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RESEARCH PAPER

HHP1 is involved in osmotic stress sensitivity in *Arabidopsis*

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Abstract

HHP1 (heptahelical protein 1), a protein with a predicted seven transmembrane domain structure homologous to adiponectin receptors (AdipoRs) and membrane progestin receptors (mPRs), has been characterized. Expression of *HHP1* was increased in response to abscisic acid (ABA) and salt/osmotic stress as shown by quantitative real-time PCR and *HHP1* promoter-controlled GUS activity. The *HHP1* T-DNA insertion mutant (*hhp1-1*) showed a higher sensitivity to ABA and osmotic stress than the wild-type (WT), as revealed by the germination rate and post-germination growth rate. The induced expression of stress-responsive genes (*RD29A*, *RD29B*, *ADH1*, *KIN1*, *COR15A*, and *COR47*) was more sensitive to exogenous ABA and osmotic stress in *hhp1-1* than in the WT. The hypersensitivity in the *hhp1-1* mutant was reversed in the complementation mutant of *HHP1* expressing the *HHP1* gene. The data suggest that the mutation of *HHP1* renders plants hypersensitive to ABA and osmotic stress and HHP1 might be a negative regulator in ABA and osmotic signalling.

Key words: ABA, HHP1, mPR, osmotic stress.

Introduction

Plants use an interconnected signalling network to cope with the abiotic stresses of drought, salinity, and cold (Chinnusamy *et al.*, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006). Osmotic stress and cold stress are perceived by either ABA-dependent or ABA-independent pathways. These pathways lead to the expression of some common downstream stress-responsive proteins such as RD29A, COR15A, KIN1, and ADH1 (Kurkela and Franck, 1990; Jarillo *et al.*, 1993; Yamaguchi-Shinozaki and Shinozaki, 1993, 1994; Stockinger *et al.*, 1997; Ishitani *et al.*, 1998). Many genes that are either involved in the signalling pathways or directly responsible for the stress tolerance are identified through transcriptome analysis. However, more signalling components that participate in the plant responses to abiotic stresses are expected, due to the complex environment that plants are facing. One such candidate is HHP1 (heptahelical transmembrane protein 1) which is induced in the transcription level with salt treatment (Hsieh and Goodman, 2005).

HHP1 is a member of the HHP family from *Arabidopsis* which comprises at least five members HHP1, HHP2, HHP3, HHP4, and HHP5 (Hsieh and Goodman, 2005). The HHP protein family is homologous to the PAQR family of proteins which include the membrane progestin receptor (mPR), adiponectin receptor (AdipoR), and YOL002c (Yamauchi *et al.*, 2003; Zhu *et al.*, 2003; Lyons *et al.*, 2004; Tang *et al.*, 2005). The wide distribution of the PAQR family members indicates that they may have diversified from a common ancestor and evolved to have different functions. The mPR protein is involved in a novel 'non-genomic' signalling pathway for steroid hormones. In animals, steroid hormones transmit signals through members of the nuclear receptor protein super-family. This is mainly attributed to the hydrophobic nature of steroids, which allows them to cross cell membranes without help from proteins. However, many lines of evidence have demonstrated the presence of an alternative signalling pathway for steroids through membrane-bound receptors (Falkenstein *et al.*, 2000). This type of signalling is not blocked by transcriptional inhibitors and is therefore described as the 'non-genomic action' of steroids. The AdipoR is the receptor of

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adiponectin which has an antidiabetic effect (Yamauchi *et al.*, 2003). The YOL002c is proposed to regulate the homeostasis of zinc ions through its role in the metabolism of sterol (Lyons *et al.*, 2004). We are interested in how the PAQR proteins have evolved across animals and plants and the possible role of PAQR homologues in salt sensing or signalling.

The mRNA expression patterns of the five *HHP* genes in response to phytohormones, sucrose, temperature, and salt have been described (Hsieh and Goodman, 2005), however, further experiments are needed to elucidate the functions of the HHP family. HHP proteins are identified from the primary structural analysis by the seven predicted transmembrane domains and the lack of similarity to G protein-coupled receptors. In *Arabidopsis*, there are at least 168 proteins which possess seven transmembrane domains based on TransMembrane Spanning (TMS) domain prediction (Ward, 2001). To avoid the complexity that was raised from the diversity of function within the HHP family and to narrow down our target, the AtHHP1 was chosen in this study, which is the closest homologue of mPR in *Arabidopsis*. The five AtHHP proteins fall into three groups based on phylogenetic analysis. Among them, AtHHP2 and AtHHP3 form one group (77.3% similarity between them), and AtHHP4 and AtHHP5 form another group (96.1% similarity between them) (Hsieh and Goodman, 2005). The sequence similarities between AtHHP1 and the other two groups are 52.2% and 44.0%, respectively (Hsieh and Goodman, 2005).

Due to the fact that expression levels of *HHP* genes are differentially regulated by the treatment of salt, an attempt has been made to explore the possible roles played by HHP1 in high salinity stress signalling. High salinity stress sensing and tolerance share some common signalling components with other abiotic stresses such as drought and cold. There are at least six signalling pathways present in plants when they are exposed to drought or high salinity stresses. Of these pathways, three are independent of ABA and the other three are ABA-dependent (Shinozaki and Yamaguchi-Shinozaki, 2007). DRE (dehydration-responsive element)/CRT (C-repeat element) and ABRE (ABA-responsive element) are the major *cis*-acting elements in the ABA-independent and ABA-dependent signalling pathways, respectively (Zhu *et al.*, 2007). Other *cis*-acting elements include MYCR (MYC transcription factor recognition sequence), MYBR (MYB transcription factor recognition sequence), NACR (NAC recognition sequence), ZFHDR (zinc-finger homeodomain recognition sequence) (Yamaguchi-Shinozaki and Shinozaki, 2006; Chinnusamy *et al.*, 2007; Zhu *et al.*, 2007). Osmotic and cold stresses lead to the expressions of several kinds of genes including the ABA-signalling genes (*ABI1*, *ABI2*, *ABI3*, and *ABI5*), ABA biosynthetic genes (*NCED3*, *AAO3*, *ABA1*, and *ABA3*), and common downstream stress-responsive genes (*RD29A*, *RD29B*, *ADH1*, *KIN1*, *COR15A*, and *COR47*) (Kurkela and Franck, 1990; Jarillo *et al.*, 1993; Yamaguchi-Shinozaki and Shinozaki, 1993, 1994; Stockinger *et al.*, 1997; Ishitani *et al.*, 1998).

In this study, it is demonstrated that the HHP1-defective mutant (*hhp1-1*) had a higher sensitivity to ABA and osmotic stress in terms of germination rate and post-germination growth rate. The higher expression of the downstream stress-responsive genes was compatible with the phenotype. It is concluded that HHP1 might play a role in osmotic stress signalling and act as a negative regulator. The possibility that HHP1 might serve as a novel signalling component of osmotic signalling is discussed.

Materials and methods

Sequence analysis

The sequences of the PAQR protein family were retrieved by a BLAST (Altschul *et al.*, 1990) search based on the sequence of HHP1 against the databases of SwissProt/TrEMBL. The transmembrane predictions and domain analysis were conducted by using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and the Pfam protein families database (Finn *et al.*, 2008). The phylogenetic dendrogram (phylogram) is generated from ClustalW2 alignment (Larkin *et al.*, 2007) following by the TreeView program (Page, 1996).

Plant materials and growth conditions

A T-DNA insertion mutant of *HHP1*, SALK_056174 (*hhp1-1*), derived from *Arabidopsis thaliana* (Col-0, used as WT), was used in this study. Seeds were surface-sterilized with 1% sodium hypochlorite and 0.5% Tween 20 and washed with sterile water. Stratification was performed by plating seeds on 1/2 MS medium containing 3% (w/v) sucrose and 0.8% phytagar and incubating them at 4 °C for 4 d, then the plates were transferred to a growth chamber at 22 °C and 50–60% relative humidity (RH) under long day conditions (16/8 h light/darkness). After 10 d, the seedlings were transferred to soil and incubated at 22 °C and 50–60% RH under the long day conditions.

Cloning of the HHP1 *CDS (coding sequence)*

Total RNA was extracted from 4-d-old WT *Arabidopsis* seedlings using TRIzol reagent (Invitrogen, USA) and converted to cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen, USA) and an oligo$(dT)_{15}$ primer. The coding sequence of *HHP1* was amplified by the polymerase chain reaction (PCR) using the primer pairs 5′-GAATTCATGGACCAAAATGGTCATAACGACGAA-3′ (*HHP1* forward) and 5′-CTCGAGTTAACAACCAACGTGGTCACGCCA-3′ (*HHP1* reverse). Then, the coding sequence was cloned into the pGEM-T vector (Promega, USA) and sequenced.

Complementation of hhp1-1

The coding sequence of *HHP1* was amplified by sticky-end PCR (Zeng, 1998) using the primer pairs 5′-CATGGACCAAAATGGTCATAACGACGAAGCA-3′ (*HHP1_C*

5′ primer_1) plus 5′-TTTAACAACCAACGTGGTCAC-GCCAGT-3′ (*HHP1_C* 3′ primer_1) and 5′-GACCAAA-ATGGTCATAACGACGAAGCAGAAA-3′ (*HHP1_C* 5′ primer_2) plus 5′-CTAGTTTAACAACCAACGTGG-TCACGCCAGT-3′ (*HHP1_C* 3′ primer_2), and cloned into the *Nco*I and *Spe*I sites of the binary vector pCAMBIA1302. The resulting construct was sequenced and transferred into *hhp1-1* plants via *Agrobacterium* GV-3101-mediated *in planta* transformation (Bent, 2000) to complement *hhp1-1*. Transformant seedlings of the complemented *hhp1-1* (c-*hhp1-1*) T_1 lines and subsequent germinations were selected for hygromycin resistance and the expression of full-length *HHP1* CDS using RT-PCR. Homozygous T_3 lines were used for further phenotypic analysis.

