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Stress induced and nuclear localized HIPP26 from *Arabidopsis thaliana* interacts via its heavy metal associated domain with the drought stress related zinc finger transcription factor ATHB29

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Abstract HIPP26 from *Arabidopsis thaliana* belongs to a novel class of plant proteins, characterized by a heavy metal associated domain and an additional isoprenylation motif. It is induced during cold, salt and drought stress. The nuclear localization of HIPP26, predicted by a NLS motif, could be confirmed in onion epidermal cells overexpressing GFP-HIPP26. Experiments with modified HIPP26 indicate that the isoprenylation plays a role in the spatial distribution in the nucleus. Using promoter-GUS constructs, a tissue specific expression pattern of HIPP26 could be shown, with high expression in the vascular tissue. By a yeast-two-hybrid approach a strong interaction of HIPP26 with the zinc finger homeodomain transcription factor ATHB29, which is known to play a role in dehydration stress response could be detected. This was confirmed by GST pull-down assays. When using a modified HIPP26 lacking the two central cysteines of the heavy metal associated domain, ATHB29 was not bound in the GST pull-down assay, indicating that this structure is necessary for the interaction. Further yeast-two-hybrid analyses testing interaction of different members of the HIPP family with related zinc finger transcription factors revealed a specific interaction of ATHB29 with several HIPP proteins. A functional relationship between HIPP26 and ATHB29 is also indicated by experiments with mutants of *HIPP26* showing altered expression levels of such genes known to be regulated by ATHB29.

Keywords Abiotic stress · Heavy metal associated domain · Isoprenylation motif · Zinc finger homeodomain transcription factor (ATHB29, ZFHD1, At1g69600)

Abbreviations

GUS	Glucuronidase
HIPP	Heavy metal associated isoprenylated plant protein
HMA	Heavy metal associated domain
NLS	Nuclear localization signal
RTQ-PCR	Quantitative RealTime PCR
smRS-GFP	Solubility modified red shifted-green fluorescent protein
Y2H	Yeast two hybrid
ZF-HD	Zinc finger-homeodomain box

Introduction

Recently we identified in barley the novel stress and senescence regulated protein HvFP1 with a heavy metal associated domain and a C-terminal isoprenylation site (Barth et al. 2004). In the model plant *Arabidopsis thaliana* sequence analyses revealed that there are a total of at least 44 genes encoding proteins which also represent these two characteristic domains. The function of most of these proteins is not yet clear.

Heavy metal associated domains (HMA, pfam00403.6) are known from animal, plant and bacterial proteins and exhibit a core sequence (M/L/IxCxxC) with two characteristic cysteines which are involved in heavy metal binding

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(Hung et al. 1998; Dykema et al. 1999). Most of the investigations on HMA domain containing proteins from various animal, yeast, bacterial and plant species indicate binding of copper to the HMA domain (e.g. Bull et al. 1993). But there are also some reports showing binding of other heavy metals like nickel or zinc (Dykema et al. 1999; Suzuki et al. 2002). Analyses of several HMA containing proteins of different organisms revealed a function in heavy metal transport and heavy metal homeostasis (Lin et al. 1997; Chu et al. 2005).

In 1999, Dykema et al. reported that there are several proteins in *Arabidopsis thaliana* which in addition to the well known HMA domain also contain at the C-terminal end an isoprenylation motif (CaaX-motif) which comprises the site where farnesyl- or geranylgeranyl transferases add an isoprenyl residue to the cysteine of the CaaX-motif. After that, carboxyl peptidases remove the three carboxy terminal amino acids and the COOH group of the prenylcysteine is methylated by a carboxyl methyl transferase (Rodriguez-Concepcion et al. 1999). Isoprenylation is a post-translational protein modification that involves the formation of a covalent thioether bond between the cysteine and farnesyl- or geranylgeranyl-residues. In general, prenylated proteins are known to play important regulatory roles in cell cycle control, signal transduction, cytoskeletal organization or intracellular vesicle transport (Crowell 2000). The lipid modification of the protein creates a hydrophobic anchor that is important for interaction of the protein with membranes or other proteins (Yalovsky et al. 1999). The knowledge about the functions of the plant proteins which contain both, the HMA and the isoprenylation site (Dykema et al. 1999) is rather incomplete. Suzuki et al. (2002) identified a cadmium binding protein with both domains (Cdi19) which is induced in response to heavy metal stress and its overexpression conferred cadmium tolerance indicating a role in heavy metal homeostasis or detoxification. To our knowledge, all other proteins of this plant protein family are not yet characterized. Some of the members also contain potential nuclear localization signals (NLS) which together with the other domains implicates a regulatory function within the nucleus.

In this report we analyze stress dependent expression of HIPP proteins and show that members of this protein family interact with the zinc finger homeodomain transcription factor ATHB29 playing a central role in dehydration-stress response (Tran et al. 2007). In addition, we present data which prove that the central cysteines of the metal binding domain are essential for this interaction. We also show that loss-of-function of HIPP26 affects expression of genes known to be regulated by ATHB29. Our data demonstrate a functional relationship of HIPP26 and transcription factor ATHB29 during stress response in plants.

Materials and methods

Plant material

Seeds of Arabidopsis (*Arabidopsis thaliana* (L.) Heynh. ecotype Columbia) were either germinated and grown on soil (for cold treatment and senescence) or hydroponically (for ABA-, salt- and drought-experiments) in 0.1 times Hoagland's No.2 basal salt mixture (#H2395 from Sigma-Aldrich, Germany). At day (12 h, 100 $\mu Em^{-2}\ s^{-1}$) temperature was 23°C and at night (12 h) 16°C. The abiotic stress treatments started 8 weeks after sowing at the beginning of the day phase. Cold treatment was as described in Barth et al. (2004). Unstressed control plants were always harvested at the same time points. For ABA treatment 50 µM (+)-*cis*, trans-abscisic acid (DUCHEFA Biochemie B.V., Haarlem, Netherlands) and for salt stress 250 mM NaCl were added to the hydroponic medium. Drought stress was performed by removing the plants from the hydroponic medium according to Philipps et al. (2006). Water content of the samples was 97.9% of control after 1.5 h, and 85.8% of control after 4 h of treatment. Copper stress was done by adding CuCl to obtain a final concentration of 50 µM in the hydroponic medium. For analysis of leaf senescence, plants were grown under standard conditions for 9d (mature leaves) or 38d (senescent leaves) on soil.

The HIPP26 Knock Out Mutant Athipp26 was provided from Martienssen Lab (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA). This Line (ET7654) was identified by Martienssen (1998) during a systematic screening of an enhancer trap transposon mutagenized *Arabidopsis* library. The sequences that indicate an insertion event in the first intron, 46 bases after the translation start ATG of HIPP26, are annotated with the genebank accession numbers AY200075 and AY200076. Due to the Landsberg erecta background of the Athipp26 line the *Arabidopsis thaliana* (L.) Heynh. ecotype Landsberg erecta (Ler-0, NASC ID: NW20, Nottingham Arabidopsis Stock Centre, United Kingdom) wildtype was used to backcross Athipp26 on wildtype to equal the genetic background of both lines. To prevent effects of possible second site mutations only homozygous plants (Athipp26 (HIPP26 knock out) and AtHIPP26 (wildtype)) of the F2 progeny were used for all comparative experiments.

Sequence analysis and construction of phylogenetic tree

The amino acid consensus sequence of the heavy metal associated domain (HMA domain, PFAM 00403) and the BLAST Servers at NCBI (Altschul et al. 1990) were used to identify putative HMA domain containing proteins from *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia.

