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Characterization of the *Arabidopsis thaliana heme oxygenase 1* promoter in response to salinity, iron deficiency, and mercury exposure

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Abstract

The *Arabidopsis heme oxygenase 1* (*HY1*) plays a significant role in the signal transduction of abiotic stimuli and hormonal response. To characterize the *HY1* promoter, an approximately 1.8 kb of it (pHY1, -1666 to +132) and its deletion fragments (5D1, -1528 to +132; 5D2, -1109 to +132; 5D3, -688 to +132; 5D4, -169 to +132; 3D1, -1666 to +100; 3D2, -1666 to -1; and 3D3, -1666 to -170), were fused to the *β-glucuronidase* (*GUS*) reporter gene and transformed into *Arabidopsis*. The transgenic plants were subjected to several environmental stimuli (especially to mild salinity, iron deficiency, and mercury exposure). The results show that the region from +1 to +100 in the 5'-untranslated region were essential for *HY1* basal promoter activity. The induced GUS activities under NaCl and H_2O_2 treatments were slowed down by the progressive 5' deletion (from -1666 to -688) and correlated with the reduced numbers of myeloblastosis (MYB) binding sites (MBSs; -1542, -1333, -1078, and -177). The MBS-free promoter construct 5D4 (-169 to +132), however, fully lost the inducibility. Therefore, we propose that the MBS elements existing in the *HY1* promoter might be crucial for salinity-induced *HY1* up-regulation in an H_2O_2-dependent fashion. Moreover, the regions from -169 to -1 and -688 to -169 were presumed as the regulatory regions of *HY1* promoter in response to iron deficiency and mercury exposure, respectively.

Additional key words: β-glucuronidase, MYB binding site, transgenic plants, 5'-untranslated region.

Introduction

Heme oxygenase (HO, EC 1.14.99.3), the rate-limiting enzyme of heme metabolism, has been characterized in a wide variety of organisms including plants (Maines 1997, Muramoto *et al*. 1999, Emborg *et al*. 2006, Shekhawat and Verma 2010). Heme oxygenase catalyzes the oxidative cleavage of heme to produce biliverdin IXα, CO, and free iron (Fe^{2+}), in the presence of reducing agent (NADPH/FNR/Fd) (Yoshinaga *et al*. 1982, Muramoto *et al*. 2002, Shekhawat and Verma 2010). In plants, an HO transcript or activity has been identified in *Arabidopsis* (Davis *et al*. 1999, Muramoto *et al*. 1999), rice (Izawa *et al*. 2000, Xu *et al*. 2012), maize (Han *et al*. 2012), wheat (Xie *et al*. 2008, Wu *et al*. 2013), alfalfa (Baudouin *et al*. 2004, Fu *et al*. 2011, Cui *et al*. 2012), tomato (Davis *et al*. 2001), soybean (Noriega *et al*. 2004, Yannarelli *et al*. 2006), cucumber (Li *et al*. 2011), sorghum (Davis *et al*. 2001), Chinese cabbage (Jin *et al*. 2012), and pine (Davis *et al*. 2001). For example, four members of HOs, which are classified into two distinct classes, have been discovered in *Arabidopsis*. Among the two classes, the HO1 sub-family consists of HY1 (HO1), HO3, and HO4; whereas HO2, which is unable to

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; 4-MU - 4-methylumbelliferone; 5'-UTR - 5'-untranslated region; DPI - diphenylene iodonium; GUS - β-glucuronidase; HO - heme oxygenase; HY1 - heme oxygenase 1; MS - Murashige and Skoog; MBS - MYB binding site; MYB - myeloblastosis; NAA - 1-naphthalene acetic acid; - PEG - polyethylene glycol; pHY1 - promoter of *heme oxygenase 1*; RD22 - responsive to dessication 22; ROS - reactive oxygen species; SA - salicylic acid; TSS - transcription start site.
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bind or degrade heme, has seemed as the only member of the HO2 sub-family (Emborg *et al*. 2006, Gisk *et al*. 2010). A *HY1* mRNA is by far the most abundant *HO* transcript, and the *HO2* mRNA has second amount, whereas both *HO3* and *HO4* express at substantially low levels (Matsumoto *et al*. 2004, Zimmermann *et al*. 2004). A great deal of evidence has indicated that *Arabidopsis HO1* (*HY1*) is involved in the signal transduction of photomorphogenesis, lateral and adventitious roots development, stomatal closure, hormonal response, and abiotic stimuli (Davis *et al*. 1999, Muramoto *et al*. 1999, Xu *et al*. 2011, Chen *et al*. 2012, Fang *et al*. 2014, Li *et al*. 2015). Among them, abiotic stimuli including salinity, iron deficiency, and mercury exposure, have been well elucidated (Huang *et al*. 2006, Han *et al*. 2007, 2014, Cao *et al*. 2011, Shen *et al*. 2011, Xie *et al*. 2011, Zhu *et al*. 2014). However, related molecular mechanisms remain elusive and, especially, corresponding responsive promoters responding to specific stimuli in plants have not been well characterized.

