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Robust root growth in *altered hydrotropic response1* (*ahr1*) mutant of *Arabidopsis* is maintained by high rate of cell production at low water potential gradient

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ABSTRACT

Hydrotropism is the directional root growth response determined by water stimulus. In a water potential gradient system (WPGS) the roots of the Arabidopsis wild type have a diminished root growth compared to normal medium (NM). In contrast, the *altered hydrotropic response1* (*ahr1*) mutant roots maintain their robust growth in the same WPGS. The aims of this work were to ascertain how *ahr1* roots could sustain growth in the WPGS, with a special focus on the integration of cellular processes involved in the signaling that determines root growth during abiotic stress and their relation to hydrotropism. Cellular analysis of the root apical meristem of *ahr1* mutant contrary to the wild type showed an absence of changes in the meristem length, the elongation zone length, the length of fully elongated cells, and the cell cycle duration. The robust and steady root growth of *ahr1* seedlings in the WPGS is explained by the mutant capacity to maintain cell production and cell elongation at the same level as in the NM. Analysis of auxin response at a transcriptional level showed that roots of the *ahr1* mutant had a lower auxin response when grown in the WPGS, compared to wild type, indicating that auxin signaling participates in attenuation of root growth under water stress conditions. Also, wild type plants exhibited a high increase in proline content while *ahr1* mutants showed minimum changes in the Normal Medium → Water Stress Medium (NM → WSM), a lower water potential gradient system than the WPGS. Accordingly, in this condition, gene expression of *Δ1-6 Pyrroline-5-Carboxylate Synthetase1* (*P5CS1*) involved in proline synthesis strongly increased in wild type but not in *ahr1* seedlings. The *ahr1* phenotype shows unique features since the mutant root cells continue to proliferate and grow in the presence of a progressively negative water potential gradient at a level comparable to wild type growing in the NM. As such, it represents an exceptional resource for understanding hydrotropism.

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1. Introduction

Plants depend on environmental cues to coordinate their growth and development to a greater extent than animals. One of the most important traits that plants have evolved is the power to sense environmental changes in order to direct their growth orientation to cope with stress. The directional growth of plant organs related to an environmental stimulus constitutes a tropism. Gravitropism and phototropism are the most studied tropisms, and many genes involved have been identified thus far (Morita, 2010). Hydrotropism (tropism towards water) has been less examined although it is essential for drought resistance (Cassab et al., 2013; Moriwaki et al., 2013; Bao et al., 2014). Hydrotropism implies the perception of a water gradient, the transduction of hydrotropic signal(s), and subsequent alteration of root growth directionality. The perception and absorption of water during hydrotropism has implications on plant survival in the presence of water-limited

Abbreviations: ABA, abscisic acid; dpg, days post germination; GUS, β, glucuronidase; MS, Murashige and Skoog medium; NM, normal medium; P5CS1, Δ1-6 pyrroline-5-carboxylate synthetase1; PRODH1, proline dehydrogenase1; QC, quiescent center; RAM, root apical meristem; WPGS, water potential gradient system; WSM, water stress medium.

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conditions. Nonetheless, the molecular mechanism controlling this tropism only started to be elucidated. The genes responsible for altered hydrotropic phenotype of two mutants, *miz1* and *miz2*, have been cloned (Kobayashi et al., 2007; Miyazawa et al., 2009). *MIZ1* encodes a protein of unknown function, which is expressed in the root cap and in the stele (Kobayashi et al., 2007). The expression analysis of this gene in the root has not revealed whether *MIZ1* is spatially regulated by moisture or dryness, and it is not clear in which root tissue *MIZ1* acts to regulate hydrotropism. The *miz2* phenotype results from the mutation in *GNOM* gene, which encodes an ADP-ribosylation factor-type G (ARF-GEF) protein regulating vesicle trafficking (Miyazawa et al., 2009). The best-characterized GNOM function is the polar targeting of PIN proteins to the plasma membrane (Geldner et al., 2003). Because the subcellular polarity of PIN proteins determines the direction of auxin flow, allelic *gnom* mutants show defects in auxin-dependent developmental processes such as embryogenesis, lateral root formation, and gravitropism (Shevell et al., 1994; Geldner et al., 2004). In partial loss-of-function of *gnom*, both gravitropism and hydrotropism are disrupted; however, the $gnom^{miz2}$ mutant showed defects in hydrotropic, but not in the gravitropic response. Current data indicate that hydrotropism utilizes a different GNOM-mediated vesicle trafficking compared to those involved in gravitropism (Miyazawa et al., 2009).

Two other hydrotropic mutants, *no hydrotropic response1* (*nhr1*) and *altered hydrotropic response1* (*ahr1*), have been characterized, but the genes responsible for the mutant phenotype have not yet been identified (Eapen et al., 2003; Saucedo et al., 2012). For the isolation of the *ahr1* mutant, we used a water potential gradient system (WSM → NM system) that consists of a vertically oriented square Petri dish where the Water Stress Medium (WSM, containing 2.5% w/v glycerol) was located at the top and the Normal Medium (NM) was at the bottom. Contrary to the wild type, primary roots of *ahr1* grow towards the source of higher water availability (NM) and developed an extensive root system over time (Saucedo et al., 2012). Roots of the *ahr1* mutant also maintain their growth towards the lower water potential gradient in the NM → WSM system (NM at the top and WSM at the bottom of square Petri dish) (Eapen et al., 2003). These reports suggest that the gene responsible for the *ahr1* mutation plays an important role in the perception of water deficiency.

In higher plants, proline accumulation is one of the main responses to abiotic stress (Sharma et al., 2011). Drought stress induces proline accumulation at high levels, for example in maize (Ober and Sharp, 1994) and Arabidopsis (Szekely et al., 2008) among other species. Proline is considered an important osmolyte that acts as a molecular chaperone stabilizing the structure of proteins, as well as a regulator of cellular redox potential and an antioxidant, controlling free radical levels (Hare et al., 1999; Hong et al., 2000). During stress responses, Δ1-6 Pyrroline-5-Carboxylate Synthetase1 (P5CS1) and Proline Dehydrogenase1 (PRODH1) synthesize and degrade proline, respectively, and P5CS1 is the rate-limiting enzyme (Delauney and Verma, 1993; Nanjo et al., 2003; Roosens et al., 1998). Proline synthesis and catabolism are required for optimal growth at low water potential since mutants in both genes show severe growth deficiencies under water stress conditions (Sharma et al., 2011), suggesting that proline biosynthesis and catabolism in Arabidopsis are required for growth at low water potential.

The roles of hormones ABA, auxin, and cytokinin in hydrotropism have been reported (Antoni et al., 2013; Eapen et al., 2003; Kaneyasu et al., 2007; Ponce et al., 2008; Saucedo et al., 2012). ABA increases the high root growth elongation phenotype of *ahr1* in the WSM → NM system since it significantly enhances the development of a long and highly branched root system. Cytokinin, however, completely inhibits this development, since *ahr1* roots develop a hydrotropic curvature in the oblique NM → WSM system similar to the wild type roots. It has been reported that auxin signaling plays an important role in hydrotropism in Arabidopsis roots, taking into account that a specific inhibitor of the auxin response (*p*-chlorophenoxyisobutylacetic acid) reduces hydrotropism, whereas inhibitors of auxin influx or efflux have no effect (Kaneyasu et al., 2007). Henceforth, we hypothesized that contrary to the wild type plant, *ahr1* mutant does not perceive low water potential gradients and the processes involved in cell division and expansion are preserved, allowing efficient root growth.

To address whether a greater root performance in the mutant under water stress conditions results from impact on cell proliferation, cell elongation, or both, we analyzed cell division and cell elongation in the primary root of the *ahr1* mutant. Additionally, we also examined the possible role of auxin in *ahr1* root phenotype when plants grew in water stress conditions. We evaluated the root growth rates and the sensitivity of *ahr1* roots to auxin under a milder water potential gradient system (WPGS), which consists of square Petri dish with the NM at the top and the WSM containing 0.4% (w/v) glycerol at the bottom.

Our results provide a novel understanding of how roots perceive the changes in water potential and point out the role of the root apical meristem (RAM) and cell production in this process. In addition, the fact that the *ahr1* roots' response to auxin at a transcriptional level is lower compared to those of wild type in the WPGS, opens new possibilities for deciphering the role of auxin in the integration of the signaling that controls abiotic stress responses and its relation to hydrotropism. We also analyzed proline content in roots of wild type and *ahr1* mutant in response to water stress. Contrary to wild type, proline was not accumulated in roots of *ahr1* seedlings grown under lower water potential. Finally, these results imply that the *ahr1* mutant represents a valuable genetic resource for the development of crops better adapted to drought.

2. Materials and methods

2.1. Plant materials, growth media and mutant screen

Wild type *Arabidopsis thaliana* (L.) Heynh. Columbia-0 (Col-0) seeds were provided by the Arabidopsis Biological Resource Center (Ohio State University). $CYCB1;1_{DB}$:*GUS* and *DR5:GUS* lines were in Col-0 background and have previously been described by Colon-Carmona et al. (1999) and Ulmasov et al. (1997). For studies of root growth dynamics presented in this work the WSM media supplemented with different amounts of glycerol were used. For analysis of the cellular bases of root growth of *ahr1* growing in the WPGS, the WSM was supplemented with 0.4% (w/v) glycerol and 0.2% (w/v) alginic acid. For preparing the WPGS, 46 mL of the NM were poured into the upper sector of a 10-cm square Petri dish containing an acrylic slab (90 mm × 10 mm × 4 mm) for separating the two media. After solidification of the NM, the slab was removed and 4 mL of the WSM were poured in the lower sector of the Petri dish. Once the WSM solidified the water potential in the WPGS was established. Seeds were placed immediately onto the NM sector in a line, 6.5 cm from the border of the WSM. Dishes were sealed with Parafilm (Sigma-Aldrich, MO, USA) and maintained in a vertical position. Plants were grown at 21 °C, 16/8 h light/dark cycle and the light intensity was 105 μmol photons m^{-2} s^{-1}. The hydrotropic response of *ahr1* mutant was also tested in a moisture gradient according to Kobayashi et al. (2007).

2.2. Water potential analysis of the WPGS

The water potential of the WPGS was measured utilizing the Osmometer model Vapro 5520 (Wescor Inc.). A piece of agar

medium, approximately 0.5 cm^2, was taken at time zero and every 2, 4, and 6 d after beginning of the experiment at three different distances (1.3 cm, 5.7 cm and 8 cm) from the top part of the vertically oriented dish. Then, the piece of agar was melted at 8 °C and 10 μL of the medium was poured into a square filter disc for the measurements performed according to the manufacturer instructions. Each measurement was repeated three times for each time point during the time-course analysis. Data are presented as the mean ± SD, $n = 10$.

