PALACKÝ UNIVERSITY OLOMOUC

Department of Botany & Laboratory of Growth Regulators

Involvement of Aquaporins in Light-regulated Sensitivity of Plants to Osmotic Stress

Ph.D. thesis

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Declaration

I hereby declare that this Ph.D. thesis is my own and was carried out entirely with help of literature cited in the list of references.

Bibliographical identification

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Abstract:

Divergent abiotic constrains induce osmotic stress on plant cells resulting in an imbalance in water homeostasis. Aquaporins, especially those residing in the plasma membrane (PIPs), play a central role in coping with the impact of the osmotic stress on plant cells. Besides, the involvement of LEA-type genes/proteins (Late Embryogenesis Abundant) and members of BURP-domain containing genes/proteins is also expected. Tomato cv. Rutgers (WT) and *7B-1* mutant seeds, which are impaired in responses to BL and less responsive to osmotic stress than WT seeds, were used as a model system. The thesis focused on determining the impact of osmotic stress, blue light (BL) and the *7B-1* mutation on the gene expression profiles of mentioned stress-responsive genes. To assess that, a quantitative RT-PCR and subsequent REST (Relative Expression Software Tool) 2009 software analysis were used. The data showed that aquaporin *PIP1;3*, *BURP-domain containing* gene *RD22-like* and *LEA-type* genes were significantly affected by osmotic stress, BL as well as the *7B-1* mutation. Interestingly, the *7B-1* mutation, BL and osmotic stress downregulated *PIP1;3* transcript level, which could improve osmotic adjustment of mutant seeds under stress conditions. On the other hand, the transcript levels of *RD-22 like* and *LEA-type* genes were induced by BL and osmotic stress, which corresponded with their expected protective role under osmotic stress. The *7B-1* mutation reduced the expression level of both *RD-22 like* and LEA-type genes. Altogether, our data indicate

the existence of a link between osmotic stress and BL signalling and the involvement of the *7B-1* mutation in this crosstalk.

Keywords: *7B-1* mutant, aquaporin, blue light, dehydrin, LEA, PIP, RD22, seed, *Solanum lycopersicum* L., tomato

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Abstrakt:

Působením nepříznivých podmínek z vnějšího prostředí dochází k narušení rovnováhy osmotických dějů v rostlinách, následkem čehož na rostlinu působí osmotický stress. Klíčovou roli ve vyrovnávání se negativním účinkům působení osmotického stresu hrají vodní kanály (akvaporiny), především ty umístěné v plazmatické membráně. Reakce rostlin k osmotickému stresu není spojena jen s akvaporiny, ale také s řadou dalších genů a proteinů, především LEA genů/proteinů (Late Embryogenesis Abundant) a zástupců ze skupiny genů/protein s BURP doménou. Modelovou rostlinou disertační práce jsou semena rajčete kultivaru Rutgers (WT) a mutanta *7B-1*, který se vyznačuje sníženou citlivostí k účinkům modrého světla a menší citlivostí klíčení k osmotickému stresu. Cílem práce je sledovat vliv osmotického stresu, modrého světla a mutace *7B-1* na expresi zmiňovaných genů zapojených v reakcích rostlin k osmotickému stresu. Exprese genů byly stanovovány pomocí kvantitativní RT-PCR a poté vyhodnocovány použitím programu REST (Relative Expression Software Tool) 2009. Výsledky ukazují, že exprese akvaporinu *PIP1;3,* genu *RD22* (zástupce ze skupiny genů obsahujícíhc *BURP* doménu) a *LEA* genů jsou signifikantně ovlivněny působením osmotického stresu, modrého světla i mutace *7B-1*. Všechny tři studované faktory (modré světlo, osmotický stres i mutace) snižovaly expresi akvaporinu *PIP1;3*, což by mohlo ukazovat na možnou cestu mutanta k lepšímu vyrovnání osmotického potanciálu při klíčení za stresových

podmínek. Naopak, působení modrého světla a osmotického stresu zvyšovalo množství transkriptu genu *RD22* a *LEA* genů, což je v souladu s předpokladem, že produkty těchto genů mají protektivní funkci. Překvapivě, mutace *7B-1* množství transkriptu genu *RD22* a *LEA* genů výrazně snižovala. Získaná data poukazují na propojenost signálních drah světla a osmotického stresu a na jejich ovlivnění mutací *7B-1*.

Klíčová slova: akvaporiny, dehydriny, klíčení semen, LEA, modré světlo, mutant *7B-1*, PIP, rajče, RD22, *Solanum lycopersicum* L.

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Contents

ABBREVIATIONS

ABA	abscisic acid
BL	blue light
CT	threshold cycle
D	darkness
DHN	dehydrin
DTT	dithoithreitol
$HgCl_2$	mercuric chloride
LEA	late embryogenesis abundant protein/gene
MS	basal Murashige and Skoog medium
PEG	polyethylene glycol
PIP	plasma membrane intrinsic protein/gene
qRT-PCR	quantitative RT-PCR
RD	responsive to dehydration
REST	relative expression software tool
RT-PCR	reverse transcription-polymerase chain reaction
SE	standard error
WT	wild-type

1 INTRODUCTION

1.1. Seed germination

The seed is the structure, which enables embryo to survive the period between seed maturation and seedling establishment (Bewley 1997). The mature tomato seed consists of an embryo embedded in a rigid endosperm and surrounded by a thin seed coat, so-called testa (Smith 1935; Varga and Bruinsma 1986) (Fig. 1). Tomato seeds develop and mature in the moist environment of the developing fruit. On the other hand, the development of the fruit is governed by the developing seeds (Varga and Bruinsma 1986).

By definition, seed germination includes the sequence of events, which begin with the uptake of water by the dry seed and end with the elongation of the embryonic axis, usually the radicle (Bewley and Black 1994). The visible sign of completion of germination is usually the penetration of the covering structures of the embryo by the radicle; the result is often called visible germination (Bewley 1997). Germination *sensu stricto* does not include subsequent seedling growth, which starts after germination, this is post-germination (Bewley and Black 1994). Germination of tomato seeds usually initiates after 48 hours of imbibition and DNA synthesis starts around 12 hours of imbibition in embryo radicles, which indicates that initial DNA synthesis is paralleled by the beginning of embryo growth through cell expansion (De Castro and Hilhorst 2000). Seed germination is governed by the growth potential of the embryo and the restraints imposed by the embryo covering layers (Bewley 1997), in other word, by interactions between the embryonic radicle tip and the enclosing endosperm cap. Weakening of the endosperm cap, by enzymatic hydrolysis, is required to allow radicle protrusion, which is generally considered as the completion of the germination, and is the main germination-limiting process in seeds of *Solanaceae* (Koornneef et al. 2002). Radicle protrusion depends on embryo growth driven by water

uptake (Kucera et al. 2005). The water uptake during seed germination is a triphasic process. It starts with a rapid initial uptake of water by the dry seed (phase I, i.e. imbibition) followed by a plateau phase (phase II). Finally, the whole process is terminated by a further increase in water uptake (phase III) accompanying the elongation of embryo axis and rupture of seed covering layers (e.g. Schopfer and Plachy 1984; Manz et al. 2005). Cell elongation driven by water uptake is necessary, and is generally accepted to be sufficient, for the completion of radicle protrusion; cell division is not essential (Barroco et al. 2005).

Testa rupture and endosperm rupture are temporally separate events during the germination of many seeds of the *Solanaceae* family (Krock et al. 2002; Leubner-Metzger 2003; Petruzzelli et al. 2003). Testa rupture of tobacco occurs at predetermined breaking points and depends on water uptake of the embryo and the endosperm. It is associated with an additional increase in seed water content in the late part of phase II water uptake (Manz et al. 2005). After testa rupture, which results from tissue dissolution, storage reserves in the micropylar endosperm cells are degraded and the radicle emerges through a hole in the endosperm (Leubner-Metzger 2003).

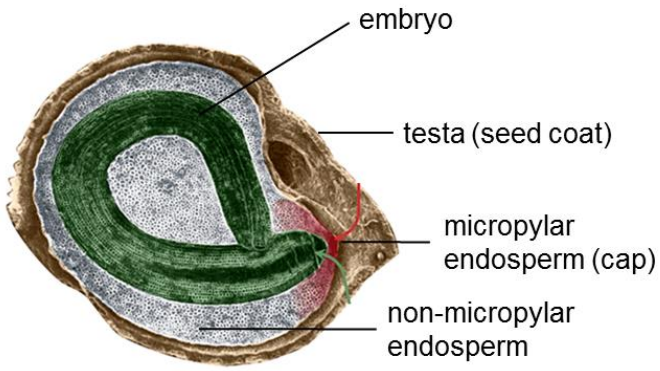

Fig. 1. Structure of mature seeds of *Capsicum annuum* (pepper). Pepper as well as tomato seeds are type members of the *Solanoideae* subgroup of *Solanaceae* family. Adapted from Finch-Savage and Leubner-Metzger (2006).

The timing of seed germination is influenced by temperature, light, water availability, soil disturbance, vegetation shading and many other factors (Franklin et al. 2005). Important role in regulation of seed germination play, of course, the balance in levels of plant hormones. Abscisic acid (ABA) is a positive regulator of induction and maintenance of seed dormancy, while it is a negative regulator of germination. On the other hand, gibberellins (GA) release dormancy, promote seed germination and counteract inhibitory effects of ABA. Gibberellins are required for embryo cell elongation, for overcoming coat restrictions to germination and for inducing endosperm weakening. Ethylene and brassinosteroids promote seed germination and also counteract the inhibitory effects of ABA on seed germination, but in most species, they appear to act after release of dormancy by gibberellins. Ethylene seems to counteract the effects of ABA on seed germination by interfering with its signalling (Kucera et al. 2005). Auxin by itself is not a necessary hormone for seed germination. However, it is present in the seed radicle tip during and after seed germination and its interactions and crosstalk with gibberellins and ethylene may influence the seed germination and establishment (Fu and Harberd 2003; Chiwocha et al. 2005). In addition, cytokinins have been implied to play an important role in regulating seed germination (Barzilai and Mayer 1964; Khan 1971; Black et al. 1974; Thomas et al. 1997). They promote seed germination, possibly through antagonizing ABA effects on germination (Khan 1971; Wang et al. 2011). Moreover, cytokinins are able to enhance seed germination by the alleviation of stresses such as salinity, drought, heavy metals and oxidative stress (Khan and Ungar 1997; Atici et al. 2005; Nikolic et al. 2006; Peleg and Blumwald 2011). Finally, it has been shown that also jasmonates have role(s) during seed germination. They can inhibit seed germination of several plant species including *Solanum lycopersicum* (Miersch et al. 2008; Oh et al. 2009).

Seed germination is a complex phenomenon that is controlled by a large number of genes, which are affected by both developmental and environmental factors (Bewley 1997;

Koornneef et al. 2002). During seed maturation, the expression of many genes is altered and specific classes of mRNAs such as those of the *LEA* (Late Embryogenesis Abundant) genes appear (Fig. 2).

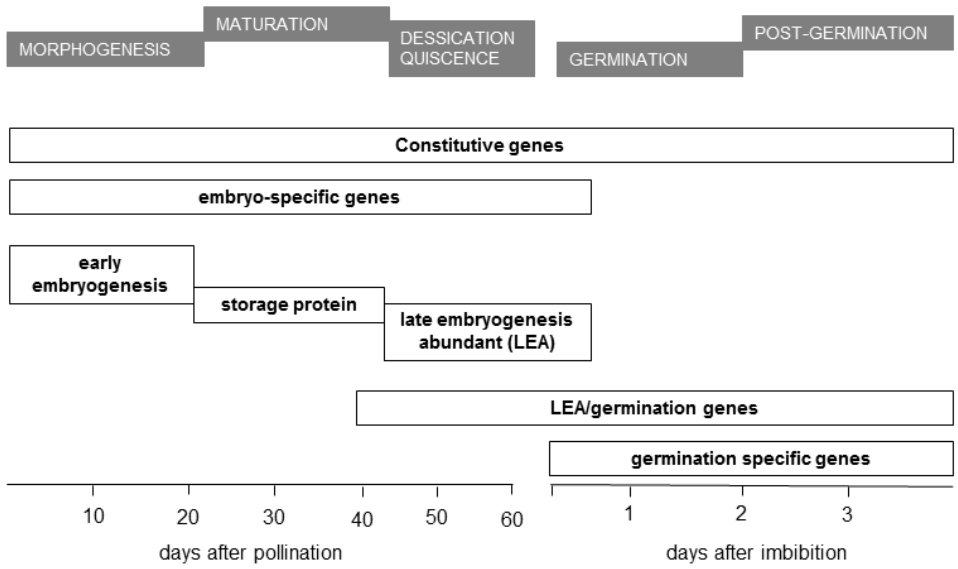

Fig. 2. Expression patterns of various gene families during seed development, germination and post-germination. Adapted from De Castro and Hilhorst (2000).

1.2. Light as a signal

Light is one of the most important environmental factors for plants. Light is not only a source of energy for photosynthesis but also an important signal that provides plants with information about their surroundings and modulates a variety of processes, from seed germination to the onset of flowering, collectively known as photomorphogenesis (Christie 2007). Plants have adopted the ability to sense multiple parameters of light, including its quality, direction, duration and intensity. Light signals are perceived through several families of photoreceptors (Fig. 3) – phytochromes (predominately absorb the red and far-red light), cryptochromes (the blue and UV-A photoreceptors), phototropins (perceive blue and UV-A

wavelengths), Zeitlupes (the blue and UV-A light photoreceptors) and UVR8 (perceive UV-B) (Galvão and Fankhauser 2015).

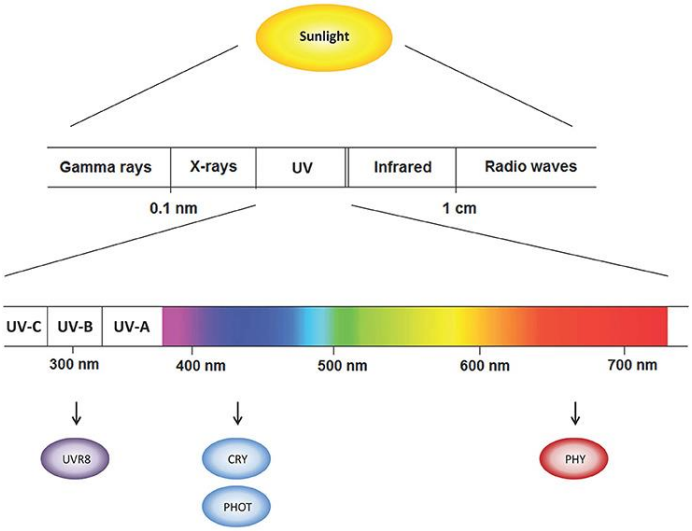

Fig. 3. The spectrum of the solar radiation focused on visible wavelengths and plant photoreceptors. In the picture, photoreceptors the UV Resistance Locus8 (UVR8), cryptochromes (CRY), phototropins (PHOT) and phytochromes (PHY) are showed. The biologically active radiation comprises the spectrum from approximately 300 to 800 nm and visible light spectrum lays in the range between 400 and 710 nm. Adapted from Zoratti et al. (2014).

In brief, plant phytochromes consist of an N-terminal photosensory region and a C-terminal regulatory region. The N-terminus is essential for light perception and photoconversion of the phytochromes whereas The C-terminal domain contains a histidine kinase-related domain which in fact, exhibits serine/threonine kinase activity. A chromophore of phytochromes is a linear tetrapyrrol phytochromobilin. Phytochromes are synthesized as a biologically inactive Pr-form. This form is converted to an active Pfr-form after red light absorption. Far-red light absorption converts Pfr-form back to Pr-form (Burgie and Vierstra 2014). *Arabidopsis thaliana* has five phytochromes phyA-E (Clack et al. 1994). Phytochromes are cytoplasmic proteins which are, at least partly, translocated to the nucleus after light absorption. In the nucleus they act on light-regulated gene expression by transcriptional, post-transcriptional and post-translational mechanisms (Rausenberger

et al. 2011; Leivar and Monte 2014). Phytochromes function in seed germination, de-etiolation, stomata development, flowering, shade avoidance, senescence and many others physiological phenomena (Kami et al. 2010)

Cryptochromes are dimeric flavoproteins comprised of the highly conserved N-terminal Photolyase Homologous Region (PHR) domain and the diverged CRY C-terminal Extension (CCE) domain. The N-terminus binds a chromophore, flavin adenine dinucleotide (FAD) (Lin et al. 1995; Malhotra et al. 1995). UV-A and blue light are primarily absorbed by the oxidized form of FAD. Upon blue light absorption, FAD undergoes photoreduction and turns into the semi-reduced FAD radical ($FADH^{\cdot}$), which absorbs a broader spectrum of light (including green light). The semi-reduced is unstable and can be transformed to the oxidized FAD in the absence of light (Bouly et al. 2007). Cryptochromes are localized in the nucleus and/or in the cytoplasm. In the nucleus, cryptochromes regulate transcription through interaction with divergent transcription factors and the proteasome. *Arabidopsis thaliana* has two cryptochrome photoreceptors, cry1 and cry2 (Yu et al. 2007). Besides, cry3 was detected in mitochondrion or chloroplasts having the function in repairing UV-induced damage on DNA (Pokorny et al. 2008). In plants, cryptochromes control a variety of responses such as de-etiolation, photoperiodic flowering, guard cell development, stomata opening, leaf senescence or pathogenic responses (Liu et al. 2016).

Phototropins are the plasma membrane-localized phosphoproteins which can be divided into an N-terminal photosensory region and a C-terminus with the Ser/Thr protein kinase domain. Blue light is perceived by two flavin mononucleotide (FMN) chromophore-binding LOV1 (Light, Oxygene, Voltage) and LOV2 domains at the N-terminus. Autophosphorylation and phosphorylations play an essential role in the phototropin signalling. Phototropins are involved in various responses like phototropism, chloroplast

movements, stomata opening, leaf photomorphogenesis or the rapid inhibition of hypocotyl growth. *Arabidopsis thaliana* has two phototropins, phot1 and phot2 (Christie 2007).

Zeitlupe family comprises ZEITLUPE, FLAVIN-BINDING, KELCH REPEAT, F-BOX and LOV KELCH PROTEIN2 proteins. They consist of an N-terminal domain with a single LOV domain (binding FMN), an F-box and a C-terminus with six Kelch repeats (serve as protein-protein interaction domain). Zeitlupes form F-box-containing E3 ubiquitin ligase complex which control directly light-induced protein degradation. Thus, these proteins combine both photoreceptor and F-box protein activity (Ito et al. 2012). They are involved in controlling of photoperiodic flowering, the circadian clock and so on (Sawa et al. 2007).

Besides being harmful, low doses of UV-B have regulatory effects on plants. Plants use the UV Resistance Locus8 (UVR8) as a UV-B photoreceptor (Rizzini et al. 2011). To perceive light, this photoreceptor uses group of closely packed tryptophan residues. Perception of UV-B light induced monomerization of UVR8, a monomer is a seven-bladed β-propeller protein (Jenkins 2014). Then, UVR8 interacts with COP1 in the nucleus, which leads to the expression and stabilization of transcription factors binding to the promotor of UV-B responsive genes (Binkert et al. 2014). UVR8 acts in various photomorphogenic responses such as the suppression of hypocotyl and root growth, the promotion of cotyledon opening and the biosynthesis of flavonoid compounds.

Genomic studies showed that light induces massive reprogramming of the plant transcriptome. Light can affect transcription through signal transduction pathways or by direct effects on transcription factors (Jiao et al. 2007). Light can induce expression of some stress-responsive genes including dehydrins. For example, exposure to white light and red light stimulate dehydrin transcript accumulation (Natali et al. 2007). As far as I know, there is no report on the effect of blue light on dehydrin gene expression. On the other hand, BL can modulate the expression of aquaporins (Kaldenhoff et al. 1995, 1996; Liang et al. 2013).

1.3. Abiotic stress

The evolution of plants has resulted in the elaboration of protective mechanisms responsible for adaptation to constantly changing environment. The response to a certain stress is usually composed of stress-specific adaptive responses as well as general responses conferring basic protection (Kültz 2005). One key strategy for plants to cope with the changing environment is the fine-tuning of gene expression in response to those changes (Baena-González 2010; Lauria and Rossi 2011). To do that, plants have evolved mechanisms that allow them to perceive the incoming stresses and then adjust adequately their physiology and metabolism via fine-tuning the gene expression patterns (Zhang et al. 2006). It is still unknown how plants sense osmotic stress, because an osmosensor has not been identified yet. However, several candidates for osmosensors have been proposed, even though their role in sensing osmotic stress has to be proven (Bartels and Sunkar 2005). It has also been suggested that aquaporins could function as osmo- and turgor sensors (Hill et al. 2004).

Stress-responsive gene expression in plants is mediated by both ABA-dependent and ABA-independent pathways (Shinozaki and Yamaguchi-Shinozaki 1997; Leung and Giraudat 1998; Thomashow 1999; Rock 2000). Until now, many osmotic stress-responsive genes have been described in plants (Shinozaki and Yamaguchi-Shinozaki 1997; Thomashow 1999; Zhang et al. 2004). These genes can be generally classified into two groups (Fig. 4). First group of so-called functional genes comprises of genes whose products are involved in protection of cells under osmotic stress and production of important metabolic proteins. The products of functional genes can be for example water channels or LEA proteins (Late Embryogenesis Abundant proteins). Second group of genes is composed of regulatory genes whose products are important members of regulatory pathways such as different transcriptional factors, protein kinases or enzymes of ABA biosynthesis (Nakashima and Yamaguchi-Shinozaki 2006).

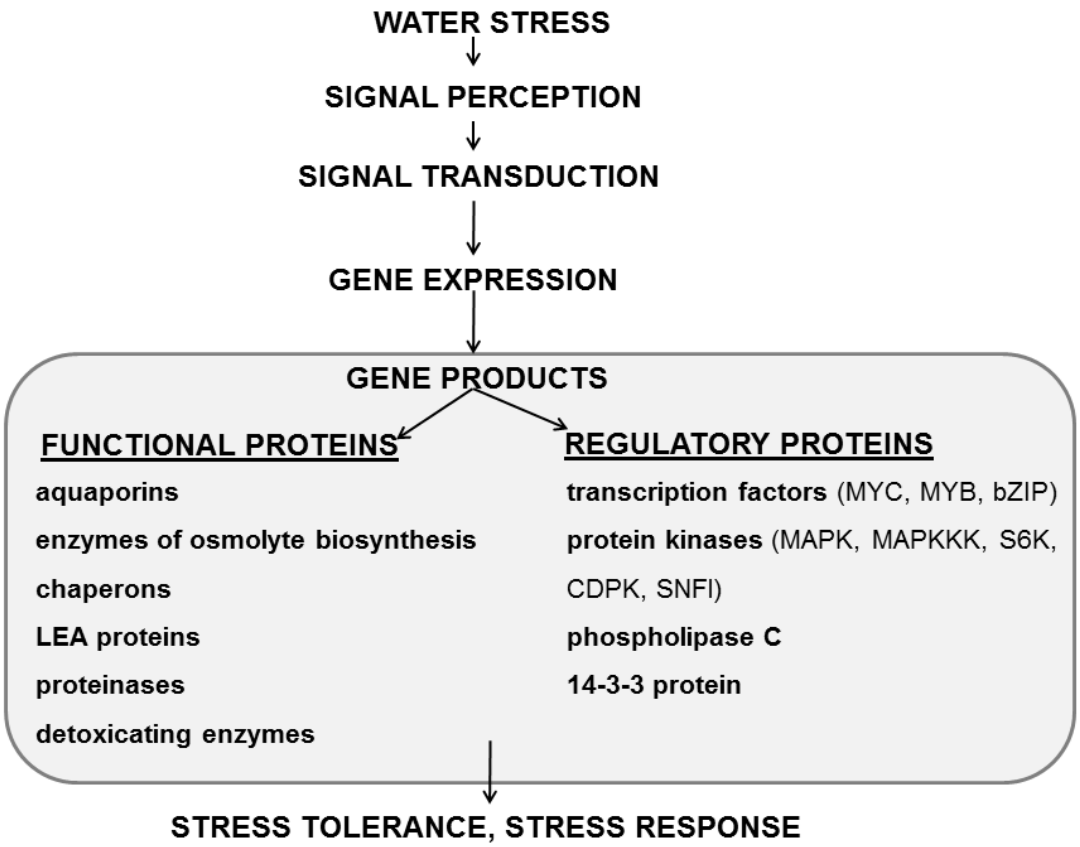

Fig. 4. The product of stress-responsive genes involved in stress tolerance and responses. Adapted from Shinozaki and Yamaguchi-Shinozaki (1997).

Phytohormones are significantly involved in fine-tuning of plant responses to abiotic challenges. They often rapidly alter gene expression by inducing or preventing the degradation of transcriptional regulators via the ubiquitin–proteasome system (Santner and Estelle 2009, 2010). Abscisic acid (ABA), which accumulates to high levels in plant tissues in response to various stresses, plays a central role in stress responses. The accumulation of ABA is a cell signalling process, encompassing perception of the stress signal followed by its transduction and regulation of expression of genes encoding enzymes of ABA biosynthesis and catabolism (Zhang et al. 2006). ABA synthesis is one of the fastest responses of plants to abiotic stress. Indeed, other hormones, in particular salicylic acid,

ethylene and jasmonate, also play significant roles in plant defence (Horváth et al. 2007; Kazan 2015).

1.4. Aquaporins

Abiotic stresses induce osmotic stress on plants and disturb the plant water homeostasis by affecting water uptake as well as distribution of water within plant body (Venkatesh et al. 2013). The fundamental task in maintaining plant water relations perform water channels (aquaporins) which represent a more rapid way of water movement across membranes compared to slow diffusion of water molecules. Of course, both paths facilitate and regulate the passive movement of water down a water potential gradient (Maurel et al. 2008). It is expected that under well-watered conditions, water moves mainly along the apoplastic pathway, however, under water stress conditions, it seems to move mainly through transcellular pathway (from cell to cell through aquaporins) since transpiration is restricted (Steudle and Peterson 1998).

Beside the role in maintaining water homeostasis, there is an opinion that aquaporins can function as osmosensors or turgosensors during plant growth and development (Hill et al. 2004). Another compelling suggestion comes from Lorenz et al. 2003 who proposed that PIP1-type aquaporins can serve as a BL-photoreceptor because of their localization in the plasma membrane and ability to bind flavins (the cofactors of BL photoreceptors). It seems that the N-terminus of PIP1 interacts with the flavin. Blue light induces a covalent bound of PIP1 and flavin (oxidized FMN, flavin mononucleotide) resulting in the signalling form of PIP1. In the dark, the bound is slowly releasing so the adduct returns to the starting form (or by photochromic reversion) in which both components are bind loosely (Hertel 2005).

Aquaporins belong to a family of major intrinsic proteins (MIPs) (Gomes et al. 2009) which can be divided into aquaporins *sensu stricto* and aquaglyceroporins. Aquaporins are especially water transporters whereas aquaglyceroproteins transport a variety of solutes such as urea or ammonia (Maurel et al. 2008; Reuscher et al. 2013). Aquaporins family is classified according to sequence similarities into five main subfamilies – the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the NOD26-like intrinsic proteins (NIPs), the small basic intrinsic proteins (SIPs) and the X-intrinsic proteins (Johanson and Gustavsson 2002; Bienert et al. 2011). Recently, 47 aquaporin genes were found in the tomato genome and based on phylogenetic analysis they were categorized into 14 PIPs, 11 TIPs, 12 NIPs, 4 SIPs and 6 XIPs (Reuscher et al. 2013).

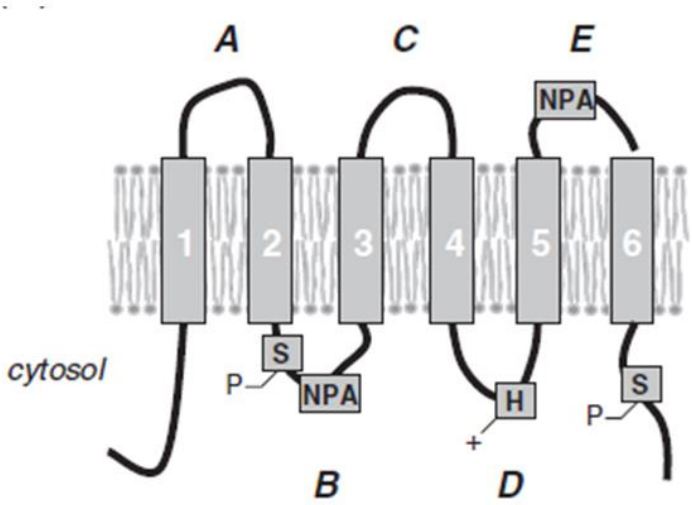

Fig. 5. The structure of an aquaporin monomer. Typically, the aquaporin monomer consists of six transmembrane helices (1–6) and five interconnecting loops (A–E) with the highly conserved NPA motifs. In the picture, the most frequent regulatory sites of PIP aquaporins are marked as well (a histidine (H) residue and two serine (S) residues, which are the main targets of phosphorylation). Adapted from Luu and Maurel (2005).

Aquaporin monomers are small membrane proteins (21-34 kDa) exhibiting a typically conserved structure with six membrane-spanning α-helices linked by five loops (Fig. 5). The N and C termini of the channel face to the cytosol (Fujiyoshi et al. 2002). Aquaporins

usually form homo- or heterotetramers, each monomer acting as functionally independent water pore (Murata et al. 2000; Fetter et al. 2004; Bienert et al. 2012).

For regulation of water movement is important both the abundance and the activity of water channels in the membrane. At the transcriptional level, the expression of *PIP* genes could be regulated by numerous environmental factors, including light (Kaldenhoff and Eckert 1999; Lorenz et al. 2003; Cochard et al. 2007; Voicu et al. 2008; Baaziz et al. 2012), drought (Lian et al. 2004; Alexandersson et al. 2005; Aroca et al. 2006; Galmés et al. 2007), cold (Jang et al. 2004), salt (Jang et al. 2004; Boursiac et al. 2005; Liu et al. 2013) and phytohormones (Kaldenhoff et al. 1993; Phillips and Huttly 1994; Morillon et al. 2001; Suga et al. 2002; Aroca et al. 2006). At the posttranscriptional level, AQP activity can be modulated by various modifications such as phosphorylation, methylation or ubiquitylation and interactions with other proteins involved in aquaporin trafficking through secretory pathway and gating (the closing and opening of the channel) (Hachez et al. 2006; Luu and Maurel 2005; Ma et al. 2008; Chaumont and Tyerman 2014).