Treatments with different concentrations of phytohormones and salt

Surface-sterilized seeds of the WT were grown on 1/2 MS agar plates as above, then, after 10 d, the seedlings (stage 1.04) were immersed in ddH_2O supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), indole 3-acetic acid (IAA), 1-aminocyclopropanecarboxylic acid (ACC), 6-benzylaminopurine (BA), kinetin, (±)-*cis*, *trans*-abscisic acid (ABA), gibberellic acid (GA_3), jasmonic acid (JA), salicylic acid (SA), 22(*S*),23(*S*)-homobrassinolide (HBL), or NaCl. After incubation for the indicated time, the seedlings were harvested, fixed in liquid nitrogen and stored at –80 °C for RNA extraction.

Effects of ABA or osmotic stress on germination rate, post-germination growth efficiency, and salt stress sensitivity

To determine the effect of ABA or osmotic stress on germination, surface-sterilized seeds of Arabidopsis WT, *hhp1-1*, and c-*hhp1-1* mutants were grown on minimal medium supplemented with different concentrations of ABA, NaCl, or mannitol at 22 °C under the long day conditions. A seed was considered as germinated when the radicle penetrated the seed coat by 1 mm. The percentage of germinated seeds was scored daily in three independent experiments (60 seeds per experiment). Seeds from WT and mutants used for phenotypic analysis were harvested and collected in the same time, and stored in a humidity-controlled dry box.

To determine the effect of ABA or osmotic stress on post-germination growth, plants grown and treated under the conditions described above were harvested at the 12th day, and the seedlings with cotyledon greening were scored in three independent experiments (60 seeds per experiment).

To determine the salt-stress sensitivity of *Arabidopsis* seedlings to NaCl, 6-d-old seedlings of the WT, *hhp1-1*, and c-*hhp1-1* mutants grown on minimal medium were transferred to minimal medium supplemented with different concentrations of NaCl and grown at 22 °C under the long day conditions. After 6 d, the survival rate of the 12-d-old seedlings was scored in three independent experiments (60 seedlings per experiment).

Chlorophyll measurement

Seedlings were treated with ABA, NaCl, or mannitol from day 1 for 12 d, then chlorophyll was extracted from the leaves with 80% acetone and the chlorophyll content was determined by measuring the absorption at 663 and 645 nm according to Arnon (Arnon, 1949).

Quantitative real-time PCR analysis of HHP1 *or stress-responsive gene expression profiles*

Total RNA was isolated from *Arabidopsis* WT, *hhp1-1*, and c-*hhp1-1* mutants that had undergone various treatments (phytohormones, osmotic stress, or cold) using the pine tree method (Chang *et al.*, 1993), followed by removal of genomic DNA with TURBO DNA-free kits (Ambion, USA). First-strand cDNA (fs-cDNA), synthesized using SuperScript™ III reverse transcriptase (Invitrogen, USA) and an oligo$(dT)_{15}$ primer, was used as the template for real-time PCR using gene-specific primers designed by using Primer Express 2.0 (Applied Biosystems). *ACTIN2* was used as the internal control in the same cDNA sample. The gene-specific primers used to produce a single amplicon of about 70 bp were: *HHP1* (5′-CCCCGTGGATGCAA-AGAG-3′ and 5′-TGAGCCTCCTAAGAAAACGAAGA-3′), *RD29A* (5′-TGATCGATGCACCAGGCGTAAC-3′ and 5′-CCTGGTGGAATAATTTCCTCCG-3′), *RD29B* (5′-CGCCACGGTCCGTTGA-3′ and 5′-TCCACCGGA-ATCCGAAAAC-3′), *ADH1* (5′-ATTCGACCAAGCTAA-GGAATTCGG-3′ and 5′-CACCTGTTGAATTGGCTTGT-CATG-3′), *KIN1* (5′-ATGCGAAAGATCAAACTCCC-CAAA-3′ and 5′-TTCGGATCGACTTATGTATCGTGA-3′), *COR15A* (5′-CAGTGAAACCGCAGATACATTGGG-3′ and 5′-GGCTTCTTTTCCTTTCTCCTCC-3′), *COR47* (5′-GGAGTACAAGAACAACGTTCCCGA-3′ and 5′-TG-TCGTCGCTGGTGATTCCTCT-3′), *ABI3* (5′-GGCAGG-GATGGAAACCAGAAAA-3′ and 5′-GGTTACCCACGT-CGCTTTGCTT-3′), *ABI5* (5′-TGCTAAAAGGACAG-GAGGAGGAGG-3′ and 5′-AACGCCACCTCCATAGCA-AACA-3′), *NCED3* (5′-ATGGCTTCTTTCACGGCAAC-3′ and 5′-GACGATAATGGCGGCTGAGTATGA-3′), *AAO3* (5′-TCGGCGAGTACATTGTATAAGCCA-3′ and 5′-AC-TTCCACCTCGCTGACTCCAACT-3′), *ABA1* (5′-GTGAT-CGGATTAACGGTCTCGTTG-3′ and 5′-TGACGCCGC-AGGAGTGAAAGTAT-3′), *ABA3* (5′-TGCTACAAGGC-TCCCCCTTT-3′ and 5′-CACAACCCTTTGCAGCATCA-3′), and *ACTIN2* (5′-TGTGGATCTCCAAGGCCGAGTA-3′ and 5′-CCCCAGCTTTTTAAGCCTTTGATC-3′).

Real-time PCR was performed according to the manufacturer's protocol using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with ABsolute™ QPCR SYBR Green Mixes (ABgene, UK) in a final volume of 20 μl with 500 nM ROX reference dye. The thermal cycling programme was 95 °C for 15 min, followed by 40 cycles of 95 °C for 20 s, 55 °C for 20 s,

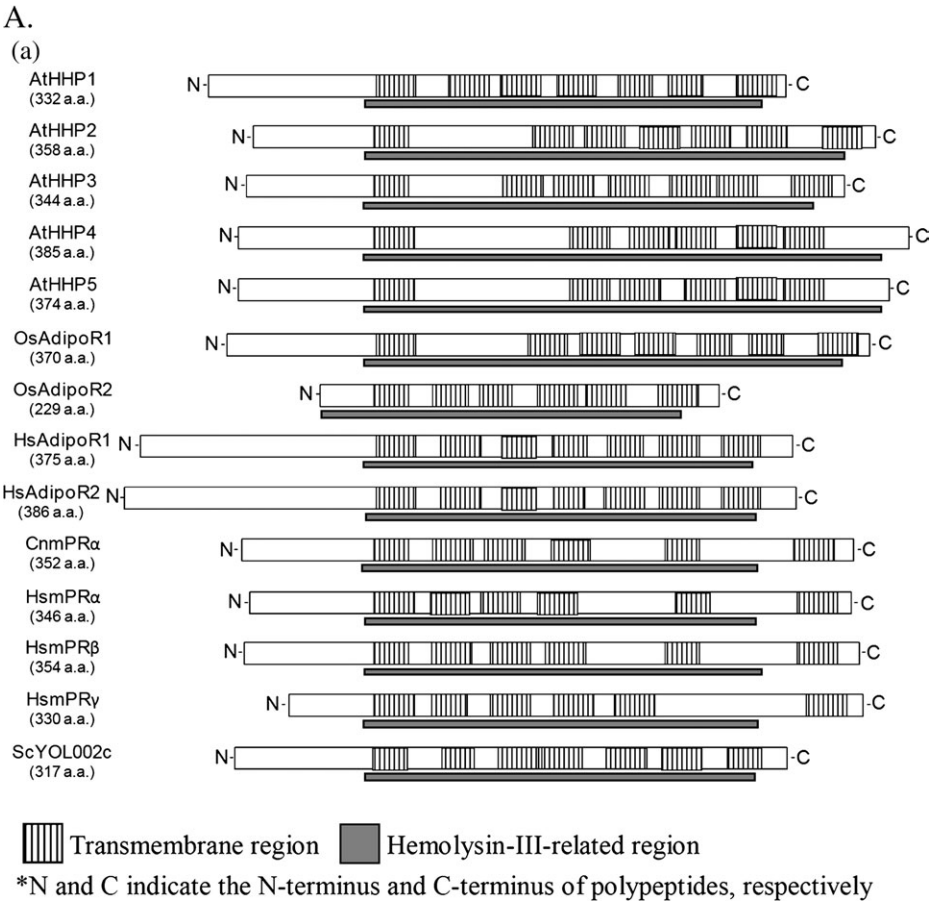

Fig. 1. (A) Predicted transmembrane regions (TM) of HHP1 and other members of the PAQR family. The 7TM and haemolysin-III-related domains are indicated (a). A phylogenetic tree was generated from the multiple alignment of five AtHHPs (AtHHP1, NP_197527; AtHHP2, NP_194814; AtHHP3, NP_565564; AtHHP4, NP_195483; AtHHP5, NP_195545), two rice putative adiponectin receptors (OsAdipoR1, BAD37427; OsAdipoR2, BAD28011), two human adiponectin receptors (HsAdipoR1, NP_057083; HsAdipoR2, NP_078827), three human membrane progestin receptors (HsmPRα, NP_848509; HsmPRβ, NP_588608; HsmPRγ, NP_060175), spotted seatrout membrane progestin receptor (CnmPRα, AAO39265), and PAQR protein from *Saccharomyces cerevisiae* (ScYOL002c, NP_014641) (b). (B) Schematic representation of the genomic organization of *HHP1*. The position of the T-DNA insertion in the *hhp1-1* mutant (SALK_056174) is indicated by black arrows. (C) RT-PCR products of *HHP1* in the WT, two lines of *hhp1-1* T11 (*hhp1-1*_T11_1 and 2) and three lines of complemented *hhp1-1* T1 (c-*hhp1-1*_T1_1, 2 and 3) mutants using specific primers of *HHP1* CDS. At, *Arabidopsis thaliana*; Cn, *Cynoscion nebulosus*; Hs, *Homo sapiens*; Os, *Oryza sativa*; Sc, *Saccharomyces cerevisiae*.

and 72 °C for 30 s. Each first-strand cDNA was analysed in triplicate by real-time PCR. All PCR products were verified to be single fragments using dissociation curves and analysis on an agarose gel stained with ethidium bromide. All the amplified fragments were cloned into pGEM-T for DNA sequencing, and the sequences were identical to those of the corresponding region of the target genes. Relative quantification of gene expression was performed by the comparative C_T method, which uses arithmetic formulae to establish standard curves (Perkin-Elmer User Bulletin 2). The relative fold expression changes were calculated as $2^{-\Delta\Delta C_T}$. Validation experiments were performed to demonstrate that the efficiencies of target (*HHP1* and stress-responsive genes) and reference (*ACTIN2*) amplification were approximately equal to those obtained using the $\Delta\Delta C_T$ calculation.

