

## Expression and Inhibition of Aquaporins in Germinating *Arabidopsis* Seeds

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Extensive and kinetically well-defined water exchanges occur during germination of seeds. A putative role for aquaporins in this process was investigated in *Arabidopsis*. Macro-arrays carrying aquaporin gene-specific tags and antibodies raised against aquaporin subclasses revealed two distinct aquaporin expression programs between dry seeds and young seedlings. High expression levels of a restricted number of tonoplast intrinsic protein (TIP) isoforms (TIP3;1 and/or TIP3;2, and TIP5;1) together with a low expression of all 13 plasma membrane aquaporin (PIP) isoforms was observed in dry and germinating materials. In contrast, prevalent expression of aquaporins of the TIP1, TIP2 and PIP subgroups was induced during seedling establishment. Mercury (5  $\mu$ M HgCl<sub>2</sub>), a general blocker of aquaporins in various organisms, reduced the speed of seed germination and induced a true delay in maternal seed coat (testa) rupture and radicle emergence, by 8–9 and 25–30 h, respectively. Most importantly, mercury did not alter seed lot homogeneity nor the seed germination developmental sequence, and its effects were largely reversed by addition of 2 mM dithiothreitol, suggesting that these effects were primarily due to oxidation of cell components, possibly aquaporins, without irreversible alteration of cell integrity. Measurements of water uptake in control and mercury-treated seeds suggested that aquaporin functions are not involved in early seed imbibition (phase I) but would rather be associated with a delayed initiation of phase III, i.e. water uptake accompanying expansion and growth of the embryo. A possible role for aquaporins in germinating seeds and more generally in plant tissue growth is discussed.

**Keywords:** Aquaporin — *Arabidopsis thaliana* — Embryo growth — Germination — Mercury — Seed.

Abbreviations: ABIS, standard nutrient agar solution; Col, Columbia; DTT, Dithiothreitol; GST, gene-specific tag; MRI, magnetic resonance imaging; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PIP, plasma membrane intrinsic protein; TIP, tonoplast intrinsic protein;  $t_{RE50}$ , time of half germination (radicle emergence) response; WS, Wassilewskija.

### Introduction

Seeds are crucial organs for plant life dispersal and survival, and a precise control of the timing of seed germination provides a strong advantage for facing adverse environmental conditions (Bewley, 1997). Seed germination is triggered by tissue imbibition (uptake of water) and, after an apparent lag phase, is followed by the elongation of the radicle and thereafter of the whole embryonic axis (Bewley and Black 1994, Bewley 1997). In some plant species such as tobacco or *Arabidopsis*, rupture of the maternal seed coat (testa) can be taken as the first external sign of initiation of germination, whereas subsequent rupture of a zygotic tissue, the endosperm, and radicle protrusion indicate the completion of the process (Leubner-Metzger et al. 1995, Liu et al. 2005). In general, the ability of seeds to germinate seems to be critically determined by a change in the balance between growth potential of the embryo and the mechanical resistance of the surrounding tissues. In lettuce, tobacco or tomato seeds, the endosperm is the major constraining structure for germination (Bewley 1997, Koornneef et al. 2002). In contrast, mutant analysis revealed that, in *Arabidopsis*, the seed germination performance can be determined by the properties of the testa (Debeaujon et al. 2000, Koornneef et al. 2002).

These purely mechanical models can be implemented with concepts of plant water relations (Gummerson 1986, Bradford 1990). Water potential gradients which represent the motive force for water flow, and ultimately tissue expansion, were used to describe irreversible growth of the embryo as an interplay between cell turgor and cell wall extensibility (Welbaum et al. 1998). According to these models, germination would be primarily controlled by intracellular solute accumulation and cell wall loosening in the elongating radicle, together with weakening of surrounding tissues by cell separation, this being due, for instance, to the activity of cell wall hydrolases (Leubner-Metzger et al. 1995, Bewley 1997, Welbaum et al. 1998). As in most initial models of plant growth, a possible

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limitation by water uptake, i.e. by cell and tissue hydraulic conductivity, was discarded (Cosgrove 1993). Yet, there have been concerns recently about the significance of growth-induced water potential gradients (Fricke 2002, Tang and Boyer 2002).

Nevertheless, extensive and kinetically well-defined water exchanges are associated with seed imbibition and subsequent embryo growth (Bewley and Black 1994, Welbaum et al. 1998). These exchanges have classically been described using gravimetric methods (Bewley and Black, 1994). More recently, nuclear magnetic resonance (NMR) spectroscopy has provided insights into changes in the physical states of seed water during germination (Gruwel et al. 2001, Krishnan et al. 2004). In particular, magnetic resonance imaging (MRI) revealed a precise spatial distribution of water in germinating seeds and strikingly different patterns between species (Jenner et al. 1988, McEntyre et al. 1998, Manz et al. 2005, Terskikh et al. 2005). Thus, a tight control of water transport operates within seeds.

Aquaporins, which mediate a large part of the cell–cell and intracellular water movements in plants, represent good candidates for such a control (Maurel and Chrispeels 2001). In plants, aquaporins occur with a high diversity of isoforms (35 in *Arabidopsis thaliana*), the most abundant aquaporins in the vacuolar and plasma membranes belonging to the tonoplast intrinsic protein (TIP) and the plasma membrane intrinsic protein (PIP) classes, respectively. Although the role of aquaporins in germination has remained largely undetermined, expression analyses of a few aquaporin isoforms have provided hints at putative functions for these proteins in seeds. For instance, Fukuhara et al. (1999) observed that aquaporin expression in seeds of *Mesembryanthemum crystallinum* was associated with embryo growth and was retarded in dormant seeds. A class of tonoplast aquaporins ( $\alpha$ -TIP and  $\beta$ -TIP, or TIP3s) is specifically expressed in seeds (Johnson et al. 1990, Höfte et al. 1992) and, based on their expression and regulation properties, we have proposed a role for these aquaporins in cell osmoregulation and maturation of the vacuolar apparatus during late seed development and during the early stages of germination (Maurel et al. 1997). A role for aquaporin-controlled water transport across cell plasma membranes during seed imbibition and early growth of the embryo has recently received some support in a study by Gao et al. (1999). These authors observed a low abundance of a PIP2 aquaporin mRNA in *Brassica* dry seeds, which was markedly increased by priming. This pre-treatment also resulted in an enhancement of germination under normal or stress conditions, and, overall, the germination efficiency was well correlated with the initial expression level of this aquaporin.

