

AtbHLH68* transcription factor contributes to the regulation of ABA homeostasis and drought stress tolerance in *Arabidopsis thaliana

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Basic Helix-Loop-Helix (bHLH) transcription factors are involved in a wide range of developmental processes and in response to biotic and abiotic stresses. They represent one of the biggest families of transcription factors but only few of them have been functionally characterized. Here we report the characterization of *AtbHLH68* and show that, although the knock out mutant did not have an obvious development phenotype, it was slightly more sensitive to drought stress than the Col-0, and *AtbHLH68* overexpressing lines displayed defects in lateral root formation and a significant increased tolerance to drought stress, likely related to an enhanced sensitivity to ABA and/or increased ABA content. *AtbHLH68* was expressed in the vascular system of *Arabidopsis* and its expression was modulated by exogenously applied ABA in an organ-specific manner. We showed that the expression of genes involved in ABA metabolism (*AtAAO3* and *AtCYP707A3*), in ABA related response to drought-stress (*AtMYC2*, *AtbHLH122* and *AtRD29A*) or during LR's development (*AtMYC2* and *AtABI3*) was deregulated in the overexpressing lines. We propose that *AtbHLH68* has a function in the regulation of lateral root elongation, and in the response to drought stress, likely through an ABA-dependent pathway by regulating directly or indirectly components of ABA signaling and/or metabolism.

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Abbreviations – 2,4-D, dichlorophenoxyacetic acid; ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; bHLH, basic HELIX-LOOP-HELIX; BR, brassinosteroid; GA₃, gibberellic acid; LR; lateral root; LRP, lateral root primordium; NAA, 1-naphthaleneacetic acid; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction.

Introduction

Basic HELIX-LOOP-HELIX (bHLH) proteins, which are present in all eukaryotic organisms, constitute the second largest family of transcription factors in Angiosperms. In *Arabidopsis thaliana*, 162 bHLH proteins have been identified and multiple sequence alignment of the bHLH domain has been used to build a tree containing 21 subfamilies (Bailey et al. 2003, Toledo-Ortiz et al. 2003). This bHLH domain is highly conserved and contains two functionally distinct regions: the basic region and the helix-loop-helix (HLH) region. The basic region is generally composed of 13 to 17 basic amino acids that are important for DNA binding (Kong et al. 2012), mainly through recognition of the E-Box (Pires and Dolan 2010), while the HLH region is mainly composed of hydrophobic amino acids forming two amphipathic α -helices connected by a loop of variable length. The HLH region is required for the formation of homo or heterodimers with one or several other bHLH proteins (Toledo-Ortiz et al. 2003, Feller et al. 2011). Depending on their dimerization properties bHLH proteins can act either as activators or repressors of transcription (Feller et al. 2011). Analysis of amino acid sequences suggests that over 20% the bHLH family members may lack the capacity to bind DNA because they have a mutation in either the E-box binding domain (in 11 cases) or amino acid E13 in the basic DNA binding domain (27 cases) (Toledo-Ortiz et al. 2003). It has been suggested that such non-DNA binding bHLH could act as negative regulators of DNA-binding bHLH members by forming inactive heterodimers (Littlewood and Evan 1995). It has also been proposed that there is a tripartite system in which a non-DNA binding bHLH acts as a transcriptional repressor by either binding directly to a DNA-binding bHLH or sequestering another non-DNA-binding bHLH, thus repressing its association with its own target (Ikeda et al. 2012). Nevertheless, it was recently shown that AtbHLH112, a putative non-DNA-binding bHLH was in fact able to bind DNA and to regulate expression of genes involved in abiotic stress tolerance by binding to their E-box and GCG-box motifs (Liu et al. 2015) suggesting that things are likely more complex than expected and that there is still a need to functionally characterize atypical bHLH proteins to better understand their role in the regulation of gene expression.

To date functions have been determined for only 30% of more than 160 predicted *bHLH* genes. They are involved in developmental processes such as root (Schlereth et al. 2010, Wang et al. 2014), stamen (Mandaokar et al. 2006), or gynoecium (Gremski et al. 2007) development; in light signaling (Ni et al.

1998, Martínez-García et al. 2000); in the regulation of various hormone signaling pathways including brassinosteroid (Wang et al. 2009), gibberellin (Wang et al. 2009, Castelain et al. 2012), jasmonic acid (Nakata et al. 2013) or abscisic acid (ABA) (Abe et al. 2003, Liu et al. 2014, Tian et al. 2015) signaling; and also the overexpression of some bHLHs confers tolerance to salt, osmotic, freezing, cold and oxidative stress (Xie et al. 2012, Babitha et al. 2013, Peng et al. 2014), and other bHLHs are involved in NaCl, drought and osmotic stress signaling (Liu et al. 2014); iron homeostasis (Long et al. 2010) or in the response to phosphate starvation (Yi et al. 2005). All these studies indicate that bHLH transcription factors are involved in the responses to biotic and/or abiotic stresses.

Among the members of the bHLH transcription factor family, *AtMYC2* (*AtbHLH006*) was first described as an ABA- and drought-responsive gene. Phenotypic analysis of *myc2* mutants and *AtMYC2* overexpressing lines suggested that *AtMYC2* is a positive regulator of ABA signaling (Abe et al. 1997, 2003). It has also been proposed that *AtMYC2* may act at the crosstalk between abiotic and biotic stress responses and at the crosstalk between several hormone signaling pathways, including those mediated by ABA (For review Fujita et al. 2011). Since the earliest report, several members of the bHLH transcription factor family have been shown to contribute to abiotic stress tolerance through the complex ABA signaling network (i.e.: *AtAIB/AtbHLH17*, *AtAIG/AtbHLH32*, *AtbHLH92*, *AtASK1/AtbHLH122*, *AtASK2/AtbHLH128* and *AtASK3/AtbHLH130*) (Kim and Kim 2006, Li et al. 2007, Jiang et al. 2009, Babitha et al. 2013, Liu et al. 2014). Moreover, it has also been shown that *AtbHLH122* is able to directly repress the expression of the *AtCYP707A3* gene that code for an enzyme involved in ABA catabolism (Liu et al. 2014).

Abscisic acid (ABA) is a phytohormone involved in many physiological processes including embryo maturation, seed germination, control of stomata closure, root growth, lateral root formation and adaptation to environmental stresses such as drought (Xu et al. 2013). For the plant to cope with the changing environment, ABA levels must be adjusted constantly by a fine-tuning of ABA biosynthesis, catabolism and conjugation during the entire plant development (Nambara and Marion-Poll 2005). ABA is transported at long distance by the vascular tissues, either from the roots or from the shoot depending on the environmental cues (Xu et al. 2013). And several transcription factors, such as AP2/ERF, MYB, NAC, HD-ZF and bHLH, have been shown to regulate the expression of genes involved in ABA homeostasis and/or signaling (Fujita et al. 2011, Xu et al. 2013).

In the frame of a functional characterization of phloem-expressed genes identified by mining several transcriptomic databases (Le Hir et al. 2008) we focused on a transcription factor of the *AtbHLH* family, *AtbHLH68*. In the present article, we show that the expression of the Arabidopsis *AtbHLH68* gene, which encodes an atypical putative non-DNA-binding bHLH belonging to the subfamily 12 of the bHLH transcription factor family of Arabidopsis (Toledo-Ortiz et al. 2003), is regulated by ABA. The

characterization of a mutant and overexpressing lines provides evidences that *AtbHLH68* could be involved in lateral root development and resistance to drought likely by controlling ABA homeostasis.

Material and methods

Plant material and growth conditions

Plants used in this study were in the *A. thaliana* Col-0 background. Seeds of *bhlh68-1* mutant line were provided by NASC, under the accession number SM_3.16293. Fifteen to 30 plants of each line were grown in soil and heterozygous/homozygous plants were genotyped using gene-specific primers in combination with a primer specific for the left border of the T-DNA insertion (Appendix S1).

Plants in soil were grown under long day conditions (16 h light/8 h dark cycles) with a light intensity of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ at temperatures of 23–25°C during the day and 18–20°C at night. For *in vitro* culture, seeds were sterilized and sown on *Arabidopsis* medium as described previously (Santoni et al. 1994).