For assessment of aquaporin activity in the intact plants, mercuric chloride is still used as a common inhibitor of aquaporins, even though it is unspecific and it has many side effects on plant cells (Javot and Maurel 2002). Mercurials cause oxidation of cystein residues within the water pore and alter the conformation in other regions of the aquaporin protein (Preston et al. 1992). Another useful tool for studying aquaporin functions is evaluation of their expression after injection into the frog (*Xenopus laevis*) oocytes (Schuurmans et al. 2003).

Generally, it is accepted that the presence of aquaporins in the membrane markedly increases its hydraulics conductivity. However, in addition to water, some aquaporins could transport small organic compounds and gases such as O_2, CO_2, H_2O_2, urea, ammonia or boric acid. The central pathways for transcellular and intracellular water transport are formed

by PIP and TIP aquaporins (Maurel et al. 2008, 2015). Nevertheless, PIP1 aquaporins show low or slight water permeability when expressed in *Xenopus* oocytes (Chaumont et al. 2000). Fetter et al. (2004) showed that co-expression of PIP1 and PIP2 aquaporins is essential to increase PIP1 water channel activity (Fetter et al. 2004). This differential behaviour of PIP1 and PIP2 aquaporins is most probably related to their differential trafficking (Zelazny et al. 2007, 2009). Still, the ability to transport other substrates rather than water is often attributed to PIP1 aquaporins. For example, *Arabidopsis thaliana* aquaporin PIP1;2 participate in transporting CO_2 (Uehlein et al. 2003, 2012) and *Nicotiana tabacum* aquaporin PIP1;3 is thought to be involved in oxygene transport. Thus, PIPs appears to play a dual function. They are important for preserving water homeostasis and play a role in regulation of photosynthesis Aquaporins participate also in cell signalling. For example, AtPIP1;4 is able to translocate extracellular hydrogen peroxide into the cytoplasm and active immune responses induced by bacteria (Tian et al. 2016).

The PIP isoforms of aquaporins has been proven to participate in responses to abiotic stresses, such as drought, salinity or chilling (Lian et al. 2004; Guo et al. 2006; Liu et al. 2007; Mut et al. 2008; Matsumoto et al. 2009). Moreover, these environmental stresses are able to modulate PIP gene expression (Aroca et al. 2012). Transcriptional regulation of aquaporin genes by plant hormones or various environmental stimuli (light, drought, nutrient status etc.) has been intensively studied, especially in *Arabidopsis thaliana* (Liu et al. 2003; Maathuis et al. 2003; Jang et al. 2004). Besides, it seems that PIPs constantly change their intracellular localization between plasma membrane and different cell compartments and stresses can promote their partitioning to plasma membrane (Luu et al. 2012). Aquaporin gene expression might be upregulated, downregulated or without any change in response to stress (Tyerman et al. 2002).

It was shown that during seed germination aquaporins participate in water uptake after radicle emergence. It seems that they are closed during imbibition of water by dry seed to prevent too rapid entrance of water to seed which could be fatal to seed (Willigen et al. 2006). Seed germination occurs by elongation of embryo cells, which is triggered by imbibition of water by dry seed. Therefore, aquaporin gene expression is low during imbibition and rises with increasing hydration of seed (Obroucheva 2012). The main changes in aquaporin composition are associated with radicle emergence, which activates the expression of PIP and TIP genes to provide sufficient water entry into elongating embryonic axis cells. Apparently, accelerated water flow via aquaporins is driven by the accumulation of endogenous osmotica, imported from storage tissues and organs, in elongating cells (Obroucheva 2013, 2017).

1.5. LEA genes/ proteins

Late Embryogenesis Abundant (LEA) genes/proteins are the most prevalent group of protective molecules expressed in response to different abiotic stresses (Bartels and Sunkar 2005). LEA proteins accumulate naturally in some desiccation tolerant structures such as the seeds, probably to protect embryo structures under conditions of water deficit (Dure et al. 1989). Moreover, they are also produced in plant vegetative tissues during exposure to different water-related stresses (drought, salinity, cold) (Roberts et al. 1993; Ingram and Bartels 1996; Bray 1997). Thus, LEA proteins can be considered as stress proteins. It is known that presence of LEA proteins correlates with the acquisition of stress tolerance (Cuming 1999; Bartels and Salaminy 2001). LEA proteins are suggested to be involved in protection of plant cells against damage induced by impairment of water balance, but their functions are still obscure (Baker et al. 1988; Dure 1993; Close 1997; Zhu et al. 2000; Ramanjulu and Bartels 2002). There are several possible activities of LEA proteins, such as interaction with RNA or DNA, water or ion binding, antioxidative activity or sugar glass

stabilization in the dry state (Tunnacliffe and Wise 2007; Tunnacliffe et al. 2010). There is, however, good evidence that some LEA proteins can stabilize membranes and/or enzymes during freezing and desiccation (Thalhammer and Hincha 2013).

There are a few features which share most LEA proteins: a high hydrophilicity, a low content of hydrophobic amino acids, a high portion of charged amino acids, a low content or complete lack of cysteine as well as tryptophan and some of LEA proteins are rich in glycine (Tunnacliffe and Wise 2007; Battaglia et al. 2008; Hundertmark and Hincha 2008). Plant LEA proteins are classified into several groups (Tab. 1), however, the grouping of proteins is not consistent in the literature (more details in Tunnacliffe and Wise 2007; Hundertmark and Hincha 2008). One of LEA groups is a family of so-called dehydrins (also known as group 2 LEA proteins or LEA D11) (Battaglia et al. 2008). Dehydrins are plant-specific proteins, whereas members of the other LEA groups are also present in other organisms (Eriksson and Harryson 2011).

Tab. 1. The different types of LEA-type protein classification. The classification of LEA protein has been rearranged several times from the time of their first description. Adapted from Amara et al. 2014.

Pfam	Dure et. al 1989	Bray 1993	Tunnacliffe, Wise 2007	Battaglia et al. 2008	Bies-Estheve et al. 2008	Hundertmark, Hincha 2008	LEA protein database (LEAPdb) 2010
PF00257	D11	group 2	group 2	group 2	group 2	dehydrin	classes 1 to 4
PF00477	D19 D132	group 1	group 1	group 1	group 1	LEA_5	
PF02987	D7 D29	group 3 group 5	group 3	group 3A group 3B	group 6	LEA_4	classes 6
PF03168	D95			group 5C	group 7	LEA_2	classes 7 and 8
PF03242	D73		LEA_5	group 5B	group 6	LEA_3	classes 9
PF03760	D113	group 4	group 4	group 4A group 4B	group4	LEA_1	classes 10
PF04927	D34	group 6	group 6	group 5A	group 5	SMPO	classes 11
PF03168				group 6 group 5A	group 8	PvLEA18	classes 12

Dehydrin name comes from their early proposed function in plants surviving drought (Close et al. 1989). In orthodox seeds (like tomato seeds), the production of DHNs is associated with the desiccation of the embryo as a part of the seed maturation process (Ingram and Bartels 1996). Dehydrins are expressed at high levels under low cellular water content or after ABA treatment. Many dehydrin genes are responsive to more than one stress (Eriksson and Harryson 2011). Dehydrins, similarly as most of other LEA proteins, belong into the group of natively unfolded or intrinsically unstructured proteins, the proteins that lack a fixed 3D structure (Dunker et al. 2002; Uversky et al. 2005), however a propensity for alphahelix formation was observed (Tunnacliffe et al. 2010). On account of this feature, dehydrins stay soluble even after boiling that is used in their purification procedures (such as one from Oliveira et al. 2007). Dehydrins are characterised by the existence of conservative sequences, which are known as K-, S- and Y-segments (Close 1996) and especially the presence of a K-segment (a highly conservative lysin-rich motif) is obligatory. Interestingly, the K-segment resembles a lipid-binding domain found in other proteins such as apolipoproteins and α-synuclein (Close 1997; Davidson et al. 1998) and it was hypothesized that dehydrins could undergo function-related conformational changes at the water/membrane interface, perhaps related to the stabilization of vesicles or endomembrane structures under stress conditions (Koag et al. 2003). Besides, it seems that DHNs accumulate near the plasma membrane or membrane-rich areas surrounding lipid and protein bodies (Asghar et al. 1994; Egerton-Warburton et al. 1997; Danyluk et al. 1998; Puhakainen et al. 2004). An S-segment consists of serine residues and it is a site of dehydrin phosphorylation (Allagulova et al. 2003), which leads to dehydrin translocation into the nucleus (Close 1996, 1997; Campbell and Close 1997). The Y-segment is usually located at the N-terminal region of dehydrins and it shares significant homology with the nucleotide-binding site of chaperones (Close 1996, 1997). Furthermore, dehydrins also contain less conservative

regions enriched with glycine and polar amino acids (Close 1997; Ismail et al. 1999). Each dehydrin can be subclassified on the basis of these domains (Close 1997).

Dehydrin encoding genes are upregulated in response to various stresses and ABA treatment (Close et al. 1989; Robertson and Chandler 1992; Reynolds and Bewley 1993; Busk and Pages 1998; Lang et al. 1998; Choi et al. 1999; Nylander et al. 2001; Jiang and Huang 2002; Welling et al. 2002). Moreover, light (white and red spectra of light) can enhance dehydrin transcripts accumulation (Natali et al. 2007). The effect of BL on expression of dehydrin genes was not studied yet.

Dehydrins (DHNs) can be found especially in the cytoplasm and the nucleus. Their distribution between cytoplasm and nucleus most probably depends on protein phosphorylation status (as mentioned above), which in turn can be controlled by stress conditions (Goday et al. 1994; Alsheikh et al. 2003; Brini et al. 2007; Mehta et al. 2009). DHNs accumulate in different tissues during plant growth and development and in response to stress (Nylander et al. 2001; Rorat 2006). They probably play a role in physical protection of the cell from water deficit or temperature changes. They are among the most frequently observed proteins in plants under water stress (Suprunova et al. 2004). Dehydrin proteins might undergo function-related conformation changes at the water/membrane interface. This could be associated with the stabilization of vesicles or endomembrane structures under stress conditions (Ismail et al. 1999; Koag et al. 2003).

1.6. BUPR domain-containing genes/ proteins

Since plant growth and productivity are significantly affected by environmental conditions, numerous stress-responsive genes have been studied and used to improve the stress tolerance of crops. A great potential in improvement of stress resistance show BURP domain-containing genes/proteins (Wang et al. 2011). The members of a plant specific BURP family

share at their C terminus a highly conserved BURP domain whose name is based on four members: **B**NM2, **U**SP, **R**D22 and **P**G1β (Hattori et al. 1998). The BURP family members can be classified into BNM2-like, USP-like, RD22-like, PG1β-like, BURP V, BURP VI and BURP VII subfamilies, while the last three subfamilies were found in rice (Granger et al. 2002; Ding et al. 2009).

Many BURP domain-containing genes have been identified in various plant species and they have diverse expression patterns. For example, expression of *RD22* (Responsive to Dehydration 22) can be induced by drought, abscisic acid (ABA) and salt stress (Yamaguchi-Shinozaki and Shinozaki 1993). BURP genes play diverse roles in the cells, *PG1β*, a β-subunit of polygalacturonase isoenzyme 1, is involved in pectin depolymerization and cell-wall softening during tomato fruit ripening (Zheng et al. 1992). However, the precise functions of BURP domain-containing genes and proteins are still obscure. The previous reports have attributed two possible functions. Firstly, they are important in plant development and metabolism, possibly, they could participate in cellular secretion pathway during embryogenesis, seed, fruit and root development (Wang et al. 2011). Secondly, many BURP domain-containing proteins are responsive to stress treatments, thus their involvement in stress adaptation can be expected (Urao et al. 1993; Iwasaki et al. 1995; Abe et al. 1997).

1.7. Tomato *7B-1* mutant

The subject of my study was a unique tomato (*Solanum lycopersicum* L.) *7B-1* mutant. *7B-1* is a spontaneous recessive single-gene mutant which was isolated as a photoperiod sensitive male-sterile line (genetic background cultivar Rutgers). In long days (16-h light/8-h dark) flowers of *7B-1* plants are male-sterile, whereas in short days (8-h light/16-h dark) they are fertile (Sawhney 1997, 2004). Since its isolation *7B-1* has been intensively studied particularly by our group and groups of Professor Vipen K. Sawhney (University

of Saskatchewan, Saskatoon, Canada) and Professor Andrea Mazzucato (University of Tuscia, Viterbo, Italy), whose researches are focused especially on plant male-sterility.

The *7B-1* mutation includes the alternations in the structure of the anthers, which have shrunken stamens and produce non-viable microspores in long days flowers (Sawhney 1997). Recently, *SlGLO2* was proposed to be a candidate gene for *7B-1* mutation (Pucci 2017). *SlGLO2* belongs to class B of MADS-box genes which are involved in stamen development (Geuten and Irish 2010). Besides, the potential role of various miRNA in regulation of male-sterility in *7B-1* mutant was studied (Omidvar et al. 2015).

On the other hand, the germination tests showed that seed germination in *7B-1* mutant is more resistant to the inhibitory effects of an osmotic stress induced by mannitol and polyethylene glycol (PEG), to various salts (including NaCl, Na_2SO_4, KCl and K_2SO_4) and to low-temperature stress compared to cv. Rutgers seeds specifically under blue-light conditions (Fellner and Sawhney 2001, 2002). This indicates that blue light is able to control or amplify the inhibitory effect of abiotic stresses on seed germination and that *7B-1* seems to be affected in blue-light perception or blue-light signalling pathways (Fellner and Sawhney 2001). Based on other experiments (Bergougnoux et al. 2012; Ježilová et al. 2012; Hlavinka et al. 2013), it was proposed that the *7B-1* mutation could impair phototropin signalling pathway. Moreover, the *7B-1* mutation has a pleiotropic effect, including increased seed size and weight. *7B-1* seeds have an elevated level of ABA but reduced levels of gibberellins, ethylene, auxin and cytokinins (Fellner et al. 2001; Fellner and Sawhney 2002; Fellner et al. 2005; Bergougnoux et al. 2012).

An important role to both seed germination and adaptation to abiotic challenges play nitric oxide (NO) (Garcia-Mata and Lamattina 2001; Bethke et al. 2004; Šírová et al. 2011; Piterková et al. 2012). NO breaks seed dormancy and stimulates seed germination in many plant species by regulating ABA catabolism and endogenous ABA levels in response

to external stimuli (Liu et al. 2010; Piterková et al. 2012). Piterková et al. (2012) found that inhibition of tomato seed germination by blue light is, at least partially, mediated by the absence of NO in seeds. Besides, a reduced level of NO is probably associated with the inhibitory effects of ABA and mannitol-induced osmotic stress on seed germination (Piterková et al. 2012). Mannitol inhibits seed germination by increasing the level of endogenous ABA (Fellner and Sawhney 2002; Piterková et al. 2012). ABA accumulation can be reduced by NO signalling, thus ABA and NO act antagonistically during seed germination (Piterková et al. 2012). It is expected that under mannitol-induced osmotic stress ABA-overproducing *7B-1* mutant seeds amasses less additional ABA than WT seeds, which results in increased accumulation of NO, and thus in higher NO-mediated catabolism of ABA (for more details see model in Piterková et al. 2012).

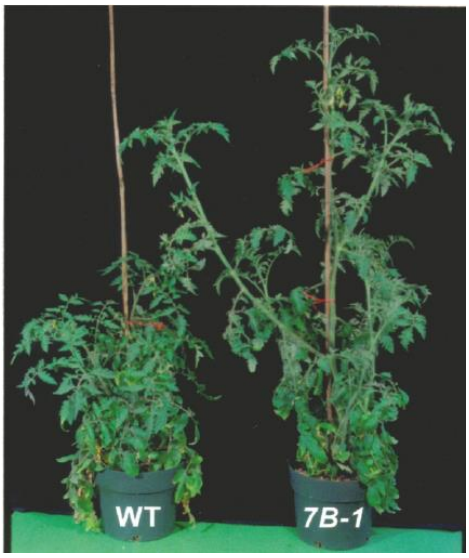

Fig. 6. Morphology of 3-month old WT and *7B-1* mutant plants. Adapted from Fellner et al. (2005).

7B-1 mutant is very interesting not only at the stage of seed germination but also because *7B-1* plants (the WT and *7B-1* mutant plants are shown in Fig. 6) possess many interesting features. *7B-1* mutant plants contain higher amount of chlorophyll (Fellner et al. 2005; Sheoran et al. 2006; Bergougnoux et al. 2009) and less anthocyanin than WT

plants (Sheoran et al. 2006; Bergougnoux et al. 2009). The mutant shows changes in stomatal conductance, photosynthetic rate and water-use efficiency (Ježilová et al. 2012). Besides, *7B-1* has a defect in light induced stomatal opening. Thus, *7B-1* stomata are almost resistant to the stimulatory effect of the blue light (BL). Experiments with fusicoccin (a fungal toxin that is able to activate the guard cell plasma membrane H^+-ATPase), with various anion channel blockers and ABA suggest that the *7B-1* mutation affects signalling involved in the inhibition of anion channels important for stomatal opening (Hlavinka et al. 2013). Finally, the *7B-1* plants were found to be less sensitive to coronatine, the phytotoxine produced by a hemi-biotrophic pathogen *Pseudomonas syringae* pv. *tomato*, in a BL-dependent manner (Bergougnoux et al. 2009). The results indicate the involvement of BL-signalling in sensitivity to biotic stress.

2 AIMS OF THE THESIS

The objective of this thesis was to investigate a role of plasma membrane aquaporins (*PIPs*) in the cross-talk between abiotic stress signalling and blue light (BL) signalling pathways during process of seed germination. The experimental strategy was based on the combination of physiological and molecular approach. Therefore, the experimental aim of the thesis was to characterize the expression of plasma membrane aquaporin during seed germination and early post-germination and to test for the existence of a BL and osmotic-stress control over these processes. The effect of mercury-induced aquaporin blockage, osmotic stress and BL on the number of germinated tomato seeds was studied as well. For this, tomato *Solanum lycopersicum* L. (cv. Rutgers) and *7B-1* mutant were used as model experimental plants.

The mechanisms involved in plant stress tolerance are very complex and many genes are believed to be involved in them. A main response to challenging conditions is the change in gene expression and synthesis of different types of protective molecules. Thus, we simultaneously studied the effect of BL and osmotic stresses on the expression profiles of one *BURP*-domain containing gene (*RD22-like* gene) and several *LEA* genes (including dehydrins) in tomato WT and *7B-1* mutant seeds. *LEA* and dehydrin expressions were quantified also at the protein level.

3 MATERIALS AND METHODS

3.1. PLANT MATERIAL

Seeds of tomato *Solanum lycopersicum* L. cultivar Rutgers (WT) and mutant *7B-1* (Sawhney 1997) were used in all experiments (for detailed mutant description see chapter Introduction).

3.2. SEED GERMINATION TESTS

The procedure of seed germination test was described in Balarynová et al. (2018) and Balarynová and Fellner (2018). In brief, the surface sterilized seeds were cultivated in conditions *in vitro* in the Petriho dishes filled with basal MS medium (Murashige and Skoog 1962) or with medium supplemented with mercuric chloride ($HgCl_2$, in concentrations 20, 30, 40, 45 or 50 μM) or mannitol (20 or 70 mM) or polyethylene glycol (PEG, 2 or 5%). The plates with seeds were cultivated in the controlled growth chambers (Microclima 1000E, Snijders Scientific B. V., Netherlands) in continuous blue light (BL) conditions or they were wrapped in tinfoil (the dark) and placed into the same growth chamber. The BL sources were blue tubes TLD-36W/18-Blue (Philips, USA) with a maximum irradiance at 460 nm. Total photon fluence rate of light was 10 $\mu mol.m^{-2}.s^{-1}$.

The percentage of seed germination ((germinated seeds/total number of seeds on the dish) x 100) was counted every day for 7 days and then the average percentage of seed germination was calculated. Seven experiments on medium supplemented with 20 and 30 μM $HgCl_2$, 4 experiments with 40, 45 and 50 μM $HgCl_2$, 11 experiments on medium supplemented with 20 and 70 mM mannitol and 8 experiments with 2 and 5 % PEG were done. For the control, seeds were sown on the basal MS was in each experiment.

3.3. GENE EXPRESSION ANALYSIS

3.3.1. Seed collection

The seed samples were collected into liquid nitrogen. To investigate expression profiles of seeds with and without radicle, the seeds with and without visible radicle were collected 96-h after seed sowing. To study the effect of aquaporin blockage by mercuric chloride ($HgCl_2$) and BL on tomato seed germination, *7B-1* and WT seeds with visible radicle (1-10 mm long) were collected after 96-hour cultivation in the presence of 30 μM $HgCl_2$ in the dark or under BL conditions. To study the effect of 20 and 70 mM mannitol or 5% PEG treatment on seed germination, the seeds with radicle up to 10 mm were collected after 96-hour cultivation on MS medium supplemented with 20 mM mannitol, 70 mM mannitol or 5% PEG. On MS medium and on MS supplemented with 70 mM mannitol, also the seeds without visible radicle were collected 96-h after seed sowing.

Seeds germinated under BL were harvested under BL conditions. Seeds germinated in the dark were harvested under green safelight. Collected seed samples were stored in -80°C until RNA isolation.

3.3.2. RNA isolation and cDNA synthesis

The seed samples were ground under liquid nitrogen into fine powder. Total RNA was extracted as described in Balarynová et al. 2018 and Balarynová and Fellner 2018. Before cDNA synthesis, residual DNA was removed from samples by Recombinant DNaseI treatment (Takara Bio Inc., Japan) and Recombinant RNase Inhibitor (Takara Bio Inc., Japan) for 60 minutes at 37°C. DNaseI was inactivated according to manufacturer´s instruction. Finally, first-strand cDNA mixtures were prepared from 0.7 μg of total RNA using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Bio).

3.3.3. Quantitative RT-PCR

The specific primers were designed by PrimerQuest® program (IDT, Coralville, USA) and their specificity was checked by observing the melting curve of the qR-PCR products. Besides, the products of PCR reaction were checked on 3% agarose gel. The sequences of primers used are given in Table 2 and in Balarynová et al. 2018.

Tab. 2. Sequences of primers used in qRT-PCR analysis. The primers were designed using PrimerQuest® program, IDT, Coralville, USA (retrieved 12 December, 2012).

Gene	Accession number	Forward primer	Reverse primer
PIP1;1	BP887068	ACAAGGACTACAAAGAGCCAC	GATTTAGAAACGCCCATGACG
LEA	NM_001251869.2	GGTCAAACACTGGAAAGCTG	TCGTCTTATCATGGGTTGCC
DHN5	XM_004230885.3	GGTAACCCTATGCACCTTACTG	CAGCACCTAACCCAGTTGTAG
DHN9	Solyc02g084840.2	TGAAGGAGAAATTGCCAGGAG	ACGGCTTCAATTCCTAGTGG
RD22-like	ACD49740.1	CAAAAGTTA AGGCTG TGGCTG	ATGACAAACAGGGACGGATC

The gene expression analysis was conducted as described in Balarynová et al. 2018 and Balarynová and Fellner 2018. Three replicates were used for each sample and melting curve was analysed at the end of PCR reaction. *PP2Acs* (protein phosphatase 2A catalytic subunit) (Lovdal and Lillo 2009) and *TIP41like* (Dekkers et al. 2012) genes were used as references. The primer efficiency was calculated from the slope of the dilution curve. The series of 50, 100, 500 and 1000-fold diluted cDNA was prepared and measured in triplicate.

The relative gene expression was calculated using REST (Relative Expression Software Tool) software (Pfaffl et al. 2002). The sample of WT seeds cultivated on MS medium in the dark was used as a control. In the experiments with dry seeds, the sample of dry WT seeds was used as a control. In experiments comparing the gene expression in germinated and non-germinated seeds, non-germinated WT seeds were used as a control.

Moreover, REST software analysed also the significant differences (at 5% significance level) amongst studied treatments (see section Statistical analysis).

3.4. STATISTICAL ANALYSIS

Since the data were not normally distributed, the significant differences between control (seeds on MS medium) and various stress treatments (20-50 μM $HgCl_2$ or mannitol/PEG induced osmotic stress treatments) were assessed using Kruskal-Wallis test (a non-parametric test) with multiple comparisons at the 0.05 significance level. The dark-grown samples were tested against D-control (seeds on MS). The BL-grown samples were tested against BL-control. For each treatment, the effect of BL compared to the dark was evaluated using Mann-Whitney U test (a non-parametric test) at the 0.05 significance level. Statistical analyses were done using the STATISTICA 12 software (StatSoft, OK, USA). The results are showed as box plots, where the lower and upper ends of the box are the first and third quartiles, respectively. The horizontal line inside the box indicates the median value (the square inside the box is a mean) and the whiskers represent the adjacent values (1.5 times interquartile range). The lines extending from the top and bottom of each box mark the adjacent values within the data set that fall within 1.5 times interquartile range. Outlier points are those whose values are either greater than upper quartile + (1.5 x interquartile distance) or less than lower quartile - (1.5 x interquartile distance).

The gene expression was quantified using REST (Relative Expression Software Tool) 2009 software (Pfaffl et al. 2002). This software takes into account the different PCR efficiencies of the studied genes, reference genes normalization and C_T-values of each sample. The sample of WT seeds cultivated on MS medium in the dark was used as a control, thus the relative expression was calculated against the expression of this sample. Beside estimation of relative expression, REST software provides a non-parametric statistical test based

on a pair wise fixed reallocation randomization test (at 0.05 significance level) to determine a significant difference between samples and control.

4 RESULTS and DISCUSSION

4.1. The expression patterns of *PIPs* during different stages of seed germination and early post-germination

Using the common aquaporin blocker, mercuric chloride ($HgCl_2$), it was confirmed that aquaporins are not involved in tomato seed germination during imbibition of water by dry seed (Balarynová and Fellner 2018). This finding is consistent with the results presented by Veselova and Veselovsky (2006); Willigen et al. (2006); Obroucheva (2012). Dry seeds are known to contain a number of aquaporins which were formed during seed development and maturation (Obroucheva and Antipova 2000; Obroucheva 2012, 2017). Likewise, my experiments confirm the presence of *PIP* transcripts in dry tomato seeds. Moreover, the gene expression analyses demonstrated that amount of *PIP* transcripts increased after radicle protrusion, during radicle elongation, indicating the role of plasma membrane aquaporins in radicle growth (Balarynová and Fellner 2018). This finding is consistent with findings of Liu et al (2007) in rice. In rice seeds, the expression of *OsPIP1;1, OsPIP1;2, OsPIP1;3, OsPIP2;1, OsPIP2;4, OsPIP2;5* and *OsPIP2;8* aquaporin genes increased strongly before radicle emergence (Liu et al. 2007). Generally, the expression of AQP genes begins when the hydration level of the dry seeds reach level of 50-55% of fresh weight (Obroucheva and Antipova 2000; Obroucheva 2012).

In tomato seeds, *PIP1;2*, *PIP1;5*, *PIP2;1* and *PIP2;3* genes were expressed more than *PIP1;1*, *PIP1;3*, *PIP1;4* and *PIP2;2* genes during tomato seed germination and early post-germination on MS medium. The gene expressions of tested *PIPs* followed a similar patterns in dark- and BL-germinated seeds over the period of 24, 72 and 96 hours after seed sowing (Balarynová and Fellner 2018). The question is whether they are different in the presence of mercury and under osmotic stress.

4.2. The expression patterns of *PIPs* in seeds without or with visible radicle

In order to compare expressions of studied *PIP* genes in germinated and non-germinated seed samples, seeds with visible radicle (1-10 mm long) and seeds without visible radicle were collected after 96-hour germination on MS medium in the dark and in BL. The analysis using the REST software for both relative quantification and statistical analysis showed that *PIP* gene expression can be upregulated (*PIP1;3*, *PIP1;5*, *PIP2;2*, *PIP2;3*), downregulated (*PIP1;2*, *PIP1;4*) or not changed (*PIP2;1*) in germinated seeds compared to seeds without visible radicle (Fig. 7). Interestingly, the amount of *PIP1;1* transcript was kept at the same level in BL-grown seeds with visible radicle as in non-germinated seeds whereas it was markedly reduced in D-grown seeds of both genotypes (Fig. 7). This could reflect the importance of PIP1;1 protein to radicle growth in BL before as well as after radicle protrusion.

Altogether, divergent trends in *PIP* gene expressions between germinated and non-germinated seeds might reflect their different functions during seed germination, radicle protrusion and elongation. Above all, *PIP1;3*, *PIP1;5*, *PIP2;2*, *PIP2;3* seemed to be involved in radicle elongation after testa penetration since their expression levels were more pronounced in seeds with visible radicle compared to non-germinated seeds (Fig. 7).

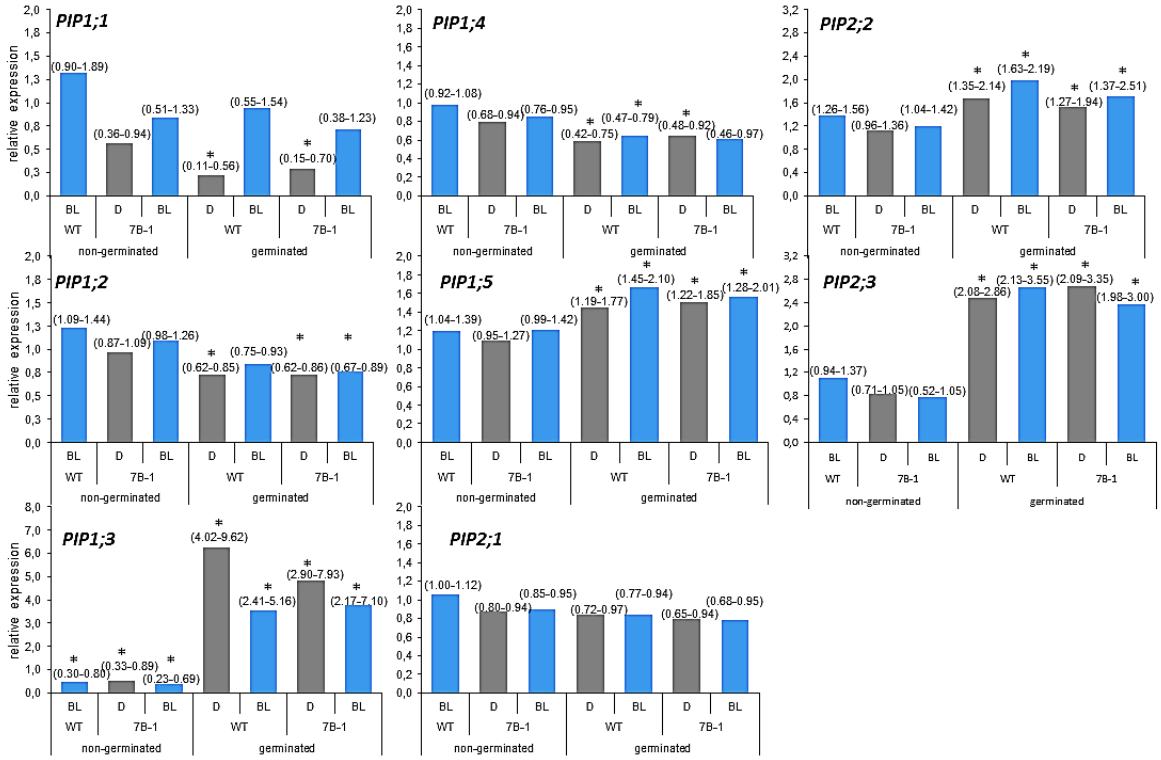

Fig. 7. The expression of *PIP* genes in WT and *7B-1* mutant seeds after 96-h cultivation in the dark (D) and in blue light (BL). The non-germinated seeds were seeds without visible radicle whereas the germinated seeds had a radicle (1-10 mm long). The data shows the relative expression computed using REST software and SE of three independent experiments. The relative expression was quantified against the expression from sample of non-germinated WT seeds in the dark (a control), whose expression had therefore the value 1. Asterisks indicate significant differences ($p<0.05$) to the control.

