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Citation style: Sadura Iwona, Libik-Konieczny Marta, Jurczyk Barbara, Gruszka Damian, Janeczko Anna. (2020). Plasma membrane ATPase and the aquaporin HvPIP1 in barley brassinosteroid mutants acclimated to high and low temperature. "Journal of Plant Physiology" Vol. 244, iss. 2 (2020), s. 1-8, art. no. 153090, DOI 10.1016/j.jplph.2019.153090

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Journal of Plant Physiology 244 (2020) 153090

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Plasma membrane ATPase and the aquaporin HvPIP1 in barley brassinosteroid mutants acclimated to high and low temperature

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ARTICLE INFO

Keywords:
Acclimation
Brassinosteroids
PM H^+-ATPase
High temperatures
HvPIP1
Low temperatures

ABSTRACT

The integral parts of the cell membranes are the functional proteins, which are crucial for cell life. Among them, proton-pumping ATPase and aquaporins appear to be of particular importance. There is some knowledge about the effect of the temperature during plant growth, including stress-inducing temperatures, on the accumulation of the membrane proteins: plasma membrane H^+-ATPase and aquaporins, but not much is known about the effect of the phytohormones (i.e. brassinosteroids (BR)) on control of accumulation of these proteins. The aim of our study was to answer the question of how a BR deficit and disturbances in the BR perception/signalling affect the accumulation of plasma membrane H^+-ATPase (PM H^+-ATPase), the aquaporin HvPIP1 transcript and protein in barley growing at 20 °C and during its acclimation at 5 °C and 27 °C. For the studies, the BR-deficient mutant 522DK (derived from the wild-type Delisa), the BR-deficient mutant BW084 and the BR-signalling mutant BW312 and their wild-type Bowman were used. Generally, temperature of growth was significant factor influencing on the level of the accumulation of the *H^+-ATPase* and *HvPIP1* transcript and the PM H^+-ATPase and HvPIP1 protein in barley leaves. The level of the accumulation of the *HvPIP1* transcript decreased at 5 °C (compared to 20 °C), but was higher at 27 °C than at 20 °C in the analyzed cultivars. In both cultivars the protein HvPIP1 was accumulated in the highest amounts at 27 °C. On the other hand, the barley mutants with a BR deficiency or with BR signalling disturbances were characterised by an altered accumulation level of PM H^+-ATPase, the aquaporin HvPIP1 transcript and protein (compared to the wild types), which may suggest the involvement of brassinosteroids in regulating PM H^+-ATPase and aquaporin HvPIP1 at the transcriptional and translational levels.

1. Introduction

The cell membrane plays an important role in the interaction of a cell with the environment and also regulates the transport of various substances inside and outside of a cell. Among others, both the lipid and protein components of the membrane alter dynamically as a result of changes in the temperature during plant growth (Los and Murata, 2004; Zheng et al., 2011). A membrane-sensing hypothesis, which assumes that membranes are the thermal sensors of cells, has even been proposed (Horváth et al., 2012). Many biochemical reactions, which are crucial for cell life, occur with the participation of membrane-bound proteins (Brown, 2011). Among the many membrane-bound proteins, proton-pumping ATPase and aquaporin appear to be very important. Plasma membrane H^+-ATPase (PM H^+-ATPase) is part of a large family of membrane proteins that are responsible for the active transport of cations or other compounds across the membranes and this process is coupled with ATP hydrolysis. The PM H^+-ATPase releases protons from a cell in order to create a proton motive force that has a membrane potential of 120–160 mV (negative inside) and a pH gradient of 1.5–2 units (acid outside) (Sze et al., 1999). It is known that PM H^+-ATPase is also involved in other functions that are crucial for appropriate plant growth including salt tolerance, intracellular pH regulation and cellular expansion (Morsomme and Boutry, 2000). Aquaporins, on the other hand, are transmembrane proteins, which were discovered as water channels that transport water through the cell membranes. In addition to water, aquaporins can transport, among others, CO_2, urea, ammonia, silicon and O_2 (Maurel et al., 2008; Kapilan et al., 2018). There is a family of major intrinsic proteins (MIP) and they are classified into five subfamilies, which include the plasma membrane intrinsic proteins (PIPs) that are located in the organs that are distinguished by huge

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https://doi.org/10.1016/j.jplph.2019.153090
Received 3 June 2019; Received in revised form 17 September 2019; Accepted 6 October 2019
Available online 28 November 2019
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fluxes of water e.g. the vascular tissues. Their main function in plants is to regulate the transmembrane water transport when the water flow needs to be modified or when it is very low (Kapilan et al., 2018). They are also known to participate in regulating the root water transport in response to different environmental factors and in facilitating water transport through the inner leaf tissues during transpiration (Maurel et al., 2008). Its transport activity may be controlled by multiple mechanisms that are involved in regulating the accumulation of transcripts or proteins or in subcellular trafficking (Maurel et al., 2008).

