RESEARCH PAPER

Disrupting the bimolecular binding of the haem-binding protein 5 (AtHBP5) to haem oxygenase 1 (HY1) leads to oxidative stress in Arabidopsis

Hye-Jung Lee¹, Nobuyoshi Mochizuki², Tatsuru Masuda³ and Thomas J. Buckhout¹,*

¹ Applied Botany, Institute of Biology, Humboldt University Berlin, Invalidenstraße 42, 10115 Berlin, Germany
² Department of Botany, Graduate School of Science, Kyoto University, Kitashirakawa, Kyoto 606–8502, Japan
³ Department of General Systems Studies, Graduate School of Arts and Sciences, University of Tokyo, Komaba 3-8-1, Tokyo, 153–8902, Japan
* To whom correspondence should be addressed: E-mail: h1131dqy@rz.hu-berlin.de

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Abstract

The Arabidopsis thaliana L. SOUL/haem-binding proteins, AtHBP5s belong to a family of five members. The Arabidopsis cytosolic AtHBP1 (At1g17100) and AtHBP2 (At2g37970) have been shown to bind porphyrins and metalloporphyrins including haem. In contrast to the cytosolic localization of these haem-binding proteins, AtHBP5 (At5g20140) encodes a protein with an N-terminal transit peptide that probably directs targeting to the chloroplast. In this report, it is shown that AtHBP5 binds haem and interacts with the haem oxygenase, HY1, in both yeast two-hybrid and BiFC assays. The expression of HY1 is repressed in the athbp5 T-DNA knockdown mutant and the accumulation of H₂O₂ is observed in athbp5 seedlings that are treated with methyl jasmonate (MeJA), a ROS-producing stress hormone. In contrast, AtHBP5 over-expressing plants show a decreased accumulation of H₂O₂ after MeJA treatment compared with the controls. It is proposed that the interaction between the HY1 and AtHBP5 proteins participate in an antioxidant pathway that might be mediated by reaction products of haem catabolism.

Key words: Arabidopsis, haem oxygenase, oxidative stress, haem-binding protein.

Introduction

Haem is prominent among the iron binding molecules in the cell. This tetrapyrrole binds ferrous iron (Fe²⁺) at four co-ordinated nitrogens in the protoporphyrin ring system and is incorporated into many apo-proteins as a prosthetic group (Kumar and Bandyopadhyay, 2005). Free haem molecules can react with oxygen at one of the two uncoordinated binding sites of Fe²⁺, producing Fe³⁺ and reactive oxygen species (ROS, Balla et al., 2003). The presence of free haem in the cytoplasm must be maintained at a low concentration (<0.1 µM) to prevent oxidative stress through the oxidation of haem iron (Khan and Quigley, 2011).

In higher plants, haem is synthesized by the tetrapyrrole biosynthesis pathway in plastids, sharing a common biosynthetic pathway with chlorophyll up to the intermediate protoporphyrin IX. At this point the pathway diverges into the Fe²⁺ and Mg²⁺ branches and continues with the ferro- or Mg-chelatase, respectively (Mochizuki et al., 2010). Haem is widely distributed in the cell; although, its allocation and trafficking into the cytosol, endoplasmic reticulum, mitochondria or other target organelles in plant cells are not well understood. The degradation of haem is mediated by haem oxygenase (HY or HO), a mixed function oxidase that catalyses the oxidative cleavage of the α methine carbon atom of haem, producing biliverdin-IXα (BV-IXα), Fe³⁺, and CO (Khan and Quigley, 2011). In higher plants BV-IXα can be further reduced to phytochromobilin (PΦB), which serves as a chromophore for phytochrome (Terry et al., 2002). The phytochrome-deficient mutants hyl1, pcd1, and se5 in...
Arabidopsis, pea (Pisum sativum L.), and rice (Oryza sativa L.), respectively, all lack haem oxygenase 1 and show an impaired conversion of haem to BV-IX\textsubscript{a}. Several haem oxygenase genes have been found in higher plants (Davis et al., 1999; Muramoto et al., 1999; Izawa et al., 2000). Arabidopsis, for example, has four HO genes, HY1 (synonymous with HO1, At2g26670), HO3 (At1g69720), and HO4 (At1g58300) that belong to the HO-1 subfamily and encode putative haem oxygenases. HO2 (At2g26550) is the sole member of the HO-2 subfamily and shows stable, high affinity binding to protoporphyrin IX \textit{in vitro} (Gisk et al., 2010). The principal difference between the HO-1 and HO-2 subfamilies is a 15 amino acid insertion in HO-2 that is rich in aspartate and glutamate and the absence of a conserved histidine that is presumably necessary for haem binding (Davis et al., 2001). In Arabidopsis (Col-0), the transcription level of HY1 is significantly higher than HO2, HO3, and HO4 in most tissues. Phenotypic studies with the \textit{hol} single mutant and \textit{ho} double or triple mutants demonstrate that the \textit{hy1} null mutant dramatically alters plant growth and development, indicating its dominant function in photomorphogenesis (Emborg et al., 2006).