Alignment of amino acid sequences was made using Clustal W method in the program MegAlign of the

'Lasergene expert sequence analysis software' (DNASTAR Inc., Madison, WI, USA). Phylogenetic trees were constructed by comparing 52 amino acid sequences on the basis of the HMA domain. Putative *cis*-elements in the promoter region of HIPP26 were identified by a web based signal scan search using the plant *cis*-acting regulatory DNA elements (PLACE) database (Higo et al. 1999).

Manipulation of DNA and RNA

Standard methods (Sambrook and Russell 2001) were employed to manipulate DNA and RNA. DNA sequencing was carried out using ABI Prism™ 370 automatic DNA-sequencer (Applied Biosystems, Foster City, CA, USA). RNA was isolated according to Chomczynski and Mackey (1995) and was quantified spectrophotometrically.

For RT-PCR of the HIPP26 cDNA (At4g38580), 0.5 µg of total RNA of 30 h cold (6°C) stressed Arabidopsis plants was processed according to the supplier's instruction using the 'OneStep RT-PCR' Kit (QIAGEN, Hilden, Germany), HIPP26cDNAfor and HIPP26cDNArev Primer (supplemental Table S1). The cDNA was purified, cloned using the 'pGEM-T Vector System I' (Promega, Madison, WI, USA), sequenced and the corresponding clone was named pGEM-HIPP26.

Gene expression analysis by quantitative RealTime-PCR

Total RNA, isolated from at least three plants per sample, was treated with DNaseI (Roche Diagnostics GmbH, Mannheim, Germany) and cDNA was synthesized using the SuperScript™III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA) according to the supplier's instructions. PCR was carried out in the iCycler (Bio-Rad, München, Germany) in a total volume of 20 µl including 1× Platinium®SYBR®Green qPCR SuperMix-UDG (Invitrogen), 0.3 µM of each gene-specific primer (supplemental Table S1) and 10 nM fluorescein (Bio-Rad) as passive reference dye for well factor calibration. Data collection was performed from three independent experiments with at least three different measurements per sample. To calculate the expression rate and standard error between the target group and the control group the Realtive Expression Software Tool (REST-384 version 2) developed by Pfaffl et al. 2002 was used.

Analysis of localization of HIPP26 using HIPP26-GFP constructs

The HIPP26 ORF was amplified by using the cDNA clone pGEM-HIPP26 and PCR primer pairs HIPP26forAB and HIPP26revA or HIPP26forAB and HIPP26revB (supplemental Table S1). The PCR products were cloned by using the 'pGEM-T Vector System I', sequenced, digested with *Bgl*II and *Bam*HI and cloned into *Bgl*II site of pKEx4tr-GFP02. The vector and the transformation procedure of onion epidermal cells were performed as described by Barth et al. (2004). GFP fluorescence was determined by FITC-filtered visual inspection under a confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

Tissue specific expression

The 1,055 bp region upstream of the translational start ATG of HIPP26 was amplified using genomic DNA isolated from *Arabidopsis thaliana* ecotype Columbia as template and HIPP26Pro for and HIPP26Pro rev primer (see supplemental Table S1) and standard PCR conditions. The resulting PCR product was cloned into the *Xba*I-*Sma*I sites of pGPTV-Kan (Becker et al. 1992) in front of the open reading frame of the *uidA* gene resulting in pGPTV-HIPP26Pro. This vector and the empty pGPTV-Kan as control were transferred to *Agrobacterium tumefaciens* strain GV3101(pMP90) (Koncz and Schell 1986). Wild-type *Arabidopsis thaliana* L. ecotype Columbia plants were transformed by the *Agrobacterium*-mediated floral dip method (Clough and Bent 1998). Seedlings and leaves of T2 and T3 lines were used for monitoring histochemically the GUS activity. The plant material was stained in a solution containing 50 mM $NaPO_4$(pH 7.2), 0.5 mM $K_3Fe(CN)_6$, 0.1% (v/v) Triton X-100, 10 mM EDTA, and 1 mM X-GlcA (#X1406, DUCHEFA Biochemie B.V., Haarlem, Netherlands). After a 1 min vacuum infiltration, the staining reaction was incubated 24 h at 37°C. Leaf pigments were removed by washing 5–10 times with 50% (v/v) ethanol for 24 h each time.

Yeast two-hybrid screen and two-hybrid assay

Standard techniques were used for the manipulation of yeast (Guthrie and Fink 1991). The Yeast Two-Hybrid screen had been carried out according to the instructions of the Matchmaker Two-Hybrid System 3 (CLONTECH, Mountain View, CA, USA). Three different HIPP26 bait constructs were amplified by PCR using the cDNA clone pGEM-HIPP26 and the Primer HIPP26forCDEF and HIPP26revC, HIPP26revD or HIPP26revE (supplemental Table S1). The PCR products were cloned, sequenced and used together with the Gal4 DNA binding domain (BD) vector pGBKT7 (CLONTECH) to prepare appropriate bait vectors. These bait vectors were transformed into the yeast strain AH109. CD4-30, the Gal4 Activation Domain (AD) cDNA library, constructed by Fan et al. (1997) was transformed into the yeast strain Y187. After mating high (plating directly onto SD/-Leu/-Trp/-Ade) and low (plating

first onto SD/-Leu/-Trp and after 4 days onto SD/-Leu/-Trp/-Ade) stringency screenings were performed. Putative positive colonies were rescued and re-tested on SD/-Leu/-Trp/-Ade/-His and for β-galactosidase activity. Interaction in yeast was confirmed by re-transformation in AH109, mating with Y187 harbouring different bait constructs and negative controls (empty pGBKT7 and pGBKT7-Lam). Confirmed positive interacting clones were sequenced by using the primer pAD-Gal4-2.1for and pAD-Gal4-2.1rev (supplemental Table S1). β-galactosidase reporter gene activity was assayed by filter lifts.

The entire coding region of further HIPP genes was also cloned into the pGBKT7 vector. The resulting plasmids were transformed in AH109 yeast cells. The coding region of several ATHB genes was cloned into the Gal4-AD vector pGAD-C1 (James et al. 1996). The resulting plasmids were transformed in Y187 yeast cells. After yeast mating high stringency yeast two hybrid assays (on SD/-Leu/-Trp/-Ade plates) were performed and β-galactosidase reporter gene activity was analysed by filter lifts.

In vitro binding assay

The entire coding region of ATHB29 (At1g69600) was amplified by RT-PCR using the 'OneStep RT-PCR' Kit (QIAGEN) and the primers ATHB29for and ATHB29rev. The cDNA clone was used as template for an in vitro transcription performed by SP6 RNA polymerase from MBI Fermentas according to the supplier's instruction. *In vitro* translation was carried out by Flexi® Rabbit Reticulocyte Lysate System (Promega) in the presence of ^{35}S-Met (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

HIPP26-4 was produced by a two step site directed mutagenesis using the pGEM-HIPP26 cDNA clone and the primer HIPP26forCDEF together with HIPP26revG, and HIPP26forG together with HIPP26revC (supplemental Table S1). Purified PCR products were mixed and amplified by PCR using the primer HIPP26forCDEF and HIPP26revC.