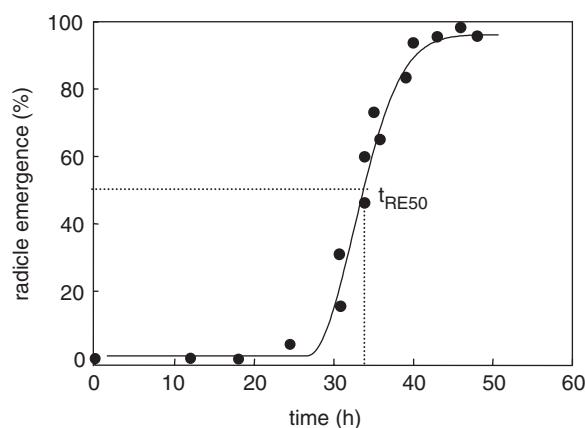
The aim of the present work was to characterize the expression of the whole aquaporin complement during seed germination and to test for the existence of a hydraulic control of this process. For this, we used *Arabidopsis* seeds as a model. Aquaporin transcript and protein abundance was investigated and the function of aquaporins was probed using the conventional aquaporin blocker, mercury. Our data suggest that, although they are not involved in early seed imbibition, aquaporins may modulate the speed of seed germination.

## Results

### *Expression of aquaporins during seed germination*

To investigate the role of aquaporins in the germination of *Arabidopsis* seeds, a good kinetic knowledge of the germination process itself and of the concomitant aquaporin expression properties was first required.

Seeds from accessions Wassilewskija (WS) and Columbia (Col) were kept on a standard nutrient agar solution (ABIS) for 48 h at 4°C to synchronize their subsequent germination behaviour and then transferred to 25°C. For WS seeds, germination, i.e. visual emergence of radicles, was observed after an ~20 h lag. Most seeds (>90%) had germinated 40–45 h after transfer (Fig. 1). The time at which 50% of viable seeds showed radicle emergence, defined as  $t_{RE50}$ , was  $35.6 \pm 4.9$  h ( $n = 7$  independent experiments with >400 seeds each). Col seeds exhibited a very similar germination behavior, although at a slightly faster rate ( $t_{RE50} = 21.9 \pm 3.4$  h;  $n = 4$ ) (see control curve in Fig. 3B). In this accession, testa rupture can be observed under a binocular microscope and typically preceded radicle emergence by  $4.7 \pm 0.3$  h ( $n = 2$ ) (Fig. 3B). Testa rupture was more difficult to score in WS seeds but occurred ~6 h ( $n = 2$ ) prior to radicle emergence.



**Fig. 1** Germination curve of WS *Arabidopsis thaliana* seeds. Seeds were stratified in the dark at 4°C for 48 h and then transferred to 25°C at time 0. The time at which 50% of the viable seed germinated (radicle emerged) is indicated as  $t_{RE50}$ .

In both WS and Col accessions, the steepness of the slope of the germination curves indicated that *in vitro* germination of *Arabidopsis* seeds can be studied as a highly synchronized process.

The properties of aquaporin expression during this process were first investigated by gene expression profiling, considering the whole set of 35 isoforms present in *Arabidopsis* (Johanson et al. 2001, Quigley et al. 2001). We previously developed macro-arrays carrying aquaporin gene-specific tags (GSTs) and established that this approach provides specific expression data for 29 individual aquaporin genes and three pairs of close homologs (Boursiac et al. 2005). cDNAs produced from RNAs extracted from dry WS seeds and 2-day-old WS seedlings were hybridized to the aquaporin macro-arrays (Fig. 2A). In dry seeds, *PIP* genes exhibited low expression levels, with only *PIP1;2* transcripts being over the detection threshold. In contrast, strong and specific expression of members of two TIP subclasses (*TIP3;1/TIP3;2* and *TIP5;1*) was detected. We note that, due to a high sequence identity, the *TIP3;1* and *TIP3;2* transcripts cannot be distinguished and hybridize to the same GST (Boursiac et al., 2005) (Fig. 2A). In 2-day-old WS seedlings, significant expression of several members of the PIP1 (*PIP1;1*; *PIP1;2*; *PIP1;4*) and PIP2 (*PIP2;1*; *PIP2;2*; *PIP2;6*; *PIP2;7*) subclasses was observed (Fig. 2A). Strong expression of *TIP1;1*, *TIP1;2* and *TIP2;1* was also observed, whereas all *TIP* transcripts prevalent in seeds were not detected. Altogether, the macro-array approach reveals two distinct aquaporin expression programs between dry seeds and young seedlings. It also allows the identification of the subclasses of PIPs (PIP1 and PIP2) and TIPs (TIP1, TIP2, and TIP3) that are characteristically expressed in either one of the two materials.