From the physiological and biochemical points of view, maintaining the integrity and fluidity of the cell membranes is very important for plants to survive under high/low temperature stress (Zheng et al., 2011). For example, according to Los and Murata (2004), low temperatures change the lipid composition of the cell membranes by increasing the content of unsaturated fatty acids, which is connected with membrane rigidification, while high temperatures cause the fluidisation of the cell membranes. However, knowledge about effect of temperature stress on the membrane proteins, including the accumulation or activity of PM H^+-ATPase and the aquaporin proteins, is rather scarce. The response of the PM H^+-ATPase to low temperatures in cucumber roots has been relatively well described (Ahn et al., 2000; Janicka-Russak et al., 2012; Muzi et al., 2016). On the other hand, cold lowers the expression of the *aquaporin* (*PIP*) transcripts (Maurel et al., 2008). Simultaneously, however, not much is known about the effect of phytohormones on the activity of these proteins (Suga et al., 2002; Falhof et al., 2016). There are only a few articles that discuss the effects of exogenously applied brassinosteroids (BR) on the levels of the PM H^+-ATPase or aquaporin (Morillon et al., 2001; Suga et al., 2002) and vacuolar V-ATPase and aquaporin (Yang et al., 2003). For example, Suga et al. (2002) investigated the effect of exogenously applied brassinosteroid (0.1 μM brassinolide, BL) as well as 1 mM gibberellic acid (GA_3) or 0.1 mM abscisic acid (ABA) on the accumulation of the plasma membrane aquaporin isoforms (RsPIPs) in radish seedlings. GA_3 and ABA lowered the amount of the RsPIP2-1 protein, while the BL did not cause any significant changes. A good model for studies of the role of hormonal regulation (combined with the impact of temperature) on the functioning of ATPase or aquaporins could be mutants with disturbances in hormonal biosynthesis. To the best of our knowledge, the number of articles that specifically discuss brassinosteroid mutants in terms of the expression of PM H^+-ATPase or aquaporins is low and among them, there are no works that are specifically dedicated to this aspect in cereal plants. Brassinosteroids are the plant steroid hormones that are responsible for the processes of plant growth and modulating the plant responses to environmental stresses (Bajguz and Hayat, 2009; Sadura and Janeczko, 2018). In our studies, barley brassinosteroid mutants (522DK, BW084 and BW312) were used (Gruszka et al., 2011; Dockter et al., 2014). The 522DK and BW084 are mutants with disturbed BR biosynthesis which results in lower level of BR in comparison to wild type. In mutant BW312 as a result of mutation, BR receptor partly losses its function (BR perception is weaken), BR signaling is disturbed and endogenous BR level increased.

Our earlier studies have shown that all of these mutants, after acclimation at 27 °C had higher tolerance to high temperature (38 − 45 °C) in comparison to their wild types (Sadura et al., 2019). Simultaneously after acclimation at 5 °C, mutants BW084 and BW312 had lower tolerance to frost in comparison to respective wild type cultivar Bowman (Sadura et al., 2019). On the other hand, frost tolerance of 522DK was similar to tolerance of its wild type cultivar Delisa. The phenomenon was partly explained by altered mutant's hormonal homeostasis (mainly ABA accumulation) but mechanism of changed tolerance remained generally unknown. Since both for frost and partly for heat tolerance important is cell water management and also cellular transport of water and other substances, in present work we would like to focus on answering the following question - how brassinosteroid deficit and disturbances in its signaling influence on PM H^+-ATPase and HvPIP1 (aquaporin) transcript and protein accumulation in barley growing at 20 °C and after plant's acclimation at 5 °C and 27 °C?

2. Materials and methods

2.1. Plant material

The seeds of the spring barley (*H. vulgare* L.) cultivars (Delisa and Bowman) and their 522DK, BW084 and BW312 mutants were derived from the collection of the Department of Genetics, University of Silesia (Katowice, Poland). The cultivars and mutant lines were selected according to the works of Gruszka et al. (2011) and Dockter et al. (2014).

The 522DK mutant was obtained from the Delisa cultivar and is characterised by a G > A substitution at position 1130 of the *HvDWARF* transcript (Gruszka et al., 2011) and at position 3031 in the gene sequence (Gruszka et al., 2016), which causes substitution of the valine-341 residue by isoleucine. The *HvDWARF* gene encodes the brassinosteroid C6-oxidase, which takes part in BR biosynthetic pathways and this is the cause of the lower content of BR (mainly castasterone) in the mutant (Gruszka et al., 2016; Sadura et al., 2019). The BW084 and BW312 mutants are Near-Isogenic Lines of Bowman cultivar (Dockter et al., 2014). The BW084 (*brh13.p*) carries the C2562 T missense mutation in the *HvCPD* gene encoding barley C-23α-hydroxylase cytochrome P450 90A1 (CYP90A1), which catalyses the early steps of the BR biosynthesis. The C2562 T mutation results in a substitution of the highly conserved amino acid residue (Pro-445 to Leu). Pro-445 is situated within the highly conserved heme binding site in the C-terminal part of the HvCPD protein (Dockter et al., 2014). Mutant is characterized with lower content of 28-homocastasterone while content of brassinolide and castasterone is even below detection limits (Sadura et al., 2019). The BW312 (*ert-ii.79*) mutant that is defective in the BR perception has a double substitution (CC1760/1761AA) in the BR receptor kinase BRI1. The substituted amino acid residue (Thr-573 to Lys) is present in the steroid-binding island domain of the BR receptor. The charged Lys-573 in the hydrophobic active site vicinity, destroys the charge neutrality, and may handicap the binding of BR molecules (Dockter et al., 2014). This mutation causes significant increase in BR (castasterone, brassinolide and 28-homocastasterone) content (Sadura et al., 2019).

2.2. Plant growth and experimental design

The seeds were put on moist filter paper on plastic vessels (10 cm × 10 cm × 3 cm) for germination in the dark at 24 °C for three days. To each vessel 40 seeds and 10 ml of water were added. After germination, the seedlings were planted into pots with soil (15–20 plants/pot 40 cm × 15 cm × 15 cm). Each pot was filled with 3 kg of soil prepared from: "Eco-ziem Universal soil" (Eko-Ziem s.c., Jurków, Poland), soil from the cultivation plots at the University of Agriculture (Kraków) and sand (1:1:0.5). The plants were kept for three weeks in a growth chamber (20 °C d/n; 16 h photoperiod). Light intensity: 170 μmol $m^{-1} \cdot s^{-1}$ emitted by HPS Philips SON-T AGRO 400 W lamps. When plants developed three to four leaves, the pots with plants were separated into two groups. The first group was acclimated to 5 °C (8 h photoperiod, 21 days). The second group was acclimated to 27 °C (16 h photoperiod for seven days). After acclimation, plants usually developed four to five leaves.

