Expression of genes encoding PIP aquaporins in tomato seeds exposed to blue radiation and mercury

J. BALARYNOVÁ and M. FELLNER*

Laboratory of Growth Regulators, Centre of the Region Haná for Biotechnological and Agricultural Research, Palacký University and Institute of Experimental Botany, Academy of Sciences of the Czech Republic, CZ-78371 Olomouc, Czech Republic

Abstract

Aquaporins control the specific transport of water and some other small molecules across membranes and are involved in various physiological processes. Plasma membrane aquaporins (PIPs) were shown to play an important role during tomato seed development. Therefore, we were interested in the participation of PIPs in seed germination and early seedling growth of wild type (WT) and the 7B-1 mutant, affected by blue light responses. We characterised the expression patterns of PIP-type aquaporin genes in these lines during different phases of seed germination and seedling growth after HgCl₂ (an aquaporin blocker) treatment. Further, we investigated whether blue radiation (BL) was involved in the regulation of these processes. Our experiments showed that 7B-1 mutant seed germination and root elongation are less responsive to HgCl₂ compared to WT. In both WT and 7B-1 mutant seeds, BL modulates the expression of PIP1;1 (upregulation) and PIP1;3 (downregulation) aquaporin isoforms. The PIP1;3 gene is downregulated not only by BL but also by HgCl₂ with a stronger effect in WT seeds. Thus, we show that BL can alter PIPs gene expression during tomato seed germination and seedling growth and that the 7B-1 mutation reduced the responsiveness to mercury blockage of aquaporins. Altogether, our data indicate that PIP aquaporins participate in tomato seed germination and radicle elongation and that the 7B-1 mutation and BL have an impact on these processes.

Additional key words: 7B-1 mutant, seed germination, Solanum lycopersicum.

Introduction

Seed germination commences with the uptake of water by dry seed and terminates with the elongation of the embryonic axis (Bewley and Black 1994), while the visible sign of completed germination is the penetration of the structures surrounding the embryo by the radicle (Bewley 1997). Seed germination is driven by the uptake of water which occurs in three phases: germination is triggered by high water uptake during imbibition (phase I) followed by a period of limited water uptake (lag phase, phase II). Finally, water uptake increases in phase III during the elongation of the radicle and the whole embryonic axis (Bewley and Black 1994). Aquaporins control water movements and maintain water homeostasis in plant cells. They represent an alternative pathway to simple diffusion of water across membranes. Via both routes, the water molecules move passively in response to water potential gradients, but the water passage through aquaporins is faster than the simple diffusion (Steudle 1994). It is expected that aquaporins are not involved in the early process of imbibition, but they are possibly important during embryo growth (Willigen et al. 2006).

Tomato aquaporins are classified into five main groups (Chaumont et al. 2001, Danielson and Johanson 2008). From 47 aquaporin genes in the tomato genome,
Materials and methods

Plants and germination: Tomato (Solanum lycopersicum L.) cv. Rutgers (wild-type, WT) and the 7B-1 mutant impaired in various responses to BL were used in this study. The seeds were surface sterilized in 2.8 % (m/v) sodium hypochlorite solution (Bochemia, Bohumin, Czech Republic) for 20 min and then rinsed with sterile distilled water. Afterwards, seeds were sown on basal Murashige and Skoog (1962; MS) medium supplemented with 0.7 % (v/v) agar in round Petri dishes (90 mm in diameter). Based on the experiment, the MS medium was supplemented with 0, 20, or 30 µM HgCl₂, or 2 mM DTT, or with a mixture of 30 µM HgCl₂ and 2 mM DTT. The dishes were placed vertically in growth chambers (Microclima 1000, Snijders Scientific, Tilburg, The Netherlands) and seeds were incubated at a temperature of 23 °C and air humidity of 75 %, under continuous BL or dark. For dark conditions, plates were wrapped in aluminium foil. BL was provided by fluorescent tubes (TLD-36W/18-Blue, Philips, Einhoven, The Netherlands) giving maximum irradiance of 10 µmol m⁻² s⁻¹ at 440 nm). The light spectrum was measured using a portable spectroradiometer (model LI-1800; Li-COR, Lincoln, NE, USA). Seed germination was recorded daily from the second to eighth day after sowing. The germination percentage [(germinated seeds/total number of seeds) × 100] was counted every day and the average was calculated. For each treatment at least six independent experiments per each time point were performed and thirty seeds per dish were sown for each treatment.