HO also participates in cell defense against oxidative stress in higher plants. It was reported that reactive oxygen species (ROS) triggered the expression of HY1 in soybean and wheat plants (Noriega et al., 2004; Chen et al., 2009; Wu et al., 2011). Similarly, the cytoprotective signal, potentiated by low concentrations of nitric oxide (NO) under UV-B irradiation, was associated with the enhanced expression of HO (Yannarelli et al., 2006; Santa-Cruz et al., 2010). This response to oxidative stress was confirmed at the transcript, protein and enzyme activity levels.

Haem is relatively hydrophobic, and it is difficult to envisage how a product of a membrane-localized ferrochelatase would have access to the soluble stromal protein, HO (Joyard et al., 2009). Furthermore, since the products of HO, Fe\textsuperscript{2+}, CO, and BV-IX\textsubscript{a}, are themselves reactive catabolites, it is essential that haem metabolism be regulated. We speculate that the transfer of haem to HO requires a carrier protein.

\textit{AtHBP2} was initially identified as a phytocrome A-induced transcript that rapidly responded to light during de-etiolation (Khanna et al., 2006). \textit{AtHBP2} encodes a p22HBP/SOUL protein and belongs to a family of six \textit{Arabidopsis} genes. The amino acid sequences of the AtHBPs are homologous to mammalian SOUL and p22HBP (haem-binding protein), which were initially purified from vertebrates and have been shown to bind cytosolic haem (Taketani et al., 1998; Zylla and Reppent 1999). These vertebrate haem-binding proteins were considered to function in transfer or delivery mechanisms of cytosolic haem to apoproteins (Jacob Blackmon et al., 2002; Babusiak et al., 2005; Dias et al., 2006). Takahashi et al. (2008) have shown that cytosolic \textit{Arabidopsis} AtHBP1 (At1g17100) and AtHBP2 (At2g37970) bound porphyrins, including haem, and they proposed that these two HBPs were cytosolic tetrapyrrole-carrier proteins.

In contrast with the putative cytosolic AtHBPs, AtHBP3 and AtHBP5 (At3g10130 and At5g20140, respectively) have N-terminal transit peptides that are predicted to target chloroplasts. However, haem binding by these proteins has not been shown. In the present report, the physiological function of AtHBP5 was characterized by genetic and biochemical methods and by mutant analysis. Our data show that AtHBP5 is a haem-binding protein that interacts with the haem oxygenase 1 (HY1) in chloroplasts and that HY1 expression is repressed in an \textit{athbp5} knockdown mutant. It is proposed that the biomolecular binding of AtHBP5 and HY1 participates in an antioxidant pathway presumably through an influence on haem catabolism.

### Materials and methods

#### Plant material, transformation methods and growth conditions

\textit{Arabidopsis thaliana} (L.) Heynh. (Columbia-0) was used for all the experiments. The T-DNA insertion line for the \textit{AtHBP5} gene, sail_1280\_C03, was obtained from NASC (Nottingham, UK). To generate \textit{AtHBP5} over-expressing plants, full-length \textit{AtHBP5} from cDNA was placed under the control of the cauliflower mosaic virus 35S-promoter and introduced into wild-type \textit{Arabidopsis} plants by the floral dip method using \textit{Agrobacterium}-mediated transformation (Clough and Bent, 1998). The genotypes of the T-DNA mutant were confirmed using PCR analysis, and immunoblots were used to identify over-expressing transgenic plants using a polyclonal anti-\textit{AtHBP5} antibody (see below). Plants were grown on ES agar medium (Gollhofer et al., 2011) at 21 °C under a 10 h light (95 μmol m\textsuperscript{-2} s\textsuperscript{-1})/14 h dark photoperiod or on soil as indicated.

#### Construction of \textit{AtHBP} expression plasmids and recombinant protein expression in \textit{E. coli}

The sequences encoding full-length \textit{AtHBP1}, 2, 3, and 5 were amplified from \textit{Arabidopsis} cDNA with the primers shown in Supplementary Table S1 at \textit{JXB} online. The amplified fragments were cloned into the pGEM-T vector (Promega), and constructs were confirmed by sequencing (SMB GmbH, Berlin, Germany). The fragments were digested with NdeI/SalI to yield \textit{AtHBP1}, \textit{AtHBP2}, and \textit{AtHBP3} or NdeI/HolI to yield \textit{AtHBP5}, and then cloned into pET-24a or pET-28a vectors (Merck Chemicals). The expression vectors containing the \textit{AtHBP} genes fused to a 6xHis tag, were introduced into \textit{E. coli} [BL21(DE3) or C43(DE3)]. Expression of the 6xHis tagged recombinant proteins was induced at 20 °C by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.05 mM. Recombinant proteins were purified using Ni\textsuperscript{2+}-NTA agarose (Qiagen) under native conditions.

