

1 ORIGINAL ARTICLE

2 **Exogenous application of abscisic acid (ABA) increases root and cell**
3 **hydraulic conductivity and abundance of some aquaporin isoforms in the**
4 **ABA deficient barley mutant Az34**

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16 Running title: ABA increases hydraulic conductivity and abundance of aquaporins in barley

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1 **Background and Aims** Regulation of water channel aquaporins (AQP) provides another
2 mechanism by which abscisic acid (ABA) may influence water flow through plants. To the
3 best of our knowledge, no studies have addressed the changes in ABA levels, the abundance
4 of aquaporins and root cell hydraulic conductivity ($L_{p_{Cell}}$) in the same tissues. Thus, we
5 followed the mechanisms by which ABA affects root hydraulics in an ABA-deficient barley
6 mutant Az34 and its parental line cv. Steptoe. We compared the abundance of AQPs and
7 ABA in cells to determine spatial correlations between AQP abundance and local ABA
8 concentrations in different root tissues. In addition, abundance of AQPs and ABA in cortex
9 cells was related to $L_{p_{Cell}}$.

10 **Methods** Root hydraulic conductivity ($L_{p_{Root}}$) was measured by means of root exudation
11 analyses and $L_{p_{Cell}}$ using a cell pressure probe. The abundance of ABA and aquaporins in root
12 tissues was assessed through immunohistochemical analyses. Isoform-specific antibodies
13 raised against HvPIP2;1, HvPIP2;2 and HvPIP2;5 were used.

14 **Key Results** Immunolocalization revealed lower ABA levels in root tissues of Az34 compared
15 with Steptoe. Root hydraulic conductivity ($L_{p_{Root}}$) was lower in Az34, yet the abundance of
16 HvPIPs in root tissues was similar in the two genotypes. Root hair formation occurred closer
17 to the tip, while the length of root hair zone was shorter in Az34, compared with Steptoe.
18 Application of external ABA to the root medium of Az34 and Steptoe increased the
19 immunostaining of root cells for ABA and for HvPIP2;1 and HvPIP2;2 especially in root
20 epidermal cells and the cortical cell layer located beneath, parallel to an increase in $L_{p_{Root}}$ and
21 $L_{p_{Cell}}$. Treatment of roots with Fenton reagent, which inhibits aquaporin activity, prevented
22 the ABA-induced increase in root hydraulic conductivity.

23 **Conclusion** Shortly after (< 2 hours) ABA application to the roots of ABA-deficient barley,
24 increased tissue ABA concentrations and AQP abundance (especially the plasma-membrane
25 localised isoforms HvPIP2;1 and HvPIP2;2) were spatially correlated in root epidermal cells
26 and the cortical cell layer located beneath, in conjunction with increased $L_{p_{Cell}}$ of the cortical
27 cells. In contrast, long-term ABA deficiency throughout seedling development affects root
28 hydraulics through other mechanisms, in particular the developmental timing of the formation
29 of root hairs closer to the root tip and the length of the root hair zone.

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31 **Keywords:** barley, aquaporins, abscisic acid, hydraulic conductivity, immunolocalization.
32 Abbreviations: ABA, abscisic acid; AQP, aquaporins; $L_{p_{Root}}$, root hydraulic conductivity;
33 $L_{p_{Cell}}$, root cell hydraulic conductivity; PB, phosphate buffer; PGT, phosphate buffer
34 containing gelatin and Tween 20.

1 **Introduction**

2 Abscisic acid has long been known to control plant water relations by closing stomata (for a
3 review, see Dodd 2005). The capacity of ABA to influence water-conducting paths within
4 plants has received less attention, with data showing a positive effect of exogenous ABA on
5 root hydraulic conductance (Ludewig et al., 1988; Zhang et al., 1995; Hose et al. 2000;
6 Mahdieh and Mostajeran 2009), no effect (Aroca et al. 2003), variable effect depending on the
7 concentration of applied ABA or a negative effect in the shoot (Pantin et al. 2013). This
8 apparently differential effect of ABA on root and shoot hydraulic conductance may simply be
9 reconciled by a unified dose-response curve to exogenous ABA (Dodd 2013). The discovery
10 of water channels known as aquaporins (AQP), capable of controlling cellular hydraulic
11 conductivity by altering membrane permeability for water, has added a new target through
12 which ABA may influence water flow through plants (for a review, see Maurel et al. 2008).
13 ABA can influence activity of aquaporins at different levels by either changing the expression
14 of genes for AQPs or post-transcriptional modifications of gene products (Maurel et al. 2008
15 and references therein). Comparing AQP gene expression in maize plants differing in ABA
16 concentration revealed a positive relationship between ABA concentration and either AQP
17 expression or root hydraulic conductance (Parent et al. 2009).