HHP1 *promoter*::GUS (HHP1::GUS) *assay*

A *HHP1* promoter fragment from 1754 bp to 1 bp upstream the translation initiation codon was amplified from genomic DNA prepared from the WT seedlings by sticky-end PCR using the primer pairs: 5′-AATTCGCTA-CAACTATGGCCA-3′ (*HHP1_P* 5′ primer_1) plus 5′-GAAGAGCTCTCGAGTATCGA-3′ (*HHP1_P* 3′ primer_1) and 5′-CGCTACAACTATGGCCATTTTA-3′ (*HHP1_P* 5′ primer_2) plus 5′-CATGGAAGAGCTCTC-GAGTA-3′ (*HHP1_P* 3′ primer_2). The PCR product was cloned into the *Eco*RI and *Nco*I sites of the binary vector pCAMBIA1381Z and the resulting construct was sequenced and transferred into WT *Arabidopsis* plants via *Agro-bacterium* GV-3101-mediated *in planta* transformation (Bent, 2000). Transformant seedlings of the *HHP1::GUS*

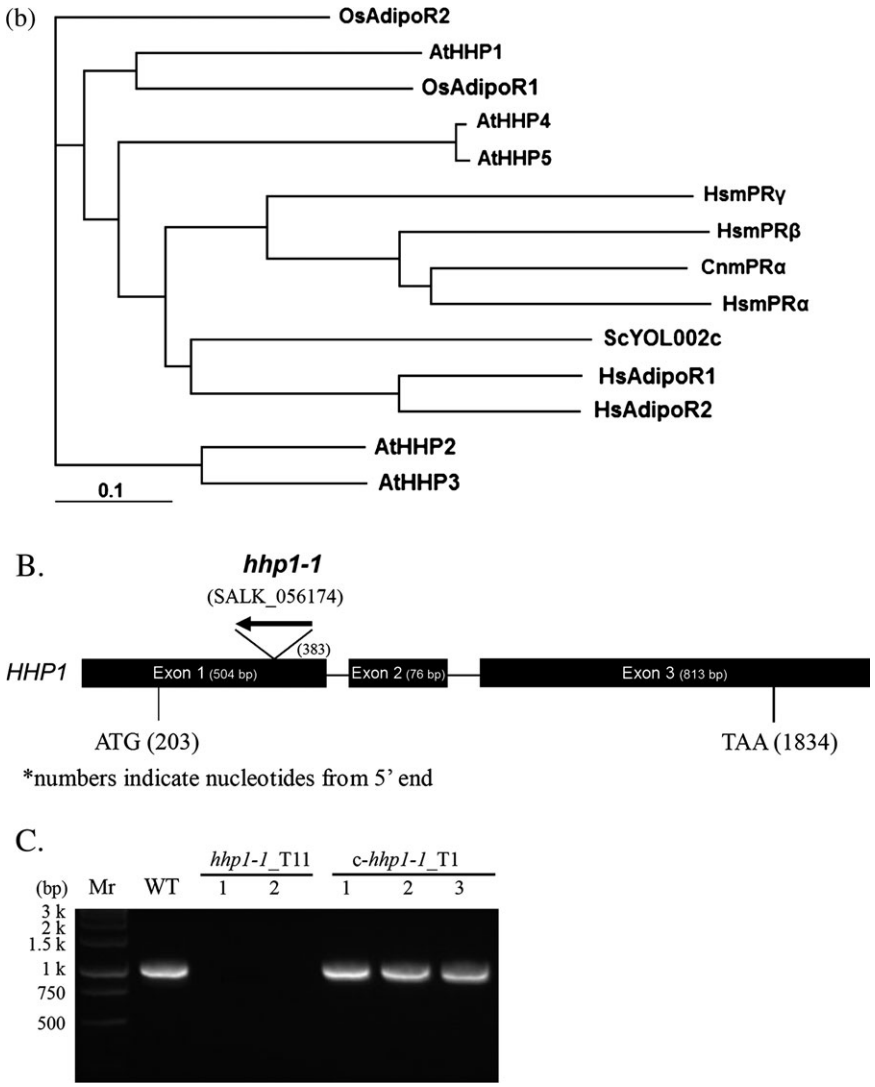

Fig. 1. (*Continued*)

T_1 lines and subsequent germinations were selected for hygromycin resistance and β-glucuronidase (GUS) activity using GUS staining. To analyse GUS activity of *HHP1::GUS* transgenic mutants, eight independent homozygous T_3 lines were first germinated and grown on 1/2 MS medium containing 3% (w/v) sucrose and 0.8% phytagar for 10 d under the growth condition as above. Seedlings were then transferred to a new liquid 1/2MS medium containing 3% (w/v) sucrose without (control) or with 50 μM each of 2,4-D, IAA, ACC, BA, kinetin, ABA, GA_3, JA, SA, or 10 μM of HBL, 100 mM or 300 mM of NaCl as above. These seedlings were treated for 5 h until assay.

For GUS staining, seedlings were vacuum-infiltrated in the X-Gluc solution (0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 50 mM phosphate buffer, pH 7.4, 0.3% Triton X-100 and 1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide) for 15 min at room temperature and then incubated at 37 °C for 5 h, followed by transferring into 70% (v/v) ethanol to remove chlorophyll. GUS staining patterns were verified in eight independent homozygous T_3 lines, and representative individuals from representative lines were chosen for photography. Seedlings were observed by a Leica $MZ7_5$ stereomicroscope and photographs were taken by an Olympus digital camera C-5050ZOOM (Olympus). Then these images were arranged by using PhotoImpact version 8.0 (Ulead Systems).

For fluorometric GUS activity assays (Jefferson *et al.*, 1987), WT and three independent homozygous *HHP1::GUS* T_3 lines were grown and treated as above. The treated seedlings were harvested and ground in 250 μl extraction buffer (50 mM NaH_2PO_4 buffer, pH 7.4, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% v/v SDS, and 0.1% v/v Triton X-100) by freezing with liquid nitrogen and grinding with a mortar and pestle. The extracts were centrifuged at 20 000 *g* at 4 °C for 15 min, then 10 μl of supernatant was transferred to 300 μl GUS assay buffer (1 mM 4-methyl umbelliferyl β-D-glucuronide (MUG) in the extraction buffer). The reaction was incubated at 37 °C for 2 h, followed by adding 300 μl of 200 mM Na_2CO_3, then the fluorescence of 4-methylumbelliferone (MU) was determined

by using a fluorimeter (Beckman Coulter DTX880 Multimode Detector) with excitation at 365 nm and emission at 455 nm. The fluorimeter was calibrated with freshly prepared MU standards of different concentrations in the extraction buffer. The concentrations of total proteins in plant extracts were determined by using Bradford's method.

Results

Isolation of T-DNA insertion mutant of HHP1 *and the complementation test*

The most distinguishing feature of HHP1 and other PAQR proteins is that they are predicted to contain seven TM domains. HHP1, consisting of 332 amino acids annotated in the TAIR (The *Arabidopsis* Information Resource) as the expressed protein containing a haemolysin-III-related domain (Fig. 1Aa). A phylogenetic tree was generated to depict the relationship between HHP1 and its homologues within the PAQR protein family (Fig. 1Ab). All of these homologues contain the haemolysin-III-related domain comprising seven predicted TMs, an N-terminal domain, a C-terminal domain, and six loops connecting TMs (Fig. 1Aa). The length of the HHP1 is comparable to those of their PAQR homologues, for example, mPR from human (NP_848509; 346 amino acids), adiponectin receptor from human (ADR1, NP_057083; 375 amino acids), and YOL002c from *Saccharomyces cerevisiae* (IZH2; 317 amino acids). The location of the seven predicted TM α-helices determines the sizes of the N- and C-terminal domains and the loops connecting TMs. HHP1 has a longer N-terminal segment comprising 96 amino acids, a shorter C-terminal segment comprising six amino acids and six short loops, ranging from five to 20 amino acids, connecting TMs. In order to investigate the physiological function of the *HHP* gene family, a search for T-DNA insertion mutants from ABRC stocks was made using the SIGnAL 'T-DNA Express' *Arabidopsis* Gene Mapping Tool (http://signal.salk.edu). Plants from seeds of the *AtHHP1* mutant (SALK_056174) were screened for homozygous single T-DNA insertions. Individual homozygous plants derived from the SALK line were identified by PCR screening using primers specific for the *AtHHP1* gene and the left border primer of the T-DNA insertion. Fifteen homozygous plants derived from the SALK_056174 T_4 lines were confirmed by RT-PCR not to express full-length *HHP1* CDS. The single T-DNA insertion lines, SALK_056174_10 T_7 were verified using inverse PCR (Does *et al.*, 1991). The T-DNA insertion position in exon 1 (SALK_056174) of *HHP1* was examined by sequencing (Fig. 1B). The plant line derived from SALK_056174_10 T_7 was named *hhp1-1* (Fig. 1B). The *hhp1-1* mutant was used as the *HHP1* knockout mutant because it did not express the full-length *HHP1* CDS as revealed by RT-PCR using specific primers of *HHP1* CDS (Fig. 1C). In addition, a WT copy of the *HHP1* CDS in the form of *35S::HHP1* was introduced back into the *hhp1-1* plants to generate transgenic mutants named as complemented-*hhp1-1* (c-*hhp1-1*) for complementation experiments. T_1 transgenic complemented *hhp1-1* plants were selected by hygromycin resistance. The expression of full-length *HHP1* CDS in mutants was analysed using RT-PCR (Fig. 1C) and the products of RT-PCR were verified by DNA sequencing. The expression of *HHP1* driven by the 35S promoter in the c-*hhp1-1* was 0.7–3-fold higher than in the WT (data not shown). The *hhp1-1* T_{11} lines and the homozygous complemented *hhp1-1* T_3 lines were used for further phenotypic analysis. Although there is no significant difference in phenotype observed between mutants and WT, *hhp1-1* exhibited some interesting features such as less apical meristem dominance, a shorter length of hypocotyl, and a more curled cotyledon (data not shown). These specific traits of *hhp1-1* implied that HHP1 might play a role in the development of *Arabidopsis*.