Samples for the analyses of the transcripts (*H^+-ATPase* and aquaporin *HvPIP1*) were collected from the plants (the central part of the second leaf) before the acclimation (20 °C), and then on the last day of the acclimation at 5 °C (21st day) and 27 °C (7th day). Additional samples were also collected at the halfway point of the acclimation (10th day at 5 °C and 3rd day at 27 °C).

To determine the accumulation of protein, two types of samples were collected. To obtain the first group of samples, the aerial parts of the seedlings were cut off and the cell membrane fraction was immediately isolated and then frozen at − 80 °C for the further analysis of

Table 1
Sequence origins and primers and probes sequences used in the study.

Gene name	GenBank ID	Forward primer	Reverse primer	TaqMan MGB Probe
HvPIP1;1	AB286964.1	CTGGCGGGCACATCAAC	CGTCAGCGACAGCTTCCT	FAM-ACCTTCGGGCTGTTCC-MGB
ATPase1	AF308816.1	AGACAGGAAGTACCTGAGAAATCCA	TGGTCTCGGCACTGTCATG	FAM-CACCAGCAGAATCC-MGB
Actin	AY145451.1	GCAACTGGGATGACATGGAGAAAAT	GCCACACGGAGCTCATTGTA	FAM-CTGGCATCACACTTTC-MGB

the proteins. To get the second group of samples, the aerial parts of the seedlings were cut off, frozen (−80 °C) and kept in order to prepare a fresh crude leaf extract immediately before the protein analysis. The aim of using this approach was to compare the results of the protein analyses that were obtained from both types of samples. Similar to the preparations of the transcripts, samples were collected from plants growing at 20 °C, 5 °C (21st day) and 27 °C (7th day). Additional samples were also collected at the halfway point of acclimation (10th day at 5 °C and 3rd day at 27 °C) in order to have the leaves cut off and frozen to get the crude extract for further protein analysis.

2.3. Biochemical analysis

2.3.1. Isolation of the cell membranes

The cell membranes were isolated based on a modified protocol of Sommarin et al. (1985) and Janeczko et al. (2008). About 100 g of plant material were homogenised using a Camry CR 4050 blender in 400 ml of a 10 mM Tris/HCl buffer (pH 7.8) containing 0.25 M sucrose, 1 mM EDTA and 2.5 mM dithiotreitol (DTT). All of the reagents were purchased from Sigma-Aldrich (Poznań, Poland). The crude extract was filtered and centrifuged for ten minutes at 10 000 × *g* (Beckmann L8-M, rotor SW 27 and 28, Palo Alto, CA, USA) to remove any residues after the plants had been homogenised. The supernatant was centrifuged for 30 min at 80 000 × *g* (Beckmann L8-M, rotor SW 27 and 28, Palo Alto, CA, USA). The obtained pellet constituted the cell membrane fraction. The samples that were obtained were used to analyse the accumulation of proteins.

2.3.2. Measurement of the protein concentration in the cell membrane fractions

The protein concentration was estimated according to Sedmak and Grossberg (1977). Two µl of a 10 % water solution of Triton X-100 (Sigma-Aldrich, Poznań, Poland) was mixed with 2 µl of the microsomal fraction and 196 µl of a buffer to isolate the cell fractions and kept for 15 min in ice. Next, 3 mL of water and 1 mL of a Bradford reagent (BioRad, Munich, Germany) were added. After 10 min, the absorbance was recorded (596 nm) using a UV/VIS spectrometer Lambda Bio 20 (Perkin Elmer, Norwalk, USA). Measurements were carried out in three replicates. Bovine serum albumin – BSA (Sigma-Aldrich, Poznań, Poland) was used as the calibration standard. The BSA for spectrophotometric measurements was diluted in the same buffer as buffer used to isolate the cell membrane fractions and 2 µl of a 10 % water solution of Triton X-100 was added.

2.3.3. Measurement of the protein concentration in the crude leaf extract

The aerial parts of the seedlings (five to six plants) were collected and then they were cut into about 0.5 cm fragments and 1 g of the sample was prepared. The samples were homogenised in 2.5 mL of a Tricine buffer containing 100 mM Tricine, 3 mM $MgSO_4$, 1 mM DTT, 3 mM EGTA, adjusted to pH 8.0 with 1 M Tris. After the homogenisation, which was carried out at 4 °C, the samples were centrifuged for five minutes at 35 060 × *g* (Hettich, Tuttingen, Germany). The supernatant was collected and the concentration of the protein in the obtained crude extract was measured according to Bradford (1976) using a Bio-Rad Protein Assay Kit with BSA as the standard. Four measurements were taken per sample.