In fact, the promoter character of a corresponding gene has been the common strategy for understanding the gene expression profile. In a promoter, *cis*-acting regulatory elements are significant molecular switches involved in the transcriptional regulation of gene expression under developmental processes, hormonal response, and environmental stimuli (Yamaguchi-Shinozaki and Shinozaki 2005). A number of *cis*-elements have been discovered to interact with a transcription factor to activate or repress gene expression, such as myeloblastosis (MYB) binding site (MBS) (Abe *et al*. 2003, Chen *et al*. 2006), abscisic acid-responsive element (ABRE) (Busk and Pagès 1998, Yoshida *et al*. 2010), and WRKY binding site (W box) (Pandey and Somssich 2009, Buscaill and Rivas 2014). The MBS (TAACTG) was identified in the maize *bronze-1* promoter (Urao *et al*. 1993). The MBS binding proteins, MYB transcription factors, are key molecules controlling development, metabolism, and response to biotic and abiotic stresses (Dubos *et al*. 2010). The MBS element in the *responsive to dessication 22* (*RD22*) promoter, which is recognized by AtMYB2, functions as an indispensable *cis*-acting element in salt-, drought-, and abscisic acid-induced gene expression (Urao *et al*. 1993, Abe *et al*. 2003). Comprehensively, analyzing the essential *cis*-acting elements of a promoter is very helpful for understanding the gene expression profile. In many cases, the leading intron in the 5'-untranslation region (5'-UTR) can greatly influence gene expression (Fiume *et al*. 2004, Liu *et al*. 2010, Parra *et al*. 2011, Wu *et al*. 2014, De La Torre and Finer 2015).

Previously, a ~1.1 kb *Arabidopsis HY1* promoter fragment was cloned, and its tissue expression was determined by histochemical analysis (Emborg *et al*. 2006, Li *et al*. 2013). However, the functional regions or *cis*-acting elements for regulating *Arabidopsis HY1* promoter activity are not well elucidated yet. Here, to characterize the regulatory region especially in response to mild salinity, iron deficiency, and Hg exposure, we generated a ~1.8 kb *HY1* promoter fragment and its deletion constructs including the 5' and 3' deletion forms. These promoter fragments were fused to the *β-glucuronidase* (*GUS*) reporter gene in the binary vector pCAMBIA1301 and transformed into the *Arabidopsis thaliana* (Col-0) wild-type. We tried to elucidate the functional regions for basal activities and the regulatory regions of *HY1* promoter in response to mild salinity, iron deficiency, mercury exposure, as well as hydrogen peroxide (H_2O_2) signal (Xie *et al*. 2011, 2014, Sewelam *et al*. 2014).

Materials and methods

Isolation of *HY1* promoter: The total genomic DNA was extracted from leaves of *Arabidopsis thaliana* L. (cv. Col-0). Primers for the *HY1* promoter cloning were designed according to the 5' flanking sequence of *HY1* obtained from the National Center for Biotechnology Information (*NCBI*; http://www.ncbi. nlm.nih.gov/). The promoter of *HY1* was amplified by polymerase chain reaction (PCR) with 25 mm^3 of a 2× *PrimeSTAR* (*TaKaRa*, Dalian, China) *GC* buffer, 4 mm^3 of a dNTP mixture (2.5 mM each), 1 mm^3 of each primer (10 μM; F: 5'-AAGCTTGATTAGTAGGGAACTTGAG-3'; R: 5'-CCATGGGGTTTGATCGGAATAGAAA-3') (Table 1 Suppl.), 0.5 mm^3 of *PrimeSTAR* HS DNA polymerase (2.5 U mm^{-3}, *TaKaRa*), 0.5 mm^3 of DNA (500 ng mm^{-3}), and 18 mm^3 of double distilled H_2O. The PCR was performed under the following conditions: pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 2 min with 30 cycles; and another extension at 72 °C for 10 min. The PCR product was purified and cloned to the vector pMD19-T (*TaKaRa*). The isolated sequence was confirmed by sequencing and designated as pHY1 (the promoter of *HY1*).