Tomato seeds were sown on the Petri dishes with MS medium supplemented with 0 or 30 µM HgCl₂ and cultured in darkness or BL as described above. After specified times (4, 8, 12, 24, 48, 72 and 96 h after sowing), non-germinated seeds were collected and excess water was removed by a filter paper. Subsequently, seeds were weighed, dried at 70 °C for 48 h and then weighed again. The water content was calculated as [(FM - DM)/FM] × 100, where FM is the fresh mass of imbibed seeds and DM is seed dry mass. The experiment was repeated twice.

Tomato seeds were sown on MS medium in square Petri dishes (120 × 120 mm) and incubated in the growth chambers in darkness as described above. After 3 d, germinated seeds were transferred onto dishes containing MS medium supplemented with HgCl₂ (0 - 30 µM) and demonstrated that continuous BL can alter aquaporin gene expression in Arabidopsis cell cultures. However, information linking these observations to provide a mechanism for BL-induced inhibition of seed germination via aquaporins is still missing.

A spontaneous recessive single gene mutant 7B-1 was selected for its male sterility (Sawhney 1997). Seed germination tests show that 7B-1 is less responsive than the corresponding wild type (WT) to the inhibitory effects of osmotic stress specifically under BL (Fellner and Sawhney 2002). This indicates that BL can modulate the inhibitory effect of abiotic stresses on tomato seed germination. Other results suggest that the 7B-1 mutation impairs BL signalling pathways, possibly the phototropin pathway (Bergougnoux et al. 2012, Hlavinka et al. 2013), and it probably affects the downstream components of the light signalling pathways (Omidvar and Fellner 2015). Recently, a genetic characterization of the 7B-1 mutant was published and SIGLO2 gene was proposed as a candidate gene underlying the 7B-1 mutation (Pucci et al. 2017). However, the precise characterisation of the 7B-1 mutation is still in progress.

The aims of our work were: 1) to investigate the responsiveness of tomato seed germination and early radicle and root elongation to mercury treatment (an aquaporin blockage); 2) to characterise the expression of PIP genes during seed germination and early radicle elongation; 3) to analyse the putative BL control over these processes.
incubated in growth chambers in the dark or under continuous BL for 7 d. Then, the seedling root length was measured with a ruler. For each HgCl<sub>2</sub> concentration, at least three independent experiments with at least seven seedlings were performed.

**RNA isolation, cDNA synthesis, and PCR:** For analysis of PIP expression in different developmental stages, the first set of seeds was harvested 24 h after sowing (non-visible radicle). The second set comprised germinated seeds collected after 72 h of cultivation (the radicle was 1 - 3 mm long). Finally, seeds with radicle up to 10 mm were harvested 96 h after sowing. To study the effect of HgCl<sub>2</sub> on PIP expression, WT and 7B-1 seeds with radicle up to 10 mm were harvested after 96-h cultivation in the dark or under BL. Seeds germinated under BL were harvested at BL, while seeds germinated in the dark were harvested under a green safelight. Dry WT and 7B-1 seeds were also extracted. All seeds were ground by a mortar and pestle to a fine powder under liquid nitrogen. Total RNA from the seeds was isolated using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Residual DNA was removed from samples by treatment with recombinant DNasel (Takara, Tokyo, Japan) and recombinant RNase inhibitor (Takara) at 37 °C for 60 min. DNasel was inactivated by phenol/chloroform extraction according to the manufacturer’s instructions. Following this, first-strand cDNA mixtures were prepared from 0.7 µg of total RNA using PrimeScript<sup>™</sup> 1<sup>st</sup> strand cDNA synthesis kit (Takara).