Effects of phytohormones on HHP1 *expression profiles*

To investigate the effects of phytohormones on the *HHP1* expression, the expression patterns of *HHP1* in 10-d-old (stage 1.04) WT *Arabidopsis* seedlings treated without (control) or with 50 μM auxin (2,4-D or IAA), cytokinin (kinetin or BA), ACC, GA_3, JA, SA, or 10 μM HBL for 3 h (Fig. 2Aa), or with 50 μM ABA for 1, 4, 8, 16, or 24 h (Fig. 2Ab) were analysed by real-time PCR. *ACTIN2* (*ACT2*) was used to normalize the expression of *HHP1* while the expression patterns were shown as the relative fold expression compared to that in the untreated WT (control). In the WT, *HHP1* expression was increased significantly by ABA (Fig. 2Ab) while the other phytohormones had little effect (Fig. 2Aa). It is interesting that *HHP1* expression was rapidly increased to 6-fold by ABA within 1 h but the degree of the induction was gradually decreased to 2-fold after longer ABA treatment (Fig. 2Ab). *HHP1* expression was also examined by β-glucuronidase (GUS) activity staining in homozygous *HHP1::GUS* T_3 mutants harbouring the *HHP1::GUS* transgene. The *HHP1* promoter-controlled GUS activity was increased in 10-d-old (stage 1.04) transgenic *HHP1::GUS Arabidopsis* seedlings treated with 50 μM ABA for 5 h as shown by histochemical (Fig. 2B) and quantitative GUS activity analyses (Fig. 2C). These results agreed with those obtained from real-time PCR.

HHP1 *expression is increased by salt stress*

To investigate the effect of salt stress on the *HHP1* expression, the expression profiles of *HHP1* in 10-d-old (stage 1.04) WT *Arabidopsis* seedlings treated without (control) or with 300 mM NaCl (Fig. 3A) for different durations were analysed by real-time PCR. *ACT2* was used to normalize the expression of *HHP1* while the results were presented as the fold expression relative to that of untreated WT (control). In the WT, the expression of *HHP1* was increased 6–12-fold by NaCl treatment (Fig. 3A). An induced expression of *HHP1* was observed within 1 h of

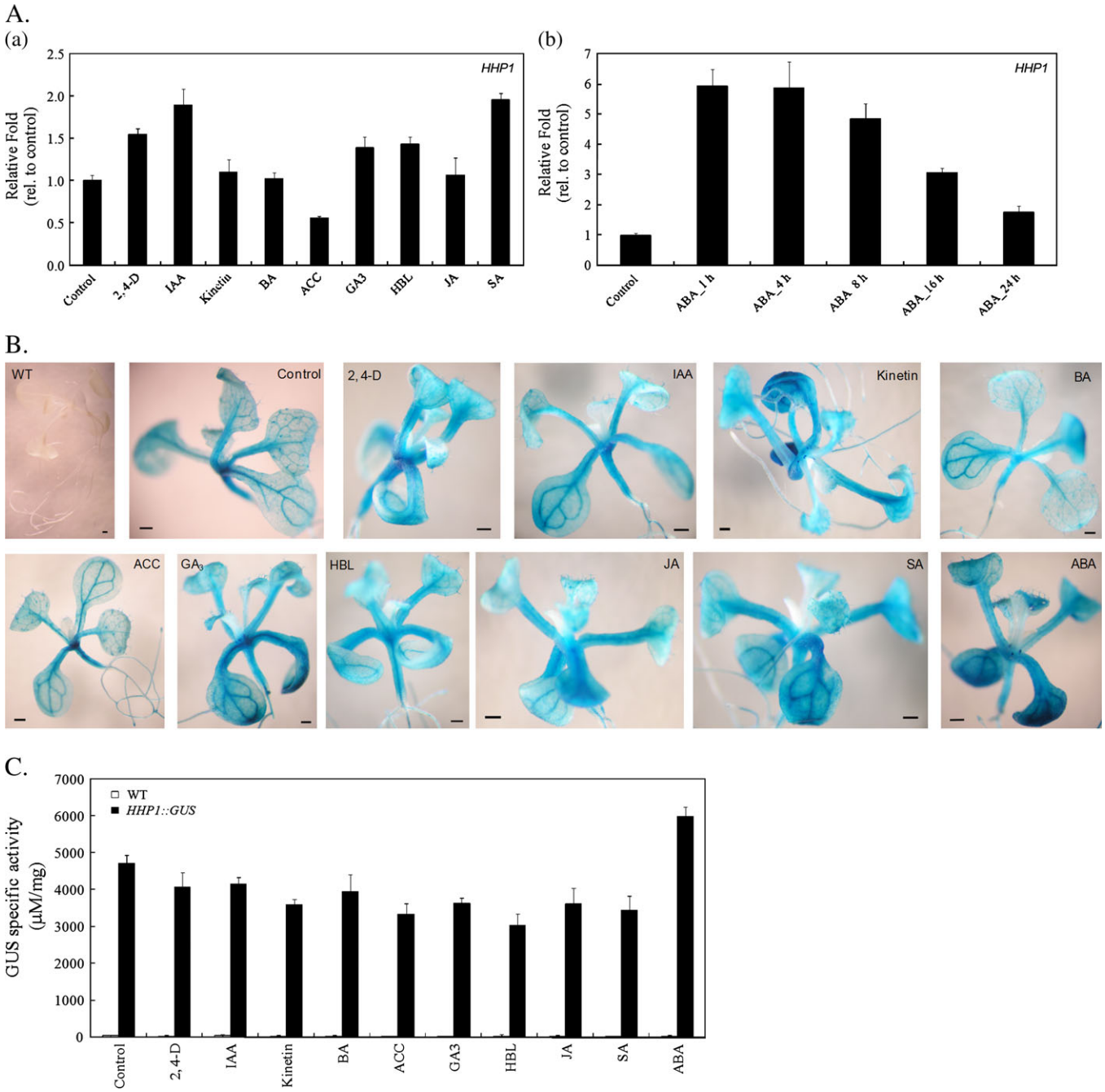

Fig. 2. Expression profiles of *HHP1* in response to various phytohormones. (A) The expression of *HHP1* in 10-d-old (stage 1.04) WT *Arabidopsis* seedlings treated without (control) or with 50 μM auxin (2,4-D or IAA), cytokinin (kinetin or BA), ACC, GA_3, JA, SA, or 10 μM HBL for 3 h (a), or with 50 μM ABA for 1, 4, 8, 16, or 24 h (b) was analysed by real-time PCR. The data are the mean ±standard error for three independent amplification reactions and representative of at least two independent biological replicates, each consisting of 10–15 seedlings. (B) Histochemical analyses and (C) quantitative analyses of β-glucuronidase (GUS) activity in 10-d-old (stage 1.04) WT or transgenic *HHP1::GUS Arabidopsis* seedlings treated without (control) or with 50 μM auxin (2,4-D or IAA), cytokinin (kinetin or BA), GA_3, ABA, ACC, JA, SA, or 10 μM HBL for 5 h. For histochemical analyses, staining was performed for 5 h. The shown GUS staining patterns are representative of a total of eight independent homozygous transgenic *HHP1::GUS* T_3 lines. Scale bars correspond to 1 mm. For quantitative analyses, the values of GUS specific activity are the mean ±standard error of fluorescence of the reaction product 4-methylumbelliferone (MU) normalized to protein concentration for three independent homozygous transgenic *HHP1::GUS* T_3 lines.

high salinity stress, and the high expression level was maintained to a longer treatment (24 h) (Fig. 3A). These results were supported by the analysis of *HHP1* promoter-controlled GUS activity. As shown in Fig. 3, strong GUS activity was present in the 10-d-old (stage 1.04) transgenic *HHP1::GUS Arabidopsis* seedlings treated with salt (100 and 300 mM NaCl) for 5 h as revealed by histochemical (Fig. 3B) and quantitative analyses (Fig. 3C).