GST-HIPP26-1 and GST-HIPP26-4 fusion constructs were prepared by cloning the coding regions of both HIPP26 versions into the *Eco*RI-*Xho*I sites of a modified pGEX-2TK (GE Healthcare Bio-Sciences). For expression of the recombinant proteins, Rosetta (DE3) pLysS (Novagen, Madison, USA) *E. coli* cells were transformed with pGEX-2TKmod, pGEX-2TKmod-HIPP26-1 and pGEX-2TKmod-HIPP26-4, and the resultant strains were grown overnight. The expression of recombinant proteins was performed by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 2 mM and further incubation for 3 h. Lysates containing GST alone or the GST-HIPP26 fusion constructs were incubated each with 70 µl pre-equilibrated Glutathione-Sepharose 4B resin (GE Healthcare Bio-Sciences) under gentle rotation at 4°C overnight. The GST, GST-HIPP26-1 and GST-HIPP26-4 Sepharose beads were washed with 1 ml binding buffer (phosphate-buffered saline including 1 mM fresh DTT and 0.05% (v/v) Nonidet P-40). For binding in vitro translated ATHB29 was then added. After a 1.5 h incubation under gentle rotation at 4°C the beads were washed with 900 µl binding buffer and 3 times with 900 µl binding buffer without Nonidet P-40 to remove unbound proteins and resuspended in SDS-PAGE sample buffer. Radioactive proteins were visualized by autoradiography after fractionation on 16% SDS-polyacrylamide gel.

Results

Characterization of HIPP proteins

Recently we identified from barley a novel nuclear localized protein with a heavy metal associated domain (HMA) and a C-terminal isoprenylation motif (*HvFP1*, Barth et al. 2004). Data bank research revealed that in *Arabidopsis thaliana* there are at least 44 genes coding for proteins containing one or more heavy metal associated domains (HMA domain, pfam00403.6) with two characteristic cysteines and additionally a C-terminal isoprenylation motif. Figure 1a shows the genetic tree of all HMA domains of the *Arabidopsis* proteins encoded by these genes. Because of the two characteristic features, the heavy metal associated domain and the isoprenylation motif, we denoted all these proteins HIPPs (heavy metal associated isoprenylated plant proteins). In Fig. 1a we list both, the AGI-code and the novel HIPP nomenclature. Based on the sequence homologies all the HIPP proteins cluster in six different groups (I–VI).

In this work we focus on cluster III of the *Arabidopsis thaliana* HIPP family showing highest homology to the cold regulated barley gene *HvFP1*. Figure 1b shows exemplarily the protein structure of one member of cluster III, HIPP26, which in addition to the HMA domain with its central HMA-motif (M/L/IxCxxC) and the typical carboxy-terminal CaaX-motif for isoprenylation and next to this CaaX motif a highly conserved sequence (conserved C-Terminus, cCT) which can be found in a large number of isoprenylated plant proteins (see alignment of all HIPPs of cluster III in supplemental Fig. 1), also contains a NLS (nuclear localization signal). Similar to the barley protein HvFP1 and the *Arabidopsis* HIPP26 some other HIPPs of cluster III contain a nuclear localization signal (NLS) made up of basic amino acids lysine, arginine and histidine (K, R and H). These are HIPP20, 21, 22, 23, 24, 25 and 27, predicting a nuclear localization of these proteins (see supplemental Fig. 1).

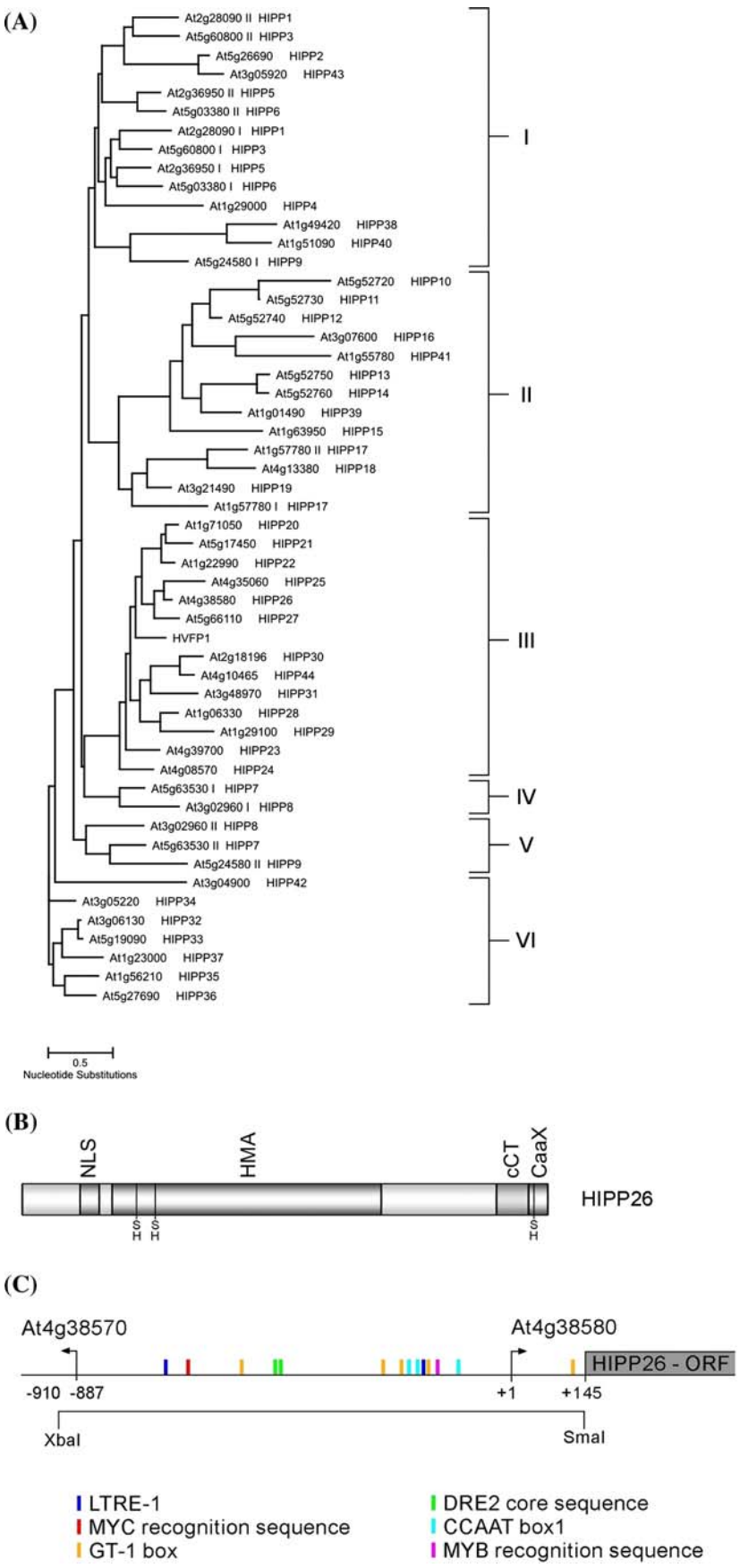


Fig. 1 **a** Phylogenetic tree of HIPP proteins (heavy metal associated isoprenylated plant proteins) from *Arabidopsis thaliana* containing at least one heavy metal associated (HMA) domain and in addition a carboxy-terminal isoprenylation motif. The tree is prepared on the basis of the different HMA domains using the Clustal W algorithm of the Megalign modul of the Lasergene software package (DNASTAR Inc., Madison, USA). In addition the AGI code is shown. The proteins cluster in six subgroups. **b** Protein structure of HIPP26 with putative NLS (nuclear localization signal), the HMA domain (heavy metal associated, with the central cysteines), the carboxy terminal isoprenylation motif (CaaX) and the highly conserved cCT domain next to the CaaX. **c** Sequence analysis of *HIPP26* promotor. Positions of putative cis regulatory elements are marked in different colours. The indicated *Xba*I-*Sma*I fragment was amplified and cloned to analyse the tissue specific expression of *HIPP26* by promoter reporter gene experiments (see Fig. 6)

Additionally a N-terminal myristoylation motif (MGxxxT/S, see Yalovsky et al. 1999) can be found in HIPP 20, 23 and 24.