18 Barley plants present an ideal model system to study the interplay between AQPs,
19 ABA and root hydraulic conductivity (L_{pRoot}) and cell hydraulic conductivity (L_{pCell}), since
20 the transcellular pathway where water crosses cell membranes through AQPs is more
21 important in these plants than in other species such as maize (Stuedle and Jeschke 1983,
22 Stuedle and Peterson 1998, Knipfer and Fricke 2010). To the best of our knowledge, the
23 effects of exogenous ABA on the relationship between AQPs and root hydraulics have not
24 been studied in barley, nor are we aware of any study where changes in ABA levels have
25 actually been followed at root tissue level and been related to changes in L_{pCell} . Thus, we
26 addressed the effects of exogenous ABA on AQPs and hydraulic conductance of barley plants
27 differing in ABA concentration. We compared abundance of ABA and AQP isoforms in root
28 tissues and L_{pRoot} in the ABA-deficient (but not insensitive) barley mutant Az34 (Walker-
29 Simmons et al. 1989, Martin-Vertedor and Dodd 2011) and its parent Steptoe. Previous
30 studies indicated that Az34 retained normal stomatal (Mulholland et al. 1996) and leaf growth
31 (Martin-Vertedor and Dodd 2011) sensitivity to addition of exogenous ABA. Moreover, we
32 examined the effects of exogenous ABA on hydraulic characteristics in roots of Az34 plants.
33 Root cortex L_{pCell} was also analysed in Az34 using the cell pressure probe. We used
34 antibodies raised against ABA (Fricke et al. 2004, Akhiyarova et al. 2006) and anti (HvPIP2)

1 AQPs antibodies raised specifically against those plasma membrane-localised barley
2 aquaporin isoforms (HvPIP2;1, HvPIP2;2, HvPIP2;5) which are candidates to facilitate water
3 flow through barley roots (Knipfer et al. 2011, Horie et al. 2011). Immuno-histochemistry
4 was used to determine spatial correlations between AQP abundance and local ABA
5 concentrations. The formation of root hairs was also determined in these regions, as these
6 cells facilitate a considerable portion of the water uptake of barley roots (Knipfer and Fricke
7 2010). We hypothesised that different mechanisms could regulate the response of Lp_{Root} and
8 Lp_{Cell} to long-term endogenous ABA concentrations (root hair development) and to transient
9 exogenous ABA treatment (AQP abundance) respectively.

10 **Materials and methods**

11 *Plant growth*

12 Seeds were germinated for 3 days in darkness at 21- 24 °C on either rafts made from sealed
13 glass tubes tied together or on a nylon mesh floated over tap water, which were then
14 suspended over 0.1 strength Hoagland–Arnon nutrient medium (0.5mM KNO₃, 0.5mM
15 Ca(NO₃)₂, 0.1mM KH₂PO₄, 0.1mM MgSO₄, 0.5mM CaSO₄) in 3-litre containers and grown
16 at an irradiance of 400 μmol m⁻² s⁻¹ and a 14-h photoperiod for further 4-5 days. In
17 experiments with exogenous hormone, ABA was added to the nutrient solution to yield 10⁻⁵
18 M concentration. Twenty minutes after addition of exogenous ABA, shoots were excised for
19 the collection of bleeding sap (representing ‘xylem exudate’) from the roots during
20 subsequent 1 h. For Lp_{Cell} determination, plants were analysed between 20 and 90 min after
21 addition of ABA.

22 Since root hairs were not visible under low magnification images of the entire root system
23 (x1), 50 images of root portions from the zone where root hairs were present were made under
24 microscope (x50) from which the image of the whole root was reconstructed in **Photoshop**
25 **Portable**.

26 *Root exudation analyses*

27 Bleeding sap flow from detached root systems was measured according to Carvajal et al.
28 (1996) with modifications described by Kudoyarova et al. (2011). In short, the aerial parts of
29 the plant were removed leaving a cylinder of leaf bases still attached to the root system. The
30 cylinder of leaf bases was connected to a thin pre-weighed capillary by means of silicon
31 tubing. Experiments started 4 h after the start of photoperiod by excising the shoot. In some
32 experiments, this was preceded by 20 minutes of ABA treatment. After 1 h, the capillary
33 containing osmotically-driven bleeding sap was disconnected from the root system and
34 weighed; the root system was also weighed to determine its fresh weight (FW). Bleeding sap

Commented [DI1]: Cite manufacturer in parentheses ?