2.3. Root growth parameters determination and microscopy

The primary root length and root hydrotropic curvature was measured on scanned images of seedlings using ImageJ software (http://rsb.info.nih.gov/ij/). The number of cortical cells within the RAM cell proliferation domain was determined on cleared root preparations in accordance with previously described criteria (Ivanov and Dubrovsky, 2013). Roots were cleared and fixed as previously reported (Dubrovsky et al., 2009) and analyzed under a Zeiss Axiovert 200 M microscope (Zeiss) equipped with differential interference contrast (Nomarski) optics. The cortical cell length was determined for 10 cells per root on cleared preparations using an ocular micrometer. The root growth parameters were evaluated for each individual root based on the model of linkage between cell cycle duration, cell production, and the rate of root growth as described in detail (Ivanov and Dubrovsky, 1997; López-Bucio et al., 2014). The cell cycle duration was evaluated using the equation $T = (N_{CPD}\ le\ ln2)/V$, where T is cell cycle duration (h), N_{CPD} is the number of cortical cells in the RAM proliferation domain, *le* is average length of 10 cortical fully elongated cells (μm), and V is the rate of root growth (μm/h) evaluated during last 24 h of growth (Ivanov and Dubrovsky, 1997). For GUS staining, roots were pre-fixed in 0.3% formaldehyde (Sigma-Aldrich, MO, USA) for 20 min at room temperature, washed in 100 mM sodium phosphate buffer, pH 7.4, and stained as described (López-Bucio et al., 2014). Photographs were taken using a Photometrics CoolSNAPcf Color Camera (Valley International Corporation, TX, USA).

2.4. Hormone treatment and statistical analysis

Indole acetic acid (IAA) (Sigma-Aldrich, MO, USA), prepared as 50 μM stock solution was diluted in 1 N NaOH (Sigma-Aldrich, MO, USA). Filter-sterilized IAA to the desired concentration (1 and 2 μM) was diluted in soft agar (0.15% w/v) and a strip of agar of 5 mm (1 mL per square dish) was carefully placed at the root tip of 2 dpg seedlings. For mock samples a drop of 1N NaOH was added to the soft agar. Data derived from all the analyses performed on *ahr1* and wild type seedlings were analyzed by two-tailed Student's *t*-test and/or two-way analysis of variance (ANOVA) with Tukey's multiple comparison test using Prism 6 program for MacOS X (GraphPad Software, Inc., CA, USA). All experiments were repeated at least twice.

2.5. Determination of proline content

The proline content was measured using the colorimetric method according to Bates et al. (1973) with minor modifications as follows: about 0.3 g fresh root tissue was ground in a mortar with liquid nitrogen. Tissue powders were suspended in 1.2 mL of 3% sulfosalicylic acid (Sigma-Aldrich, MO, USA) and incubated in boiling water for 10 min. After centrifugation at 3000×*g* for 10 min, 1 mL supernatant was vigorously mixed with 1 mL acid ninhydrin (Sigma-Aldrich, MO, USA) and 1 mL glacial acetic acid (Sigma-Aldrich, MO, USA). The mixtures were incubated at 100 °C for 1 h and the reaction was stopped in an ice bath for 10 min. The reaction mixtures were extracted with vigorous shaking with 2 mL toluene (Sigma-Aldrich, MO, USA), the upper colored phase was recovered and absorbance was read at 520 nm, using toluene as a blank. Proline (Sigma-Aldrich, MO, USA) concentration was determined from a standard curve made by the same procedure. Data were calculated on a fresh weight basis (FW).

2.6. Determination of water content

Four dpg wild type and *ahr1* plants were grown in the NM or in the NM → WSM. Fresh weight was determined for three biological replicas with 50 seedlings each. Weight in mg was registered using an analytical balance and compared between two growth systems and *ahr1* and wild type seedlings. Error bars represent SD (Student's *t*-test: $^*P < 0.05$).

2.7. RNA isolation and real time PCR gene expression analysis

Total RNA was isolated from 200 roots of Col-0 or *ahr1* 5 dpg seedlings grown in the NM or in the NM → WSM using Trizol reagent (Thermo Fisher Scientific, MA, USA). Two units of DNase (Promega, Madison, WI) and 2 μL of RiboLock (Thermo Fisher Scientific, MA, USA) were added to 40 μg of RNA and incubated 30 min at 37 °C. DNase was inactivated for 15 min at 65 °C. For cDNA synthesis 2 μg of total RNA, 1 μL of 500 μg/mL of oligo (dT25)VN and 1 μL (200 Units/μL) of Moloney murine leukemia virus Retroranscriptase (MoMLVRT) (Invitrogen, CA, USA) were used (Castillo-Olamandi et al., 2007). Real-time PCR using GreenMaster (Jenna Bioscience, Jena, Germany) was performed on Qiagen Rotor 6000 (Hilden, Germany) detector system following the PCR conditions: 95 °C 10 min, 40 cycles (95 °C, 10 s; 55 °C, 15 s; 65 °C, 16 s). Primers used for gene expression analyses are listed in Supplementary Table S1 in the online version at DOI: 10.1016/j.jplph.2016.11.003. Real time RT-PCR analyses were always performed on three biological replicates with three technical replicates each, using 18S rRNA (At3G41768) as a housekeeping gene. Fold change of each gene analyzed was calculated as $2^{-\Delta\Delta CT}$ method. The threshold cycle (CT) was automatically determined for each reaction by the system set with default parameters.

3. Results

3.1. Ahr1 *mutant maintains root growth under different low water potential gradients*

Considering that the root growth of wild type is severely inhibited in the WSM → NM system after 3 dpg and with the aim to prevent sudden root growth inhibition and evaluate the changes in meristem length and other growth parameters (Saucedo et al., 2012), we replaced the WSM → NM system with a mild water potential system named WPGS (Fig. 1A). To determine the water stress conditions that allowed wild type and *ahr1* seedling growth, we tested several glycerol concentrations in the WSM sector of the WPGS. When the WSM sector contained 0.25% (w/v) glycerol, the root growth of the *ahr1* mutant was similar to that in the wild type seedlings (Fig. 1B). However, when seedlings grew in the WPGS prepared with a WSM sector containing 0.3%, 0.4% or 0.5% (w/v) glycerol wild type roots still grew, although to a lesser extent than *ahr1* roots, therefore changes in meristem activity and growth rate could be determined (Fig. 1C–E). Based on this data, the WPGS prepared with a WSM sector containing 0.4% (w/v) glycerol was chosen for experiments as both wild type and *ahr1* mutant roots maintained their growth for at least 6 dpg (Fig. 1F). This design reduced the steepness of the water potential gradient compared to the previously used WSM → NM. The water potential gradient of the WPGS was -0.25 ± 0.08 MPa in the NM sector, where the seeds were sown ($t = 0$). At the middle of the plate the value was -0.23 ± 0.07 MPa