4.3. The effect of mercuric chloride on the *PIP* gene expression

Previous study of Shiota et al. (2006) showed the role of aquaporins in tomato seed development and proposed their involvement in tomato seed germination. Involvement of *PIPs* in seeds germination is also expected in seeds of rice, pea, *Arabidopsis thaliana* or *Brasicca napus* (Gao et al. 1999; Schuurmans et al. 2003; Willigen et al. 2006; Liu et al. 2007). Since mercuric chloride ($HgCl_2$) is a common aquaporin blocker used in plant physiology, the effect of this blocker on the average percentage of tomato seed germination was studied to show whether the aquaporin blockage affect tomato seed germination.

The increasing concentrations of $HgCl_2$ (20, 30, 40, 45 and 50 μM) were applied to tomato WT and *7B-1* mutant seeds (Fig. 8) germinating in the dark as well as in BL. For WT seeds (Fig. 8A), the inhibitory effect of 30 μM and higher concentrations of $HgCl_2$ was showed to be significant compared to corresponding control on MS medium in both the dark and BL. Seed germination in WT seeds was markedly reduced by 20 μM $HgCl_2$ in the dark as well as in BL but this trend was not proved to be statistically significant. Blue light decreased significantly the percentage of germinated WT seeds only on MS medium (not marked in Fig. 8A). Similarly, *7B-1* mutant seed germination was lowered significantly by mercury concentrations 30 μM and higher in the dark (Fig. 8B). Interestingly, under BL conditions, the inhibitory effect of mercury on *7B-1* mutant seeds was significant only on 45 and 50 μM $HgCl_2$. Blue light decreased significantly the percentage of germinated *7B-1* mutant seeds only on MS medium (not marked in Fig. 8B). The mercury-induced reduction of seed germination could be accounted for by blockage of mercury-sensitive aquaporins, which was proposed several times (Martinez-Ballesta et al. 2003; Boursiac et al. 2005). The data demonstrated the lower responsiveness of *7B-1* mutant seed germination to mercuric chloride compared to WT seed germination, which was markedly reduced by mercury-induced aquaporin blockage. This could suggest the higher amount of aquaporins in *7B-1* mutant seeds. Interestingly, the data did not show the difference in sensitivity of tomato seed germination to $HgCl_2$ between dark and BL conditions.

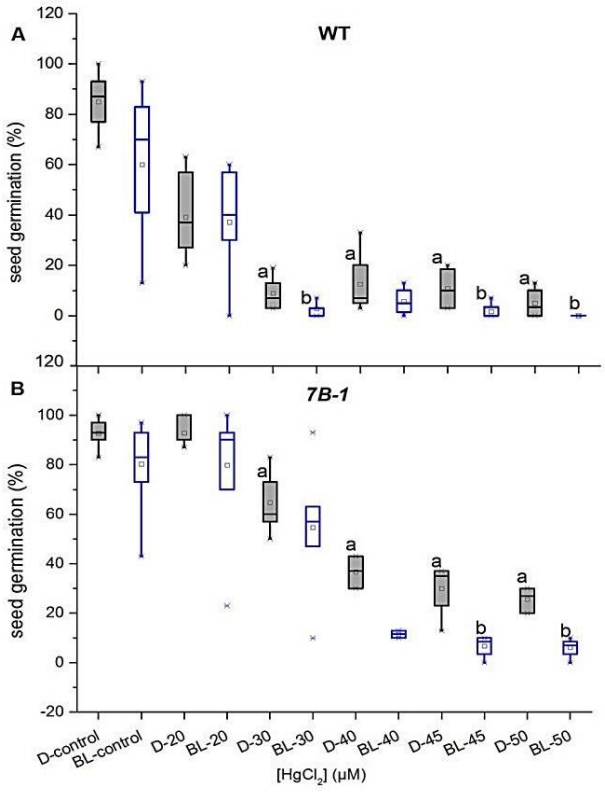

Fig. 8. The germination of tomato cv. Rutgers (WT) (A) and *7B-1* mutant (B) seeds incubated for 7 days in the dark (D) or in blue light (BL) in the presence of increasing concentrations of mercuric chloride ($HgCl_2$). The boxes borders are the first and third quartiles with a median value of seed germination marked inside the box. The whiskers indicate outliers (1.5 times interquartile range). For each treatment, at least four independent experiments were done. Thirty seeds were sown on every dish. The significant differences tested using Kruskal-Wallis test at 0.05 significance level to D- control are marked as a, the significant differences to BL-control are marked as b.

At the transcript level, a semi-quantitative RT-PCR screening for changes of *PIP* gene expressions induced by mercury treatment (30 μM $HgCl_2$) reported a decrease in *PIP1;3* gene expression in WT and *7B-1* mutant seeds germinated in the presence of mercury (other *PIPs* seemed to be impervious to $HgCl_2$, Balarynová and Fellner 2018). This trend was confirmed using a quantitative RT-PCR (Fig. 9). It was showed to be significant for WT seeds germinated in the dark as well as in BL and marginally significant (P=0.05) for *7B-1* mutant seeds germinated in the dark. A decrease in *PIP1;3* expression did not reach a statistical significance at 0.05 level (P=0.085) in *7B-1* mutant seeds germinated under BL condition.

Nevertheless, *7B-1* mutant seed germination seemed to be less responsive to $HgCl_2$ than WT seed germination and even *PIP1;3* gene transcript level in *7B-1* seemed to be less reduced by $HgCl_2$ than *PIP1;3* transcript level in WT seeds. The importance of *PIP1;3* gene (together with *PIP1;1*) in seed germination was showed in rice seeds (Liu et al. 2007). Could the *PIP1;3* transcript level in germinated seeds correspond with the responsiveness of seed germination to mercury?

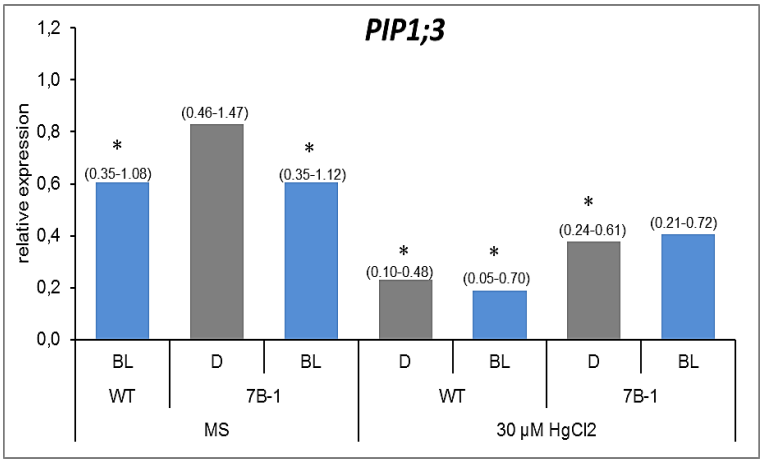

Fig. 9. The relative expression of *PIP1;3* gene in WT and *7B-1* mutant seeds after treatment with 30 µM mercuric chloride ($HgCl_2$). The seeds germinated for 96 h in the dark (D) or under blue light (BL). The expression was measured by qRT-PCR and quantified against the expression from sample of WT seeds germinated in the dark (a control, its expression had the value 1) using REST. The data shows the relative expression and SE of at least three independent experiments. Asterisks indicate significant differences ($p<0.05$) to the control.

4.4. The effect of the *7B-1* mutation on *PIP* gene expression

At the level of seed germination, the *7B-1* mutant seeds always germinated more than corresponding WT seeds (Figs. 8 and 13). This fact leads to the question if there is any significant impact of the *7B-1* mutation on the expression of *PIP* genes. Comparative study of trends in the expression of various *PIP* genes during different stages of seed germination and early post-germination (in seeds harvested 24, 72 and 96 hours afer seed sowing) showed that the patterns of *PIP* gene expression were similar in WT and *7B-1* seeds (Balarynová

and Fellner 2018). Interestingly, dry *7B-1* mutant seeds contained lower amount of *PIP* transcripts than dry WT seeds (Balarynová and Fellner 2018).

A REST analysis of seeds germinated in the dark and BL showed that *PIP1;2* gene (Fig. 10) was expressed differently in the *7B-1* mutant compared to WT seeds. The *PIP1;2* gene expression was stimulated in WT seeds germinated under BL, whereas this stimulation was not found in *7B-1* seeds germinated under the same conditions. The explanation of the role of this aquaporin in the ability of the *7B-1* mutant to germinate more effectively than WT under BL is not clear. One of the possible explanations could be that *PIP1;2*, as one of the most expressed gene in tomato seeds (Balarynová and Fellner 2018), may participate in preservation of water for radicle growth during seed germination. Thus, *7B-1* mutant seeds may be prepared for germination better than WT seeds.

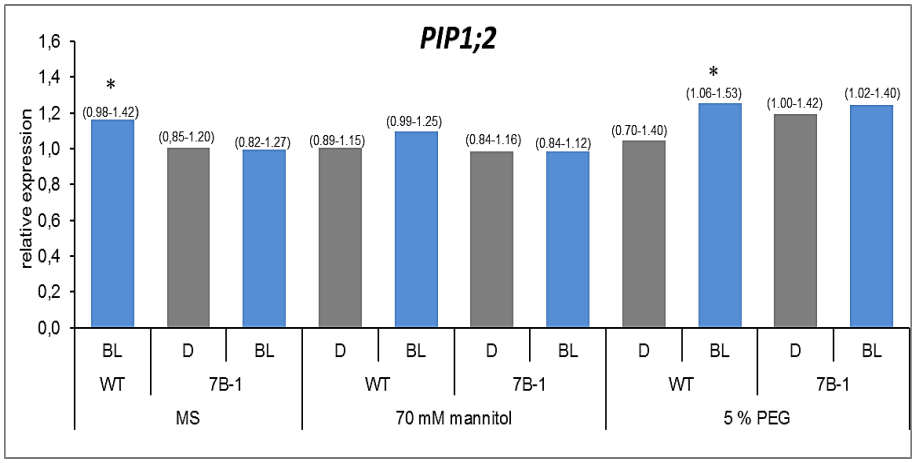

Fig. 10. The relative expression of *PIP1;2* gene in WT and *7B-1* mutant seeds under osmotic stress induced by mannitol and PEG treatment. The seeds germinated for 96 h in the dark (D) or under blue light (BL). The expression was measured by qRT-PCR and quantified against the expression from sample of WT seeds germinated in the dark (a control, its expression had the value 1) using REST. The data shows the relative expression and SE of at least three independent experiments. Asterisks indicate significant differences ($p<0.05$) to the control.

For the sake of completeness, the significant impact of the *7B-1* mutation on *PIP1;3* gene expression (Fig. 11) was found. In dark-germinated seeds under osmotic stress induced

by both 70 mM mannitol and 5% PEG, the *7B-1* mutation decreased the expression of *PIP1;3* in *7B-1* seeds. The effect of the *7B-1* mutation under BL conditions is not clear. It seemed that *PIP1;3* gene expression in BL-germinated seeds is reduced by BL and osmotic stress in both WT and *7B-1* mutant seeds.

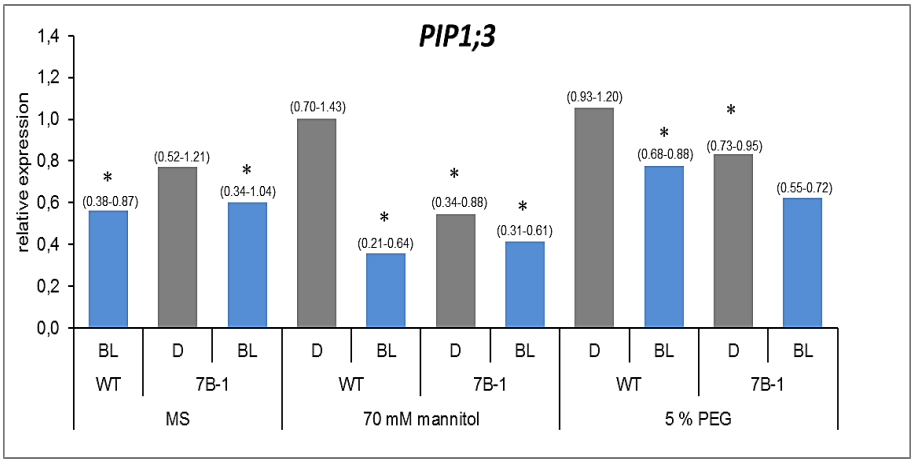

Fig. 11. The relative expression of *PIP1;3* gene in WT and *7B-1* mutant seeds under osmotic stress induced by 70 mM mannitol and 5% PEG treatment. The seeds germinated for 96 h in the dark (D) or under blue light (BL). The expression was measured by qRT-PCR and quantified against the expression from the sample of WT seeds germinated in the dark (a control, its expression had the value 1) using REST. The data shows the relative expression and SE of at least three independent experiments. Asterisks indicate significant differences ($p<0.05$) to the control.

4.5. The effect of blue light on *PIPs* gene expression

Seed-germination assays showed that BL had a significant impact on WT and *7B-1* mutant seed germination. The seed germination was always lowered by BL compared to the dark in both genotypes (Figs. 8 and 13).

REST software analysis highlighted the *PIP1;1* (Fig. 12), *PIP1;2* (Fig. 10) and *PIP1;3* (Fig. 11) genes as the BL-responsive plasma membrane aquaporins. Interestingly, blue light upregulated as well as downregulated the expression of mentioned *PIP* genes.

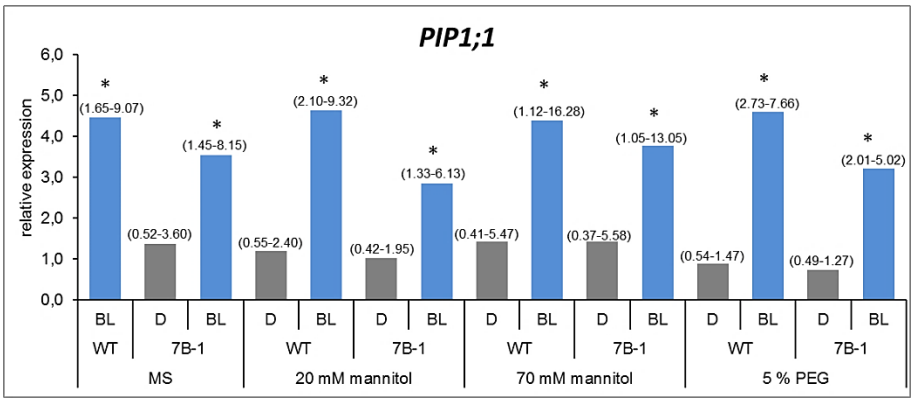

Fig. 12. The relative expression of *PIP1;1* gene in WT and *7B-1* mutant seeds under osmotic stress induced by mannitol and PEG treatment. The seeds germinated for 96 h in the dark (D) or under blue light (BL). The expression was measured by qRT-PCR and quantified against the expression from the sample ofWT seeds germinated in the dark (a control, its expression had therefore the value 1) using REST. The data shows the relative expression and SE of at least three independent experiments. Asterisks indicate significant differences ($p<0.05$) to the control.

The transcription of *PIP1;1* gene was induced by BL in both WT and *7B-1* mutant seeds (Fig. 12). The trend of BL-induced transcript accumulation was not changed even by the mannitol- or PEG-induced osmotic stress. There are several reports indicating responsivity of *PIP1;1* gene expression to abiotic stresses, for example in leaves of *Posidonia oceanica* (Serra et al. 2013), in roots as well as aerial parts of *Arabidopsis thaliana* plants (Jang et al. 2004) or in leaves and root of rice (Liu et al. 2013). The involvement of the PIP1;1-encoded protein in the fruit ripening was shown in *Fragaria x ananassa* (Mut et al. 2008). Also, PIP1;1 is positively involved in the cell expansion of *Rosa hybriad* petals (Chen et al. 2013). However, as far as I know, there is no a mention concerning ability of BL to stimulate *PIP1;1* gene expression. Our results could indicate the role of *PIP1;1* in radicle elongation under BL conditions. The fact that tomato seedlings growing in BL had longer roots than dark-grown seedlings (Balarynová and Fellner 2018) might hint that also radicle elongation could be stimulate by BL and thus that *PIP1;1* may be involved in BL-stimulated radicle and root elongation. Besides, the expression of *PIP1;1* gene and corresponding protein

in roots was detected in *Arabidopsis thaliana* (Santoni et al. 2003; Jang et al. 2004), rice (Liu et al. 2013) or grapevine (Vandeleur et al. 2009).

The transcript level of *PIP1;2* gene was stimulated significantly by BL in WT seeds (Fig. 10). This trend was obvious also under osmotic stress conditions, however, it reached statistical significance only under PEG-induced osmotic stress. Interestingly, the *PIP1;2* gene expression was affected significantly not only by BL but also by the *7B-1* mutation (conferring the lower sensitivity to BL), as mentioned above. This phenomenon clearly indicates the connection between the *7B-1* mutation and BL sensing/signalling. The fact that *PIP1;2* gene transcript level is modulated by both the *7B-1* mutation and BL raised the question whether the products of these genes could play a role in BL sensing or signalling? It was reported that *7B-1* mutation confers the defects in phototropin responses (Hlavinka et al. 2013; Bergougnoux and Fellner, unpublished data) and the possible function of PIP aquaporins as BL sensors (similar to phototropins) was proposed by Lorenz et al. (2003). On the other hand, in in walnut (*Juglans regia*) leaves light increased abundance of *PIP2;1* and *PIP2;2* (Cochard et al. 2007). In tomato seeds, the light-mediated changes in *PIP2;1* and *PIP2;2* were not detected. Moreover, the *PIP1;2* gene is intensively studied for BL-mediated control of its expression in plant leaves (Cochard et al. 2007), as discussed in Balarynová et al. (2018).

Finally, BL not only increased, but also decreased *PIP* gene expression. The expression of *PIP1;3* gene was reduced significantly by continuous BL in both WT and *7B-1* mutant seeds on MS as well as on the medium containing mannitol or PEG (Fig. 11).

4.6. The effect of osmotic stress on *PIPs* gene expression

Seed-germination tests showed that in the dark, WT seed germination (Fig. 13A) was reduced significantly only by 70 mM mannitol, while *7B-1* mutant seed germination (Fig. 13B) was decreased significantly by both 70 mM mannitol and 5% PEG (this effect was marginally significant, p-value 0.049). In BL, not only 70 mM mannitol, but also 5% PEG (in the dark the effect of 5% PEG was slightly not significant, p-value 0.060) had a significant impact on WT seed germination (Fig. 13A).

On both MS controls, *7B-1* mutant seeds germinated more than WT seeds, which is quite different from findings of Fellner and Sawhney (2001, 2002) who found that the percentage of WT and *7B-1* mutant seed germination is similar on MS controls. These discrepancies could be explained by different growth conditions, especially by using a different light source. Besides, Fellner and Sawhney did not find the difference in WT and *7B-1* mutant seed germination after mannitol treatments (100-140 mM) in the dark, which is inconsistent with the results of my experiments presented in (Fig. 13).

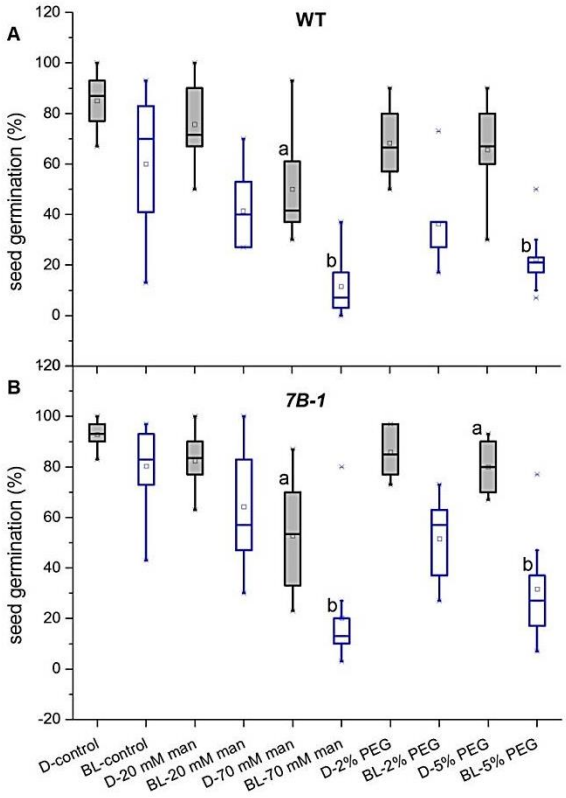

Fig. 13. The germination of tomato cv. Rutgers (WT) (A) and *7B-1* mutant (B) seeds incubated for 7 days in the dark (D) or in blue light (BL) under osmotic stress induced by mannitol and PEG. The boxes borders are the first and third quartiles with a median value of seed germination marked inside the box. The whiskers indicate outliers (1.5 times interquartile range). For each treatment, at least eight independent experiments were done. Thirty seeds were sown on every dish. The significant differences tested using Kruskal-Wallis test at 0.05 significance level to D- control are marked as a, the significant differences to BL-control are marked as b.

Mannitol in concentration 70 mM seemed to be efficient to inhibit WT and *7B-1* seed germination in the dark as well as in BL (Fig. 13). At the transcriptional level, the quantification and statistical evaluation of the gene expression data by REST showed the significant impact of 70 mM mannitol treatment on the amount of *PIP1;3* transcript (Fig. 11). The level of *PIP1;3* transcript was reduced significantly in WT seeds germinated under 70 mM mannitol-induced osmotic stress in BL (Fig. 11). On the other hand, *PIP1;3* was downregulated in both dark and BL germinated seeds in the *7B-1* mutant. This could be a mechanism to limit the water loss from the seeds. The expression of the rest of the studied

PIP genes remained stable after 70 mM mannitol treatment. Therefore, it can be assumed the importance of the stable expression of these aquaporins for seed germination and radicle elongation under stress conditions to maintain osmotic balance in germinating seeds.

At the transcript level, the effect of 20 mM mannitol on expression of *PIP* genes was studied. After three experiments, it seemed that 20 mM mannitol-induced osmotic stress did not affect significantly (at the 0.05 significance level) WT and *7B-1* mutant seed germination as well as expression of *PIP* genes (Fig. 12 for *PIP1;1*, Tables 1 and 2 Suppl. for other genes).

It was reported that effect of mannitol on plants is significantly less severe than the effect of PEG (Slama et al. 2007). Therefore, to screen for changes of *PIP* gene expression induced by osmotic stress, I investigated not only the effect of mannitol, but also the effect of PEG treatment on their transcript levels. Based on results of seed germination tests (Fig. 13), 5% PEG was used to induce osmotic stress on germinated WT and *7B-1* mutant seeds in the dark and under BL conditions. Using REST software, the expression levels of *PIP1;1* (Fig. 12), *PIP1;2* (Fig. 10), *PIP1;4*, *PIP1;5*, *PIP2;1*, *PIP2;2* and *PIP2;3* (Tab. 2 Suppl.) genes were found not to be changed by PEG treatment in both WT and the *7B-1* mutant seeds in the dark as well as in BL. The inhibitory effect of PEG on *PIP1;3* gene expression was detected in the dark-germinated *7B-1* seeds. Interestingly, this effect was not found in WT seeds geminated in the dark, and the effect of PEG was not so obvious in WT and *7B-1* mutant seeds germinated under BL conditions (Fig. 11).

The responsiveness of *PIP1;3* gene expression to BL as well as to osmotic stress corresponded with inhibitory effect of these stressors on tomato seed germination, which makes this gene very interesting for further research on possible interconnection of BL- and osmotic-stress signalling pathways.

Interestingly, both *7B-1* mutation and mannitol-induced osmotic stress had a similar impact on aquaporin transcript levels (downregulation). A possible consequence of a decrease in *PIP* transcript abundance caused by the mutation and osmotic stress might be a lower abundance of PIP proteins in the membranes, which could be a way leading to better osmotic adjustment of mutant seeds under osmotic stress compared to WT seeds. Thus, the seeds conserved more water for radicle protrusion and growth. Of course, this assumption needs experimental approval.

Altogether, both PEG (5%) and mannitol (70 mM) reduced WT and *7B-1* seed germination (Fig. 13) and expression of plasma membrane aquaporin *PIP1;3* (Fig. 11, Tab. 3). The results indicate that the *PIP1;3* gene may play significant roles in regulating osmotic balance of germinating seeds when they encounter environmental stresses. The sensitivity of *PIP1;3* gene expression to various abiotic stresses was shown several times especially in *Arabidopsis thaliana* and rice. Interestingly, both upregulation (for example, Jang et al. 2004; Lian et al. 2004, 2006; Abdelkader et al. 2012) and downregulation (for example, Lian et al. 2004; Alexandersson et al. 2005; Boursiac et al. 2005; Katsuhara et al. 2014) of its transcript level were found in the presence of various abiotic. Also, expression of *PIP1;3* gene was induced in response to embolism in *Populus trichocarpa* (Secchi and Zwieniecki 2010). It was shown to be turgor responsive and involved in regulating water status of *Arabidopsis thaliana* pollen (Shagan et al. 1993; Becker et al. 2003). Besides, *PIP1;3* was abundant in *Populus balsamifera* or *Arabidopsis thaliana* leaves (Jang et al. 2004) and it is expected to play a role in water redistribution in leaf tissues (Almeida-Rodriguez et al. 2010). Moreover, in *Nicotiana tabacum* leaves, PIP1;3 seemed to be involved in oxygen transport, which indicated its role in photosynthesis and cell signalling (Zwiazek et al. 2017).

Tab. 3. A summary of the impact of blue light (BL), the *7B-1* mutation and various stress treatments ($HgCl_2$, an aquaporin inhibitor; mannitol and polyethylene glycol, PEG, induced osmotic stress) on the expression of tested *PIP* genes. The table is a brief summary of the findings discussed above. The symbols used means upregulation (↑), downregulation (↓) and no significant effect (-) on *PIP* gene expression.

	gene							
treatment	***PIP1;1***	***PIP1;2***	***PIP1;3***	***PIP1;4***	***PIP1;5***	***PIP2;1***	***PIP2;2***	***PIP2;3***
BL	↑	↑	↓	–	–	–	–	–
7B-1 mutation	–	↓	↓	–	–	–	–	–
30 µM $HgCl_2$	–	–	↓	–	–	–	–	–
20 mM mannitol	–	–	–	–	–	–	–	–
70 mM mannitol	–	–	↓	–	–	–	–	–
5 % PEG	–	–	↓	–	–	–	–	–

4.7. Non-germinated seeds under osmotic stress

Changes in *PIP* genes expression were monitored also in non-germinated seeds (that means in seeds without visible radicle collected 96 hours after seed sowing) after mannitol treatment (Tab. 3 Suppl.). The expression analysis showed that mannitol affected significantly only the expression of *PIP1;1* gene (Fig. 14) in non-germinated seeds, more specifically, mannitol reduced the expression of *PIP1;1* in *7B-1* mutant seeds in the dark. Interestingly, this trend is similar to the trend observed in germinated seeds in which *PIP1;1* gene expression was also decreased in the dark. The response of non-germinated *7B-1* seeds to osmotic stress is difficult to explain, however, it could be a way to adapt the challenging environment during seed germination.

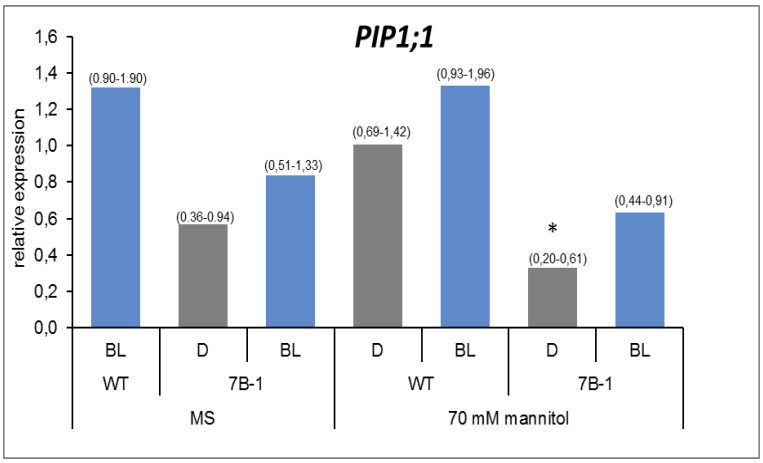

Fig. 14. The relative expression of *PIP1;1* gene in non-germinated WT and *7B-1* mutant seeds after 96-h germination in the dark (D) or in blue light (BL). The expression was measured by qRT-PCR and quantified against the expression from the sample of non-germinated WT seeds in the dark (a control, its expression had therefore the value 1) using REST. The data shows the relative expression and SE of at least three independent experiments. Asterisks indicate significant differences ($p<0.05$) to control.