The *cis*-acting regulatory elements of pHY1 were analyzed by using a search tool in the *PlantCARE* database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; Lescot *et al*. 2002).

Expression vector of pHY1 construction and its deletion constructs: To comprehensively analyze the expression characteristics of the *HY1* promoter, forward primers (F0, F1, F2, F3, and F4) and reverse primers (R0, R1, R2, and R3) were designed and used to isolate the 5' deletion fragments 5D1 (F1 and R0; -1528 to +132), 5D2 (F2 and R0; -1109 to +132), 5D3 (F3 and R0; -688 to +132), and 5D4 (F4 and R0; -169 to +132), and

3' deletion fragments 3D1 (F0 and R1; -1666 to +100), 3D2 (F0 and R2; -1666 to -1), and 3D3 (F0 and R3; -1666 to -170) (Table 1 Suppl.). All the deletion fragments were cloned to the pMD19-T vector and sequenced to confirm no nucleotide mutation. Afterwards, pHY1 and the deletion fragments were ligated in *Hin*dIII and *Nco*I sites of the binary expression vector pCAMBIA1301 to replace the cauliflower mosaic virus 35S (CaMV35S) promoter. The constructs were transformed into the *Agrobacterium tumefaciens* L. strain EHA105. The pCAMBIA1301 harboring the CaMV35S promoter was used as a positive control.

Acquisition of transgenic plants: Flowering *Arabidopsis thaliana* plants were transformed by the floral dip method (Clough and Bent 1998). The harvested seeds were selected on a 1/2 Murashige and Skoog (1962; MS) agar medium (pH 5.8) with 50 mg dm^{-3} hygromycin, and positive transformants were confirmed by PCR. At least 12 independent transgenic lines were obtained for each promoter construct. Three independent T3 homozygous lines for each promoter construct were selected and used in further analysis.

Growth conditions and treatments: All *Arabidopsis thaliana* lines used in this study were of Col-0 background. The seeds were surface sterilized with a 0.25 % (m/v) sodium hypochlorite solution for 15 min, rinsed several times with sterilized deionized water, and then cultured on a 1/2 solid MS medium with 1 % (m/v) sucrose at pH 5.8 in 90 mm Petri dishes. The plates were kept at 4 °C for 2 d for vernalization, and then transferred into a growth chamber with a photon flux density of 150 μmol m^{-2} s^{-1}, a 16-h photoperiod, and a temperature of 22 °C.

To characterize expression patterns of pHY1 and its deletion constructs, 10-d-old seedlings (stage 1.02; Boyes *et al*. 2001) were exposed to a variety of environmental stimuli including salinity, osmotic stress, high or low temperature, and different metal ions. For osmotic stress, the seedlings were picked up from the solid medium and roots were dipped in a liquid 1/2 MS medium with 30 mM mannitol and 2 % polyethylene glycol (PEG) for 6 h. For mild salinity, 10 mM NaCl with or without pre-incubation with 20 μM diphenylene iodonium [DPI, an inhibitor of NADPH oxidase; also regarded as inhibitor of reactive oxygen species (ROS) production] was used in the MS medium. Abnormal temperatures - cold stress with 4 °C and heat stress with 37 °C - were applied for 6 h. For metal ions disequilibrium, 50 μM $CdCl_2$, 50 μM $HgCl_2$, 50 μM $Pb(NO_3)_2$, 100 μM $CuSO_4$, 100 μM $ZnSO_4$, or 50 μM $CoCl_2$ were added to the MS medium for 6 h. For iron deficiency condition, Fe-EDTA was removed for 24 h. The seedlings were also treated with 0.5 mM H_2O_2, 40 nM 2,4-dichlorophenoxyacetic acid (2,4-D), 100 nM 1-naphthalene acetic acid (NAA), or 500 μM salicylic acid (SA) in the indicated time points to detect *HY1* promoter- or its deletion-driven *GUS* reporter gene expression.