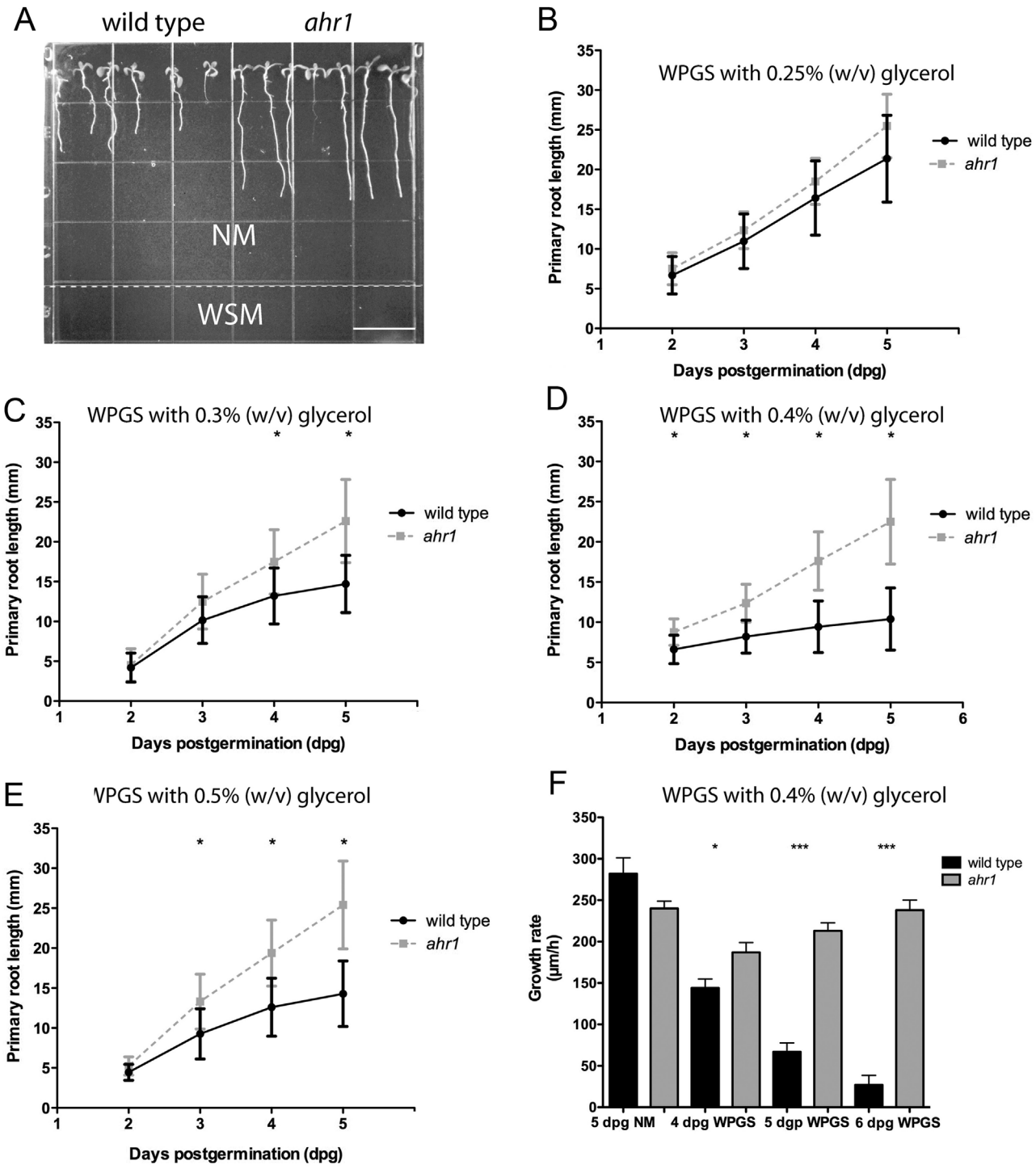

Fig. 1. The water potential gradient system (WPGS) used for root growth analysis and the rate of root growth in wild type and *ahr1* grown in the WPGS prepared with different glycerol concentrations. (A) Wild type and *ahr1* mutant seedlings at 5 dpg grown in the WPGS prepared with 0.4% (w/v) glycerol. A dotted line shows the border between the upper sector with the normal medium (NM) and the lower sector with the water stress medium (WSM). Scale bar = 13 mm. (B–E) WPGS with four different concentrations of glycerol (0.25%, 0.3%, 0.4% and 0.5% [w/v]) in the WSM were tested and the dynamics of root growth of the *ahr1* mutant was compared with those of the wild type grown in the WPGS. In the medium containing 0.4% (w/v) glycerol (D), the wild type root showed continuous growth for 5 d, while in the other media, its growth was inhibited earlier (0.5% [w/v]) (E), or was not inhibited at all (0.25% and 0.3% [w/v]) (B–C). (F) The growth rate of wild type and the *ahr1* mutant in the WPGS prepared with 0.4% glycerol. The primary root growth rate in *ahr1* was significantly higher than those of the wild type when seedlings grew in the WPGS and no differences were observed when wild type and *ahr1* seedlings grew in the NM. Five dpg *ahr1* roots grew at a similar rate in the NM, while root growth in the wild type decreased 90% in the WPGS compared to those in the NM. Combined data of two independent experiments, mean + SD, $n = 30$. Statistically significant differences were determined using Student's *t*-test: *, $P < 0.05$, *** and $P < 0.001$.

and at the bottom of the plate (in the WSM sector) the value was -0.45 ± 0.03 MPa. The water potential values in the WPGS with 2 dpg seedlings a value of -0.29 ± 0.02 MPa was obtained in the NM sector where seeds were sown. The water potential at the middle of the plate was -0.30 ± 0.05 MPa, and decreased to -0.32 ± 0.06 MPa in the WSM sector of the plate. Therefore, differences in root growth capabilities between *ahr1* and wild type observed in a previous study (Saucedo et al., 2012) which were about 2 fold, were not

caused by stressful growth conditions but were related to the greater overall performance of the mutant roots growing in a substrate with a pronounced low water potential gradient.

3.2. Hydrotropism of ahr1 roots is altered in the presence of a moisture gradient

The hydrotropic response of *ahr1* roots in the hydrotropic moisture gradient assay generated with K_2CO_3 in a closed chamber was also analyzed (Kobayashi et al., 2007). The root curvature of this mutant was significantly reduced compared to those of the wild type, while root elongation was similar in both genotypes: the hydrotropic curvature (angle) of *ahr1* roots increased about 2.6 fold while those of the wild type raised around 7.5 fold compared to water control, indicating that *ahr1* roots showed altered responsiveness to the moisture gradient. As a non-hydrotropic control we used *miz1* root seedlings (Fig. 2). As expected, a significantly negative hydrotropic curvature and root elongation was observed in the *miz1* mutant roots compared to wild type. Root elongation was similar in the three genotypes that grew 24 h in the presence of the moisture gradient generated by K_2CO_3 (Fig. 2C).

3.3. The ahr1 mutant maintains cell division in the root apical meristem of seedlings grown in the WPGS

The *ahr1* mutant has been previously reported to display a normal morphology of the root tip when grown in the WSM → NM (Saucedo et al., 2012), suggesting that the RAM of *ahr1* is able to maintain its activity under water stress conditions, contrary to wild type. We confirmed these observations when the seedlings grew in the WPGS (Supplementary Fig. S1 in the online version at DOI: 10.1016/j.jplph.2016.11.003). Also these observations suggest that not only cell proliferation processes but also cell elongation processes are not modified in *ahr1* when water availability declines over time, contrary to the wild type behavior.

To demonstrate this, we measured a) the length and number of cells in the RAM, b) the length of the elongation zone, c) the whole growing part of the root (combined length of the RAM and the elongation zone), and d) the length of fully elongated cells. The cell production rate and the duration of the cell division cycle were also determined. For these analyses the *ahr1* mutant and the wild type were grown in the WPGS with 0.4% (w/v) glycerol in the WSM sector and in the NM as a control. In non-stressful conditions (NM only) no significant differences were observed in the RAM length and cell number of both wild type and *ahr1* roots (Table 1). In contrast, after 6 dpg wild type plants grew at a lesser extent in the WPGS, the RAM length and cell number decreased compared to *ahr1* (Fig. 3A–B and Supplementary Fig. S1A–D in the online version at DOI: 10.1016/j.jplph.2016.11.003 and Table 1). This analysis indicated that the RAM length and its cell number in the *ahr1* mutant were not affected by the water stress conditions after 5 dpg growing in the WPGS, moreover, they increased over time (6 dpg), contrary to the wild type.

It is known that rapid cell elongation starts simultaneously in all the tissues (Ivanov and Dubrovsky, 2013). The length of the elongation zone in root seedlings grown in the NM showed a similar behavior in the wild type and the *ahr1* mutant (Fig. 3C). However, in the WPGS, the elongation zone of the wild type was significantly diminished compared to the *ahr1* mutant (Table 1). The 6 dpg *ahr1* root elongation zone was about 6 times longer and similar to that in roots grown in the NM (Table 1). The end of the elongation zone is hallmarked with the beginning of cell differentiation, which in the epidermis can be appreciated by root hair bulge formations. To have another independent parameter showing the beginning of differentiation, we also measured the distance between the first differentiated protoxylem recognizable at the tip and the meristem border towards the base of the root on cleared preparations. In seedling roots growing in the NM this distance was similar in the wild type and in the *ahr1* mutant (Table 1 and Fig. 3D). On the contrary, in seedlings grown in the WPGS, this distance was considerably reduced in the wild type whereas in the *ahr1* mutant it was not affected. Also, the length of fully elongated cortical cells diminished significantly in the wild type growing in the WPGS at 5 and 6 dpg whereas cell length in the *ahr1* mutant roots was equally maintained in seedlings grown in the NM and in the WPGS (Table 1 and Fig. 3E).

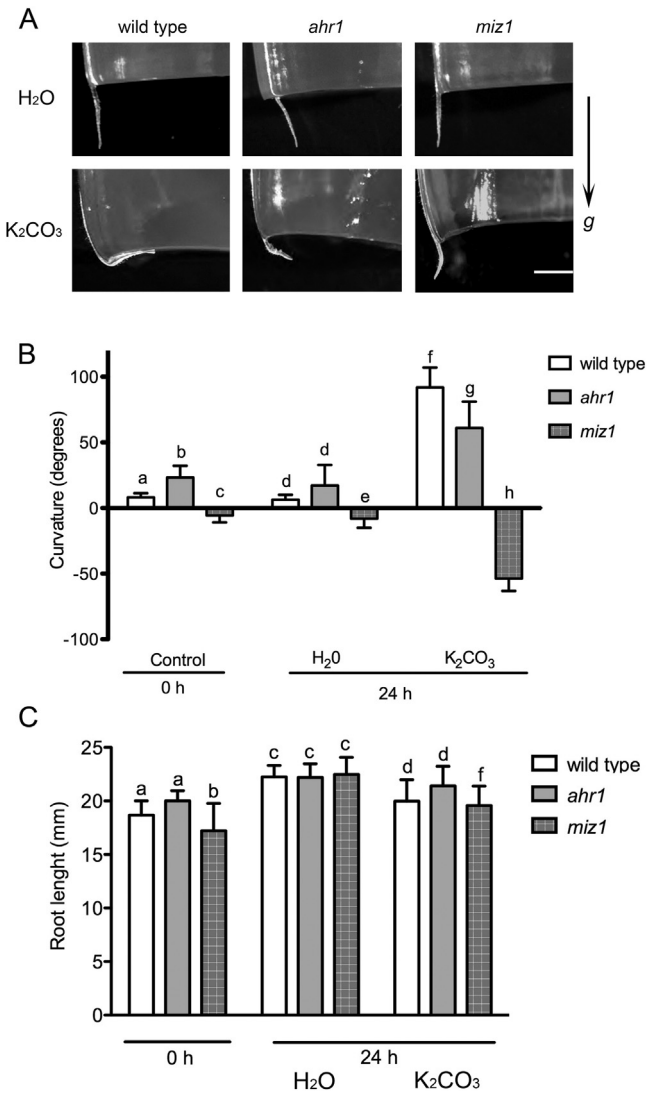

Fig. 2. The hydrotropic response of *ahr1* root seedlings in a humidity-based experimental system of hydrotropism. (A) Changes in the hydrotropic curvature with a moisture gradient established with a saturated solution of K_2CO_3 in 3 dpg *ahr*1, wild type and *miz1* root seedlings, and orthogravitropic root growth with a humidity-saturated environment with H_2O. (B) The hydrotropic curvature of *ahr1*, wild type and *miz1* root seedlings after 24 h of incubation in H_2O or in saturated solution of K_2CO_3. (C) Changes in root length after 24 h in the chamber with H_2O or with saturated solution of K_2CO_3. Data represent the mean of two independent experiments and error bars denote SD, $n = 10$. Data were analyzed by two-way ANOVA and Tukeyís multiple comparison test analyzed SD. Different letters denote statistically significant difference ($P < 0.05$, <0.01, and <001). Arrow (*g*) indicates the direction of the gravity vector. Scale bar in (A) = 1 mm.