4.8. *RD22-like* gene expression profile

The results of microarray analysis provided for us by Andreas Madlung team (University of Puget Sound, Tacoma, WA, USA) showed that the gene showing similarity to *Arabidopsis thaliana RD22* (SGN-U580823, GenBank accession number ACD49740.1) is overexpressed significantly in WT seeds germinated in BL compared to the *7B-1* mutant seeds germinated under the same conditions. The microarray assay compared germinated WT and *7B-1* mutant seeds (with radicle up to 10 mm) harvested after 96 h germination under continuous BL (unpublished data). The subsequent qRT-PCR analysis proved that the transcriptional level of *RD22-like* gene (Fig. 15) was induced significantly under BL and osmotic stress conditions in WT seeds. On the basal medium, the *7B-1* mutation reduced significantly the amount of *RD22-like* gene transcript in the dark as well as in BL. On the other hand, under osmotic stress, the expression of *RD22-like* gene was stimulated in the *7B-1* mutant seeds in BL

conditions, while the expression level in that condition was always lower than in WT (Fig. 15).

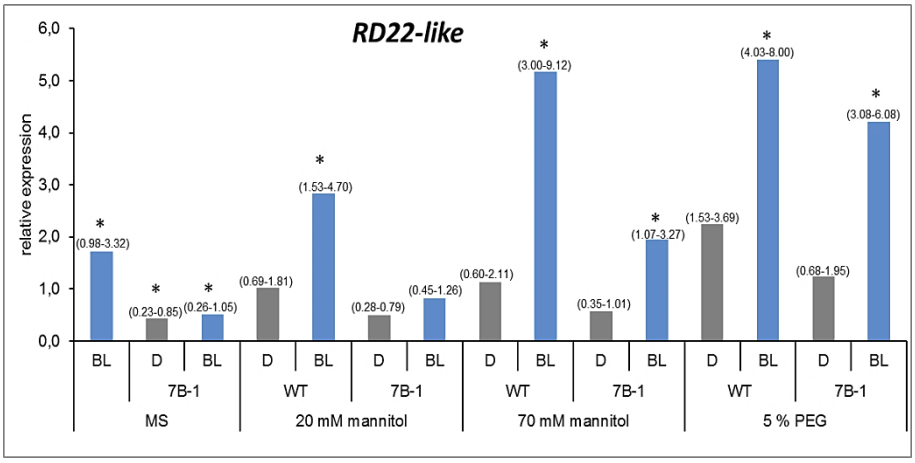

Fig. 15. The relative expression of *RD22-like* gene in WT and *7B-1* mutant seeds under osmotic stress induced by mannitol and PEG treatment. The seeds germinated for 96 h in the dark (D) or under blue light (BL). The expression was measured by qRT-PCR and quantified against the expression from the sample of WT seeds germinated in the dark (a control, its expression had therefore the value 1) using REST. The data shows the relative expression and SE of at least three independent experiments. Asterisks indicate significant differences ($p<0.05$) to control.

The stress-inducible patterns of *RD22* gene were showed in several plant species. The expression of *Arabidopsis thaliana RD22* gene was reported to be enhanced by water deficit, salinity and ABA (Urao et al. 1993; Yamaguchi-Shinozaki and Shinozaki 1993; Abe et al. 1997). In multree (*Morus multicaulis*), the *RD22* gene expression was induced by drought, low temperature or salinity (Wang et al. 2014). In *Vitis vinifera*, salinity stimulated the expression of *RD22* gene (Jamoussi et al. 2014). The protective role of the product of this gene is expected. Wang et al. 2012 reported the protective function of *Glycine max RD22* under abiotic stress conditions (Wang et al. 2012). Similarly, the protective role of grapevine *RD22* was showed in transgenic tobacco lines exposing to salinity (Jamoussi et al. 2014). Therefore, the participation of the product of *RD22-like* gene could be expected in tomato seeds as well.

The induction of *RD22* gene has been used as a marker for stress response pathway in plants (Song et al. 2009; Xu et al. 2010; Harshavardhan et al. 2014). From this point of view, it is interesting that the *7B-1* mutation conferring the lower sensitivity to abiotic stresses downregulated significantly the expression of *RD22-like* gene in the seeds in the dark as well as in BL under normal conditions (MS). Under osmotic stress, the stimulation of *RD22-like* gene expression was not so strong in the *7B-1* seeds compared to WT seeds (Fig. 15). Could this indicate that the *7B-1* mutant seeds are prepared to deal with osmotic stress? They could contain higher amount of osmoprotectants or other proteins responsible for protection of the cells against adversities, such as heat shock proteins, aquaporins or LEA proteins (Harshavardhan et al. 2014).

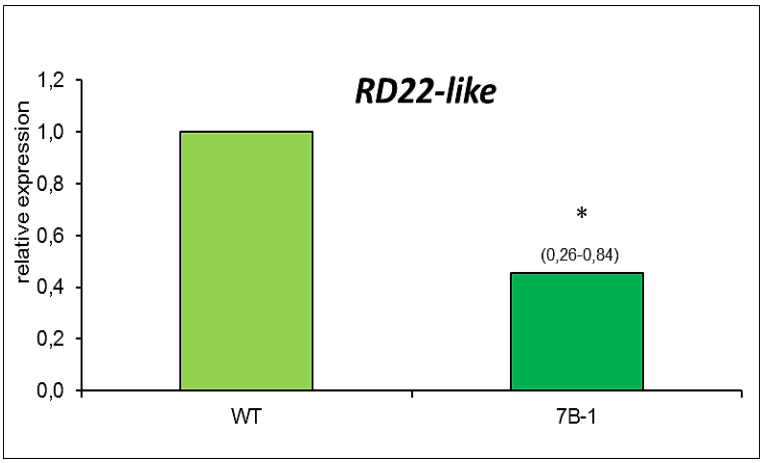

Fig. 16. The relative expression of *RD22-like* gene in dry WT and *7B-1* mutant seeds. The expression was measured by qRT-PCR and quantified against the expression from dry WT seeds using REST. The data shows the relative expression and SE of three independent experiments. Asterisks indicate significant differences ($p<0.05$) to control.

On the other hand, RD22 participate in a variety of developmental responses including fruit and seed development (Batchelor et al. 2002; Ruiz et al. 2013, Yamaguchi-Shinozaki and Shinozaki 1993). The expression analysis of dry seeds showed that there is a pool of *RD22-like* transcript in both WT and *7B-1* mutant seeds (Fig. 16). The *7B-1* mutant seeds contained significantly lower amount of *RD22-like* transcript than seeds in WT.

Light induction of RD22 was studied just in connection with the antagonistic response of stomata to light and ABA. Both factors (light and ABA) stimulate the expression level of *RD22* gene in *Arabidopsis thaliana* (Goh et al. 2003; 2009). There is no comprehensive study concerning the light stimulation of *RD22*.

4.9. LEA and dehydrin expression profiles

LEA proteins were originally described in plant seeds (Dure et al. 1981) and they were found to be related to seed development (Dure 1993; Close 1996, 1997; Battaglia et al. 2008). Although their function is still not completely understood, it has been suggested that they could facilitate water uptake during seed imbibition under osmotic stress. LEA proteins together with sugars may act as a seed hydration buffer (Walters et al. 1997; Hara 2010). Therefore, they could promote seed germination by altering the capacity of the seed to absorb water during water imbibition (Calestani et al. 2015). For this reason, they may participate in *7B-1* mutant seed resistance to BL-induced inhibition of seed germination and to osmotic stress.

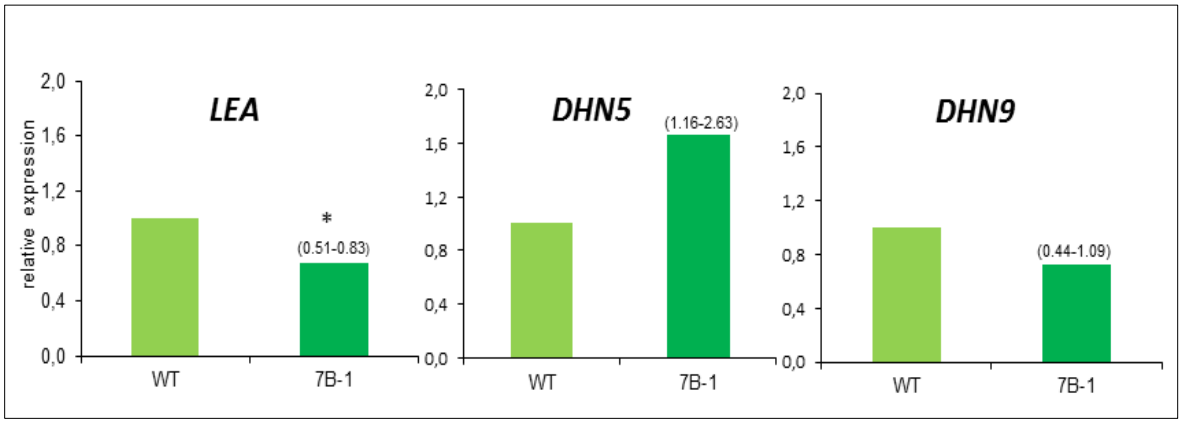

Fig. 17. The relative expression of *LEA-type* genes in WT and *7B-1* mutant seeds imbibed for 2 hours in the dark. The expression was measured by qRT-PCR and quantified against the expression from the sample of imbibed WT seeds using REST. The data shows the relative expression and SE of three independent experiments. Asterisks indicate significant differences ($p<0.05$) to control.

Firstly, the difference in amount of *LEA* and dehydrin (*DHN5* and *DHN9*) transcripts was compared in WT and *7B-1* mutant seeds imbibed for 2 hours in the dark (Fig. 17). The results showed that WT seeds contained significantly more *LEA* transcript than *7B-1* mutant seeds whereas both genotypes had the same amount of dehydrin transcripts. The data indicated that there was a pool of *LEA* and dehydrin transcripts in both WT and *7B-1* mutant seeds at the beginning of seed germination. For the sake of completeness, the expression patterns of *LEA-type* genes (Fig. 18) in imbibed and germinated seeds (seeds after 96-h germination, with visible radicle) showed that *DHN9* gene is prevalent in imbibed seeds, while the *LEA* gene is dominant in germinated seeds. The *DHN5* gene expression is the lowest in imbibed as well as germinated seeds. There was no difference between WT and *7B-1* mutant seeds in these trends.

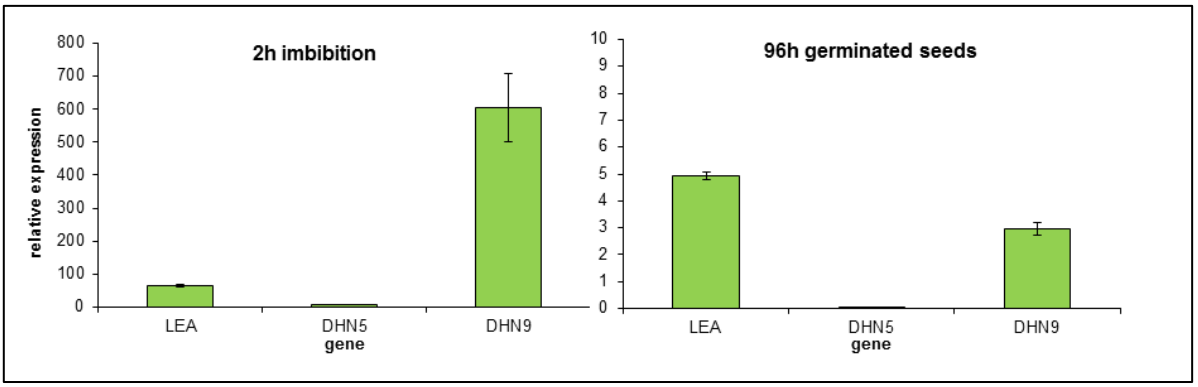

Fig. 18. The relative expression of *LEA-type* genes in WT seeds collected after 2-h imbibition and 96-germination in the dark. The relative expression analysed by qRT-PCR was calculated using 'delta C_T-method' (the expression was relative to references - *PP2Acs* and *TIP41like* genes, Pfaffl 2001). The values represent geomean and SE of three independent experiments.

With the aim to assess the impact of BL, the *7B-1* mutation and osmotic stress (70 mM mannitol and 5% PEG) on *LEA* and dehydrin gene expression (Fig. 19), a REST analysis from the RT-qPCR data was used. BL enhanced significantly the expression of *LEA* gene in WT seeds but significantly reduced it in *7B-1* seeds. In the mutant, the trend of *LEA* transcript accumulation under BL was obvious only under osmotic stress. On the other hand,

the expressions of both dehydrin genes in WT were induced markedly by BL while more pronouncedly under osmotic stress. Interestingly, a great increase in dehydrin gene expressions under osmotic stress was observed also in *7B-1* seeds. The responsiveness of *DHN5* and *DHN9* genes to both mannitol- and PEG-induced osmotic stress was highly significant. The impact of osmotic stress on *LEA* gene expression was less prominent indicating that in tomato seeds, dehydrins played a dominant role in coping with osmotic stress.

Interestingly, BL or osmotic stress (in WT seeds) or combination of both BL and osmotic stress (in *7B-1* mutant seeds) enhanced *LEA* and dehydrin genes expression (Fig. 19). This could be associated with the predicted protective role of the products of these genes. Natali et al. (2007) reported that exposure to white and red light stimulates dehydrin transcript accumulation in *Helianthus annuus*, and modulation of transcripts by blue and red light has been found for *LEA* genes (Crosatti et al. 1999). In both cases, light has a stimulatory effect on transcript accumulation, which is in agreement with my results. Unfortunately, as far as I know, there is no report related to the effect of BL on dehydrin expression. Nevertheless, I showed that also dehydrins are responsive to BL. In conclusion, both environmental challenges (mannitol- and PEG-induced osmotic stress and BL), which reduced tomato seed germination, increased expression of *LEA* and *DHN* genes encoding the putative protective molecules involved in protection against damages induced by stresses.

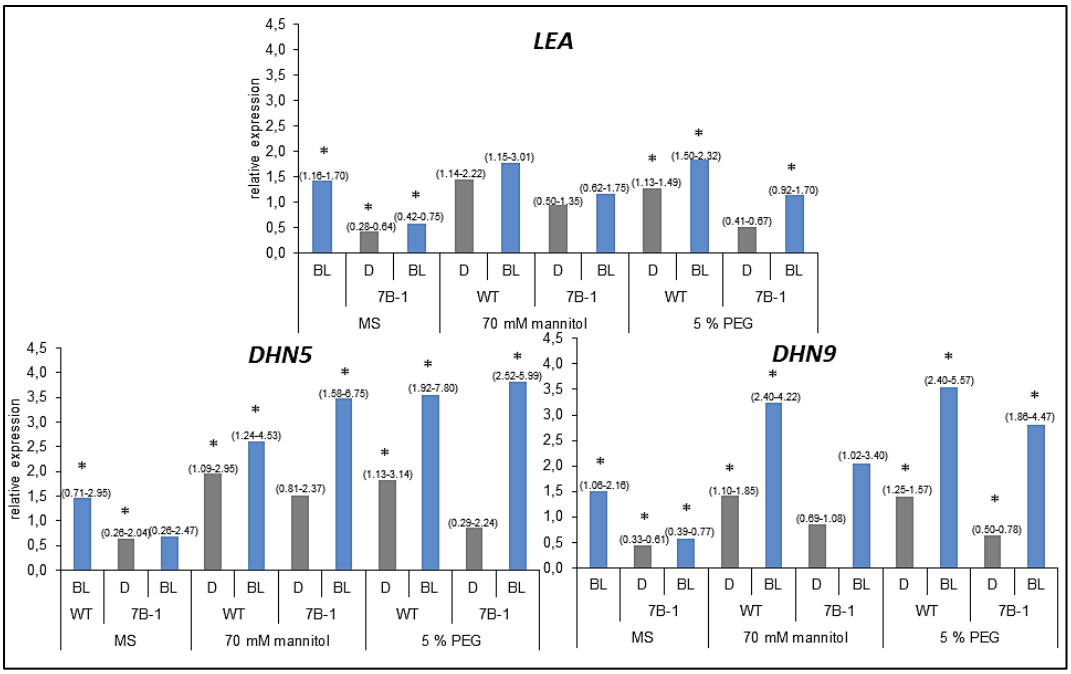

Fig. 19. The relative expression of *LEA-type* genes in WT and *7B-1* mutant seeds under osmotic stress induced by mannitol and PEG treatment. The seeds germinated for 96 h in the dark (D) or under blue light (BL). The expression was measured by qRT-PCR and quantified against the expression from the sample of WT seeds germinated in the dark (a control, its expression had therefore the value 1) using REST. The data shows the relative expression and SE of at least three independent experiments. Asterisks indicate significant differences ($p<0.05$) to control.

Despite the fact that *7B-1* mutant seeds are more tolerant to osmotic stress and BL and that the higher content of *LEA*/*DHN* transcripts can be expected, the transcription of *LEA* and *DHN* genes was significantly lower in *7B-1* seeds (Figs. 17 and 19). On the contrary, an improved plant stress tolerance is often positively related to the expression levels of LEA, especially DHNs, transcripts or proteins, and usually, more tolerant genotypes have higher levels of *DHNs* than the less tolerant ones (Porat et al. 2004; Kosová et al. 2010). May *7B-1* mutant seeds express more DHN/LEA proteins, which could be reason for lower expression of these genes? This assumption was assessed using mass spectrometry, however, the data are not complete yet and they are preparing for publication.

In the literature, there are several reports concerning the association of dehydrins with membranes (Hara et al. 2003; Xu et al. 2013). They are the peripheral membrane proteins (Qian et al. 2008, Hassan et al. 2013). It seems that dehydrin proteins accumulate close to membranes during osmotic stress, and they probably bind to membrane through the K segment (Koag et al. 2009) and protect membrane against the detrimental effect of osmotic stress (the participation of S segments together of the K segment is expected) (Graether and Boddington 2014). Thus, dehydrins stabilize cell membranes during dehydration (Dure 1993; Close 1996; Koag et al. 2009; Hara 2010). They are thought to retain water and stabilize the macromolecules as well as membranes (Melloul et al. 2013). In fact, the interaction between dehydrins and plasma membrane aquaporins (PIPs) cannot be excluded. Both protein families reside at the membranes and both are crucial for stress responses. There are a few studies focusing on the role of aquaporins and dehydrins in stress tolerance (Takahashi et al. 2013; Chen and Arora 2014; Hamla et al. 2014).

5 Conclusions

Plants survive in constantly changing environment because of carefully orchestrated responses to diverse challenges during their lifecycles. The responses to stress factors involve abundant players from different protein families. Coping with the stressors is often associated with aquaporins, especially those residing in the plasma membrane. Our group work with very interesting model plant species, tomato *7B-1* mutant, which is known by lower responsiveness of its seed germination to different stresses including osmotic stress and blue light-induced inhibition of seed germination. Besides, the *7B-1* mutation impairs BL signalling pathways. Thus, the main aim of our research is to understand the mechanism involved in the ability of the *7B-1* mutant to handle various stresses. This thesis focused on the characterization of physiological and transcriptional response of WT and *7B-1* mutant seeds to mercury, an inhibitor of aquaporins and to various osmotic stress treatments in the dark and under blue light.

The data showed that *7B-1* mutant seed germination was always less responsive than WT seed germination to various stress treatments applied. Thus, the *7B-1* mutant seeds germinated more pronouncedly than WT seeds under all tested conditions. However, the expression of the studied genes in *7B-1* mutant seeds was the same or even lower as in WT seeds. Interestingly, the *7B-1* mutation decreased significantly the expression level of *LEA*, dehydrins, *RD22-like* and *PIP1;3* genes. Besides, the *7B-1* mutation affected the BL-induced stimulation of *PIP1;2* gene expression, which indicates the connection between the *7B-1* mutation and BL-signalling. Therefore, the *7B-1* mutation, conferring the lower responsiveness to BL and osmotic stress, reduced the transcript level of stress-responsive genes. The following study should focus on characterization of protein abundance of stress-responsive proteins in WT and *7B-1* seeds to validate the possible effect of the *7B-1* mutation on protein accumulation. This is almost done for LEA-type proteins in WT and *7B-1*

seeds under osmotic stress induced by 70 mM mannitol, but the data are preparing for publication (Balarynová et al., unpublished data). In addition, BL alone had a significant impact on transcript level of several genes. Mostly, it induced the gene expression, but reduced the percentage of seed germination of both WT and *7B-1* mutant seeds with more pronounced effect on WT seed germination. Particularly, a combination of osmotic stress treatment and BL enhanced considerably the expression level of *RD22-like* and *LEA-type* genes (especially dehydrins). On the other hand, *PIP* genes *PIP1;1* and *PIP1;2* were induced just by BL, whereas *PIP1;3* gene was affected by BL, osmotic stress as well as the *7B-1* mutation. The *PIP1;3* transcript level was reduced by any treatment used. In the next studies, it will be interesting to see how the PIP1;3 protein level and localization in the germinated seeds is affected by BL and osmotic stress in both WT and *7B-1* mutant seeds.

The future research could also focus on possible interaction between LEA-type proteins and plasma membrane aquaporins (PIPs). Both protein families reside at/near the membranes and both are crucial for stress responses. The cooperation between PIP a LEA proteins in alleviation of impact of osmotic stress cannot be excluded. The cell wall-localization associated with the apoplast is expected for of RD22 proteins (Zheng et al. 1992; Wang et al. 2012; Phillips and Ludidi 2017). Whether this group of stress-responsive proteins could interact with plasma membrane is not known. To investigate the behaviour of RD22 proteins upon environmental challenges is the way to better understanding their function and their interaction with other protective molecules.

Interestingly, it was reported recently that *SlGLO2* gene, belonging to class B of MADS-boxe genes (Geuten and Irish 2010), is most likely a candidate gene for the *7B-1* mutation. The mutants in B-class MADS-box genes are often sensitive to various environmental factors (Pucci 2017). Similarly, the *7B-1* mutant is known by its lower sensitivity to BL (Fellner and Sawhney 2001) and a photoperiod responsiveness (Sawhney

1997, 2004). It was shown that the promoter regions of B-class MADS-box genes contain the regulatory elements connected with the perception of environmental conditions including light (Pucci et al. 2017). The future project could be therefore focused on possible role of B-class MADS-box proteins in blue light signalling and/or cross-talk between light and abiotic stress signalling.

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7 Supplementary materials

Tab. 1 Suppl. The relative expression of *PIP1;2* and *PIP1;3* genes in the WT and *7B-1* mutant seeds germinated under osmotic stress induced by 20 mM mannitol. The qRT-PCR data were quantified using REST software**.**

			PIP1;2				*PIP1;3*			
			R	**SE range**	**p-value**	**result**	**R**	**SE range**	**p-value**	**result**
MS	***WT***	**D**								
		BL	**1,09**	0,672 - 1,747	0,552	**no**	**1,15**	0,871 - 1,513	0,145	**no**
	7B-1	**D**	**1,02**	0,646 - 1,697	0,908	**no**	**1,03**	0,808 - 1,369	0,675	**no**
		BL	**1,04**	0,686 - 1,700	0,806	**no**	**1,08**	0,806 - 1,429	0,371	**no**
20 mM mannitol	***WT***	**D**	**0,86**	0,714 - 1,153	0,311	**no**	**1,12**	0,864 - 1,435	0,351	**no**
		BL	**0,91**	0,845 - 1,009	0,206	**no**	**1,16**	0,921 - 1,388	0,204	**no**
	7B-1	**D**	**0,80**	0,612 - 1,109	0,322	**no**	**1,04**	0,773 - 1,396	0,724	**no**
		BL	**0,90**	0,688 - 1,293	0,636	**no**	**0,96**	0,673 - 1,628	0,794	**no**

Tab. 2 Suppl. The relative expression of *PIP1;4, PIP1;5, PIP2;1, PIP2;2* and *PIP2;3* genes in WT and *7B-1* mutant seeds germinated under various osmotic stress treatments in the dark (D) and in blue light (BL). The qRT-PCR data were quantified using REST software at the 0.05 significance level.

			PIP1;4				*PIP1;5*				*PIP2;1*				*PIP2;2*				*PIP2;3*			
			R	SE range	p-value	result	R	SE range	p-value	result	R	SE range	p-value	result	R	SE range	p-value	result	R	SE range	p-value	result
MS	*WT*	D																				
		BL	**1,09**	0,672 - 1,747	0,552	no	**1,15**	0,871 - 1,513	0,145	no	**1,00**	0,829 - 1,223	0,968	no	**1,18**	0,872 - 1,467	0,113	no	**1,07**	0,834 - 1,377	0,387	no
	7B-1	D	**1,02**	0,646 - 1,697	0,908	no	**1,03**	0,808 - 1,369	0,675	no	**0,94**	0,740 - 1,173	0,398	no	**0,91**	0,673 - 1,294	0,342	no	**1,09**	0,867 - 1,444	0,310	no
		BL	**1,04**	0,686 - 1,700	0,806	no	**1,08**	0,806 - 1,429	0,371	no	**0,94**	0,744 - 1,185	0,407	no	**1,02**	0,756 - 1,340	0,887	no	**0,96**	0,773 - 1,196	0,532	no
20 mM mannitol	*WT*	D	**0,86**	0,714 - 1,153	0,311	no	**1,12**	0,864 - 1,435	0,351	no	**0,91**	0,726 - 1,123	0,658	no	**0,89**	0,688 - 1,204	0,584	no	**0,97**	0,787 - 1,188	0,799	no
		BL	**0,91**	0,845 - 1,009	0,206	no	**1,16**	0,921 - 1,388	0,204	no	**0,93**	0,728 - 1,125	0,610	no	**1,08**	0,928 - 1,243	0,440	no	**0,84**	0,701 - 1,045	0,201	no
	7B-1	D	**0,80**	0,612 - 1,109	0,322	no	**1,04**	0,773 - 1,396	0,724	no	**0,90**	0,694 - 1,151	0,514	no	**0,82**	0,554 - 1,144	0,651	no	**0,89**	0,813 - 1,030	0,402	no
		BL	**0,90**	0,688 - 1,293	0,636	no	**0,96**	0,673 - 1,628	0,794	no	**0,99**	0,740 - 1,334	0,965	no	**0,67**	0,464 - 0,932	0,122	no	**0,80**	0,663 - 1,017	0,253	no
70 mM mannitol	*WT*	D	**1,17**	0,593 - 2,093	0,601	no	**1,07**	0,897 - 1,395	0,559	no	**1,08**	0,938 - 1,242	0,256	no	**1,07**	0,752 - 1,541	0,676	no	**1,03**	0,857 - 1,296	0,792	no
		BL	**1,11**	0,615 - 1,890	0,706	no	**1,06**	0,836 - 1,401	0,676	no	**0,98**	0,882 - 1,158	0,884	no	**0,96**	0,633 - 1,415	0,890	no	**0,89**	0,682 - 1,126	0,403	no
	7B-1	D	**1,35**	0,782 - 2,741	0,298	no	**1,08**	0,839 - 1,311	0,441	no	**0,99**	0,851 - 1,104	0,924	no	**0,89**	0,648 - 1,154	0,418	no	**0,96**	0,757 - 1,189	0,722	no
		BL	**1,06**	0,607 - 2,142	0,854	no	**1,07**	0,807 - 1,529	0,610	no	**0,96**	0,847 - 1,110	0,530	no	**0,87**	0,632 - 1,068	0,322	no	**0,79**	0,624 - 0,978	0,060	no
5 % PEG	*WT*	D	**1,07**	0,865 - 1,251	0,507	no	**0,92**	0,738 - 1,092	0,664	no	**0,84**	0,760 - 0,910	0,054	no	**0,94**	0,798 - 1,022	0,627	no	**1,00**	0,783 - 1,247	0,980	no
		BL	**1,03**	0,976 - 1,107	0,613	no	**1,15**	1,170 - 1,432	0,051	no	**0,88**	0,785 - 0,968	0,166	no	**1,41**	1,267 - 1,594	0,107	no	**1,13**	0,998 - 1,261	0,356	no
	7B-1	D	**1,08**	0,962 - 1,183	0,306	no	**0,96**	0,882 - 1,030	0,560	no	**0,83**	0,728 - 0,880	0,051	no	**1,00**	0,940 - 1,077	0,845	no	**0,99**	0,828 - 1,162	0,967	no
		BL	**0,86**	0,753 - 0,979	0,123	no	**1,10**	0,997 - 1,203	0,213	no	**0,65**	0,587 - 0,742	0,053	no	**0,98**	0,934 - 1,027	0,589	no	**0,89**	0,731 - 1,071	0,400	no

Tab. 3 Suppl. The relative expression *PIP* genes quantified in non-germinated WT and *7B-1* mutant seeds germinated in the dark (D) and in blue light (BL). The qRT-PCR data were quantified and analysed using REST software at the 0.05 significance level.