1 from each capillary was diluted five times to provide sufficient sample for measurement of
2 osmotic potential using a freezing point depression osmometer (Osmomat 030, Germany). In
3 preliminary experiments, proportionality of the effect of dilution on the obtained values was
4 checked. Root hydraulic conductivity, L_{pRoot} was calculated according to equation:
5 $L_{pRoot} = J / ((\Psi_s - \Psi_x) \times FW)$ where J is the bleeding sap flow rate and $(\Psi_s - \Psi_x)$ the difference in
6 osmotic pressure between xylem sap and root medium: a root solute reflection coefficient of
7 1.0 was used (Knipfer and Fricke 2010). To inhibit AQP activity, hydroxyl radicals (*OH)
8 were produced through the Fenton reaction ($Fe^{2+} + H_2O_2 = Fe^{3+} + OH^- + *OH$) by mixing equal
9 volumes of 6 mM H_2O_2 and 6 mM $FeSO_4$ (Ye and Steudle, 2006), correspondingly. Roots of
10 barley plants were placed in the solution. Preliminary experiments showed that inhibition of
11 transpiration by the Fenton reagent was reversible, since transpiration returned to the pre-
12 treatment levels within 30 min after substitution of the culture medium for the one without
13 Fenton reagent.

14 *Cell pressure probe analyses*

15 Turgor, halftime of water exchange ($T_{1/2}$), cell elastic modulus (ϵ) and L_{pCell} was determined
16 through the cell pressure probe technique as described previously (e.g. Fricke and Peters
17 2002, Knipfer et al. 2011, Suku et al. 2014). Cell osmotic pressure, which is required for
18 calculation of L_{pCell} was determined through picolitre osmometry of sap extracted from cells
19 (Fricke and Peters 2002), and the dimension of cells (volume, surface area) was determined
20 through free-hand cross-sections assuming that cells were shaped like cylinders (data not
21 shown). The cells which were analyzed in the root hair region were cortex cells, and were
22 located in the two cortical cell layers beneath the epidermis.

23 Generation of antibodies against AQPs, protein expression in oocytes, and Western analysis
24 Polyclonal antibodies for HvPIP2s were raised in rabbits against synthetic oligopeptides
25 (Medical & Biological Laboratories Co., Japan) corresponding to the amino acid sequences in
26 the N- region of HvPIP2;1 (Katsuhara et al. 2002), HvPIP2;2 (Horie et al. 2011), and
27 HvPIP2;5 (EVMETGGGGDFAAKD, in the present study).

28 The specificity of HvPIP2;5 antibodies was tested through expression of HvPIP2;5
29 isoform in oocytes of the toad *Xenopus laevis* and subsequent analysis of membrane protein
30 fraction through Western analyses. Expression of HvPIP2;5 in *Xenopus* oocytes was
31 performed according to Katsuhara et al. (2002). Briefly, the coding region of HvPIP2;5
32 cDNAs was sub-cloned into pXβG-ev1, corresponding cRNA was synthesized and injected
33 into oocytes. Total membranes of oocytes expressing HvPIP2;5 protein were extracted
34 according to Leduc-Nadeau et al. (2007) All membrane protein corresponding to one oocyte

1 was used as a sample and subjected to the solubilization, SDS-PAGE, and the Western
2 blotting as described previously (Katsuhara et al., 2002).

3 *Immunoassay of ABA.*

4 ABA was immunoassayed as previously described (Vysotskaya et al., 2009) in the roots of
5 control plants of Steptoe and Az34 and those exposed to 10^{-5} M ABA in solution. Aqueous
6 residues of ethanol extracts were diluted with distilled water, acidified with HCl to pH 2.5 and
7 partitioned twice with peroxide-free diethyl ether (ratio of organic to aqueous phases was
8 1:3). Subsequently, hormones were transferred from the organic phase into 1% sodium
9 hydrocarbonate (pH 7-8, ratio of organic to aqueous phases was 3:1), acidified with HCl to
10 pH 2.5, re-extracted with diethyl ether, methylated with diazomethane and immunoassayed
11 using antibodies to ABA (Veselov et al. 1992). ABA recovery calculated in model
12 experiments was about 80%. Reducing the amount of extractant, based on the calculated
13 distribution of ABA in organic solvents, increased the selectivity of hormone recovery and the
14 reliability of immunoassay. The reliability of the immunoassay for ABA was enabled by both
15 specificity of antibodies and purification of hormones according to a modified scheme of
16 solvent partitioning (Veselov et al. 1992).

17 *Immunolocalization of ABA and AQPs*

18 Immunolocalization was carried out on root sections prepared from the root hair zone (3-5
19 mm from the root tip, Fig. 1). Specific rabbit antisera against ABA and HvPIP AQPs were
20 used for immunolocalization of these antigens. Sections of roots were harvested from control
21 plants and from those exposed to an ABA solution for about 1 h. To prevent ABA washing
22 out from tissues during fixation and dehydration, root tip segments 3-5 mm in length were
23 fixed in 4% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, Sigma, United States) for 4 h
24 (under vacuum during the first 30 min of fixation) and then with 4 % formaldehyde for a
25 night as described earlier (Vysotskaya et al. 2007). In this process, ABA carboxylic groups
26 were linked with protein amino groups. Following fixation in formaldehyde, root segments
27 were dehydrated in ethanol solutions of increasing grades (up to 96%). Segments were then
28 embedded in methacrylate resin (JB-4, Electron Microscopy Sciences, United States) as
29 recommended by the manufacturers. Histological sections (1.5 μ m thin) were cut with a
30 rotation microtome (HM 325, MICROM Laborgeräte, Germany) and placed on slides.