Cell cycle duration was determined in the RAM of wild type and *ahr1* seedlings as previously described (Ivanov and Dubrovsky, 1997; López-Bucio et al., 2014). In the NM, both 5 dpg wild type and *ahr1* showed a similar duration of cell cycle, whereas when grown in the WPGS, the wild type showed a significant, two-fold, increase

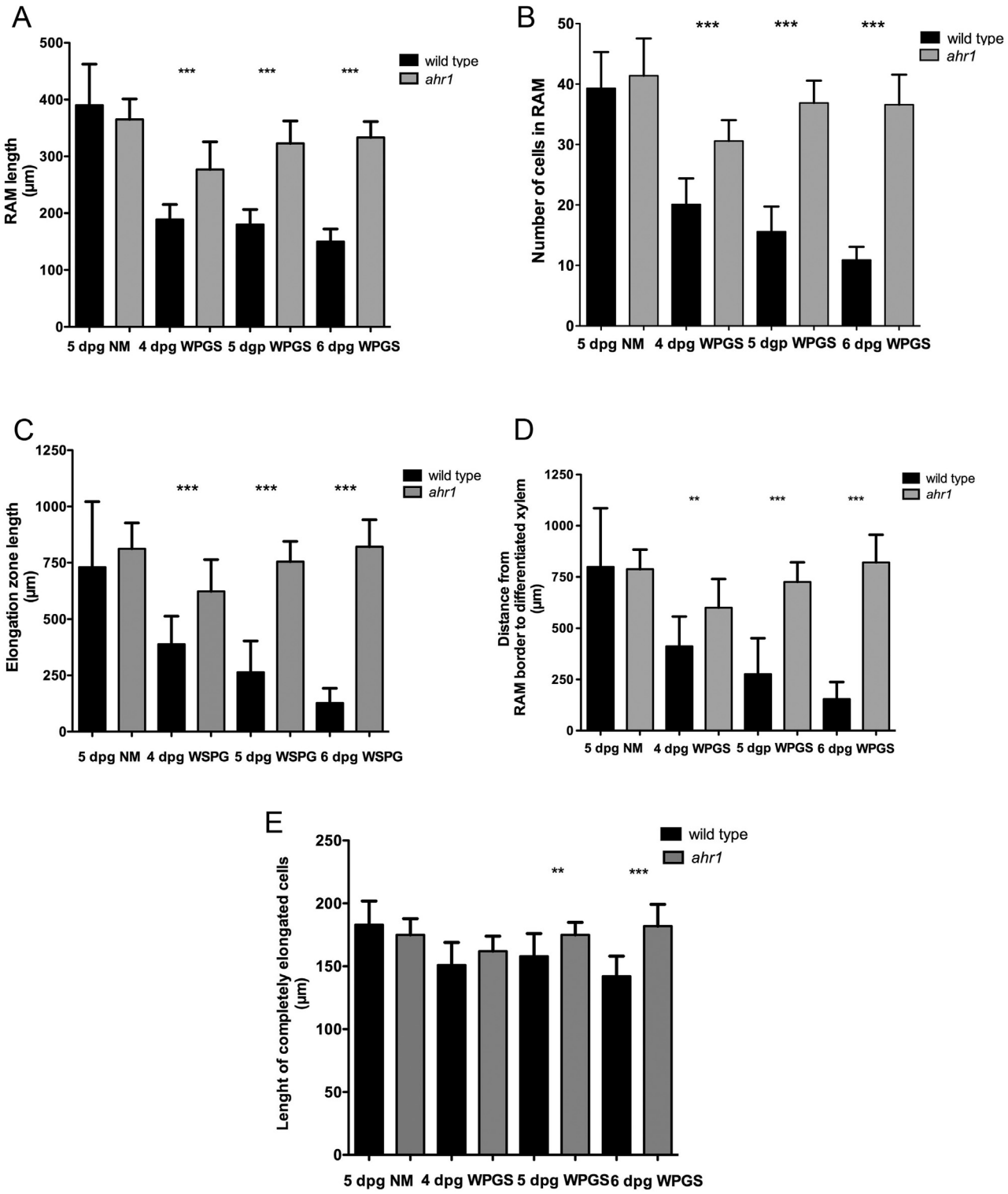

Fig. 3. The increased root growth rate in *ahr1* mutant grown in the WPGS correlated with the increased meristem length, the higher number of cells in RAM, the greater elongation zone length, the longer distance from RAM border to xylem, and the increased length of elongated cells, compared to those of the wild type. Quantifications of the root meristem length, the meristem cell number, the elongation zone (EZ) length, the fully elongated cortical cell length, in wild type (black bars) and *ahr1* mutant (gray bars) were analyzed in seedlings at 4 dpg, 5 dpg and 6 dpg grown in the WPGS and at 5 dpg in seedlings grown in the NM. (A) The root apical meristem length determined for the cortical cell file within both, the cell proliferation and the transition domains. (B) Number of cells in RAM for a cortical cell file within both, the cell proliferation and transition domains. (C) The length of the elongation zone. (D) The distance from the RAM border toward the differentiated xylem element. (E) The length of fully elongated cortical cells. Data are mean + SD, $n = 30$ from two independent experiments. Statistically significant differences were determined using Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

in cell cycle duration at 6 dpg (Fig. 4E). To support this quantitative analysis we introduced a G2/M transition marker, *Cyclin B1;1*$_{DB}$*:GUS* (Colon-Carmona et al., 1999,) in the *ahr1* background. As expected, both the RAM length and level of GUS expression in *ahr1* grown in the NM were similar to those in wild type seedlings grown under the same conditions (Fig. 4A–B). However, the GUS

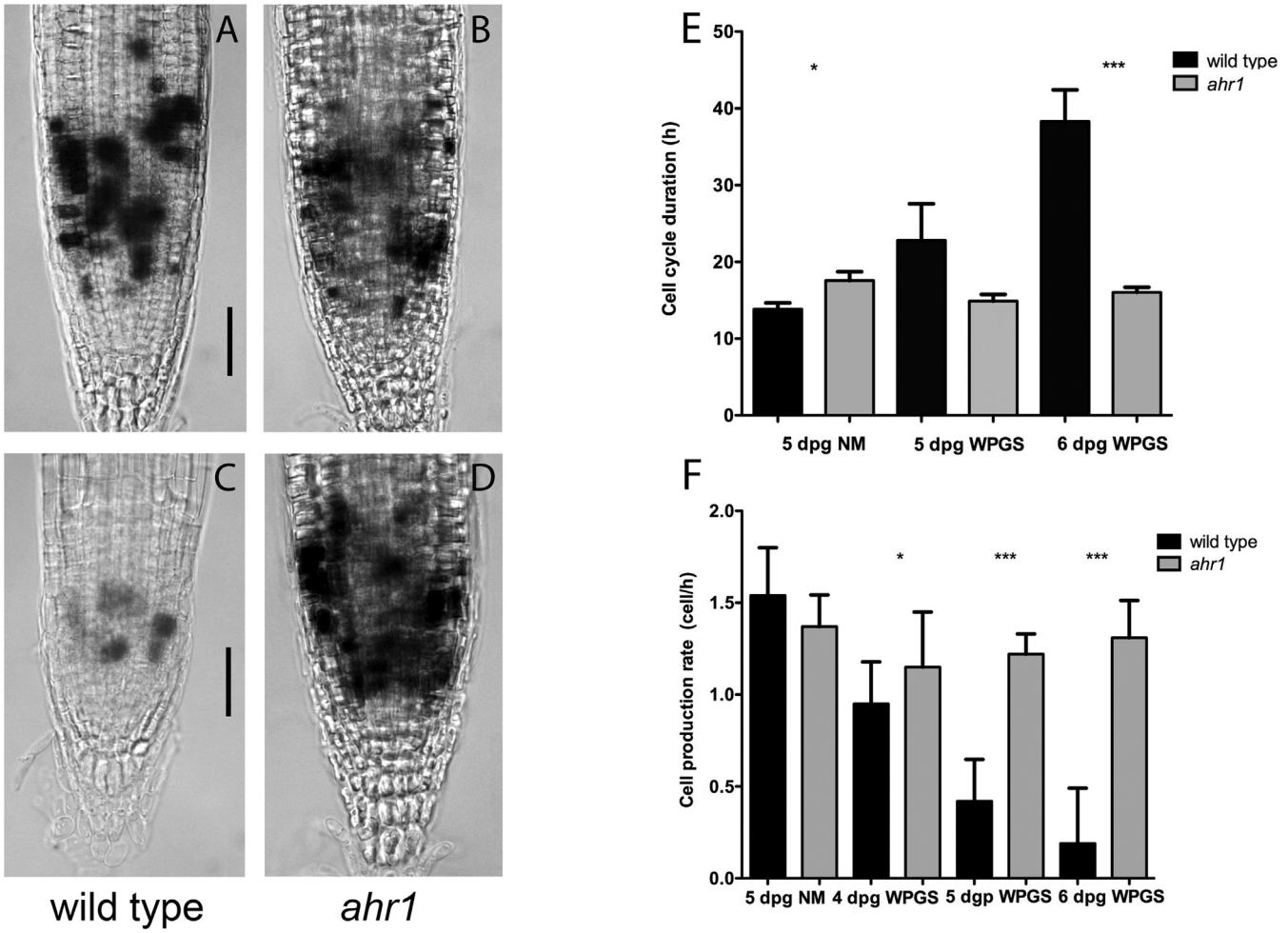

Fig. 4. The increased root growth rate in *ahr1* mutant grown in the WPGS correlated with a greater cell production. *CyclinB1:GUS* expression in the 5 dpg wild type (A) and in the *ahr1* mutant (B), grown in the NM and in 5 dpg wild type (C) and in the *ahr1* mutant (D) grown in the WPGS. Scale bar = 40 μm. The images are representative of two different experiments ($n = 24$). (E), The cell cycle duration was calculated as described in Materials and Methods and (F) The cell production rate analyzed in seedlings at 4 dpg, 5 dpg and 6 dpg in the WPGS and 5 dpg in the NM in *ahr1* (gray bars) and the wild type (black bars). Each point represents the mean + SD of 30 roots. Data are mean + SD, $n = 30$ from two independent experiments. Statistically significant differences were determined using Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 1
RAM length and number of cells in the RAM of wild type and *ahr1* roots grown on WPGS.

	Wild type	*ahr1*
RAM length (μm)		
5 dpg	180 ± 26	365 ± 36
6 dpg	150 ± 22	323 ± 28
Number of Cells in RAM		
5 dpg	15.6± 4	36.9 ± 4
6 dpg	10.9 ± 2.2	37 ± 5
Root elongation zone length (μm)		
5 dpg	264 ± 139	755 ± 90
6 dpg	127 ± 65	821 ± 120
Distance of RAM border from differentiated xylem (μm)		
5 dpg	276 ± 175[a]	726 ± 95[a]
6 dpg	154 ± 83[a]	821 ± 134[a]
Length of completely elongated cells		
5 dpg	158 ± 18	175 ± 10
6 dpg	142 ± 16	182 ± 17

[a] $P > 0.05$, Student's *t*-test.

expression in *ahr1* was maintained in the WPGS contrary to the wild type (Fig. 4C–D). The RAM length and cell proliferation in the wild type grown in the WPGS were clearly diminished compared to the *ahr1* mutant grown under the same conditions (Fig. 4F). Therefore, our quantitative and qualitative analyses indicated that cells in the RAM of wild type roots perceived a much greater water stress than those in the *ahr1* mutant. As cell cycle duration increased in wild type seedling grown in the WPGS, we can expect that overall RAM cell production decrease significantly under low water potential. Indeed, the average cell production rate in roots of wild type seedlings grown in the WPGS was 13 times lower than in *ahr1* (Fig. 4F). Importantly, both cell production and cell cycle time in *ahr1* grown under the WPGS conditions were the same as those in the plants grown in the NM (Fig. 4F). Therefore, contrary to wild type, when water availability declined, *ahr1* roots were able to maintain normal root growth. Cellular analysis showed that this ability of the mutant roots was related to the absence of changes in meristem length, elongation zone length, fully elongated cell length, and cell cycle duration. All of these parameters measured in the mutant were similar in the NM and the WPGS and similar to the wild type grown in the NM. Therefore, more vigorous root growth in the *ahr1* mutant in the WPGS can be explained by the fact that a low water potential may not be perceived at the cellular

level in the RAM. Alternately, root growth can be compensated by a not yet described mechanism.