For ABA/NAA response assays, plants were germinated and grown on vertical plates containing the appropriate concentration of ABA or NAA, which was added to the medium after autoclaving. The root length of 12-day-old seedlings was measured on photographs taken with a 450D digital camera (Canon, New York, NY) using the ImageJ software package (Abramoff et al. 2004). Based on the different lateral root developmental stages described in (1997), roots at stage VII to Emergence were treated as lateral root primordia (Appendix S1A–B and D–E) and all lateral roots post emergence were treated as lateral roots independently of length (Appendix S1C and F). Lateral root primordia and lateral roots were scored directly on the main root using a stereomicroscope (Zeiss, Thornwood, NY). At least 30 plants were used for each experiment and each experiment was repeated 3 times.

Generation of *bHLH68* promoter:GUS fusions and 35S:CDS overexpressing lines

To generate *bHLH68*_{pro}:GUS line a 1291 bp of the upstream region of each gene was amplified from *Arabidopsis* Col-0 genomic DNA by PCR (for details Appendix S4). To generate 35S_{pro}:*HLH68* (*bHLH68ox*) lines, the entire coding sequence of each gene was amplified by PCR from Col-0 cDNA extracted from 7-day-old *A. thaliana* seedlings. We used Finnzyme's Phusion high-fidelity DNA polymerase (Thermo Scientific, Wilmington, DE) for PCR amplification. The PCR products were first sub-cloned into the Gateway entry vector pENTR/D TOPO (Invitrogen, Carlsbad, CA), then recombined with the destination binary vector pKGWFS7 or pB7WG2D (Karimi et al. 2007) and used to produce *bHLH68*_{pro}:GUS or 35S_{pro}:*bHLH68* lines respectively. All constructs were transferred into *Agrobacterium* strain GV3101::pMP90. The floral-dip method was applied to Col-0 plants to produce the transgenic lines (Clough and Bent 1998). The GUS expression pattern was observed in the progeny of 15 independent T1 lines. One representative mono-insertional homozygous line was retained for further observation. For the

overexpressing lines, 15 independent T1 lines were observed. The overexpression of *AtbHLH68* was checked by qPCR analysis and two independent mono-insertional homozygous lines were kept for further observations (Fig. 1).

RNA extraction and cDNA synthesis

Total RNA samples were collected from 10-day-old Col-0, mutant or *35S_{pro}::bHLH68* seedlings grown *in vitro* under long day conditions on *A. thaliana* medium (Santoni et al. 1994). For hormone response analysis, total RNA was extracted from Col-0 seedlings grown for 7 days under long day conditions prior to transfer into liquid medium for 24 h before a 5-h exposure to 50 μ M of abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC), brassinolide (BL), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin, gibberellic acid (GA₃) or, as control, no hormone. For ABA response experiment, total RNA was extracted from Col-0 seedlings grown for 10 days under long day conditions in presence or not of 0.5 μ M of ABA. Samples were ground using a MM 301 vibration mill (Retsch GmbH, Haan, Germany) at a frequency of 25 Hz for two min with 3 mm tungsten carbide beads (Qiagen, Venlo, The Netherlands). TRIzol® reagent (Invitrogen, Carlsbad, CA) was used to extract total RNA. RNA quality and quantity was measured using a NanoDrop ND 1000 (Thermo Scientific, Wilmington, DE). 5 μ g of RNA was treated with DNase I kit (Fermentas, Glen Burnie, MD). First strand cDNA was synthesized using a Superscript Reverse Transcriptase II kit (Invitrogen, Carlsbad, CA) with oligo-d(T)₁₂V primers. The RNA strand was removed by treatment with RNase H (Fermentas, Glen Burnie, MD). cDNAs were diluted 30-fold prior to use for quantification of relative gene expression levels by qPCR.

Gene expression analysis

Relative quantification of gene expression was carried out by qPCR. qPCR reactions were performed in triplicate in a 96-well transparent plate on a Bio-Rad CFX96 Real-Time PCR machine (Bio-Rad, Hercules, CA) in 20 μ l mixtures each containing 10 μ l MESA Fast qPCR™ Mastermix Plus for SYBR Assay (Eurogentec, Liège, Belgium), 0.2 μ l forward and reverse primer (30 μ M each), 4.8 μ l sterile water and 5 μ l of a 1/30 dilution of cDNA. The following qPCR program was applied: initial denaturation at 95°C for 5 min, followed by thirty-nine cycles of 95°C for 10 s, 60°C for 20 s, 72°C for 30 s. Melting curves were derived after each amplification by increasing the temperature in 0.5°C increments from 65°C to 95°C. The C_q values for each sample were acquired using the Bio-Rad CFX Manager 3.0 software. The specificity of the amplification was also assessed for each gene using dissociation curve analysis. A unique peak on the dissociation curve was confirmed for each gene. The C_q values were carefully checked and in some cases, one value was discarded because it differed from the other two replicates by > 0.5. For each primer pair used (see Appendix S5 in Supporting Information), amplification efficiencies were calculated using a 10-fold serial dilution series of cDNA. The primer pair amplification efficiency varied

between 78 and 115% (Appendix S5). Four genes were tested as potential reference genes: *AtAPT1* (At1g27450), *AtTIP41* (At4g34270), *AtEF1 α* (At5g60390) and *AtUBQ5* (At3g62250). For each experiment, the geNorm algorithm (Vandesompele et al. 2002) was used to determine the gene most stably expressed under the specific experimental conditions. Depending on the experiment *AtTIP41*, *AtUBQ5* or *AtAPT1* was chosen.

The relative expression level for each sample was calculated according to the Pfaffl method (Pfaffl 2001) using the following formula: $\text{average } E_t^{\Delta Cq(A-B)} / \text{average } E_r^{\Delta Cq(A-B)}$, where E_t is the amplification efficiency of the target gene primers, E_r is the reference gene primer efficiency, A represents the sample to which the quantification is relative, and B is the other sample. Average values were obtained for two or three biological replicates, depending on experiment, and the results were plotted on a logarithmic scale normalized to the Col-0 values, which were arbitrarily set to 1.

Drought stress experiments

Firstly, a mild drought stress was applied according to the protocol published by Bouchabké-Coussa et al. (2008). Briefly, after stratification for 4 days at 4°C, seeds were sown on propagation plugs (4 × 4 cm clods – 70% blond peat, 20% perlite and 10% vermiculite, Fertiss[®]). Plants were grown under long day conditions with a 16 h photoperiod, in a controlled environment chamber (22°C, 70% RH, PPFD approximately 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and watered with nutritive solution as described in Bouchabke et al. (2008). The relationship between soil volumetric water content and soil suction was previously assessed (Bouchabke et al. 2008). During all the growth period prior to starting the stress experiments the propagation plugs were saturated with nutritive solution in order to reach 100%. Once saturation was reached (time 0) stress experiments could start. Propagation plugs were weighed daily from t_0 . Once the target saturation was reached, this was then maintained for the duration of the experiment. For the control plants the soil water content was fixed at 60% of the substrate maximal water content (SMCW), while for the plants subjected to the stress, the soil water content was fixed at 30% SMCW. An individually monitored stress was applied. Each propagation plug, within a set watering regime, was maintained at the same saturation level based on its actual weight. In this way, the substrate saturation of all the genotypes within a watering regime was identical regardless of their water consumption. After 10 days of treatment, photographs were taken and analyzed the ImageJ software package to measure the area of green tissue.

Secondly, a more severe drought treatment was applied to the different genotypes analyzed. The plants were sown in soil (12 plants per tray) and grown with normal watering for 15 days before the water was withheld for 20 days. Photographs of the plants were taken with a 450D digital camera (Canon, New York, NY).

Measurement of ABA content

About 10 mg (FW) of shoot or root parts of Col-0, *bhlh68-1*, *bHLH68ox-1* and *bHLH68ox-2* seedlings, grown for 10 days in *in vitro* conditions, were harvested into liquid nitrogen and ABA content was quantified according to Druart et al. (2007) with the exception that the mass spectrometry analysis was performed on an Agilent 7000 GC-QqQ-MS instrument (Agilent, Santa Clara, CA) running in multiple-reaction monitoring mode (MRM). The following MRM-transition stages were monitored for quantification of ABA: m/z 190 → 162; ²H₆-ABA (internal standard), m/z 194 → 166.