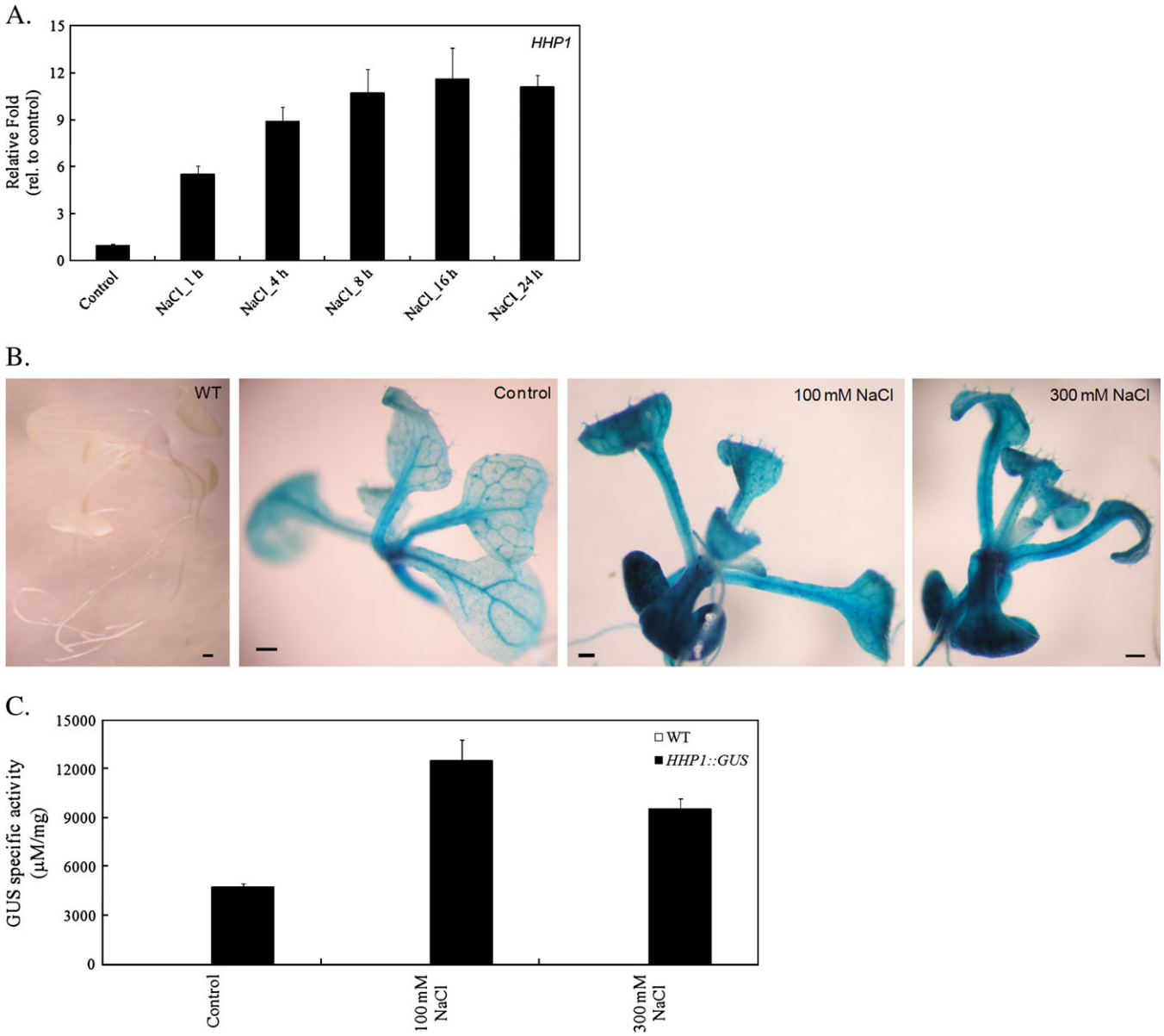

Fig. 3. Expression profiles of *HHP1* in response to salt stresses. (A) The expression of *HHP1* in 10-d-old (stage 1.04) WT *Arabidopsis* seedlings treated without (control) or with 300 mM NaCl for different duration was analysed by real-time PCR. The data are the mean ±standard error for three independent amplification reactions and representative of two independent biological replicates, each consisting of 10–15 seedlings. (B) Histochemical analyses and (C) quantitative analyses of GUS activity in 10-d-old (stage 1.04) WT or transgenic *HHP1::GUS Arabidopsis* seedlings treated without (control) or with salt stress (100 and 300 mM NaCl) for 5 h. For histochemical analyses, staining was performed for 5 h. The GUS staining patterns shown are representative of a total of eight independent homozygous transgenic *HHP1::GUS* T_3 lines. Scale bars correspond to 1 mm. For quantitative analyses, the values of GUS specific activity are the mean ±standard error of fluorescence of the reaction product MU normalized to protein concentration for three independent homozygous transgenic *HHP1::GUS* T_3 lines.

The hhp1-1 *mutant shows higher sensitivity to ABA and osmotic stress*

Our results showed that *HHP1* expression was significantly increased by ABA (Fig. 2) or salt stress (Fig. 3). These results were supported by the fact that the promoter region (–1 to –1754) of *HHP1* contains ABA-responsive element (ABRE)-related element, Dc3 promoter-binding factor (DPBF) element, DRE/CRT (dehydration-responsive element/C-repeat), LTRE (low temperature responsive element), PRE (pro- or hypoosmolarity-responsive element) and MYB and MYC recognition sequences (MYB/C RS), all of which are *cis*-acting elements often found in ABA- or salt-responsive genes (Finkelstein *et al.*, 2002; Chinnusamy *et al.*, 2004; Yamaguchi-Shinozaki and Shinozaki, 2005). The presence of these elements was predicted using PLACE Web Signal Scan (a database of plant *cis*-acting regulatory DNA elements) (Higo *et al.*, 1999). These results implied that HHP1 might be involved in the ABA response and salt stress sensitivity.

ABA plays essential roles in several physiological functions (embryogenesis, seed germination, seed dormancy, lateral root formation, leaf transpiration, fruit ripening, and the transition from vegetative to reproductive growth) and tolerance to abiotic and biotic stresses (Finkelstein *et al.*, 2002; Chinnusamy *et al.*, 2004; Bishopp *et al.*, 2006; De Smet *et al.*, 2006). To elucidate the relationship between HHP1 and ABA, the ABA sensitivity of the *hhp1-1* mutant was investigated using bioassays, including germination efficiency (radicle emergence rate), post-germination growth (cotyledon greening) and chlorophyll content (Fig. 4). In the absence of exogenous ABA, the WT, *hhp1-1*, and c-*hhp1-1* showed similar germination (Fig. 4A), whereas, in the presence of exogenous ABA, especially at 1 μM, the *hhp1-1* showed lower germination efficiency than the WT and c-*hhp1-1* (Fig. 4A, B); at 1 μM ABA, 77% of *hhp1-1* seeds germinated by day 4 compared to 96% of WT and 97% of c-*hhp1-1* (Fig. 4A). During early growth (cotyledon greening) of seedlings exposed to different concentrations of ABA, the *hhp1-1* was more sensitive than the WT and c-*hhp1-1* to exogenous ABA in terms of growth retardation after germination; at 1 μM ABA, 11% of *hhp1-1* seedlings showed cotyledon greening by day 12 compared to 48% of WT and 49% of c-*hhp1-1* (Fig. 4C). To examine ABA sensitivity more precisely, the chlorophyll content of seedlings grown in the presence of ABA from day 1 to day 12 was measured. As shown in Fig. 4D, the reduction in chlorophyll content under the ABA stress was more marked in the *hhp1-1* than in the WT and c-*hhp1-1*. In summary, the *hhp1-1* was more sensitive to ABA than the WT and c-*hhp1-1*. These results suggest that HHP1 may be involved in the response to ABA or play a negative regulatory role in ABA signalling during seed germination and early growth in *Arabidopsis*.

In order to determine whether HHP1 was involved in osmotic stress sensitivity, germination efficiency, post-germination growth efficiency, chlorophyll content, and salt-stress sensitivity were examined in the WT, *hhp1-1*, and c-*hhp1-1* treated with different concentrations of NaCl or mannitol. No significant difference in germination efficiency was seen without NaCl treatment (Fig. 5Aa), but the *hhp1-1* was more sensitive than the WT and c-*hhp1-1* to the germination delay caused by 150 mM NaCl (Fig. 5Ab); at 150 mM NaCl, 70% of *hhp1-1* seeds had germinated by day 4 compared to 87% of WT and 86% of c-*hhp1-1* (Fig. 5Aa). When grown in the presence of NaCl, the *hhp1-1* showed more growth retardation immediately after germination (Fig. 5Ac) and retained less chlorophyll (Fig. 5Ad) than the WT and c-*hhp1-1*. In the salt-stress sensitivity test, the *hhp1-1* had a lower survival rate than the WT and c-*hhp1-1* when

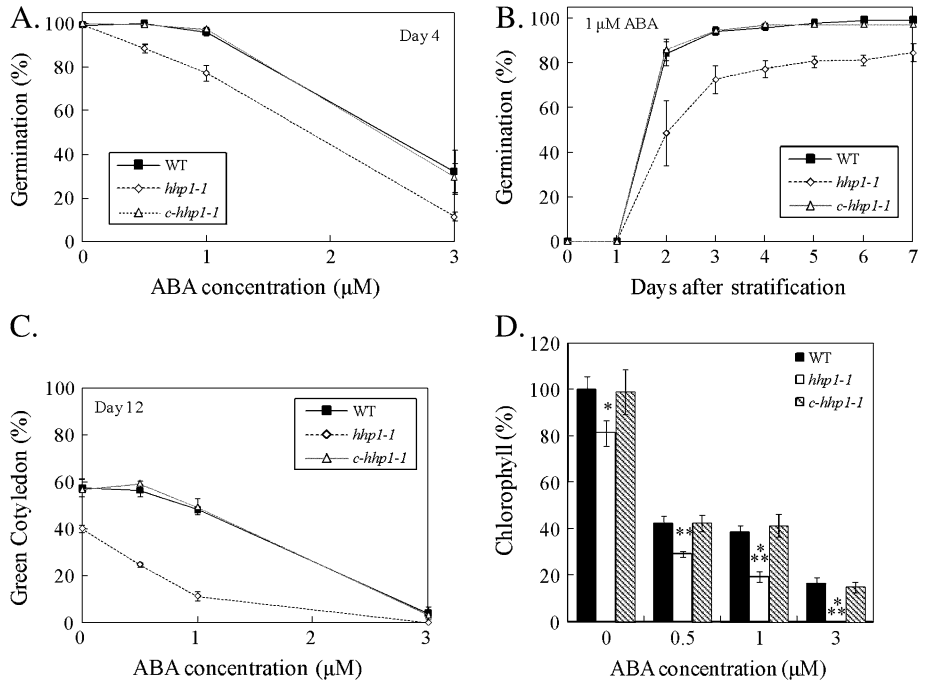

Fig. 4. The *hhp1-1* mutant is more sensitive to ABA than the WT and c-*hhp1-1* mutant. (A, B) Germination efficiency of WT (black squares), *hhp1-1* (white diamonds), or c-*hhp1-1* (white triangles) seeds grown on minimal medium supplemented with different concentrations of ABA (0, 0.5, 1, or 3 μM) for 4 d after stratification (A) or on minimal medium supplemented with 1 μM ABA for 1–7 d after stratification (B). (C) Post-germination growth efficiency of WT, *hhp1-1*, or c-*hhp1-1* seedlings grown on minimal medium supplemented with different concentrations of ABA for 12 d after stratification. (D) Chlorophyll content of WT, *hhp1-1*, or c-*hhp1-1* seedlings grown on minimal medium supplemented with different concentrations of ABA for 12 d after stratification; the ABA-untreated WT content is taken as 100%. The values are the mean ±standard error for three independent experiments, with approximately 60 seeds used per experiment. The asterisks indicate the level of significance of differences between the WT and *hhp1-1* or c-*hhp1-1* under the same growth conditions. (*P <0.05; **P <0.01; ***P <0.005, Student's t test).