3.4. *Root growth in* ahr1 *is less sensitive to auxin compared to wild type*

Auxin is required for the RAM maintenance and activity (Leyser, 2005), and its inhibitory effect on root elongation is well known (Ivanchenko et al., 2010; Leyser, 2002). To address the question of whether the lack of perception of low water potential gradient in the mutant is related to changes in auxin signaling, we also analyzed *ahr1* mutant behavior when auxin was applied externally. We performed a growth assay in the presence of 1 and 2 μM IAA. An IAA-containing soft agar block, with 1 or 2 μM IAA, was placed directly on 2-dpg seedling root tips growing in both the NM and the WPGS to prevent the additional effect of auxin on the shoot growth. Fig. 5A showed primary root growth of *ahr1* and wild type in control conditions in both the NM and the WPGS (mock). Four dpg *ahr*1 seedlings showed similar reduction of root length in the presence of 1 and 2 μM IAA in both the NM and the WPGS (Fig. 5B). However, root length of 4 dpg wild type seedlings was significantly inhibited with 1 μM IAA in the WPGS, indicating that wild type roots were sensitive to both auxin treatments and the WPGS conditions. Root growth of both 6 dpg *ahr1* and wild type seedlings grown in the NM in the presence of 1 μM of IAA was significantly lower compared to 4 dpg seedlings (Fig. 5C), indicating that root sensitivity to auxin differed with age. At 6 dpg *ahr1* mutant seedlings grown in the WPGS showed a significant decrease in root length in the presence of 1 μM of IAA compared to those untreated (Fig. 5C), but similar inhibition of root length was observed in those treated with auxin in the NM. There was no significant reduction in root length in 6 dpg wild type seedlings grown in the WPGS, but root length inhibition of wild type roots in the NM supplemented with 2 μM IAA was similar to those wild type roots grown in the WPGS without auxin. These results indicated that IAA at these concentrations equally inhibited *ahr1* root growth in the NM system and the WPGS. These results also showed that 6 dpg wild type seedling roots were more sensitive to the conditions of the WPGS than to 1 μM IAA in the NM compared to those of *ahr1* in the WPGS (Fig. 5B–C). Additionally, the fact that root elongation in *ahr1* was equally sensitive to IAA treatment in the NM and the WPGS suggests that auxin signaling might not be involved in maintenance of vigorous root growth in the WPGS.

The role of auxin in the maintenance of more vigorous root growth in the *ahr1* mutant in the WPGS was analyzed at a transcriptional level. Therefore, we monitored this auxin response using *DR5:GUS* expression (Ulmasov et al., 1997). In agreement with published data, (Blilou et al., 2005; Dubrovsky et al., 2011; Sabatini et al., 1999) in wild type seedlings grown in the NM, the expression of *DR5:GUS* auxin reporter was observed in the QC, the root cap, and provascular tissues (Fig. 6A). Nevertheless, in the *ahr1* mutant grown in the NM, a consistent decreased level of GUS staining was observed in all these compartments compared to the wild type ($n = 24$, Fig. 6B), indicating that basal auxin transcript levels were down regulated as a consequence of *ahr1* mutation. In the *ahr1* mutant grown in the WPGS, *DR5:GUS* expression was even more reduced than in the NM (Fig. 6D). In 19 out of 24 plants analyzed, no GUS expression was detected in the provascular tissues of *ahr1*. In wild type, 24 plants of 25 analyzed showed GUS expression in this compartment. Interestingly, expression of the *DR5:GUS* auxin reporter in wild type roots grown in the WPGS was shifted towards the shoot and root cap cells showed low levels of *DR5:GUS* expression (Fig. 6C). The fact that altered *DR5* activity was detected mainly in the provascular tissues of the RAM suggested possible accumulation of auxin in this compartment when wild type seedlings grew in the WPGS. This analysis suggested that the more vigorous root

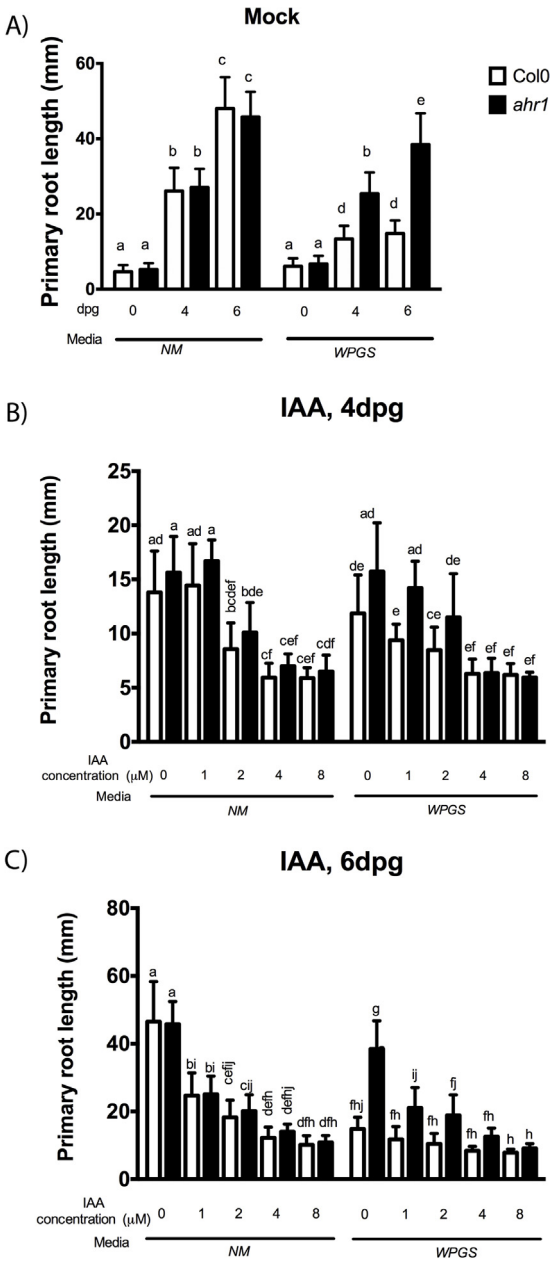

Fig. 5. The root growth of the *ahr1* mutant was less sensitive to low concentrations of auxin in both the NM and the WPGS. The effect of 1 μM and 2 μM of IAA in the root growth of the *ahr1* mutant and the wild type was tested in the NM and the WPGS. (A) The effect of mock treatment (growth media plus 50 μL of 1 N NaOH) on primary root lengths of 4 and 6 dpg *ahr1* mutant and wild type seedlings in the NM and the WPGS. *ahr1* mutant roots grow significantly more than those of the wild type in the WPGS. From left to right $n = 44, 45, 44, 45, 34, 34, 39, 40, 39, 40, 28$, and 29. (B) In the NM, root growth of *ahr1* and wt 4 dpg seedlings treated with 1 μM IAA was not inhibited compared to those untreated seedlings. *ahr1* mutant and wild type root growth was equally inhibited by 2 μM IAA. In the WPGS, the growth of *ahr1* mutant roots was only inhibited at 2 μM IAA compared to those of the wild type. From left to right $n = 28, 28, 18, 16, 27, 28, 16, 18, 12, 11, 21$, and 23. (C). The root growth of both 6 dpg *ahr1* mutant and wild type seedlings in the NM showed inhibition at 1 and 2 μM IAA compared to those untreated. In the WPGS, the growth of 6dpg *ahr1* roots was significantly inhibited at 1 μM IAA compared to those of wild type. IAA treatment similarly inhibited root growth of *ahr1* in the NM and the WPGS. In wild type roots, the root elongation was similarly diminished with 2 μM IAA in the NM and in those untreated in the WPGS. Data are mean of three independent experiments. Data were analyzed by two-way ANOVA and Tukeyís multiple comparison test. Different letters denote statistically significant difference ($P < 0.05$). Error bars denote SD.

