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ORIGINAL RESEARCH

Accumulation of High Levels of ABA Regulates the Pleiotropic Response of the *nhr1 Arabidopsis* Mutant

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Abstract Plants have evolved a variety of mechanisms for responding to environmental cues, which allows them to survive in the presence of limited resources or environmental stresses. One of the most significant growth adaptations plants have attained is tropism, a growth response that involves bending of plant organs toward or away from a stimulus. Roots exhibit hydrotropism in response to moisture gradients, which is thought to be critical in acquiring water and establishing their stand in the soil. However, the mechanism underlying hydrotropism remains unsolved. Here, we report that the no hydrotropic response (*nhr1*) mutant of *Arabidopsis*, which is impaired in hydrotropism, is tolerant to drought. The no hydrotropic response phenotype of *nhr1* was repressed by AbamineSG, an inhibitor of abscisic acid (ABA) biosynthesis, indicating that ABA negatively regulates hydrotropism. Furthermore, the content of ABA was higher in *nhr1* compared to those of wild type (wt). However, the higher ABA levels in *nhr1* plants were not due to higher transcript levels of 9-*cis*-epoxycarotenoid dioxygenase (*NCED3*), since these were diminished compared to those of wt. Our results indicated that the root hydrotropic response of the *nhr1* mutant is modulated by ABA and that the higher ABA levels of the mutant might confer it drought resistance.

Keywords ABA-hypersensitive mutant · AbamineSG · Abscisic acid (ABA) · *Arabidopsis thaliana* · Hydrotropism · Drought tolerance

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Introduction

The most recent predictions indicated that the percentage of the areas with extreme drought in the world would increase from 3% to 50% for the 2050. This drastic increase, an outcome of global warming, would wreak havoc on water availability, farm incomes, agricultural productivity, and food security around the globe (Battisti and Naylor 2009). Therefore, understanding the mechanisms of drought tolerance and breeding for drought-resistant crop plants has been the major goal of plant biologists. Up to now, drought tolerance is recalcitrant to molecular genetics study mostly due to our limited awareness of specific traits linked to drought tolerance. Besides, it is difficult to achieve in nature drought stress treatments in a quantitative and reproducible way. These difficulties have considerably hampered research on drought tolerance. Hence, the

biological basis for drought tolerance is basically unknown, and few drought tolerance determinants have been identified (Nelson et al. 2007; Bohnert et al. 1995). One way to minimize the negative impact of drought on yield is to manipulate root system architecture toward distribution of roots in the soil that optimizes water and nutrient uptake (de Dorlodot et al. 2007). Overall root system depth determines the efficiency of exploration for shallow resources such as phosphorous (Lynch and Brown 1997) and deep resources such as water (Ho et al. 2005, 2004). The differential growth of roots directed by a moisture gradient is called hydrotropism, a long-recognized, but not well-understood, plant behavior (Cassab 2008; Darwin 1881). Perception of moisture gradients, as other stimuli (gravity, obstacles, light, nutrients, etc.) begins in the root cap, which assesses toward what stimuli will direct root growth according to the necessities of the plant. Hydrotropism in the model plant *Arabidopsis* is easily observed despite gravity, and thus, it has been feasible to isolate mutants affected in their hydrotropic responses (Kobayashi et al. 2007; Miyazawa et al. 2009; Eapen et al. 2003). Analysis of the no hydrotropic response (*nhr1*) and *mizu-kussei1* and *2* (*miz1, miz2*) mutants has revealed new insights of the mechanism of hydrotropism. Both *nhr1* and *miz1* are unable to develop a hydrotropic response and are affected in another tropic response (gravitropism and phototropism). This indicates that these genes are not exclusive components of hydrotropism and supports the notion that the assessment mechanism of the root cap integrates not only different stimuli but also different genes in order to produce a final tropic response. *MIZ1* encodes a protein containing an uncharacterized domain, the MIZ domain, which is conserved among land plants but is absent in green algae, cyanobacteria, or animals (Kobayashi et al. 2007). Additionally, MIZ1 is expressed in the root cap, mature region of roots and hydathodes. Nonetheless, there is little understanding of the function of *MIZ1* in hydrotropism. The third mutant, *miz2*, is affected in *gnom*, a guanine nucleotide exchange factor for ADP-ribosylation factor-type G proteins, that lacks hydrotropic but not gravitropic response, implying distinct roles of vesicular trafficking between hydrotropism and gravitropism in roots (Miyazawa et al. 2009). Hydrostimulated *nhr1* mutant seedlings maintained starch in amyloplasts of root cap columella cells in contrast to those of wt, which might help it in sustaining cell turgor and water stress (Ponce et al. 2008). Hence, others and we hypothesize that hydrotropism might contribute to drought avoidance in plants (Kobayashi et al. 2007). In addition, it has been reported that ABA is a critical regulator of the mechanism that integrate hydrotropism and gravitropism (Ponce et al. 2008). ABA, locally added to seeds or roots of *nhr1*, considerably improved the no hydrotropic response of the mutant by increasing the root downward growth toward the zone with less water availability (Eapen et al. 2003). Interestingly, roots of wt seeds treated locally with ABA in the test system for hydrotropism instead of responding to hydrotropism; they were strongly gravitropic and showed downward growth into the medium with less accessible water (Ponce et al. 2008). Therefore, ABA is a critical regulator of hydrotropism and gravitropism. This regulation is valuable when plants contend short or prolonged periods of unfavorable growth conditions since induced root downward growth might be important for trailing humidity gradients in soil. Thus, studies that identify molecular regulators of hydrotropism will help to improve the performance of crops grown under periodic drought conditions. ABA is also a significant player in the response of plants to drought. In particular, drought can result in the closure of stomata and increased biosynthesis of ABA (Shinozaki et al. 2003; Finkelstein et al. 2002). Several drought-inducible genes are induced by exogenous ABA treatment, whereas others are not affected. There is evidence that demonstrated the presence of both ABA-independent and ABA-dependent regulatory systems governing drought-inducible gene expression (Shinozaki and Yamaguchi-Shinozaki 2007; Yamaguchi-Shinozaki and Shinozaki 2005). However, although increased expression of these genes has been shown to confer drought tolerance in a number of plant species (Zhang et al. 2004), other less-desirable developmental phenotypes, such as stunted growth, are often associated with their high constitutive expression (Dai et al. 2007; Kasuga et al. 1999). Thus far, traditional forward genetics approaches can be useful for isolating plant phenotypes specifically conferred by drought stress. However, few genetic screens have been reported for the isolation of drought tolerance determinants in roots (Xiong et al. 2006; Eapen et al. 2003; Takahashi et al. 2003). Because roots are the location where plants first encounter drought stress, it is possible that roots may be able to sense and adapt to this stress condition. Thus far, considerable progress has been made in understanding root growth under water stress (Sharp et al. 2004a). However, there has been no genetically defined drought-adaptive response in root development reported thus far. Inhibition of lateral root development is a typical adaptive response of root to drought stress (Xiong et al. 2006; Vartanian et al. 1994) since plants will increase the uptake of water by restricting the proliferation of lateral roots and allocating more resources to the growth of primary roots toward the subsoil. This drought response is partially mediated by ABA. Mutants with altered responses to drought or ABA in lateral root development have been isolated (Xiong et al. 2006). The *dig3* mutant is practically insensitive to the ABA inhibition of lateral root development and is also highly susceptible to drought stress. The *nhr1* mutant,