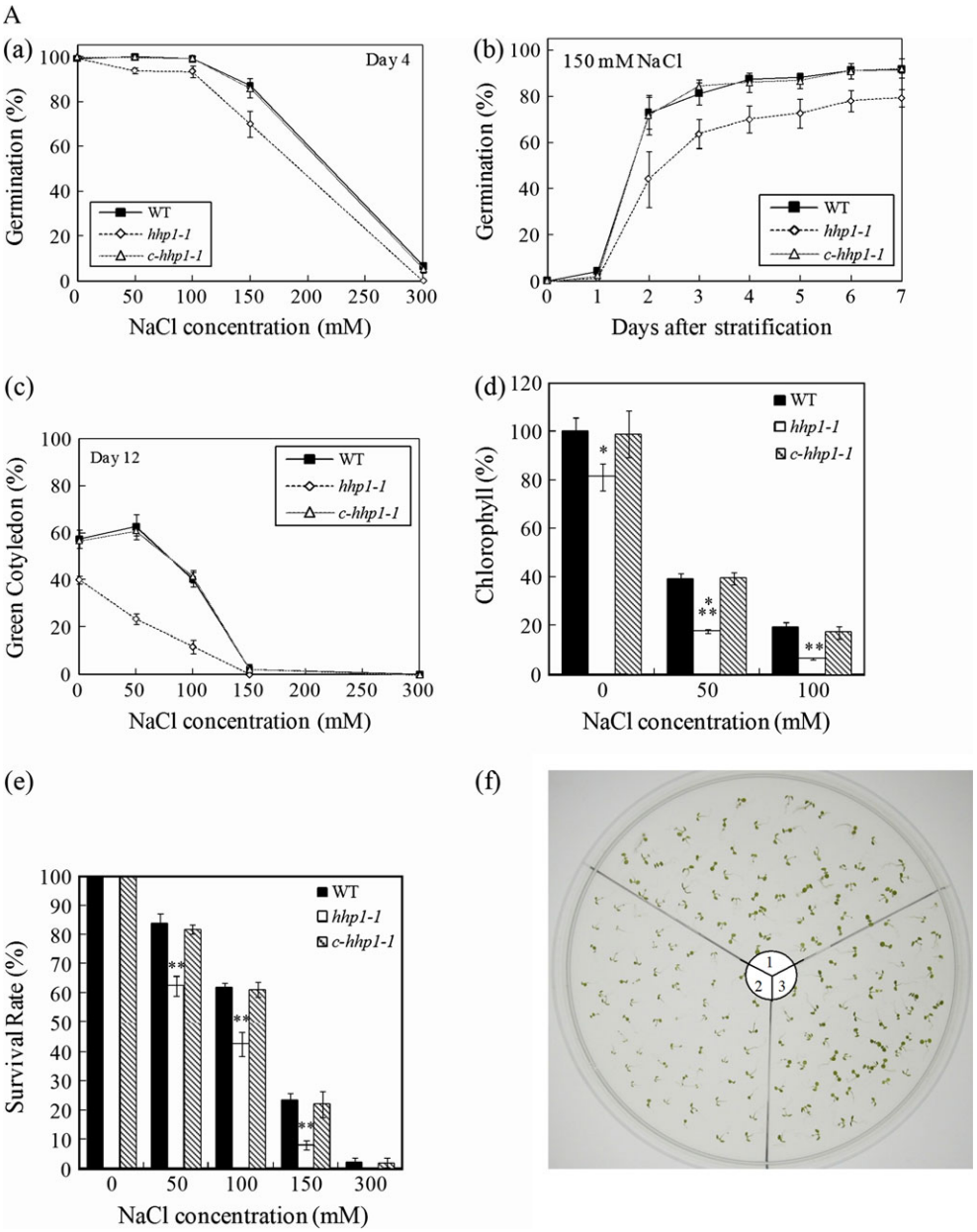

Fig. 5. The *hhp1-1* mutant is more sensitive to osmotic stress than the WT and c-*hhp1-1* mutant. (Aa, Ab) Germination efficiency of WT (black squares), *hhp1-1* (white diamonds), or c-*hhp1-1* (white triangles) seeds grown on minimal medium supplemented with different concentrations of NaCl (0, 50, 100, 150, or 300 mM) for 4 d after stratification (Aa) or on minimal medium supplemented with 150 mM NaCl for 1–7 d after stratification (Ab). (Ac) Post-germination growth efficiency of WT, *hhp1-1*, or c-*hhp1-1* seedlings grown on minimal medium supplemented with different concentrations of NaCl for 12 d after stratification. (Ad) Chlorophyll content of WT, *hhp1-1*, and c-*hhp1-1* seedlings grown on minimal medium supplemented with different concentrations of NaCl for 12 d after stratification; the NaCl-untreated WT content is taken as 100%. (Ae) Survival rate of WT, *hhp1-1*, or c-*hhp1-1* seedlings grown on minimal medium supplemented with different concentrations of NaCl from day 7 to day 12 after stratification. (Af) Photograph of WT (1), *hhp1-1* (2), and c-*hhp1-1* (3) seedlings treated with 50 mM NaCl from day 7 to day 12 after stratification. (Ba, Bb) Germination efficiency of WT, *hhp1-1*, or c-*hhp1-1* seeds grown on minimal medium supplemented with different concentrations of mannitol (0, 100, 200, 300, 400, or 500 mM) for 4 d after stratification (Ba) or on minimal medium supplemented with 200 mM mannitol for 1–7 d after stratification (Bb). (Bc) Post-germination growth efficiency of WT, *hhp1-1*, or c-*hhp1-1* seedlings grown on minimal medium supplemented with different concentrations of mannitol for 12 d after stratification. (Bd) Chlorophyll content of WT, *hhp1-1*, or c-*hhp1-1* seedlings grown on minimal medium supplemented with different concentrations of mannitol for 12 d after stratification; the mannitol-untreated WT content is taken as 100%. The values are the mean ±standard error for three independent experiments, with approximately 60 seeds used per experiment. The asterisks indicate the level of significance of differences between the WT and *hhp1-1* or c-*hhp1-1* under the same growth conditions (*P <0.05; **P <0.005; ***P <0.0005, Student's t test). (C) Expression profiles of *HHP1* in response to sucrose, KCl, LiCl and mannitol. The expression of *HHP1* in 10-d-old (stage 1.04) WT *Arabidopsis* seedlings, which were grown on minimal medium (a)

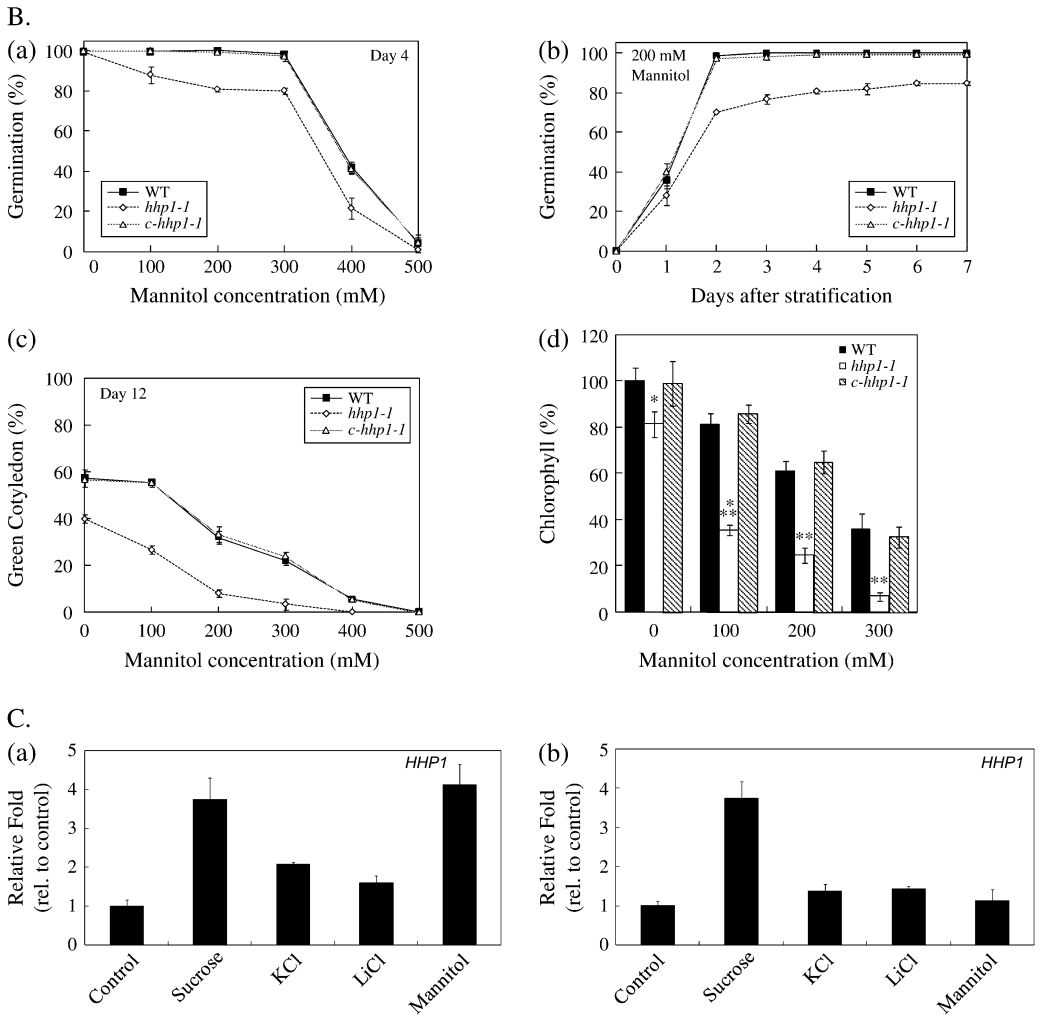

Fig. 5. (*Continued*)