The coding sequence for the mature \textit{AtHBP5} protein excluding the N-terminal 52 amino acids was amplified with the appropriate primers (see Supplementary Table S1 at \textit{JXB} online), and the fragment cloned into the pGEM-T. After validation of the amplified nucleotide sequence, the fragment was inserted into pET-24(+) and transformed into \textit{E. coli} BL21 (DE3). Synthesis of the 6xHis-tagged mature \textit{AtHBP5} protein was induced at 30 °C by the addition of IPTG to a final concentration of 0.2 mM, and the recombinant protein was purified using an IMAC cartridge (Bio-Rad) under denaturing conditions. The purified \textit{AtHBP5} protein was used as an antigen for production of the anti-\textit{AtHBP5} antibodies (Operon, Japan).

#### Haem binding assay

Haem binding was performed followed the method of Mills and Payne (1995). Briefly, the proteins \textit{AtHBP1}, \textit{AtHBP2}, \textit{AtHBP3}, and \textit{AtHBP5} were diluted in resuspension buffer (20 mM TRIS-HCl, pH 8.0, 150 mM NaCl) and incubated with 50 μl of pre-equilibrated hemin agarose (H6390, Sigma-Aldrich; packed volume, >4 μmol ml\textsuperscript{-1}) for 0.5 h at room temperature on a rotary shaker. The mixture was centrifuged for 5 min at 750 g and the pellet washed three times with high-salt buffer (20 mM TRIS-HCl, pH 8.0, 20 mM EDTA and 1 M NaCl) to remove non-specifically bound proteins. The resin mixture was washed once in an equilibration solution, and finally suspended in 100 μl SDS sample buffer. The hemin-bound protein was subjected to SDS-PAGE analysis, followed by immunoblotting and detection with horseradish peroxidase (HRP)-conjugated anti-His antibody (Sigma-Aldrich).
Screening for protein-protein interactions by yeast two-hybrid analysis

The coding regions of AtHBP1, AtHBP2, AtHBP3, AtHBP5, and HY1 (At2g26670) were amplified without the predicted signal peptide from Arabidopsis cDNA with the appropriate primers (see Supplementary Table S1 at JXB online) by PCR. The amplified fragments were cloned into the TA-cloning vector and sequenced. The fragments were digested and subcloned into pBTH117c vector that contained the LexA DNA-binding and into pGAD10 that contained the GAL4 activation domain (Waneker et al. 1997). These vectors were used for yeast two-hybridization. Saccharomyces cerevisiae strain L40ccua was co-transformed with bait and prey vectors by the lithium acetate method, and the positive transformants were verified by growing on selection medium (SC-trp-leu-his-ura medium, and β-galactosidase activity was assayed using the X-gal filter lift method. The actively growing colonies were blotted onto nitrocellulose filters. The filters were frozen in liquid nitrogen, thawed at room temperature for 30 s, and placed on Whatman 3MM paper saturated with X-gal solution for detection of β-galactosidase activity.

Split yellow fluorescence protein (YFP) for protein interaction analysis

For the in vivo protein interaction, the bimolecular fluorescence complementation (BiFC) method described by Walter et al. (2004) was adapted. The modified YFP split binary vectors, pSPYNE-35S/pUC-SPYNE, which encodes the N-terminal fragment of YFP, and pSPYCE-35S/pUC-SPYCE, encoding the C-terminal fragment of YFP both under the control of the 35S promoter, were used. The open reading frames of AtHBP5 and HY1 were cloned using the appropriate primers (see Supplementary Table S1 at JXB online) and verified by sequencing. In parallel, cloning of HY1 and AtHBP5 genes without the predicted N-terminal transit peptide was also conducted using the primers listed in Supplementary Table S1 at JXB online. The amplified fragments were digested with SmaI and subcloned into the corresponding binary vectors. The combinations of AtHBP5_YFP and HY1_YFP or AtHBP5_YFP and HY1_YFP BiFC binary vectors were transiently expressed in tobacco (Nicotiana benthamiana) leaves. Three days after transformation, the infiltrated tobacco leaves were observed under a confocal laser scanning microscope (TCS SP2, Leica).