whose no hydrotropic response phenotype is enhanced by ABA, is a good candidate for examining the correlation of hydrotropism with drought tolerance. In this work, we analyzed the hydrotropic response of the *nhr1* mutant in the presence of an ABA biosynthetic inhibitor and the effect of ABA during its growth and development. We found that the lack of hydrotropic response of the *nhr1* mutant was re-established in the presence of AbamineSG, a specific inhibitor of ABA biosynthesis (Kitahata et al. 2006; Han et al. 2004). In addition, *nhr1* contained higher levels of ABA than the wt, confirming that ABA is a critical regulator of hydrotropism (Ponce et al. 2008). We also examined *nhr1* sensitivity to ABA during germination, seedling establishment, and survival after drought. Every phenotype of the *nhr1* mutant recorded during growth and development was delayed or decreased in presence of ABA. Hence, our results suggest that the mutant is hypersensitive to ABA. After presenting further data on *nhr1* drought insensitivity in soil, we will suggest that the hydrotropism signaling could interplay with drought stress signaling.

Materials and Methods

Plant Materials and Growth Conditions

Seeds of *Arabidopsis thaliana* ecotype Columbia-0 (wt, Col-0) and *nhr1* mutant (background Col-0) were used. Seeds were surface-sterilized for 5 min with 1.25% (*v*/*v*) NaOCl containing 0.1% (*v*/*v*) triton X-100 for 4 min then rinsed five times with sterile water and sown in 0.1% (*w*/*v*) sterile agar. All seeds were stratified at 4°C for 3 days and then planted on square or round Petri dishes containing 0.5× MS medium (Murashige and Skoog 1962) supplemented with 0.5% (*w*/*v*) sucrose and 1% (*w*/*v*) agar (pH 5.7), which is named normal medium (NM) in this work. The test medium (TM) for hydrotropism was made as Eapen et al. (2003) reported. Briefly, the TM was formed for two medium types; the area of Petri dish was divided in two equal parts: on the upper zone, the NM was poured, and on the lower zone was filled with water-stress medium (WSM=NM plus 2.5% (*v*/*v*) glycerol and 0.5% (*w*/*v*) alginic acid) in an amount of 50–50% (*v*/*v*). Plates were then transferred to a growth chamber (22°C with a 16:8 light/dark cycle).

Germination, Seedling Establishment, and Seedling Survival Assay

Germination was considered when radicules have emerged, and it was scored every 12 or 24 h for 4 to 5 days until no further germination was observed. For seedling establishment, seedlings with green and expanded cotyledons (180°) were scored every 12 or 24 h for 5 to 6 days. The seedlings were considered alive (survival) if they were etiolated for a period of 10 days after the ABA treatment.

ABA and AbamineSG Treatments

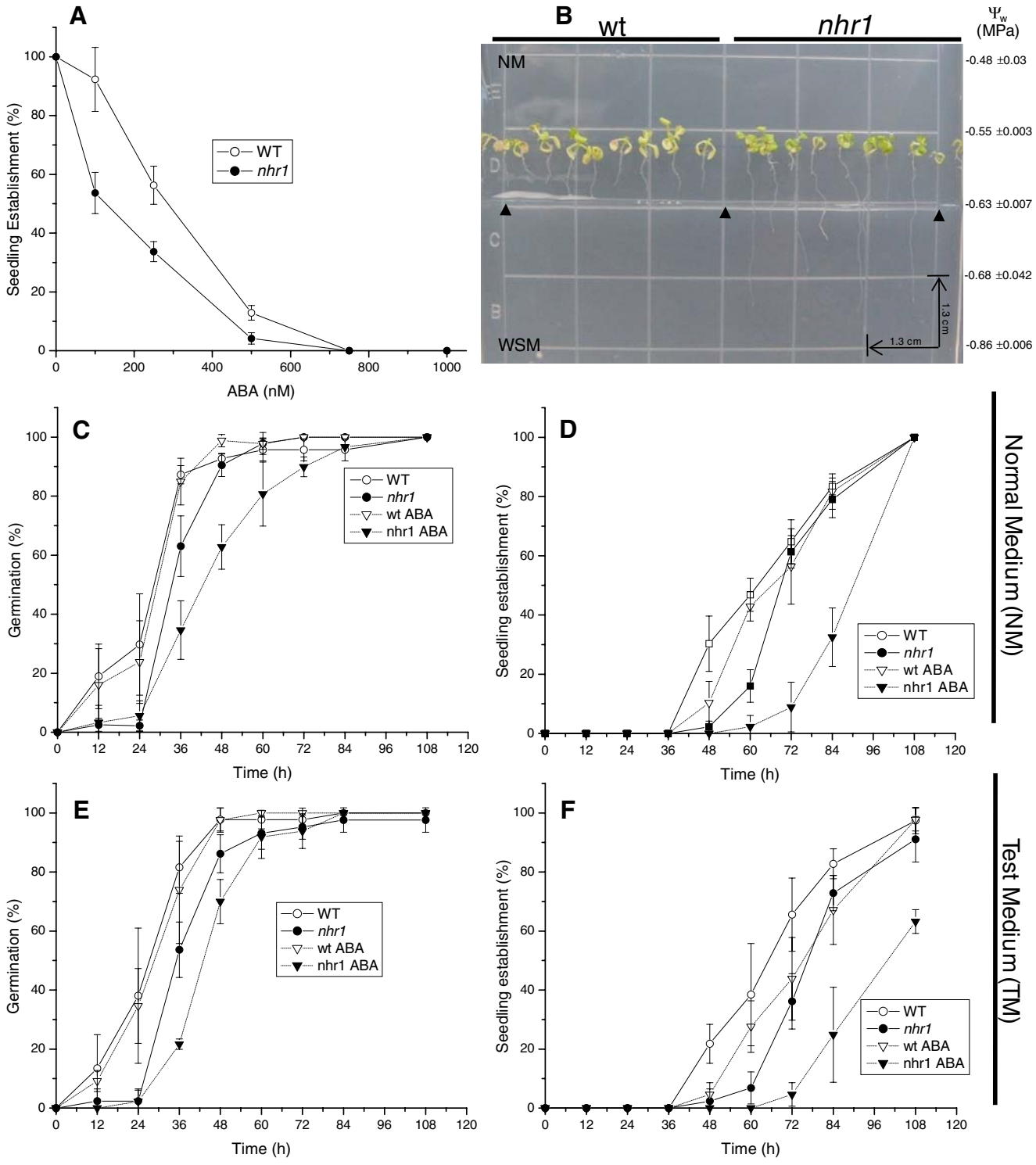
For the ABA assay, seeds were sown on NM as described above, and different concentrations of filter-sterilized ABA (Sigma-Aldrich, St. Louis, MO, USA) were added to the plate before the agar solidified. Control plates contained NM plus ABA solvent (final concentration of methanol/water/acetic acid at 0.18/0.018/0.002%, *v*/*v*/*v*). For the AbamineSG treatment, seeds were germinated in the TM for hydrotropism (Eapen et al. 2003) supplemented with 100 μM AbamineSG (Kitahata et al. 2006); control plates included AbamineSG solvent (ethanol). The hydrotropic response was evaluated after 2 weeks.