			PIP1;2				*PIP1;3*				*PIP1;4*				*PIP1;5*			
			R	**SE range**	**p-value**	**result**	**R**	**SE range**	**p-value**	**result**	**R**	**SE range**	**p-value**	**result**	**R**	**SE range**	**p-value**	**result**
MS	***WT***	**D**																
		BL	**1,23**	1,098 - 1,441	0,092	**no**	**0,49**	0,301 - 0,803	0,010	**DOWN**	**0,98**	0,916 - 1,078	0,885	**no**	**1,20**	1,043 - 1,386	0,102	**no**
	7B-1	**D**	**0,96**	0,874 - 1,088	0,687	**no**	**0,52**	0,333 - 0,885	0,019	**DOWN**	**0,80**	0,675 - 0,939	0,073	**no**	**1,09**	0,954 - 1,268	0,496	**no**
		BL	**1,09**	0,982 - 1,255	0,394	**no**	**0,40**	0,229 - 0,689	0,005	**DOWN**	**0,86**	0,763 - 0,953	0,130	**no**	**1,20**	0,990 - 1,423	0,203	**no**
70 mM mannitol	***WT***	**D**	**1,16**	0,997 - 1,403	0,212	**no**	**0,94**	0,554 - 1,411	0,801	**no**	**1,14**	0,969 - 1,456	0,591	**no**	**1,24**	1,167 - 1,345	0,056	**no**
		BL	**1,22**	1,122 - 1,391	0,101	**no**	**0,51**	0,309 - 0,788	0,060	**no**	**0,95**	0,747 - 1,209	0,842	**no**	**1,23**	1,034 - 1,383	0,151	**no**
	7B-1	**D**	**0,95**	0,841 - 1,062	0,497	**no**	**0,39**	0,300 - 0,572	0,000	**DOWN**	**0,79**	0,652 - 1,001	0,270	**no**	**1,12**	0,871 - 1,341	0,438	**no**
		BL	**1,15**	1,018 - 1,329	0,105	**no**	**0,50**	0,360 - 0,714	0,046	**DOWN**	**0,89**	0,804 - 0,956	0,060	**no**	**1,29**	1,028 - 1,559	0,208	**no**

			PIP2;1				*PIP2;2*				*PIP2;3*			
			R	**SE range**	**p-value**	**result**	**R**	**SE range**	**p-value**	**result**	**R**	**SE range**	**p-value**	**result**
MS	***WT***	**D**												
		BL	**1,06**	1,000 - 1,122	0,221	**no**	**1,38**	1,255 - 1,564	0,072	**no**	**1,10**	0,943 - 1,367	0,601	**no**
	7B-1	**D**	**0,88**	0,799 - 0,940	0,124	**no**	**1,12**	0,962 - 1,356	0,492	**no**	**0,84**	0,712 - 1,054	0,378	**no**
		BL	**0,88**	0,807 - 0,920	0,105	**no**	**1,20**	1,042 - 1,422	0,184	**no**	**0,78**	0,524 - 1,054	0,581	**no**
70 mM mannitol	***WT***	**D**	**1,07**	1,020 - 1,139	0,051	**no**	**1,06**	0,901 - 1,261	0,752	**no**	**0,98**	0,831 - 1,260	0,905	**no**
		BL	**1,01**	0,892 - 1,165	0,948	**no**	**1,52**	1,418 - 1,679	0,068	**no**	**0,89**	0,780 - 0,994	0,192	**no**
	7B-1	**D**	**0,94**	0,884 - 0,982	0,100	**no**	**1,09**	0,980 - 1,270	0,472	**no**	**0,76**	0,678 - 0,852	0,012	**no**
		BL	**0,94**	0,884 - 0,992	0,237	**no**	**1,32**	1,226 - 1,491	0,074	**no**	**0,79**	0,665 - 0,997	0,253	**no**

8 Appendixes

Appendix 1

Changes in plasma membrane aquaporin gene expression under osmotic stress and blue light in tomato

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ORIGINAL ARTICLE

Changes in plasma membrane aquaporin gene expression under osmotic stress and blue light in tomato

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Abstract

Divergent abiotic stresses induce osmotic stress on plant cells resulting in an imbalance in water homeostasis which is preserved by aquaporins. Since the plasma membrane aquaporins (PIPs) were shown to be involved in seed development and responses to abiotic stresses, we focused on determining the contribution of mannitol-induced osmotic stress, blue light (BL), and *7B-1* mutation to their gene expression in tomato (*Solanum lycopersicum* L.) seeds. To assess that, we used a quantitative RT-PCR to determine the expression profiles of genes encoding PIPs. Subsequently, a multiple linear regression analysis was used to evaluate the impact of studied stressors (mannitol and BL) and *7B-1* mutation on *PIP* gene expressions. We found that mannitol-induced osmotic stress and *7B-1* mutation (conferring the lower responsiveness to osmotic stress- and BL-induced inhibition of seed germination) decreased expression of *PIP1;3*, *PIP2;3* and *PIP1;2*, *PIP2;1* genes, respectively. This might be a way to retain water for radicle elongation and seed germination under the stress conditions. Interestingly, the expression of *PIP1;3* gene was downregulated not only by osmotic stress, but also by BL. Altogether, our data indicate the existence of a link between osmotic stress and BL signalling and the involvement of the *7B-1* mutation in this crosstalk.

Keywords Tomato · Seed · Aquaporins · Blue light · *7B-1* mutant · Mannitol · *PIP*s

Introduction

Plant growth and development are often severely affected by abiotic stresses. Abiotic stresses are known to induce osmotic imbalance in plants and disturb the plant water homeostasis. Aquaporins, the membrane intrinsic proteins facilitating and regulating the passive movement of water molecules down a water potential gradient, play a central role in maintaining a turgor and water transport in plants (Maurel et al. 2015). Above all, the plasma membrane intrinsic protein (PIP) aquaporins have been proven to participate in responses to abiotic stresses, such as drought, salinity, or chilling (Lian et al. 2004; Liu et al. 2007). Moreover, *PIP* gene expression can be regulated by these abiotic stresses (Aroca et al. 2012) as well. In addition, there is an opinion that PIP1-type aquaporins can function as sensors of blue light (BL) because of their localization in the plasma membrane and ability to bind flavins (the cofactors of BL photoreceptors) (Kaldenhoff and Eckert 1999; Lorenz et al. 2003). Furthermore, the expression of *PIP1;2* aquaporin was showed to be enhanced by BL in *Arabidopsis thaliana* cell tissue cultures (Kaldenhoff et al. 1995, 1996).

In plant seeds, aquaporins play a central role in the physiology of water economy (Maurel et al. 2015; Obroucheva 2013; Obroucheva et al. 2017). Embryo growth during seed germination is driven by water uptake which is necessary for embryo cell elongation and expansion. In general, seed germination follows three phases. In phase I, imbibition of water, reactivation of metabolism and at the end of this phase, cell elongation, and radicle emergence occurs. In phases II and III, the embryonic axis grows further as cells elongate and divide to establish the seedling (Toole et al. 1956). Aquaporins are not involved in the early imbibition of dry seed by water, but they play a role in embryo growth (Obroucheva 2012; Willigen et al. 2006). It was reported that

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the expression of aquaporin genes is low during seed imbibition and starts when a seed hydration level of 50–55% (fresh weight) is achieved (Obroucheva 2012). The expression of aquaporin genes is strongly activated at radicle emergence and just after it. Despite dry seeds contain the transcripts of aquaporin genes and aquaporin themselves, it seems that the aquaporins function only after radicle protrusion to provide enhanced water inflow to growing cells (Obroucheva 2013). In addition, Shiota et al. (2006) showed that plasma membrane aquaporins are expressed during tomato seed development. Apparently, they participate in the delivery of water and possibly some other compounds into the developing seeds (Obroucheva 2013).

Seed germination is one of the physiological processes which are influenced by light. Although very little is known about perception of blue light (BL) by seeds (Goggin and Steadman 2012), it was shown that BL mostly reduces seed germination in tomato and that tomato seeds under osmotic stress germinate better in the dark than in BL (Fellner and Sawhney 2002; Piterková et al. 2012). Besides, BL can modulate transcription of many genes including plant aquaporins (Baaziz et al. 2012; Kaldenhoff et al. 1995, 1996) and water stress acts on aquaporin function at the level of transcription and gating through direct effects on osmotic gradient (Luu and Maurel 2005).

Tomato (*Solanum lycopersicum* L.) is one of the most important crop species. The *7B-1* mutant displays male sterility under long days, but under short days, it produces fertile flowers (Sawhney 1997; Sheoran et al. 2009). Compared with the corresponding WT (cv. Rutgers), the *7B-1* shows reduced de-etiolation of hypocotyl growth associated with hypersensitivity to, and high endogenous levels of, ABA (Fellner et al. 2001). The *7B-1* mutation impairs various BL responses, such as BL-induced stomata opening (Hlavinka et al. 2013; Ježilová et al. 2012), hypocotyl phototropism (Bergougnoux and Fellner, unpublished results) and chloroplast movement (Špundová and Savara, unpublished results). It suggests that *7B-1* mutation may somehow affect phototropin signalling, while the possibility that *7B-1* is a CRY mutant is not likely (Sheoran et al. 2006). In long-day conditions, *7B-1* seed germination was found to be less responsive than corresponding WT to various salts (including NaCl, Na_2SO_4, KCl, and K_2SO_4), osmotic stress and to low-temperature stress (Fellner and Sawhney 2001). Interestingly, Fellner and Sawhney (2002) also reported that seed germination in *7B-1* is more tolerant to exogenous ABA and osmotic stress specifically under BL. Moreover, we reported that BL amplifies the inhibitory effect of osmotic stress on tomato seed germination (Fellner and Sawhney 2002; Piterková et al. 2012). Recently, we identified number of potentially novel miRNAs, which are associated with enhanced tolerance of *7B-1* to abiotic stress under BL (Omidvar et al. 2015). Our data also showed that response to different lights and stresses in *7B-1* and WT involves remodelling DNA methylation, highlighting the differences in epigenetic and transcriptional regulation of light and stress responses between *7B-1* and WT (Omidvar and Fellner 2015). Specifically in BL conditions, *7B-1* is less sensitive to biotic stress as well (Bergougnoux et al. 2009). Recently, a genetic characterization of the *7B-1* mutant was published and *SlGLO2* gene was proposed as a candidate gene underlying the *7B-1* mutation (Pucci et al. 2017). *SlGLO2* belongs to class B MADS-box genes which are involved in stamen development (Pucci et al. 2017). However, a role of *SlGLO2* in BL- and/or stress signalling is not known and it is under investigation.

Number of reports describing a direct contribution of BL in the ability of plants to tolerate abiotic stress is very limited. Therefore, *7B-1* mutant seems to be a suitable model to study a possible interaction between BL and osmotic stress. Various environmental factors regulate aquaporins mostly at the transcriptional level (Liu et al. 2013). For this reason, we hypothesized that the tolerance of *7B-1* mutant seeds to BL and osmotic stress may involve differential expression of aquaporin genes. Shiota et al. (2006) showed that genes of PIP aquaporin family (namely *PIP1;2*, *PIP1;3*, *PIP1;4*, *PIP1;5*, *PIP2;1*, *PIP2;2,* and *PIP2;3*) participate in tomato seeds development, while expression of some *PIPs* can be regulated by abiotic stresses (Aroca et al. 2012). Thus, we focused on this aquaporin family and we studied the expression of these seven genes in *7B-1* seeds as a function of mannitol-induced osmotic stress and BL conditions. We revealed that the gene expression patterns of *PIP1;2*, *PIP1;3*, *PIP2;1,* and *PIP2;3* were altered in response to BL, osmotic stress, and/or by *7B-1* mutation. Interestingly, both *7B-1* mutation and osmotic stress decreased expression of some of the studied *PIPs*, most probably to preserve enough water for radicle elongation under stress conditions. As *7B-1* mutation causes defects in BL responses, and expression of *PIP1;2* and *PIP2;1* is regulated by BL, we hypothesized that these genes may play roles in BL sensing or signalling.

Materials and methods

Plant material and stress treatment

Solanum lycopersicum L. (tomato) seeds of cultivar Rutgers (WT) and recessive single gene *7B-1* mutant (Sawhney 1997) were used. *7B-1* seeds are less responsive than WT to BL-induced inhibition of seed germination and to various abiotic stresses specifically under BL (Fellner and Sawhney 2001, 2002; Piterková et al. 2012).

All experiments were performed in in vitro conditions. Sterilized seeds (2.8% sodium hypochlorite solution, Bochemie, Czech Republic) were incubated in squared Petri dishes

filled with basal MS medium (Murashige and Skoog 1962) or with MS medium supplemented with 20 or 70 mM mannitol. The edge of each plate was twice sealed with an air permeable tape (Batist, Czech Republic). The dishes with seeds were placed in vertical position into controlled growth chambers (Microclima 1000E, Snijders Scientific B. V., The Netherlands) in continuous BL conditions or they were wrapped in tinfoil (to simulate the darkness) and placed into the same chamber. BL was provided by blue tubes TLD-36W/18-Blue (Philips, USA) with a maximum irradiance at 460 nm. Total photon fluence rate of light was 10 µmol m^{-2} s^{-1}. The light spectrum was measured using a portable spectroradiometer (model LI-1800; Li-COR, NE, USA).

Number of germinated seeds was counted the seventh day after sowing and the average percentage of seed germination was calculated. At least 11 experiments were done for each treatment. To show a trend in course of seed germination, the number of germinated seeds was scored from second to seventh day as well. A control on MS was sown for each experiment. For each treatment, there were at least four independent experiments per each time point.

Gene expression analyses

The WT and *7B-1* mutant seeds germinated on basal MS medium or MS medium supplemented with 70 mM mannitol were collected (96 h after sowing, radicle 1–10 mm long) into liquid nitrogen. Seeds from BL were harvested under BL and seeds from darkness under green safelight. Harvested seeds were ground under liquid nitrogen and total RNA was isolated with the Isolate II RNA Plant Kit (Bioline) using RLS buffer according to a manufacturer´s instruction. At the last step, RNA was eluted with 40 µl of RNase-free water. As soon as isolation was done, residual DNA was removed from samples by recombinant DNaseI treatment (Takara Bio Inc., Japan) and recombinant RNase inhibitor (Takara Bio Inc., Japan) for 60 min at 37 °C. DNaseI was inactivated by heat treatment according to manufacturer´s instruction. Following this, first-strand cDNA mixtures were prepared from 0.7 µg of total RNA using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Bio). The gene expression was analysed using SensiFAST™ SYBR Lo-ROX Kit (Bioline) on the CFX96 Touch™ (BioRad, USA). The PCR reaction mixture was prepared according to manufacturer's protocol—the reaction mixture contained 10 µl of SensiFast SYBR^R Lo-ROX Mix, 0.8 µl of each primer (10 µM), 4.4 µl of sterile RNase-free water, and 4 µl of cDNA template (diluted 1/50). The final reaction volume was 20 µl. Each sample was measured in triplicate. The following PCR running conditions were used—an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 15 s. Melting curve was analysed at the end of PCR reaction. The experiments were repeated five times.

PP2Acs (protein phosphatase 2A catalytic subunit) (Lovdal and Lillo 2009) and *TIP41-like* (Dekkers et al. 2012) genes were used as reference genes. The sequences of *PIP* primers used are given in Table 1. The primers were designed using PrimerQuest® program (IDT, Coralville, USA). The primer efficiencies were calculated from the slope of the dilution curve and they ranged from 0.9 to 1.0.

Statistical analysis

The significant differences between control (seeds on basal MS medium) and mannitol treatments were tested using Kruskal–Wallis test (a distribution-independent test) with multiple comparisons at the 0.05 significance level, since the data were not normally distributed. The analysis was done separately for dark- and BL-grown samples. For each treatment, the significance of differences between the dark and BL was assessed using Mann–Whitney *U* test (a non-parametric test) at the 0.05 significance level. Statistical analyses were performed using the STATISTICA 12 software (StatSoft, OK, USA). The results of germination assay are presented as box plots, where the lower and upper ends of the box are the first and third quartiles, respectively. The horizontal line inside the box is the median value (the square inside the box represents a mean) and the whiskers indicate the adjacent values (1.5 times interquartile range).

The relative gene expression of target gene was determined against the expression level of both reference genes with taking into account the different PCR efficiencies of

Table 1 Sequences of *PIP* primers used in RT-qPCR analyses

Gene	Accession number	Forward primer	Reverse primer
Sl*PIP1;2*	BP884557	TCCTATTTTGGCACCTCTTCC	ATCCCATGCCTCGTCTTTG
Sl*PIP1;3*	AW625013	ACGAAAGGTGATGGTCTTGG	GGGAACGTGTGAATCTCTAGC
Sl*PIP1;4*	AF218774	GAAGAGGATGTGAAGGTTGGAG	ACCAAGAATGTAGCTCACCAG
Sl*PIP1;5*	X73848	CACCAGCTCCATTGTTTGAAC	AACCCCTTGAATACCCACTG
Sl*PIP2;1*	BI929127	AGGTGGAGGTGCTAACTTTG	AACACAGGGACATGGGAATC
Sl*PIP2;2*	BG128835	CAGCATGGCAAAGATTATGTGG	CGTAGAGGAAAAGCAGAGTAGC
Sl*PIP2;3*	AW224678	TTCACCTTGCCACTATTCCG	AGAAAATCCAGTGTTCGTCCC

genes (Pfaffl 2001). Then, a geomean of the calculated relative expressions was used as a dependent variable to design a matrix for multiple linear regression analysis. The independent variables (predictors) designed as a mutation, light, and stress could take on one of two values, 1 or 0, to indicate the presence or absence, respectively, of the effect of the predictor (the binary system). To find the significance of the effect of predictors and to quantify it, the multiple linear regression analysis was done using the QC.Expert 2.9 software (TriloByte Statistical Software, Czech Republic).

Results

The main goal of this work was to investigate how osmotic stress (induced by 70 mM mannitol) and BL influence the expression profile of *PIP* genes in *7B-1* mutant seeds and its corresponding WT and how their gene expression patterns associate with reduced *7B-1* sensitivity to osmotic stress and BL. We focused on the effect of two different mannitol treatments and BL on seed germination in both genotypes. Then, we studied changes in the expression of *PIP* genes in seeds induced by 70 mM mannitol, BL and *7B-1* mutation.

Seed germination assays

In the dark as well as in BL, WT seed germination was significantly affected by treatment with 70 mM mannitol, but not by 20 mM mannitol (Fig. 1a). Similarly, seed germination in *7B-1* mutant was reduced significantly by 70 mM mannitol in the dark as well as in BL (Fig. 1b). However, in the dark, 70 mM mannitol tended to reduce *7B-1* mutant seed germination less than WT seed germination, but this difference did not reach statistical significance (at the 0.05 significance level). In both the dark and BL, 20 mM mannitol had not significant effect on *7B-1* mutant seed germination (Fig. 1b).

For each treatment, the effect of BL on WT and *7B-1* mutant seed germination compared to D was assessed using Mann–Whitney test (at the 0.05 significance level). The effect of BL was always significant (not marked in Fig. 1).

The trends of kinetics of seed germination shown in Fig. 2 indicate that WT and *7B-1* mutant differed just very slightly in the kinetics of seed germination on the basal MS medium in the dark. On the other hand, BL reduced markedly kinetics of WT seed germination (Fig. 2a), whereas in *7B-1* mutant, its effect was not so pronounced (Fig. 2b).

Changes in aquaporin gene expression under blue light and osmotic stress

In this study, we focused on investigation into the effect of BL, osmotic stress induced by 70 mM mannitol and *7B-1* mutation on RNA levels of plasma membrane aquaporins (namely, *PIP1;2*, *PIP1;3*, *PIP1;4*, *PIP1;5*, *PIP2;1*, *PIP2;2,* and *PIP2;3*) in germinated seeds (radicle up to 10 mm) cultivated for 96 h.

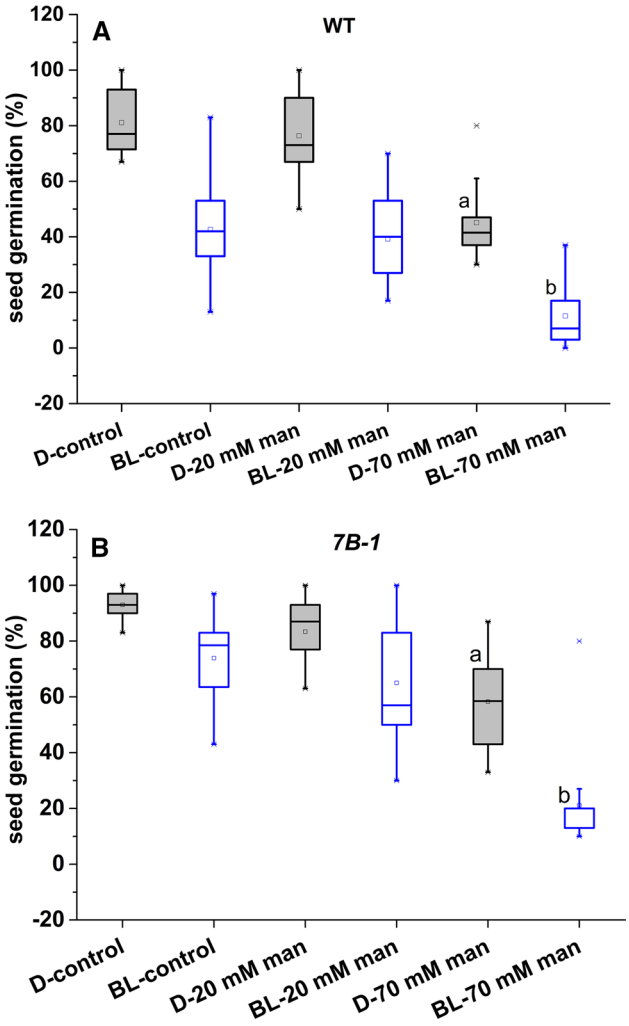

Fig. 1 Effect of mannitol on germination of tomato cv. Rutgers (WT) (**A**) and *7B-1* mutant (**B**) seeds incubated for 7 days in the dark (D) or under continuous blue light (BL). Seeds germinated on the basal MS medium (MS) serve as a control. The significant difference of mannitol treatments compared to corresponding control on medium without mannitol was tested using Kruskal–Wallis test at 0.05 significance level, a: in the dark, significantly different from D-control, b: in BL, significantly different from BL control. For each treatment, at least 11 experiments were done

The multiple linear regression analysis showed that none of studied predictors (blue light, osmotic stress, and *7B-1* mutation) reached statistical significance (at 0.05 level) for *PIP1;4*, *PIP1;5,* and *PIP2;2* genes (Table 2). In other words, expression of none of these genes was affected significantly by BL, osmotic stress, or by *7B-1* mutation. With the predictors mutation and stress held fixed, BL affected significantly the expression of *PIP1;2* (upregulation),

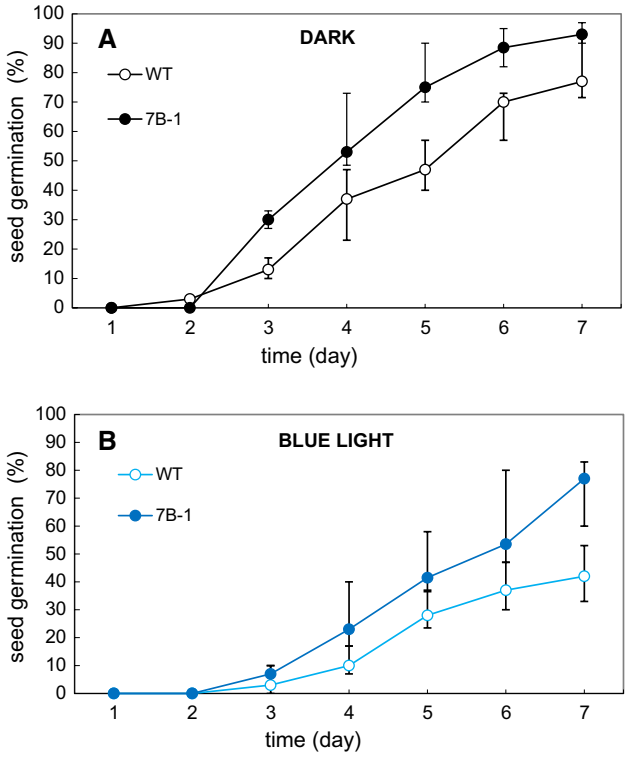

Fig. 2 Kinetics of WT and *7B-1* mutant seed germination on MS medium (control) and on MS medium supplemented with 70 mM mannitol during 7-day cultivation in the dark (**A**) or blue light (**B**). The data show medians and the error bars represent the first and third quartiles. For each treatment, there were at least four independent experiments per each timepoint. Thirty seeds per each dish were sown for each treatment

PIP1;3 (downregulation), and *PIP2;1* (downregulation) genes (Table 3). As Table 3 further shows, *7B-1* mutation decreased significantly the expressions of *PIP1;2* and *PIP2;1* genes when all the other predictor variables (BL and osmotic stress) were held fixed. Finally, held all the other predictors constant, the mannitol-induced osmotic stress reduced significantly the expression levels of *PIP1;3* and *PIP2;3* genes (Table 3).

In other words, the results of multiple linear regression analysis can also be presented as the equation of multiple linear regression involving the predictors with significant impact on the expression of each gene (Table 4).

Discussion

Expression of the aquaporins is divergently regulated by various environmental factors as a part of an adaptation mechanism by which plants reduce water losses through efflux to cope with stress conditions. Since the specific function of plant aquaporins under different stress and light conditions is still unclear, the comprehensive studies of aquaporin gene expression in divergent plant species are crucial to improve our understanding of their function in responses to various challenges. We wanted to assess statistically the potential impact of 70 mM mannitol-induced osmotic stress, BL and *7B-1* mutation on *PIPs* gene expression. The expression patterns could provide useful indications about the functions of the studied aquaporins. Tomato *7B-1* mutant seed germination, showing BL-specific reduced responsiveness to osmotic stress (Fellner and Sawhney 2002), is a valuable model system to study the possible involvement of aquaporins in light-regulated stress signalling.

Various responses of aquaporins to osmotic stress suggest that each aquaporin isoform could contribute differently to water transport and regulation of water homeostasis in germinated seeds. Several studies demonstrated that plants downregulate the aquaporin expressions to avoid excessive loss of water in challenging environment (Alexandersson et al. 2005; Jang et al. 2004; Lian et al. 2006). Here, we found that the expressions of *PIP1;3* and *PIP2;3* genes were downregulated by osmotic stress induced by 70 mM mannitol and it was associated with reduction of seed germination. All these literature data suggest that some aquaporins may help to adapt or tolerate the stress by reducing their expression. On the other hand, other aquaporins might help in maintaining the normal physiological processes in seeds, and thus, their expressions remained stable under the stress conditions. In our study, this was the case of *PIP1;2*, *PIP1;4*, *PIP1;5*, *PIP2;1,* and *PIP2;2* aquaporin genes. On the contrary, some reports show that the expression of *PIPs* can

Table 2 Results of a multiple linear regression analysis for genes *PIP1;4*, *PIP1;5*, and *PIP2;2*, whose gene expression was not affected significantly by blue light (BL), *7B-1* mutation, or mannitol-induced osmotic stress

Predictors	*PIP1;4*		*PIP1;5*		*PIP2;2*	
	Regression coefficient	*p* value	Regression coefficient	*p* value	Regression coefficient	*p* value
Constant	**1.57**	0.0000	**12.51**	0.0000	**1.04**	0.0000
BL	− 0.17	0.3859	0.75	0.3042	− 0.05	0.3949
Mutation	0.30	0.1224	− 0.28	0.7027	− 0.11	0.0666
Stress	0.06	0.7334	− 0.14	0.8433	− 0.06	0.2950

Significant values are shown in bold

In the table, the value of regression coefficients and corresponding *p* values are shown

Table 3 Results of a multiple linear regression analysis showing the effect of blue light (BL), *7B-1* mutation and mannitol-induced osmotic stress on the expression of *PIP* genes

Predictors	*PIP1;2*		*PIP2;1*		*PIP1;3*		*PIP2;3*	
	Regression coefficient	*p* value	Regression coefficient	*p* value	Regression coefficient	*p* value	Regression coefficient	*p* value
Constant	**8.35**	0.0000	**10.09**	0.0000	**0.63**	0.0000	**10.32**	0.0000
BL	**0.73**	0.0126	**− 0.52**	0.0321	**− 0.21**	0.0001	− 1.01	0.0723
Mutation	**− 0.59**	0.0417	**− 0.53**	0.0294	− 0.07	0.1302	− 0.57	0.3032
Stress	− 0.20	0.4734	0.07	0.7645	**− 0.10**	0.0435	**− 1.52**	0.0083

Significant values are shown in bold

In the table, the value of regression coefficients and corresponding *p* values are shown. The constant is the expression level of the gene when all of the studied predictors are equalled to zero

Table 4 Equations of a multiple linear regression for *PIP1;2*, *PIP1;3*, *PIP2;1,* and *PIP2;3* genes

Gene	Equation of multiple linear regression
PIP1;2	$R = 8.35 + 0.73$ (light) $- 0.59$ (mutation)
PIP1;3	$R = 0.63 - 0.21$ (light) $- 0.10$ (stress)
PIP2;1	$R = 10.09 - 0.52$ (light) $- 0.53$ (mutation)
PIP2;3	$R = 10.32 - 0.52$ (stress)

Shown are the values of regression coefficient of the predictors (light, mutation, or/and mannitol-induced osmotic stress) with significant (at the 0.05 significance level) impact on gene expression (*R*)

also be upregulated by abiotic stresses, suggesting that these aquaporins can facilitate water transport under stress conditions (Jang et al. 2004; Liang et al. 2013; Liu et al. 2013).

Although the difference in percentage of WT and *7B-1* mutant seed germination under 70 mM mannitol was not so distinct as reported for higher mannitol concentrations (Fellner and Sawhney 2002), *7B-1* mutation had a statistically significant negative effect on expression of aquaporin genes *PIP1;2* and *PIP2;1*. This indicates the tendency of *7B-1* mutant seeds to preserve water for radicle growth during seed germination. Thus, the mutation acted on *PIP* gene expressions in the similar way as osmotic stress (conserve the water under the stress) making *7B-1* mutant pre-adapted to osmotic stress. This is further supported by the elevated level of ABA in *7B-1* mutant (Fellner and Sawhney 2001, 2002).

Blue light tended to modulate *PIP* gene expression levels in WT and *7B-1* mutant seeds (Balarynová and Fellner, unpublished results). Here, we confirmed statistically this trend and found that BL enhanced *PIP1;2* and decreased *PIP2;1* and *PIP1;3* gene expressions in tomato seeds. It is known that BL reduces tomato seeds germination (radicle protrusion) (Piterková et al. 2012 and here Fig. 2). On the other hand, BL stimulates tomato root elongation (Balarynová and Fellner, unpublished results); thus, the same stimulatory effect could be expected for radicle. Therefore, the effect of BL on expression of *PIPs* genes involved in radicle elongation during seed germination could be dual. *PIPs* expression should be sufficient to ensure the radicle elongation but not so high to prevent immoderate loses of water which is important for cell elongation.

Literature reporting participation of aquaporins in seed germination or radicle growth is very limited (Obroucheva 2012; Obroucheva et al. 2017, Willigen et al. 2006). Interaction between light and aquaporins was studied especially in plant leaves because of their putative involvement in regulation of water transport and turgor in leaf cells. It was showed that plasma membrane aquaporins play a role in response of the leaf hydraulic conductance to light which stimulates leaf conductance to water. The light stimulation of leaf hydraulic conductance was not associated with stomatal opening. For example, *Juglans regia* aquaporin *JrPIP2;1* and *JrPIP2;2* transcript abundances are related to the dynamics of water transport in leaves (Cochard et al. 2007). Interestingly, BL and green light seem to stimulate leaf hydraulic conductance the most effectively (Baaziz et al. 2012). In our experiments, BL reduced significantly the percentage of seed germination and it was associated with downregulation of *PIP1;3* and *PIP2;1* genes. These results suggest that these PIPs might play an important role in water transport during seed germination. On the other hand, expression of *PIP1;2* gene was induced by BL. Aquaporin PIP1;2 seems to be important element of a whole plant water status in *A. thaliana*. Its expression and activity was reported to be control by multiple factors including BL (Kaldenhoff et al. 1993; Postaire et al. 2010). Besides, *PIP1;2* was one of the most transcribed aquaporin in germinated tomato seeds (Tables 3, 4). Thus, we can expect its significant contribution to maintain appropriate water status in tomato seeds under BL conditions. Moreover, *PIP1;2* may compensate for the reduced expression of *PIP1;3* and *PIP2;1* genes. Finally, *PIP1;2* gene could perform another roles in seeds (see below), because it is believed that PIP2 aquaporins are better water channels than PIP1 aquaporins (Maurel et al. 2015).