Identification of T-DNA mutant and semi-quantitative reverse transcriptase (RT)-PCR analysis

The AtHBP5 T-DNA insertion mutant was initially grown on soil and selected for herbicide resistance by spraying with BASTA (0.01%). The mutants surviving the herbicide treatment were screened by PCR genotyping using gene-specific primers (see Supplementary Table S1 at JXB online). Total RNA was extracted from T-DNA mutant plants using TRIzol (Bioline), and 1 μg RNA was treated for 30 min with Dnase I as described in the Fermentas manual. cDNA was synthesized using Revert Aid M-MuLV Reverse Transcriptase (Fermentas). Semi-quantitative PCR was used for the analysis of gene expression and PCR primers were designed with PerlPrimer software (see Supplementary Table S1 at JXB online; Marshall, 2004).

Plant treatment and detection of H2O2

Arabidopsis seeds were surface-sterilized for 3 min with 4% (v/v) NaOCl and rinsed five times with sterile dH2O. Seeds were plated on ES-agar and vernalized for 5 d at 4 °C in the dark. One-week-old athbp5, AtHBP5 over-expressing transformants, and wild-type seedlings were treated with 200 μM MeJa by spraying and incubated for 3 d. Diaminobenzidine (0.1% DAB) solutions (D4168, Sigma-Aldrich) were prepared in dH2O. The MeJa-treated seedlings were incubated in the DAB solution at room temperature in the dark until brown spots appeared. The reaction was stopped by immersion of the seedlings in boiling ethanol (96%) for 10 min and the DAB-stained seedlings were photographed. Determination of H2O2 in plant tissues was conducted using the method of Zhou et al. (2006). Briefly, 0.3 g of MeJa-treated seedlings was ground in liquid N2 to which 3 ml of 5% TCA and 0.1 g activated charcoal (Sigma-Aldrich) were added. The homogenate was cleared by centrifugation, and the pH of the extract was adjusted to 8.4 with ammonia hydroxide. The filtered extract was divided into aliquots of 1 ml. Catalase was added to one aliquot, which was subsequently used as a blank. The H2O2 content in the remaining aliquots was determined colorimetrically at 505 nm.

Determination of haem, chlorophyll and carotenoid

The haem content of intact plants was determined by the chemiluminescence of HRP using the method of Espinas et al. (2012). Chlorophyll and carotenoid content were determined according to Melis et al. (1987) and according to Lichtenthaler (1987), respectively.

Results

Phylogenetic analysis of SOUL/haem-binding proteins (HBPs)

In a search of the NCBI (National Center for Biotechnology Information) database for plant genes containing a SOUL/haem-binding protein (HBP) domain, 62 proteins from flowering plants, a conifer, and a club-moss were found. These were subjected to a phylogenetic analysis (see Supplementary Fig. S1 at JXB online). Five Arabidopsis p22HBP/SOUL proteins were found in both A. thaliana and A. lyrata, and in two subspecies of rice (O. sativa Japonica and Indica groups). An additional gene locus in Arabidopsis, At1g78450, which showed high sequence similarity to SOUL/HBPs, has been identified as a pseudogene (Takahashi et al., 2008) and was therefore not included in the analysis. An unrooted, radial phylogenetic tree of higher plant p22HBP/SOUL amino acid sequences was constructed using the Neighbor–Joining method, and the reliability of the phylogenetic tree was confirmed using a bootstrap re-sampling strategy (Tamura et al., 2011). The resulting tree was divided into five clearly distinct clades, of which four contained at least one Arabidopsis p22HBP/SOUL (see Supplementary Fig. S1 at JXB online). Interestingly, Arabidopsis sequences were not found in clade 4, which, however, contained representatives of all other genera analysed (see Supplementary Fig. S1 at JXB online). Clade 2 included nine sequences from flowering plants but lacked sequences from Picea and Selaginella (see Supplementary Fig. S1 at JXB online). The members of clades 1, 2, 3, and 4 formed a clade of clades, whereas clade 5 displayed a sister group-like relationship among members.

The recombinant proteins AtHBP1, AtHBP2, and AtHBP5 bind haem in vitro

The haem binding activity of the putative AtHBPs was initially investigated. The full-length AtHBP1, AtHBP2, AtHBP3, and AtHBP5 coding sequences were cloned from wild-type Arabidopsis cDNA and expressed in E. coli. Although most His-tagged recombinant AtHBPs were present in inclusion bodies from the bacterial cells, it was possible to express the soluble AtHBPs under non-denaturing conditions by growing cells
at 20 °C. Recombinant AtHBPs were detected using an anti-His tag antibody after separation by SDS-PAGE. For binding assays using a batch procedure, pre-equilibrated hemin-agarose was added to the purified recombinant AtHBPs and non-hemin-bound proteins were subsequently removed by washing with a high salt buffer. Bound AtHBPs were eluted with SDS sample buffer, separated by SDS-PAGE (12% acrylamide), blotted onto a PVDF membrane, and immunologically detected.