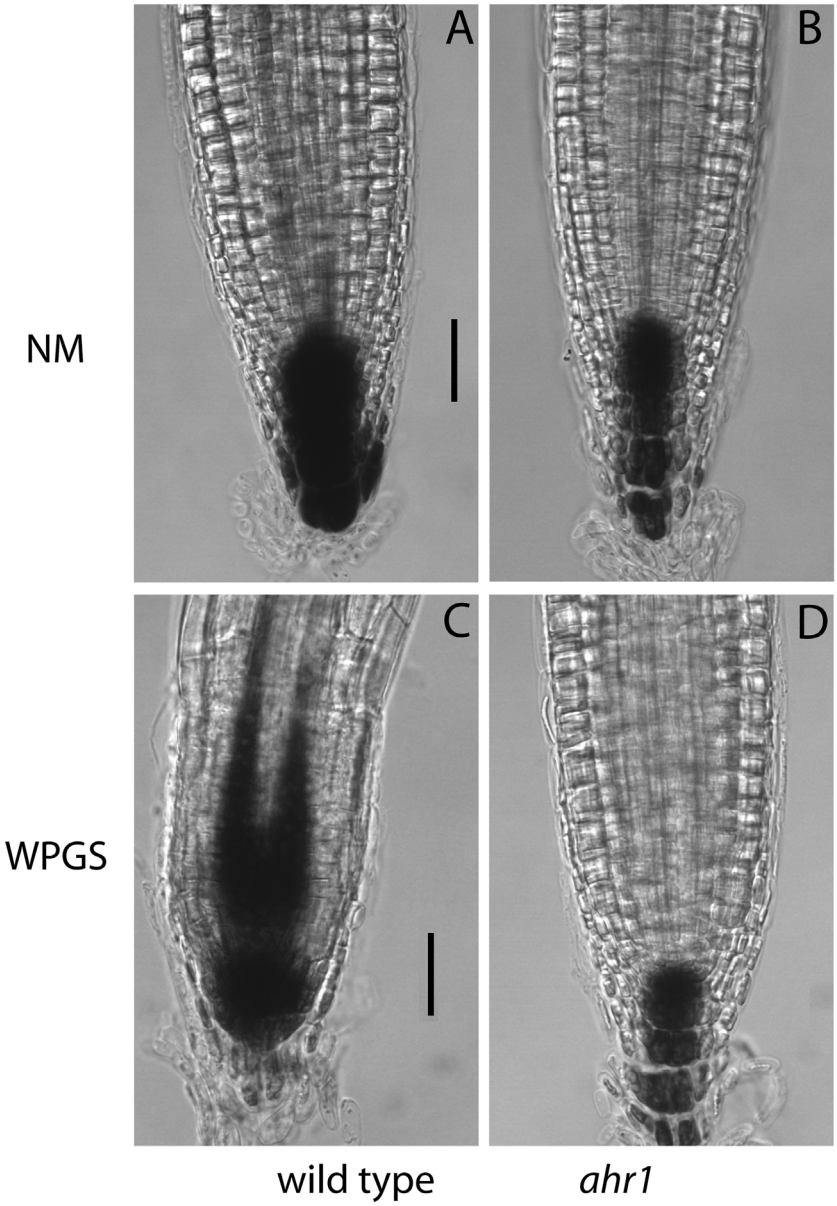

Fig. 6. Expression of *DR5:GUS* auxin reporter in 5 dpg root seedlings of the *ahr1* mutant is diminished compared to the wild type growing in normal (A, B) and the WPGS (C, D) media. In wild type root seedlings grown in the WPGS (C) the expression of the *DR5:GUS* auxin reporter was shifted (toward the shoot) and reduced in the root cap compared to those growing in the NM (A). Roots of *ahr1* seedlings growing in the WPGS (D) showed a reduction of the *DR5:GUS* auxin reporter staining compared to those growing in the NM. (B). All panels are under the same magnification; scale bar = 40 μm. The images are representative of two independent experiments ($n = 24$).

growth of *ahr1* seedlings grown in the WPGS is partially dependent on auxin transcription down regulation.

3.5. *Proline content in* ahr1 *does not change in low water potential system*

Considering that proline accumulation is required for Arabidopsis maintenance of growth at low water potential (Sharma et al., 2011), proline amounts of *ahr1* and wild type seedlings were evaluated, in the NM → WSM. The proline content was measured in roots of wild type and *ahr1* 5 dpg seedlings (Fig. 7A). Results showed 3.5-fold increase of proline in the wild type roots in the NM → WSM compared to NM (82.8 ± 2.8 and 25.2 ± 0.77 mg g^{-1} FW). In contrast, proline amounts in *ahr1* roots had only a small increase in the NM → WSM system (43.01 ± 0.85 and NM 35.1 ± 1.3 mg g^{-1} FW, respectively) (Fig. 7A).

To support these observations the relative expression of genes related to proline metabolism was analyzed by real time RT-PCR in roots of 5 dpg seedlings, grown in the NM and the NM → WSM (Fig. 7C–D). The relative expression of the biosynthetic gene *P5CS1* was 11-fold higher in wild type roots in response to the low water potential gradient when compared with *ahr1* mutant roots (Fig. 7C). No significant changes in the expression of the catabolic *PRODH1*gene were observed in roots of wild type or *ahr1* in response to the NM → WSM (Fig. 7D).

To examine if the changes in proline correlated with alterations in water content, the fresh weight was measured in wild type and in *ahr1* seedlings, grown in the NM and in the NM → WSM as an indirect method (Fig. 7B). An increment of 1.4-fold in fresh weight in wild type seedlings in the NM → WSM compared with the NM (99.2 ± 2.9 and 70.6 ± 3.7 mg, respectively) was observed. The fresh weight of *ahr1* showed no significant change in both the

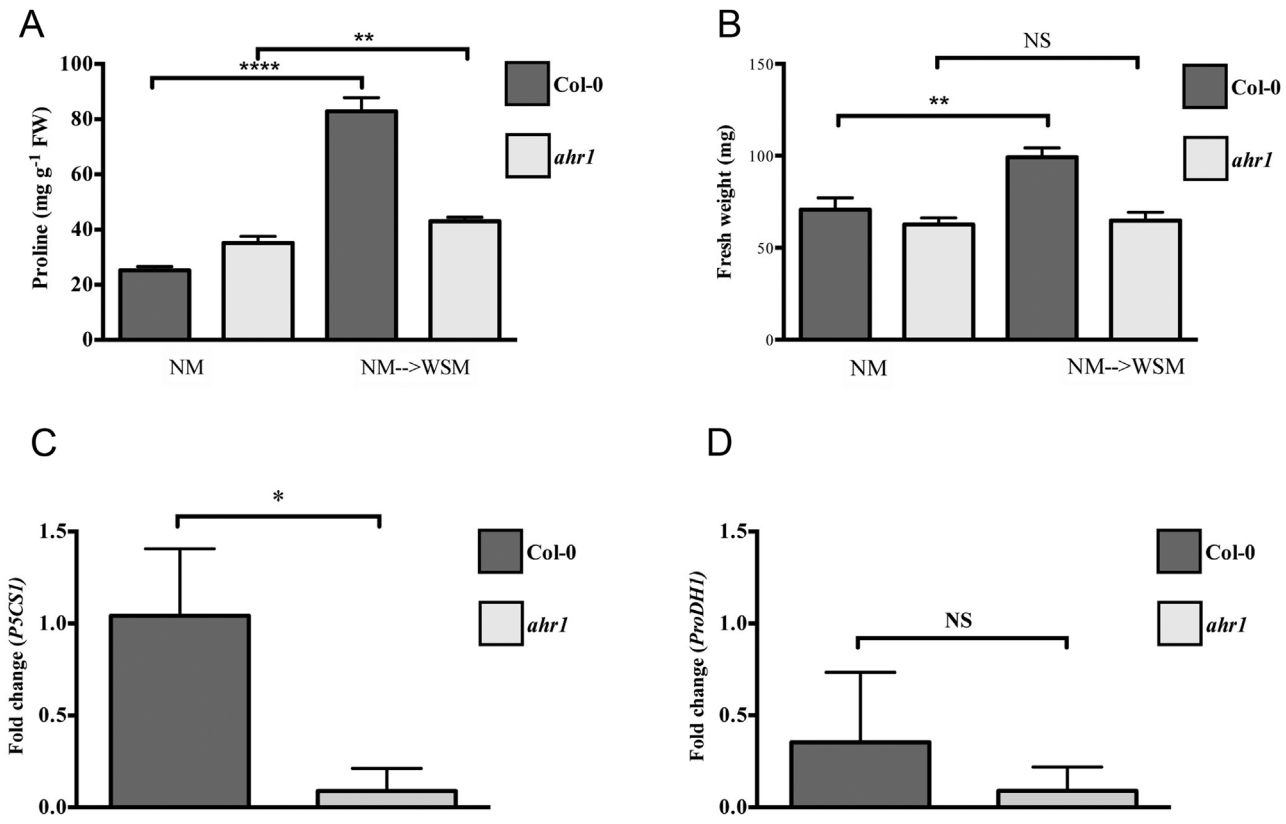

Fig. 7. No changes in water content and proline accumulation in *ahr1* root seedlings were observed. (A) Quantification of fresh weight in wild type and *ahr1* seedlings. Seeds were germinated in the NM or the NM → WSM systems and fresh weight was recorded in 5 dpg seedlings. Data shown are means of 3 independent experiments ± SD, including 50 seedlings in each experiment. (B) Free proline content in wild type and *ahr1* roots in response to grow in the NM and in the NM → WSM system. (C and D) Expression patterns of genes related to proline biosynthesis (*P5CS1*) and degradation (*PRODH1*). Total mRNA of 5 dpg roots of wild type (dark gray bars) and *ahr1* seedlings (light gray bars) grown in the NM or in the NM → WSM were used to analyzed (C) *P5CS1* and (D) *PRODH1* gene expression by real time RT-PCR. Fold-change values are the mean ± SD from three biological replicates each with three technical replicates. Asterisks indicate significant differences ($P < 0.05$) according to Student's *t*-test.

NM and the NM → WSM (62 ± 2.0 and 64.8 ± 2.5 mg, respectively) (Fig. 7B).

In conclusion, wild type seedlings increase *P5CS1* gene expression, and proline content when grown at low water potential as was previously described (Sharma and Verslues, 2010). Instead *ahr1* showed neither increase in *P5CS1* gene expression, or in proline content in this condition. As stated before, *ahr1* mutant seems to be less sensible to low water potential gradients, and as consequence, proline accumulation is not necessary to maintain water content or to sustain growth.

4. Discussion

One of the main questions of hydrotropism research is to comprehend how the root tip perceives the information on availability of water in its environment, how root adapts to situations where water is limited, and how it displays a differential growth response. Our study of the *ahr1* mutant intends to shed light on some of the aspects of root hydrotropic and growth responses in plants grown under low water potential gradient conditions.

The *ahr1* mutant has a higher tolerance to steeper water potential since its roots in vertically oriented Petri dishes grow downward in both the WSM → NM and the NM → WSM systems. However, this mutant is incapable of growing in a water stress medium containing 2% glycerol (WSM), indicating that *ahr1* is still able to sense the water stress. In contrast, wild type roots are unable to grow in both systems (Eapen et al., 2003; Saucedo et al., 2012). One purpose of this work was to analyze the root meristematic activity of the mutant and wild type plants grown in a system that permitted to maintain growth. The WPGS designed in this work fulfilled these requirements.

Considering that auxin can inhibit or stimulate cell elongation in a dose and organ dependent manner (Thimann, 1938) and that recent evidence demonstrates also a significant intermediary role for auxin in environmental adaptation in plants (Kazan, 2013), the role of auxin in vigorous root growth was also analyzed in *ahr1* seedlings grown in the WPGS. Taking into account that proline accumulation is one of the metabolic responses during water stress, we evaluated this metabolite in both mutant and wild type roots, in the lower water potential medium (NM → WSM).

4.1. *The* ahr1 *mutant maintains cell division in the root apical meristem in the WPGS*

In this work, we studied the RAM behavior in wild type during root growth accompanied with progressive and controlled decrease of water potential in the substrate. Our studies demonstrate that the capacity of wild type Arabidopsis to grow towards low water potentials is rather limited in the WPGS medium. In contrast, roots of the *ahr1* mutant growing in this medium display a totally opposite behavior: the root growth rate was about 9 times greater (Fig. 1F), the length of RAM was 55% longer (Fig. 3A), the number of cells in RAM was about 3 times greater (Fig. 3B), the root elongation zone length was 6.5 longer (Fig. 3C), the fully elongated cell length in roots was 22% longer (Fig. 3E), and the cell production was 7 times higher (Fig. 4B) compared to wild type. We can conclude

that the cell proliferation-related processes have a greater impact than the cell elongation in *ahr1* roots to maintain growth at the low water potential-gradient conditions studied here. The only parameter analyzed in this work that was not importantly affected in the wild type compared to the *ahr1* mutant grown in the WPGS was the length of completely elongated cells (Fig. 3E). Also, the RAM in wild type grown in the WPGS steadily diminished its size but was able to maintain slow growth for at least 10 days (data not shown). Therefore, indeterminate root growth was maintained even under these unfavorable conditions. These observations agree with previous studies by Vartanian et al. (1994) on drought rhizogenesis in Brassicaceae. These authors observed that during progressive drought stress, Arabidopsis roots preserved meristematic cells and a new functional root system was formed upon rehydration (Vartanian et al., 1994).