GUS activity assays

For histochemical GUS staining, samples were incubated for 20 min in 80% acetone at -20°C and washed three times in distilled water. They were vacuum infiltrated for 10 minutes in GUS buffer (0.1 M phosphate buffer pH 7, 0.1% Triton X-100, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA and 1 mg ml⁻¹ X-Gluc) before overnight incubation in the same buffer in the dark at 37°C. All samples were cleared in 8:3:1 chloral hydrate:glycerol:distilled water (w/v/v) before observation. For flower stem sections, 1 cm long segments were taken from the bases of flower stems from 30-day-old plants. After staining, the segments were embedded in 8% agarose and 30 μm sections were prepared with a VT100S vibratome (Leica microsystems, Wetzlar, Germany). Pictures were taken with either an Axioplan 2 digital camera equipped with Axiovision software (Zeiss, Thornwood, NY) or a 450D digital camera (Canon, New York, NY).

For the fluorescence assay of GUS activity, the substrate 4-methylumbelliferyl-β-D-glucuronide (4-MUG) was used with protein extracts of both shoot and root parts of *bHLH68_{pro}:GUS* plants grown in the absence or presence of 0.5 μM ABA for 10 days.

Statistical analysis

Differences between Col-0 plants and lines in which *AtbHLH68* was knocked out or overexpressed were assessed using Student's *t*-test. For the drought stress experiment, a two-way ANOVA combined with Tukey's multiple comparison post-test was used for comparisons of multiple means; it was performed using GraphPad Prism version 6.0d for Mac (Graphpad, La Jolla, CA).

Results

Overexpression of *AtbHLH68* induces defects in root development

In order to explore the biological functions of the *AtbHLH68* transcription factor, we isolated T-DNA insertion lines SM_3.16293 (*bhlh68-1*) for the *AtbHLH68* gene (Appendix S2A). We also generated transgenic plants in which the *AtbHLH68* gene was constitutively over-expressed under the control of the

CaMV 35S promoter. Molecular characterization of these lines showed a significant over-expression of the target gene in *bHLH68ox-1* and *bHLH68ox-2* compared to Col-0 and the absence of full-length cDNA in *bhlh68-1* mutant confirming that it is a knock out (KO) mutant (Fig. 1A and B)

When the seedlings were grown *in vitro* no obvious developmental phenotypic difference between the mutant and the Col-0 was observed (Appendix S2B–E). In contrast, there was a significant difference in root development between the overexpressing lines and Col-0 (Fig. 1C–I). The primary root of both overexpressing lines was significantly shorter than that of Col-0 (Fig. 1C–F) and displayed a yellowish color (Fig. 1C–E). In addition, the *bHLH68ox* lines developed almost no elongated lateral roots (LR) (Fig. 1C–G). Therefore, we counted the lateral root primordia (LRP) from stage VII to stage Emergence according to Malamy and Benfey (1997). We observed 4 times more LRP in the overexpressing lines compared to Col-0 (Fig. 1H) but their growth was arrested indicating the lack of emerged and elongated LRs was not due to the absence of lateral root primordia (LRP) but likely to a defect in emergence / elongation of the initiated LRP.

When the plants were grown in soil the KO mutant developed a rosette, which was slightly but significantly bigger than that of Col-0 (Fig. 1I–J). In contrast, plants of the overexpressing lines grew slower in soil and remained smaller (Fig. 1I–J).

LR formation in *Arabidopsis* is controlled by several hormones the main one being auxin but abscisic acid (ABA) was also shown to play a role in the control of LRP elongation (Casimiro et al. 2001, De Smet et al. 2003, Duan et al. 2013). Therefore, we tested the behavior of the mutant and overexpressing lines on media containing variable concentrations of the synthetic auxin NAA or of ABA (Fig. 2). In the presence of NAA we observed no difference in term of primary root elongation between Col-0, the *bhlh68-1* mutant and overexpressing lines which displayed similar inhibition of the primary root growth (Fig. 2A). LR density was increased in both Col-0 and the *bhlh68-1* mutant, although to a significantly less extent in the mutant suggesting that this later could be slightly resistant to exogenous auxin compared to Col-0 (Fig. 2B). In absence of auxin the LR density of the overexpressing lines is very low compared to Col-0 (Fig. 2B), and in presence of 0.1 μ M of NAA a slight but significant increase of LR density was observed for *bHLH68ox* (Fig. 2B). No significant effect of auxin could be observed for the other overexpressing line *bHLH68ox-1* (Fig. 2B). However, 0.1 μ M of NAA strongly stimulated the formation of LRPs in both overexpressing lines (Fig. 2C) and the LRP density was similar to the LR density of Col-0, suggesting that the overexpressing lines display a similar sensitivity to auxin than the Col-0 as for the LRP initiation, but that these LRPs are unable to emerge and elongate.

Because increased ABA concentration is known to block the emergence and elongation of LRP (De Smet et al. 2003) we tested the effect of exogenously applied ABA on both mutant and overexpressing lines. One μ M of exogenously applied ABA induced a reduction of about 45% of the PR length and a

decrease of the LR density in both Col-0 and *bhlh68-1* seedlings (Fig. 2D), whereas at the same concentration the overexpressing lines showed a 80% reduction of their root length (Fig. 2D). In addition, 1 μ M of ABA decreased the LRP density in the overexpressing lines while it had no effect on Col-0 seedlings (Fig. 2F). These results indicate that the overexpression of *AtbHLH68* confers enhanced sensitivity to exogenously applied ABA, suggesting that *AtbHLH68* could be involved in the control of ABA signaling and/or homeostasis.

The expression of genes involved in ABA homeostasis is altered in both the *bhlh68-1* mutant and the *AtbHLH68* overexpressing lines

To check a potential role of *AtbHLH68* in the control of ABA homeostasis we quantified the ABA content in both shoot and root parts of 10-day-old seedlings grown *in vitro* (Fig. 3A). No statistically significant difference in ABA content was observed in the *bhlh68-1* mutant compared to Col-0 for the root part, whereas in the shoot part, a slight tendency to a decrease in ABA content was observed even if this difference was not significant (Fig. 3A). In contrast the overexpressing lines *bHLH68ox-1* and *bHLH68ox-2* displayed a significant increase in the endogenous content of ABA compared to Col-0 in the shoot and in the root (Fig. 3A).

Endogenous ABA concentrations is principally controlled by the balance between biosynthesis and catabolism, therefore we determined in both shoots and roots of the overexpressing lines the expression of *AtALDEHYDE OXIDASE 3* (*AtAAO3*) and *AtABSCISIC ACID 8'HYDROXYLASE 3* (*AtCYP707A3*), which are respectively involved in the biosynthesis and catabolism of ABA (Fig. 3B). *AtAAO3* was significantly up-regulated in the shoot and the root of both *bHLH68ox* lines while it was down-regulated in the shoot of the mutant line and unchanged in the mutant root (Fig. 3B). *AtCYP707A3* was undetectable in both the shoot and the root of the *bhlh68-1* mutant compared to Col-0 (Fig. 3B), suggesting a strong down-regulation of *AtCYP707A3* expression in the entire mutant seedlings. In contrast *AtCYP707A3* was up-regulated in the shoot of the overexpressing lines and slightly down-regulated or not affected in *bHLH68ox-1* and *bHLH68ox-2* respectively (Fig. 3B). These results suggest that *AtbHLH68* plays a role in the regulation of ABA homeostasis. The fact that we could not detect any changes in the ABA content of the mutant despite a down-regulation of *AtAAO3* and *AtCYP707A3* gene expression might be due to compensatory regulatory mechanisms.