2.3.4. Accumulation of the transcripts of H^+-ATPase and HvPIP1: RNA isolation, cDNA synthesis and real-time PCR reaction

The accumulation of the *ATPase* and *HvPIP1* transcripts was measured using real-time quantitative PCR amplification and the analyses were carried out using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Samples (approximately 0.05 g of the central part of the second leaf) were collected and frozen in liquid nitrogen. Next, the mRNA was isolated using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Subsequently, the genomic DNA was eliminated by adding 2 µl of mRNA (corresponding to approximately 600 ng of RNA) to 2 µl of the gDNA Wipeout Buffer and 10 µl of RNase-free water (included in a QuantiTectReverse Transcription Kit, Qiagen, Hilden, Germany). After 2 min of incubation at 42 °C, the obtained mixture was added to a reverse-transcription master mix (containing 1 µl of Quantiscript Reverse Transcriptase, 4 µl of Quantiscript RT Buffer and 1 µl of RT Primer Mix) (QuantiTectReverse Transcription Kit, Qiagen, Hilden, Germany) and a reverse-transcription reaction was performed at 42 °C (15 min). The concentration and quality of the RNA and cDNA were estimated using a Q5000 UV/VIS spectrophotometer (Quawell, San Jose, CA, USA). The PCR amplifications of the *ATPase1* and *HvPIP1;1* transcripts were performed in triplicate, as described by Jurczyk et al. (2012). The primers and probes for the target genes and an endogenous control gene were designed using Primer Express Software v 3.0.1 (Applied Biosystems, Foster City, CA, USA) and their sequences are listed in Table 1. The levels of the *ATPase1* and *HvPIP1;1* transcripts accumulations were determined relative to *Actin* as the reference gene for five biological replicates (five different cDNAs from five different plants).

2.3.5. Analysis of the accumulation of the cell membrane proteins PM H^+-ATPase and HvPIP1 using immunoblotting

Equal amount of proteins present in the extracts isolated from analysed material was loaded on 12 % polyacrylamide gels and electrophoretically separated as described by Laemmli (1970). The separated proteins were blotted to a nitrocellulose membrane for 2 h at 45,5 mA (7 − 9 V) using a BioRad semi-dry transfer. The membranes were blocked overnight with low-fat milk powder that had been diluted in a TBS-T buffer (containing 0.9 % NaCl and 10 mM Tris). Next, the membranes were washed with a TBS-T buffer four times for 5 min and then incubated in the appropriate primary antibody (Agrisera) (anti-PIP1, 1:2000, for 1 h 30 min; anti-H^+ATPase, 1:2000, for 1 h 30 min). Then, the membranes were washed again with the TBS-T buffer (four times for 5 min) and incubated in Alkaline Phosphatase – conjugated secondary anti-rabbit antibody (Sigma-Aldrich) (1:2000, for 1 h 30 min). Three independent replicates were performed. Quantification of analysed protein level was performed on the basis of staining intensity of bands corresponding with PIP1 or PM H^+-ATPase, performed by ImageJ software (NIH, USA).

2.4. Statistical analysis

Statistical analysis (ANOVA, post hoc test) was performed using Statistica 13.1 (StatSoft, Tulsa, OK, USA). When more than two groups were compared, the Duncan's test was used (for Bowman, BW084, BW312). When two groups were compared (Delisa, 522DK), the Student's *t*-test was used. Values marked with the same letters in the figures did not differ significantly. Additionally, the accumulations of

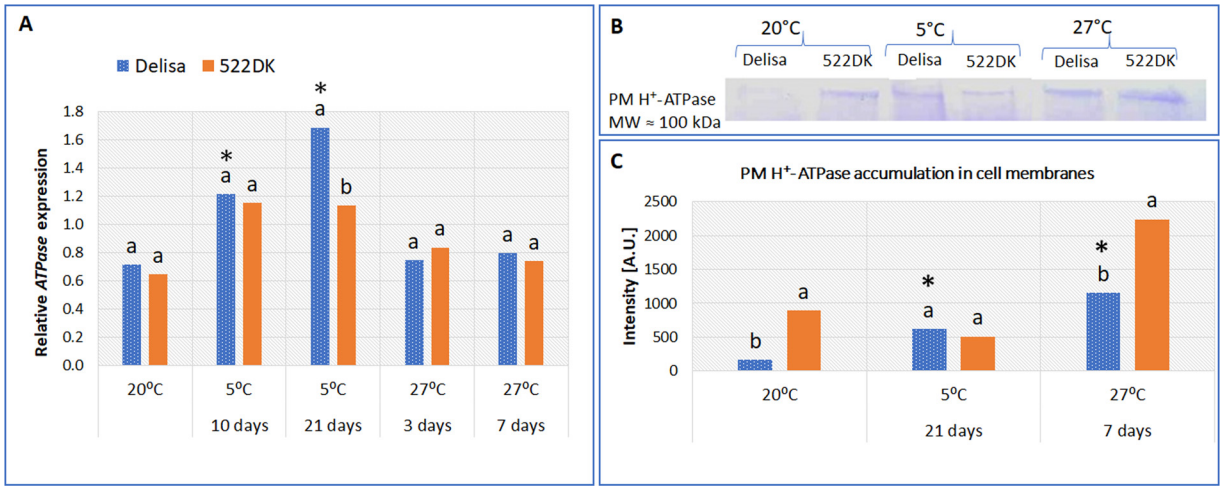

Fig. 1. Changes in the relative *ATPase* transcript level (A) and the PM H^+-ATPase protein accumulation (B, C) in the barley cell membrane fractions of the wild-type Delisa and its mutant (522DK) at 20 °C and after acclimation at 5 °C and 27 °C. The results of the accumulation of the transcript are presented as the fold change in the expression of a specific gene in the given samples compared to the endogenous reference gene – *actin*. The visualised band corresponded with the protein identified using the anti- PM H^+-ATPase antibody. MW – molecular weight standard (Thermo Scientific PageRuler Prestained Protein Ladder), 20 µg of proteins from the cell membrane fractions was applied on each lane. The amount of accumulated PM H^+-ATPase was estimated based on densitometric analysis of the band intensity staining. The values are presented as arbitrary units (A.U.), which correlated with the area under the densitometric curves. Significant differences between cv. Delisa and its mutant 522DK – (Student's *t*-test, $P \leq 0.05$) for each temperature are indicated by different letters. Moreover, the accumulation of the transcript and protein concentration in the Delisa cultivar at different temperatures were compared. The comparisons were performed in pairs (for 20 °C and 5 °C; 20 °C and 27 °C) (Student's *t*-test, $P \leq 0.05$) and significant differences are indicated by an "*".

the transcripts and proteins in the Delisa and Bowman cultivars at different temperatures were compared. The comparisons were performed in pairs (for 20 °C and 5 °C; 20 °C and 27 °C) (Student's *t*-test, $P \leq 0.05$) and significant differences are indicated by an asterisk (*).