β-Glucuronidase assay: Histochemical staining assays were examined according to the method of Jefferson *et al*. (1987). Seedling or transgenic plant tissues at different development stages were collected and stained with a solution containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (*Sigma*, St. Louis, USA), a 100 mM phosphate buffer (pH 7.0), 10 mM Na_2-EDTA, 1 mM K_3FeCN_6, 1 mM K_4FeCN_6, and 0.5 % (v/v) *Triton X-100* for 12 h at 37 °C. The stained tissues were then decolorized with 75 % (v/v) ethanol. The pictures of the samples were visualized and photographed using a *Leica M125* stereoscopic microscope (*Leica*, Wetzlar, Germany).

Assays of GUS were also performed according to the fluorometric method described by Jefferson *et al*. (1987). Extracts for GUS assay were obtained from the seedlings with 0.6 cm^3 of an extraction buffer containing a 50 mM phosphate buffer (pH 7.0), 0.1 % sodiumdodecyl sulphate, 10 mM Na_2-EDTA, 0.1 % *Triton X-100*, and 0.1 % β-mercaptoethanol. A 0.1 cm^3 of the GUS extract was mixed with 0.9 cm^3 of a 37 °C pre-warmed extraction buffer containing 1 mM 4-methylumbelliferyl-β-D-glucuronide (*Sigma*, St. Louis, USA), and keeping at 37 °C for 0, 15, 30, 45, and 60 min. A 0.2 cm^3 of the reaction mixture was transferred to 0.8 cm^3 of 0.2 M Na_2CO_3 to stop the enzymatic reaction. The GUS activity was measured with 365 nm excitation and 455 nm emission with an *Infinite® M 200 PRO* fluorescence detector (*TECAN*, Männedorf, Switzerland) with a multifunctional microplate reader. The total protein in the GUS extract was measured by the Bradford (1976) method.

Transcription analysis by real-time PCR: The total RNA was isolated according to the manufacturer's instructions using a *Trizol Plus* reagent (*Biouniquer Technology*, Nanjing, China). The extracted 1 μg total RNA from different samples was reverse transcribed to cDNA by AMV reverse transcriptase XL (*TaKaRa*) in a 20-mm^3 reaction volume. Real-time PCR reactions were performed using the *StepOne* real-time PCR system (*Applied Biosystems*, Foster City, USA) with a *SYBR Green* (*TaKaRa*) mix according to the manufacturer's instructions. A forward primer: 5'-GCATGGTAG ATCTGAGAACCG-3' and a reverse primer: 5'-CCA ACGCTGATCAATTCCACA-3' were used for *GUS* reporter gene expression. An *HY1* transcription analysis was determined with a forward primer: 5'-AAC CCACTTTTCCTTCGACCT -3' and a reverse primer: 5'-CAGTAGTAGCCGCAACCACC-3'. An *RD22* transcription analysis was measured with a forward primer: 5'-CCAGGAGCAAACCCTTTCGT-3' and a reverse primer: 5'-TCCTCAGCGTTAAACCGGACA-3'. A relative gene transcription compared to the pHY1

construct or indicated control sample was presented by normalization to *Actin2* (GenBank accession number NM_121018).

Statistical analysis: Data are presented as means ± SDs of three independent experiments. For statistical analysis, Student's *t*-test taking $P < 0.05$ as a significant level was carried out.

Results

In order to comprehensively investigate the expression profile of the *A. thaliana HY1*, a putative promoter fragment with 1798 bp of the 5' flanking region of the translation start site (TSS), was cloned *via* PCR by using the sequence specific primers (F0 and R0; Table 1 Suppl.). The cloned sequence designated as the full-