31 Sections were treated for 30 min with 0.1 M Na-phosphate buffer (pH 7.3) containing 0.2%
32 gelatin and 0.05% Tween 20 (PGT), washed with distilled water and incubated for 2 h in a
33 moist chamber at room temperature with immune rabbit anti-ABA or anti-HvPIP2 sera (20 μ l)
34 diluted with PGT at the ratio of 1:50 or 1:200 (when anti-ABA serum was applied to the

1 sections of Steptoe roots in some cases). To check specificity of immunostaining, some
2 sections were treated with non-immune serum at similar dilution. To visualize antibodies
3 bound to either ABA or AQPs, sections were treated for 1 h in a moist chamber with the
4 second goat antibodies raised against rabbit immunoglobulin labelled with colloidal gold
5 (1:40 in PGT, Aurion, USA). After three washes with phosphate buffer (PB), samples were
6 post-fixed in 2% glutaraldehyde in PB for 5 minutes. Then the sections were washed with
7 distilled water, incubated with silver enhancer (Aurion, USA) for 30 min. Excess silver was
8 removed with distilled water and sections examined under a light microscope (Carl Zeiss
9 Jena, Germany) equipped with an AxioCam MRc5 digital camera (Carl Zeiss Jena,
10 Germany).

11 Intensity of immunostaining of plasmalemma aquaporins was estimated from 8-bit grayscale
12 images using ImageJ software (1.48, National Institutes of Health). Circles of fixed
13 dimensions were marked along cell membranes of epidermis and cortical cell layer beneath
14 epidermis with regular intervals around entire perimeter of root section and mean pixel
15 intensities were measured within the regions of interest (ROI). Staining values, obtained by
16 determining the pixel intensity for every circle, were averaged for each of root section (about
17 160 circles per image of one root section). Intensity of root section staining for ABA was
18 measured by using the “Freehand Selections” Tool of the same software by selecting the
19 entire area of root sections and measuring mean pixel intensities within the ROI. Images were
20 taken from 9 independent sections per genotype or ABA-treatment. Intensity of staining was
21 expressed in arbitrary units, maximal staining taken for 100 %, minimal – for 0.

22 *Statistics*

23 Data were expressed as means \pm SE, which were calculated in all treatments using MS Excel.
24 Significant differences between means were analyzed by t-test and two-way analysis of
25 variance (ANOVA) with genotype and ABA treatment as main factors, and an LSD (least
26 significance difference) test to discriminate means.

27 **Results**

28 Root hydraulic conductivity (L_{pRoot}) was about 2 times lower in Az34 than Steptoe (Table 1).
29 Inhibiting AQP activity by producing reactive hydroxyl radicals during the Fenton reaction
30 decreased hydraulic conductivity of ABA-treated and untreated plants of both genotypes
31 (Table 1).

32 Root fresh weight did not differ significantly ($p>0.1$, t-test) between the two genotypes
33 ($[63 \pm 4]$ and $[54 \pm 6]$ mg in Steptoe and Az34, respectively), thus the difference in L_{pRoot}
34 between the two genotypes could not be attributed to the difference in root mass. Root hairs

1 appeared closer to the root tip (1.1 ± 0.1 mm from root tip) in Az34 compared with Steptoe
2 (2.3 ± 0.2 mm from root tip). Moreover, the length of the root hair zone was shorter in Az34
3 than in Steptoe (Fig. 1) ($[13 \pm 1]$ and $[32 \pm 3]$ mm in Az34 and Steptoe, respectively, the
4 difference being significant at $p=0.001$).

5 Bulk ABA concentration in Az34 roots was only a third of the concentration in
6 Steptoe. ABA treatment increased root ABA concentrations by 5-fold in both genotypes
7 (Table 1). ABA treatment increased Lp_{Root} of both genotypes similarly by 2-fold or more (no
8 significant genotype \times ABA interaction: $p=0.13$, two way ANOVA). ABA treatment of Az34
9 raised endogenous root ABA concentrations and Lp_{Root} . Application of exogenous ABA to the
10 root medium of Az34 plants increased the Lp_{Cell} of root cortex cells almost three-fold ($p <$
11 0.001) (Table 2). This was due to a much decreased $T_{1/2}$ ($p < 0.001$) while changes in ϵ were
12 minor and not significant. Exogenous ABA had no effect on the turgor of cortical cells.