From our data, it is clear that the wild type Arabidopsis reduced their root growth in the WPGS was mainly caused by inhibition of cell proliferation and a decrease in cell production. This conclusion was confirmed by a much lower incidence of *Cyc B1;1:GUS* (Fig. 4A) found in this and previous studies (Ji et al., 2014) when plants grew under water deficit conditions. It was considered that water deficit induces premature differentiation of the RAM and that its cells become differentiated (Ji et al., 2014). Therefore, these roots maintain root indeterminacy and when transferred to optimal growth condition, the normal RAM can be reestablished (Vartanian et al., 1994; Xiong and Sheen, 2013). Importantly, the mutant *ahr1* studied here, showed no decrease in the RAM length or increased cell cycle duration when grown in the WPGS. Based on these results we suggest that the *AHR1* gene is involved in modulation of the RAM activity when seedlings perceive a low water potential gradient.

Previous studies have shown that some cultivars of maize growing in drying soil are able to continue root elongation at low water potential that are low enough to inhibit shoot growth completely (Sharp et al., 1988). It was also observed that at greater water deficit, a progressive decrease of the elongation zone takes place (Sharp et al., 1988) and the elongation of the root is maintained preferentially, in the distal (toward the apex) portion of the elongation zone (Sharp et al., 1988, 2004; Sharp and LeNoble, 2002). A decrease in the elongation zone length was also found in rice (Lynch, 2013) and in our study of Arabidopsis wild type roots (Fig. 3C). In Arabidopsis under mild water stress, cell elongation and even cell production can be stimulated (van der Weele et al., 2000). Under conditions studied here (WPGS) no such stimulation was observed. It has been proposed that root growth rate at a certain water stress is determined mainly by the supply of cells to the elongation zone (van der Weele et al., 2000). It is important to note that in our study, water deficit was progressively increasing while root continued its growth, contrary to many studies on the effect of water deficit when a certain water potential gradient is maintained. Therefore, the exerted water stress in the WPGS may have a more severe effect on root growth and may explain why we observed deceleration of wild type root growth.

4.2. Response of ahr1 mutant roots to moisture gradients

The root hydrotrotropic curvature of *ahr1* mutant seedlings in the hydrotropic assay using a moisture gradient (Kobayashi et al., 2007) was significantly reduced compared to those of the wild type indicating that *ahr1* roots showed an altered hydrotropic response (Fig. 2). This result reiterated that *ahr1* mutants displayed altered hydrotropic response. The altered hydrotropic response of *ahr1* roots might be related to their low perception of water stress and scarce proline accumulation under lower water potential conditions.

4.3. Root growth in ahr1 is less sensitive to auxin compared to wild type

Our data showed that in the NM, 2 μM IAA was sufficient to inhibit root growth response in *ahr1* mutant and wild type 5 dpg seedlings. In the WPGS, roots of wild type seedlings were more sensitive to IAA and their growth inhibition started at 1 μM. Instead *ahr1* was less sensitive to the added auxin since root growth inhibition response started at 2 μM. In older wild type and *ahr1* seedlings grown in the NM (6 dpg), the auxin inhibition of root growth was slightly lower than at 4 dpg. In the WPGS *ahr1* was able to sustain growth at both auxin concentrations, compared to wild type roots (Fig. 5C). These results imply that *ahr1* root growth is less sensitive to auxin than wild type when seedlings grow under a low water potential gradient.

Moreover, the vigorous root growth observed in the *ahr1* mutant grown in the WPGS might be regulated by auxin response at a transcriptional level since there was a pronounced lower auxin response (*DR5* activity) in seedlings grown in the WPGS (Fig. 6D). Lower auxin transcriptional response might be less inhibitory for root growth and this may explain why the cellular parameters studied in *ahr1* was maintained in the WPGS at the level similar to wild type in the NM. Pharmacological studies of the rate of the root hydrotropic reaction in response to inhibitors of auxin transport and anti-auxins showed that auxin response but not transport was involved in hydrotropic root growth (Kaneyasu et al., 2007). Our analysis of *DR5* activity in wild type and *ahr1* suggests that the modulation of auxin response at transcriptional level might be related to perception of the hydrotropic signal. The role of polar auxin transport during the hydrotropic response has not been totally understood (Cassab et al., 2013; Moriwaki et al., 2013; Shkolnik et al., 2016). Nonetheless, a recent report states that auxin is a negative regulator of hydrotropism since by blocking TIR1-dependent auxin signaling the hydrotropic root curvature is obstructed (Shkolnik et al., 2016). Our study also underlines the importance of auxin in the root capability to restrain its growth in low water potential gradient conditions.

To our knowledge *ahr1* is the first mutant in vascular plants whose root cells continuously divide and elongate in the presence of a low water potential gradient. Analysis of cellular bases of the root growth in *ahr1* suggests a role of *AHR1* gen in cell-proliferation processes. Also *AHR1* gen may be involved in the regulation of the redox state as may suggest the absence of proline accumulation in seedlings grown in the NM → WSM.

4.4. ahr1 neither induce P5CS1 nor increase proline accumulation when grown in a low water potential system

A proline increment in response to abiotic stress has been observed in Arabidopsis seedlings (Sharma and Verslues, 2010). Levels of biosynthetic P5CS1 and catabolic PRODH1 proteins regulate the proline accumulation being partially transcriptional regulated by *P5CS1* and *PRODH1* gene expression (Sharma and Verslues, 2010). In this work we observed an increment of proline content in roots of wild type seedlings grown in the NM → WSM that correlates with up-regulation of *P5CS1* gene (Fig. 7A, C). It has been previously suggested that the main source of proline biosynthesis at low water potentials is the photosynthetic tissue and that this synthesis generates NADP to maintain a higher NADP/NADPH ratio. Then proline metabolism might buffer the redox status under stress. Proline can be transported to the growing regions of the root and catabolyzed in the mitochondria by PRODH1 to support growth (Sharma et al., 2011). Based on the up-regulation of *P5CS1* expression patterns and the accumulation of proline content in roots of wild type seedlings observed in this work, we suggest that Arabidopsis roots induced proline synthesis when growing in the

NM → WSM system. Proline content in roots of wild type seedlings could be the result of two different processes: proline transport from the shoot and/or its synthesis in the root.

A model has been proposed for proline growth inhibition in Arabidopsis. In this model proline accumulation induces an increase in cytosolic Ca^{2+}, NADPH oxidase activity and reactive oxygen species (ROS) that leads to Salicylic Acid (SA) accumulation (Chen et al., 2011). Expression of *NADPHC oxidase* gene, involved in superoxide ion ($O_2^{\bullet-}$) synthesis was up regulated in wild type roots grown in the NM → WSM. Moreover $O_2^{\bullet-}$ was slightly accumulated in wild type roots grown in the NM → WSM as reveled by nitroblue tetrazolium detection (Supplementary Fig. S2 in the online version at DOI: 10.1016/j.jplph.2016.11.003). The *ahr1* mutant did not accumulate proline and $O_2^{\bullet-}$ and expression of *NADPHC oxidase* gene was not up regulated in roots of seedlings grown in the NM → WSM, indicating that proline accumulation is negatively regulated in *ahr1* mutant and, in consequence, its roots maintain normal growth. Therefore, the gene responsible for the *ahr1* phenotype might be regulating directly or indirectly proline accumulation in wild type plants and root growth inhibition under water deficit conditions. Then it might be possible that proline regulates the inhibition of root growth in wild type plants grown in the NM → WSM by a similar mechanism to that proposed for SA accumulation in response to pathogens. Proline and ROS accumulation, as well as NADPH oxidase activation (Chen et al., 2011) may be a common response to several stresses. As no relationship between SA and hydrotropic response has been described, a common activation pathway may not be ruled out.

5. Conclusion

The aims of this work were to ascertain how *ahr1* roots could sustain growth in the WPGS, with a special focus on the integration of cellular processes involved in the signaling that determines root growth during abiotic stress and their relation to hydrotropism. The *ahr1* phenotype shows unique features since the mutant root cells continue to proliferate and grow in the presence of a progressively negative water potential gradient at a level comparable to wild type growing in the NM. As such, it represents an exceptional resource for understanding hydrotropism. To our knowledge *ahr1* is the first mutant in vascular plants whose root cells continuously divide and elongate in the presence of a low water potential gradient.

The altered hydrotropic response phenotype of *ahr1* roots was confirmed in the hydrotropic moisture gradient indicating that their hydrotropic curvature was decreased compared to the wild type. This seems to indicate that the tolerance of *ahr1* roots to grow in the presence of water potential gradients correlated with a weak hydrotropic response. When wild type, *miz1* and *MIZ1OE* plants were grown in the hydrostimulated chambers and subjected to drought stress for 12 d, the survival rate of *miz1-1* mutant plants significantly diminished, compared with wild-type plants (Iawata et al., 2013). In contrast, *MIZ1OE* plants showed an enhanced tolerance to drought stress and had a significantly greater survival rate than wild type plants (Iawata et al., 2013). Since *miz1* mutant roots were not resistant to water limited conditions indicated that a lack of hydrotropism correlates with a lack of drought tolerance. So, we will expect a moderate to low tolerance to drought of *ahr1* roots. Contrary to *miz1* roots that lack a hydrotropic curvature, *ahr1* grows in water potential gradients and shows a lower hydrotropic response This might suggests that *ahr1* mutant roots perceive differences in substrate water potential or relative humidity but cannot develop a typical hydrotropic curvature.

In addition, the fact that the *ahr1* roots' response to auxin at a transcriptional level is lower compared to those of wild type in the WPGS, opens new possibilities for deciphering the role of auxin in the integration of the signaling that controls abiotic stress responses and its relation to hydrotropism. Proline was not accumulated in roots of *ahr1* seedlings grown under lower water potential contrary to wild type indicating that the altered hydrotropic response of *ahr1* roots might be related to their low perception of water stress. Currently, drought stress represents the most important confrontation for global food security given the impact of climate change and extreme current and pending weather events. Hence, the capacity of *ahr1* roots to grow in medium with low water potential gradient might confer drought tolerance by gaining access to deeper water resources since higher growth rate is achieved by exposing their roots to water deficit. Finally, the capacity of sensing low water potential gradient and responding to it by increasing growth rate of *ahr1* roots represent an important resource for improvement of drought tolerance in major crops.