The expression of *AtbHLH68* is repressed by exogenously applied ABA or GA

To support the role of *AtbHLH68* in the regulation of ABA homeostasis and / or signaling, we checked if *AtbHLH68* gene expression is regulated by ABA. We quantified the expression of the *AtbHLH68* gene in entire seedlings grown *in vitro* and transferred in the presence or absence of 50 μ M of different hormones during 5 h (Fig. 3C). The accumulation of *AtbHLH68* transcript was significantly reduced in the presence

of ABA or gibberellic acid (GA₃) but no significant effect of auxin, ethylene, cytokinin or brassinosteroid was observed (Fig. 3C). In a parallel experiment we quantified the GUS activity of the *bHLH68_{pro}:GUS* transcriptional fusion in shoot and root parts of 10-day-old seedlings of a stable transformant grown *in vitro* in the presence or absence of 0.5 μ M of ABA (Table 1). Under these conditions the GUS activity in the *bHLH68_{pro}:GUS* line was significantly induced by exogenous ABA in the roots while it was strongly repressed in the shoots (Table 1). This differential regulation of *AtbHLH68* expression was confirmed by qPCR in Col-0 seedlings grown *in vitro* in presence or absence of 0.5 μ M of ABA (see Appendix S3). We confirmed that *AtbHLH68* gene relative transcript amount is lower in the shoots in presence of ABA while it is increased in the roots (Appendix S3). These results suggest that *AtbHLH68* expression is differentially regulated by ABA in the shoots and in the roots.

***AtbHLH68* overexpressing lines are more tolerant to drought stress while the mutant is more sensitive**

Since ABA is an important regulator of abiotic stresses, such as drought, we investigated the behavior of the mutant and overexpressing lines in response to drought treatments (Fig. 4). First, the plants were submitted to a mild drought treatment as described by Bouchabke-Coussa et al. (2008). To quantify the effect of this drought treatment we measured the green area of the lines of treated and non-treated lines. As already described above, the *bhlh68-1* mutant had a slight but significant larger green area than Col-0, while *bHLH68ox-1* line had a significantly reduced green area (Fig. 4A). In drought condition, a significant decrease in green area was observed for all the genotypes compared to untreated lines (Fig. 4A). Nevertheless, the ratio between the green area in drought condition and the green area in control condition was significantly reduced and increased for the *bhlh68-1* mutant and the *bHLH68ox-1* line respectively (Fig. 4B) indicating that the mutant was more sensitive to the mild drought treatment while the over-expressing line was more resistant.

To confirm this differential tolerance to drought, the different lines were sown in soil and the plants grown for 2 weeks before withholding water for 20 days (Fig. 4C). In these conditions the overexpressing lines stayed green and turgid while the leaves of Col-0 plants and the *bhlh68-1* mutant became purple and wilted (Fig. 4C). The *bhlh68-1* mutant was also slightly more sensitive to drought than Col-0 when submitted to this drastic drought treatment. Taken together, these observations suggest that overexpression of *AtbHLH68* makes *Arabidopsis* plants more tolerant to drought.

Knockout or over-expression of *AtbHLH68* affects the expression of ABA and drought stress signaling related genes

In order to check if ABA signaling could be altered in the *bHLH68ox* lines we quantified the expression of eight genes involved in ABA and / or drought stress signaling, *AtABII* (*ABA INSENSITIVE 1*; Casimiro et

al. 2001), *AtABI3* (*ABA INSENSITIVE 3*; Brady et al. 2003), *AtABF3* (*ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 3*; Yoshida et al. 2010), *AtRD29A* (*RESPONSIVE TO DESICCATION 29A*; Msanne et al. 2011), *AtMYC2/AtbHLH006* (Abe et al. 2003), *AtbHLH122/AtABA INDUCIBLE GENE1* (*AtAGII*; Liu et al. 2014), *AtKIN2* (*KINASE 2*; Kurkela and Borg-Franck 1992) and *AtWRKY63* (Ren et al. 2010) in both shoot and root parts of Col-0 and overexpressing lines seedlings grown *in vitro* (Fig. 5A, B). In the *bHLH68ox-1* and *-2* shoots, the relative transcript amount of the eight genes was up-regulated compared to Col-0 shoots. The opposite was observed for three of them (*AtbHLH122*, *AtRD29A* and *AtWRKY63*) which expression was significantly down-regulated in the mutant *bhlh68-1* (Fig. 5A). In the roots the expression of *AtABI3* was down-regulated while the expression level of *AtRD29A* did not change and that of the six remaining genes was up-regulated compared to Col-0 roots (Fig. 5B). In contrast, the expression of most genes was unchanged in the root of the mutant *bhlh68-1* except that of *AtABI3* and *AtWRKY63*, which was slightly up-regulated (Fig. 5B). Collectively, these findings suggest that *AtbHLH68* is involved in the regulation of ABA signaling pathway either directly or indirectly.

***AtbHLH68* is expressed in the vascular tissue of all the organs**

We also analyzed the expression pattern of *AtbHLH68* in organs and tissues. RNA from *Arabidopsis* rosette leaves, cauline leaves, floral stem, flowers, young siliques as well as apical and root parts of *in vitro* grown seedlings, was subjected to qPCR (Fig. 6A). We showed that *AtbHLH68* is expressed in all organs tested and the highest transcript abundance was observed in the floral stem (Fig. 6A). In addition we produced an *Arabidopsis* transgenic line expressing *bHLH68_{pro}:GUS* construct and checked for GUS activity in the different organs (Fig. 6B–J). GUS activity driven by the promoter of *AtbHLH68* was specifically observed in the vascular system of the cotyledons and in the central cylinder of the main root of 10-day-old seedlings grown *in vitro* (Fig. 6B and 6C). A GUS activity was also detected as early as in the very initial lateral root primordia (Fig. 6D) as well as in the central cylinder of the lateral root in the later stages of its development (Fig. 6E–F). However, no GUS signal was observed in the lateral root meristem (Fig. 6E–F). Additionally, GUS activity was detected in the vascular system of rosette leaves, cauline leaves and flowers of 4-week-old plants grown in soil (Fig. 6G, H and J). In the floral stem, sections revealed the expression of *bHLH68_{pro}:GUS* in the phloem cells (Fig. 6I). These data showed that the expression of *AtbHLH68* is restricted to the vascular system.

Discussion

Drought tolerance is a physiological process that is controlled by ABA, and the results we provide in this article suggest that the transcription regulator *AtbHLH68* could play a role in drought responses either through ABA signaling and/or through the regulation of ABA homeostasis in *Arabidopsis*.

We observed that the knockout mutant *bhlh68-1* was more sensitive to drought stress than Col-0,

while the overexpressing line was more tolerant. In addition we observed that the expression of two DNA-binding bHLH (encoded by *AtMYC2* and *AtbHLH122*), which are positive regulators of drought response (Abe et al. 2003, Liu et al. 2014), that of *RESISTANT TO DESSICATION STRESS 29A* (*AtRD29A*), which is strongly induced by ABA, drought and salt stress (Yamaguchi-Shinozaki and Shinozaki 1993, Msanne et al. 2011) as well as the expression of *AtWRKY63* involved in ABA signaling (Ren et al. 2010) were up-regulated in the shoot part of the overexpressing lines (Fig. 5). With the exception of *AtMYC2*, these genes were in contrast down-regulated in the knockout mutant *bhlh68-1*. The *AtWRKY63* protein was shown to bind to the promoter of the *AtABF2* gene (Ren et al. 2010) and the expression of *AtABF2* and downstream genes such as *AtRD29A* and *AtCOR47* was down-regulated in the mutant *abo3/wrky63*, which was ABA-hypersensitive and less tolerant to drought than Col-0 (Ren et al. 2010). Additionally, the transcriptional induction of *AtWRKY63/AtABO3* by ABA was impaired in *abi1*, *abi2* and *abi5* mutant lines, which places the *AtWRKY63* gene downstream of *AtABI1*, *AtABI2* and *AtABI5* but upstream of *AtABF2*, *AtRD29A* and *AtCOR47* (Rushton et al. 2012). Here we observed a down-regulation of *AtWRKY63* as well as that of its downstream target *AtRD29A* in the apical part of the *bhlh68-1* mutant. The opposite was observed in the overexpressing lines (Fig. 5A). However, in the root parts there was no positive correlation between *AtWRKY63* and *AtRD29A* expression (Fig. 5B) suggesting that the target of *AtWRKY63* in the root might be different than in the shoot. Although the phenotype of the mutant *bhlh68-1* was subtler than that of the over-expressing lines, we could observe a deregulation of the ABA signaling pathway likely *via* the action of *AtWRKY63* in the shoot part. Additional genes related to drought stress or ABA signaling such as *AtABI1*, *AtABF3* and *AtKIN2* were up-regulated in the overexpressing lines but their expression was not modified in the mutant (Fig. 5A–B). *AtABI1* encodes a protein phosphatase 2C and the mutant in this gene displays reduced drought rhizogenesis (Casimiro et al. 2001). *AtABF3* belongs to the *AtAREB/AtABF bZIP* factor subfamily and is regulated by ABA and water stress (Yoshida et al. 2010). The expression of *AtKIN2* gene was shown to be regulated by cold and ABA (Kurkela and Borg-Franck 1992). Therefore, the over-expression of *AtKIN2* in the overexpressing line is likely due to the constitutive accumulation of ABA.