ABA Level Quantification

The ABA levels were quantified as reported by Kitahata et al. (2006). Rosette leaves collected after 39 days after germination and extracted with 5 ml of methanol–water–acetic acid (90:9:1, *v*/*v*/*v*). Internal standards of $^{13}C_2$-ABA were added at the beginning of the extraction. Following the extraction, 17.5 ml of water was added. The samples were clarified by centrifugation at 15,000 rpm for 10 min. Oasis HLB cartridges (Waters, Mississauga, Canada) were conditioned with methanol and equilibrated with methanol–water–acetic acid (9:90:1, *v*/*v*/*v*). Then, the

Fig. 1 Seed vigor and seedling establishment of the *nhr1* mutant are affected by ABA. **a** Effect of ABA (0, 100, 250, 500, 750, and 1,000 nM) in wt and *nhr1* seedling establishment in NM after 5 days of sowing. **b** TM for hydrotropism containing in the upper zone NM and in the lower zone a WSM with 2.5% (*w*/*v*) glycerol (Eapen et al. 2003). The lack of hydrotropic response (downward root growth maintenance) is observed only in *nhr1*, whereas those wt roots developed a curvature (hydrotropic response) and remained in the zone with higher water potential and consequently did not show any further downward growth. Photograph was recorded at 12 days after sowing. *Numbers on the right part* denote the values of water potential along the TM taken the same day that the picture was taken. *Arrowheads* delimited the border between NM and WSM. **c** Germination and **d** seedling establishment in NM. **e** Germination and **f** seedling establishment in TM. Evaluations were done every 12 h up to 108 h after sowing. Germination and seedling establishment were measured and plotted as a percentage of the total number of seeds germinated or established. *Open circles* correspond to wt, *closed circles* correspond to *nhr1* mutant without ABA (control), and *open triangles* and *closed triangles* correspond to wt and *nhr1* on medium supplemented with 100 nM ABA. The control medium contained the ABA solvent at the same concentration than those containing ABA. The plots and the photograph are representative experiments of five different assays (n=45 with three replicates, mean ± SD)

samples were loaded onto the cartridges and washed with methanol–water–acetic acid (9:90:1, *v*/*v*/*v*). ABA was eluted with 1 ml methanol–water–acetic acid (90:9:1, *v*/*v*/*v*) and collected in clean tubes; 5 µl of each sample was loaded onto an HPLC equipped with a c18 column (150×2 mm; Shiseido, Tokyo, Japan) using a flow rate of 0.2 ml min^{-1} and a binary solvent system comprising methanol and water with 0.1% formic acid. The compounds were analyzed by tandem mass spectrometry with MRN in negative ion mode.



Analysis of Roots and Bolting Day

The length of the primary roots and the length of visible lateral roots were measured using National Institute of Health (NIH) Image J software version 1.34 (rsb.info.nih.gov/ij/). The data were transferred to a Microsoft Excel spreadsheet, and the average length of primary and lateral roots plus SD were calculated and plotted (Origin 6.1 program). Bolting day was considered when the bolt reached 1 cm of length. Afterward, leaf number was measured.

Photography of Seedlings and Plants

Photographs of roots were taken with a Sony DSC-F717 (Tokyo, Japan) digital camera and images were processed using the Microsoft program editor.

Drought Stress and Transpirational Water Loss Assay

For drought stress, seedlings of 14 days after germination were transplanted into pots (9.5×9.5×8.5 cm) filled with metro-mix 200 (Sun Grow, Bellevue, WA, USA). During 3 weeks, plants were constantly watered then, pots were not watered up to total water loss and had a water potential of −0.75±0.047 MPa, whereas control plots (well watered) had a water potential of −0.36±0.049 MPa. Afterward, all pots were rewatered simultaneously for 1 week and plants were observed. Plants were considered dead if they did not recover after the rewatering period. The data of water potential in plant tissue and medium culture were obtained using PSYPRO, a water potential system (Wescor Inc., Logan, UT, USA). For transpirational water loss assay, leaves of 3-week-old mutant and wt plants growing in pots were detached and placed in a weighing boat, and changes in fresh weight over time were monitored using an electronic balance.

Semiquantitative RT-PCR

The total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) from rosette leaf (0.2 g) at 35 days after germination. The protocol followed was that recommended by the supplier. RNA integrity was checked by electrophoresis in agarose gels (1.2%, *w/v*) using TBE buffer and ethidium bromide staining. The RT-PCR was performed using one-step kit of end point (Invitrogen). The gen-specific primer pairs used were as follows: for NCED3 (AT3G14440; 5′–3′): ATG GCT TCT TTC ACG GCA AC and (5′–3′): TCT CGT GGC TGA CAA GGA AA; for 18S rRNA (AT2G01010 and AT3G41768; 5′–3′): TCA GAC TGT GAA ACT GCG AAT GG and (5′–3′): CCG TGT CAG GAT TGG GTA ATT TGC. The amplified fragments were visualized by electrophoresis in agarose gels (1.5%, *w/v*) in TBE buffer and ethidium bromide. All primers were synthesized by the Unidad de Síntesis y Secuenciación, Instituto de Biotecnología, Universidad Nacional Autónoma de México.

Results

The *nhr1* Mutation Delayed Seed Germination and Seedling Establishment

Since the no hydrotropic phenotype of the *nhr1* mutant was enhanced by ABA (Ponce et al. 2008), we decided to test processes that characteristically are ABA regulated, such as germination efficiency and postgermination growth of *nhr1* seedlings (Koornneef et al. 1984) in the presence or absence of ABA. ABA, at a concentration of 100 nM, induced 50% of inhibition of *nhr1* seedling establishment (Fig. 1a) and seed germination (data not shown), whereas wt was not affected at this concentration. In the absence of ABA, *nhr1* showed a significant delayed in both germination (emergence of radicle; Fig. 1c, e) and seedling establishment (expansion of green cotyledons; Fig. 1d, f) compared to wt in both NM and TM (Fig. 1c, d). For instance, the highest germination value was achieved 36 h earlier in untreated *nhr1* seeds compared to those treated with ABA (Fig. 1c). In addition, 80% of seedling establishment occurred approximately 12–18 h earlier in untreated *nhr1* mutants in contrast to those treated. In the TM for hydrotropism with a substrate water potential gradient (Fig. 1e–f), the no hydrotropic response of *nhr1* corresponded to roots that showed continuous growth, and the hydrotropic response of wt corresponded to roots that showed curvature and cessation of growth (Fig. 1b). However, in the TM plus ABA, the highest germination value of *nhr1* was observed 36 h after those without ABA. On the other hand, seedling establishment was extremely slow in *nhr1* mutants treated with ABA in the TM since about 70% of untreated plants were already established and only 25% of ABA treated seedlings showed their cotyledons expanded (Fig. 1f). These results indicate that *nhr1* mutant is sensitive to exogenous ABA during germination and seedling establishment. Therefore, we considered to *nhr1* as a putative ABA-hypersensitive mutant and decided to study ABA-related functions.

