reaction towards lower versus higher NaCl concentrations. Elevated Ca^{2+} is an immediate early signal that may also trigger gene expression in response to NaCl (Kiegle *et al.*, 2000). Lynch *et al*. (1989) observed a dependence of cytosolic calcium on the concentration of NaCl, which resulted in a differentiation similar to this study's analyses. Up to 90 mM, NaCl did not significantly increase intracellular calcium, whereas between 90 and 120 mM NaCl a sharp rise was provoked that was sustained at 150 and 180 mM NaCl.

ABA regulation

The transient induction of *ZmPIP* members by 100 mM NaCl could be mediated by ABA. Indeed, exposure to 1 μM ABA transiently induced *ZmPIP2;4* and another member, *ZmPIP1;2*. Thus, the salt-induction of *ZmPIP2;4* was correlated to its induction by ABA, whereas an ABA-independent pathway seems to activate the salt enhancement of *ZmPIP1;1* and *ZmPIP1;5*. However, the same ABA level was determined after 2 h for the 200 mM NaCl experiment, and it was not associated with the transcriptional induction of any of the *ZmPIP* members. Therefore, either other factors overruled the ABA regulation of *ZmPIP2;4* in the 200 mM NaCl experiment or the ABA-dependence of *ZmPIP2;4* induction is more complex and can sense the ABA concentration and/or its changes. Indeed, ABA was further enhanced after 200 mM NaCl application in contrast to 100 mM NaCl.

Interestingly, a short-term treatment of maize roots with low concentrations of ABA induced cellular and root hydraulic conductance (Hose *et al*., 2000; Wan *et al*., 2004). Thus, the transcriptional level of two specific PIP isoforms, *ZmPIP2;4* and *ZmPIP1;2*, could be involved in this hormone-dependent up-regulation, besides potential, as yet unknown, regulation at the protein level.

Previous reports also indicated positive or negative effects of ABA on the transcription of aquaporin genes, which did not give a consistent view (Weig *et al.*, 1997; Hoth *et al.*, 2002; Seki *et al.*, 2002*b*; Jang *et al.*, 2004). However, in all these analyses high, unphysiological concentrations of ABA up to 100 μM had been applied. Although such concentrations may facilitate the discovery of truly regulated genes, unspecific pharmaceutical effects can not be excluded. Indeed, 100 μM ABA massively and apparently unspecifically repressed most *ZmMIP* genes in roots after 24 h (Table 1).

Conclusion

The comparison of the effects of two different NaCl concentrations on the expression of maize *MIP* genes revealed differential and gene-specific effects. This emphasizes isoform-specific functions in relation to tissue water permeation or growth as well as differential responses to varying extents of the same abiotic stressor. However, these findings may also mean that there are different roles for aquaporins than those related to the simple permeation of water or other uncharged molecules through cellular membranes. Hill *et al*. (2004) recently challenged the importance of a 'simple permeation hypothesis'. They suggested an additional function of aquaporins as osmotic or turgor sensors. Regarding the transiently induced *ZmPIP* isoforms, a putative role or participation as such sensors would mean that they were induced under conditions when the osmotic potential was lowered to a level allowing water uptake and turgor re-establishment. Conversely, a widespread suppression of these functions would be provoked after 24 h under severe salt stress, although only specific isoforms could be involved in sensing. However, in these cases, as well as in other reports, there is currently no experimental verification of such an additional role of aquaporins.

Supplementary data

There are four figures and three tables which constitute supplementary data to this manuscript. These can be found at JXB online.

[‡] However, Wang *et al.* (2003) used a selection of ten cDNA clones (>400 bp) that allowed cross-hybridization and thus could not obviate the detection of altered transcription from homologous members.

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