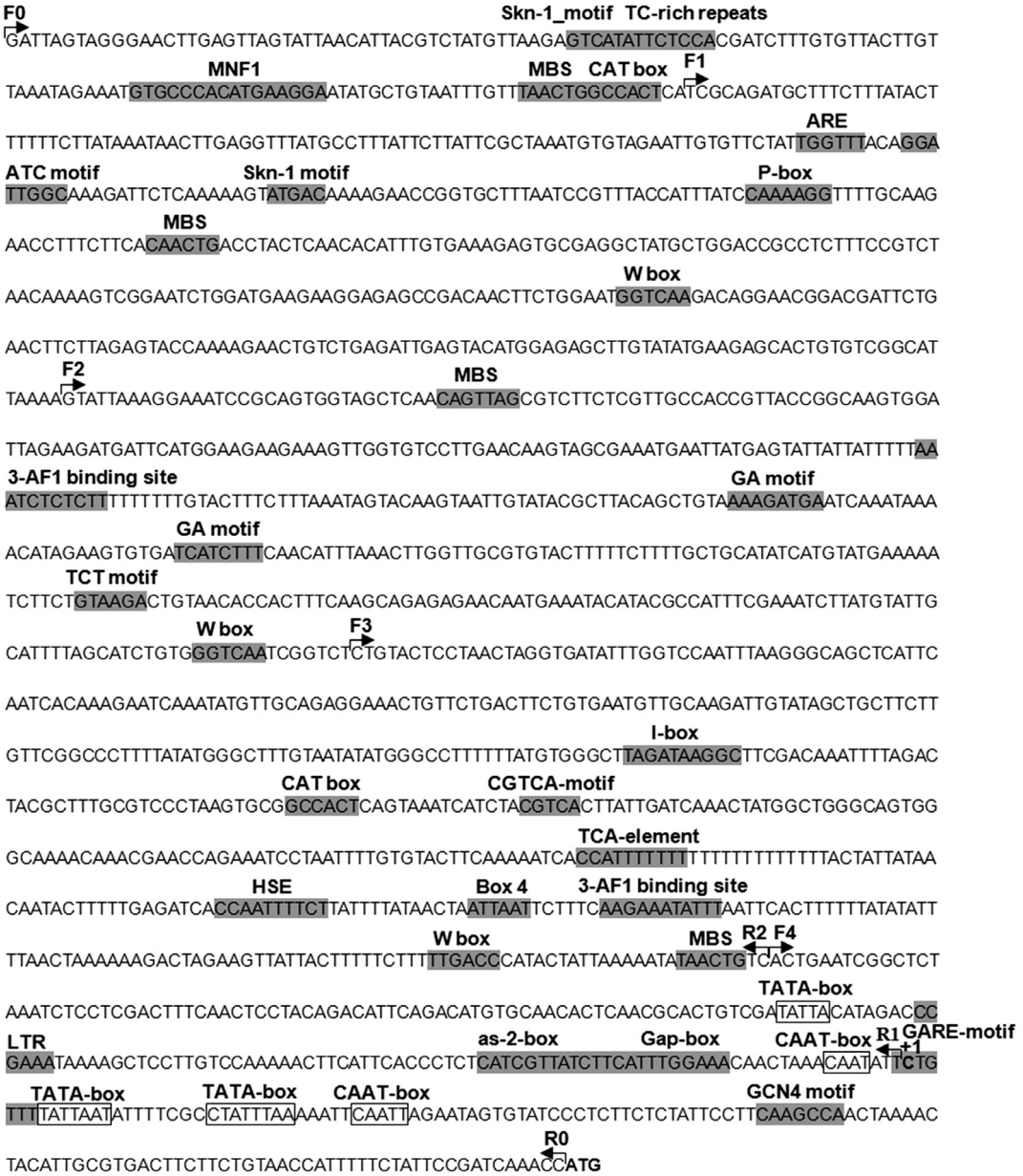

Fig. 1. The nucleotide sequence of the *heme oxygenase 1* promoter (pHY1). The sequence 1 798 bp upstream of the ATG starting codon was cloned and shown. The transcription start site (TSS), defined as +1, is presented at 132 bp upstream of the start codon site (ATG). The positions and direction of primers are shown as the *crooked arrow*. The pHY1 was analyzed by using *PlantCARE*, and putative *cis*-elements were *boxed* or *shadowed*.

length *HY1* promoter pHY1 was confirmed by nucleotide sequencing.