13 Root sections treated with non-immune serum were weakly stained (Fig. 2A). ABA
14 immunolocalization showed strong labelling of cells in all root tissues of Steptoe (Fig. 2B).
15 Staining for ABA was much lower in root cells of Az34, which was most noticeable in the
16 weakly labelled cortex (Fig. 2C). Application of ABA to Az34 plants increased
17 immunostaining of cortical cells, especially those which were located closer to the root
18 periphery (intensity of root immunolabelling for ABA was statistically higher in ABA treated
19 plants at $p<0.05$) (Fig. 2D, Table 3). The strong labelling of ABA in sections of Steptoe roots
20 prior to application of ABA impaired the detection of differences in labelling between ABA-
21 treated and untreated plants (data not shown). Dilution of anti-ABA serum decreased
22 immunostaining of Steptoe roots, but enabled detection of increased staining of the ABA-
23 treated Steptoe roots (Table 3). Even after dilution of the serum, the sections were more
24 strongly stained for ABA in the case of Steptoe compared to Az34 (despite the use of more
25 concentrated serum in the case of the mutant).

26 Western-blotting showed specificity of antibodies raised against a synthetic
27 oligopeptide corresponding to the amino acid sequences in the N- region of HvPIP2;5 (Fig.
28 3). These antibodies recognized the band in membrane proteins of oocytes expressing
29 HvPIP2;5 and did not recognize other PIP2 proteins. The specificity of antibodies used to
30 detect HvPIP2;1 and HvPIP2;2 has been shown previously (Horie et al. 2011).

31 Staining of sections, which was indicative of the presence of candidate PIP2 AQPs,
32 was hardly visible on sections treated with non-immune serum (Fig. 4D (Steptoe) and Fig. 5D
33 (Az34)). This changed when antibodies against HvPIP2 AQPs were used for immunostaining.
34 Cell boundaries, which included the plasma membrane, were clearly visualized due to

1 immunolabelling of plasma membrane AQPs (Fig. 4A-C, Fig. 5A-C). Labelling of boundaries
2 of, or next to metaxylem cells was most intense. Cytoplasm was also immunostained for
3 AQPs, though less than cell boundaries. Immunostaining was rather low for HvPIP2;5 and
4 stronger for HvPIP2;1 and HvPIP2;2.

5 Root sections of Az34 and Steptoe did not differ visibly in the immunostaining for any
6 of the HvPIP2 AQPs (cf. Fig. 4 and the left part of Fig. 5 A, B and C). This was supported by
7 a quantitative analysis of immunostaining intensity using ImageJ software (Table 3) ABA-
8 treatment increased immunostaining of roots cells of Az34 and Steptoe for HvPIP2;1 and
9 HvPIP2;2 antibodies (Table 3, means for immunostaining intensity along cell membranes of
10 root periphery different at $p < 0.05$), whereas the level of staining did not change for the
11 HvPIP2;5 antiserum. The increase in immunostaining was most pronounced at the root
12 periphery (Fig. 5).

13 **Discussion**

14 *Comparing Az34 with Steptoe*

15 Previous measurements of bulk root ABA concentrations showed that ABA levels in Az34
16 plants were 70% lower than in their wild-type parent Steptoe (Kudoyarova et al. 2014). The
17 present study extends these analyses to the tissue level and shows that all major tissues which
18 are located along the radial path of water movement across roots have lower ABA levels in
19 Az34 compared with Steptoe.

20 Initially, the longer-term, and possibly developmental, effect of differences in root
21 ABA levels on root water uptake properties were compared between genotypes. The
22 decreased root hydraulic conductivity of Az34 (compared to Steptoe) was accompanied by a
23 decreased ABA concentration consistent with the role of ABA in regulating water uptake
24 across roots (Parent et al. 2009). However, the abundance of HvPIP2;1 and HvPIP2;2 was
25 similar in roots of both genotypes and did not match the genotypic difference in L_{pRoot} . The
26 latter could mean either that longer-term lowered levels of ABA reduce activity of AQPs at
27 the post-translational level, or that the reduction in L_{pRoot} does not involve changes in AQP
28 activity.

29 Root hydraulic conductivity depends not only on AQP activity, but also on other root
30 attributes such as root hair development. The length of the root hair zone, which can be
31 extremely important to overall water uptake by the plant (Segal et al. 2008) was shorter in
32 Az34 compared with Steptoe and this may have also contributed to a reduced ability of Az34
33 roots to take up and conduct water. This observation agrees with earlier reports that
34 accumulation of ABA under moderate drought enhances root hair development (Xu et al.,

1 2013). Further experiments are needed to study the mechanism(s) responsible for the
2 decreased L_{pRoot} in Az34 compared with Steptoe.