A web-based signal scan of the 1,055 bp upstream sequence of *HIPP26* using the plant *cis*-acting regulatory DNA elements (PLACE) database (Higo et al. 1999) revealed the presence of a high number of potential *cis*-acting regulatory DNA elements (see listing in supplemental Table S2). In Fig. 1c we show the predicted positions of the typical stress responsive elements LTRE-1 (low temperature responsive element, Dunn et al. 1998), DRE2 (dehydration responsive element, Dubouzet et al. 2003), GT-1 box (involved in pathogen- and salt-induced gene expression, Park et al. 2004), CCAAT box (involved in heat shock response, Haralampidis et al. 2002), MYC recognition site (binding site of ICE1, a major transcriptional activator of cold responsive genes, Chinnusamy et al. 2003) and MYB recognition sites (important for dehydration-inducible expression, Abe et al. 1997).

Expression patterns during cold stress

Expression of HIPPs of cluster III in response to cold treatment at an early (6 h treatment) and a later (30 h treatment) phase was analyzed by quantitative RealTime-PCR (Fig. 2a). Expression levels of the genes is represented as fold-difference over the control, which is considered as 1. Although all used primers in the PCR yielded a product when using genomic DNA, transcripts of two genes (*HIPP28* and *HIPP29*) were not detectable with this sensitive method, indicating that these two genes are not expressed at least under the conditions used.

The cold treatment results in the induction of four genes from this cluster, HIPP23, HIPP24, HIPP25 and HIPP26. HIPP23 and HIPP26 are highly induced within the first 6 h of the cold treatment. In later stages of the stress treatment the expression levels of these genes decrease again indicating a fast and transient expression pattern during cold stress. HIPP25 shows induction only in the early phase. HIPP24 is highly up-regulated in later stages of the cold treatment showing high relative expression levels at 30 h treatment. All other HIPP genes of cluster III are either not induced or even repressed in response to the cold stress.

HIPP26 is that cold induced gene, which shows the highest homology to the known barley *HvFP1* (Barth et al. 2004). Its fast and transient induction in early phases of the stress treatment and the presence of a nuclear localization signal (NLS) in addition to the two conserved domains (HMA and isoprenylation motif), hint at a possible regulatory function of this protein. Because of these features we further concentrated on the functional analyses of HIPP26 (At4g38580) in this report.

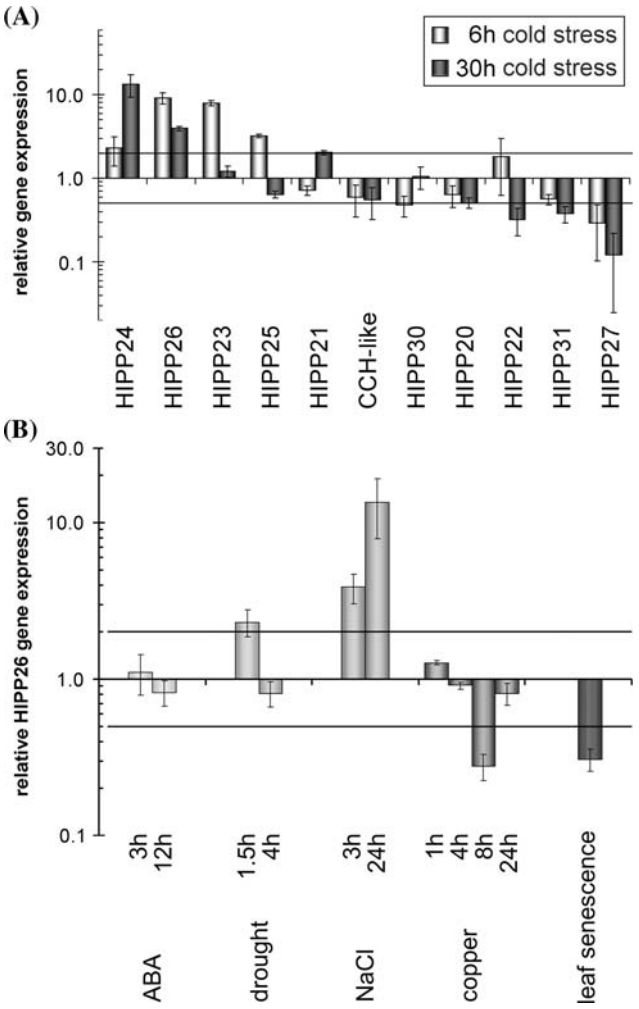


Fig. 2 **a** Quantitative RealTime-PCR expression analysis of HIPPs from cluster III (see Fig. 1a) in cold treated Arabidopsis plants (6 or 30 h). The level of mRNAs in each case is normalized to that of 18S rRNA and the expression levels are represented as fold-difference over the control, which is considered as 1. Error bars indicate the standard error ($n = 3$). **b** Quantitative RealTime-PCR (RTQ-PCR) expression analysis of HIPP26 in Arabidopsis plants exposed to either drought, salt (250 mM NaCl) or copper (50 μM CuCl) stress or to 50 μM ABA for different periods of time. In addition, the relative expression of HIPP26 in senescent leaves compared to that in mature leaves is also shown. For growth conditions and treatments see Materials and Methods. The level of mRNA in each case is normalized to that of 18S rRNA and the expression levels are represented as fold-difference over the control (without treatment for ABA, drought, salt and copper at each time point or the mature leaf for senescence), which is considered as 1. Error bars indicate the standard error ($n = 3$)

Expression of HIPP26 in response to abscisic acid, drought, salt, heavy metal and leaf senescence

The barley gene *HvFP1* showed a complex expression pattern with induction at different abiotic stress conditions (cold, drought and heavy metal), during leaf senescence and in response to abscisic acid (Barth et al. 2004).

Therefore, expression of *HIPP26* was further analyzed under these conditions by quantitative RealTime PCR. As shown in Fig. 2b, ABA has no influence on the expression of *HIPP26*. While drought stress shows only a slight effect, salt stress clearly results in increased transcript levels of *HIPP26*. Copper treatment and also leaf senescence rather inhibited expression of *HIPP26*. These data prove that *HIPP26* is as the barley *HvFP1* clearly induced by cold. But in contrast to *HvFP1*, which is also induced in response to ABA and copper treatment and during leaf senescence, *HIPP26* is not induced under these conditions, indicating only a partial overlap in the induction pathways of the barley and the *Arabidopsis* gene.

Confirmation of nuclear localization

The NLS indicates nuclear localization of HIPP26. In order to confirm this, we transformed epidermal onion cells by particle bombardment with two different smRS-GFP-HIPP26 constructs, where HIPP26 is fused to the C-terminal end of smRS-GFP. Subcellular localization of the chimeric proteins was then analyzed by confocal laser scanning microscopy (Fig. 3). The upper panel (a) shows fluorescence of the GFP control alone without HIPP26. It reveals typical GFP fluorescence in the onion epidermal cells. When transformed with the GFP fusion to the wild-type-HIPP26 (GFP-HIPP26-1, Fig. 3b), green fluorescence can be detected exclusively within the nucleus. This green fluorescence is concentrated in several speckle-like structures within the nucleus. In addition to the GFP-HIPP26-1 construct which allows isoprenylation at the intact C-terminal end, we also performed the experiment with a modified HIPP26 in which the C-terminal cysteine was exchanged to a glycine. The corresponding GFP-HIPP26-2 construct therefore can not be isoprenylated. The results are shown in Fig. 3c. This chimeric protein is also exclusively located within the nucleus of the onion epidermal cells. But, in contrast to the intact HIPP26, this protein which can not be isoprenylated is more uniformly located in the nucleus showing high green fluorescence in two defined round areas which were identified to be the nucleoli. Our results prove on one hand the nuclear localization predicted by the NLS-motif, and on the other hand indicate that the isoprenylation may be important for a specific localization of HIPP26 within the nucleus.