3. Results and discussion

3.1. Accumulation of the H^+-ATPase transcript and protein in the barley BR mutants and wild type plants growing at 20 °C and acclimated at 5 °C and 27°C

In our studies, low temperature (5 °C) gradually increased the relative transcript level of *H^+-ATPase* in both the Delisa and Bowman barley cultivars compared to the temperature 20 °C (Fig. 1A and 2 A). Finally, after 21 days at 5 °C, relative transcript level in both cultivars was doubled compared to the level that had been observed at 20 °C. A similar, although much weaker, tendency was observed for all of the tested mutants with a brassinosteroid deficit or signalling disturbances. According to literature, the exposure of cucumber roots to low temperatures resulted in a gradual decrease of the *H^+-ATPase* gene expression from day 1–6 of chilling, compared to the control plants (Ahn et al., 2000). Similarly, a decreased relative *H^+-ATPase* expression was obtained by Janicka-Russak et al. (2012) for cucumber roots that had been exposed to a low temperature for three days. However, prolonged exposure to a low temperature (for 6 days) increased the expression levels of four of the six genes encoding PM H^+-ATPase that were tested compared to the control plants.

The BR mutants derived from the Bowman cultivar, BW084 and

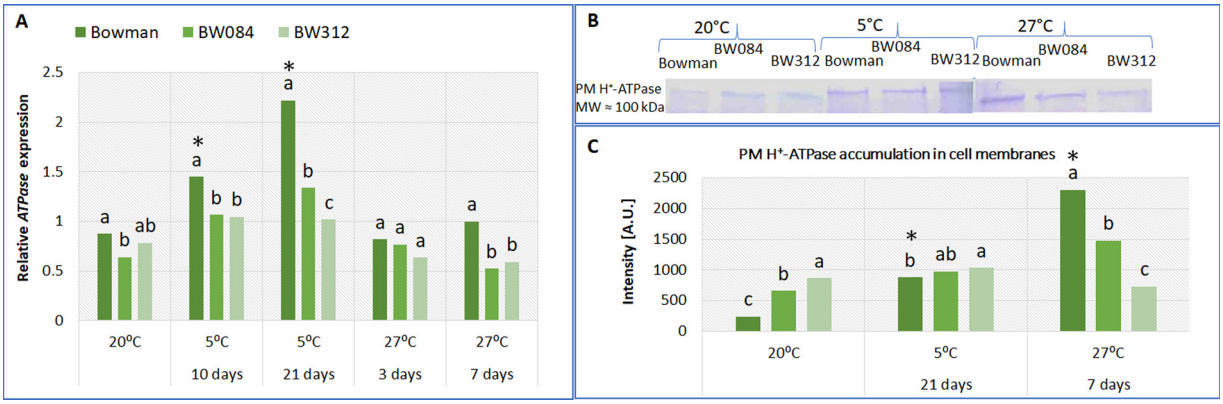

Fig. 2. Changes in the relative *ATPase* transcript level (A) and the PM H^+-ATPase protein accumulation (B, C) in the barley cell membrane fractions of the wild-type Bowman and its mutants (BW084 and BW312) at 20 °C and after acclimation at 5 °C and 27 °C. The results of the accumulation of the transcript are presented as the fold change in the expression of a specific gene in the given samples compared to the endogenous reference gene – *actin*. The visualised band corresponded with the protein that was identified using the anti- PM H^+-ATPase antibody. MW – molecular weight standard (Thermo Scientific PageRuler Prestained Protein Ladder), 20 µg of proteins from the cell membrane fractions was applied on each lane. The amount of the accumulated PM H^+-ATPase was estimated based on a densitometric analysis of the band intensity staining. The values are presented as arbitrary units (A.U.), which correlated with the area under the densitometric curves. Significant differences between cv. Bowman and its mutants (BW084 and BW312) – (Duncan's test, $P \leq 0.05$) for each temperature are indicated by different letters. Moreover, the accumulations of the transcript and protein in the Bowman cultivar at different temperatures were compared. The comparisons were performed in pairs (for 20 °C and 5 °C; 20 °C and 27 °C) (Student's *t*-test, $P \leq 0.05$) and significant differences are indicated by an "*".

BW312, showed a lower (few percent to about 50 %) level of the *H*$^+$-*ATPase* transcript accumulation than their respective cultivar at all tested temperatures but especially at 5 °C (Fig. 2 A). Mutant 522DK had a statistically significant lower level of the transcript than Delisa only at 5 °C (Fig. 1A). Then in the case of the mutants with BR biosynthesis disturbances, the effect was more spectacular for mutant BW084 than for mutant 522DK. It should be kept in mind that the mutants represent defects at various stages of the BR biosynthesis, as they carry mutations in two different loci (Dockter et al., 2014; Gruszka et al., 2016). BW084 has a mutation in early stages of the biosynthesis of BR and produces two main BR (brassinolide or castasterone) at a level that is sometimes impossible to detect (Sadura et al., 2019). Mutant 522DK has a defect in the late stage of BR biosynthesis and only produces a lower content of BR (on average 30 %) at 5 °C (Sadura et al., 2019). Generally the effect (lowered transcript level) was especially prominent under the stress conditions (5 °C) and in this case the effect was independent on the genetic background of the analyzed mutants. The lower level of the transcript accumulation in BR-deficient mutants may suggest that BR are one of regulators that are necessary for controlling the accumulation of the *H*$^+$*-ATPase* transcript. The effect, which was significant also in the case of mutant BW312, means that BR signalling is also important for regulation of accumulation of this transcript.