In conclusion, these results suggest that at least a part of the transcriptional network involved in drought-stress response was activated in the *AtbHLH68* overexpressing lines and repressed in the mutant line.

Interestingly, we demonstrated that *AtbHLH68* is expressed in the vascular tissues, both in the shoot and in the roots, during the whole plant development. In the floral stem the promoter expression was restricted to the phloem tissue (Fig. 6). The vascular tissues are the primary site of ABA production, with many genes, involved in biosynthesis, catabolism or transport of ABA, such as *AtNCED3*, *AtABA2*, *AtAAO3*, *AtCYP707A3* and *AtABCG25* being expressed in the vascular parenchyma cells or in the phloem

companion cells (Endo et al. 2008, Okamoto et al. 2009, Kuromori et al. 2014). We hypothesized that *AtbHLH68* could either positively regulate the expression of genes related to ABA metabolism or inhibit negative regulators of such genes in the vascular tissue and thereby affect ABA homeostasis. The response of the mutant and overexpressing lines to drought stress could indeed be a consequence of a perturbed ABA homeostasis. Although we did not observe a significant change in the knock out mutant, plants overexpressing *AtbHLH68* contained significantly more ABA compared to Col-0 (Fig. 3A). In the shoot part, this increased endogenous ABA content coincided with an up-regulation of the expression of *AtAAO3* and *AtCYP707A3* (Fig. 3B), which encode the enzyme catalyzing the last step of ABA biosynthesis (Seo et al. 2000) and the enzyme catabolizing ABA (Saito et al. 2004) respectively. In the roots the accumulation of ABA was likely emphasized by the down-regulation of the ABA degrading enzyme *AtCYP707A3*. In the mutant line in contrast we observed a down-regulation of the biosynthesis gene *AtAAO3* in the shoot part and a strong down-regulation of the *AtCYP707A3* gene since we could not detect any expression in both shoot and roots (Fig. 3B). In this case the reduced biosynthesis might be compensated by a reduced degradation in order to maintain a Col-0 level of ABA in the mutant. These results strongly suggest that *AtbHLH68* controls ABA homeostasis as shown for at least one additional *bHLH* transcription factor, *AtbHLH122*, which is involved in the tolerance to abiotic stresses by modulating ABA catabolism or ABA mediated response (Kim and Kim 2006, Liu et al. 2014). *AtbHLH122* protein was shown to directly interact with the promoter of the *AtCYP707A3* gene to repress its expression, which led to an increased ABA content (Liu et al. 2014). Nevertheless, here we show that when *AtbHLH122* is strongly down-regulated in the shoot of the *bhlh68-1* mutant line (Fig. 5A) in which the expression of *AtCYP707A3* became undetectable and that in the shoot of the *AtbHLH68* overexpressing lines *AtbHLH122* is up-regulated (Figure 5A) like the expression of *AtCYP707A3* (Fig. 3B). In contrast, we indeed observed a down-regulation of *AtCYP707A3* in the root of both the mutant and one of the overexpressing line (Fig. 3B) in which *AtbHLH122* is up-regulated (Fig. 5B). These results suggest that the physical interaction between *AtbHLH122* protein and the promoter of *AtCYP707A3* might be organ-dependent Like *AtbHLH68*, *AtbHLH122* was preferentially expressed in the vascular tissues (Liu et al. 2014) and we observed that its expression was strongly up-regulated in both shoots and roots of the *AtbHLH68* overexpressing lines suggesting that *AtbHLH122* could be a potential target of *AtbHLH68*, as it is known that bHLH proteins interact with other bHLH proteins (Toledo-Ortiz et al. 2003). We can speculate that *AtbHLH122* and *AtbHLH68* could interact directly or indirectly to regulate the expression of genes involved in ABA homeostasis in the vascular tissue.

In contrast to what was observed in the shoot part, the expression of *AtMYC2*, *AtbHLH122* and *AtRD29A* was differentially regulated in the roots (Fig. 5B). The expression of *AtbHLH122* and *AtMYC2* was still strongly up-regulated in the roots of both overexpressing lines, while *AtRD29A* expression was

not modified in either of the lines. In the mutant line *AtMYC2* expression was not affected in shoot and root, but *AtbHLH122* was up-regulated while *AtRD29A* expression was not affected when both genes were down-regulated in the shoot part. These results suggest that *AtbHLH68* could target different ABA-dependent or ABA-independent pathways controlling drought in the root or in the shoot. Interestingly the phenotype of the *AtbHLH68* overexpressing lines is reminiscent of that of lines overexpressing another *bHLH* transcription factor, *AtbHLH112*, described in (Wang et al. 2014, Liu et al. 2015). Both *AtbHLH68* and *AtbHLH112* over-expressing lines have defective lateral root development [Fig. 2 and Wang et al. (2014)]. In both cases the number of emerged LR was reduced while the number of arrested LRP was increased, but in the case of the *AtbHLH112* over-expressing line the LR phenotype could be rescued by exogenously applied auxin (Wang et al. 2014), while this was not the case of *AtbHLH68* overexpressing lines (Fig. 2). The transition from lateral root primordial (LRP) emergence to LR elongation results from the activation of meristematic initials in the LR meristem (Malamy and Benfey 1997), and this step is negatively regulated by exogenously applied ABA (De Smet et al. 2003). In this context, the high ABA content in the root of the *bHLH68ox* lines would be consistent with the arrest of LRP emergence. Interestingly, *AtbHLH68* is expressed throughout the lateral root development as early as in stage I LRP (Fig. 6) and its expression is induced in the root by exogenously applied ABA (Table 1 and Appendix S3), strongly suggesting that *AtbHLH68* plays a role in the control of LRP in response to ABA. Therefore, the LR elongation phenotype observed could be the consequence of a regulatory loop involving *AtbHLH68* that could directly or indirectly regulate the expression of genes involved in ABA homeostasis (as suggested above) and in return, the resulting ABA accumulation could promote the expression of *AtbHLH68* in roots maintaining the inhibitory effect on LRP elongation. Interestingly, the expression of *AtABI3*, that is involved in ABA signaling and is also necessary for proper auxin responsiveness for LR initiation (Brady et al. 2003), was de-regulated in the overexpressing lines, suggesting that the auxin responsiveness of LRP and therefore the cross talk between ABA signaling and auxin action could be impaired in these lines. On the other hand, the expression of *AtMYC2* gene, which is ABA-regulated and known to be a positive regulator of LR formation (Yadav et al. 2005), was upregulated in the roots of the overexpressing lines. Overall our data suggest that the overexpression of *AtbHLH68*, which induces a constitutive ABA accumulation, impacts the LR elongation by disturbing both ABA and auxin signaling during this process. Although overexpression of *AtbHLH68* suppressed LR emergence, the *bhlh68-1* mutant showed only marginal differences in our analysis when compared to Col-0 regarding the LR phenotype (Fig. 2) as well as the ABA content in the root (Fig. 3A). This might be attributable to functional redundancy between the different members of the bHLH transcription factors family. However, it is interesting to mention that after treatment with 0.1 μ M NAA, *bhlh68-1* mutant displayed slightly but significantly less sensitivity to NAA than Col-0 (Fig. 2B), implying that *AtbHLH68* may also play a