3 *Effects of exogenous ABA on root (cell) hydraulics in Az34*

4 By comparing root hydraulics of Az34 and Steptoe in the presence and absence of
5 exogenously-added ABA, short-term effect of differences in root ABA levels on root water
6 uptake properties were revealed. Thus the response of the two genotypes to exogenous ABA
7 was similar. These experiments excluded the possibility that ABA affects root hydraulics
8 through developmental changes and provided a more convenient model for relating tissue
9 ABA levels to AQP abundance and hydraulics at a cellular level. Comparison of the
10 distribution of immunostaining between cells shows some similarity in the increased labelling
11 of ABA and AQPs in response to application of ABA. ABA-staining increased especially in
12 the root periphery, at the epidermis and cortical cell layers located beneath, and in these
13 tissues staining of HvPIP2;1 and HvPIP2;2 increased too. In addition, the stimulation of
14 L_{pRoot} by exogenous ABA (Table 1) was accompanied by enhanced L_{pCell} (Table 2). As L_{pCell}
15 reflects membrane transport properties, these results indicate that exogenous ABA stimulates
16 L_{pRoot} by stimulating the water transport properties of root cells, or at least those root cortex
17 cells which were analyzed in the present study. Furthermore, since membrane water transport
18 properties involve AQP function, and since exogenous ABA increased immunostaining of
19 HvPIP2;1 and HvPIP2;2 in cortex cells, we conclude that exogenous ABA increased L_{pRoot}
20 through an increase in the abundance, and activity, of at least two HvPIP2 isoforms
21 (HvPIP2;1, HvPIP2;2), but not that of HvPIP2;5 in root cortex cells. Parent et al. (2009)
22 showed up-regulation of expression of all ZmPIP AQP isoforms by ABA in maize plants. The
23 present data show that ABA also increases the protein level of some (HvPIP2;1, HvPIP2;2)
24 yet not all PIP AQPs (HvPIP2;5).

25 ABA can induce expression of AQP genes (Maurel et al. 2008). We do not know
26 whether, in shorter term experiments like ours (about 1 h), any changes in AQP expression
27 are likely to influence corresponding protein levels. Rather, effects of ABA at the post-
28 transcriptional level of AQPs are more likely to occur. As ABA can regulate the activity of
29 AQPs through their phosphorylation (Chaumont and Tyerman 2014 and references therein),
30 rapid ABA-induced changes in hydraulic conductivity could be explained through this
31 mechanism. In addition, ABA has been suggested to alter the conformation and gating and,
32 through this, water permeability of AQPs (Wan et al., 2004).

33 The present data show that application of exogenous ABA to the ABA-deficient barley
34 mutant Az34 and its parental cultivar Steptoe increases root and root cell hydraulic

1 conductivity parallel to an increased abundance of particular PIP2 AQP isoforms in root
2 epidermal and cortex cells closer to the root periphery. The difference in root hydraulic
3 conductivity between Az34 and its ABA-sufficient parent, Steptoe, may also involve
4 developmental effects of ABA on the timing and formation of root hairs during root
5 development.

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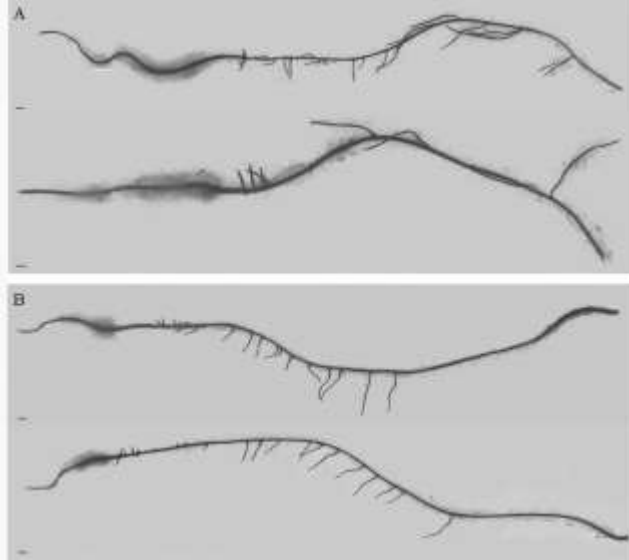
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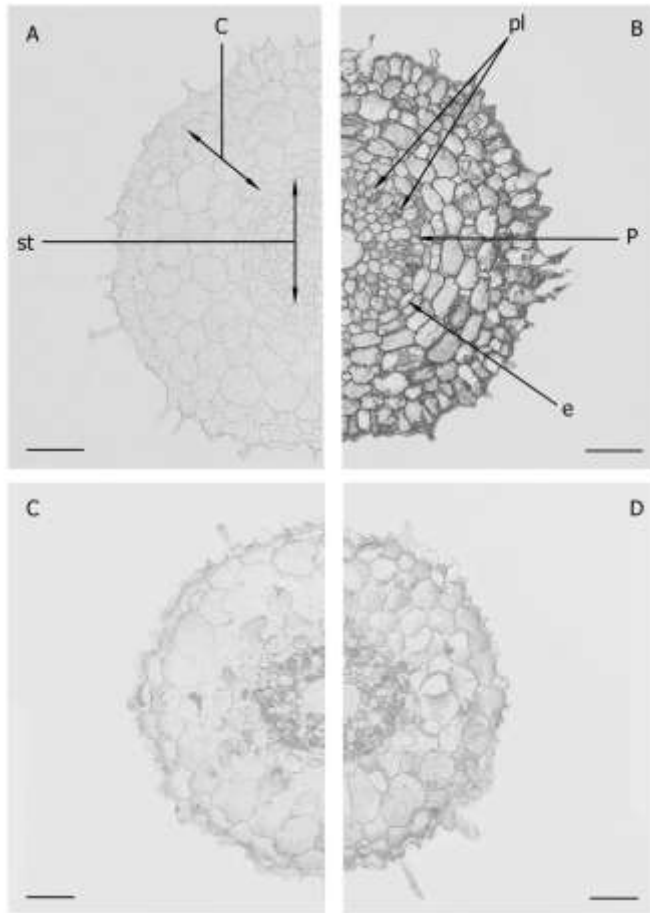
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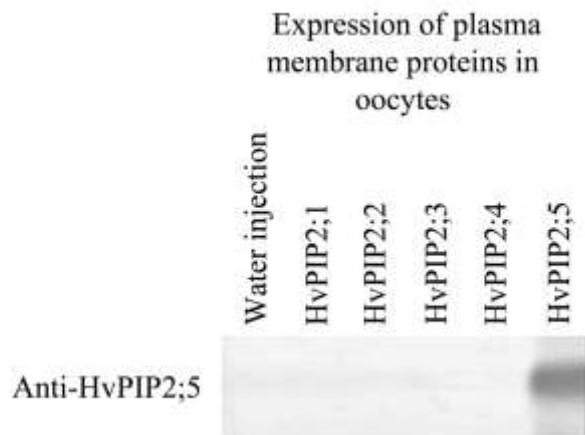