The *nhr1* Mutant Is Sensitive to ABA

It was recently reported that inhibition of lateral root development by drought is an adaptive response to this stress (Xiong et al. 2006) and that ABA plays an important role in lateral root formation in *Arabidopsis* (De Smet et al. 2006). Since *nhr1* showed inhibition during early stage development by ABA, it is possible that postgermination growth is

affected too. To further verify the effect on the ABA responses at the adult stage, we examined the number of visible lateral roots and their total length in both optimal and stressful conditions. Growth of the primary root of *nhr1* was undistinguishable from those of wt (Fig. 2a) under optimal conditions plus 50 μM ABA, since no statistical differences were observed between both genotypes; however, the mean value was slightly larger in *nhr1* than in wt. When total lateral root length and number of lateral roots (density) were compared between the mutant and the wt grown in optimal conditions, *nhr1* seedlings showed a significant reduction. However, 50 nM of ABA reduced drastically (14%) lateral root development in *nhr1* (Fig. 2b). Thus, the *nhr1* mutation also affects lateral root development and ABA seems to be involved. An ABA-hypersensitive mutant (*ahg2*) of *Arabidopsis* that showed very poor germination and seedling establishment in the presence of ABA has been reported earlier (Nishimura et al. 2005). Since *nhr1* showed similar ABA responses as *ahg2*, we decided to test the ABA effect during plant survival. For this, we examined seedling growth in the presence of ABA on NM and the survival of the mutant was evaluated. Seven-day-old seedlings were placed on an ABA-containing plate and grown for 10 days of treatment. Seedling development was inhibited in both wt and *nhr1* genotypes in the presence of ABA, but *nhr1* seedlings became etiolated at 3 μM ABA (Fig. 2c). Furthermore, wt seedlings showed 100% survival in contrast with *nhr1* seedlings, which displayed 76% survival; nonetheless, only 13% of *nhr1* seedlings survived to the treatment of 5 μM ABA in clear difference with the 70% value of

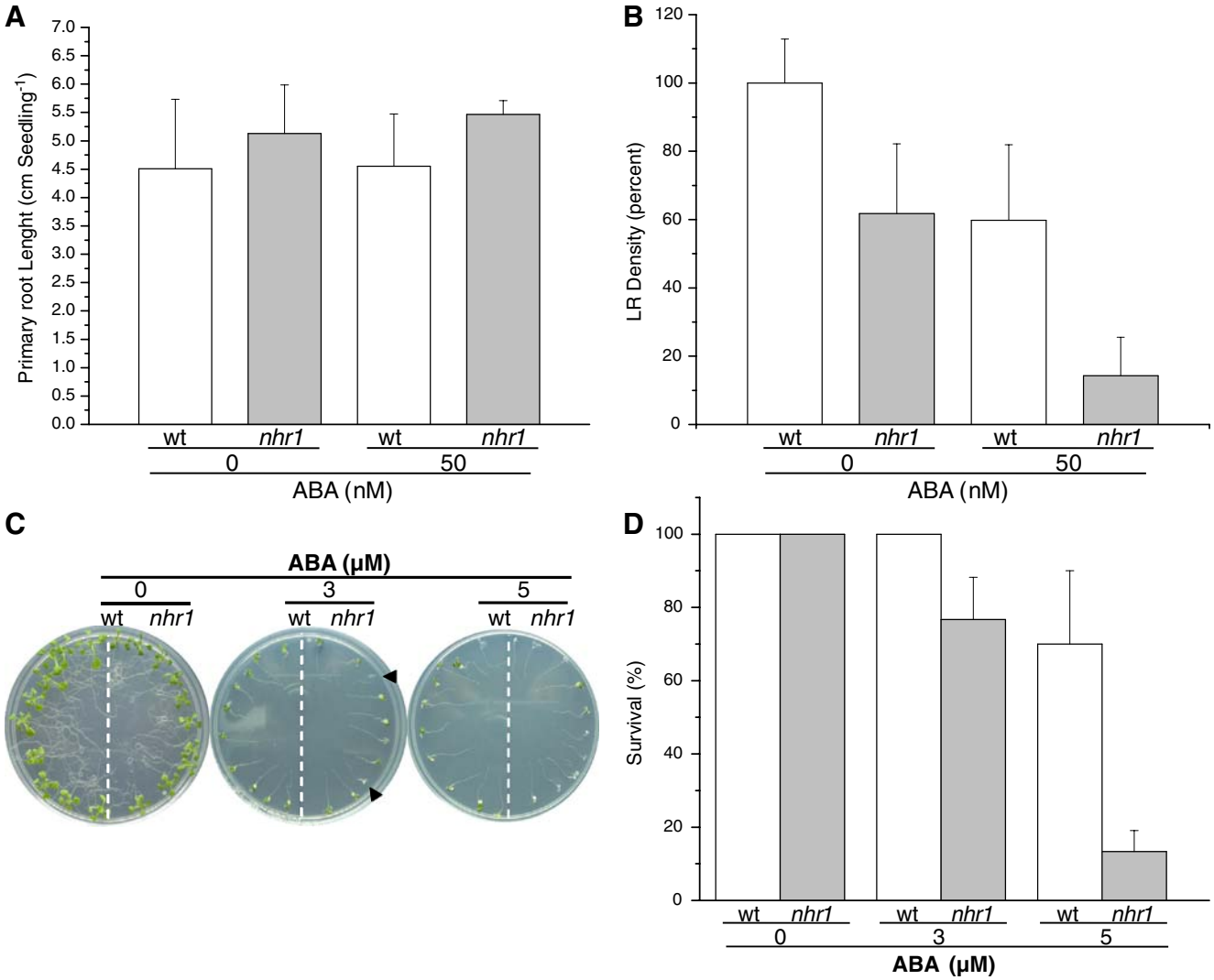


Fig. 2 *nhr1* seedlings are hypersensitive to ABA in lateral root development and survival. **a** Primary root length and **b** lateral root density of wt and *nhr1* 12-day-old seedlings grown in NM supplemented without or with ABA. **c** Qualitative and **d** quantitative analysis of wt and *nhr1* survival after 10 days of growing in the presence of ABA. Seven-day-old seedlings grown in NM were transferred to NM plates containing different concentrations of ABA, and data were taken after 10 days in 17-day-old seedlings. Data are representative of three different assays (n=35 with five replicates for **a** and **b**, n=10 with three replicates for **c** and **d** (mean ± SD))

those of wt (Fig. 2d). These results indicated that *nhr1* is hypersensitive to ABA at postgermination growth.

NHR1 Locus Affects Flowering

The *nhr1* plants grew slower than wt in pots, and we noticed that *nhr1* plants were delayed in the timing of bolting (change from vegetative to reproductive stage) compared to those of wt (Fig. 3a) and also showed a significant increase in the number of rosette leaves (wt 8.5±0.15 and *nhr1* 10.1±0.57). Furthermore, the rosette diameter of *nhr1* plants was as well larger than those of wt (data no shown). To determine whether this delay was due specifically to ABA disrupting *NHR1* function rather than to a pleiotropic effect of ABA that coincidentally affected *NHR1* expression, we treated by aspersion with 10 μM ABA *nhr1* and wt plants every 48 h during 35 days. Control plants were treated only with methanol/water/acetic acid without ABA. Afterward, estimation of bolting time was made after 45 days and was delay by 50% in untreated *nhr1* plants compared to those of wt. However, addition of ABA to *nhr1* plants caused a further delay in bolting time since this was postponed by 90% in contrast to those of wt. At final day of assay, ABA delayed the bolting time stronger in the *nhr1* (56.1±11.5) than wt (25.8±1.5; Fig. 3a, b). Furthermore, rosette sizes of wt and *nhr1* were smaller in ABA treated than in untreated plants (Fig. 3b) during the first 35 days of treatment; however, *nhr1* rosettes continued to grow up to bolting or just increased the size of its rosette (data no shown). We also evaluated the percentage of bolting in plants grown during 28 days in NM without and with 100 nM of ABA. We found that wt plants showed an elevated percentage of bolting in both the absence (86.7± 13.3) and presence of ABA (84.4±3.8), compared to those of *nhr1* plants, which showed a significant lower percentage of bolting in both the absence (46.7±6.7) and presence of ABA 4.4±3.8. Our results suggest that the delay in bolting of *nhr1* plants is probably due to an abnormal flowering control by ABA-disrupting *NHR1* function.