When the levels of the accumulation of the PM H$^+$-ATPase protein in the Delisa and Bowman cultivars was compared at 20 °C and 5 °C, a few times higher accumulation of this protein was observed at 5 °C (Figs. 1B and C and 2 B and C) and these data are in agreement with the literature. Kim et al. (2013) found that the leaves and roots of camelina (*Camelina sativa* L.) and rapeseed (*Brassica napus* L.) that had been exposed to low temperature (2 °C/1 day) accumulated a higher amount of the PM H$^+$-ATPase protein than the plants that had not been exposed to temperature stress. Prolonged low temperature treatment changed the accumulation of the PM H$^+$-ATPase protein in both species, however it was dependent on whether the roots or leaves were examined. Low temperature also increased the accumulation of PM H$^+$-ATPase in the plasma membrane fraction of *A. thaliana* plants and this effect corresponded with the results obtained for the relative expression of the *H*$^+$-*ATPase* genes – exposure to low temperature increased their levels (Muzi et al., 2016). This is especially in agreement with our results obtained for cv. Delisa and Bowman because, as was mentioned, the accumulation of *H*$^+$*-ATPase* transcript (simultaneously with the PM H$^+$-ATPase protein accumulation) was clearly higher after exposure to 5 °C (if compare to 20 °C).

All of the BR mutants grown at 20 °C accumulated a few times more of the PM H$^+$-ATPase protein in their membrane fraction than their respective cultivars (Figs. 1B and C and 2 B and C), but at 5 °C the effect was not noted (522DK) or hardly visible in the case of BW084 and BW312. It could suggest that, in some temperature conditions (especially at 20 °C), BR are negative regulators of the production of this protein in barley. We suggest that this BR-dependent regulation of the PM H$^+$-ATPase protein accumulation may occur in this case rather at the translational level.

There are not many studies related to effect of the plant exposure to higher temperatures on the accumulation of the PM H$^+$-ATPase transcript or protein. That is why our work provides new information in this field. The temperature of 27 °C that was tested in our experiment was not as extreme and was maintained for a longer period. The accumulation of the *H*$^+$*-ATPase* transcript remained unchanged in the Delisa and Bowman cultivars at 20 °C and at 27 °C (Figs. 1A and 2 A). However, the accumulation of the PM H$^+$-ATPase protein was few times higher at 27 °C than at 20 °C in both cultivars (Figs. 1B and C and 2 B and C). The effect of much higher temperature was earlier studied by Liu et al. (2009). Authors found that in pea leaves, heat (38 °C) caused an increase in the accumulation of the PM H$^+$-ATPase transcript and protein after 1 h of high-temperature treatment and that after a longer period (3 h) its amount decreased.

The BR deficiency that was connected with disturbances at the late stage of biosynthesis (in 522DK mutant) had no effect on the accumulation of the *H*$^+$*-ATPase* transcript at 27 °C (after 3 or 7 days) (Fig. 1A). The mutant was however characterized by increased accumulation of the PM H$^+$-ATPase protein (Fig. 1 B and C). The accumulation of the transcript in the BW084 mutant (strong BR deficit) and the BW312 mutant (BR perception disturbances) growing at 27 °C was unchanged after 3 days but lowered after 7 days in comparison to Bowman (Fig. 2A). It was accompanied also by lowered level of PM H$^+$-ATPase protein in comparison to Bowman wild type (Fig. 2B and C). Comparing results obtained for two BR deficient mutants 522DK and BW084 we can say that the observed opposite effect (especially for protein accumulation) was probably dependent on the genetic background of the analyzed mutants.

Not much is known about the role of BR in regulating the functioning of PM H$^+$-ATPase. Jakubowska and Janicka (2017) found that the relative expression of the *H*$^+$*-ATPase* genes in cucumber roots that had been treated with 10 nM 24-epibrassinolide (one of the brassinosteroids) markedly increased expression of three out of seven genes compared to the control (not treated with 24-epibrassinolide). In general, this may be in agreement with our findings where the BR-deficient mutants (especially BW084 in which the defect occurs at the early step of the BR biosynthesis) showed lower accumulation of the *H*$^+$*-ATPase* transcript.

Jakubowska and Janicka (2017) also found that both the control cucumber roots and the roots that had been treated with 24-epibrassinolide accumulated a similar amount of the PM H$^+$-ATPase protein. In this context, our data are slightly different because depending on the temperature, genotype and the mutation, the plants had different accumulations of the PM H$^+$-ATPase protein (most often increased or decreased, rarely unchanged). This difference may be a result of different approaches – exogenous BR treatment in the study by Jakubowska and Janicka (2017) and use of the BR-deficient and insensitive mutants in our experiments. The difference may also stem from different plant species and organs tested.

3.2. Accumulation of the aquaporin (HvPIP1) transcript and protein in barley BR mutants and wild type plants growing at 20 °C and acclimated at 5 °C and 27 °C

In both the Delisa and Bowman cultivars, there was a marked decrease in the relative *HvPIP1* expression during the cold acclimation (5 °C) compared to temperature 20 °C (Figs. 3A and 4 A). Many earlier studies have shown that cold decreases the expression of the *aquaporin* (*PIP*) transcripts in plant roots or shoots (for review see Maurel et al. (2008)), thus our results correspond with reported observations. On the other hand, not much is known about the effect of higher temperatures on the accumulation of the *PIP* transcripts in plants and known studies usually concerned the impact of temperature about 40 °C. Our studies of barley cv. Delisa and cv. Bowman that had been exposed 3 or 7 days to a less extreme temperature 27 °C, showed that the accumulation of the *HvPIP1* transcript (compared to 20 °C) was finally increased by a third (cv. Delisa) or doubled (cv. Bowman). Earlier, Christou et al. (2014) described the effect of heat stress (40 °C) on the accumulation of the *PIP* transcripts in strawberry roots. They found a rapid accumulation of the *PIP* transcript after 1 h of the stress treatment. However, the accumulation of the *PIP* transcript after 8 h of the heat treatment was similar to that of the control. In our work, all of the tested mutants showed similar tendencies in the *HvPIP1* transcript accumulation as their respective cultivars after both cold acclimation and after exposure to a higher temperature (27 °C) in comparison to 20 °C.