Interaction partners of HIPP26

A yeast two hybrid (Y2H) screening was used to identify HIPP26 interaction partners and GST pull-down experiments were done for verification (Fig. 4). In the Y2H experiments we used three different constructs as bait, the wildtype HIPP26 (HIPP26-1), HIPP26-2, in which the C-terminal cysteine is exchanged to a glycine and thereby isoprenylation is impossible, and HIPP26-3 with a deleted C-terminus, which can also not be isoprenylated. The screens using the GAL4-cDNA library CD4-30 (Fan et al. 1997) obtained from the ABRC stock centre were performed on the basis of GAL4 dependent transcriptional activation of three different reporter genes. An interaction of the three HIPP26 constructs with suitable library proteins was detected by a strong nutritional selection on synthetic dropout media lacking adenine (e.g. in −LTA and −LTAH, Fig. 4b) via activation of the ADE2 reporter gene. β-galactosidase and the HIS3 reporter genes were also used for the characterisation of positive interacting clones. 3-aminotriazole (3-AT), a competitive inhibitor of the HIS3 protein was used to enhance the stringency of the nutritional selection on synthetic dropout media lacking histidine (−LTAH, Fig. 4b). All results clearly prove a specific interaction of HIPP26 with ATHB29, a zinc-finger homeodomain box transcription factor (At1g69600, Tan and Irish 2006). A clear interaction is detected even in the presence of 12.5 mM 3-AT. The Y2H-screening indicated another zinc-finger homeodomain box protein to be a putative interaction partner of HIPP26. This is ATHB21. But the interaction seems to be not as strong as with ATHB29, since the detected β-galactosidase activity and growth of yeast under the stringent conditions with the inhibitor 3-AT are clearly reduced.

The interaction between HIPP26 and ATHB29 was confirmed by an independent experiment. A GST-HIPP26 construct was overexpressed in *E. coli* cells and by the GST-tag immobilized on glutathione-sepharose. Additionally, a GST-HIPP26 construct was investigated in which the two central cysteines of the M/L/IxCxxC core sequence were exchanged by two glycines (HIPP26-4, Fig. 4a). ATHB29 was *in vitro* transcribed and in the presence of ^{35}S-labelled methionine translated and then added to the glutathione-sepharose coupled with the suitable GST fusion proteins. The left part of Fig. 4c shows as input control 8% of the radiolabelled protein. The right part shows a radioactive band only in that experiment where the wildtype HIPP26 was used. Neither in the GST control, nor in the experiment with the modified HIPP26, ATHB29 could be pulled down.

Our data from the two independent approaches indicate that HIPP26 interacts with the zinc-finger homeodomain box transcription factor ATHB29. For this interaction an intact heavy metal associated domain including the two central cysteines is important.

Protein–protein-interactions of ATHB transcription factors and HIPP proteins

ATHB29 belongs to a *Arabidopsis* zinc finger homeodomain box transcription factor subfamily (ZF-HDs)

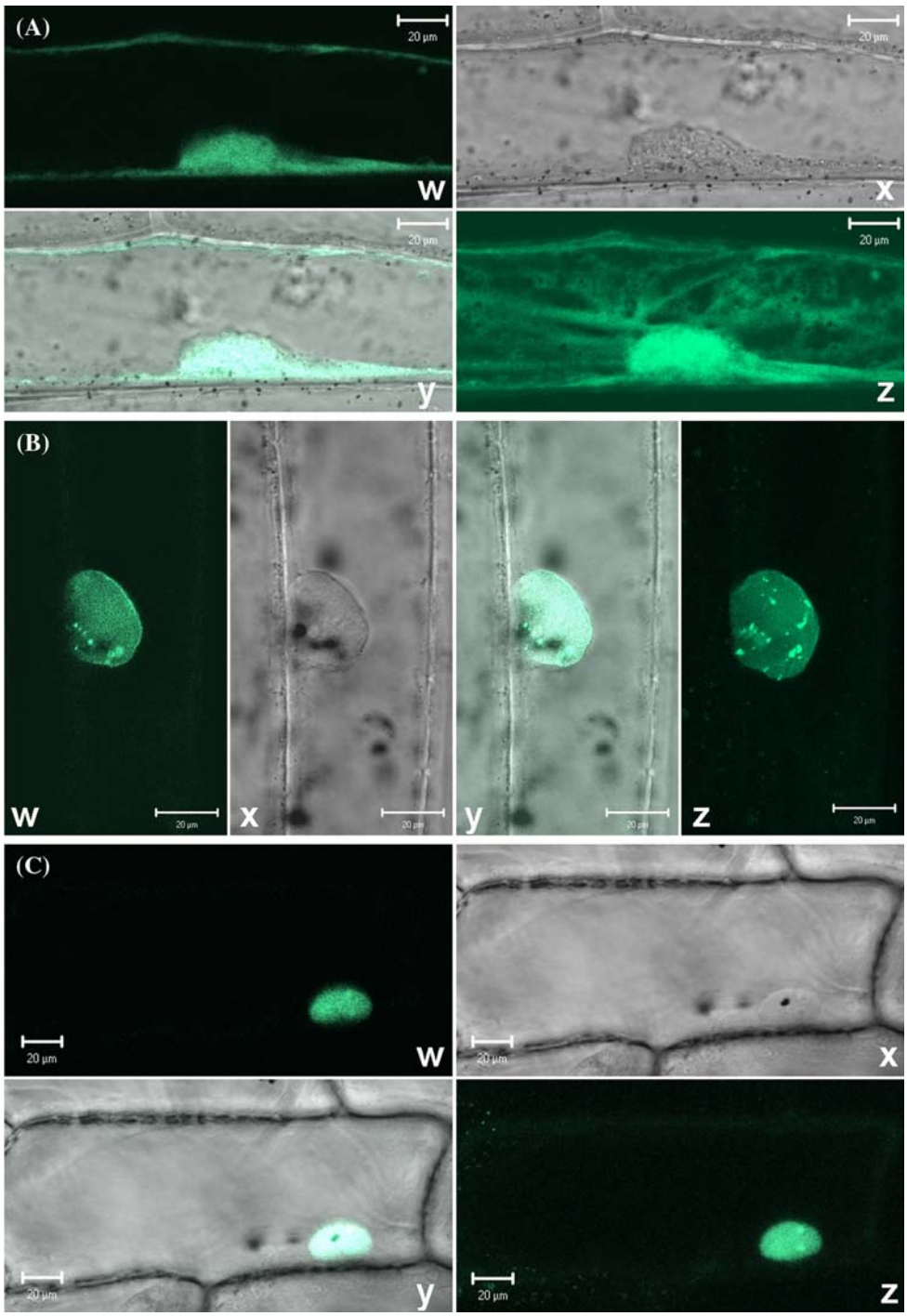
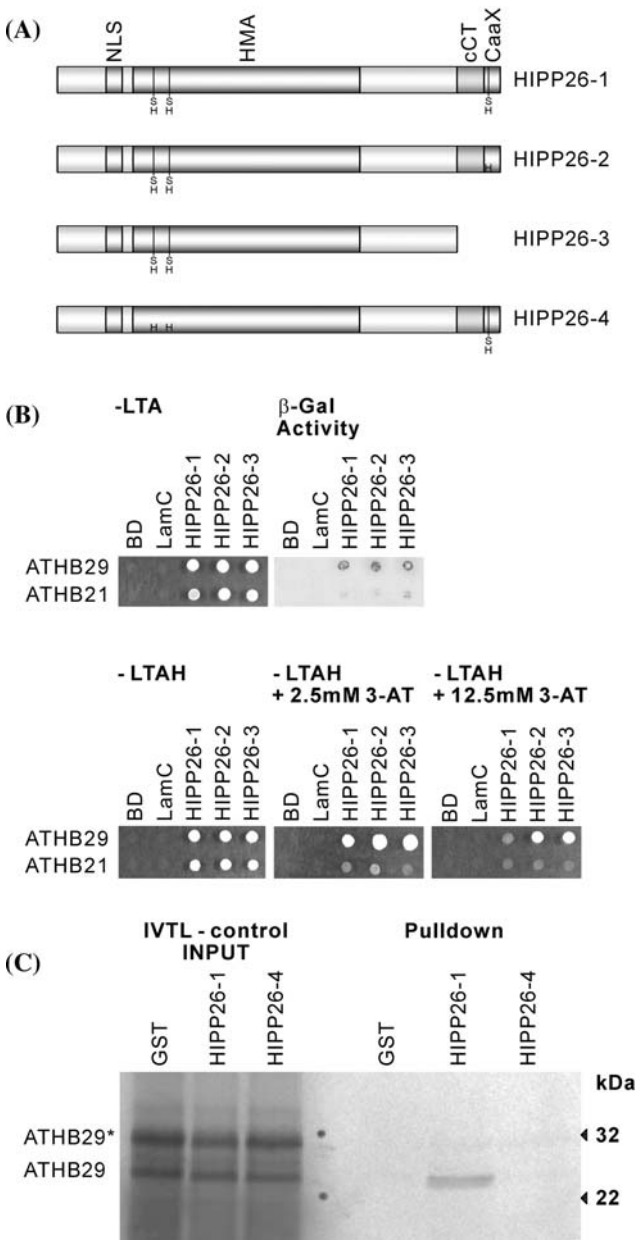


