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 superfamily. 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...
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 (AB12, AB13, and AB15), ABA biosynthetic genes (NCED3, AA03, AB13, and AB13), 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 HHPl-defective mutant (hhpl-I) 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 HHPl might play a role in osmotic stress signalling and act as a negative regulator. The possibility that HHPl 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 HHPl 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 HHPl, SALK_056174 (hhpl-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 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 HHPl 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 HHPl was amplified by the polymerase chain reaction (PCR) using the primer pairs 5’-GAATT-CATGGACAAAAATGGTCAATAAAGCGAA3’ (HHPl forward) and 5’-CTCGAGTTAACAACCAACGTGGT- CATGG-3’ (HHPl reverse). Then, the coding sequence was cloned into the pGEM-T vector (Promega, USA) and sequenced.

Complementation of hhpl-1

The coding sequence of HHPl was amplified by sticky-end PCR (Zeng, 1998) using the primer pairs 5’-CATGG-ACAAAAATGGTCAATAACGACGAGCA-3’ (HHPl_C

| 1590 | Chen et al. |
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 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 (phytotoxins, 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'-CCCGGTGATGCCAG-AGAG-3' and 5'-TGAGCCCTCTAAAGAAAAGAGA-3'), RD29A (5'-TATGAGTGCACAGGTTCAC-3' and 5'-CCTGGGTGAATATTTCTCGG-3'), RRD29B (5'-CGCCACGTCGTTGTA-3' and 5'-CCACGGAATTCG-3'), HHP1 (5'-AGAC-3' and 5'-AGAG-3'), NCED3 (5'-ATGGCCAGAGGAAACGTTCCGG-3' and 5'-GGCTCTCTTCTTCTCTTCC-3'), KIN1 (5'-ATGCGAAAGATCAACTCCCACAAA-3' and 5'-TTCCGATCGACTTATGATCGTA-3'), COR15A (5'-CAGTGAAAAGCCGACATACATTGGG-3' and 5'-GGCTCTTCTCTTCTTCTCC-3'), COR47 (5'-GGAGTACAAGAACGCAATCCGCGA-3' and 5'-TGTCGTCGCTGGTATCTC-3'), ABI3 (5'-GGCAGCAGCATCCACAGA-3' and 5'-GTGGATCTCCAAGGCCAGTA-3'), and NCED3 (5'-ATGGCCAGAGGAAACGTTCCGG-3' and 5'-GGCTCTCTTCTTCTTCTCC-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,
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 \(\Delta C_T\) calculation.

**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 seabream membrane progestin receptor (CmmPRα, AA039265), 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.

**HHP1 promoters::GUS**

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’-AATTCGCTACAACCTATGGGCCA-3’ (HHP1 P 5’ primer_1) plus 5’-GAAGAGCTCTCGAGTATCGA-3’ (HHP1 P 3’ primer_1) and 5’-CGCTACAACCTATGGCCATTTTA-3’ (HHP1 P 5’ primer_2) plus 5’-CATGGAAGAGCTCTCGAGTATCGA-3’ (HHP1 P 3’ primer_2). The PCR product was cloned into the EcoRI and NcoI sites of the binary vector pCAMBIA1381Z and the resulting construct was sequenced and transferred into WT Arabidopsis plants via Agrobacterium GV-3101-mediated in planta transformation (Bent, 2000). Transformant seedlings of the **HHP1::GUS**
T₁ 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 T₃ 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, GA₃, 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 K₃Fe(CN)₆, 0.5 mM K₄Fe(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 T₃ lines, and representative individuals from representative lines were chosen for photography. Seedlings were observed by a Leica MZ75 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).

Fig. 1. (Continued)
by using a fluorimeter (Beckman Coulter DTX880 Multi-mode 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 haemolsysin-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 haemolsysin-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 (ADMR, 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 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 the primers specific for the AtHHP1 gene and the left border of the T-DNA insertion. Fifteen homozygous plants derived from the SALK_056174 T3 lines were confirmed by RT-PCR not to express full-length HHP1 CDS. The single T-DNA insertion lines, SALK_056174_10 T1, 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 T1 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 3SS::HHP1 was introduced back into the hhp1-1 plants to generate transgenic mutants named as complemented-hhp1-1 (c-hhp1-1) for complementation experiments. T1 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 T1 lines and the homozygous complemented hhp1-1 T3 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, GA3, 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 T3 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
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. 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, GA3, 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), GA3, 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 T3 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 T3 lines.
The hhp1-1 mutant shows higher sensitivity to ABA and osmotic stress

Our results showed that HHPI 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 HHPI 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 HHPI 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

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**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)
6-d-old seedlings treated with different NaCl concentrations for 6 d; fewer than 63% of hhpl-1 seedlings survived at 50 mM NaCl compared to 84% of WT and 82% of c-hhpl-1 seedlings (Fig. 5Ae, Af). Taken together, these results showed that the hhpl-1 was more sensitive to salt than the WT and c-hhpl-1. Similarly, the germination rate of hhpl-1 was greatly reduced by 200 mM mannitol, while that of the WT and c-hhpl-1 was not severely affected (Fig. 5Ba, Bb). The hhpl-1 plants showed greater growth retardation and retained less chlorophyll in the presence of mannitol than the WT and c-hhpl-1 (Fig. 5Bc, Bd). The profound sensitivity to mannitol indicated that hhpl-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., 2002a, 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, and c-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), ABI1 (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-I, while the induction intensity of these eight genes was significantly stronger in the hhp1-I than in the WT and c-hhp1-I (Fig. 6). Compared with the WT and c-hhp1-I, the ABA-induced expression of the ABA biosynthetic genes NCED3 (Ca), AAO3 (Cb), and ABA3 (Cd) was enhanced in hhp1-I, but such enhancement was not so obvious for the expression of ABA1 (Cc) (Fig. 6). In summary, the upregulated gene expression of the stress-responsive genes, ABA-signalling genes and ABA biosynthetic genes under ABA or salt treatments in hhp1-I 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.
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., 2002b; 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., 2002b; 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., 2002b), 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 region of **HHP1** 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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