In our studies, lower accumulation of the *PIP* transcript in both cultivars (at 5 °C compared to 20 °C) was generally accompanied by a similar accumulation of the HvPIP1 protein in Delisa at 20 °C and 5 °C (Fig. 3B and C) and a slightly higher accumulation of the HvPIP1 protein at 5 °C than at 20 °C in Bowman (Fig. 4B and C). An accumulation of the protein HvPIP1 is not always correlated with the same

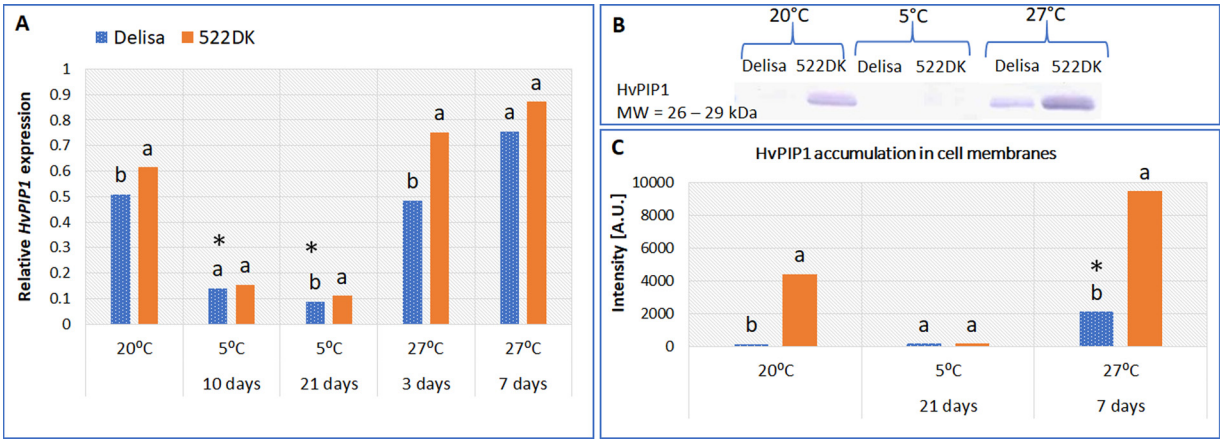

Fig. 3. Changes in the relative *HvPIP1* transcript level (A) and the HvPIP1 protein accumulation (B, C) in barley cell membrane fractions of wild type Delisa and its mutant (522DK) at 20 °C and after acclimation at 5 °C and 27 °C. The results of the accumulation of the transcript are presented as the fold change in the expression of a specific gene in the given samples compared to the endogenous reference gene – *actin*. The visualised band corresponded with the protein that was identified using the anti-HvPIP1 antibody. MW – molecular weight standard (Thermo Scientific PageRuler Prestained Protein Ladder), 7.5 µg of proteins from the cell membrane fractions was applied on each lane. The amount of accumulated HvPIP1 was estimated based on a densitometric analysis of the band intensity staining. The values are presented as arbitrary units (A.U.), which are correlated to the area under the densitometric curves. Significant differences between cv. Delisa and its mutant 522DK – (Student's *t*-test, $P \leq 0.05$) for each temperature are indicated by different letters. Moreover, the accumulations of the transcript and protein in the Delisa cultivar at different temperatures were compared. The comparisons were performed in pairs (for 20 °C and 5 °C; 20 °C and 27 °C) (Student's *t*-test, $P \leq 0.05$) and significant differences are indicated by an "*".

accumulation of the transcript. Aroca et al. (2005) showed that maize roots accumulated significantly more of the PIP protein after chilling than the control plants, but the opposite effect was observed for the expression levels of the *PIP* genes. However, at 27 °C (compared to 20 °C) both cultivars accumulated markedly higher amount of HvPIP1 protein (Fig. 3B and C and 4 B and C), which also correspond with higher *HvPIP1* transcript accumulation.

In our experiment, the BR-deficient mutant 522DK generally had a higher accumulation of the aquaporin transcript than Delisa, while mutant BW084 (and signalling mutant BW312) showed the opposite tendency (Figs. 3A and 4 A). However, both BR-deficient mutants had a significantly increased accumulation of the HvPIP1 protein in the membrane fraction (at 20 °C and 27 °C, Figs. 3B and C and 4 B and C). Mutant BW084 had a slightly lower accumulation of HvPIP1 at 5 °C than the Bowman cultivar, while in 522DK there was no difference compared to Delisa. BW312 accumulated a higher level of the HvPIP1 protein at 20 °C but a lower level at 5 °C and 27 °C compared to Bowman (Fig. 4B and C). To the best of our knowledge, very little is known about the effect of BRs on the accumulation of aquaporins in plants. Morillon et al. (2001) showed that brassinolide may control the activity of aquaporins in *A. thaliana*. On the other hand, brassinolide had no significant effect on the accumulation of the aquaporin proteins in the cell membrane fraction of radish seedlings (Suga et al., 2002).