Kinetic aspects of the transition in aquaporin expression profiles during seed germination were studied by following the abundance of proteins, a more significant parameter than transcript abundance. For this, we used antibodies specific for some of the aquaporin subclasses identified above. In particular, two anti-peptide antibodies were used, which recognize several PIP1 isoforms (PIP1;1, PIP1;2, PIP1;3 and PIP1;4) and several PIP2 isoforms (PIP2;1, PIP2;2 and PIP2;3), respectively (Santoni et al. 2003). In dry WS seeds, both PIP1 and PIP2 isoforms were barely immunodetected, suggesting low expression levels relative to total protein (Fig. 2B; data not shown). Expression increased most notably after germination (Fig. 2B) and was clearly detectable at 60 h after transfer at 25°C, i.e. at 1 d after radicle emergence. A serum raised against bean seed-specific  $\alpha$ -TIP (TIP3) (Johnson et al. 1989) detected *Arabidopsis* homologs in dry seeds, the expression of which was significantly decreased shortly after germination (Fig. 2B). In contrast, TIPs detected by

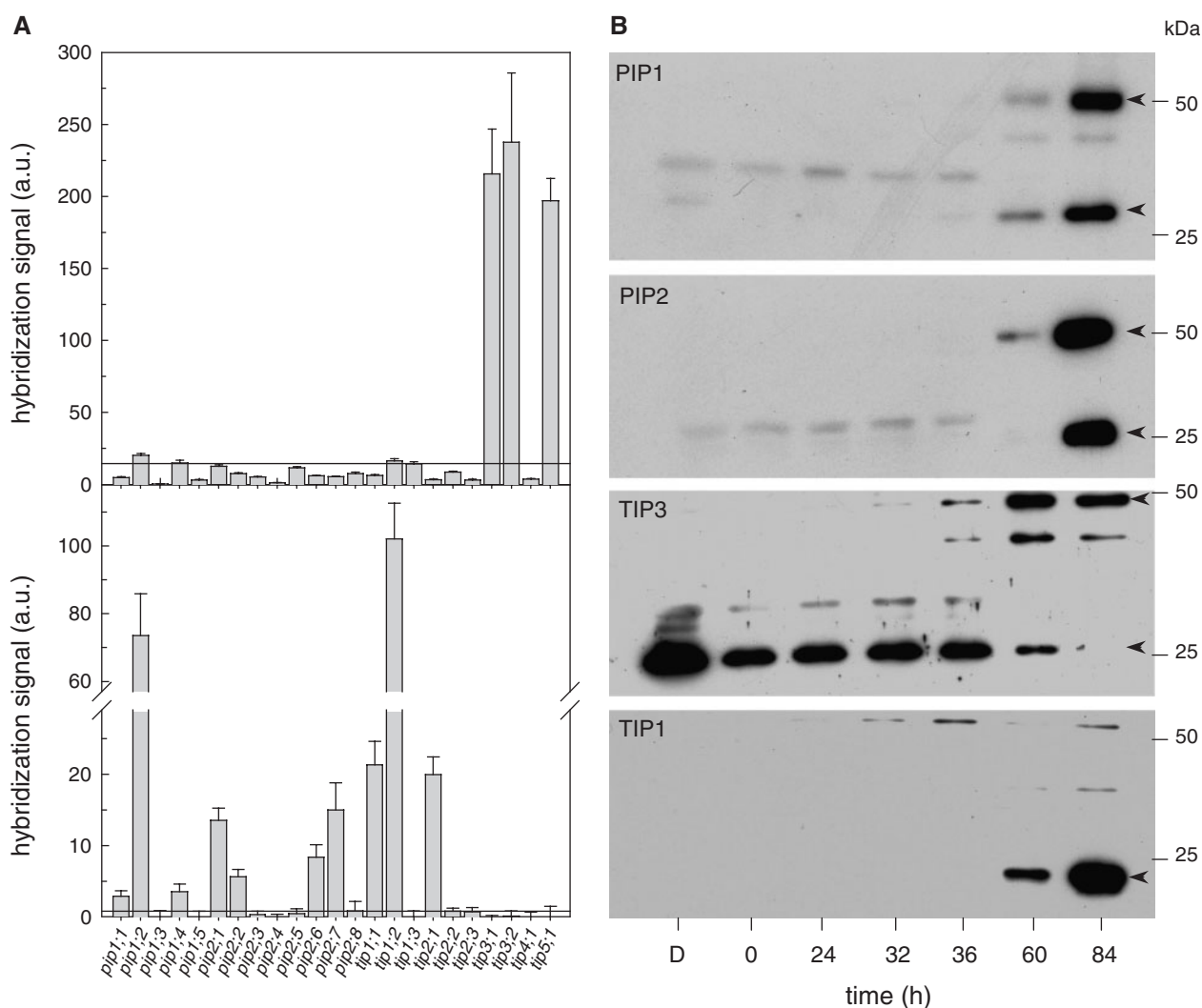
antibodies raised against a TIP1 homolog of radish (Maeshima 1992) showed increased expression after 60 h at 25°C, i.e. at 1 d after germination (Fig. 2B). Expression of this class of TIPs is closely related to cell expansion in *Arabidopsis* and radish (Ludevid et al. 1992, Maeshima 1992). Altogether, the data suggest that PIPs are not very abundant in dry and germinating seeds, whereas certain TIPs are highly expressed. Strong expression of specific PIP and TIP isoforms is induced shortly after germination and is associated with embryo growth.