positive role in the induction of additional LRs under an auxin treatment. It has long been established that a close relationship exists between drought response and lateral root development. The root system is very sensitive to water deficit, under drought stress conditions. Indeed, depth and elongation of the primary root and formation of the lateral roots are adjusted to sustain the viability of plants (Deak and Malamy 2005, Gubler et al. 2005, Yu et al. 2008). Notably, the number of lateral roots and their growth are significantly reduced under water stress conditions (Deak and Malamy 2005) in contrast to the primary root which development is not systematically affected by drought. More recently it was shown that reduced lateral root branching density significantly improves drought tolerance in maize plants (Zhan et al. 2015). In this process, abscisic acid plays a major role since one of the consequence of drought is to induce ABA production. Then ABA activates the expression of the Kip-RELATED PROTEIN2 gene (*AtKRP2*) which functions as a cyclin-dependent kinase inhibitor and regulates the G1-S transition of the cell cycle in the pericycle lateral root founder cells (Himanen et al. 2002). This gene is repressed by auxin but activated by abscisic acid (ABA), which is consistent with the promotive role of auxin and the repressive role of ABA in lateral root initiation (De Smet et al. 2003, Verkest et al. 2005). Therefore, we suggest that the constitutive reduction of the lateral root number in the overexpressing lines, which is likely due to the increased level of ABA in the roots, could explain at least partially the increased drought tolerance of the *AtbHLH68* overexpressing lines.

Author contributions

C.B. conceived the study. M.C., R.L.H., S.D. and C.B. designed the experiments. M.C., D.C., R.L.H., T.M. performed the experiments. R.L.H., S.D. and C.B. analyzed the data. R.L.H., and C.B wrote the manuscript, and all authors read and approved the final manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Stages of lateral root primordium and lateral root development.

Appendix S2. Phenotypic characterization of *bhlh68-1* mutant line.

Appendix S3. *AtbHLH68* expression is differentially regulated by ABA in shoot and root parts.

Appendix S4. List of primers used in this study.

Appendix S5. Efficiencies of the primer pairs used in this study.

Appendix S6. Accession numbers.

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Accepted Article

Figure legends

Fig. 1. Phenotypic characterization of *bHLH68ox* lines. (A) Relative expression of *AtbHLH68*. mRNAs were extracted from Col-0, *bHLH68ox-1*, *bHLH68ox-2* seedlings grown *in vitro* for 10 days under long day conditions. The mRNA contents are expressed relative to those of *AtTIP41* and normalized to the value of Col-0 seedlings, which was arbitrarily set to 1. Values represent means \pm SE from three biological replicates. * indicate a significant difference ($P < 0.05$) from Col-0 according to Student's *t*-test. (B) The presence of full-length mRNA was checked in *bhlh68-1* mutant as described in Experimental procedures. The *Acyl Protein Thioesterase 1 (AtAPT1)* was used as an internal control. (C-E) Morphology of 12-day-old *in vitro* seedlings of the different genotypes. In panel C, the small white squares point to panel D (Col-0 root) or panel E (overexpressing line root). The scale bar represents 1 cm. (F-I) Primary root length, lateral root number and lateral root primordia number in Col-0, *bHLH68ox-1* and *bHLH68ox-2* overexpressing plants respectively. * indicates significant difference ($P < 0.05$) from Col-0 according to Student's *t*-test. Error bars indicate the SD ($n > 30$). (J) Morphology of 4-week-old plants grown in soil. The scale bar represents 1 cm.

Fig. 2. Effect of exogenously applied NAA or ABA on root length and the density of lateral roots or lateral root primordia in Col-0, *bhlh68-1* and *bHLH68ox* lines. (A–F) Seedlings were grown in *in vitro* conditions for 12 days on a medium containing either 0.01 μM or 0.1 μM of NAA (A–C) or 0.3 μM or 1 μM of ABA (D–F). The primary root length was measured (A and D). The number of lateral roots (LR) or the number of lateral root primordia (LRP) were also recorded and presented as the ratio of the number of LRs per cm of primary root length (B and E) and number of LRPs per cm of primary root length (C and F). Error bars indicate the SD ($n = 20$). * indicates a significant difference ($P < 0.05$) between the genotype without hormone treatment (designed “control”) and with hormone treatment according to Student's *t*-test. The circles indicate a significant difference ($P < 0.05$) between the overexpressing line and the WT in the same condition according to Student's *t*-test.

Fig. 3. Altered expression of *AtbHLH68* affects ABA homeostasis. (A) Quantification of the ABA content in shoot parts and root parts of 10-day-old seedlings grown *in vitro*. Values represent mean \pm SE from six biological replicates. * indicates significant difference ($P < 0.05$) from the Col-0 according to Student's *t*-test. (B) Relative expression of *AtAAO3* and *AtCYP707A3* in shoots and roots. mRNAs were extracted from seedlings grown *in vitro* for 10 days. The expression level of each gene is relative to that of the reference gene *AtAPT1* (shoot parts) or *AtUBQ5* (root parts) and normalized to that in Col-0 seedlings,

which was set to 1. Values represent mean \pm SE from three technical replicates. Quantifications performed on two independent growth experiments gave similar results. * indicate significant difference ($P < 0.05$) from Col-0 according to Student's *t*-test. (C) Relative expression of *AtbHLH68* under different hormone treatments. mRNAs were extracted from Col-0 seedlings grown *in vitro* for 7 days prior to a 5-h exposure to 50 μ M ABA, ACC, BRs, 2,4-D, kinetin or GA₃. The expression level of each gene is relative to that of the reference gene *AtTIP41* and normalized to that in Col-0 seedlings, * indicates significant difference ($P < 0.05$) from the Col-0 according to Student's *t*-test. ND: not detected.

Fig. 4. Deregulation of *AtbHLH68* expression affects drought resistance. (A) Box plot representing the average green area (GA) of the Col-0, the mutant *bhlh68-1*, and the over-expressing line *bHLH68ox-1* grown under normally watered conditions (black boxes) or under mild drought stress (red boxes). Values are mean \pm SD; a one-way ANOVA combined to a Tukey's multiple-comparison post-test was used to analyze the different genotypes in the different conditions. * indicates values significantly different from the Col-0 and # indicates significant difference between treated and non-treated plants ($P < 0.05$, $n \geq 30$). (B) Box plot representing the ratio of the green area of treated plants / green area of non-treated plants. Asterisks indicate significant difference ($P < 0.05$) from Col-0 according to Student's *t*-test. (C) Photos of Col-0, *bhlh68-1* and *bHLH68-ox1* plants subjected to a severe drought stress. All plants were grown in soil with sufficient water for 15 days before water was withheld for 20 days. Bar = 5 cm.

Fig. 5. The expression of some ABA signaling genes is altered in *AtbHLH68* mutant and overexpressing lines. Relative expression of *AtMYC2*, *AtbHLH122*, *AtABI3*, *AtRD29A*, *AtWRKY63*, *AtABII*, *AtABF3*, and *AtKIN2* in shoots (A) and in roots (B) of the mutant line *bhlh68-1* and the over-expressing lines *bHLH68ox-1* and *bHLH68ox-2*. mRNAs were extracted from seedlings grown *in vitro* for 10 days. The expression level for each gene is relative to that of the reference gene *AtAPT1* (shoot) or *AtUBQ5* (root) and normalized to that in the Col-0 seedlings, which was set to 1. Values represent mean \pm SD from three technical replicates. Quantifications performed on two independent growth experiments gave similar results. * indicates significant difference ($P < 0.05$) from Col-0 according to Student's *t*-test.