Using this procedure, the recombinant AtHBP5 protein was detected in the fraction bound to hemin-agarose (Fig. 1A, lane ‘HA’). Binding of AtHBP1 and AtHBP2 to hemin-agarose was also observed (Fig. 1A, lane ‘HA’), confirming the previous observation of Takahashi et al. (2008). The His-tag antibody recognized the recombinant AtHBP3 protein in the second high-salt wash fraction (‘HS2’), but no signal was detected in the bound fraction (Fig. 1A, lane ‘HA’). Thus, it was concluded that the AtHBP5 bound to hemin-agarose, while the binding of AtHBP3 was non-specific.

To verify the specificity of the haem-AtHBP5 protein complex, the absorbance spectrum of haem upon addition of the purified AtHBP5 protein was monitored. The recombinant AtHBP5 protein, lacking the predicted signal peptide, was cloned and expressed in E. coli. The AtHBP5 protein was resuspended in TRIS buffer (20 mM TRIS-HCl, pH 6.5), incubated with 5-fold excess of hemin for 20 min on ice, and the spectrum analysed. Upon addition of the AtHBP5 protein, the broad Soret band of free hemin was intensified and λ_max shifted from 398 nm to 413 nm. In addition, Q-bands appeared around 540 nm (Fig. 1B). This shift was attributed to the co-ordination of hemin by the AtHBP5 protein. A similar spectral shift in the Soret region has been previously observed for the Fe^{3+}-haem–SOUL complex (Sato et al., 2004). When the reaction was performed at pH 2.2, the peak in the Soret region was widened but λ_max was largely unchanged (Fig. 1B). These data supported haem binding by AtHBP5.

Protein–protein interaction between AtHBP5 and HY1

The AtHBP5 protein had an N-terminal extension, which was not found in AtHBP1, AtHBP2 or mammalian homologues. AtHBP5

![Fig. 1. (A) Haem-binding activity of recombinant AtHBPs. Total protein extracts from E. coli following IPTG treatment (fraction ‘A’) were chromatographed on a Ni^{2+}-agarose column. The column was washed with loading buffer and eluted with imidazole buffer (‘FL’=flow-through fraction, ‘W’ and ‘E’=wash and eluted fractions, respectively). The eluted fractions were concentrated and bound in a batch method to hemin-agarose beads. The beads were washed twice with high-salt-containing buffer and eluted with SDS sample buffer. ‘S’ is the soluble fraction after incubation with hemin-agarose beads, ‘HS1’ and 2 are high-salt wash fractions, ‘HA’ the eluted HBP fraction, and M is the molecular mass marker. (B) Spectrophotometric analysis of haem binding to AtHBP5. The purified AtHBP5 protein was incubated with a 5-fold excess of hemin for 20 min on ice and the spectrum analysed (b). The spectrum of hemin alone (a) or hemin plus AtHBP5 at pH 2.2 (c) are also shown.](image-url)
Bimolecular interaction between AtHBP5 and HY1 was predicted to be localized in chloroplasts (Emanuelsson et al., 2007) and was identified in chloroplasts by mass spectrometry (Zybaiov et al., 2008). As HY1 is a crucial enzyme in haem homeostasis, the possibility was considered that AtHBP5 might be involved in the delivery of haem to HY1 in chloroplasts.

The protein–protein interaction between AtHBP5 and HY1 was examined in a yeast two-hybrid system. The bait or prey plasmids containing HY1 and AtHBPs were co-transformed into an L40ccua yeast strain as described (see Materials and methods). The co-transformed yeast cells were tested for the activation of the LacZ reporter gene, as well as for growth on media lacking tryptophan, leucine, histidine, and uracil. After 24 h incubation, the X-gal product was visible only in yeast cells co-expressing HY1 and AtHBP5 fusion proteins. The activity of the reporter gene was silent in the other yeast colonies (Fig. 2A).

Similar results were obtained when the bait and prey vectors were reversed. Thus, the results of the yeast two-hybrid analysis supported a protein–protein interaction between HY1 and AtHBP5.

To verify the interaction of HY1 and AtHBP5 in living plant cells, a bimolecular fluorescence complementation (BiFC) assay was used. YFP sequences encoding either the N-terminal 155 amino acids or the C-terminal 86 amino acids were fused to the full-length HY1 or AtHBP5 genes (AtHBP5-YFPn and HY1-YFPc or AtHBP5-YFPc and HY1-YFPn). Tobacco leaves were transiently co-transformed and yellow fluorescence was observed using an excitation wavelength from 490–510 nm. In leaves that co-expressed with AtHBP5-YFPc and HY1-YFPn, a yellow fluorescent signal co-localized with the red autofluorescence of chlorophyll (Fig. 2B). Tobacco leaves transformed with any combination of empty binary vectors showed chlorophyll fluorescence but no YFP signal (Fig. 2C). These results confirmed a protein–protein interaction between HY1 and AtHBP5 in chloroplasts.