To screen changes of PIP gene expression induced by 30 µM HgCl<sub>2</sub> and the differences in the amount of PIP transcripts in WT and 7B-1 mutant seeds, a reverse transcription (RT) semiquantitative (sq)PCR was used. Gene expression was analysed with primers specific for nine tomato PIP genes (Shiota <it>et al.</it> 2006). The reaction mixture [0.1 mm<sup>3</sup> of GoTaq® DNA polymerase (Promega, Madison, USA), 4 mm<sup>3</sup> of 5× colorless GoTaq® reaction buffer (Promega), 1 mm<sup>3</sup> of each primer (10 µM), 2 mm<sup>3</sup> of dNTP mixture (1 mM), 9.8 mm<sup>3</sup> of sterile RNase-free water and 1 mm<sup>3</sup> of cDNA template] volume was 20 mm<sup>3</sup>. DNA was denatured at 94°C for 3 min, followed by 25 or 30 cycles of amplification (94 °C for 30 s, 53 - 60 °C for 30 s and 72 °C for 1 min) and final extension at 72 °C for 5 min. LeEF1 (Elongation Factor 1α) (Shiota <it>et al.</it> 2006) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; primer kindly provided by V. Bergougnoux: F-AACCGGTGTCCTCAGTACAGGA, R-CACCCA CAAACAACTATGGGACAT) were used as references. The PCR products were separated on 1 or 1.5 % (m/v) agarose gel with ethidium bromide staining. The bands were evaluated using ImageJ program (Collins 2007) and gene expression relative to both reference genes and to control sample. The experiments with HgCl<sub>2</sub> were repeated four times, experiments with dry seeds three times.

To assess the expression of PIPs during different stages of seed germination and early post-germination and to study the effect of 30 µM HgCl<sub>2</sub> on PIP1;1 and PIP1;3 gene expression, RT-qPCR was used. The gene expression was analysed using SYBR Premix Ex Taq™ (perfect real-time kit; Takara) using a Mx3000P™ thermal cycler (Agilent Technologies, Palo Alto, USA). The PCR reaction mixture was prepared according to the manufacturer’s protocol, containing 12.5 mm<sup>3</sup> of SYBR Premix Ex Taq™, 0.5 mm<sup>3</sup> of each primer (10 µM), 0.5 mm<sup>3</sup> of ROX Reference Dye II, 9 mm<sup>3</sup> of sterile RNase-free water and 2 mm<sup>3</sup> of cDNA template (diluted 1/50). The final reaction volume was 25 mm<sup>3</sup>. Each sample was measured in triplicate. The following PCR running conditions were: an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 20 s. Melt curves were analysed at the end of PCR reactions. PP2Acs (Protein phosphatase 2A catalytic subunit) (Lovdal and Lillo 2009) and TIP41-like (Dekkers <it>et al.</it> 2012) genes were used as references. The sequences of aquaporin primers are given in Balarynova <it>et al.</it> 2018. The specific primers for PIP1;1 gene (accession number BP887068) were F-ACAAGGATGTTGTTCTGACAAGGA, R-CACCCA AACAAAATCATGGGACAT. The primers were designed using PrimerQuest<sup>®</sup> program (IDT, Coralville, USA) and their efficiencies were calculated from the slope of the dilution curve. The series of 50, 100, 500, and 1000-diluted cDNA was prepared and measured in triplicate. The relative expression of PIP1;1 and PIP1;3 genes under mercury treatment was quantified against the expression in untreated WT seeds in the dark using delta-delta C<sub>T</sub> method and the experiment was repeated four times. The expression of individual PIPs in seeds collected 24, 72, and 96 h after sowing was calculated using delta C<sub>T</sub> method (the expression was relative to reference genes) (Pfaffl 2001). For PIP1;1, PIP1;3, PIP1;4, and PIP2;2 genes, whose expressions were low, the relative expression was quantified also using delta-delta C<sub>T</sub> method (against both WT and reference genes). The experiment was repeated three times.