Fig. 6. *AtbHLH68* is expressed in all the organs and preferentially in the vascular system. (A) Relative expression of *AtbHLH68* in different organs. mRNAs were extracted from different parts of Col-0 plants grown in soil for 4 weeks (RL, rosette leaves; CL, cauline leaves; FS, floral stem; F, flowers; YS, young siliques) or from Col-0 seedlings grown *in vitro* for 10 days (A, apical parts; R, root parts). The expression level for *AtbHLH68* is relative to that of the reference gene *AtTIP41*. Values represent mean \pm SD from three technical replicates. Quantifications performed on two independent growth experiments gave similar

results. (B–J) *bHLH68_{pro}GUS* plants were subjected to GUS staining and signals were detected in the vascular system of cotyledons (B), in the central cylinder of the main root (C) as well as in the lateral root at different developmental stages (D–F) of seedlings grown *in vitro*. Black arrows point to the lateral root primordia. In plants grown in soil, a GUS signal was also detected in the vascular system of rosette leaves (G) and cauline leaves (H). In a transverse section of a floral stem, a GUS signal is observed in the phloem cells (I). ep, epidermis; co, cortex; ph, phloem; xy, xylem; if, interfascicular fibers. In flowers, a GUS signal is detected in the vascular system of the petals as well as in the stamens (J).

Table 1. Effect of ABA treatment on the promoter activity of *bHLH68*. Seedlings were grown for 10 days *in vitro*, on a medium containing, or lacking, 0.5 μ M of ABA. GUS activity was quantified as described in Materials and Methods. Data are mean \pm SD from three biological replicates. * indicates significant difference (* $P < 0.05$, *** $P < 0.001$) from the control treatment (without ABA) according to Student's *t*-test.

Organ	ABA treatment	GUS activity (pmol of hydrolyzed MUG min ⁻¹ μ g ⁻¹ proteins)
Root	–	716.13 \pm 13.64
Root	+	907.09 \pm 111.59*
Shoot	–	3457.19 \pm 421.46
Shoot	+	465.09 \pm 144.25***

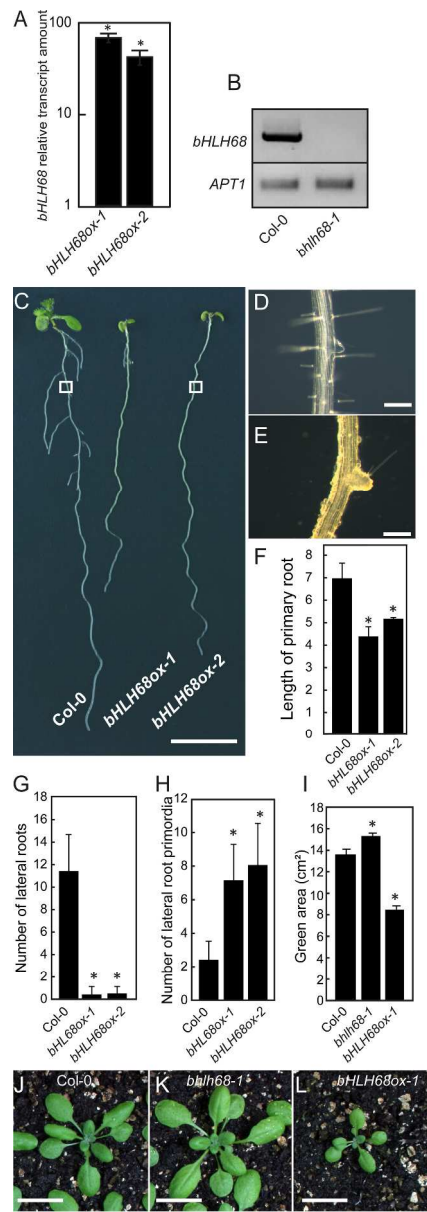


Fig. 1. Phenotypic characterization of bHLH68ox lines.
Fig. 1

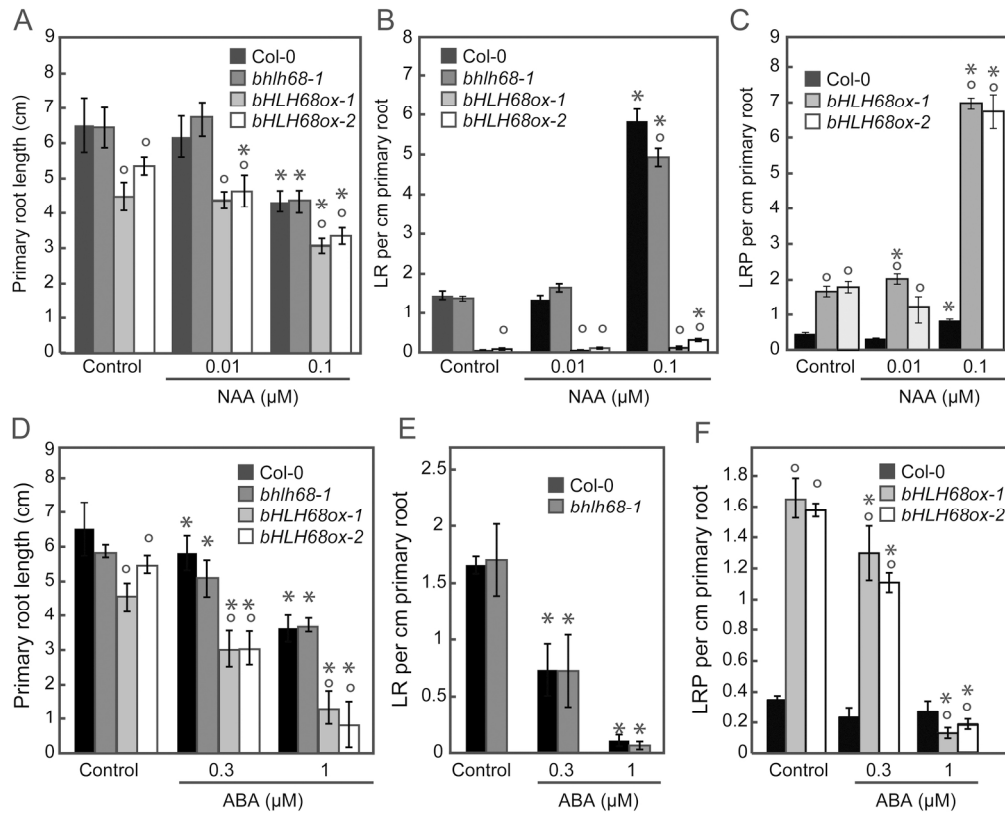


Fig. 2. Effect of exogenously applied NAA or ABA on root length and the density of lateral roots or lateral root primordia in wild type Col-0, *bhlh68-1* and *bHLH68ox* lines.

Fig. 2

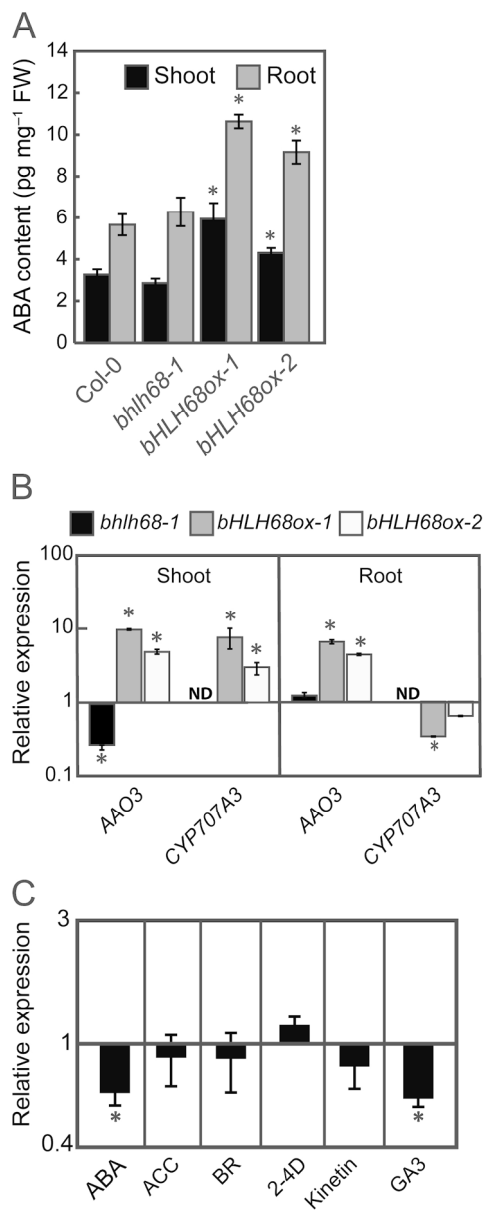


Fig. 3. Altered expression of bHLH68 affects ABA homeostasis.