Inhibition of ABA Biosynthesis Triggers the Hydrotropic Response of *nhr1* Roots

ABA is synthesized from C_{40}-carotenoids, such as 9-*cis*-neoxanthin and 9′-*cis*-violaxanthin via the oxidative cleavage catalyzed by NCED (Schwartz et al. 1997). AbamineSG is a novel inhibitor of ABA biosynthesis that targets the activity of NCED and does not cause lethal damage (Kitahata et al. 2006). Because the no hydrotropic phenotype of the *nhr1* mutant is increased by the addition of ABA (Ponce et al. 2008), we speculate that inhibition of ABA accumulation will elicit a hydrotropic response in *nhr1* roots. Indeed, 27%

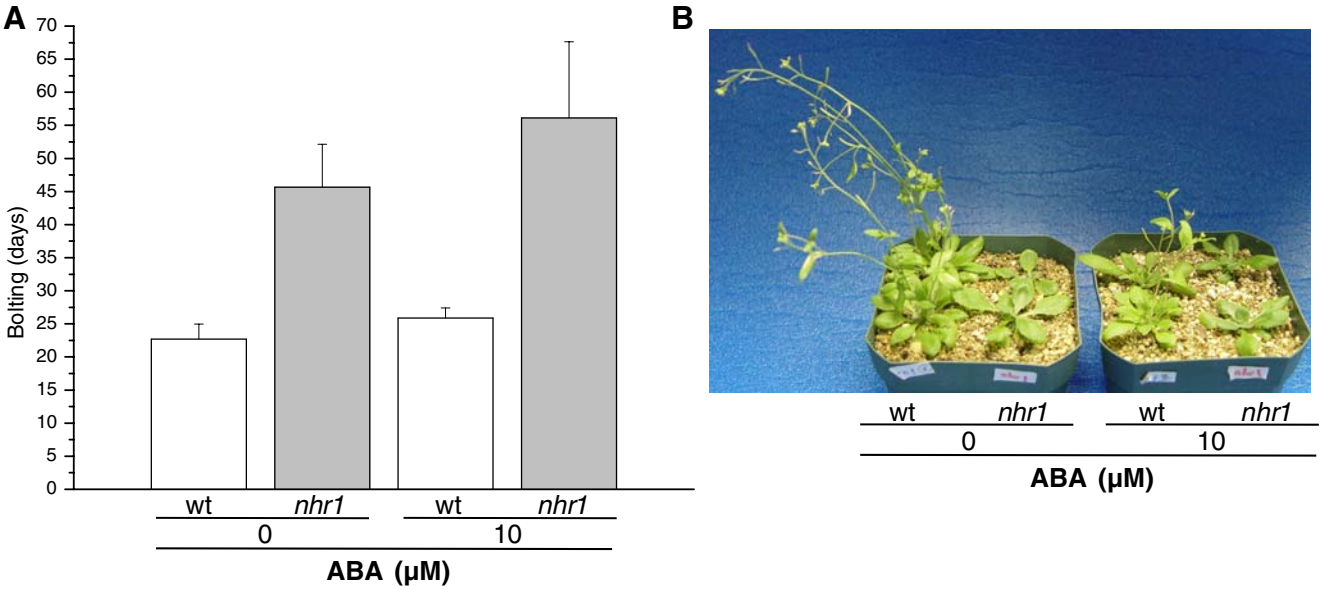


Fig. 3 Bolting time is delayed in *nhr1* plants and increased by the addition of ABA. **a** Days to bolting for wt and *nhr1* without and with 10 μM ABA sprayed on rosette leaves during 35 days. Plants were scored when the bolt reached 1 cm of length. **b** Comparison of wt (*left*) and *nhr1* mutant (*right*) plants grown on soil. Photograph was taken when both genotypes were 40 days old and grown under long day conditions for 5 days without any further ABA treatment. *nhr1* was in the vegetative phase and continued to develop rosette leaves, while wt had already flowered and produced siliques. Wt exhibited the morphological features, such as the rosette, elongated stem, cauline (stem) leaves, and siliques, whereas the *nhr1* mutant showed a considerable delay for the transition to the reproductive phase (n=20 plants). Data and photograph are representative of four independent tests. ABA was prepared fresh for each application. Plot represents mean ± SD