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References

Antoni, R., Gonzalez-Guzman, M., Rodriguez, L., Peirats-Llobet, M., Pizzio, G.A., Fernandez, M.A., et al., 2013. PYRABACTIN RESISTANCE1-LIKE8 plays an important role for the regulation of abscisic acid signaling in root. Plant Physiol. 161, 931–941.

Bao, Y., Aggarwal, P., Robbins II, N.E., Sturrock, C.J., Thompson, M.C., Tan, H.Q., et al., 2014. Plant roots use a patterning mechanism to position lateral root branches toward available water. Proc. Natl. Acad. Sci. U. S. A. 111, 9319–9324.

Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39, 205–207.

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., et al., 2005. The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. Nature 433, 39–44.

Cassab, G.I., Eapen, D., Campos, M.E., 2013. Root hydrotropism: an update. Am. J. Bot. 100, 14–24.

Castillo-Olamandi, L., Bravo-García, A., Morán, J., Rocha-Sosa, M., Porta, H., 2007. AtMCP1b, a chloroplast-localised metacaspase, is induced in vascular tissue after wounding or pathogen infection. Funct. Plant Biol. 34, 1061–1071.

Chen, J., Zhang, Y., Wang, C., Lu, W., Jin, J.B., Hua, X., 2011. Proline induces calcium-mediated oxidative burst and salicylic acid signaling. Amino Acids 40, 1473–1484.

Colon-Carmona, A., You, R., Haimovitch-Gal, T., Doerner, P., 1999. Technical advance: spatio-temporal analysis of mitotic activity with a labile cyclin-GUS fusion protein. Plant J. 20, 503–508.

Delauney, A.J., Verma, D.P.S., 1993. Proline biosynthesis and osmoregulation in plants. Plant J. 5, 215–223.

Dubrovsky, J.G., Soukup, A., Napsucialy-Mendivil, S., Jeknic, Z., Ivanchenko, M.G., 2009. The lateral root initiation index: an integrative measure of primordium formation. Ann. Bot. 103, 807–817.

Dubrovsky, J.G., Napsucialy-Mendivil, S., Duclercq, J., Cheng, Y., Shishkova, S., Ivanchenko, M.G., et al., 2011. Auxin minimum defines a developmental window for lateral root initiation. New Phytol. 191, 970–983.

Eapen, D., Barroso, M.L., Campos, M.E., Ponce, G., Corkidi, G., Dubrovsky, J.G., et al., 2003. A no hydrotropic response root mutant that responds positively to gravitropism in Arabidopsis. Plant Physiol. 131, 536–546.

Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., et al., 2003. The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell 112, 219–230.

Geldner, N., Richter, S., Vieten, A., Marquardt, S., Torres-Ruiz, R.A., Mayer, U., et al., 2004. Partial loss-of-function alleles reveal a role for GNOM in auxin transport-related, post-embryonic development of Arabidopsis. Development 131, 389–400.
Hare, P.D., Cress, W.A., van Staden, J., 1999. Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. J. Exp. Bot. 50, 413–434.
Hong, Z., Lakkineni, K., Zhang, Z., Verma, D.P., 2000. Removal of feedback inhibition of delta(1)-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol. 122, 1129–1136.
Iawata, S., Miyazawa, Y., Fujii, N., Takahashi, H., 2013. MIZ1-regulated hydrotropism functions in the growth and survival of *Arabidopsis thalia*na under natural conditions. Ann. Bot. 102, 103–114.
Ivanchenko, M.G., Napsucialy-Mendivil, S., Dubrovsky, J.G., 2010. Auxin-induced inhibition of lateral root initiation contributes to root system shaping in *Arabidopsis thaliana*. Plant J. 64, 740–752.
Ivanov, V.B., Dubrovsky, J.G., 1997. Estimation of the cell-cycle duration in the root meristem: a model of linkage between cell-cycle duration, rate of cell production, and rate of root growth. Int. J. Plant Sci. 158, 757–763.
Ivanov, V.B., Dubrovsky, J.G., 2013. Longitudinal zonation pattern in plant roots: conflicts and solutions. Trends Plant Sci. 18, 237–243.
Ji, H., Liu, L., Li, K., Xie, Q., Wang, Z., Zhao, X., et al., 2014. PEG-mediated osmotic stress induces premature differentiation of the root apical meristem and outgrowth of lateral roots in wheat. J. Exp. Bot. 65, 4863–4872.
Kaneyasu, T., Kobayashi, A., Nakayama, M., Fujii, N., Takahashi, H., Miyazawa, Y., 2007. Auxin response, but not its polar transport, plays a role in hydrotropism of Arabidopsis roots. J. Exp. Bot. 58, 1143–1150.
Kazan, K., 2013. Auxin and the integration of environmental signals into plant root development. Ann. Bot. 112, 1655–1665.
Kobayashi, A., Takahashi, A., Kakimoto, Y., Miyazawa, Y., Fujii, N., Higashitani, A., et al., 2007. A gene essential for hydrotropism in roots. Proc. Natl. Acad. Sci. U. S. A. 104, 4724–4729.
López-Bucio, J.S., Dubrovsky, J.G., Raya-Gonzalez, J., Ugartechea-Chirino, Y., López-Bucio, J., de Luna-Valdez, L.A., et al., 2014. *Arabidopsis thaliana* mitogen-activated protein kinase 6 is involved in seed formation and modulation of primary and lateral root development. J. Exp. Bot. 65, 169–183.
Leyser, O., 2002. Molecular genetics of auxin signaling. Annu. Rev. Plant. Biol. 53, 377–398.
Leyser, O., 2005. Auxin distribution and plant pattern formation: how many angels can dance on the point of PIN? Cell 121, 819–822.
Lynch, J.P., 2013. Steep, cheap and deep: an ideotype to optimize water and N acquisition by maize root systems. Ann. Bot. 112, 347–357.
Miyazawa, Y., Takahashi, A., Kobayashi, A., Kaneyasu, T., Fujii, N., Takahashi, H., 2009. GNOM-mediated vesicular trafficking plays an essential role in hydrotropism of Arabidopsis roots. Plant Physiol. 149, 835–840.
Morita, M.T., 2010. Directional gravity sensing in gravitropism. Annu. Rev. Plant Biol. 61, 705–720.
Moriwaki, T., Miyazawa, Y., Kobayashi, A., Takahashi, H., 2013. Molecular mechanisms of hydrotropism in seedling roots of *Arabidopsis thaliana* (Brassicaceae). Am. J. Bot. 100, 25–34.
Nanjo, T., Fujita, M., Seki, M., Kato, T., Tabata, S., Shinozaki, K., 2003. Toxicity of free proline revealed in an Arabidopsis T-DNA-tagged mutant deficient in proline dehydrogenase. Plant Cell Physiol. 44, 541–548.
Ober, E.S., Sharp, R.E., 1994. Proline accumulation in maize (*Zea mays* L.) primary roots at low water potentials (I. Requirement for increased levels of abscisic acid). Plant Physiol. 105, 981–987.
Ponce, G., Rasgado, F.A., Cassab, G.I., 2008. Roles of amyloplasts and water deficit in root tropisms. Plant Cell Environ. 31, 205–217.
Roosens, N.H., Thu, T.T., Iskandar, H.M., Jacobs, M., 1998. Isolation of the ornithine-delta-aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana*. Plant Physiol. 117, 263–271.
Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., et al., 1999. An auxin-dependent distal organizer of pattern and polarity in the Arabidopsis root. Cell 99, 463–472.
Saucedo, M., Ponce, G., Campos, M.E., Eapen, D., García, E., Luján, R., et al., 2012. An altered hydrotropic response (*ahr1*) mutant of Arabidopsis recovers root hydrotropism with cytokinin. J. Exp. Bot. 63, 3587–3601.
Sharma, S., Verslues, P.E., 2010. Mechanisms independent of abscisic acid (ABA) or proline feedback have a predominant role in transcriptional regulation of proline metabolism during low water potential and stress recovery. Plant Cell Environ. 33, 1838–1851.
Sharma, S., Villamor, J.G., Verslues, P.E., 2011. Essential role of tissue-specific proline synthesis and catabolism in growth and redox balance at low water potential. Plant Physiol. 157, 292–304.
Sharp, R.E., LeNoble, M.E., 2002. ABA, ethylene and the control of shoot and root growth under water stress. J. Exp. Bot. 53, 33–37.
Sharp, R.E., Silk, W.K., Hsiao, T.C., 1988. Growth of the maize primary root at low water potentials: I. Spatial distribution of expansive growth. Plant Physiol. 87, 50–57.
Sharp, R.E., Poroyko, V., Hejlek, L.G., Spollen, W.G., Springer, G.K., Bohnert, H.J., et al., 2004. Root growth maintenance during water deficits: physiology to functional genomics. J. Exp. Bot. 55, 2343–2351.
Shevell, D.E., Leu, W.M., Gillmor, C.S., Xia, G., Feldmann, K.A., Chua, N.H., 1994. EMB30 is essential for normal cell division, cell expansion, and cell adhesion in Arabidopsis and encodes a protein that has similarity to Sec7. Cell 77, 1051–1062.
Shkolnik, D., Krieger, G., Nuriel, R., Fromm, H., 2016. Hydrotropism: root bending does not require auxin redistribution. Mol. Plant. 9, 757–759.
Szekely, G., Abraham, E., Cseplo, A., Rigo, G., Zsigmond, L., Csiszar, J., et al., 2008. Duplicated P5CS genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis. Plant J. 53, 11–28.
Thimann, K.V., 1938. Hormones and the analysis of growth. Plant Physiol. 13, 437–449.
Ulmasov, T., Murfett, J., Hagen, G., Guilfoyle, T.J., 1997. Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9, 1963–1971.
van der Weele, C.M., Spollen, W.G., Sharp, R.E., Baskin, T.I., 2000. Growth of *Arabidopsis thaliana* seedlings under water deficit studied by control of water potential in nutrient-agar media. J. Exp. Bot. 51, 1556–1562.
Vartanian, N., Marcotte, L., Giraudat, J., 1994. Drought rhizogenesis in Arabidopsis thaliana (differential responses of hormonal mutants). Plant Physiol. 104, 761–767.
Xiong, Y., Sheen, J., 2013. Moving beyond translation: glucose-TOR signaling in the transcriptional control of cell cycle. ABBV Cell Cycle 12, 1989–1990.