#### *Reversible effects of mercury on seed germination*

Mercury, a general blocker of aquaporins in various organisms, was used as a tool to probe the function of these proteins in seeds. Growth of WS and Col seeds in ABIS complemented with 5  $\mu$ M HgCl<sub>2</sub> delayed radicle emergence by  $27.8 \pm 5.3$  h (results from  $n=3$  independent germination assays, each on three independent seed lots) and  $\sim 25$  h ( $n=2$ ), respectively. Germination curves of control and mercury-treated seeds showed fairly similar slopes at  $t_{RE50}$ , indicating that the treatment did not interfere with seed lot homogeneity (Fig. 3A, B). In addition, the radicles of control and mercury-treated WS seeds were histologically indistinguishable at the time of emergence ( $t_{RE50}$ ), when the effects of the blocker were scored (Fig. 4A, i and ii). However, at the late stages of the assay, i.e. at 60 h and beyond, mercury-treated seedlings showed anatomical abnormalities. Root emergence was delayed with respect to cotyledon emergence (Fig. 4B, ii) and in the longer term seedlings showed an absence of root hairs. These abnormalities may reflect noxious and unspecific effects of mercury due to the long-term (days) nature of the treatment. Nevertheless, the delay in germination induced by mercury in WS and Col seeds was reduced to  $4.6 \pm 5.4$  h and  $\sim 4$  h, respectively, when seeds were treated with both 5  $\mu$ M HgCl<sub>2</sub> and 2 mM dithiothreitol (DTT), again without any change in the slope of the germination curves (Fig. 3A, B). In addition, exposure of Col seeds to 5  $\mu$ M HgCl<sub>2</sub> delayed testa rupture by 8–9 h ( $n=2$  independent experiments) and this delay was reduced by >50% when seeds were treated with 5  $\mu$ M HgCl<sub>2</sub> and 2 mM DTT (Fig. 3B). These data suggest that mercury reduces the speed of seed germination (testa rupture and radicle emergence) by acting on the early phases of embryo growth. These early effects are primarily due to oxidation of cell components, possibly aquaporins, without irreversible alteration of cell integrity.

#### *Effects of mercury on seed water uptake*

Modulation of the speed of seed germination by mercury could be mediated by direct effects on the water uptake capacity of seeds, early during seed imbibition or later during embryo growth. The kinetics of water uptake in



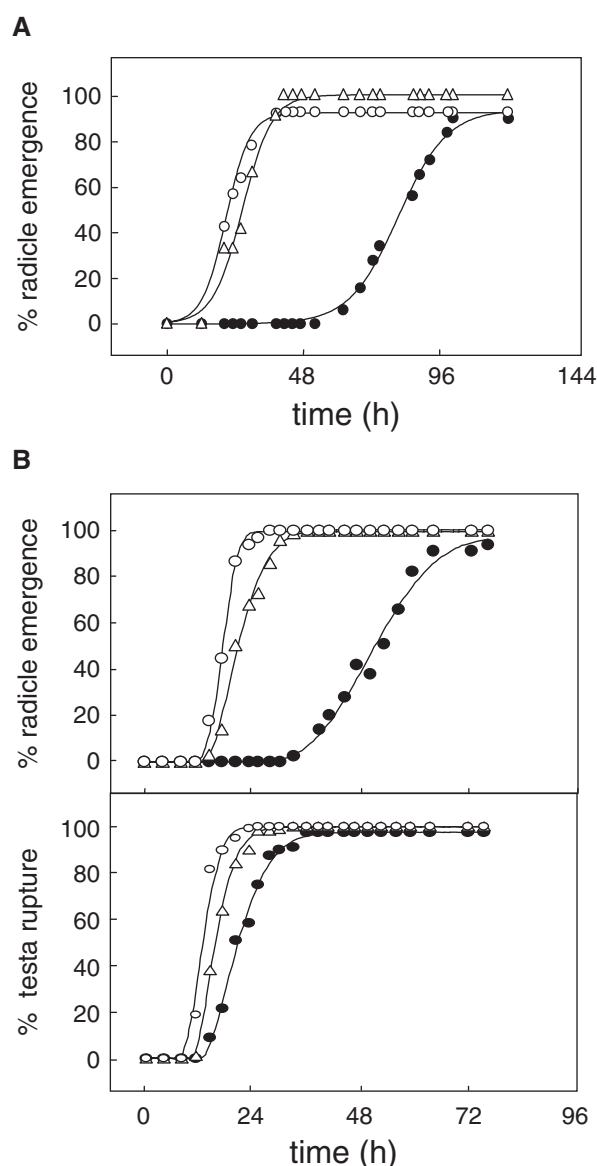
**Fig. 2** Aquaporin expression during germination of WS *Arabidopsis* seeds. (A) Macro-array expression profiling of *PIP* and *TIP* genes in dry seeds (upper panel) and 2-day-old germinating seedlings (lower panel). In these experiments, full (100%) germination was observed at 46 h after transfer of seeds to 25°C and seedlings were collected at 96 h. Hybridization signals (in arbitrary units;  $\pm$ SD) from two independent biological experiments, each from two independent membranes, were averaged. Signals from GSTs of the *PIP* and *TIP* subfamilies only are shown. The horizontal line indicates the mean level of unspecific hybridization signals +2 SD (see Materials and Methods) and corresponds to the threshold above which a signal is considered as significantly over background. Note that hybridization signals obtained from the two materials cannot be quantitatively compared but indicate clearly distinct aquaporin expression profiles. (B) Immunodetection of aquaporins reactive to antibodies raised against a PIP1;1 peptide (PIP1), a PIP2;2 peptide (PIP2), bean  $\alpha$ -TIP (TIP3) and a TIP1 homolog of radish (TIP1). Seeds were stratified at 4°C for 48 h and then transferred to 25°C. Total proteins were extracted from dry seeds (D) or at the indicated time after transfer to 25°C, and analyzed by immunoblot (5  $\mu$ g of total protein per lane). The signals at  $\sim$ 25 and  $\sim$ 50 kDa (arrowheads) represent specific signals for aquaporins migrating as monomers or dimers, respectively. The doublet at 40–50 kDa in the TIP3 blot was not clearly identified but may reflect an aggregation of TIP3, during protein isolation specifically in germinating seedlings. In contrast, the bands at  $\sim$ 30 kDa in the PIP1 and PIP2 blots are not specific. Mean water contents for dry seeds and for the four time points until 36 h (100% germination) were (in g H<sub>2</sub>O g DW<sup>-1</sup>): 0.0502, 1.693, 1.729, 2.306 and 3.014, respectively.

dry and germinating seeds were thus characterized as the time-dependent changes in seed fresh weight with respect to dry weight.