6-d-old seedlings treated with different NaCl concentrations for 6 d; fewer than 63% of *hhp1-1* seedlings survived at 50 mM NaCl compared to 84% of WT and 82% of c-*hhp1-1* seedlings (Fig. 5Ae, Af). Taken together, these results showed that the *hhp1-1* was more sensitive to salt than the WT and c-*hhp1-1*. Similarly, the germination rate of *hhp1-1* was greatly reduced by 200 mM mannitol, while that of the WT and c-*hhp1-1* was not severely affected (Fig. 5Ba, Bb). The *hhp1-1* plants showed greater growth retardation and retained less chlorophyll in the presence of mannitol than the WT and c-*hhp1-1* (Fig. 5Bc, Bd). The profound sensitivity to mannitol indicated that *hhp1-1* was more sensitive to general osmotic stress. In summary, these results suggest that HHP1 may contribute to osmotic stress sensitivity and play a negative regulatory role in seed germination and early growth in *Arabidopsis*.

Hsieh and Goodman (2005) reported that the expression of *HHP1* in the WT was unaffected by exogenous mannitol. However, it was found here that *HHP1* showed different expression patterns in response to exogenous mannitol in the WT seedlings when grown under different growth conditions, in particular, in the presence of sucrose. The expression patterns of *HHP1* in 10-d-old (stage 1.04) WT seedlings, which were grown on either minimal medium (Fig. 5Ca) or 1/2 MS medium containing 3% (w/v) sucrose (Fig. 5Cb), and treated with 100 mM sucrose, 300 mM KCl, 40 mM LiCl, or 300 mM mannitol for 4 h, were analysed by real-time PCR (Fig. 5C). *ACT2* was used to normalize the expression of *HHP1* and the results were presented as the fold expression relative to that of untreated WT (control). Irrespective of the growth conditions, the *HHP1* expression was increased 4-fold by 100 mM sucrose. The exogenous

or on 1/2 MS medium containing 3% (w/v) sucrose (b), treated without (control) or with 100 mM sucrose, 300 mM KCl, 40 mM LiCl or 300 mM mannitol for 4 h was analysed by real-time PCR. The data are the mean ±standard error for three independent amplification reactions and representative of two independent biological replicates, each consisting of 10-15 seedlings.

mannitol indeed had an inducing effect on the expression of *HHP1* in WT seedlings grown in the minimal medium (Fig. 5Ca), but when grown in the presence of sucrose (Fig. 5Cb), the expression level was not changed in response to mannitol. In addition, K^+ (300 mM KCl) and Li^+ (40 mM LiCl), two analogues of Na^+, had no significant inducing effect on the expression of *HHP1* (Fig. 5C), indicating that the NaCl effect on *HHP1* expression was mainly due to the osmotic change. In summary, *hhp1-1* grown in the absence of sucrose showed profound sensitivity to salt and mannitol, implying that *hhp1-1* was more sensitive to general osmotic stress (Fig. 5A, B).

Expression profiles of stress-responsive genes in the WT, hhp1-1 *and c-*hhp1-1 *in response to exogenous ABA and osmotic stress*

To gain further insights into the molecular basis of the observed phenotypes and the role of HHP1 in ABA and osmotic stress signalling, the expression profiles of several well-characterized stress-responsive genes (*RD29A*, *RD29B*, *ADH1*, *KIN1*, *COR15A*, and *COR47*), ABA-signalling genes (*ABI3* and *ABI5*), ABA biosynthetic genes (*NCED3*, *AAO3*, *ABA1*, and *ABA3*) (Xiong *et al.*, 2002*a, b*; Chinnusamy *et al.*, 2004; Nambara and Marion-Poll, 2005; Barrero *et al.*, 2006; Valliyodan and Nguyen, 2006; Hanson *et al.*, 2008) were investigated by real-time PCR in the 10-d-old (stage 1.04) WT, *hhp1-1*, and c-*hhp1-1* seedlings, treated with 300 mM NaCl for 1.5 h or with 100 μM ABA for 3 h (Fig. 6). *ACT2* mRNA levels were used to normalize the expression of these genes and gene expression in the untreated WT was used as a calibrator. Without treatment, the expression levels of these stress-responsive genes, ABA-signalling genes, or ABA biosynthetic genes were generally low in the WT, *hhp1-1*, and c-*hhp1-1*. With ABA or salt treatment, the expression of *RD29A* (Aa), *RD29B* (Ab), *ADH1* (Ac), *KIN1* (Ad), *COR15A* (Ae), *COR47* (Af), *ABI3* (Ba) and *ABI5* (Bb) exhibited a similar up-regulated pattern in the WT, *hhp1-1*,

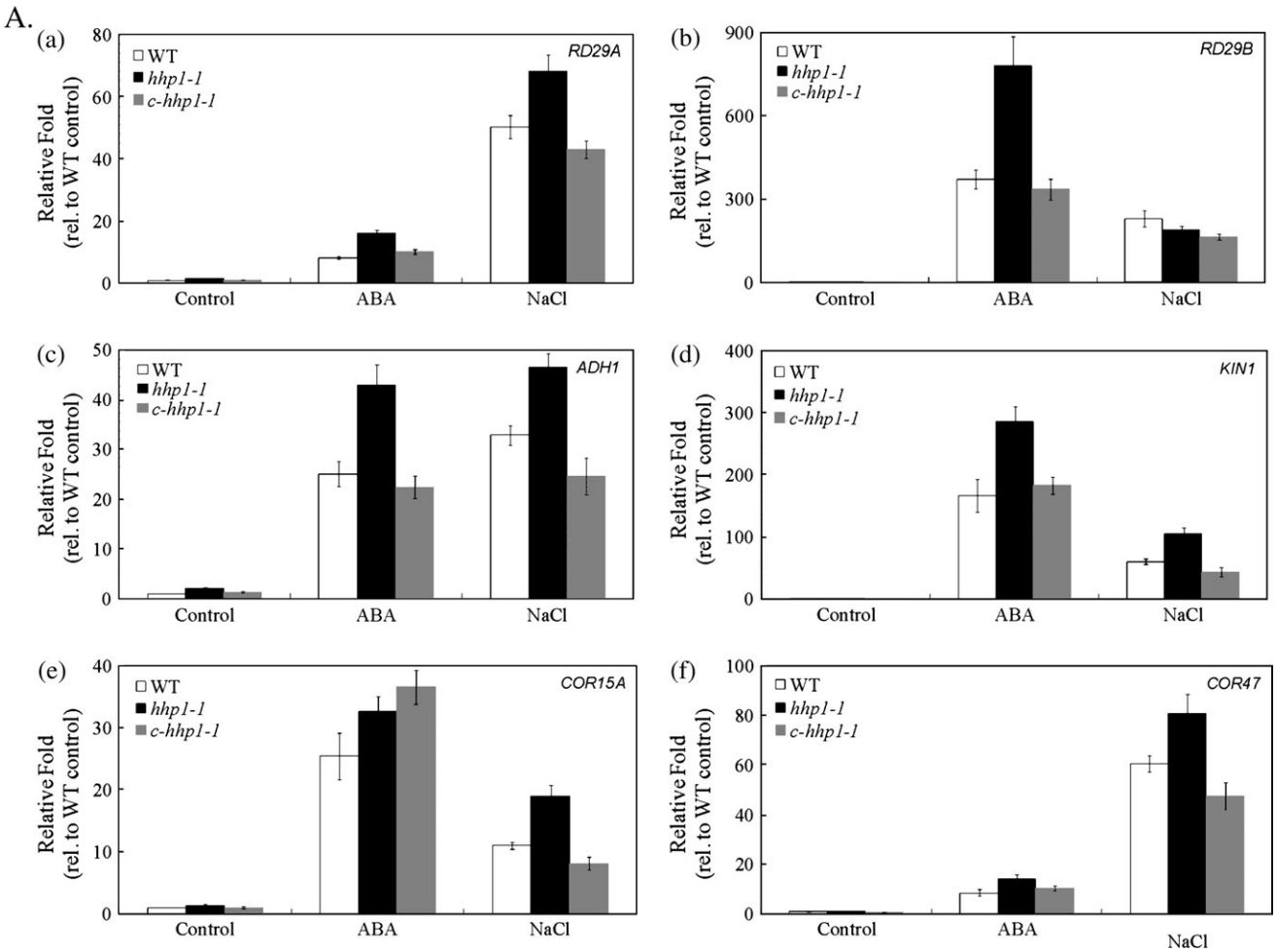

Fig. 6. Expression profiles of stress-responsive genes (A), ABA-signalling genes (B) and ABA biosynthetic genes (C) in the WT, *hhp1-1*, and c-*hhp1-1* in response to exogenous ABA and salt stress. The expressions of *RD29A* (Aa), *RD29B* (Ab), *ADH1* (Ac), *KIN1* (Ad), *COR15A* (Ae), *COR47* (Af), *ABI3* (Ba), *ABI5* (Bb), *NCED3* (Ca), *AAO3* (Cb), *ABA1* (Cc), or *ABA3* (Cd) in the 10-d-old (stage 1.04) seedlings of WT, *hhp1-1*, and c-*hhp1-1*, grown on the minimal medium, treated without (control) or with 100 μM ABA for 3 h or 300 mM NaCl for 1.5 h were analysed by real-time PCR. The data are the mean ±standard error for three independent amplification reactions and representative of two independent biological replicates, each consisting of 10–15 seedlings.

and c-*hhp1-1*, while the induction intensity of these eight genes was significantly stronger in the *hhp1-1* than in the WT and c-*hhp1-1* (Fig. 6). Compared with the WT and c-*hhp1-1*, the ABA-induced expression of the ABA biosynthetic genes *NCED3* (Ca), *AAO3* (Cb), and *ABA3* (Cd) was enhanced in *hhp1-1*, but such enhancement was not so obvious for the expression of *ABA1* (Cc) (Fig. 6). In summary, the up-regulated gene expression of the stress-responsive genes, ABA-signalling genes and ABA biosynthetic genes under ABA or salt treatments in *hhp1-1* was enhanced (Fig. 6). This implies that HHP1 might be a negative upstream regulator in the ABA biosynthetic and signalling pathways in response to exogenous ABA and osmotic stress.