Fig. 3 Confocal, digital images of onion epidermal cells 12–24 h after bombardment with wolfram particles loaded with constructs **a** smRS-GFP control, **b** smRS-GFP attached to the N-terminal end of the wildtype HIPP26 (smRS-GFP-HIPP26-1) and **c** smRS-GFP attached to the N-terminal end of a modified HIPP26 where the C-terminal cysteine is exchanged to a glycine, preventing prenylation. In addition to the GFP-fluorescence (w) and the differential interference contrast (DIC) images (x) also a merged picture of both channels (y) and a 3D-projection of the GFP channel (from 20 slices, each 1,5 µm) through the whole cell (z) are shown

consisting of at least 14 members (ATHB21–ATHB34, Tan and Irish 2006). In order to test the specificity of the protein–protein interaction between members of this family with the different HIPP proteins of cluster III, yeast-two-hybrid assays were performed. Figure 5 indicates that ATHB29, in addition to HIPP26, also strongly interacts with HIPP20, HIPP21, HIPP23, HIPP24, HIPP27 and HIPP30. The only other interactions detected were between HIPP30 and ATHB21 and ATHB30.

Tissue specific expression of HIPP26

The 1,055 bp region upstream of the translational start ATG of HIPP26 (see Fig. 1c) was fused to the GUS gene and *Arabidopsis* plants were transformed with this construct. A–C (Fig. 6) shows controls transformed with the empty vector (pGPTV-KAN) which demonstrate no blue coloration. D and E show whole 10 days old Arabidopsis plants transformed with the HIPP26 promotor-GUS



◀ **Fig. 4** **a** Constructs used for yeast two hybrid analyses (HIPP26-1, HIPP26-2 and HIPP26-3) and for GST-pulldown assay (HIPP26-1 and HIPP26-4). **b** Yeast two hybrid analyses using HIPP26 constructs as bait and rescued, sequenced and retransformed positive interacting clones, isolated after a high stringency screening of the GAL4-cDNA library CD4-30 (Fan et al. 1997), as prey, were performed using three different reporter genes (ADE2, HIS3,*β*-GAL) and detection by strong nutritional selection (−LTA or −LTAH) or *β*-GAL activity staining. 3-aminotriazole (3-AT) was used to enhance the stringency of the HIS3 detection. As negative controls the GAL4 DNA binding domain (BD) and the BD fused with human LamC are also shown. **c** GST-pulldown assays with overexpressed HIPP26-1 and HIPP26-4 fused to GST and in vitro transcribed and translated ATHB29. The GST-HIPP constructs were immobilized on glutathione-sepharose and then binding of the ^{35}S labelled ATHB29 was investigated. Left side: input control; 8% of total radiolabelled protein. Black dots indicate molecular weight markers at 32 and 22 kDa. ATHB29* marks a byproduct with a higher molecular weight than the expected 26 kDa of the wildtype ATHB29. Right side: radioactive proteins bound to the GST-HIPP26 constructs on the glutathione sepharose were separated by polyacrylamide gel electrophoreses and detected with a fluorescent image analyser (FLA-3000 Series, Fujifilm Europe, Düsseldorf, Germany). GST: in this case GST was added to the glutathione sepharose as negative control

construct. GUS activity in these plants is clearly localized at the vascular tissue in the whole plant. In the petioles and upper part of the stem a more intense blue coloration can be detected. In the older plants (8 weeks old) GUS activity is also present in the vascular veins shown in the leaves and petioles. These data prove a distinct expression pattern of HIPP26 in specific tissues within the plant.

Loss-of-function of HIPP26 affects expression of genes induced by ATHB29

To further analyze the functional relationship between HIPP26 and ATHB29, expression of known target genes of ATHB29 was analyzed in wild type and mutant plants Athipp26, not expressing *HIPP26* (for Northern analyses see supplemental Fig. 2). Three genes, At1g13320 (PDF1, a 65 kDa subunit of protein phosphatase 2A), At3g53090 (UPL7, ubiquitin-protein ligase 7) and At4g26410 (unknown protein), were due to genome-wide transcript analyses for identification and testing of standard genes (Czechowski et al. 2005) chosen as reference genes. Their expression levels are not changed in plants overexpressing ATHB29 (Tran et al. 2007). At1g13320 was used as reference to normalise the quantitative RealTime PCR data of the genes of interest. The data illustrated in Fig. 7 show that the relative gene expression rates of At3g53090 and At4g26410 are not changed in mutant line Athipp26 when compared to the wild type. In contrast, expression levels of six stress-related genes which have previously been shown to be highly up-regulated after overexpression of ATHB29 (Tran et al. 2007), are clearly down regulated in Athipp26 plants. In addition, we analyzed expression of two known cold-regulated genes At5g15970 (cor6.6) and At5g52310 (cor78) which are down-regulated in ATHB29 overexpression plants (Tran et al. 2007). Both ATHB29 down-regulated genes show no significant difference in mRNA levels in HIPP26 mutant and wild type. Our data show that loss-of-function of HIPP26 inhibits ATHB29-dependent induction of stress-related target genes.