**Statistical analysis:** The STATISTICA 12 software (StatSoft, Tulsa, OK, USA) was used to perform all statistical analyses. The significant differences among the different HgCl<sub>2</sub> treatments were assessed using Kruskal-Wallis test with multiple comparisons at the 0.05 significance level. To compare the difference between WT and 7B-1 mutant root length in the dark versus BL, the Mann-Whitney U-test was used (significance level 0.05). Mann-Whitney U-test was also performed to test the differences in root length of WT and 7B-1 seedlings after various HgCl<sub>2</sub> treatments. The results of germination assay are presented as box plots. The bottom and the top of the box are made by the first
and third quartiles and the central tendency of the variable is shown as a median (the band inside the box, the square inside the box represents a mean). The whiskers indicate outliers (1.5 times interquartile range). The box plots were done using OriginPro (OriginLab, Northampton, MA, USA). For the gene expression analyses, the number of biological repeats was not sufficient to prove statistical significance. In those cases, the original values of relative expression calculated using delta-CT method or delta-delta CT-method (Pfaffl 2001) are given in the Supplement to demonstrate that the gene expression of individual replicates show the same trends (Table 1 - 5 Suppl.). These values were used to calculate the geometrical means and SE.

**Results**

To study the interaction between aquaporins and BL during seed germination, WT and 7B-1 mutant seeds were germinated *in vitro* in the presence of HgCl₂, a generally utilized inhibitor of aquaporins (Macey 1984). In the absence of HgCl₂ (control), 87% of WT seeds germinated after 7 d in the dark (D-control) while WT seed germination was slightly, but not significantly, reduced to 82% in continuous BL (BL-control) (Fig 1A). Application of 20 µM HgCl₂ reduced WT seed germination to 37 and 40% in the dark as well as under BL, respectively, compared to the corresponding control and 30 µM HgCl₂ inhibited WT seed germination to 7 and 3% in the dark and under BL, respectively. In the dark as well as under BL, inhibition of WT seed germination by HgCl₂ was completely restored when the seeds were treated simultaneously with 2 mM dithiothreitol (DTT) but DTT alone did not have any significant effect on seed germination. WT seed

---

Fig. 1. Germination of tomato wild type (WT) (A) and 7B-1 mutant (B) seeds in the dark (D) or under blue light (BL) for 7 d as affected by HgCl₂ and/or 2 mM dithiothreitol (DTT). The boxes represent the first and third quartiles and a median value of seed germination (the band inside the box, the square inside the box represents a mean). The whiskers indicate outliers (1.5 times interquartile range), the small individual crosses showing outliers outside 1.5 times interquartile range. For each treatment, at least six independent experiments were done. Thirty seeds were sown on every dish.

---
gen germination was also 90% on medium supplemented with DDT alone or with both 2 mM DTT and 30 µM HgCl₂ in the dark while it reached 77% after the same treatment under BL (Fig 1A).

Germination of 7B-1 mutant seeds (Fig 1B) reached 93 and 89% on control medium in the dark and under BL, respectively. Germination of 7B-1 mutant seeds was not affected significantly by 20 µM HgCl₂ in the dark and BL but 30 µM HgCl₂ decreased 7B-1 seed germination to 60% in the dark and to 57% under BL compared to the corresponding control. Seed germination of 7B-1 mutant was also not affected by treatment with 2 mM DTT alone (seed germination was 93% in the dark and 90% under BL) and 97% of 7B-1 mutant seed germinated on medium with both 2 mM DTT and 30 µM HgCl₂ in the dark and 87% under BL.