As shown previously in many plant species, WS *Arabidopsis* seeds germinating on ABIS exhibited typical triphasic kinetics of water uptake (Fig. 5). When seeds were transferred to ABIS at 4°C, in the absence or presence of

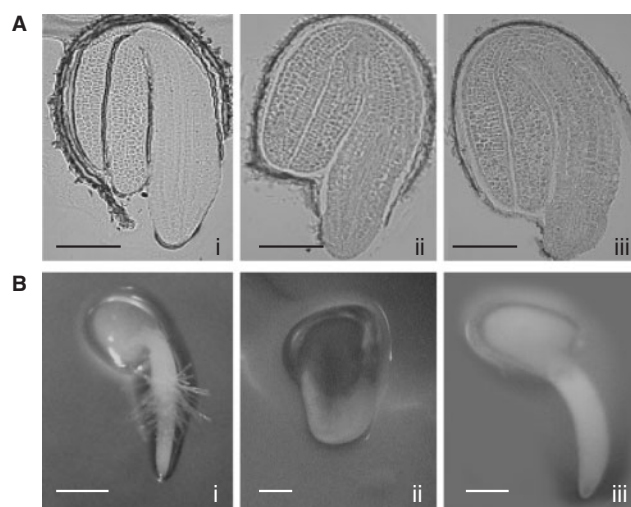
5  $\mu$ M HgCl<sub>2</sub>, initial rapid water absorption by dry seeds (phase I) was completed within 1 h, with similar half-times of  $22.6 \pm 2.5$  and  $20.9 \pm 3.2$  min, respectively (Fig. 5, inset). In the subsequent phase (phase II), which encompassed the remaining period at 4°C and the first hours following transfer to 25°C, seed water content remained constant at approximately 1.57–1.67 g H<sub>2</sub>O g DW<sup>-1</sup> and no significant





**Fig. 3** Effects of mercury on radicle emergence and testa rupture. Representative germination curves of WS (A) or Col (B) seeds, either untreated (open circles), or treated with 5  $\mu\text{M}$   $\text{HgCl}_2$  (filled circles) or 5  $\mu\text{M}$   $\text{HgCl}_2$  + 2 mM DTT (open triangles). The radicle emergence and testa rupture curves of (B) were obtained in the same germination experiment.

effect of a mercury treatment was observed (Fig. 5; Table 1). The third phase of water uptake is associated with germination (radicle emergence), and further expansion and growth of the embryo. An exponential function, fitted to the phase III of kinetic curves, was used to estimate seed water content at  $t_{\text{RE}50}$ . Values were similar in control and mercury-treated seeds ( $2.63 \pm 0.10$  and  $2.28 \pm 0.31$  g  $\text{H}_2\text{O}$  g  $\text{DW}^{-1}$ , respectively), and were in each case significantly higher by at least 0.6 g  $\text{H}_2\text{O}$  g  $\text{DW}^{-1}$  than the water content values of seeds during phase II (Table 1). However, phase



**Fig. 4** Morphology of WS seeds germinated in various growth conditions. (A) Seeds at radicle emergence were observed by light microscopy of 10  $\mu\text{m}$  thick longitudinal paraplasm sections. Seeds were germinated in a standard nutrient solution in the absence (i) or in the presence of 5  $\mu\text{M}$   $\text{HgCl}_2$  (ii), or 5  $\mu\text{M}$   $\text{HgCl}_2$  + 2 mM DTT (iii). Bars = 100  $\mu\text{m}$ . (B) Whole seedlings were observed at 1 d after radicle emergence with a binocular microscope. The growth conditions and conventions were the same as in (A). Note that root emergence was delayed with respect to cotyledon emergence in seedlings treated with 5  $\mu\text{M}$   $\text{HgCl}_2$  (ii). Bars = 300  $\mu\text{m}$ .

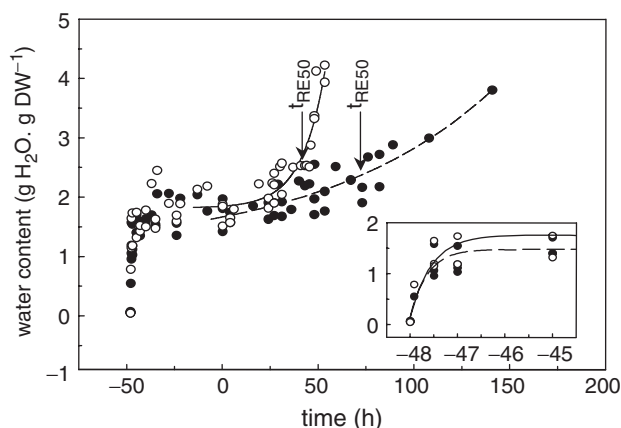
III water uptake in mercury-treated seeds was delayed with respect to water uptake in control untreated seeds (Fig. 5) and at the half germination response of control seeds, for instance, mercury-treated seeds exhibited a statistically lower water content (Table 1). Overall, the delay in phase III water uptake displayed by mercury-treated seeds paralleled their delayed germination response.

In conclusion, mercury treatment of seeds which delayed germination by supposedly blocking aquaporins delayed the onset of phase III. We note, however, that some basic aspects of seed water relations were not altered since, in all conditions studied, phase III similarly coincided with radicle emergence and the half germination response was associated with a typically conserved water content. The end of phase III corresponds to the seedling growth processes following radicle emergence. A decreased rate of water uptake during phase III was observed after mercury treatment. This may be related to the noxious effects of this treatment on seedling establishment (Fig. 4B).