A fluorescence signal could not be detected in leaves co-expressing C-terminal YFP-HY1 and N-terminal YFP-AtHBP5 fusion proteins (AtHBP5-YFPn and HY1-YFPc). It was speculated that this lack of signal was caused either by the

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**Fig. 2.** Demonstration of a protein–protein interaction between HY1 and AtHBP5 by yeast-two-hybrid (A) and BiFC (B) assays. (A) Shown are results of the two-hybrid assay using HY1 as bait and the HBPs as prey and vice versa. SC, selection medium lacking leu, trp, his, and uracil (ura) as indicated. LacZ is the activity of a reporter gene encoding β-galactosidase. (B) A BiFC assay showing co-expressed of HY1 fused at the N-terminus and AtHBP5 at the C-terminus to split YFP. (C) Results from a BiFC analysis in cells transformed with empty binary vectors. The YFP fluorescence is displayed as yellow or green colours for a more distinct merged signal.
incorrect refolding of the YFP fusion protein or by rapid turn-over of the complex. On the other hand, when the BiFC experiment was conducted with HY1 and AtHBP5 proteins that lacked the 50 and 52 N-terminal amino acid sequences, respectively, the YFP-derived fluorescence signal was visible in the cytoplasm of the tobacco epidermal cells but not in plastids (see Supplementary Fig. S2 at JXB online). These results confirm the interaction of HY1 with AtHBP5.

Characterization of an athbp5 T-DNA mutant

To clarify the physiological function of AtHBP5 further, a T-DNA mutant with an insert in the 3rd intron of AtHBP5 was obtained, and five homozygous lines were selected by PCR screening. No amplification product was observed with endogenous primers in homozygous athbp5 T-DNA lines (Fig. 3A, left panel). The AtHBP5 transcript in the T-DNA mutants was investigated using semi-quantitative RT-PCR analysis with gene-specific primers. PCR products corresponding to the AtHBP5 gene were observed with template cDNA from the wild type and a heterozygous (athbp5 11–2) but not in homozygous athbp5 lines, indicating that the AtHBP5 transcript was greatly reduced in the homozygous T-DNA mutant.

To address the transcripational activity of HY1 in the athbp5 T-DNA mutant, semi-quantitative-PCR analysis was conducted using HY1 gene-specific primers in wild-type, heterozygous, and homozygous athbp5 plants. As shown in Fig. 3A (right panel), the expression of HY1 was decreased in the athbp5 lines, whereas the heterozygous T-DNA mutant, athbp5_11-2, showed no apparent difference in expression of HY1 when compared with the wild-type plants. Down-regulation was not observed for HO3 in all cases investigated. These data indicated that the

Fig. 3. Genotyping PCR and RT-PCR analysis of the athbp5 mutant and the response of this mutant to oxidative stress. (A) The top panel illustrates the location of the T-DNA insertion in the AtHBP5 gene. The bottom-left panel shows the results of a PCR analysis on genomic DNA. Signals for AtHBP5 were observed only in the wild-type and the athbp5 11–2 heterozygous line. No PCR product was observed in the homozygous lines. The lower-right PCR gel shows the results of a semi-quantitative RT-PCR using AtACT2 as a standard. ACT2=At3g18780, HY1=At2g26670, and HO3=At1g69720. (B) Seven-day-old Arabidopsis seedlings were treated with 200 μM MeJA to induce oxidative stress. The seedlings were stained in DAB buffer for 16 h in darkness. The presence of H2O2 is indicated by brown-coloured staining of the leaves (arrows). The quantitative analysis of H2O2 is also shown in (B) (lower left).
Fig. 4. Analysis of AtHBP5 over-expressing transgenic plants using an anti-AtHBP5 antibody and the response of OE plants to oxidative stress. (A) The transgenic Arabidopsis plants were transformed with full-length AtHBP5 under the control of a 35S promoter. Fifteen µg of total protein were loaded per lane, and the protein expression level of AtHBP5 in leaves was analysed using rabbit anti-AtHBP5 antiserum (lower panel). The mature AtHBP5 protein was detected at the predicted molecular mass of 37.2 kDa. A Ponceau-stained membrane is shown as a loading control. (B) Seven-day-old Arabidopsis seedlings were treated with 200 µM MeJA to induce oxidative stress. The seedlings were stained in DAB buffer for 21 h in darkness. The presence of H2O2 is indicated by brown-coloured staining of the leaves (arrows). (C) Quantification of AtHBP5 and HY1 transcription by semi-quantitative RT-PCR on untreated AtHBP5 over-expressing transgenic plants. Description of genes: EF1α=At5g60390, AtHBP5=At5g20140, and HY1=At2g26670. (D) Quantitative analysis of the H2O2 concentration in the over-expression AtHBP5 lines.
transcript level of HY1 is specifically down-regulated in the absence of AtHBP5 and might have resulted in decreased haem oxygenase activity.