The dynamics of seed germination in response to the aquaporin blocker were assessed by determining the rate of germination over 8 days after sowing (DAS) (Fig. 2). Mercury treatment markedly delayed WT seed germination. In the control medium and in the dark, average germination of WT and 7B-1 seeds reached the threshold of 25% before 3 d after sowing (DAS; Fig. 2A,C). In the presence of 20 µM HgCl₂, germination of WT seeds reached 25% by 4th DAS. At 30 µM HgCl₂, germination of WT seeds failed to reach 25%. When 20 µM HgCl₂ was applied, 7B-1 germination reached the threshold at approximately the 3rd DAS in the dark, while 30 µM HgCl₂ delayed mutant seed germination, reaching 25% at 4th DAS. In the control medium and under BL, WT and 7B-1 mutant germination reached the 25% also by the 3rd DAS (Fig. 2B,D). Treatment with 20 µM HgCl₂ decreased the rate of WT seed germination under BL achieving the 25% threshold by 5th DAS, whereas the effect of 30 µM HgCl₂ was so pronounced that WT seed germination did not reach 25%. Treatment with 20 µM HgCl₂ did not alter the kinetics of 7B-1 seed germination under BL, although 30 µM HgCl₂ markedly delayed 7B-1 seed germination under BL reaching the 25% threshold by 5th DAS.

The higher resistance of 7B-1 seed germination to inhibition by mercury led us to test water uptake by dry WT and 7B-1 seeds in the absence or presence of 30 µM HgCl₂. In both genotypes, mercury had no effect on fresh mass increase during 96-h incubation of non-germinated seeds. As expected, BL did not affect the imbibition of water by dry WT and 7B-1 mutant seeds (Fig. 1 Suppl.).

![Fig. 2](https://via.placeholder.com/150)

**Fig. 2.** The kinetics of WT (A,B) and 7B-1 (C,D) seed germination on MS medium (control) and on MS supplemented with 20 or 30 µM HgCl₂ during 8-d cultivation in the dark (D) or under blue light (BL). The data show medians and the error bars represent the first and third quartiles. For each treatment there were at least six independent experiments per each time point. Thirty seeds per each dish were sown for each treatment.

To test the sensitivity of developing roots to the aquaporin blocker HgCl₂, root elongation of seedlings growing on different HgCl₂ concentrations was studied. In the dark control, roots of 7-d-old WT seedlings were approximately 65 mm long. Root growth of WT seedlings was strongly inhibited by all applied concentrations of HgCl₂ (Fig. 3A). BL stimulated significantly root growth in WT, whereas application of
HgCl₂ inhibited root growth counteracting the positive effect of BL (Fig. 3B). HgCl₂ from 12 µM concentration inhibited significantly the length of WT roots in the dark and BL. On control medium, roots in dark-grown 7B-1 seedlings (Fig. 3A) reached a similar length as WT roots but the inhibitory effect of HgCl₂ on root elongation was markedly lower than in WT plants. In the dark at all tested HgCl₂ concentrations, 7B-1 mutant roots were significantly longer than those of WT seedlings. BL stimulated root growth in 7B-1 plants (Fig. 3B) in a similar way as in WT plants but the WT seedlings had significantly longer roots than 7B-1 mutant seedlings growing on control MS medium. Responsiveness of the BL-incubated mutant roots to HgCl₂ was significantly lower than in BL-incubated WT roots (Fig. 3B). Concentrations of HgCl₂ 12 µM and higher inhibited significantly the length of 7B-1 roots in the dark, while under BL, significant reduction in root length of 7B-1 control occurred at 15 µM HgCl₂ and higher concentrations.

Fig. 3. The effect of increasing HgCl₂ concentration on root length of 7-d-old WT and 7B-1 mutant seedlings growing in the dark (A) or under blue light (B). The results represent the medians and the error bars are the first and third quartiles. The significant difference among various treatments compared to MS medium without HgCl₂ (control, mercury concentration 0) was tested using Kruskal-Wallis test (0.05 < P), where a indicates a significant difference against WT control, b marked the significant difference compared to 7B-1 control. At least seven seedlings were measured per treatment and the experiment was repeated three times.

For a better understanding of the possible involvement of aquaporins in seed germination and early radicle elongation, we investigated the expression profiles of nine PIP genes (PIP1;1 to PIP1;5 and PIP2;1 to PIP2;4) (Shiota et al. 2006) in dry, imbibed and germinated seeds and in seeds cultured in the absence or presence of HgCl₂.

In dry WT and 7B-1 seeds, transcripts of PIP1;3 and
genes were not detected. Expression of all other aquaporin genes tested showed a trend of lower expression in 7B-1 than in WT seeds (Fig. 2 Suppl.).