## Discussion

### *Expression of aquaporins during seed germination*

The maturation and germination of seeds require dramatic changes in tissue water content, and therefore an



**Fig. 5** Effects of a mercury treatment on water uptake in WS seeds. WS seeds were germinated on a standard agar nutrient medium in the absence (open circles) or in the presence (filled circles) of  $5\ \mu\text{M HgCl}_2$ . Water uptake was measured gravimetrically on pooled seed lots ( $\sim 100$  seeds per point) during the initial 48 h stratification period at  $4^\circ\text{C}$  (negative times), and thereafter for up to 150 h after transfer at  $25^\circ\text{C}$  (time 0). Three independent seed lines were measured per treatment. The first 3 h of water uptake (phase I) are magnified in the inset. Water uptake during phases I (inset) and III was adjusted with exponential functions. The  $t_{\text{RE}50}$  for each treatment is arrowed.

important functional role for aquaporins can be expected in this organ. In the present work, we analyzed expression of the whole aquaporin complement in *Arabidopsis* seeds. Macro-arrays were first used to identify which aquaporin classes were the most abundantly expressed in seeds and seedlings, whereas Western blot analysis was used to follow in closer detail the kinetic changes of representative aquaporin isoforms.

Altogether, our analyses revealed high expression levels of a restricted number of TIP isoforms (TIP3;1 and/or TIP3;2, and TIP5;1) in dry seeds and a complete change in expression pattern during germination, with prevalent expression of aquaporins of the TIP1 and TIP2 subgroups. The latter two classes have typically high expression levels in leaves and roots (Alexandersson et al. 2005, Boursiac et al. 2005) and are associated with cell expansion and vascular tissue development, respectively (Ludevid et al. 1992, Daniels et al. 1996). TIP3 ( $\alpha$ -TIP) homologs have already been reported as specific for maturing and dry seeds in several species (Johnson et al. 1989, Höfte et al. 1992, Maeshima et al. 1994, Oliuissou and Hakman 1995). Here, we show that a high TIP3 protein abundance was maintained until completion of germination. Whereas AtTIP5;1 had previously been described as flower and even pollen specific (<http://bbc.botany.utoronto.ca/~bvinegar/cgi-bin/efp/efpWeb.cgi>; Alexandersson et al. 2005), we observed here a high transcript abundance in dry seeds. Altogether, the data suggest that TIP5;1

**Table 1** Time-dependent mean water content of WS seeds germinating in the absence (untreated) or in the presence of mercuric chloride ( $+5\ \mu\text{M HgCl}_2$ )<sup>a</sup>

Time after transfer to $25^\circ\text{C}$ <sup>b</sup>	Untreated	$+5\ \mu\text{M HgCl}_2$
0 h	$1.57 \pm 0.20^e$ <i>a, A</i> <sup>d</sup>	$1.67 \pm 0.17$ <i>a, A</i>
$t_{\text{RE}50}$ of untreated seeds	$2.63 \pm 0.10$ <i>b, A</i>	$1.95 \pm 0.22$ <i>ab, B</i>
$t_{\text{RE}50}$ of mercury-treated seeds	ND <sup>e</sup> <i>b</i>	$2.28 \pm 0.31$

<sup>a</sup>Full water uptake kinetics were determined for the indicated growth conditions (see Fig. 3 for reference experiment). Seed water content at the indicated  $t_{\text{RE}50}$  was calculated from an exponential fit of the phase III data (see Fig. 5).

<sup>b</sup>The table reports on mean water contents at the initial time of transfer at  $25^\circ\text{C}$ , at  $t_{\text{RE}50}$  of the earlier germinating condition (untreated seeds), and at  $t_{\text{RE}50}$  of the second germinating condition (mercury-treated seeds).

<sup>c</sup>Values are in  $\text{g H}_2\text{O g}^{-1}\text{ DW} \pm \text{SD}$  ( $n=3$  independent germination experiments).

<sup>d</sup>Letters indicate statistically different values at  $P < 0.05$ ,  $n=3$ . Italicized lower case letters denote differences within a column (same treatment). Roman upper case letters denote differences along a row (same time after transfer at  $25^\circ\text{C}$ ).

<sup>e</sup>ND, not determined.

expression might be associated with tissue desiccation. It will be interesting to investigate further the subcellular expression and function of this aquaporin in seeds, in particular with respect to those of TIP3s, these isoforms being specific for protein storage vacuoles.

In contrast to tonoplast aquaporins, a surprisingly low content of PIP aquaporins was revealed in dry and germinating seeds, and enhanced expression occurred only during the late phase of embryo growth. With the exception of a study in pea (Schuurmans et al. 2003), earlier work has already pointed to low or undetectable levels of transcripts for certain PIPs in dry seeds of several plant species (Daniels et al. 1994, Gao et al. 1999, Suga et al. 2001). It is still unclear how such low expression levels of PIPs can be sufficient to achieve basic transcellular water transport in seed materials.

#### *Mercury reduces the speed of seed germination*

Although a few mercury-resistant aquaporins have been described in plants (Daniels et al. 1994, Biela et al. 1999), mercury blocks most aquaporins by oxidizing cysteine residues in the vicinity of the pore (Daniels et al. 1996, Maurel and Chrispeels 2001). When applied at low concentration ( $5\ \mu\text{M HgCl}_2$ ), mercury induced a marked delay in germination (radicle emergence) by 25–30 h (Fig. 3). A lower but significant delay in testa

rupture was also observed, suggesting that embryo growth was somehow inhibited in its very early stages and that the effects of mercury were probably prolonged during the overall process leading to radicle emergence. We note that some stimuli, such as ABA and gibberellins, act on germination of tobacco and tomato seeds, respectively, but at later stages since they block radicle emergence without altering the timing of testa rupture (Leubner-Metzger et al. 1995, Manz et al. 2005, and reference therein).

Most importantly, the effects of mercury on *Arabidopsis* seed germination were largely reversed by the scavenging molecule, DTT. We are aware that mercury has potentially toxic side effects and that the use of this compound cannot by itself provide definitive evidence for aquaporin function. However, our study was focused on the earlier processes which determine radicle emergence, and up to this stage the morphology of control and mercury-treated seeds was indistinguishable (Fig. 4A). We also noted that in all control or treated conditions, the cumulated percentage of germinated seeds rose from about 10% to >90% within a few hours. Therefore, mercury induced over the short term a true delay in the germination response and did not alter its developmental sequence. In contrast, mercury treatment had, in the long term, clear detrimental and retarding effects on seedling growth and development (Fig. 4B). The latter observations are, however, not directly related to germination.