Aquaporins transport not only water but also other solutes of great physiological importance such as carbon dioxide, hydrogen peroxide or oxygen. For example, AtPIP1;2 seems to be a CO_2 transporter (Uehlein et al.

2003, 2012) and *Nicotiana tabacum* aquaporin PIP1;3 is thought to be involved in oxygen transport. Thus, PIPs appear to play a dual function. They are important for preserving water homeostasis and also participate in regulation of photosynthesis or cell signalling. AtPIP1;4 is able to translocate extracellular hydrogen peroxide into the cytoplasm and active immune responses induced by bacteria (Tian et al. 2016).

There is a hypothesis that PIP1 aquaporins function as sensors of BL, which is supported by their localization in the plasma membrane and some of their structural features (Kaldenhoff and Eckert 1999; Lorenz et al. 2003). Interestingly, in our experiments, we showed that expression of *PIP1;2* is significantly stimulated by BL but significantly reduced in *7B-1* mutant (Table 3). Then, the reduction of *PIP1;2* expression in the mutant is in agreement with the fact that *7B-1* mutation confers reduced plant and/or seed responsiveness to BL (Fellner et al. 2001; Fellner and Sawhney 2002; Hlavinka et al. 2013; Ježilová et al. 2012). Therefore, we agree that PIP aquaporins could be more than water transporters in seeds.

Altogether, we showed that both osmotic stress and *7B-1* mutation decreased the expression levels of four tested *PIP* genes, probably to protect seed against excessive water loss during the seed germination under the osmotic stress. Moreover, BL affected *PIP* transcription levels indicating the connection between BL signalling and aquaporins. Thus, the inhibitory effect of BL and osmotic stress on tomato seed germination could be, at least partly, related to changes in aquaporin transcripts abundance. As *7B-1* mutation causes defect in phototropin responses (Hlavinka et al. 2013; Bergougnoux and Fellner, unpublished data), we hypothesize that *PIP1;2* and *PIP2;1* may play a role in BL sensing or signalling. This is supported by the fact that the expression of both genes is regulated by BL. Other experiments based on analysis of tomato phototropin mutants would support or disprove this hypothesis.

Author contribution statement JB performed and designed all experiments and wrote the manuscript. JD made data analyses and edited the manuscript. MF designed the experiments and edited the manuscript.

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Appendix 2

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

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

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Abstract

Aquaporins control the specific transport of water across membranes and are involved in various physiological processes. Plasma membrane aquaporins (PIPs) were shown to play a physiological role during tomato seed development. Therefore, we were interested in the participation of PIPs in seed germination and early post-germination. The aim of our study was to investigate the responsiveness of seed germination and early post-germination in WT and the *7B-1* mutant, affected in blue light responses, to $HgCl_2$ (an aquaporin blocker) and to characterise the expression patterns of *PIP*-type aquaporin genes in these lines during different phases of seed germination and early post-germination, and after mercury treatment. Furthermore, we wished to investigate whether blue-light (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 $HgCl_2$ 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. *PIP1;3* gene is downregulated not only by BL but also by $HgCl_2$ with a stronger effect in WT seeds. Thus, we show that BL can alter *PIPs* gene expression during tomato seed germination and early post-germination 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 early radicle elongation and that the *7B-1* mutation and BL have an impact on these processes.

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

Abbreviations: BL – blue light; C_T – threshold cycle; D – darkness; DAS – day after sowing; DTT - dithiothreitol; $HgCl_2$ - mercuric chloride; MS - the basal Murashige and Skoog medium; PIPs – plasma membrane intrinsic proteins; qRT-PCR - quantitative RT-PCR; RT-PCR - reverse transcription-polymerase chain reaction; WT – wild-type.

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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 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, 14 were categorised as Plasma membrane Intrinsic Proteins (PIPs) (Reuscher *et al.* 2013). It was shown that PIPs play an important homeostatic role during seed development (Shiota *et al.* 2006) and they were proposed to be involved in rehydration during seed germination (Gao *et al.* 1999, Suga *et al.* 2001, Schuurmans *et al.* 2003).

A useful tool to study a contribution of PIPs in seed germination and early post-germination is treatment with mercuric chloride ($HgCl_2$). Although a few mercury-resistant aquaporins have been described in plants (Daniels *et al.* 1994, Biela *et al.* 1999, Willigen *et al.* 2006), mercury compounds are traditionally used as blockers of aquaporins and water transport (Maggio and Joly 1995, Daniels *et al*. 1996, Chaumont *et al*. 1998, Biela *et al.* 1999, Hukin *et al*. 2002, Willigen *et al.* 2006, Beaudette *et al*. 2007). Mercury derivatives act by oxidizing cysteine residues within the water pore and altering the conformation in other regions of the protein

without affecting the integrity of the cell. The blockage of aquaporins by $HgCl_2$ can be reversed by reducing agents such as dithiothreitol (DTT) (Preston *et al.* 1992).

The expression of aquaporins can be modulated by various environmental factors including light (Chaumont and Tyerman 2014, Li *et al.* 2014), but there is no report of the regulation of aquaporin expression by light during seed germination. We demonstrated earlier that continuous blue light (BL) reduces tomato seed germination and that tomato *7B-1* mutant seeds are less responsive to BL-induced inhibition of seed germination (Piterková *et al.* 2012). Kaldenhoff *et al.* (1995, 1996) 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.

7B-1 is a spontaneous recessive single gene mutant selected for its male sterility under long days (Sawhney 1997). Seed germination tests show that *7B-1* is less responsive than the corresponding 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 *SlGLO2* 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: i) to investigate the responsiveness of tomato seed germination and early radicle and root elongation to mercury treatment (an aquaporin blockage); ii) to characterise the expression of *PIP* genes during seed germination and early radicle elongation; iii) to analyse the putative BL control over these processes. We used tomato seeds of cv. Rutgers (WT) and the *7B-1* mutant impaired in various responses to BL (Fellner and Sawhney 2002, Bergougnoux *et al.* 2009, 2012, Ježilová *et al.* 2012, Piterková *et al.* 2012, Hlavinka *et al.* 2013). Our results suggest that aquaporins are not involved in early seed imbibition, but in radicle elongation during tomato seed germination. Moreover, seed germination and radicle/root elongation of BL-insensitive *7B-1* mutant are less responsive to $HgCl_2$ compared to WT. Our data indicate that BL modulates the expression of *PIP1;1* (the trend of upregulation) and *PIP1;3* (the trend of downregulation) aquaporin isoforms in both WT and *7B-1* mutant seeds. There is an indication that *PIP1;3* gene expression is downregulated also by $HgCl_2$ with stronger effect in WT seeds. Our data show that *PIP* aquaporins participate in tomato seed germination and early radicle elongation and that the *7B-1* mutation and BL influence these processes.

Materials and Methods

Plant material

Tomato (*Solanum lycopersicum* L.) cv. Rutgers (wild-type, WT) and the *7B-1* mutant were used in this study. *7B-1* is impaired in various responses to BL (Fellner and Sawhney 2002, Sheoran *et al.* 2006, Bergougnoux *et al.* 2009, 2012, Ježilová *et al.* 2012, Piterková *et al.* 2012, Hlavinka *et al.* 2013, Omidvar and Fellner 2015). The germination of *7B-1* seeds is less sensitive than WT to diverse abiotic stresses (Fellner and Sawhney 2001) including osmotic stress specifically under BL conditions (Fellner and Sawhney 2002).

Germination assay

Tomato seeds were surface sterilized in 2.8 % sodium hypochlorite solution (Bochemia, Czech Republic) for 20 minutes and then rinsed with sterile distilled water. Afterwards, seeds were sown on basal Murashige and Skoog medium (MS) (Murashige and Skoog 1962) supplemented with 0.7% (w/v) agar in round Petri dishes (90 mm in diameter). Based on the experiment, the MS medium was supplemented with mercuric chloride ($HgCl_2$, 20 or 30 μM), dithiothreitol (DTT, 2 mM) or with a mixture of 30 μM $HgCl_2$ and 2 mM DTT. The dishes were placed vertically in growth chambers (Microclima 1000; Snijders Scientific B.V., The Netherlands) and seeds were incubated at 23°C in continuous BL or in darkness. For dark conditions, plates were wrapped in aluminium foil. BL was provided by fluorescent tubes (Philips TLD-36W/18-Blue; maximum irradiance of BL was at 440 nm; 10 mmol m^{-2} s^{-1} total photon flow rate). The light spectrum was measured using a portable spectroradiometer (model LI-1800; Li-COR, NE, USA). Seed germination was recorded daily from the second to eighth day after sowing. The percentage germination, (germinated seeds/total number of seeds) x100, was counted every day and the average was calculated. A control on MS was sown for each experiment. For each treatment at least six independent experiments per each time point were performed and thirty seeds per dish were sown for each treatment.

Water uptake

Tomato seeds were sown on the Petri dishes with MS medium or MS medium supplemented with 30 μM $HgCl_2$ and cultured in darkness or BL as described above. After specified times (4, 8, 12, 24, 48, 72 and 96 hours after sowing), non-germinated seeds were collected and excess water was removed by a filter paper. Subsequently, seeds were weighed, dried for 48 hours at 70ºC and then weighed again. The percentage of water content was calculated as ((FW-DW)/FW) x100, where FW is the fresh weight of imbibed seeds and DW is seed dry weight. The experiment was repeated twice.

Growth experiments

Tomato seeds were sown on MS medium in square Petri dishes (120 x 120 mm) and incubated in the growth chambers in darkness as described above. After three days, germinated seeds were transferred onto dishes containing MS medium supplemented with $HgCl_2$ (10 - 30 µM). Seeds transferred onto medium without $HgCl_2$ served as controls. Seeds were then incubated in growth chambers in the dark or in continuous BL for seven days. Then, the seedling root length was measured with a ruler to the nearest millimetre. For each $HgCl_2$ concentration at least three independent experiments on at least seven seedlings were performed.

Sampling, RNA isolation and cDNA synthesis

Tomato seeds were sown on the MS medium or on MS medium with $HgCl_2$ (30 µM) and cultivated in darkness or BL as described above. WT and *7B-1* mutant seeds were collected into liquid nitrogen. For analysis of *PIP* expression in different stages of seed germination and early post-germination, the first set of seeds was harvested 24 hours after sowing (termed as 24; non-visible radicle). The second set comprised germinated seeds collected after 72-hour cultivation just after radicle penetration (the radicle was 1-3 mm long). Finally, seeds with radicle up to 10 mm were harvested 96 hours after sowing. To study the effect of $HgCl_2$ on *PIP* expression, WT and *7B-1* seeds with radicle up to 10 mm were harvested after 96-hour cultivation in the dark or in BL. Seeds germinated under BL were harvested in BL conditions, while seeds germinated in the dark were harvested under a green safelight. Ungerminating seeds dry WT and *7B-1* seeds were also extracted for RNA isolation.

Collected seeds were ground by a mortar and pestle to a fine powder under liquid nitrogen. Total RNA from the seeds was isolated using the Qiagen RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer´s protocol. Residual DNA was removed from samples by treatment with Recombinant DNaseI (Takara Bio Inc., Japan) and Recombinant RNase Inhibitor (Takara Bio Inc., Japan) for 60 minutes at 37°C. DNaseI 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™ 1st strand cDNA Synthesis Kit (Takara Bio Inc., Japan).

RT-PCR

To screen for changes of *PIP* gene expression induced by30 µM $HgCl_2$ and the differences in amount of *PIP* transcripts in dry WT and *7B-1* mutant seeds, a semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was used. Gene expression was analysed with primers specific for nine tomato *PIP* genes (Shiota *et al.* 2006). The reaction volume was 20 µl. DNA was denatured at 94ºC for 3 min, followed by 25 or

30 cycles of amplification (94ºC for 30 sec, 53-60ºC for 30 sec and 72ºC for 1 min) and concluded with final extension at 72ºC for 5 min. *LeEF1* (*Elongation Factor 1α*) (Shiota *et al.* 2006) and *GAPDH* (*Glyceraldehyde-3-Phosphate Dehydrogenase*; primers kindly provided by V. Bergougnoux: F-AACCGGTGTCTTCACTGACAAGGA, R-CACCCACAACAAACATGGGAGCAT) were used as references. The PCR products were separated on 1 or 1.5 % agarose gel with ethidium bromide. The bands were evaluated using ImageJ processing program (Collins 2007) and gene expression relative to both reference genes and to control sample (WT seeds germinated on MS in the D or dry WT seeds) was calculated. The experiments with $HgCl_2$ were repeated four times, experiments with dry seeds three times.

qRT-PCR

To assess the expression of *PIPs* during different stages of seed germination and early post-germination and to study the effect of 30 µM $HgCl_2$ on *PIP1;1* and *PIP1;3* gene expression, quantitative RT-PCR (qRT-PCR) was used. The gene expression was analysed using SYBR Premix Ex Taq™ (Perfect Real Time) Kit (Takara Bio Inc., Japan) with a Mx3000P™ Thermal Cycler (Agilent Technologies, USA). The PCR reaction mixture was prepared according to the manufacturer's protocol, containing 12.5 µl of SYBR Premix Ex Taq™, 0.5 µl of each primer (10 µM), 0.5 µl of ROX Reference Dye II, 9 µl of sterile RNase-free water and 2 µl of cDNA template (diluted 1/50). The final reaction volume was 25 µl. Each sample was measured in triplicate. The following PCR running conditions were used: an initial denaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 20 sec. Melt curves were analysed at the end of PCR reactions. *PP2Acs* (*Protein Phosphatase 2A catalytic subunit*) (Lovdal and Lillo 2009) and *TIP41-like* (Dekkers *et al.* 2012) genes were used as references. The sequences of aquaporin primers are given in Balarynová *et al.* 2018. The specific primers for *PIP1;1* gene (accession number BP887068) were F- ACAAGGACTACAAAGAGCCAC, R-GATTTAGAAACGCCCATGACG. The primers were designed using PrimerQuest® program (IDT, Coralville, USA) and their efficiencies were calculated from the slope of the dilution curve. The series of 50, 100, 500 and 1000-fold 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_T-method', the experiment was repeated four times. The expression of individual *PIPs* in seeds collected 24, 72 and 96 hours after sowing was calculated using 'delta C_T-method' (the expression was relative to reference genes) (Pfaffl 2001). For *PIP1;1*, *PIP1;3*, *PIP1;4* and *PIP2;2* genes, whose expression levels were low, the relative expression was quantified also using 'delta-delta C_T-method'(against both the sample of WT MS D 24-hours and reference genes). The experiment was repeated three times.

Statistical analysis

The STATISTICA 12 software (StatSoft, OK, USA) was used to perform all statistical analyses. The significant differences among the different mercury treatments (in germination assay and growth responses) 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 mercury 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, 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-C_T method or delta-delta C_T-method (Pfaffl 2001) are given in the Supporting information to demonstrate that the gene expression of individual replicates show the same trends (**Table 1-5 Suppl.**). These values were used to calculate the geomeans and SE.

Results

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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 $HgCl_2$, a generally utilized inhibitor of aquaporins (Macey 1984). In the absence of $HgCl_2$ (control), 87% of WT seeds germinated after 7 days in the dark (D-control) while WT seed germination was slightly, but not significantly, reduced to 82% in continuous BL (BL-control) (**Fig 1A**). 20 μM $HgCl_2$ reduced significantly WT seed germination in the dark as well as in BL, to 37 and 40 %, respectively, compared to the corresponding control on medium without mercury. 30 μM $HgCl_2$ inhibited significantly WT seed germination to 7 and 3% in the dark and under BL, respectively, compared to the corresponding control. In the dark as well as in BL, inhibition of WT seed germination by mercury was completely restored when the seeds were treated simultaneously with 2 mM dithiothreitol (DTT). We also validated that DTT alone did not have any significant effect on seed germination in the dark and in BL. Sown on medium supplemented with DTT, 90 % of WT seeds germinated in both the dark and BL. WT seed germination was also 90 % on medium

supplemented with both 2 mM DTT and 30 µM $HgCl_2$ 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 in BL, respectively. Germination of *7B-1* mutant seeds was not affected significantly by 20 µM $HgCl_2$ in the dark and BL. 30 µM $HgCl_2$ decreased significantly *7B-1* seed germination to 60% in the dark and to 57% in BL compared to the corresponding control on MS. As for the WT, *7B-1* mutant seed germination was not affected by treatment with 2 mM DTT alone (seed germination was 93% in the dark and 90% in BL). Likewise, 97% of *7B-1* mutant seed germinated on medium with both 2 mM DTT and 30 µM $HgCl_2$ 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**). For easier comparison of individual treatments, 'a threshold' of 25% of seed germination was chosen.

Mercury treatment markedly delayed WT seed germination. In the dark control, average germination of WT seeds reached the threshold of 25% before the 3rd DAS (**Fig 2A**). In the presence of 20 µM $HgCl_2$, germination of WT seeds reached 25% approximately by the 4th DAS. At 30 µM $HgCl_2$, germination of WT seeds failed to reach 25%, While under BL control, WT seed germination reached the 25% threshold by approximately the 3rd DAS (**Fig 2B**). Treatment with 20 µM $HgCl_2$ decreased the rate of WT seed germination under BL achieving the 25% threshold by the 5th DAS, whereas the effect of 30 µM $HgCl_2$ was so pronounced that WT seed germination did not reach 25 %.

Under dark control conditions, *7B-1* seed germination exceeded 25% before the 3rd DAS (**Fig 2C**). When 20 µM $HgCl_2$ was applied, *7B-1* germination reached the threshold at approximately the 3rd DAS in the dark, while 30 µM $HgCl_2$ delayed mutant seed germination, reaching 25% approximately at the 4th DAS. When grown in BL, *7B-1* mutant seed germination reached the 25% threshold by approximately the 3rd DAS (**Fig 2D**). Treatment with 20 µM $HgCl_2$ did not alter the kinetics of *7B-1* seed germination in BL, although 30 µM $HgCl_2$ markedly delayed *7B-1* seed germination under BL reaching the 25% threshold by the 5th DAS.

The distinct 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 $HgCl_2$. In both genotypes, mercury had no effect on fresh weight increase during 96-hour 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.**).

To test the sensitivity of developing roots to the aquaporin blocker $HgCl_2$, root elongation of seedlings growing on different $HgCl_2$ concentrations was studied. In the dark control, roots of 7-day-old WT seedlings were approximately 65 mm long. Root growth of WT seedlings was strongly inhibited by all applied concentrations of $HgCl_2$ (**Fig 3A**). BL stimulated significantly root growth in WT, whereas application of mercury resulted in root growth inhibition counteracting the response to BL (**Fig 3B**). $HgCl_2$ 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 to those of the WT. The inhibitory effect of mercury on root elongation was markedly lower than in WT plants. In the dark at all tested mercury concentrations, *7B-1* mutant roots were significantly longer than those of WT seedlings. BL stimulated root growth in *7B-1* plants (**Fig 3B**) in similar way as in WT plants but the WT seedlings had significantly longer roots than *7B-1* mutant seedlings growing on MS medium without mercury (BL-control). Responsiveness of the BL-incubated mutant roots to $HgCl_2$ was significantly lower than in BL-incubated WT roots (**Fig 3B**). Concentrations of 12 μM $HgCl_2$ and higher inhibited significantly the length of *7B-1* roots in the dark, while in BL, significant reduction in root length of *7B-1* control occurred at 15 μM $HgCl_2$ and higher concentrations.

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 $HgCl_2$.

In dry WT and *7B-1* seeds, transcripts of *PIP1;3* and *PIP2;4* 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 possible involvement of 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 hours (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 level of expression after radicle protrusion (**Fig 4, Fig. 4 Suppl.**). The expression of each gene tended to be the lowest 24 hours after sowing (during water imbibition) and then it increased during germination and the early post-germination phase (72 and 96 hours after sowing). This trend was the same for both WT (**Fig 4** and **Fig. 3 Suppl.**) and *7B-1* mutant seeds in the dark as well as BL (**Fig. 3 Suppl.**).

Our data showed a trend indicating that the aquaporin genes *PIP1;2*, *PIP1;5*, *PIP2;1* and *PIP2;3* were expressed predominantly during seed germination and early post-germination (**Fig 4**). Expression of *PIP1;2* and *PIP2;1* genes tended to be highest at 72 hours after seed sowing, whereas *PIP1;5* and *PIP2;3* expression peaked one day later (96 hours after seed sowing).

Interestingly, expression of *PIP1;1* and *PIP1;3* was modulated by BL (**Figs 5**). In both genotypes, the expression of *PIP1;1* gene showed a marked stimulation by BL in germinated seeds harvested 96 hours after sowing (**Fig 5** and **Fig. 4 Suppl.)**. An increase in *PIP1;1* gene expression under BL was also observed, but less pronounced, in WT seeds harvested at 72 hours, but not at 24 hours after seed sowing. Promotion of *PIP1;1* gene expression by BL was not observed in *7B-1* mutant seeds collected after 24 and 72 hours (**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 hours 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 hours after seed sowing (**Fig. 4 Suppl.**).

To investigate resistance of *7B-1* seed germination to $HgCl_2$at gene expression level, we studied expression of the *PIP* genes in germinated seeds (radicle length up to 1 cm) incubated for 96 hours in the absence or presence of 30 μM $HgCl_2$ in the dark or in BL. From the nine *PIP* genes tested, only the expression of *PIP1;3* was affected by $HgCl_2$ (**Fig 5**). The level of *PIP1;3* gene expression was reduced by mercury in both WT and *7B-1* mutant seeds in the dark as well as in BL. The mercury-induced inhibition of *PIP1;3* transcript accumulation was less pronounced in *7B-1* than in WT seeds. $HgCl_2$ had no effect on expression of *PIP1;1* **(Fig 5)**, *PIP2;1* and *PIP2;3* (**Fig. 5 Suppl.**). However, the expression of *PIP1;2*, *PIP1;4*, *PIP1;5* and *PIP2;2* genes was elevated in WT seeds incubated in the presence of mercury under BL conditions (**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 prospective involvement of PIP aquaporins in tomato seed germination and early post-germination at both the physiological and transcript levels using the standard water channel blocker $HgCl_2$. We used the tomato *7B-1* mutant as a potentially informative model since 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 in tomato seed germination. Moreover, seed germination in the *7B-1* mutant was more tolerant to $HgCl_2$ than in the WT. Mercury caused not only reduction of the final portion of germinated seeds, but also delayed germination (radicle emergence), as has been reported for *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 compare to the WT. This suggests that the *7B-1* mutation may cause an increase in aquaporin amount or enhance their activity. However, we found that aquaporins were unlikely to be regulated by *7B-1* at the transcript 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 at the outset of seed germination (during water imbibition) and on the possible effects of $HgCl_2$ and BL on this process. The rate of water absorption was 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 *PIPs* 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 was 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.

The role of *PIPs* in tomato seed development was shown recently. Shiota *et al.* (2006) reported the expression of seven *PIPs* (*PIP1;1*, *PIP1;2*, *PIP1;3*, *PIP1;4*, *PIP1;5*, *PIP2;1* and *PIP2;2*) in developing tomato seeds and they found that the number of aquaporin molecules increases and is maintained at high levels in mature seeds. Thus, we focused on characterization of the expression patterns of *PIP*s during tomato WT and

7B-1 mutant seed germination and early post-germination. We found that transcripts of the tested *PIPs*, apart from *PIP1;3* and *PIP2;4*, preexisted in the dry seeds. Surprisingly, dry seeds of *7B-1* contained lower amounts of *PIP* 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 suggests that it is probably not involved in seed germination.

The amount of *PIP* transcripts tended to increase together with radicle protrusion. Thus, a role for *PIPs* 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 PIPs 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 turgor pressure (Chaumont 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 PIPs in tissue elongation has been suggested several times (Maurel *et al.* 2002, Fricke and Chaumont 2007, Liu *et al.* 2008).

Various trends in expression patterns were observed for the tested *PIP* 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, while *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 *PIP* transcripts accumulation was found between WT and mutant seeds in both the dark and BL. Furthermore, not even mercury treatment (30 μM $HgCl_2$) had a substantial effect on *PIP* gene expression, with only one exception. The amount of *PIP1;3* transcript was downregulated by mercury (**Fig 5**). Moreover, *PIP1;3* gene 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* gene 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 PIPs to find out whether they differ in WT and *7B-1* seeds differ at the protein level.

Specifically in BL, $HgCl_2$ enhances slightly the expression of *PIP1;2*, *PIP1;4*, *PIP1;5* and *PIP2;2* genes in WT seeds. How can this stimulation of these genes by mercury and BL be explained? It is possible that mercury-induced blockage of aquaporins together with BL-induced stress required increased transcription of these genes to compensate for the aquaporin blockage in order to sustain the appropriate embryo elongation. *7B-1* mutant seeds were not so greatly affected by the treatment with mercury, so there was no need to increase aquaporin 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 post-germination 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 $HgCl_2$ 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 $HgCl_2$, supporting the expectation that aquaporins are involved in this process (Hukin *et al.* 2002, Javot and Maurel 2002). 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 $HgCl_2$, *7B-1* roots were always longer that those of WT seedlings under BL conditions 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 in BL *PIP1;1* could be involved in root elongation. 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 $HgCl_2$ 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.

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Fig. 1. The graphs show germination of tomato cv. Rutgers (WT) **(A)** and *7B-1* mutant **(B)** seeds incubated for 7 days in the dark (D) or in blue light (BL) and reversibility of mercury inhibition by 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.

Fig. 2. The graphs show kinetics of WT (A and B) and *7B-1* (C and D) seed germination on MS medium (control) and on MS supplemented with 20 or 30 μM $HgCl_2$ during 8-day cultivation in the dark (D) or 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.

Fig. 3. The graphs show the effect of increasing mercury concentration on root length of 7-day-old WT and *7B-1* mutant seedlings growing in the dark (A) and 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 $HgCl_2$ (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.

Fig. 4. The graph shows the expression patterns of investigated *PIPs* during cv. Rutgers (WT) seed germination and early post-germination in the dark. RNA was isolated from seeds harvested 24 (24), 72 (72, seeds with radicle 1-3 mm long) and 96 (96, seeds with radicle 1-10 mm long) hours after seed sowing on MS medium. The expression was analysed by qRT-PCR. The relative expression was calculated using 'delta C_T-method' (the expression was relative to references – *PP2Acs* and *TIP41like* genes). The values represent geomean and SE of three independent experiments.

Fig. 5. The relative expression of *PIP1;1* and *PIP1;3* genes in cv. Rutgers (WT) and *7B-1* mutant seeds after 96-hour cultivation on MS medium (control) and on MS medium with 30 μM mercuric chloride ($HgCl_2$) in the dark (D) or under blue light (BL). The expression was measured by qRT-PCR. The relative expression was quantified against the expression in WT seeds in the dark using the 'delta-delta C_T-method' and values represent the geomean and SE of four experiments.

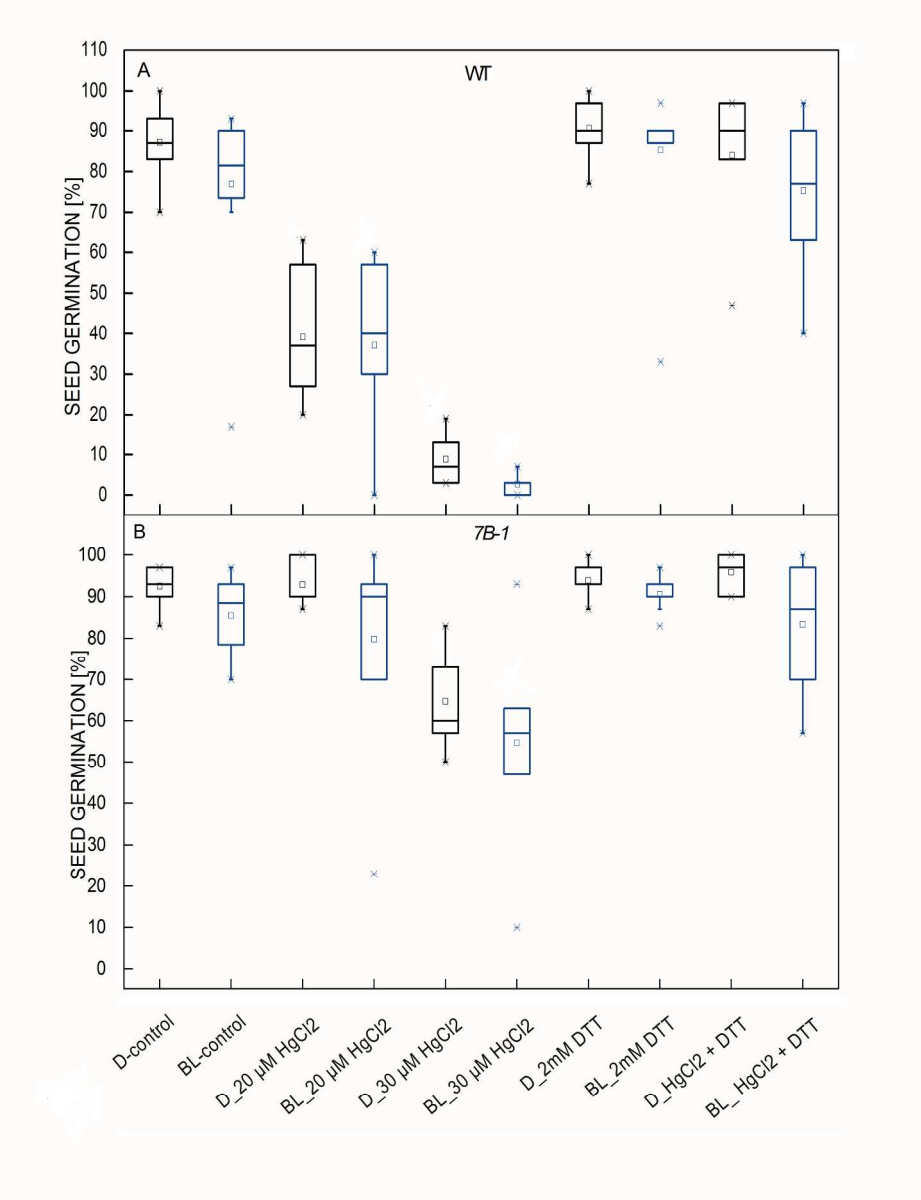

Fig. 1. The graphs show germination of tomato cv. Rutgers (WT) (A) and 7B-1 mutant (B) seeds incubated for 7 days in the dark (D) or in blue light (BL) and reversibility of mercury inhibition by 2 mM dithiothreitol (DTT). The boxes represent the first and third quartiles and a median value of seed germination (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.