To investigate a possible involvement of the PIP aquaporins in BL-regulated seed germination and early radicle growth, we studied expression of the PIP genes in seeds incubated in the dark or in BL for 24, 72, and 96 h (see Materials and Methods). The only gene that was not expressed during seed germination and early post-germination was PIP2;4. Furthermore, we found that all other genes tested showed a trend of the highest expression after radicle protrusion (Fig 4, Fig. 4 Suppl.). The expression of each gene tended to be the lowest 24 h after sowing (during water imbibition) and then it increased during germination and the early seedling growth (72 and 96 h after sowing). This trend was the same for both WT and 7B-1 mutant seeds in the dark as well as under BL (Fig. 3 Suppl.). Expression of PIP1;2 and PIP2;1 genes tended to be highest at 72 h after seed sowing, whereas PIP1;5 and PIP2;3 expression peaked one day later (96 h after seed sowing, Fig. 4).

Interestingly, expression of PIP1;1 and PIP1;3 was modulated by BL (Fig. 5). In both genotypes, the expression of PIP1;1 gene showed a marked stimulation by BL in germinated seeds harvested 96 h after sowing (Fig. 5 and Fig. 4 Suppl.). A slight increase in PIP1;1 gene expression under BL was also observed in WT seeds harvested at 72 h, but not at 24 h after sowing. Promotion of PIP1;1 gene expression by BL was not observed in 7B-1 mutant seeds collected after 24 and 72 h (Fig. 4 Suppl.). The expression of PIP1;3 was regulated by BL in the opposite way showing slight downregulation by BL in germinated seeds of both genotypes at 96 h after sowing (Fig. 5). However, no effect of BL on PIP1;3 gene expression was observed in WT and 7B-1 seeds collected 24 or 72 h after sowing (Fig. 4 Suppl.).

To investigate resistance of 7B-1 seed germination to HgCl2 at gene expression level, we studied expression of the PIP genes in germinated seeds (radicle length up to 1 cm) incubated for 96 h in the absence or presence of 30 μM HgCl2 in the dark or under BL. From the nine PIP genes tested, only the expression of PIP1;3 was affected by HgCl2 (Fig. 5). The PIP1;3 expression was reduced by HgCl2 in both WT and 7B-1 mutant seeds in the dark as well as under BL but the inhibition of PIP1;3 was less pronounced in 7B-1 than in WT seeds. HgCl2 had no effect on expression of PIP1;1 (Fig. 5), PIP2;1 and PIP2;3 (Fig. 5 Suppl.). However, the expressions of PIP1;2, PIP1;4, PIP1;5, and PIP2;2 genes were elevated in WT seeds incubated in the presence of HgCl2 under BL (Fig. 5 Suppl.).

Discussion

Water uptake and its distribution to individual cells are essential physiological processes that are also the key factors during seed germination. The main role in plant-water relations is attributed to aquaporins which form the water selective pores in the membranes (Chaumont and Tyerman 2014). In this study, we investigated the prospective involvement of PIP aquaporins in tomato seed germination and early seedling growth at both the physiological and transcription levels using the standard water channel blocker HgCl2. We used the tomato 7B-1 mutant as its seed germination shows reduced responsiveness to the inhibition imposed by BL and
various abiotic stresses compared to its corresponding WT (Fellner and Sawhney 2001, 2002).

Our experiments showed that HgCl$_2$ suppressed significantly a final percentage of WT seed germination, indicating the importance of mercury-sensitive aquaporins. Moreover, seed germination in the 7B-1 mutant was less affected by HgCl$_2$ than the WT. Application of HgCl$_2$ caused not only reduction of the final portion of germinated seeds, but also delayed germination (radicle emergence), similarly as it has been reported in *Arabidopsis* (Willigen *et al.* 2006). Delayed radicle emergence was even more inhibited by HgCl$_2$ under BL, and this effect of BL was observed in the WT as well as in the less BL-responsive 7B-1 mutant. Our results demonstrated the lower responsiveness of 7B-1 mutant seed germination to aquaporin blockage compared to the WT. This suggests that the 7B-1 mutation may cause an increase in aquaporin amount or enhance their activity. However, we think that aquaporins were unlikely to be regulated by 7B-1 at the transcription level.