#### *Water relations of germinating seeds*

Triphasic kinetics of water uptake are a landmark feature of germinating plant seeds (Bewley and Black 1994, Bewley 1997). Here, these kinetics were characterized under conditions of mercury inhibition to explore which mechanisms may be altered.

We first found that phase I was unchanged by mercury treatment and thus that aquaporins are probably not involved in the early imbibition of *Arabidopsis* seeds. This may come as a surprise since this process involves the fastest and most drastic changes in tissue hydration observed during germination. In contrast, Veselova et al. (2003) have proposed, based on inhibition studies using the mercurial *p*-chloromercuriphenylsulfonic acid, that aquaporins might be important for primary imbibition of pea seeds. Pea seeds are much larger than those of *Arabidopsis*. They have a 10- to 20-fold longer phase I and may thus require a facilitated water path for tissue imbibition. Accordingly, strong expression of a PIP1 homolog has been reported in this species (Schuurmans et al. 2003).

Our study also established that certain water relation parameters are critically linked to germination. For instance, radicle emergence was closely linked to a

conserved threshold in seed water content, that we estimated through the seed water content at  $t_{RE50}$ . The finding that this threshold was similar between control and mercury-treated seeds suggested that there was nothing inherently different in terms of germination but rather that a triggering mechanism and/or the germination process itself had been retarded. Thus, the modulation of germination speed by mercury must be exerted during phase II or early phase III. In the long term (in late phase III), mercury treatment also reduced the rate of water uptake, but this phenomenon directly reflected its late, non-specific effects on seedling growth.

#### *Putative function of aquaporins in seeds*

While our data indicate that aquaporins are not involved in the early imbibition of seeds, they suggest a possible role for these proteins in embryo growth. Aquaporins may control the rate of water uptake following phase II and therefore would control the onset of phase III. Alternatively, aquaporins may contribute to the growth process itself by supplying water to the expanding tissues of the embryo.

We have proposed a role for TIPs in cell osmoregulation and maturation of the vacuolar apparatus during late seed development (thus explaining why some TIP isoforms are highly abundant in dry seeds) and during the early stages of germination (Maurel et al. 1997). These functions may support optimal water uptake and growth of the embryo. Aquaporins may also contribute to transcellular water transport in germinating seeds and therefore may facilitate water supply to the expanding tissues. This function may be dominated by PIPs because of the presumably limiting role of plasma membrane water transport (Maurel et al. 2002). In fact, there are numerous reports of growth-induced water potential gradients in the root and leaf elongation zone of plants, suggesting that hydraulic limitations may exist during plant tissue growth (Fricke 2002, Tang and Boyer 2002). A general role for aquaporins in expansion growth has been suggested, based mostly on correlative evidence, i.e. strong aquaporin expression in expanding tissues (Ludevid et al. 1992, Balk and de Boer 1999). It was also shown that diurnal movements of tobacco leaves, a process generated by spatially differential growth rates in the petiole, could be attenuated by antisense inhibition of the plasma membrane NtAQP1 aquaporin (Siefritz et al. 2004).

Much remains to be learned about cell-specific functions of aquaporins in germinating seeds. Recent MRI analysis of germinating tobacco seeds revealed that, in species with endospermic seeds such as tobacco and *Arabidopsis*, the expanding micropylae is a preferential site for water mobilization (Manz et al. 2005). Therefore, when considered spatially, imbibition of the seed and initiation

of embryo growth are very heterogeneous. This observation further supports the idea that aquaporins may provide a hydraulic control of germination. In the future, it will be critical to see how the spatial pattern of aquaporin expression (i.e. hydraulic conductivity of cells) can fit the hydration pattern (concentrations of mobile water) revealed by MRI, therefore leading to a comprehensive understanding of water transport in seeds.

In conclusion, our data suggest a putative role for aquaporins in controlling the speed of *Arabidopsis* seed germination, by possibly acting on the early phases of embryo growth. In contrast, a role in seed imbibition was ruled out. These assumptions are based on the effects of mercury which induced a true, reversible delay in germination without altering its developmental sequence nor some basic aspects of seed water relations. There are many environmental cues, including light, temperature and anoxia, that are known to interfere with seed germination (Al-Ani et al. 1985, Bewley and Black 1994, Welbaum et al. 1998). The effects of these cues on the function of seed aquaporins and therefore a possible hydraulic control of seed germination by these factors will have to be investigated.

## Materials and Methods

### Germination assays

Seeds from *A. thaliana*, ecotype WS or Col, were surface sterilized in a solution of 86% ethanol, 3.4 g l<sup>-1</sup> Bayrochlor, 0.0002% Clean N (Colgate-Palmolive) for 6 min, thoroughly washed with sterile water and plated in clear polystyrene plates (12 × 12 cm) onto a nutrient medium [ABIS: 5 mM KNO<sub>3</sub>, 2 mM MgSO<sub>4</sub>, 1 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 50 μM Fe-EDTA, microelements as described by Murashige and Skoog (1962), 10 g l<sup>-1</sup> sucrose, 7 g l<sup>-1</sup> agar, 1 mM MES and 2.5 mM K<sub>2</sub>HPO<sub>4</sub> + KH<sub>2</sub>PO<sub>4</sub>, pH 6.1], either standard or modified as indicated. The plates containing four seed lines in rows with >80 seeds per line were sealed with an air-permeable tape, left at 4°C in the dark for 48 h, and then transferred at dawn to a culture room at 25 ± 2°C with a 16 h light (100–120 μmol m<sup>-2</sup> s<sup>-1</sup>)/8 h dark cycle. Seed germination was scored with the help of a binocular microscope, as the apparent emergence of a radicle from the seed coat, at regular intervals until no further seeds on the same plate had germinated over a 24 h period. Testa rupture was similarly scored under a binocular microscope as a longitudinal crack of the seed coat. Germination curves representing the time-dependent cumulated percentage of radicles emerged from >160 seeds were adjusted with a Weibull sigmoid function (Brown and Mayer 1988) using Sigma Plot (SPSS Science). The fitted curves were used to estimate t<sub>RE50</sub> as the time at which radicle emergence had occurred in one half of the viable seeds, i.e. in those seeds which finally did germinate. Seed lines with a final rate of germination <90% were discarded.