Discussion

In 1999 Dykema et al. identified a new class of proteins which exhibit two conserved domains, a heavy metal associated domain and an isoprenylation motif at the

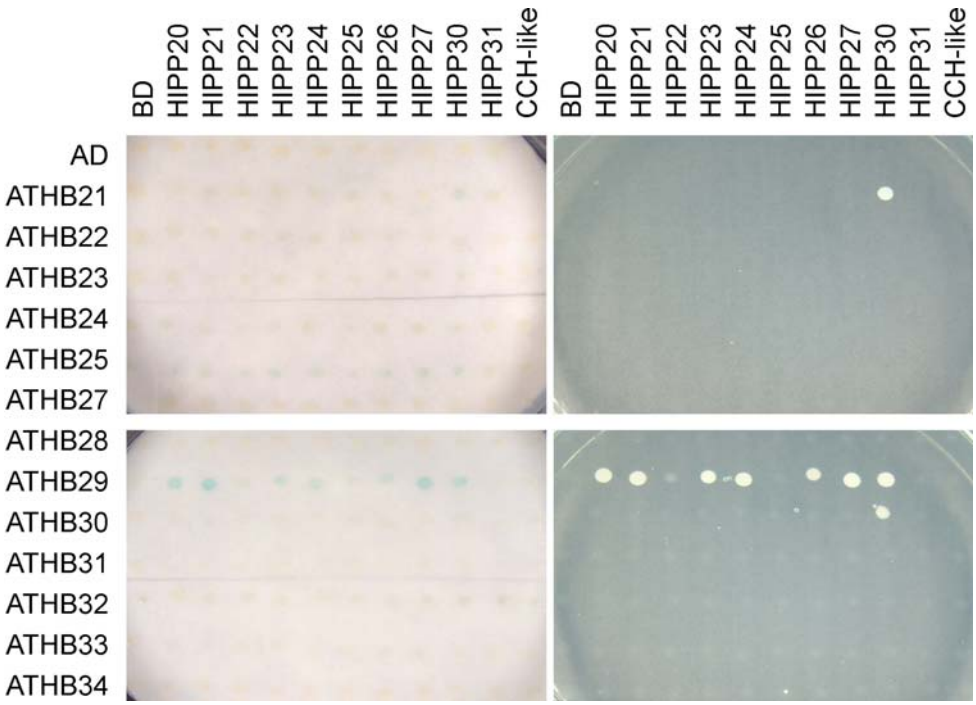


Fig. 5 Yeast two hybrid analyses of protein–protein interactions between members of the ATHB family and different HIPP proteins of cluster III. Left panel: The blue coloration in the filter lift assay indicates protein–protein interactions within the diploid yeast cells through the activation of the *lac*Z reporter genes. *Right panel*: Growth on the synthetic dropout medium lacking amongst others adenine illustrates interactions through the activation of the auxotrophic reporter gene *ADE2*. BD: empty Gal4 DNA binding domain vector control (pGBKT7); AD: empty Gal4 transcription activation domain vector control (pGAD-C1)

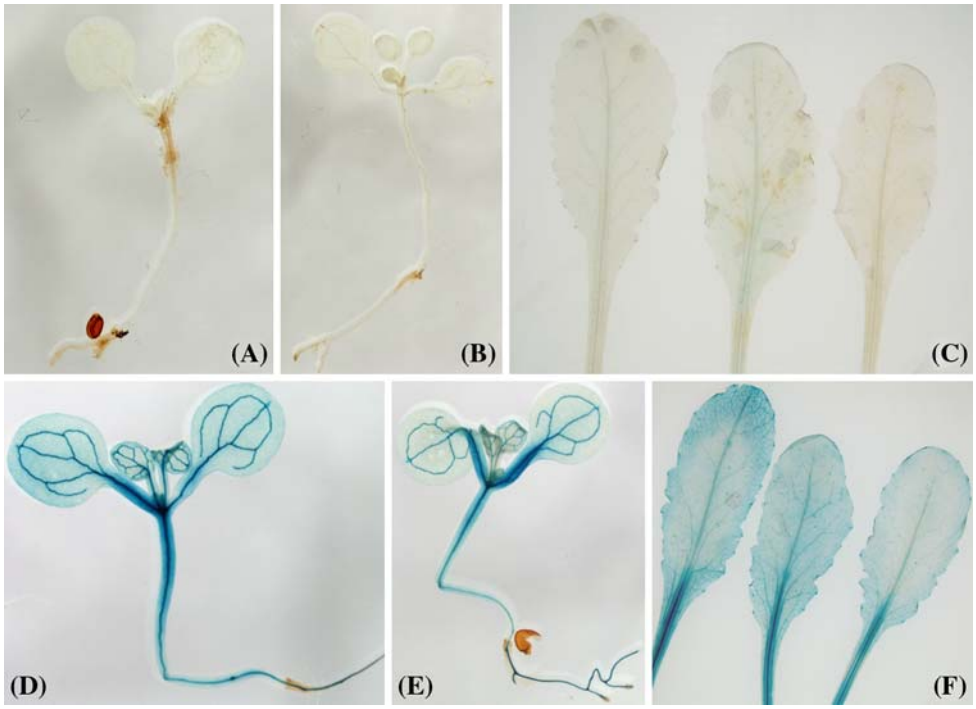


Fig. 6 Tissue specific expression of GUS under the control of the *HIPP26* promotor. **a** and **b** show 10 days old Arabidopsis plants and **c** shows leaves of 8 weeks old Arabidopsis plants, all transformed with the empty vector (pGPTV-KAN) as controls. **d** and **e** show 10 days old Arabidopsis plants and **f** leaves of 8 weeks old Arabidopsis plants, in each case transformed with the HIPP26 promotor-GUS construct. GUS activity was visualized by treatment with X-GlcA (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid)

C-terminal end. This family of heavy metal associated isoprenylated plant proteins (HIPPs) embraces at least 44 proteins in *Arabidopsis thaliana* (see Fig. 1a). The heavy metal associated domain (HMA) with its central motif M/L/IxCxxC which via forming cysteinyl sulphur ligands is responsible for heavy metal binding is reported to be involved in metal transport and metal homeostasis processes (Dykema et al. 1999; Harrison et al. 2000). Known HMA domain proteins in plants are the copper chaperones CCH, AtATX1 and AtCCS. CCH was identified as functional ortholog of the yeast copper chaperone *Anti-oxidant 1* (ScATX1) by Himelblau et al. (1998). AtATX1 delivers copper to RAN1, a copper-transporting P-type ATPase involved in ethylene response (Puig et al. 2007). AtCCS feeds copper to the copper/zinc superoxide dismutase (Chu et al. 2005).

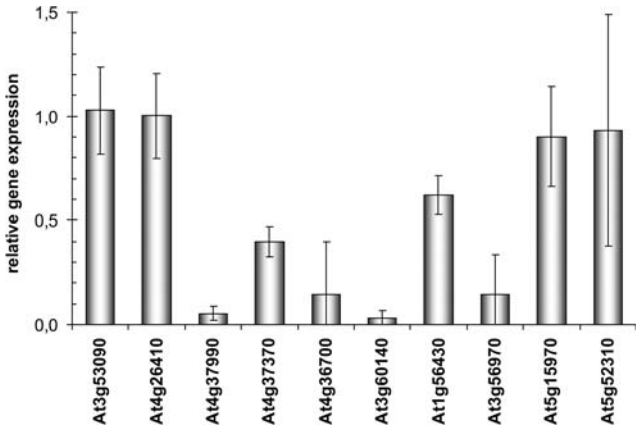


Fig. 7 Loss-of-function of HIPP26 affects expression of target genes of ATHB29 (At4g37990, At4g37370, At4g36700, At3g60140, At1g56430 and At3g56970; Tran et al. 2007) which are induced by overexpression of ATHB29. The cor6.6 (At5g15970) and cor78 (At5g52310) genes that are negatively influenced in ATHB29 overexpression lines (see supplemental data of Tran et al. 2007) are not affected. Expression levels of ATHB29 target genes and of two additional reference genes (At3g53090 and At4g26410) were analyzed in wild type and Athipp26 mutant plants by quantitative RealTime-PCR. The level of mRNA in each case is normalized to that of At1g13320 as reference gene (Czechowski et al. 2005). The results are represented as relative gene expression ratio for the Athipp26 mutants versus the wild type. Error bars indicate the standard error ($n = 3$)

HIPPs contain in addition to the HMA domain an isoprenylation motif at the C-terminal end which can be attacked by farnesyl- or geranylgeranyl-transferases (Rodriguez-Concepcion et al. 1999). Isoprenylation is a characteristic post-translational modification of many regulatory proteins which attaches a hydrophobic side chain to the protein and by this enables specific interactions with either membranes or other proteins (Crowell 2000). Except for the cadmium binding protein Cdi19 (HIPP6) which was shown to be involved in heavy metal homeostasis and/or detoxification (Suzuki et al. 2002), nothing is known about the function of the proteins that belong to the HIPP family.