Fig. 3

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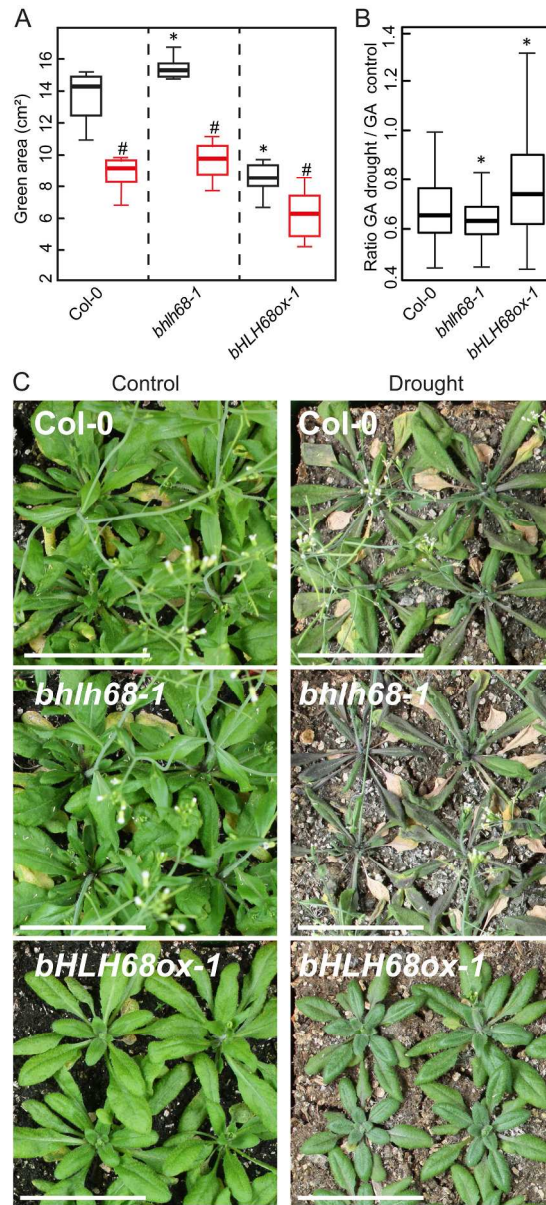


Fig. 4. Deregulation of bHLH68 expression affects drought resistance
Fig. 4

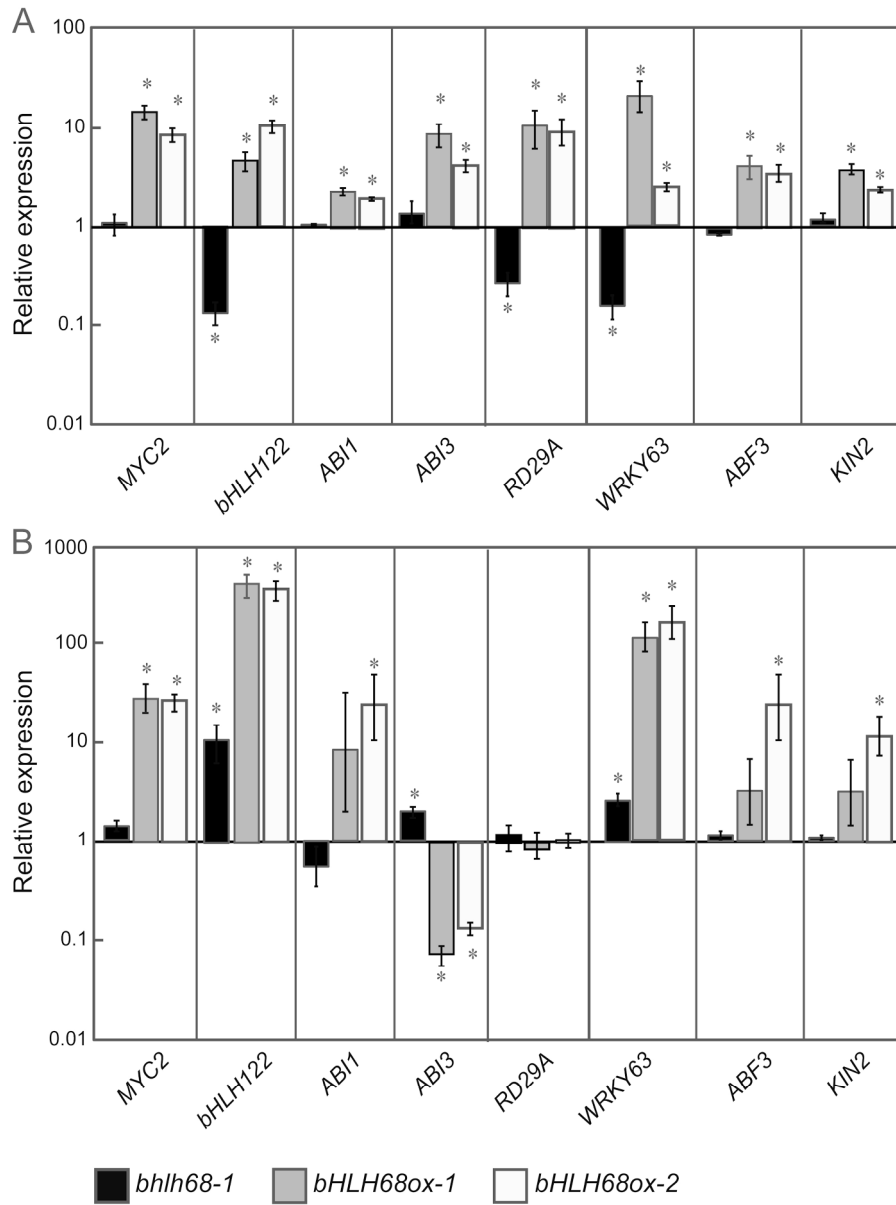


Fig. 5. The expression of some ABA signalling genes is altered in *AtbHLH68* mutant and overexpressing lines
Fig. 5

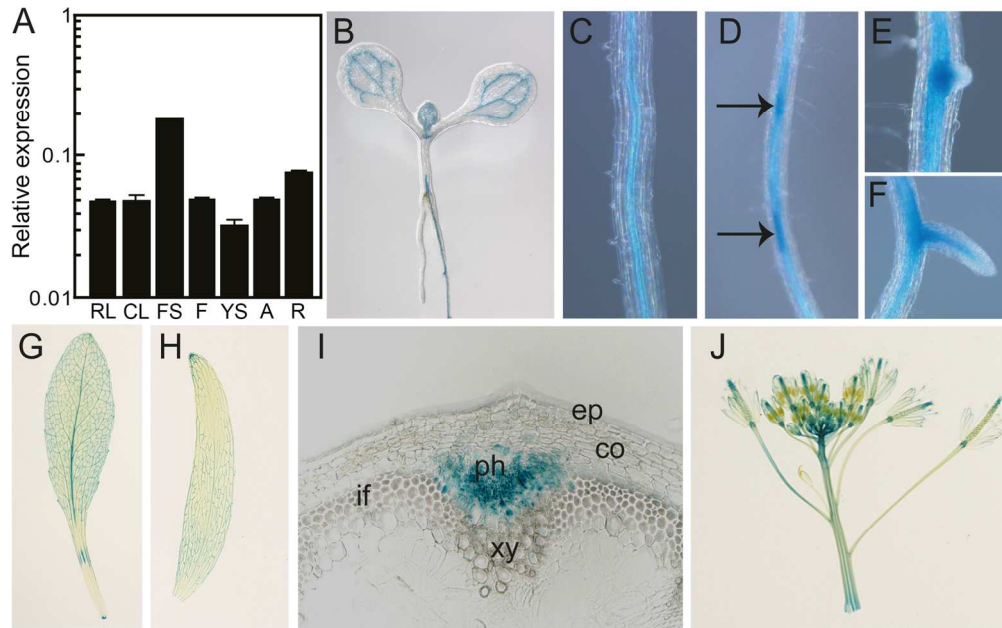


Fig. 6. bHLH68 is expressed in all the organs and preferentially in the vascular system
Fig. 6