Radicle emergence and testa rupture assays were performed in triplicate in control ABIS or in ABIS complemented with mercuric chloride (5 μM HgCl<sub>2</sub>), a reducing agent (2 mM DTT) or a combination of the latter two treatments. These compounds were added under sterile conditions to a previously autoclaved medium. Preliminary dose–response experiments allowed the determination

of 5 μM HgCl<sub>2</sub> as the minimal mercury concentration which induced a significant delay in germination with reduced long-term secondary effects on seedling morphology.

### Macro-array analysis of aquaporin expression

The development of an aquaporin macro-array has been described in detail elsewhere (Boursiac et al. 2005). This macro-array carries GSTs specific for 29 individual aquaporin genes and three pairs of close homologs (*TIP3;1/TIP3;2*, *NIP1;1/NIP1;2* and *NIP4;1/NIP4;2*). Total RNAs were extracted from seeds or seedlings using a phenol–chloroform extraction (Downing et al. 1992) followed by a clean-up using an RNeasy Mini kit (Qiagen, Hilden, Germany). All procedures for cDNA labeling and hybridization, and data analysis were as described (Boursiac et al. 2005). In particular, the unspecific hybridization signal was calculated as the mean signal from a set of negative control genes (Boursiac et al. 2005) and used to determine the threshold above which a gene can be considered as significantly expressed over background.

### Western blot analysis

Proteins were extracted essentially as described by Damerval et al. (1986). Briefly, seeds were ground in liquid nitrogen and homogenized in cold acetone with 10% trichloroacetic acid and 0.07% β-mercaptoethanol. Samples were incubated for 30 min at –20°C to allow proteins to precipitate, and centrifuged at 4°C for 10 min at 28,000 × g. The pellet was resuspended in cold acetone containing 0.07% (v/v) β-mercaptoethanol and recentrifuged. The vacuum-dried pellet was solubilized in a lysis buffer [10% (w/v) glycerol, 32 mM DTT, 2% (w/v) SDS, 60 mM Tris–HCl, pH 6.8] for 2 h with shaking at room temperature. The samples were then centrifuged at 10,000 × g for 10 min and the supernatant (protein fraction) was transferred to a clean tube and quantified using the Bradford method. Proteins (5 μg) were separated by electrophoresis on 12% SDS–polyacrylamide gels and transferred onto Immobilon<sup>TM</sup> membranes (Millipore) for 1.5 h at 75 V in the presence of 10 mM Tris, 100 mM glycine, 10% methanol. The membrane was blocked for 1 h at room temperature with shaking in a modified phosphate-buffered saline (PBS) solution (PBSTB) containing 1% Tween-20 and 1% bovine serum albumin (BSA), and further incubated overnight at 4°C with shaking, in the presence of the primary antibody. Antibodies raised against a PIP1;1 or a PIP2;2 peptide (Santoni et al. 2003) were used at a 1:5,000 dilution in PBSTB, whereas antibodies raised against bean α-TIP (TIP3) (Johnson et al. 1989) or a radish γ-TIP (TIP1) homolog (Maeshima 1992) were used at a 1:3,000 dilution. The membranes were rinsed with PBSTB and then incubated with an anti-rabbit horseradish peroxidase secondary antibody (1:20,000 dilution) in PBSTB for 1 h at room temperature. After rinsing in PBS, the blots were visualized using chemiluminescence (SuperSignal, Pierce).

### Anatomy

Seeds were fixed in 4% (w/v) paraformaldehyde in 0.1 × PBS (pH 7.5) overnight at 4°C. The seeds were then dehydrated through an alcohol series (50, 70, 95 and 100% ethanol each, 100% isopropanol and 100% butanol) and embedded in paraplast paraffin wax at 58°C for 48 h with gentle agitation. Wax-embedded transverse sections were cut (10 μm thickness) on a Leitz Wetzlar rotary microtome (Vienna, Austria), and attached to 3-aminopropyltriethoxysilane-coated glass microscope slides by heating to 58°C for 1 h. After dewaxing with xylene and rehydrating through a decreasing ethanol series, sections were



viewed and photographed with an Olympus BX61 light microscope equipped with an Olympus Color View II digital camera.

#### Water uptake experiments

Dry seeds were surface sterilized as described above except that seeds were rinsed in ethanol instead of water. Seeds were then air-dried, and placed on ABIS plates at 4°C. At regular time intervals, ~100 seeds were collected, dabbed dry on a filter paper and immediately weighed on a Sartorius M500P microbalance (Göttingen, Germany). Radicle emergence was scored in parallel on independent plates. To calculate their water content on a dry weight basis, all seed samples were oven-dried at 96°C for 24 h, and weighed. These experiments were performed in triplicate. The initial and second time-dependent increases in water content (phases I and III) were analyzed individually by fitting the experimental data with exponential functions using SigmaPlot.

#### Acknowledgments

C.V.W. was the recipient of a post-doctoral fellowship from the French Ministry of Research. We are grateful to Masayoshi Maeshima and Maarten Chrispeels for the gift of anti-TIP antibodies, Patrick Doumas for help in macro-array preparation, and François Parcy for advice on seed RNA extraction. This work was supported in part by a grant from the French Ministry of Research (ACI2003 'Biologie du Développement et Physiologie Intégrative').

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(Received June 14, 2006; Accepted July 21, 2006)