In this report we aim at a better understanding of the role of HIPP proteins in stress response in plants. We focus on HIPP proteins of group III (Fig. 1a), which show the highest homologies to the recently identified nuclear, stress responsive protein HvFP1 from *Hordeum vulgare* (Barth et al. 2004). The barley *HvFP1* also contains a HMA domain and an isoprenylation motif. It is expressed during cold and drought stress, in response to abscisic acid and during leaf senescence (Barth et al. 2004). Sub-cluster III of the Arabidopsis HIPP proteins consists of 13 HIPP proteins with up to now unknown functions. Our expression studies show that some, but not all of these HIPPs in sub-cluster III are also cold induced (HIPP23, HIPP24, HIPP25, HIPP26). Because of the high homology to HvFP1 and because of the clear cold dependent expression, HIPP26 was chosen for further detailed functional analyses. *HIPP26* is induced during cold, salt and drought stress. Promotor analyses revealed consequently the presence of salt, cold and drought stress related *cis*-elements like GT-1 boxes, LTRE-1 and DRE2 elements, MYC and MYB recognition sites. But in contrast to *HvFP1* the *HIPP26* gene is not induced in response to ABA and during leaf senescence, indicating a more specialized regulation of the different *HIPP* genes in Arabidopsis when compared to the barley gene *HvFP1*. Published expression data of Arabidopsis genes at https://www.genevestigator.ethz.ch (Zimmermann et al. 2004) support our finding that *HIPP26* is induced in response to cold and salt, and to a lower extent also to drought.

HIPP26 like some other HIPPs exhibits a NLS. We could confirm the predicted nuclear localization of HIPP26 by confocal laser scanning microscopy of onion epidermal cells transformed with GFP-HIPP26 constructs (Fig. 3). These data indicate that HIPP26 is a nuclear protein and functions within the nucleus. For the exact spatial localization of HIPP26 within the nucleus, the isoprenylation seems to be important. When using the complete construct with the intact isoprenylation motif, a speckle-like nuclear localization can be observed indicating site-specific distribution for HIPP26 in the nucleus. When isoprenylation is impaired, a more uniform distribution with highest levels in the nucleoli could be observed. This finding could indicate that the farnesyl- or geranylgeranyl-residue of HIPP26 probably by its hydrophobic nature determines the correct spatial arrangement of this protein within the nucleus. That farnesylation directs proteins to the nucleus was recently reported by Galichet et al. (2008).

Plants respond to stress in a complex temporal, but also spatial pattern. Protection mechanisms against stress induced damages often preferentially take place in those areas in plants which are very sensitive towards the stress. It is known that especially the vascular tissue, together with meristematic tissue, is sensitive against abiotic stressors like low temperature or high salt (Nylander et al. 2001). Consequently, there are many reports which show that genes involved in the response against these abiotic stressors are preferentially expressed in these tissues. For example many authors could show that stress induced dehydrins accumulate in the vascular tissue and bordering parenchymal cells (e.g. Nylander et al. 2001; Godoy et al. 1994; Houde et al. 1995; Danyluk et al. 1998; Bravo et al. 1999). Other examples for stress related genes which are preferentially expressed in the vascular tissue are the drought, salt and cold stress regulated potassium channel gene GORK (Becker et al. 2003), freezing tolerance associated proteins (Houde et al. 1995) and rice C2H2-type zinc finger protein playing a role in salt tolerance (Huang et al. 2007). The novel stress responsive gene *HIPP26* also follows this pattern of preferential expression in the sensitive vascular tissue. Analyses

of known expression-data using the genevestigator tool (https://www.genevestigator.ethz.ch, Zimmermann et al. 2004) revealed a strong coexpression of *HIPP26* and the found interactor *ATHB29* (see below) in the vascular parenchyma.

Our detailed yeast-two-hybrid analyses indicate that HIPP26 strongly interacts with ATHB29, a zinc finger-homeodomain transcription factor (ZF-HD proteins). This was confirmed by a GST pull-down approach. Zinc finger-homeodomain transcription factors constitute a unique subclass of plant homeodomain proteins (Tan and Irish 2006). Yeast two-hybrid analyses revealed that the ZF-HD proteins tend to form homo- and heterodimers which might play an important role in the interaction with the DNA and therefore might be involved in transcriptional regulation (Tan and Irish 2006). It could be shown that the homeodomain inherits DNA binding and the zinc-finger domain with its zinc is important for the dimerization (Windhövel et al. 2001).

Our knowledge about the functions of the different ZF-HD proteins during plant development and stress response is still rather incomplete. It was shown that several ZF-HD proteins are expressed during floral development (Tan and Irish 2006). Other proteins of this class of transcription factors (GmZF-HD1 and GmZF-HD2) are involved in pathogen response (Park et al. 2007). A recent publication of Tran et al. (2007) proves a role of the zinc finger homeodomain transcription factor ATHB29 (At1g69600, also termed ZFHD1) in regulation of dehydration-inducible gene expression. ATHB29 binds to the ZFHD recognition sequence in the promoter region of dehydration-inducible genes. These authors also show that ATHB29 is induced by drought, high salinity and abscisic acid. Microarray analyses of transgenic plants overexpressing ATHB29 further revealed that several stress inducible genes were up regulated and the transgenic plants show an improvement of drought stress tolerance (Tran et al. 2007). These data clearly indicate a role of the zinc finger homeodomain transcription factor ATHB29, which as shown in this publication itself interacts with the novel heavy metal associated isoprenylated protein HIPP26, in regulation of stress response of plants. Further on, we could show that ATHB29 interacts with several HIPP proteins of cluster III (HIPP20, HIPP21, HIPP23, HIPP24, HIPP26, HIPP27, HIPP30) and that, with the exception of HIPP30, all these proteins interact strongly with this member of the ZF-HD family which plays a central role in dehydration stress response (Tran et al. 2007) indicating a functional link between HIPPs and ATHB29 during stress response in plants. This functional relationship is confirmed by our results showing that loss-of-function of HIPP26 in mutant line Athipp26 specifically inhibits expression of stress-responsive genes upregulated by ATHB29.

Taken together our data indicate that the cold, salt and drought stress induced nuclear protein HIPP26 functionally interacts with the ZF-HD transcription factor ATHB29 which is involved in dehydration stress response of plants (Tran et al. 2007). Although not yet confirmed, the presence of the HMA domain in HIPP26 argues for binding of heavy metals like copper or zinc to HIPP26. In this aspect the interaction with the zinc finger transcription factor is very interesting. As shown in Fig. 4, this interaction depends on the presence of an active heavy metal binding domain. Prevention of metal binding by mutation of the two metal binding cysteines impedes the interaction. Our results indicate that HIPP26 via its heavy metal binding domain regulates the zinc finger transcription factor. A mechanism based on the interaction of a metal bound to a HMA domain with a regulatory factor is already known from bacterial systems where the HMA domain protein copZ delivers copper to the repressor copY, and by displacing the structurally needed zinc from copY by copper inactivates this repressor, which then leads to transcription of the cop operon responsible for copper homeostasis (Cobine et al. 1999; Solioz and Stoyanov 2003). The exact mode of action of the two regulatory factors HIPP26 and ATHB29 in response of the plants to different stress conditions has to be solved in future experiments.

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