For a comparison, the accumulation of PM H^+-ATPase and HvPIP1 was also analysed for the leaf crude extracts. Such analysis would be faster and simpler than analysis using procedure with cell membrane isolation. The results however differed from those obtained for the cell membrane fraction (Figs. 1 and 2, Supplementary Material). Accumulation of PM H^+-ATPase and HvPIP1 (or other proteins) is usually calculated per total protein concentration in sample. Protein concentration in pure membrane fraction is however differ than in crude leaf extract (Figs. 3 and 4, Supplementary Material). Moreover in

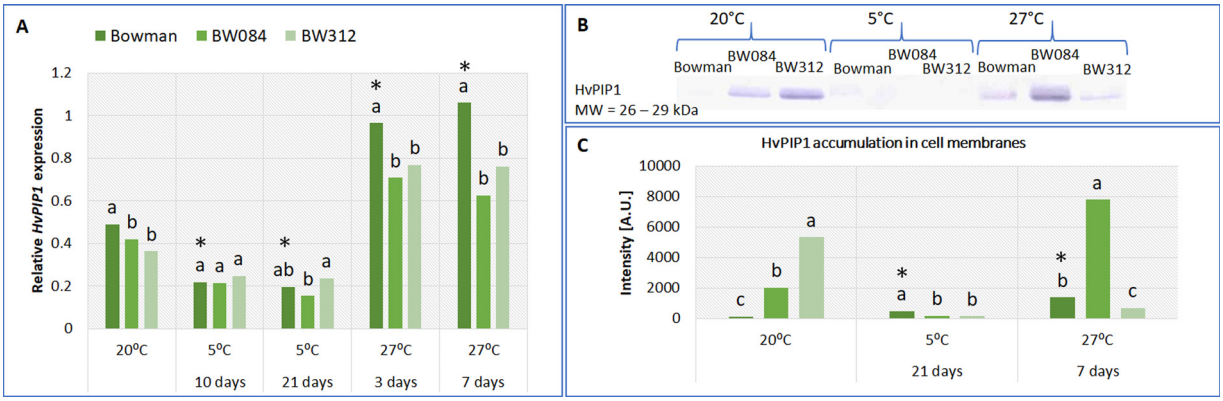

Fig. 4. Changes in the relative *HvPIP1* transcript level (A) and the HvPIP1 protein accumulation (B, C) in the barley cell membrane fractions of the wild-type Bowman and its mutants (BW084 and BW312) at 20 °C and after acclimation at 5 °C and 27 °C. The results of the accumulation of the transcript are presented as the fold change in the expression of a specific gene in the given samples compared to the endogenous reference gene – *actin*. The visualised band corresponded with the protein identified using the anti-HvPIP1 antibody. MW – molecular weight standard (Thermo Scientific PageRuler Prestained Protein Ladder), 7.5 µg of proteins from the cell membrane fractions was applied on each lane. The amount of accumulated HvPIP1 was estimated based on a densitometric analysis of the band intensity staining. The values are presented as arbitrary units (A.U.), which correlated with the area under the densitometric curves. Significant differences between cv. Bowman and its mutants (BW084 and BW312) – (Duncan's test, $P \leq 0.05$) for each temperature are indicated by different letters. Moreover, the accumulations of the transcript and protein in the Bowman cultivar at different temperatures were compared. The comparisons were performed in pairs (for 20 °C and 5 °C; 20 °C and 27 °C) (Student's *t*-test, $P \leq 0.05$) and significant differences are indicated by an "*".

the case of the leaf extract, we additionally dealt with the cytoplasmic proteins. The difference in the accumulation of PM H^+-ATPase and HvPIP1 in dependency on analyze type is surely result of the different concentration of protein in the cell membrane fraction and the crude leaf extract. Analyses of the accumulation of the membrane proteins such as ATPase or PIP should be then rather conducted on the isolated membrane fraction and should be calculated more precisely per membrane protein concentration (additionally measured after the addition of a surfactant). ATPase or PIP analyze in the crude leaf extract seems to be less reliable, because the results per soluble proteins are mainly calculations of those proteins that are present in the cytoplasm.

To conclude, in barley wild types, temperature of growth/acclimation is significant factor influencing on the level of the accumulation of the *ATPase* and *HvPIP1* transcript and the H^+-ATPase and HvPIP1 protein. The barley mutants with the BR deficiency and with BR signalling disturbances were characterised by the altered levels of *H^+-ATPase* transcript, the aquaporin *HvPIP1* transcript and the accumulation of the proteins (compared to wild types) (Table 1 A and B, Supplementary Material), which may suggest the involvement of BRs in regulating the accumulation of PM H^+-ATPase and aquaporin HvPIP1 on the transcriptional and translational levels. The phenomenon was dependent on temperature of plant growth/acclimation but there was not observed pattern of changes which could help to explain described earlier (Sadura et al., 2019) altered tolerance of mutants to heat shock or frost.

Author contributions

IS and AJ made isolation of cell membrane fraction and analyzed protein concentration in isolated fraction. IS and MLK prepared samples of crude leaf extracts and analyzed protein concentration in this material. IS and MLK made analysis of PM H^+-ATPase and HvPIP1 proteins accumulation after method optimization. IS made quantification of the results by densitometry using ImageJ software. IS and BJ measured accumulation of the transcripts *H^+-ATPase* and *HvPIP1*. DG provided seeds of mutants for experiment and gave suggestion about interpretation of data. AJ was coordinator of the project 2015/17/B/NZ9/01695, designed experiments and choose all other scientists to cooperation. All authors analyzed and interpreted data. IS wrote the manuscript under AJ supervision. All authors read article and suggested corrections.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

Acknowledgements

The work was funded by a grant from the National Science Centre (2016–2018) No. 2015/17/B/NZ9/01695 (Poland).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2019.153090.

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