Accumulation of H₂O₂ in the athbp5 T-DNA mutant treated with MeJA

Haem oxygenase1-deficient, murine embryonic fibroblasts (MEFs) had notably increased free radical production when the fibroblasts were cultured in the presence of the oxidants, hemin and H₂O₂ (Poss and Tonegawa, 1997). These data together with results of others (True et al., 2007) indicated a potentially important antioxidant function for haem oxygenase, particularly under conditions of oxidative stress. To understand the effect of repressed HY1 expression in athbp5 mutant plants, the mutants and the wild-type were treated with methyljasmonate (MeJA) which, when applied in excess, induced cell death through the accumulation of ROS (Zhang and Xing, 2008). H₂O₂ was detected by DAB staining. In the mutant leaves, H₂O₂ production was apparent as brown-colored staining on the leaf margins, while in wild-type seedlings little or no leaf staining was observed (Fig. 3B). The direct measurement of H₂O₂ confirmed the results of DAB staining. The H₂O₂ concentration was increased between 2- and 3-fold in the T-DNA mutant. These data indicated that the T-DNA insertion mutant showed enhanced susceptibility to oxidative stress, which might have been caused by loss of ROS detoxification activity in the mutant.

AtHBP5 over-expression enhanced antioxidant resistance under oxidative stress

The accumulation of H₂O₂ was observed in the athbp5 mutant following treatment with MeJA. This result indicated that the ROS concentration in the cell was altered when AtHBP5 or HY1 expression was compromised. To verify the transcriptional regulation of HY1 by AtHBP5, AtHBP5 over-expressing (OE HBP5) transgenic plants were constructed. The increased concentration of AtHBP5 protein was demonstrated by a Western blot using an anti-AtHBP5 antibody. The mature AtHBP5 protein was detected at the predicted molecular mass (37.2 kDa) in both wild-type and OE HBP5 transgenic lines (Fig. 4A, lower panel). We observed an increased AtHBP5 protein in over-expressing lines compared with the wild-type.

One-week-old seedlings of OE HBP5 transgenic lines were subjected to MeJA treatment. As shown in Fig. 4B, the brown staining on the leaf margins was only faintly visible in the over-expressing lines, whereas brown staining was extensive in the wild-type plants. Direct measurement showed an 80–90% decrease in H₂O₂ in the over-expressing plants (Fig. 4D). As predicted, over-expression of AtHBP5 resulted in plants that were less sensitive to MeJA treatment compared with the T-DNA mutant. Thus, the over-expression of AtHBP5 protein increased the ROS scavenging activity. To examine whether the over-expression may consequently modulate the transcriptional activity of HY1, an analysis of transcript abundance was conducted on the OE HBP5 plants (Fig. 4C). Consistent with the over-expression of AtHBP5 (Fig. 4A, lower panel), the transcription level of AtHBP5 was increased in the OE HBP5 transgenic lines. It was also observed that the transcription of HY1 was slightly increased compared with that of the wild-type. Considering the striking decrease of mRNA expression of HY1 in the athbp5 mutant, this difference may not be significant, but it is important to note that the over-expression of AtHBP5 had an impact on the accumulation of ROS in cells.

Analysis of haem and photosynthetic pigments

Finally, the haem and pigment contents in the mutant and over-expressing lines were investigated. Whereas the chlorophyll and carotenoid concentration and the chlorophyll a/b ratio in the mutant and over-expressing lines were unaltered, the haem content was increased in the athbp5 mutant but not in the over-expression lines. Mutation of AtHBP5 resulted in a 25% increase in haem content in mutant plants (Fig. 5). Thus, it is plausible that the higher accumulation of H₂O₂ in the athbp5 mutant was caused by an accumulation of haem and/or a decrease in haem catabolites.

Discussion

Despite the clear distinction between SOUL proteins and p22HBP in mammals, this distinction was not readily apparent in plants because of the lack of a conserved His residue and the absence of the distinct expression profiles of Arabidopsis AtHBPs. Therefore, all of the p22HBP/SOUL homologues in Arabidopsis have simply been referred to as haem-binding proteins (HBPs). In this report, the haem-binding ability of AtHBP5 has been demonstrated, and the results of Takahashi et al. (2008) have been confirmed for AtHBP1 and 2.