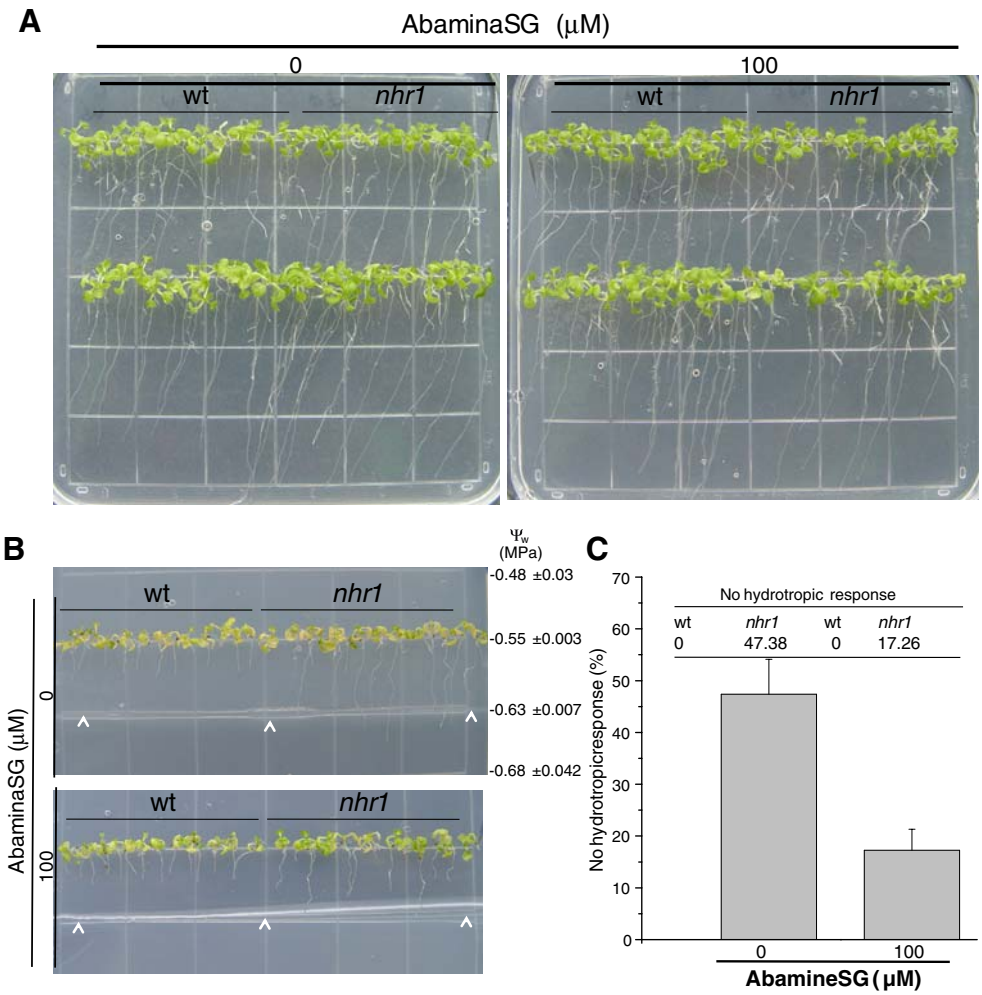


Fig. 4 The inhibition of ABA biosynthesis by AbamineSG increased the hydrotropic response of *nhr1* roots. **a** Two-week-old wt and *nhr1* seedlings grown in NM in the absence and presence of 100 μM AbamineSG. **b** Qualitative and **c** quantitative hydrotropic response of wt and *nhr1* seedlings grown for 2 weeks in the TM for hydrotropism in the absence and presence of 100 μM AbamineSG. The inhibitor did not have any adverse effect on plant growth in neither aerial part nor roots at both conditions evaluated. For **a** $n=30$ seedlings with three replicates and for **b** $n=15$ seedlings with three replicates. Two autonomous assays were done. **c** Mean and ± SD

of *nhr1* roots showed a hydrotropic response when grown on AbamineSG in the TM for hydrotropism (Fig. 4b, c). However, no phenotypic changes were observed in AbamineSG-treated *nhr1* seedlings grown in NM (Fig. 4a). AbamineG did not have any other collateral effect on plant growth and development in the shoot or root during the 2 weeks of treatment. These results indicated that the no hydrotropic response phenotype of *nhr1* roots probably depends on ABA. On the other hand, we suspected that the levels of ABA in the *nhr1* mutant were higher than in the wt, and hence, we decided to measure by RT-PCR the expression of *NCED3* in *nhr1* and wt plants. We found that inflorescences of 35-day-old wt plants grown in pots as well as 11-day-old wt seedlings showed higher transcript levels of *NCED3* than those of *nhr1* (Figs. 5a, b and 6). However, ABA levels in rosette leaves of *nhr1* were higher than those of wt (Fig. 5c). On the contrary, transcript levels of *NCED3* were more elevated in roots and cotyledons of *nhr1* seedlings grown in the TM for hydrotropism than in those of wt (Fig. 6c, d). The differences observed in the level of expression of *NCDE3* may reflect that this stress-responsive gene was more responsive to stress signaling of the *nhr1* mutant growing in the TM.

nhr1 Mutant Is Drought Tolerant

If the restriction of lateral root growth denotes an adaptation to drought (Xiong et al. 2006), one would expect that plants which showed a decrease in the number and length of lateral roots would consequently have lower transpiration rates. This assayed was done using isolated leaves whose fresh weights were recorded at different times. During the course of 180 min, *nhr1* leaves lost, on average, 10% (*w*/*v*) less water than those of the wt (Fig. 7a). We consequently tested the sensitivity to drought of *nhr1* mutant plants in soil (Fig. 7b). Under optimal conditions, *nhr1* mutant plants were similar to those of wt, except that these already bolted. To test tolerance to drought, 7-day-old wt and *nhr1* mutant seedlings of a similar size grown in NM were transferred to soil. Pots with 27-day-old *nhr1* and wt plants were saturated with water, and hence, their initial water content in the soil/pot was alike. These pots were

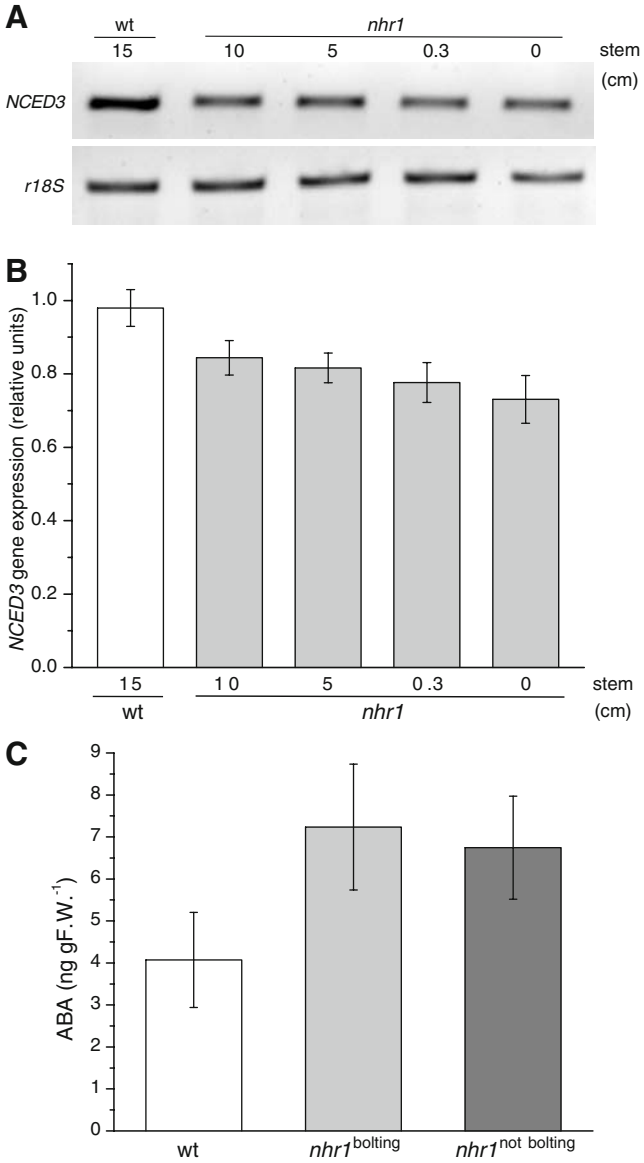


Fig. 5 *NCED3* gene expression and ABA accumulation in rosette leaves of *nhr1* and wt plants. **a** Gene expression of *NCED3* in leaves of 35-day-old plants with inflorescences of different lengths of 15 cm (wt) or 10 to 0 cm (*nhr1*). Gene expression was evaluated in rosette leaves by RT-PCR. The transcript of the *RIBOSOMAL 18S RNA (r18SRNA)* gene was amplified and used as an internal control. The gene expression was evaluated from total RNA pools isolated independently from two experiments; the typical result of an ethidium bromide-stained agarose gel is presented. **b** Densitometry examination of *NCED3* in the RT-PCR analysis. Every *NCED3* band was correlated to *r18S* gene expression, and then the *NCED3* wt band was normalized to 1 and compared to the others. Values in the plot are mean ± SD (n=4 in wt and *nhr1* for each stem length). **c** Accumulation of ABA in wt and *nhr1* leaves of 39-day-old day plants (n=5 in wt and n=10 in *nhr1*, mean ± SE)

subsequently covered with plastic wrap to avoid evaporation. Withholding water set up drought treatments. During the treatments, plants were continuously supervised for changes in growth, leaf color, and turgor maintenance. Once water was completely lost from pots, wt plants were all dried up whereas *nhr1* plants were still turgid (Fig. 7b). After 7 days of rewatering, mutant *nhr1* plants were capable to recover (data no shown). This is consistent with the elevated transpirational water loss observed in wt leaves in contrast to those of *nhr1*. Since ABA levels in the *nhr1* were higher than in the wt and the normal accumulation of ABA is necessary for root growth maintenance during water deficit conditions (Sharp et al. 2004b), then stress tolerance in *nhr1* may arise from a root system able to access more water resources or to keep it better than those of wt. It seems that *nhr1* plants avoided water loss by maintaining cell turgor, since they showed under drought stress no differences in leaf water potential in clear contrast with those of wt (Fig. 7c). These results indicate that *nhr1* plants are more competent to preserve water when subjected to drought stress.