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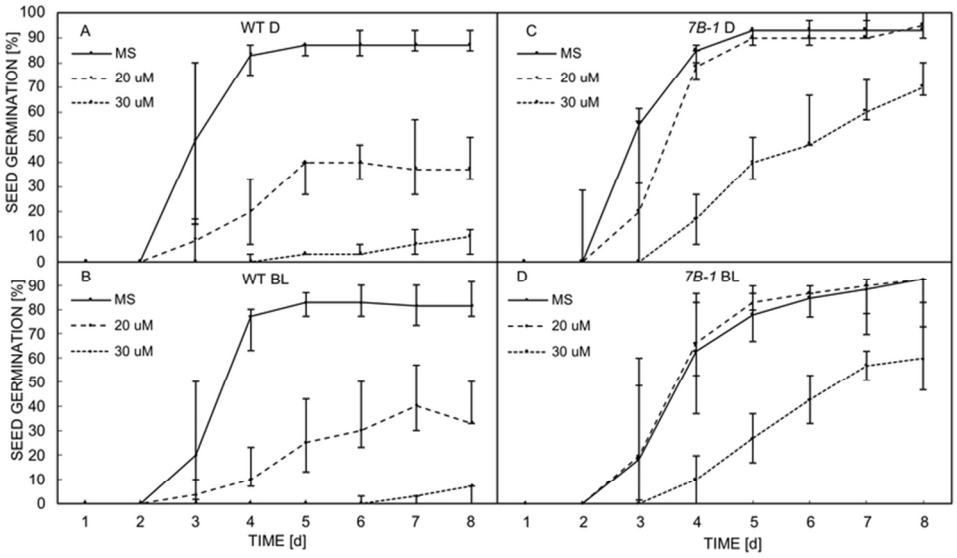

Fig. 2. The graphs show kinetics of WT (A and B) and 7B-1 (C and D) seed germination on MS medium (control) and on MS supplemented with 20 or 30 μM HgCl2 during 8-day cultivation in the dark (D) or blue light (BL). The data show medians and the error bars represent the first and third quartiles. For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

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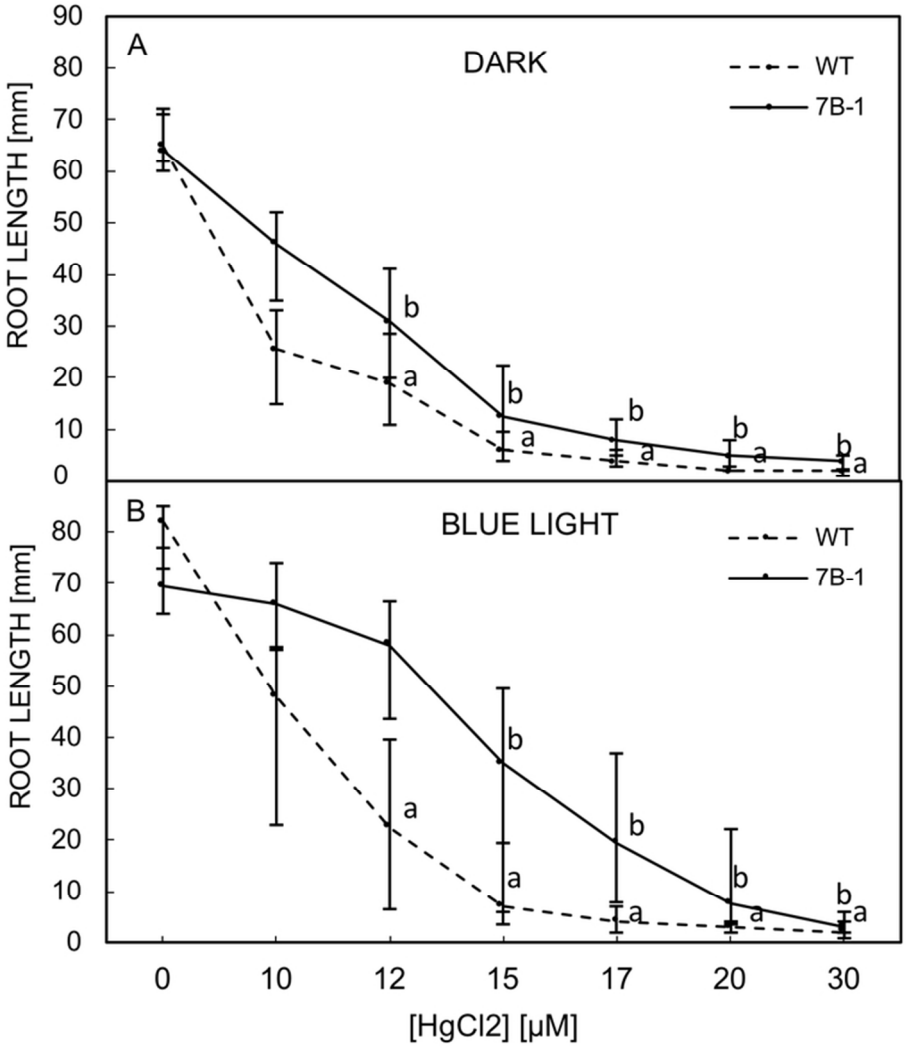

Fig. 3. The graphs show the effect of increasing mercury concentration on root length of 7-day-old WT and 7B-1 mutant seedlings growing in the dark (A) and blue light (B). The results represent the medians and the error bars are the first and third quartiles. The significant difference among various 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.

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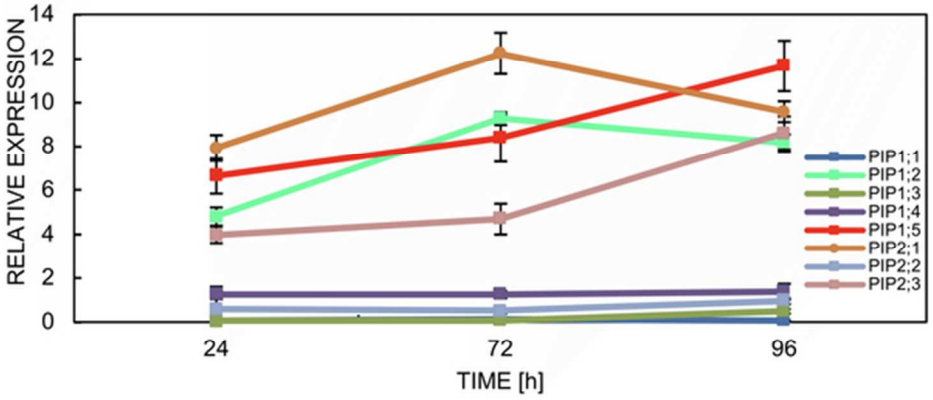

Fig. 4. The graph shows the expression patterns of investigated PIPs during cv. Rutgers (WT) seed germination and early post-germination in the dark. RNA was isolated from seeds harvested 24 (24), 72 (72, seeds with radicle 1-3 mm long) and 96 (96, seeds with radicle 1-10 mm long) hours after seed sowing on MS medium. The expression was analysed by qRT-PCR. The relative expression was calculated using 'delta CT-method' (the expression was relative to references – PP2Acs and TIP41like genes). The values represent geomean and SE of three independent experiments.

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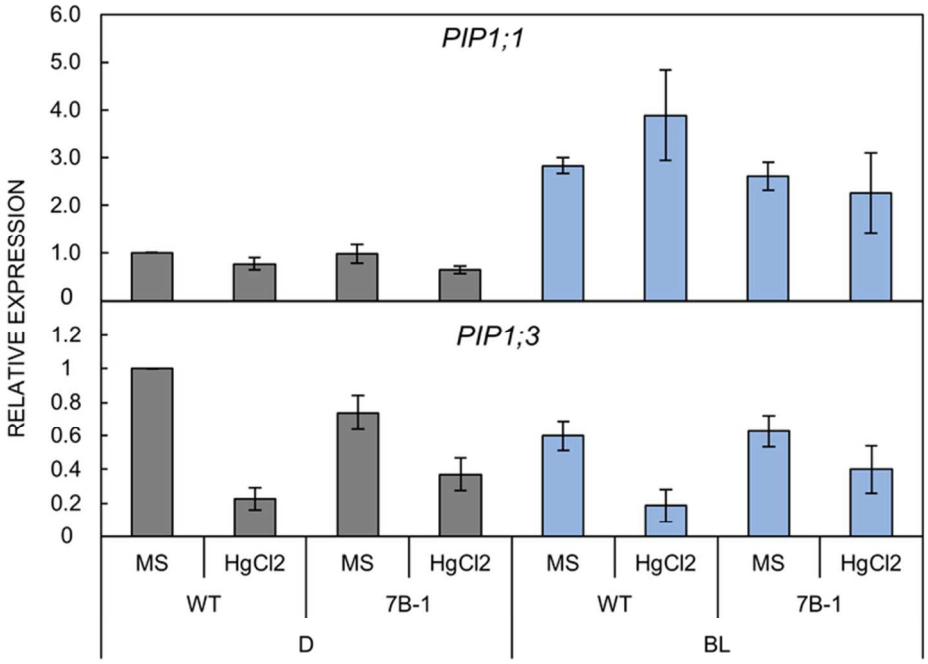

Fig. 5. The relative expression of PIP1;1 and PIP1;3 genes in cv. Rutgers (WT) and 7B-1 mutant seeds after 96-hour cultivation on MS medium (control) and on MS medium with 30 μM mercuric chloride (HgCl2) in the dark (D) or under blue light (BL). The expression was measured by qRT-PCR. The relative expression was quantified against the expression in WT seeds in the dark using the 'delta-delta CT-method' and values represent the geomean and SE of four experiments.

69x52mm (300 x 300 DPI)

Fig. 1 Suppl. The effect of mercuric chloride (30 μM $HgCl_2$) on imbibition of water in tomato cv. Rutgers (WT) and *7B-1* mutant seeds incubated in the dark (D) or under blue light (BL) conditions. Data show average fresh weight increase (FW increase) and SE from 2 independent experiments.

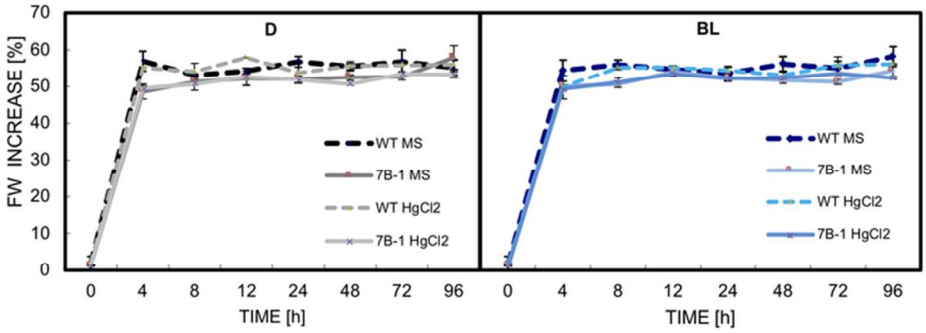

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Fig. 2 Suppl. The relative expressions of tomato *PIP* genes in dry cv. Rutgers (WT) and *7B-1* mutant seeds analyzed by RT-PCR. The expressions of *PIP1;3* and *PIP2;4* were not detected in dry seeds. The relative expression was quantified against both the expression from WT seed sample and expression of reference genes (*GAPDH* and *EF1*). Values represent geomean (of expression relative to *GAPDH* and expression relative to *EF1*) and SE of three experiments.

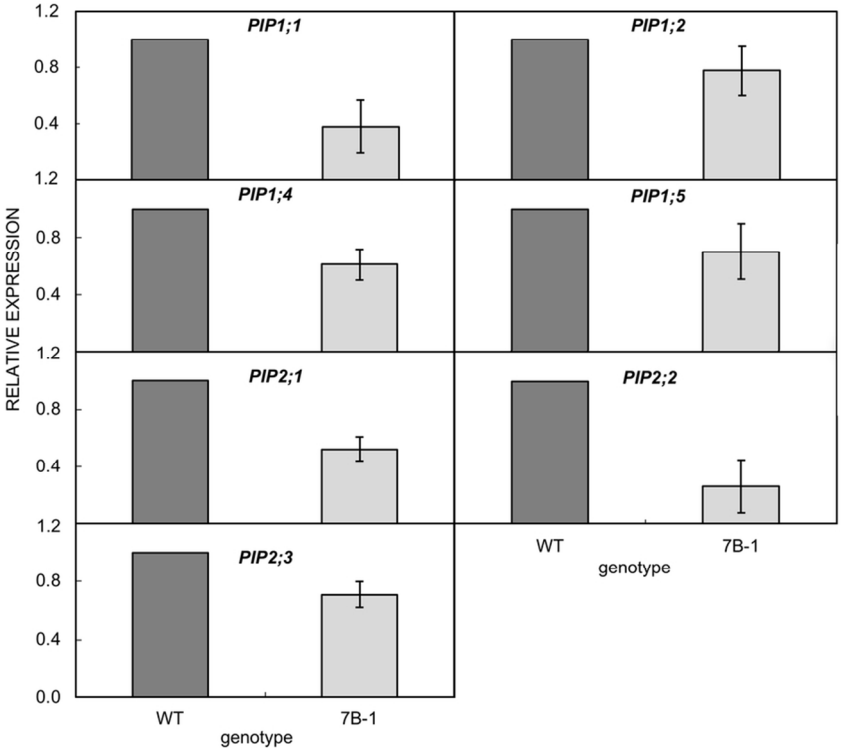

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Fig. 3 Suppl. The expression patterns of investigated *PIPs* during seed germination and early post-germination in cv. Rutgers (WT) and *7B-1* mutant seeds incubated in the dark (D) or in blue light (BL). RNA was isolated from seeds harvested 24 (24), 72 (72, seeds with radicle 1-3 mm long) and 96 (96, seeds with radicle 1-10 mm long) hours after seed sowing on MS medium. The expression was analyzed by qRT-PCR. The relative expression was calculated using 'delta C_T-method' (the expression was relative to reference genes). The values represent geomean and SE of three independent experiments.

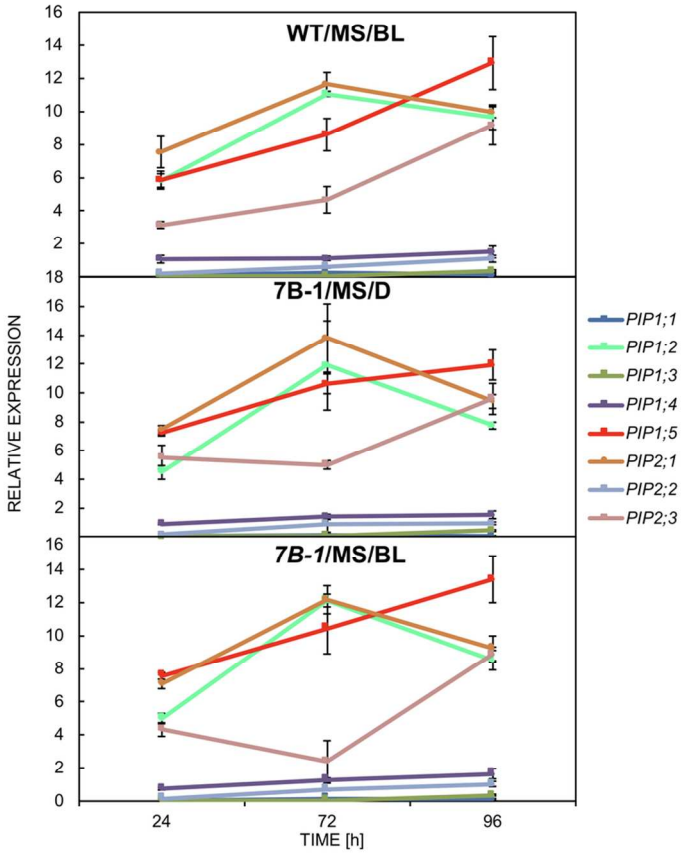

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Fig. 4 Suppl. The relative expression of *PIP1;1*, *PIP1;3*, *PIP1;4* and *PIP2;2* genes in cv. Rutgers (WT) and *7B-1* mutant seeds harvested 24 (24), 72 (72, seeds with radicle 1-3 mm long) and 96 (96, seeds with radicle 1-10 mm long) hours after sowing on MS medium in the dark (D) and blue light (BL). The expression was measured by qRT-PCR. The relative expression was quantified using 'delta-delta C_T-method' (it means against both the expression from sample of WT seeds incubated for 24 hours on MS in the dark and the expression of reference genes). Values represent geomean and SE of three experiments.

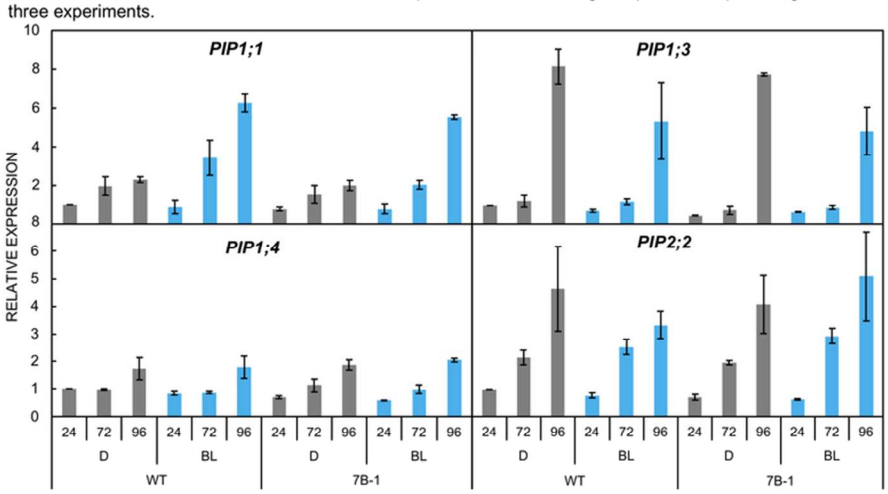

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Fig. 5 Suppl The relative expression of *PIP1;2*, *PIP1;4*, *PIP1;5*, *PIP2;1*, *PIP2;2* and *PIP2;3* genes in cv. Rutgers (WT) and *7B-1* mutant seeds after 96-hour cultivation on the MS medium (MS) or on MS medium with 30 μM mercuric chloride ($HgCl_2$) in the dark (D) and blue light (BL). The expression was measured by RT-PCR. The relative expression was quantified against both the expression from WT seed sample in the dark (WT MS D) and expression of reference genes. Values represent geomean and SE of four experiments.

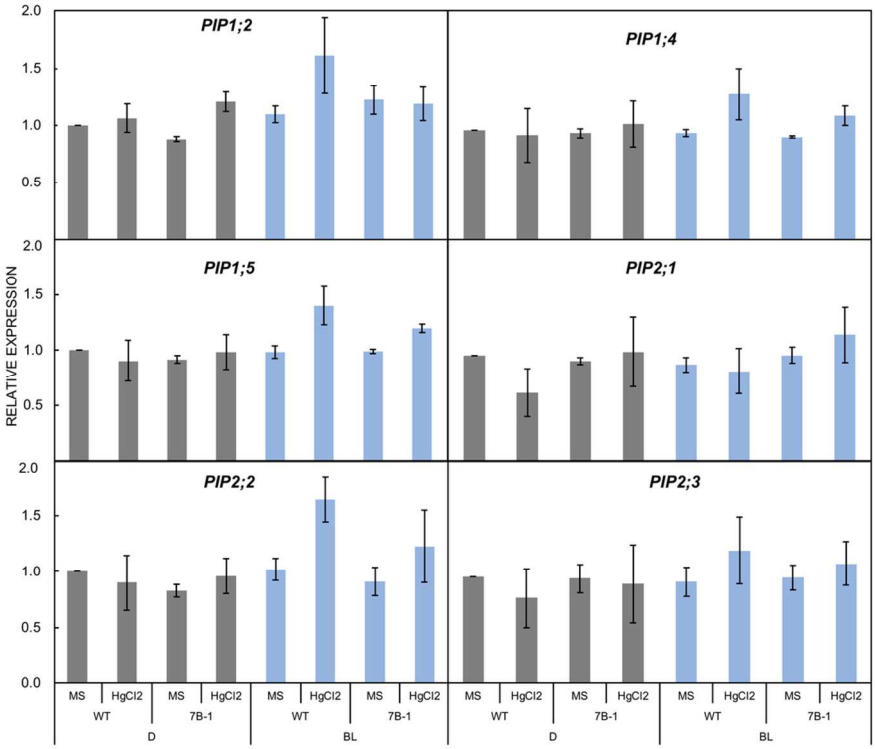

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Table 1 Suppl. The individual values of relative expressions of tomato *PIP* genes in dry *7B-1* mutant seeds analyzed by RT-PCR. The expressions of *PIP1;3* and *PIP2;4* were not detected in dry seeds. The relative expression was quantified against both the expression from WT seed sample (which has therefore always the same value, it equals 1,00), and expression of two reference genes (*GAPDH* and *EF1*). Values represent geomean (of expression relative to *GAPDH* and expression relative to *EF1*).

	relative expression		
gene	**1st set**	**2nd set**	**3rd set**
PIP1;1	0.56	0.00	0.57
PIP1;2	0.44	0.93	0.97
PIP1;4	0.80	0.62	0.42
PIP1;5	0.49	1.02	0.74
PIP2;1	0.49	0.55	0.65
PIP2;2	0.47	0.00	0.18
PIP2;3	0.66	0.57	0.68

Table 2 Suppl. The individual expressions of investigated *PIPs* during cv. Rutgers (WT) and *7B-1* mutant seed germination and early post-germination in both the dark (D) and blue-light (BL) conditions. RNA was isolated from seeds harvested 24 (24), 72 (72, seeds with radicle 1-3 mm long) and 96 (96 seeds with radicle 1-10 mm long) hours after seed sowing on MS medium. The expression was analyzed by qRT-PCR. The relative expression was calculated using 'delta CT-method' (the expression was relative to references – *PP2Acs* and *TIP41like* genes). These values were used to calculate average relative gene expression (in form of geomean) of studied genes in each sample presented in Fig.3 Suppl. and in Fig 4.

stage/gene		DARK										BLUE LIGHT									
		WT					*7B-1*					WT					*7B-1*				
24	***PIP1;1***	0.07	0.06	0.08			0.05	0.06	0.06			0.09	0.09	0.03			0.04	0.08	0.05		
	PIP1;2	5.63	4.68	4.24			5.35	3.71	4.57			6.46	5.03	6.05			5.22	5.37	4.42		
	PIP1;3	0.05	0.04	0.06			0.02	0.02	0.03			0.04	0.03	0.02			0.03	0.03	0.04		
	PIP1;4	1.65	1.08	1.09			0.99	0.83	0.87			1.20	0.78	0.98			0.91	0.67	0.67		
	PIP1;5	8.27	6.31	5.61			7.58	7.28	6.82			6.98	5.41	5.34			7.74	7.77	7.16		
	PIP2;1	9.10	7.67	7.13			7.54	7.92	6.90			9.54	7.01	6.45			6.69	7.64	6.94		
	PIP2;2	0.35	3.32	0.18			0.21	0.21	0.12			0.26	0.14	0.17			0.21	0.14	0.12		
	PIP2;3	4.37	4.42	3.29			5.67	4.22	7.07			3.24	2.78	3.43			4.51	3.63	4.99		
72	***PIP1;1***	0.10	0.18	0.14			0.09	0.15	0.09			0.20	0.32	0.21			0.14	0.15	0.14		
	PIP1;2	9.24	8.76	9.81			8.69	10.37	16.67			11.05	11.32	10.81			11.70	11.80	12.86		
	PIP1;3	0.05	0.07	0.06			0.05	0.04	0.03			0.05	0.04	0.08			0.05	0.04	0.04		
	PIP1;4	1.57	1.07	1.15			1.50	1.08	1.71			1.40	0.94	1.04			1.45	0.93	1.43		
	PIP1;5	10.53	8.20	6.81			11.30	9.93	10.50			9.55	8.71	7.03			13.62	9.82	8.51		
	PIP2;1	14.14	11.05	11.71			12.17	11.43	18.91			12.03	11.22	10.73			13.88	11.07	11.68		
	PIP2;2	0.75	0.38	0.47			0.65	0.45	1.87			0.72	0.55	0.54			0.87	0.64	0.61		
	PIP2;3	6.17	4.06	4.14			4.65	5.36	4.80			6.25	3.51	4.62			0.77	4.23	3.81		
96	***PIP1;1***	0.07	0.05	0.08	0.01	0.02	0.09	0.10	0.13	0.03	0.02	0.31	0.13	0.37	0.09	0.06	0.25	0.15	0.32	0.07	0.05
	PIP1;2	9.44	8.52	7.88	7.11	8.00	8.30	7.92	7.03	8.14	7.41	7.67	8.96	10.04	10.50	9.86	8.98	8.68	6.43	9.94	9.04
	PIP1;3	0.68	0.45	0.85	0.54	0.20	0.47	0.31	0.64	0.24	0.65	0.29	0.27	0.49	0.45		0.53	0.22	0.51	0.30	
	PIP1;4	1.57	1.63	2.82	0.82	0.87	1.75	2.20	2.26	0.91	1.10	1.67	1.64	1.31	0.75		2.36	2.09	2.35	1.01	1.02
	PIP1;5	14.79	10.69	14.51	9.39	10.05	14.16	8.78	14.55	12.09	11.03	14.71	8.83	16.46	16.22	11.25	14.07	10.88	15.25	13.76	10.46
	PIP2;1	9.44	10.73	10.46	9.46	8.05	9.40	10.67	8.88	10.29	8.02	9.69	9.81	9.53	11.27	9.55	10.13	8.56	8.43	11.80	7.76
	PIP2;2	1.13	0.81	1.41	0.84	0.75	0.88	0.92	1.07	1.17	0.75	0.96	0.84	1.85	1.17	0.86	1.46	0.78	1.51	0.81	0.83
	PIP2;3	9.05	7.27	11.34	8.83	7.22	7.75	8.68	13.46	10.75	8.31	7.25	6.70	11.90	12.41	9.03	9.16	7.33	10.00	9.70	8.38

Table 3 Suppl. The individual values of relative expression of *PIP1;1*, *PIP1;3*, *PIP1;4* and *PIP2;2* genes in cv. Rutgers (WT) and *7B-1* mutant seeds harvested 24 (24), 72 (72, seeds with radicle 1-3 mm long) and 96 (96 seeds with radicle 1-10 mm long) hours after seed sowing on MS medium in the dark (D) or blue-light (BL). The expression was measured by qRT-PCR. The relative expression was quantified against the expression from sample of WT seeds incubated for 24 hours in the dark (that is the reason why the expression of this sample is always 1,00) using 'delta-delta CT-method'. Presented values were used to calculate geomeans presented in Fig. 4 Suppl..

genotype	dark/ light	stage	gene											
			PIP1;1			*PIP1;3*			*PIP1;4*			*PIP2;2*		
WT	**D**	**24**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
		72	1.49	3.01	1.69	1.06	1.89	0.91	1.04	0.98	0.95	2.63	1.73	2.16
		96	2.43	2.51	2.03	9.38	9.04	6.44	2.62	1.53	1.29	8.07	3.70	3.30
	BL	**24**	1.26	1.48	0.36	0.57	0.73	0.60	0.89	0.72	0.96	0.98	0.65	0.75
		72	2.94	5.44	2.56	0.95	1.43	1.23	0.94	0.86	0.85	3.08	2.47	2.10
		96	5.82	7.21	5.89	9.77	5.00	3.15	2.68	1.54	1.41	3.02	3.85	2.86
7B-1	**D**	**24**	0.68	0.98	0.74	0.44	0.54	0.48	0.79	0.76	0.59	0.66	0.95	0.61
		72	1.29	2.57	1.12	1.00	1.08	0.42	1.61	0.99	0.91	1.80	2.02	1.87
		96	1.65	1.93	2.50	7.66	7.82	7.70	2.11	2.06	1.51	6.17	4.22	2.57
	BL	**24**	0.57	1.31	0.66	0.69	0.73	0.61	0.61	0.62	0.55	0.69	0.65	0.61
		72	2.02	2.45	1.69	1.00	1.04	0.71	1.31	0.85	0.88	3.46	2.86	2.50
		96	5.46	5.42	5.77	6.04	6.79	2.79	2.19	1.95	2.01	8.68	3.53	4.26

Table 4 Suppl. The individual relative expressions of *PIP1;2*, *PIP1;4*, *PIP1;5*, *PIP2;1*, *PIP2;2* and *PIP2;3* genes in cv. Rutgers (WT) and *7B-1* mutant seeds after 96-hour cultivation on the basal MS medium (MS) or on medium with 30 μM mercuric chloride ($HgCl_2$) in the dark (D) or blue-light (BL). The expression was measured by RT-PCR. The relative expression was quantified against both the expression from WT seed sample (which has therefore always the same value, it equals 1,00), and expression of two reference genes (*GAPDH* and *EF1*). Values represent geomean (of expression relative to *GAPDH* and expression relative to *EF1*).

	gene		*PIP1;2*				*PIP1;4*				*PIP1;5*				*PIP2;1*				*PIP2;2*				*PIP2;3*			
MS	**D**	**WT**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
		7B-1	0.93	0.87	0.83	0.89	1.00	1.08	0.93	0.90	0.94	0.88	0.84	1.00	1.01	0.96	0.85	0.98	0.83	0.87	0.96	0.68	1.04	1.38	0.78	0.84
	BL	**WT**	0.98	1.15	1.28	0.99	1.05	1.00	0.94	0.92	1.14	1.01	0.94	0.87	1.01	0.95	0.72	1.00	1.06	0.90	1.31	0.87	1.05	1.32	0.68	0.87
		7B-1	1.02	1.18	1.60	1.15	0.96	0.93	0.92	0.95	1.01	1.03	0.98	0.94	1.05	0.93	0.86	1.20	0.71	1.18	1.14	0.73	1.01	1.32	0.77	0.95
$HgCl_2$	**D**	**WT**	0.99	1.37	1.20	0.79	0.61	1.73	0.93	0.84	0.45	1.05	1.32	1.08	0.31	0.81	1.09	1.00	0.54	1.68	0.93	0.78	0.36	1.68	0.83	0.83
		7B-1	1.30	1.40	1.15	1.01	0.73	1.71	0.94	1.09	0.60	1.03	1.10	1.36	0.41	1.06	2.01	1.29	0.73	1.44	0.81	1.02	0.48	2.15	0.85	0.87
	BL	**WT**	2.44	1.55	2.02	0.88	2.02	1.48	1.06	1.00	1.70	1.16	1.77	1.10	1.16	0.49	1.09	1.00	2.05	1.71	1.84	1.13	1.36	2.23	0.90	0.90
		7B-1	1.23	1.61	0.91	1.10	1.01	1.40	1.04	1.14	1.19	1.12	1.30	1.19	0.81	0.91	1.94	1.43	1.43	2.22	0.87	0.82	0.95	1.76	0.84	1.15

Table 5 Suppl. The individual relative expressions of *PIP1;1* and *PIP1;3* genes in cv. Rutgers (WT) and *7B-1* mutant seeds after 96-hour cultivation on the basal MS medium (MS) or on medium with 30 μM mercuric chloride ($HgCl_2$) in the dark (D) or blue-light (BL). The expression was measured by qRT-PCR. The relative expression was quantified against the expression from sample of WT seeds incubated for 24 hours in the dark (that is the reason why the expression of this sample is always 1.00) using 'delta-delta CT-method'.

	gene		*PIP1;1*				*PIP1;3*						
MS	**D**	**WT**	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
		7B-1	1.23	0.77	0.68	1.48	0.59	1.33	0.82	0.52	0.69	0.70	0.77
	BL	**WT**	2.90	2.87	2.40	3.19	0.49	0.85	1.04	0.44	0.42	0.61	0.58
		7B-1	2.84	2.16	2.25	3.40	0.43	1.16	0.64	0.51	0.77	0.49	0.60
$HgCl_2$	**D**	**WT**	0.85	0.84	0.46	1.09	0.38	0.33	0.08	0.27			
		7B-1	0.59	0.75	0.51	0.82	0.44	0.56	0.55	0.14			
	BL	**WT**	2.44	2.72	5.78	5.92	0.11	0.08	0.29	0.50			
		7B-1	1.79	1.81	1.59	5.06	0.64	0.77	0.16	0.33			

APPENDIX 3

Hormonal regulation of aquaporins – an unpublished review

HORMONAL REGULATION OF AQUAPORINS

Jana Balarynová, Martin Fellner

Introduction

It is quite difficult to define plant hormones (phytohormones) and there is no absolute definition. Phytohormones can be defined as a group of naturally occurring, organic substances which influence physiological processes at concentrations far below those where either nutrients or vitamins would affect these processes (Davies 2013). The changes in hormone concentrations can dramatically alter plant growth and development and balance among different hormonal classes is essential. Hormones are produced in different tissues and then distributed by the vascular system through the plant body (Spaepen 2015). Phytohormones play central role in every aspect of plant physiology and integrate responses of plant growth and development to multiple environmental factors. Similarly, water channels (aquaporins) are important players in all aspect of plant life and mediate responses to the surrounding environment. Besides, the plant aquaporins can be gated by several factors including phytohormones. PIP aquaporins enable the plant to modulate their root hydraulic conductivity in response to a variety of environmental challenges (Chaumont et al., 2005; Maurel et al., 2008). The plant growth and development depend on stringent regulation of cellular water movement and homeostasis.