A phylogenetic analysis in plants grouped the HBPs into five clades based on sequence homology. Sequences in clades 1 and 2 are predicted to be localized in chloroplasts. AtHBP5 is located in clade 1 and its localization in chloroplasts supported experimentally (Fig. 2). Localization was assigned to secretory pathways for the sequences in clade 5, and no prediction could be made for the sequences in clade 3. All the sequences clustering in clade 1, including AtHBP5, contained an N-terminal NTF2-like domain, belonging to the nuclear transport factor2-like (NTF2-like) superfamily (see Supplementary Fig. S1B at JXB online). The NTF2-like domain was found in numerous proteins with widely divergent cellular functions (Dóczi et al., 2007), but the functional significance of this structure in HBPs is not known.

In this report, AtHBP5 has been identified as another member of the haem-binding protein family (Fig. 1A, 1B), whereas AtHBP3 was unable to bind to the hemin-agarose affinity column. Thus, these two proteins are not likely to have redundant functions in the cells. As a novel haem-binding protein, AtHBP5 is located in the chloroplast. A protein–protein interaction between HY1 and AtHBP5 (Fig. 2A) was demonstrated experimentally and, further, it was shown that, depending on the presence of chloroplast signal sequences, this interaction occurred in chloroplasts (Fig. 2B) or in the cytoplasm (see Supplementary Fig. S2 at JXB online).

Haem is produced from protoporphyrin IX and Fe²⁺ by ferrochelatase, located in thylakoid and chloroplast envelope...
membranes (Roper and Smith, 1997). The product of ferrochelatase is released either within the chloroplast or exported to the cytoplasm. In the former case, haem would serve as a substrate for the haem oxygenase. Our data showing the formation of an AtHBP5–HY1 complex indicate a link between the membrane-bound protein ferrochelatase and the stomatal enzyme HO. The substrate binding mechanisms, based on published protein structures, indicate that AtHBP5 may form a hydrophobic pocket with relatively high binding affinity for haem ($K_d \approx 21$ pM; Sato et al., 2004). By contrast, HY1 is believed to employ a His residue to co-ordinate ligand binding ($K_d \approx 1.6 \mu M$; Gisk et al., 2010). The haem binding to AtHBP5 could buffer the free haem at a low concentration and efficiently deliver haem to HY1.

In transcriptional analyses, HY1 transcript abundance was decreased in the athbp5 T-DNA mutant (Fig. 3A). Regardless of the decreased HY1 mRNA abundance in athbp5 plants, the mutant did not phenotypically copy the hy1 mutant, and elongation of hypocotyls that were grown in the dark, red, far-red or blue light was not different from the wild-type (see Supplementary Fig. S3 at JXB online). The fact that the athbp5 mutant did not phenocopy hy1 was not likely due to multiple isoforms of the haem oxygenase. HY1 expression was greatly decreased but not eliminated in the athbp5 mutant (Fig. 3A). Perhaps this relatively low level of expression was sufficient to ameliorate the athbp5 phenotype. On the other hand, haem accumulation as a result of decreased transcriptional activity of AtHBP5 and HY1 might have acted as a feedback inhibitor of glutamyl-tRNA reductase, which is the critical step in the regulation of tetrapyrrole biosynthesis.

The transcriptional co-expression of HY1 was observed in both the athbp5 mutant and in the over-expression lines. The signal pathway for this co-regulation remains enigmatic. A widespread co-expression of AtHBP5 and HY1 is not supported by published transcriptome analyses (e.g. Genvestigator, data not shown). This is perhaps not surprising, since the phenotype of the athbp5 mutant does not overlap with that of hy1 (=gun2). A possible mechanism for the repression of HY1 in athbp5 could be similar to the transcription factor-mediated HO-1 regulation by Bach1-Maf dimer formation found in mammals; however, at present, we have no supporting evidence for such a mechanism (reviewed by Gozzelino et al., 2010).

The athbp5 mutant showed significantly higher production of H$_2$O$_2$ compared with the wild-type following MeJA treatment.
Supplementary data can be found at JXB online.

Supplementary Table S1. Primers used for PCR experiments described.

Supplementary Fig. S1. A phylogenetic tree of 62 higher plant SOUL/haem-binding proteins and sequence alignment.

Fig. 6. Model depicting the binding of AtHBP5 and HY1 and proposed down-stream reaction leading to increased resistance to oxidative stress. In the absence of AtHBP5, the expression of HY1 is repressed by an unknown mechanism leading to the accumulation of haem and presumable to a decrease in haem breakdown products. The resulting reduction in the haem catabolites, BV-IXα and CO, would render the plants more susceptible to oxidative stress resulting from MeJA treatment.

Supplementary Fig. S2. BiFC analysis of AtHBP5 and HY1 binding in vivo.

Supplementary Fig. S3. Effect of light on hypocotyl elongation in the athbp5 T-DNA mutant.

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