To prevent the potential toxic effects of mercury, we used relatively low concentrations of HgCl$_2$, which were reported not to have any poisonous effects on seed germination (Willigen *et al.* 2006). Besides, we were able to restore seed germination by application of a reducing agent DTT. The reversibility of mercury inhibition of seed germination by DTT showed that HgCl$_2$ did not cause irreversible disrupting of cell integrity (Willigen *et al.* 2006).

Our experiments imply that mercury-sensitive aquaporins are involved in tomato seed germination. Thereupon, we focused on water uptake during seed imbibition and on the possible effects of HgCl$_2$ and BL on this process. The rate of water absorption is not altered by mercury during imbibition of *Arabidopsis* (Willigen *et al.* 2006) and pea seeds (Veselova and Veselovsky 2006). Our experiments confirmed that also in tomato, aquaporins do not participate in water uptake during seed imbibition. It was originally suggested that *PAPs* could play a role in rehydration during seed germination because their expression is elevated in germinating seeds in comparison to dry seed (Gao *et al.* 1999, Suga *et al.* 2001, Schuurmans *et al.* 2003). Further support for this notion is that commencement of seed germination includes extensive changes in water content which can be provided sufficiently only by rapid uptake through aquaporins. However, according to Veselova and Veselovsky (2006) aquaporins are closed during imbibition to prevent rapid water uptake by dry seeds which could damage them. In fact, it is likely that during imbibition water enters the seeds just by diffusion.

Thus, we focused on the characterization of the expression patterns of *PAPs* during tomato WT and 7B-1 mutant seed germination and early post-germination. We found that transcripts of the tested *PAPs*, apart from *PIP1;3* and *PIP2;4*, preexisted in the dry seeds. Surprisingly, dry seeds of 7B-1 contained lower amounts of *PAP* transcripts than did dry WT seeds despite the fact that 7B-1 seeds coped better than WT seed with mercury-inhibition. The fact that *PIP1;3* was not expressed in dry seeds, but it was detected in imbibed and germinating seeds, primarily during radicle elongation, indicates the importance of *PIP1;3* in this process. The absence of *PIP2;4* expression in dry, imbibed, or germinating seeds suggest that it is probably not involved in seed germination.

The amount of *PAP* transcripts tended to increase together with radicle protrusion. Thus, a role for *PAPs* in radicle elongation could be anticipated. This is supported by the facts that HgCl$_2$ decreased seed germination, but did not affect water imbibition of dry WT and 7B-1 seeds, which is consistent with a role for *PAPs* during radicle growth, but not during water imbibition. Besides, the expression of the studied aquaporin genes was highest in the seeds with a visible radicle. Indeed, cell elongation requires continuous uptake of water to maintain pressure potential (Chauumont and Tyerman 2014). The elongation of radicle cells is generally accepted to be sufficient for the completion of radicle protrusion, while cell division is not essential (Barroco *et al.* 2005), and a role for *PAPs* in tissue elongation has been suggested several times (Maurel *et al.* 2002, Fricke and Chauumont 2007, Liu *et al.* 2008). Various trends in expression patterns were observed for the tested *PAP* genes suggesting their different roles in various phases of seed germination and early post-germination. *PIP1;2*, *PIP1;5*, *PIP2;1* and *PIP2;3* transcripts were prevalent, whereas *PIP1;1*, *PIP1;3*, *PIP1;4*, and *PIP2;2* transcripts were much less abundant in germinating seeds. Although the seed germination assays demonstrated lower responsiveness of 7B-1 mutant seed germination to aquaporin blockage compared to WT, no fundamental difference in *PAP* transcripts accumulation was found between WT and mutant seeds in both the dark and under BL. Furthermore, not even mercury treatment (30 $\mu$M HgCl$_2$) had a substantial effect on *PAP* gene expression, with only one exception. The amount of *PIP1;3* transcripts was downregulated by mercury (Fig. 5). Moreover, *PIP1;3* expression showed a moderate downregulation also by BL during radicle elongation. The same trend was found also in 7B-1 seeds, although the effect of mercury seemed to be more obvious in WT than in 7B-1 seeds. The relevance of effect of BL on *PIP1;3* expression will be studied in detail using various BL intensities to confirm its impact on *PIP1;3* gene in tomato. It would be interesting to investigate further the protein expression of individual *PAPs* to find out whether they differ in WT and 7B-1 seeds.
Specifically, HgCl₂ enhances slightly the expression of PIP1;2, PIP1;4, PIP1;5 and PIP2;2 genes in WT seeds under BL. It is possible that mercury-induced blockage of aquaporins together with BL and this required increased transcription of these genes to compensate for the aquaporin blockage in order to sustain the appropriate root elongation. 7B-1 mutant seeds were not so greatly affected by the treatment with mercury, so there was no need to increase aquaporin gene transcription.