1
2 Fig.1. Image of the roots of Steptoe (A) and Az34 (B) plants (Scale bar – 1 mm);
3



1
 2 Fig. 2 Immunolocalization of ABA in root sections (3-5 mm from the root tip) of Steptoe (A
 3 and B) and Az34 (C and D) treated (D) and untreated (A, B, C) with 10^{-5} M ABA. Similar
 4 dilutions of anti-ABA serum were applied to the sections of either Steptoe or Az34. A-
 5 section of Steptoe roots treated with normal non-immune serum. COR-cortex; P-pericycle; pl-
 6 phloem; st-stele; e-endodermis (Scale bar – 100 μ m).
 7

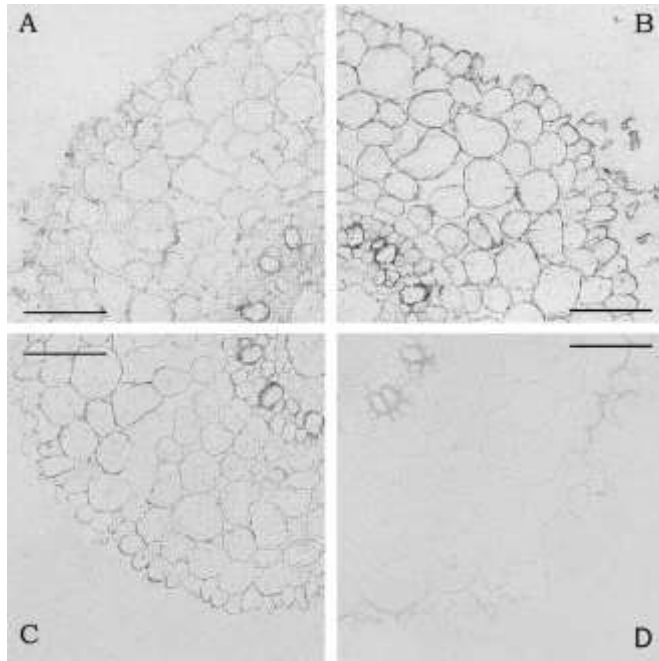
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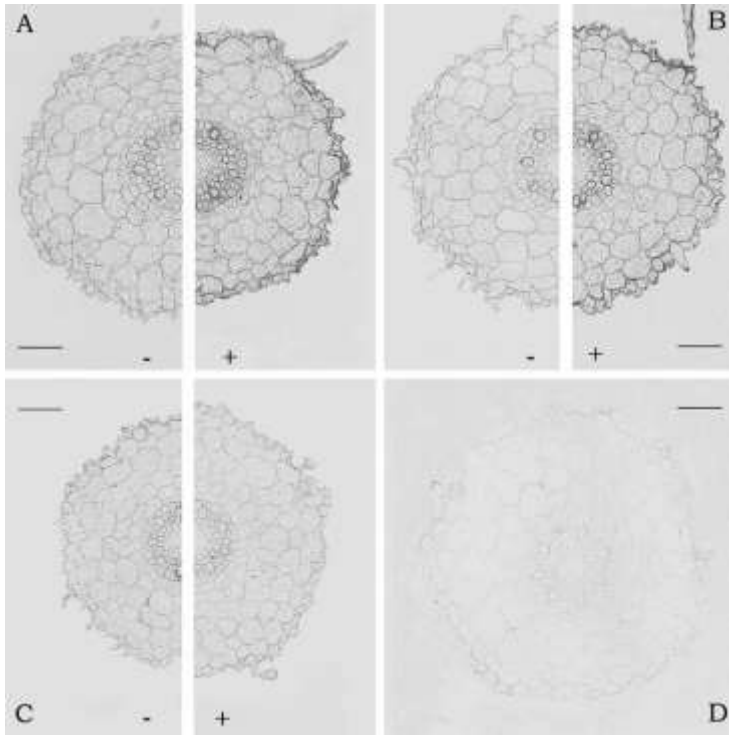
3 Fig. 3 Western blot analysis of membrane proteins of oocytes expressing cRNA of the coding
4 region of HvPIP2;5 using antibodies against synthetic oligopeptides corresponding to the
5 amino acid sequences in the N- region of HvPIP2;5.