Discussion

Here, we show that hydrotropism is modulated by ABA and that the lack of hydrotropism displayed by *nhr1* roots is a drought tolerance determinant. When plants colonized the land, they had to give up unlimited access to water and dealt with a drier environment (Eapen et al. 2005). The first vascular plants had humble roots, since portions of the underground system of stems allegedly served physiologically as roots. Roots were acquired later in the evolution of vascular plants (Niklas 1997). Henceforth, it is not surprising that some early determinants were traits that confer adaptations for overcoming drought. Once plants acquired a root proper and a root cap, the different tropisms evolved and the terrestrial landscape was tamed for a more efficient exploitation and occupation of new areas. In other words, once hydrotropism was attained, new traits allow the plant to look for water and explore new horizons in the soil. The *NHR1* gene, which seems to play an important role in hydrotropism and its disruption, renders drought tolerance. Two other mutants that do not display hydrotropism, *miz1* and *miz2*, were not tested in drought tolerance assays (Miyazawa et al. 2008; Kobayashi et al. 2007); however, analysis derived from microarray data revealed that *MIZ1* expression is upregulated only in roots closely after exposure osmotic and salt stresses (Kobayashi et al. 2007).

Promotion of dormancy is a general function of ABA (Gubler et al. 2005). The release of seed dormancy is regulated by complex interactions between environmental and genetic factors that are poorly understood. In the natural environment, the decay of dormancy for *Arabidopsis* seeds is promoted by stratification. The regulation of ABA metabolism might play a key role in dormancy release following after-ripening (Ali-Rachedi et al. 2004).

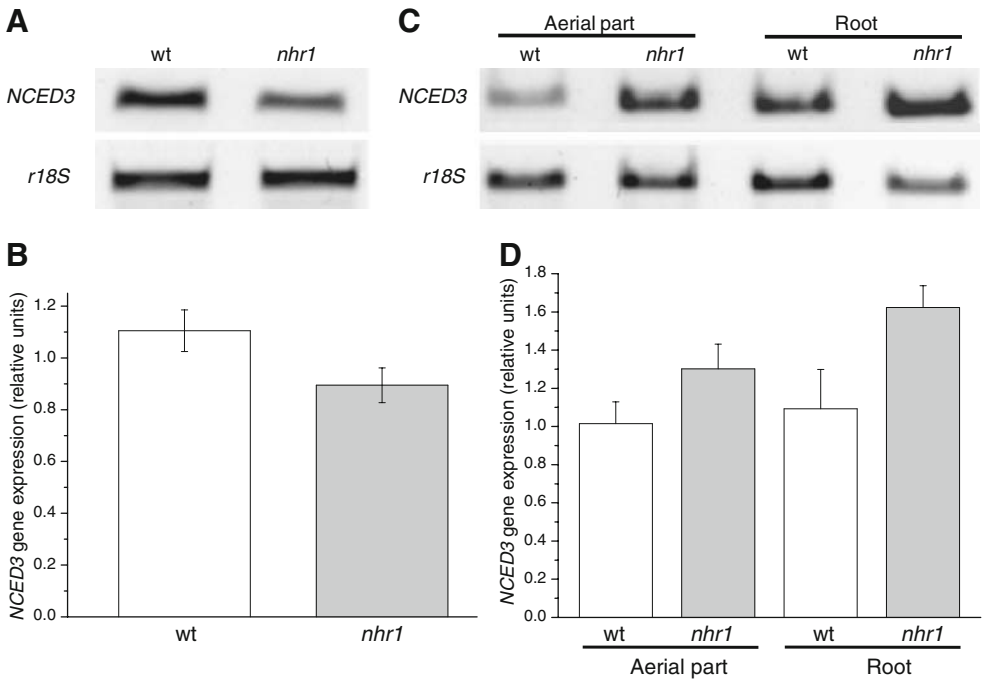


Fig. 6 Estimation of *NCED3* mRNA level in *nhr1* and wt seedlings by RT-PCR. **a** Total RNA was isolated from 11-day-old seedlings grown on NM. *NCED3* transcript levels were estimated by RT-PCR after 32 cycles with specific primers for *NCDE3*. *r18S*RNA transcript levels served as an equal loading standard. The gene expression was evaluated from two total RNA pools isolated independently, the analysis was repeated five times, and the typical result of an ethidium bromide-stained agarose gel is presented. **b** Relative transcript level of *NCED3* was determined by densitometry and is represented as relative units respect to wt sample normalized to 1; *r18S* transcript level was used as the internal control. Values are mean ± SD (n=5 in wt and *nhr1*). **c** *NCED* and *r18SRNA* transcript level from wt and *nhr1* seedling grown in TM for 12 days. The seedlings were dissected in aerial (cotyledons, leaves, and hypocotyls) and root parts. Gene expression was evaluated from two total RNA pools isolated independently with six times repeated, and the typical result of an ethidium bromide-stained agarose gel is displayed. **d** Relative transcript level of *NCED3* in seedlings grown in TM was determined by densitometry and is represented as relative units respect to wt sample normalized to 1; *r18S* transcript level was used as the internal control. Values are mean ± SD

In after-ripened seeds of both barley and *Arabidopsis*, the decline in ABA content precedes germination. Similar changes in ABA content have also been reported in dormant seeds following stratification. Following exposure of imbibed dormant *Arabidopsis* seeds to 4°C and transfer to 20°C, ABA content declined rapidly before germination. Unstratified seeds remained dormant during imbibition and retained a high concentration of ABA in the seed. Changes in the ABA content of imbibing seeds following dormancy release are likely to reflect changes in the balance between ABA synthesis and catabolism, with synthesis dominating in dormant seeds and catabolism dominating in nondormant seeds. Dormancy release by after-ripening and stratification presumably causes a switch to ABA catabolism, resulting in a decrease in ABA content in the embryo. Mutant *nhr1* seeds showed a considerable delay in seed germination and establishment in contrast with those of wt (Fig. 1). Seed germination and establishment was further postponed if seeds were treated with ABA (Fig. 1). Furthermore, *nhr1* plants contained higher concentration of ABA than wt plants (Fig. 5c). Hence, elevated levels of ABA in *nhr1* seeds during imbibition may hinder germination capacity. However, we have not determined the content of ABA in *nhr1* seeds and cannot rule out that other factors, such as ABA catabolism or sensitivity, affect germination capacity.