Alteration of aquaporin gene expression by BL was reported by Kaldenhoff et al. (1995, 1996), who found out that BL induces the transcription of aquaporin genes AthH2 and AthH3 in Arabidopsis cell cultures. However, a comprehensive study of BL effects on aquaporin function during seed germination and early seedling growth was still missing. In our study, PIP1;3 gene expression was affected by BL (see above). Besides, BL stimulated the expression of PIP1;1 in both WT and 7B-1 seeds. The facts that the PIP1;1 expression is highest in seeds with elongating radicles and that PIP1;1 transcript accumulation is stimulated by BL could suggest the involvement of this gene in BL-induced growth of WT and 7B-1 roots as discussed below.

To investigate the physiological role of aquaporins in root elongation, we studied the effect of HgCl₂ on root length of WT and 7B-1 mutant seedlings. We showed that root elongation of WT and 7B-1 mutant seedlings was suppressed by HgCl₂, supporting the expectation that aquaporins are involved in this process (Hukin et al. 2002, Javot and Maurel 2009). However, 7B-1 mutant roots were less responsive to mercury inhibition than those of WT. In both genotypes, root growth was stimulated significantly by BL compared to dark-growth seedlings. The effect of light on root elongation is well known in many plant species and the possible pathway through which roots perceive light to promote their elongation is described for Arabidopsis (Dyachok et al. 2011). 7B-1 seedling growing on MS under BL conditions had shorter roots than WT seedling growing under the same conditions. This clearly demonstrates that the 7B-1 defect in BL signalling pathways results in lower responses to BL stimuli. On the contrary, in the presence of HgCl₂, 7B-1 roots were always longer than those of WT seedlings under BL, indicating the lower responsiveness of 7B-1 root elongation to mercury treatment.

In summary, expression analyses of PIP genes indicate that, apart from PIP2;4, all tested PIPs participate in tomato seed germination and early radicle elongation. PIPs seemed to be involved in radicle elongation during seed germination with no indication of role in rapid water imbibition at the commencement of seed germination. We showed that the BL-induced expression of PIP1;1 correlated with BL-induced stimulation of root elongation, indicating that PIP1;1 could be involved in root elongation under BL. Furthermore, the relationship between the lower responsiveness of 7B-1 mutant seeds to mercury-induced inhibition of aquaporins and lower sensitivity of PIP1;3 gene expression to HgCl₂ in 7B-1 seeds suggested a role of PIP1;3 in tomato radicle elongation. Thus, we showed that BL can alter PIP gene expression (PIP1;1 and PIP1;3) during tomato seed germination and early post-germination and that the 7B-1 mutation (primarily considered to affect BL sensing) causes a lower responsiveness to mercury blockage of aquaporins.

References

Daniels, M.J., Mirkov, T.E., Chrispeels, M.J: The plasma
membrane of *Arabidopsis thaliana* contains a mercury-insensitive aquaporin that is homologous to the tonoplastic water channel protein TIP. - Plant Physiol. 106: 1325-1333, 1994.