Auxin

Although auxin plays essential roles in many developmental processes, only few examples are present in literature relating auxin effects on aquaporin expression and activity. The hormone auxin is among others a key regulator of lateral root development affecting tissue plasticity (through cell-wall enzymes), water supply (through aquaporins) and turgor maintenance to promote emergence of lateral roots. In the Arabidopsis roots, the most aquaporin genes (PIPs

and TIPs) were downregulated, except AtPIP1;3 and AtPIP2;4 which showed up to twofold induction, during lateral root formation in an auxin-dependent manner (Perét et al. 2012). Interestingly, it was found that auxin inhibits the endocytosis of PIP2 in Arabidopsis roots (Paciorek et al. 2005). Besides, auxin regulated the tissue distribution of AQPs and thus, it controlled root tissue hydraulics. IAA probably acts through an Auxin Response Factor 7 (ARF7)-dependent pathway to modify the aquaporin expression at both transcriptional and translational levels (Perét et al. 2012). On the other hand, Werner et al. (2001) found that auxin induced expression of LeAQP2 and TRAMP aquaporins during interaction of tomato plant with the parasitic plant *Cuscuta reflexa*. Auxin level is increased at the attachment sites of the parasite to the host (Werner et al. 2001). Similarly, treatment with auxin significantly enhanced the expression of tonoplast aquaporin *PgTIP1* in *Panax ginseng* callus lines (Lin et al. 2007). In strawberry, *Fragaria × ananassa (Weston) Duchesne ex Rozier*, the expression of a fruit specific aquaporin, FaPIP1;1, increases during fruit ripening and was significantly repressed by the presence of auxins. The growth of fruits required maintenance of appropriate turgor and the high expression of FaPIP1;1 in ripening fruit could indicate its importance for faster water movements (Mut et al. 2008). On the other hand, for process of ripening is necessary a gradual decline of auxins supply from achenes in the later stage of growth, because auxins at high levels delays the progress of fruit ripening (Perkins- Veazie 1995).

Gibberellic acid

Gibberellic acid (GA_3) is known especially as a growth promoting plant hormone. Involvement of aquaporins, particularly those of PIP and TIP families, in cell elongation is also well established. Therefore, the effect of gibberellins on amount of aquaporins in plant cell could be expected. Correspondingly, GA_3 increased the mRNA level of TIP1;1 (Phillips and Huttly 1994) or PIP1;2 (Kaldenhoff et al. 1996) in *Arabidopsis thaliana*. In rice (*Oryza sativa* L.), expressions of OsPIP1a (RWC1) and OsPIP2a were stimulated by treatment with

gibberellins, most probably as a result of water deficit generated by gibberellin-induced growth (Malz and Santer 1999; Sun et al. 2004). Moreover, accumulation of GA_3 on the abaxial side of rice leaves induced expression of an *OsRWC3* gene, which results in stimulation of local growth and gravitropic bending of a leaf (Hu et al. 2007). Gibberellins also modulate the expression of aquaporin γTIP which contribute to pea fruit growth (Ozga et al. 2002). However, in radish (*Raphanus sativus* L.) hypocotyls, gibberellins (namely GA_3) downregulated the expression levels of several aquaporin genes and proteins, especially those of PIP2 subgroup and TIP1;1 (Suga et al. 2002). Treatment with GA_3 did not stimulate the elongation of hypocotyls of seedlings, but induces the synthesis in the cell wall space as well as release of reactive oxygen intermediates (Schopfer et al. 2001; Suga et al. 2002). In this context, it is expected that the aquaporins function as H_2O_2 transporters (Henzler and Steudle 2000).

Ethylene

Ethylene was found to play an important role in modulating water transport in plants, perhaps by influencing aquaporins. In hypoxic aspen seedlings (*Populus tremuloides* Michx.), ethylene treatment increases significantly the root water conductivity, which is mediated by aquaporins. Since ethylene induces protein phosphorylation, it is expected that enhancement of aquaporin activity is caused by ethylene-induced phosphorylation (Kamaluddin and Zwiazek 2002; Raz and Fluhr, 1993). On the other hand, in *Medicago falcata* L seedlings, phosphorus deficiency induces production of ethylene, which reduces root water conductivity. This effect of ethylene on aquaporins is attributed to its ability to affect concentration of free Ca^{2+} ions, which are known to gate the water channels (Li et al. 2009). Ethylene inhibits expansion of petals in rose (*Rosa hybrida* L.) by, at least partly, supressing the expression of *RhPIP2;1* and *RhPIP1;1* genes (Ma et al. 2008; Chen et al. 2013). In addition, ethylene decreased the expression of *RhTIP1;1*, which was accompanied with an acceleration of the

rose flower opening (Xue et al. 2009). However, little is known about the mechanism of regulation of cell expansion by ethylene. Aquaporins in rubber tree, *Hevea brasiliensis* (Willd. ex Adr. Juss.) Muell. Arg., are differently regulated by ethylene in the inner bark tissue and laticifers. *HbPIP2;1* was upregulated by ethylene treatment in both the laticifers and the inner tissue whereas *HbTIP1;*1 was upregulated in the latex cells and decreased in the inner bark. On the contrary, *HbPIP1;1* was repressed by ethylene in both tissues. Mentioned aquaporins are probably important players in ethylene-induced enhancement of latex yield by facilitating latex flow and its prolongation (Tungngoen et al. 2009). Ethylene seems to play a role in grape berries (*Vitis vinifera* L.) expansion and softening process affecting aquaporin transcript accumulation (Chervin et al. 2008). In pear, *Pyrus pyrifolia* (Burm. f.) Nakai, the expression of *PpPIP1*, which participates in mediating water flow during pear fruit development, was enhanced by ethylene indicating its involvement in the response of pear fruit to ethylene (Shi et al. 2014).

Salicylic acid

Salicylic acid is associated especially with the defence of plants and plant immune responses. However, it can also regulate plant water transport. Salicylic acid inhibits root hydraulic conductivity in *Arabidopsis thaliana* involving a hydrogen peroxide (H_2O_2)-induced relocalization of PIPs from the plasma membrane to intracellular membrane compartments (Boursiac et al. 2008). Sánchez-Romera et al. (2016) proposed that the production of SA could participate in the reduction of hydraulic conductivity under drought conditions. It was shown that SA delays pear fruit senescence and it decreased the amount of *PpPIP1* transcript during pear fruit development. Thus, the aquaporin could play an essential role in hormonal signalling during fruit ripening (Imran et al. 2007; Shi et al. 2014). A poplar (*Populus alba* × *P. tremula* var. *Glandulosa*) aquaporin, *PatPIP1*, was expressed in poplar roots and leaves and its abundance was enhanced by abiotic stresses and plant hormones including SA.

It was proposed that *PatPIP1* plays an important role in the defence of plants against water stress (Bae et al. 2011).

Methyl jasmonate

Methyl jasmonate is able to enhance hydraulic conductivity in roots of *Arabidopsis thaliana*, *Solanum lycopersicum* and *Phaseolus vulgaris* plants most probably by increasing phosphorylation of aquaporins (Sánchez-Romera et al. 2014, 2016). Gene expression of a poplar (*Populus alba* × *P. tremula* var. *Glandulosa*) aquaporin *PatPIP1* involved in the defence of plants against water stress is strongly increased in response to jasmonic acid (Bae et al. 2011).

Abscisic acid

Abscisic acid (ABA) could probably binds directly to the channel, but the responsiveness of each aquaporin isoform to ABA is different. Besides, the expression of aquaporins can be regulated via ABA-dependent or ABA-independent signalling pathways. In addition, it was shown that ABA decreased phosphorylation status, which is one of the most important regulation mechanisms of aquaporin activity, of conserved serine residues in C-terminal region of several PIPs in *Arabidopsis thaliana* plants (Kline et al. 2010). It is known that ABA modifies hydraulic properties, especially of roots. However, the direction of this modification is quite variable. ABA mostly increases hydraulic conductivity, but there are several works indicating reduction or no change of hydraulic properties of studied plant organs after ABA treatment.

ABA decreased the amount of mRNA and protein of several aquaporins (especially of *RsPIP2s* and *RsTIP1;1*) in radish seedlings. The authors expect that it could be connected with the response to osmotic stress because the reduction of aquaporins was more pronounced under osmotic stress (Suga et al. 2002). In *Craterostigma plantagineum* callus and seedlings,

the expression level of *CpTIP* was downregulated by ABA (Mariaux et al. 1998). Likewise, in Arabidopsis roots the expression of *PIP1;5* was decreased by ABA treatment (Jang et al. 2004).

In *Arabidopsis thaliana* cell cultures, the level of *PIP1b* (*PIP1;2*) transcript was markedly increased by ABA (Kaldenhoff et al. 1993, 1996). Similarly, the level of *TIP1;1* gene was enhanced by ABA in shoots and roots of rice seedlings (Liu et al. 1994). In *Craterostigma plantagineum* callus and seedlings, ABA increased level of aquaporin *CpPIPa2* mRNA (Mariaux et al. 1998). The hydraulics conductance of the ABA-deficient tomato mutants, *sitiens* and *flacca,* was lower than in corresponding WT (Nagel et al. 1994; Tal and Nevo 1973) indicating the stimulatory effect of ABA on plant hydraulics conductivity. It may be that ABA induces transcription of aquaporins or regulates gating of aquaporins in the membranes (Hose et al., 2000; Javot and Maurel, 2002; Thompson et al. 2007; Wan et al., 2004). Jang et al. (2004) showed that ABA enhanced the expression of At*PIP1;1, PIP1;4, PIP2;2, PIP2;4* and *PIP2;7* genes in aerial parts of *Arabidopsis thaliana* seedlings. Likewise, in Arabidopsis roots the expression of *PIP1;1, PIP1;2, PIP1;4, PIP2;3, PIP2;6, PIP2;7* genes was increased by ABA treatment (Jang et al. 2004). In *Zea mays* L. cv. Helix seedlings, ABA induced the expression of *ZmPIP1;2* and *ZmPIP2;4* genes (Zhu et al 2005) and transient increase in maize hydraulic conductivity was reported also by previous studies of Hose et al., 2000 and Wan et al., 2004. During ABA-triggered stomatal closure in *Arabidopsis thaliana,* ABA induces water permeability of guard cells and transport of hydrogen peroxide by inducing aquaporin PIP2;1. The regulation of PIP2;1 in guard cells by ABA seemed to be posttranslational, through the change of phosphorylation status of PIP2;1 protein (Grondin et al. 2015). In rice (*Oryza sativa* L. cv. Zhonghan 3), ABA enhanced the expression of *OsPIP1;2, OsPIP2;5* and *OsPIP2;6* genes in roots and *OsPIP1;2, OsPIP2;4* and *OsPIP2;6* genes in leaves (Lian et al. 2005).

The presented examples showed that the ABA inducibility of aquaporins is variable and involved their upregulation as well as downregulation. In addition, there are several examples indicating insensitivity of aquaporins to ABA. For example, Jang et al. 2004 showed that the gene expression of AtPIP2;5 was not affected by ABA treatment. In *Craterostigma plantagineum* callus and seedlings, the levels of CpPIPa6, CpPIPa7 and CpPIPc aquaporins were not affected by ABA treatment (Mariaux et al. 1998).

Brassinosteroids

Modulation of aquaporin activity by brassinosteroids was also reported. Morillon et al. (2001) showed that brassionolide, one of the active brassinosteroids, enhances the water permeability of protoplast membranes in *Arabidopsis thaliana*. The authors proposed that aquaporins could be involved in the brassinolide-mediated increase in water permeability although the direct effect of brassinolide on aquaporins was not reported. Furthermore, the accumulation of *Brassica napus* aquaporin gene *BNPIP1* was reported after brassinosteroid (24-epibrassinolide, EBR) treatment in *Brassica napus* seedlings (Kagale et al. 2007). The applications of different concentrations of brassinosteroid HBR (28-homobrassinolid) induced *HvPIPs* (*HvPIP1;1*, *HvPIP1;2*, *HvPIP1;3*, *HvPIP1;5*) expression in barley (*Hordeum vulgare* L. cv. "Hilal") roots (Marakli and Gozukirmizi 2017). In both *Brassica napus* and *Hordeum vulgare* brassinosteroids were investigated in connection with the increased resistance of treated plants to abiotic stresses. On the other hand, in radish (*Raphanus sativus* L.) seedlings, the transcript and protein level of any of the aquaporins was changed by brassinolide (Suga et al. 2002)

Cytokinins

Several lines of evidence suggest the effect of cytokinins on hydraulics since these hormones are vital for plant growth and development. However, their interaction with aquaporins is not

known yet. There are some clues indicating that the expression of aquaporins is changed in response to cytokinin treatment. For example, *PgTIP1* was slightly upregulated by kinetin in *Panax ginseng* callus lines (Lin et al. 2007). On the contrary, the expression of *RhTIP* was not significantly affected by cytokinin treatments (thidiazuron and benzyladenin) in cut rose.

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Laboratory of Growth Regulators

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INVOLVEMENT OF AQUAPORINS IN LIGHT-REGULATED SENSITIVITY OF PLANTS TO OSMOTIC STRESS

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Content

1. Introduction

Plants survive in constantly changing environment because of carefully orchestrated responses to diverse challenges during their lifecycles. Many stress-responsive genes have been described in plants (Shinozaki and Yamaguchi-Shinozaki 1997; Zhang et al. 2004). These genes can be generally classified into two groups. First group of so-called functional genes comprises the genes whose products are involved in protection of cells against osmotic stress and in production of important metabolic proteins. The products of functional genes can be for example water channels (aquaporins) or LEA proteins (Late Embryogenesis Abundant proteins). Second group of genes is composed of regulatory genes whose products are important members of regulatory pathways such as different transcriptional factors, protein kinases or enzymes of ABA biosynthesis (Nakashima and Yamaguchi-Shinozaki 2006).

Coping with the abiotic constrains is often associated with aquaporins, especially those residing in the plasma membrane (PIP, Plasma membrane Intrinsic Proteins). Aquaporins are the transmembrane proteins facilitating and regulating the passive movement of water molecules down a water potential gradient. They are vital for maintaining a turgor and water transport in plants (Maurel et al. 2008). In plant seeds, aquaporins play a central role in the physiology of water economy (Maurel et al. 2008, 2015; Obroucheva 2013, Obroucheva et al. 2017). Embryo growth during seed germination is driven by water uptake, which is necessary for embryo cell elongation and expansion. Moreover, PIP aquaporins have been proven to participate in responses to abiotic stresses, such as drought, salinity or chilling (Guo et al. 2006; Lian et al. 2004; Liu et al. 2007). On the other hand, *PIP* gene expression can be regulated by these abiotic stresses (Aroca et al. 2012) as well.

Late embryogenesis abundant genes/proteins (LEA) are frequently expressed in response to different abiotic stresses (Bartels and Sunkar 2005). LEA proteins accumulate naturally in some desiccation tolerant structures such as the seeds (Dure et al. 1989). Furthermore, they are also produced in plant vegetative tissues during exposure to different water-related stresses (drought, salinity, cold) (Roberts et al. 1993; Ingram and Bartels 1996; Bray 1997). LEA proteins are suggested to be involved in protection of plant cells against damage induced by impairment of water balance (Zhu et al. 2000; Ramanjulu and Bartels 2002).

Finally, the abiotic stresses often induce the expression of BURP domain-containing genes. The members of the plant specific BURP family share at their C terminus a highly conserved BURP domain whose name is based on four members: **B**NM2, **U**SP, **R**D22 and **P**G1β (Hattori et al. 1998). This thesis focused on the expression profile of tomato *RD22-like* gene. In *Arabidopsis thaliana*, the expression of *RD22* (Responsive to Dehydration 22) can be induced by drought, abscisic acid (ABA) and salt stress (Yamaguchi-Shinozaki and Shinozaki 1993). BURP genes play diverse roles in the cells, however, the precise functions of BURP domain-containing genes and proteins are still unclear. Two possible functions have been proposed. Firstly, they are important in plant development and metabolism, possibly, they could participate in cellular secretion pathway during embryogenesis, seed, fruit and root development (Wang et al. 2011). Secondly, many BURP domain-containing proteins are responsive to stress treatments, thus their involvement in stress adaptation can be expected (Urao et al. 1993; Iwasaki et al. 1995; Abe et al. 1997).

The subject of the thesis is the tomato (*Solanum lycopersicum* L.) *7B-1* mutant and its corresponding WT cultivar Rutgers. *7B-1* is a spontaneous recessive single gene mutant selected for its male sterility under long days (Sawhney

1997). Seed germination tests show that *7B-1* is less responsive than the corresponding WT to the inhibitory effects of osmotic stress specifically under blue light (BL) (Fellner and Sawhney 2002). This indicates that BL can modulate the inhibitory effect of abiotic stresses on tomato seed germination. It was shown 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 *SlGLO2* gene was proposed as a candidate gene underlying the *7B-1* mutation (Pucci et al. 2017).

2. Aims of the thesis

The objective of this thesis was to investigate a role of plasma membrane aquaporins (*PIPs*), *RD22-like* gene (*BURP*-domain containing gene) and several *LEA* genes in the cross-talk between abiotic stress signalling and blue light (BL) signalling pathways during process of seed germination and early post-germination. For this, tomato (*Solanum lycopersicum*) cv. Rutgers and *7B-1* mutant, which is less responsive than WT to BL-induced inhibition of seed germination and to various abiotic stresses specifically under BL, were used as model experimental plants. The thesis focused on determining the impact of osmotic stress, BL and the *7B-1* mutation on the gene expression profiles of mentioned stress-responsive genes. To assess that, we used a quantitative RT-PCR and subsequent REST (Relative Expression Software Tool) 2009 software (Pfaffl et al. 2001) analysis.

In summary, the main aims of the Ph.D. thesis were:

- Physiological study of the effect of mannitol- and PEG-induced osmotic stress on the percentage of tomato WT and *7B-1* mutant seed germination in the dark and under continuous BL.
- The analysis of the potential impact of mannitol- and PEG-induced osmotic stress, BL and the *7B-1* mutation on the transcript levels of *PIP* genes, *RD22-like* gene and *LEA-type* genes in germinated and non-germinated WT and *7B-1* mutant seeds.

3. Survey of the key results

Most of the data summarized in the Ph.D. thesis were published in two research papers published in the journals with peer review – Biologia Plantarum (Balarynová and Fellner 2018) and Acta Physiologiae Plantarum (Balarynová et al. 2018). The first publication, Balarynová and Fellner 2018, focuses particularly on the participation of plasma membrane aquaporina (PIPs) during different phases of seed germination and early post-germination in the dark and under blue light (BL) conditions. The various concentrations of an aquaporin blocker, mercuric chloride ($HgCl_2$), were applied and their effect on the percentage of WT and *7B-1* mutant seed germination was studied in the dark and in BL. The importance of PIP aquaporins was showed during tomato seed germination and early radicle elongation. PIP aquaporins participated especially during radicle elongation. Interestingly, the results did not show the difference in sensitivity of tomato seed germination to $HgCl_2$ between dark and BL conditions. The data demonstrated the lower responsiveness of *7B-1* mutant seed germination to mercuric chloride compared to WT seed germination. At the transcript level, mercury decreased expression of *PIP1;3* gene in WT as well as *7B-1* mutant seeds (other *PIPs* seemed to be impervious to $HgCl_2$), while the *PIP1;3* expression in *7B-1* seemed to be less reduced by $HgCl_2$ than in WT seeds.

The second publication, Balarynová et al. 2018, deals particularly with determination of the effect of 70 mM mannitol-induced osmotic stress, BL and the *7B-1* mutation on expression of *PIP* genes in tomato seeds. The presence of 70 mM mannitol in the cultivation medium reduced significantly the percentage of both WT and *7B-1* mutant seed germination. Besides, it was showed that mannitol-induced osmotic stress and the *7B-1* mutation reduced the expression levels of several genes, which could be a way to retain water for radicle

elongation and seed germination under the stress conditions. Indeed, the expression of *PIP1;3* gene was decreased not only by osmotic stress, but also by BL, which could indicate the existence of a link between osmotic stress and BL signalling pathways.

In the thesis, the effect of another osmotica, polyethylene glycol (PEG), was screened at the physiological as well as transcriptional level. 5% PEG was showed to reduce significantly germination of WT and *7B-1* mutant seeds in the dark and in BL, with stronger effect under BL conditions. On the other hand, at the transcript level, the inhibitory effect of PEG on *PIP1;3* gene expression was detected in the dark-germinated *7B-1* seeds. Interestingly, this effect was not found in WT seeds geminated under the same conditions. The effect of PEG was not so obvious in WT and *7B-1* mutant seeds germinated under BL conditions.

Finally, the part of the thesis monitored expression profiles of *RD22-like* gene and *LEA-type* genes, which are known to participate in alleviation of adverse effect of abiotic stresses on plant cells. Interestingly, *RD22-like* as well as *LEA*-type transcript levels were significantly induced by mannitol/PEG-induced osmotic stress and BL, whereas they were reduced by the *7B-1* mutation.

4. Conclusions and perspectives

The data presented in the thesis showed that *7B-1* mutant seed germination was always less responsive than WT seed germination to various stress treatments applied. The *7B-1* mutation decreased significantly the expression levels of *LEA*, *RD22*-like and *PIP1;3* genes. Besides, the *7B-1* mutation affected the BL-induced stimulation of *PIP1;2* gene expression, which confirms the connection between the *7B-1* mutation and BL-signalling. Therefore, the *7B-1* mutation, conferring the lower responsiveness to BL and osmotic stress, reduced the transcript levels of stress-responsive genes. The following study should focus on characterization of protein abundance of stress-responsive proteins in WT and *7B-1* seeds to validate the possible effect of the *7B-1* mutation on protein accumulation. This is almost done for LEA-type proteins in WT and *7B-1* seeds under osmotic stress induced by 70 mM mannitol and the data are preparing for publication. In addition, BL alone had a significant impact on transcript level of several genes, namely on *PIP1;1*, *PIP1;2*, *PIP1;3*, *RD22-like* and *LEA-type* genes. Mostly, it induced the gene expression (only the expression level of *PIP1;3* was downregulated by BL), but reduced the percentage of seed germination of both WT and *7B-1* mutant seeds with more pronounced effect on WT seed germination. Particularly, a combination of osmotic stress treatment and BL enhanced considerably the expression level of *RD22-like* and *LEA*-type genes. On the other hand, *PIP* genes *PIP1;1* and *PIP1;2* were induced only by BL, whereas *PIP1;3* gene was affected by BL, osmotic stress as well as the *7B-1* mutation. The *PIP1;3* transcript level was reduced by any treatment used. In the next studies, it will be interesting to see how the PIP1;3 protein level and localization in the germinated seeds is affected by BL and osmotic stress in both WT and *7B-1* mutant seeds.

The future research could focus on potential interaction between LEA-type proteins and plasma membrane aquaporins (PIPs). Both protein families reside at/near the membranes and both are crucial for stress responses. The cooperation between PIP a LEA proteins in alleviation of impact of osmotic stress cannot be excluded. The cell wall-localization associated with the apoplast is predicted for of RD22 proteins. The interaction of RD22 proteins with aquaporins or LEA type proteins has not been studied yet. Thus, the followed-up studies could investigate this possibility too. To screen the behaviour of RD22 proteins upon environmental challenges is the way to better understand their function and their interaction with other protective molecules.

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Balarynová J, Fellner M (2018). Expression of genes encoding PIP aquaporins in tomato seeds exposed to blue light and mercury. Biologia Plantarum, in press.

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Balarynová J, Humplík JF, Kadlecová A, Prčina M, Fellner M (2011). Involvement of aquaporins in tomato (*Solanum lycopersicum* L.) seed germination under various light conditions. ASPB Plant Biology 2011, Minneapolis, MN, USA.

Humplík JF, **Balarynová J**, Bergougnoux V, Fellner M (2011). Effects of different anion channel inhibitors on the germination of the *7B-1*, a tomato (*Solanum lycopersicum* L.) mutant defective in BL signalling. XXIV. SPPS Congress 2011, Stavanger, Norway.

Balarynová J, Fellner M (2013). Effect of mercury on plasma membrane aquaporins during tomato seed germination and post-germination. European Biotechnology Congress 2013, Bratislava, Slovakia.

Balarynová J, Fellner M (2013). Role of *PIP1;3* and *BURP* genes in seed germination responses to osmotic stress and blue light. 7th EPSO Conference 2013, Porto Heli, Greece.

Balarynová J, Lenobel R, Fellner M (2015). Expression of dehydrins in tomato seeds which are less sensitive to abiotic stresses. EMBO Conference: Signalling in Plant Development, Brno, Czech Republic

Oral presentations

Balarynová J, Fellner M (2014). Resistance of the tomato *7B-1* mutant to osmotic stress. 12th PhD Student Conference of Plant Experimental Biology 2014, Olomouc, Czech Republic.

Balarynová J, Fellner M (2014). Expression of stress-associated genes in tomato *7B-1* mutant. Growth Regulators 2014, Kouty nad Desnou, Czech Republic

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Balarynová J, Lenobel R, Fellner M (2016). Effect of blue light and osmotic stress on dehydrin expression in tomato *7B-1* mutant. Growth Regulators on the Way 2016, Malá Morávka, Czech Republic

7. Summary in Czech (souhrn)

Název práce: Zapojení akvaporinů ve světlem regulované citlivosti rostlin k osmotickému stresu
Autor: Mgr. Jana Balarynová

Disertační práce se zabývá studiem klíčení semen rajčete (*Solanum lycopersicum* L.) kultivaru Rutgers (WT) a mutanta *7B-1*. Tento unikátní mutant se vyznačuje sníženou citlivostí klíčení semen k inhibičnímu účinku modrého světla a menší citlivostí klíčení k osmotickému stresu. Práce se zaměřuje na zapojení vodních kanálů (akvaporinů) plazmatické membrány (PIPs) v různých fázích klíčení semen a za působení osmotického stresu ve tmě a na modrém světle. Ukázalo se, že akvaporiny jsou důležité především v průběhu prodlužování radikuly, a nikoliv imbibice vody suchým semenem. Působení osmotického stresu signifikantně snižovalo procento klíčení semen obou genotypů, především semen WT. Překvapivě, na transkripční úrovni byla působením osmotického stresu ovlivněna pouze exprese genu *PIP1;3*. Množství transkriptu genu *PIP1;3* bylo také výrazně snižováno působením modrého světla, a to jak v semenech WT, tak semenech mutanta *7B-1*. Modré světlo samotné pak zvyšovalo expresi akvaporinů *PIP1;1* (v semenech obou genotypů) a *PIP;2* (jen v semench WT). Naopak, mutace *7B-1*, pokud měla vliv, tak expresi akvaporinů snižovala (*PIP1;2* a *PIP1;3*).

Reakce rostlin k osmotickému stresu není spojena jen s akvaporiny, ale také s řadou dalších genů/proteinů, především LEA genů/proteinů (Late embryogenesis abundant) a zástupců ze skupiny genů/protein s BURP doménou. Výsledky ukazují, že exprese genu *RD22* (zástupce ze skupiny genů obsahujících BURP doménu) a *LEA* genů jsou signifikantně ovlivněny působením osmotického stresu, modrého světla i mutace *7B-1*. Působení

modrého světla a osmotického stresu zvyšovalo množství transkriptu genu *RD22-like* a *LEA* genů, což je v souladu s předpokladem, že produkty těchto genů mají protektivní funkci. Překvapivě, mutace *7B-1* množství transkriptu těchto genů výrazně snižovala. Získaná data poukazují na propojenost signálních drah světla a osmotického stresu a na jejich ovlivnění mutací *7B-1*.