ABA is undoubtedly a critical regulator of hydrotropism (Ponce et al. 2008; Eapen et al. 2005). Here, we also showed further evidence that supports that the no hydrotropic response phenotype of *nhr1* roots depends on the presence of ABA since its absence activates the development of a hydrotropic response (Fig. 4). The *nhr1* plant has higher content of ABA than those of wt, indicating that elevated concentration of ABA inhibits hydrotropism, an observation that extends the previously examined effect of ABA in promoting gravitropism rather than hydrotropism in wt roots (Ponce et al. 2008). The observation that *nhr1* roots developed a significant lower number of lateral roots and that it was hypersensitive to ABA during seedling development (Fig. 2) might indicate that the elevated content of ABA in these plants (Fig. 5c) acted as a negative regulator of lateral root emergence (reviewed in De Smet et al. 2006). Nonetheless, the presence of AbamineSG did not promote the emergence of lateral roots in *nhr1* seedlings growing in optimal conditions (Fig. 4a), indicating that

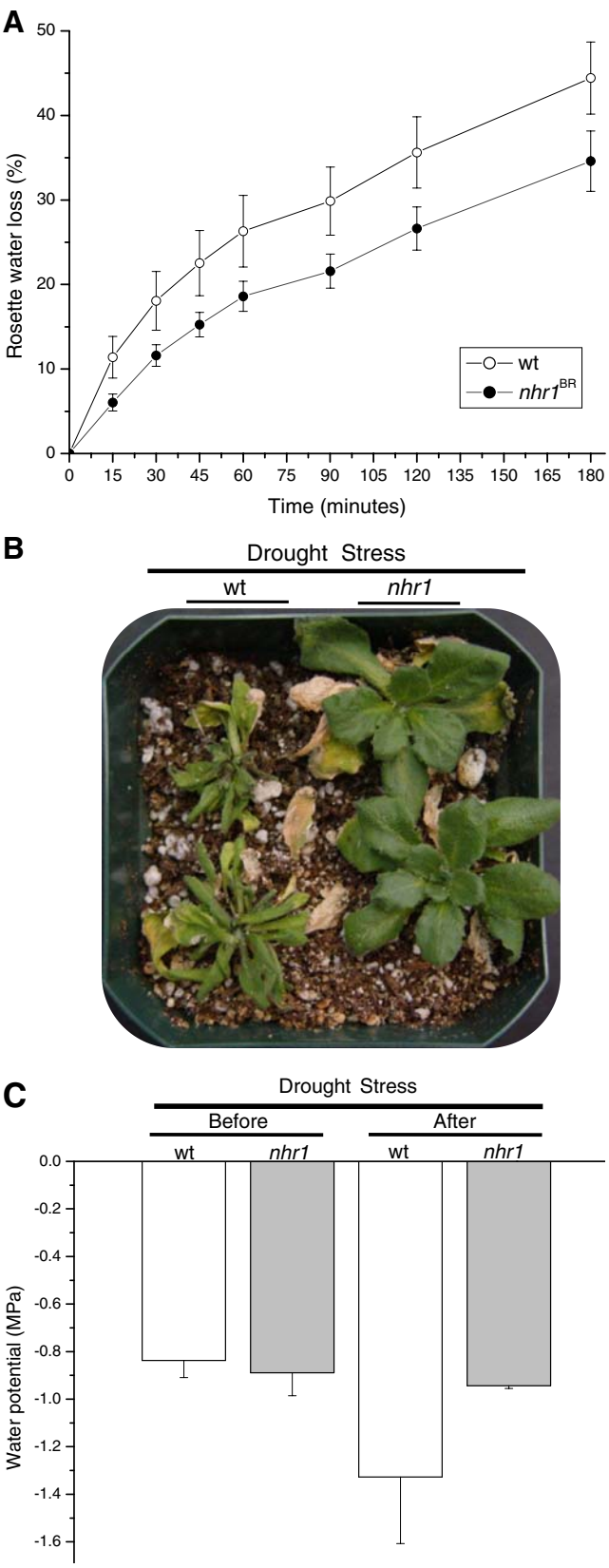


Fig. 7 *nhr1* mutant is tolerant to drought stress. **a** Transpirational rate of rosette leaves of wt and *nhr1* plants. **b** wt and *nhr1* plants submitted to drought stress. Plants were regularly watered until they were 35 days old, and then the drought stress treatment commenced and pots were not watered up to total water loss. **c** Water potential of wt and *nhr1* plants before and after drought stress. Three experiments were done independently and the plot is a representative result (n=10, mean ± SD)

ABA levels are not directly associated with this particular phenotype of *nhr1*.

In *nhr1* plants, flowering time was delayed (Fig. 3), implying that some of the multiple pathways that promote or repress flowering cannot reach a threshold level for switching the shoot apical meristem from producing leaves to forming flowers (Bernier et al. 1993). *nhr1* also plants contained high levels of ABA, and the addition of ABA significantly affected flowering time in *nhr1* compared to wt. Hence, the high content of ABA in the *nhr1* mutant could be responsible of the delay in bolting, but other factors could also be involved and should be deciphered in future experiments. Flowering (bolting) represents a crucial transition from a vegetative to a reproductive phase of the plant life cycle. The correct timing of this transition is essential to maximize reproductive success given the requirement for synchronous flowering in out-crossing species and the dependence on favorable conditions for optimal seed set (Simpson and Dean 2002).

We also hypothesized that the drought tolerance displayed by *nhr1* plants (Fig. 7) could be associated to the significant increases in the levels of ABA in this mutant. The lower transpirational water loss of *nhr1* leaves during drought (Fig. 7) is indicative of enhanced tolerance, rather than avoidance or escape responses. Dormant seeds, which are desiccation tolerant, also contain high levels of ABA (Ali-Rachedi et al. 2004). Drought induces an increase in ABA, and there is typically a corresponding increase in expression of the rate-limiting ABA biosynthetic enzymes NCED3 and NCDE1 (Iuchi et al. 2001). In fact, overexpression of *AtNCED3* in transgenic *Arabidopsis* caused an increase in endogenous ABA level and promoted transcription of drought- and ABA-inducible genes. Furthermore, plants overexpressing *AtNCED3* showed a reduction in transpiration rate from leaves and an improvement in drought tolerance (Iuchi et al. 2001). Additionally, the altered expression of *ASCORBATE PEROXIDASE 2* (*alx8*) mutant has constitutively increased ABA content, increased expression of genes responsive to high light stress, and is reported to be drought tolerant (Rossel et al. 2006). The higher levels of ABA in *alx8*, nonetheless, are not due to higher abundance of *NCED3* mRNA levels (Rossel et al. 2006) as in the case of the *nhr1* mutant (Figs. 5 and 6). Nonetheless, we cannot eliminate the possibility that the increased ABA content in *nhr1* is due to

a rise of transcription in some of the nine *NCED* gene family members. We consider that the high levels of ABA in *nhr1* might be due to either a posttranscriptional regulation of *NCED* genes or to a deficiency in ABA catabolism or ultimately to an alteration in ABA sensitivity. Future research must be aimed at the dissection of these issues as well as how does ABA levels change in *nhr1* during drought. Under nonstressed conditions, stress response genes, such as the *NCDE3*, was downregulated and ABA was accumulated (Fig. 6). From our current genetic study of hydrotropism, we conclude that *NHR1* positively regulates drought tolerance and hydrotropism mediated by ABA. The identification of *NHR1* will potentially help us to identify the link of root hydrotropism with drought tolerance and how it is controlled by ABA.

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