6



1
2 Fig. 4 Immunolocalization of AQPs in roots (3-5 mm from the root tip) of Steptoe plants
3 using antibodies against HvPIP2;1 (A), HvPIP2;2 (B) and HvPIP2;5 (C). (D) - sections
4 treated with non-immune serum (Scale bar – 50 μ m).
5

1



2

3 Fig. 5 Immunolocalization of AQPs in roots (3-5 mm from the root tip) of Az34 plants using
4 antibodies against HvPIP2;1 (A), HvPIP2;2 (A) and HvPIP2;5 (C) AQPs treated (+) and
5 untreated (-) with 10⁻⁵ M ABA. (D) - sections treated with non-immune serum (Scale bar - 50
6 μm).

7

1 Table 1 Root hydraulic conductance ($\text{mg h}^{-1} \text{g}^{-1}$ root fresh weight MPa^{-1}) and ABA
 2 concentration (pmol g^{-1} root fresh weight) of barley plants treated with 10^{-5} M ABA, Fenton
 3 reagent and control. Significantly different means for each variable are labelled with different
 4 letters (n=5, LSD test).

5

Characteristics	Genotype, treatment	-ABA	+ABA
Hydraulic conductance	Step toe, - Fenton	290 ± 35^c	610 ± 55^d
	Az34, - Fenton	136 ± 21^b	390 ± 40^{cd}
	Step toe, +Fenton	170 ± 23^b	184 ± 30^b
	Az34, +Fenton	68 ± 8^a	89 ± 14^a
ABA concentration	Step toe	23 ± 3^b	117 ± 19^c
	Az34	8 ± 1^a	38 ± 11^b

6

7

1 Table 2 Water relations parameters of root cortical cells of the ABA-deficient barley mutant
 2 Az34 in the absence (-ABA) and presence (+ABA) of exogenous ABA in the root medium
 3 (10 μ M ABA). Plants were analysed between 20 min to 2 h following the addition of ABA to
 4 the root medium. Cells were located within the root hair zone. Results are averages and SE of
 5 (n=) 23 cell analyses, which were obtained from the analysis of four roots each. ***, p <
 6 0.001 (Student's t-test)
 7

Variable	-ABA	+ABA	p-value
Cell turgor (MPa)	0.48 \pm 0.02	0.49 \pm 0.01	0.673
Cell elastic modulus (MPa)	1.62 \pm 0.19	1.34 \pm 0.11	0.212
Cell half-time of water exchange, T1/2 (s)	9.48 \pm 0.89	5.11 \pm 0.63	<0.001***
Cell hydraulic conductivity, Lp (m s ⁻¹ MPa ⁻¹)	1.90 \pm 0.27 x 10 ⁻⁷	4.54 \pm 0.60 x 10 ⁻⁷	<0.001***

8
 9

1 Table 3. Intensity of staining for ABA and PIP2 aquaporins (means \pm SE, arbitrary units,
 2 maximal staining taken for 100 %, minimal for 0%) of control and ABA-treated Az34 roots.
 3 Anti-ABA serum was 4-times diluted when applied to the sections of Steptoe roots as
 4 compared to the procedure of ABA immunolocalisation in Az34. Significantly different
 5 means for each variable within a row are labelled with different letters (n=5, LSD test)
 6

Staining for	Steptoe		Az34	
	Control	ABA-treated	Control	ABA-treated
ABA	41 \pm 7 ^b	65 \pm 9 ^c	21 \pm 7 ^a	79 \pm 5 ^c
PIP2;1	23 \pm 5 ^a	75 \pm 4 ^b	26 \pm 9 ^a	68 \pm 13 ^b
PIP2;2	21 \pm 4 ^a	85 \pm 7 ^b	12 \pm 7 ^a	87 \pm 12 ^b
PIP2;5	73 \pm 8 ^a	56 \pm 5 ^a	61 \pm 12 ^a	45 \pm 19 ^a

7