Discussion

HHPs are members of the PAQR family

The diverse functions of the members of the PAQR family in mediating progestin and adiponectin signals, in the binding of osmotin, and in zinc homeostasis and fatty acid metabolism show that these proteins have been employed widely during evolution. The PAQR family is predicted to have a 7TM topology, but lacks any significant sequence similarity with GPCRs. Based on phylogenetic analyses, it is subdivided into three subgroups of adiponectin receptor-related, mPR-related, and haemolysin-III-related (Fernandes *et al.*, 2005). In this study, we characterized the HHP1 in *Arabidopsis* that is the most closely related to the adiponectin receptor-related PAQR subgroup. Whether HHPs and other PAQR members have the structure and function similar to GPCRs is intriguing. Seatrout mPR has been predicted to have an N-terminal outside topology, as in the GPCRs (Zhu *et al.*, 2003) whereas the adiponectin receptor has been shown to adopt an N-terminal inside topology, as revealed by epitope tagging experiments (Yamauchi *et al.*, 2003). Topology prediction by TMHMM method favoured an N-terminal inside topology for HHPs (Hsieh and Goodman, 2005), so the involvement of G-protein signalling, as suggested for the mPR, may not be applied to HHPs.

HHP1 is involved in the ABA and osmotic stress response

Sensitivity/susceptibility to abiotic stresses is a very complex phenomenon and there are intricate signalling pathways

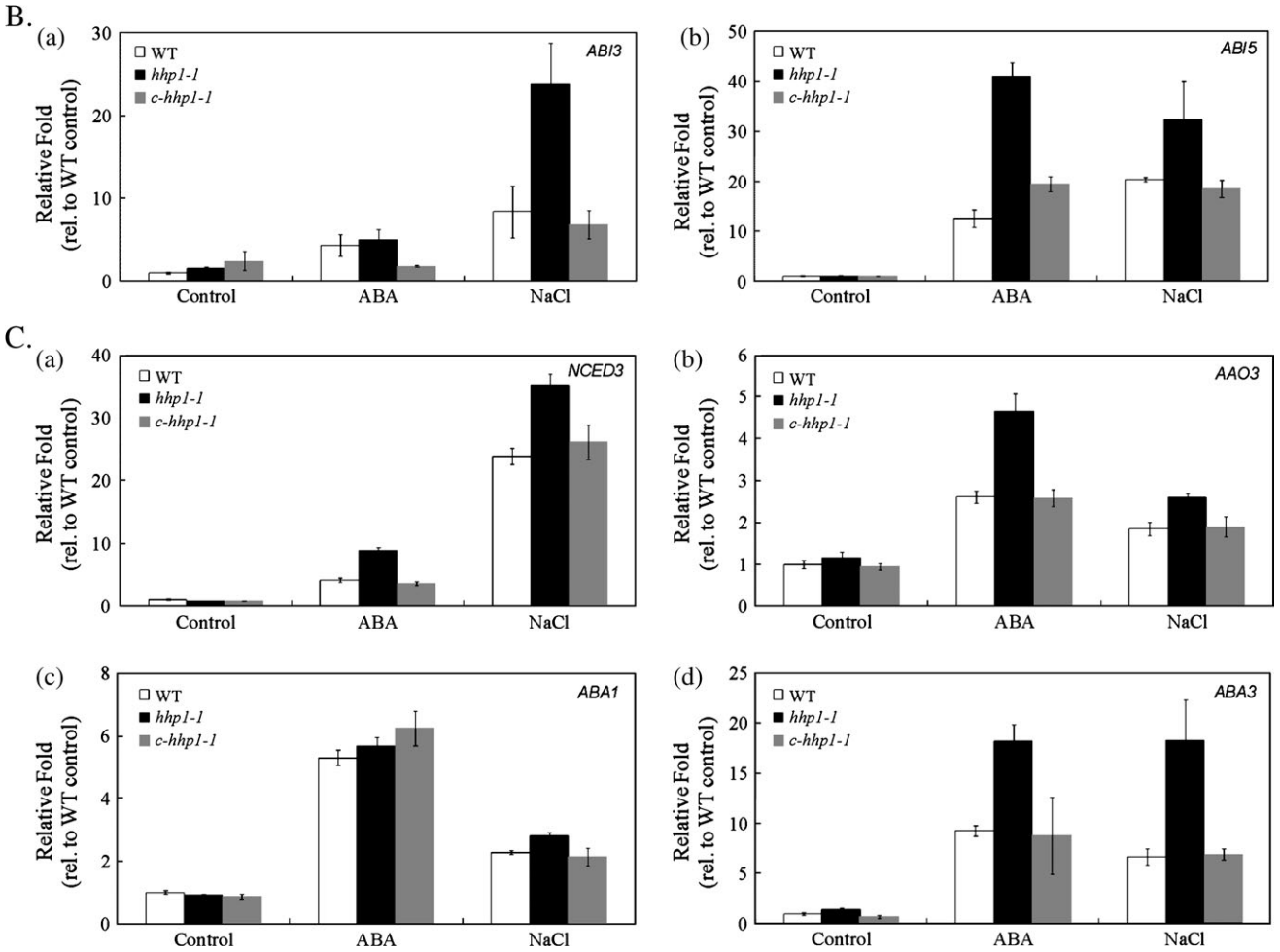

Fig. 6. (*Continued*)

which enable plants to tolerate, and adapt in response to, exogenous osmotic stress. The elucidation of the mechanism of osmotic stress signalling may be beneficial in breeding by improving stress tolerance in crops (Fujita *et al.*, 2006; Xiong *et al.*, 2002*b*; Apse and Blumwald, 2002). Phytohormones, such as SA, JA, ethylene, and ABA, regulate the protective responses of plants against biotic and abiotic stresses via signalling cross-talk. Both ABA-dependent and ABA-independent signalling pathways appear to be involved in osmotic stress sensitivity (Chinnusamy *et al.*, 2004; Fujita *et al.*, 2006; Xiong *et al.*, 2002*b*; Schroeder and Nambara, 2006). In this study, it is shown that HHP1 was a novel component of an ABA-regulated osmotic stress response in several lines of evidence. First, the promoter region of *HHP1* was found to contain an ABRE-related element and DPBF, MYB, and MYC recognition sequences, which are the downstream targets of ABA-dependent osmotic stress signalling (Chinnusamy *et al.*, 2004; Xiong *et al.*, 2002*b*), and the expression of *HHP1* was induced by ABA and osmotic stress (Figs 2, 3), indicating that *HHP1* could be a downstream stress-responsive gene of an ABA-dependent signalling pathway. Second, the fact that the *hhp1-1* mutant was more sensitive to ABA and osmotic stress (Figs 4, 5) implies that HHP1 may be involved in stress sensitivity and act as a negative regulator in response to ABA and osmotic stress in *Arabidopsis*. Third, inducing expression of stress-responsive genes, ABA-signalling genes, and ABA biosynthetic genes by ABA and osmotic stress was more sensitive in *hhp1-1* (Fig. 6). Taken together, HHP1 is likely to be an upstream negative regulator in the ABA and osmotic signalling pathways.

The possible relationship between HHP1 and osmotin

It is very interesting to note that osmotin, an antifungal basic PR5 protein from tobacco that exhibits a similar overall structural fold to adiponectin, causes apoptosis of yeast via YOL002c, an adiponectin receptor homologue (Narasimhan *et al.*, 2005). Osmotin is induced by exogenous salt and contributes to salt sensitivity (Singh *et al.*, 1987, 1989; Kononowicz *et al.*, 1992; Onishi *et al.*, 2006). Our results showed that *HHP1* was induced significantly by salt (Fig. 3) and belongs to the adiponectin receptor-related PAQR subgroup. Two predicted proteins (OSM34 and OLP) homologous to osmotin in *Arabidopsis* were recently studied by our group. It would be very interesting to elucidate whether HHP1 and osmotin work together, and what is the relationship among HHP1, OSM34, and OLP under osmotic stress.

The physiological role of HHP1 in Arabidopsis *may provide a new aspect for elucidating the cross-talk among various abiotic stresses*

Plants sense diverse abiotic stresses, such as salt, drought, and cold, by complicated signalling networks. These networks often employ some overlapping components and some common downstream tolerance-related proteins (Chinnusamy *et al.*, 2004; Bartels and Sunkar, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006; Shinozaki and Yamaguchi-Shinozaki, 2007). Although HHPs, homologous to seatrout mPR, were viewed as putative receptors involved in non-genomic actions at the beginning of this study, our results indicate that HHP1 may play an important part in ABA-regulated osmotic stress sensitivity.

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