Nucleotide constituent analysis reveals that pHY1 exhibited 64.96 % of A+T (30.42 % of A and 34.54 % of T) and 35.04 % of G+C (16.57 % of G and 18.47 % of C), consistent with the A+T rich character of a promoter region in plants (Joshi 1987). The TSS, defined as +1, was presented at 132 bp upstream of the start codon site (ATG). The predictive TATA-box, a core motif for gene expression in eukaryotes, was found at -91 (TAATA, minus strand), +7 (TATTAAT, plus strand), and +23 (TATTTAA, plus strand) (Fig. 1). The putative enhancer element CAAT-box was located at -7 (CAAT, plus strand) and +35 (CAATT, plus strand).

To comprehensively analyze the *HY1* promoter sequence, we examined the *cis*-acting elements of the cloned putative promoter sequence (pHY1) by searching for the plant promoter analysis database *PlantCARE*. As expected, many regulator motifs involved in stresses and hormonal responses including 5'-GATAAG-3' motif (known as the I-box, part of a light responsive element), MBS, salicylic acid responsive element (TCA-element), *etc*., were found suggesting that the *HY1* gene is under complex regulation (Fig. 1).

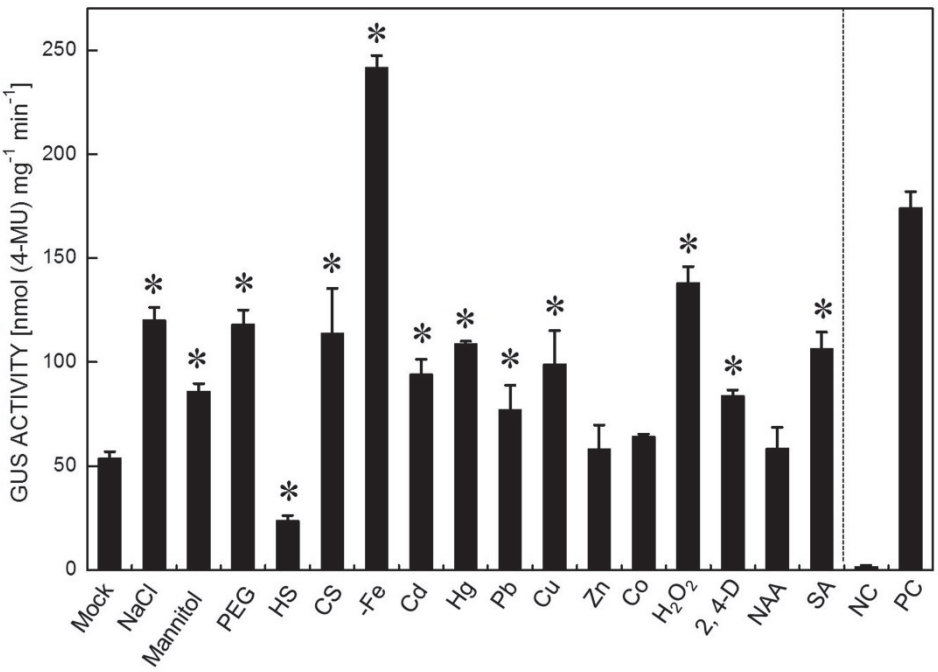

Fig. 2. Activity of β-glucuronidase (GUS) in response to environmental stimuli and hormones. Ten-day-old *Arabidopsis* transgenic seedlings carrying *heme oxygenase 1* promoter grown on 1/2 Murashige and Skoog agar medium were treated with 10 mM NaCl, 30 mM mannitol, 2 % (v/v) PEG, heat stress at 37 °C (HS), cold stress at 4 °C (CS), 50 μM $CdCl_2$ (Cd), 50 μM $HgCl_2$ (Hg), 50 μM $Pb(NO_3)_2$ (Pb), 100 μM $CuSO_4$ (Cu), 100 μM $ZnSO_4$ (Zn), 50 μM $CoCl_2$ (Co), 40 nM 2,4-dichlorophenoxyacetic acid (2,4-D), 100 nM 1-naphthalene acetic acid (NAA), and 500 μM salicylic acid (SA) for 6 h, or iron deficiency (-Fe) for 24 h and 0.5 mM H_2O_2 treatment for 3 h. The plants grown without the treatments were used as control (mock). Wild-type and CaMV35S promoter transformed plants were defined as negative control (NC) and positive control (PC), respectively. Means ± SDs of three independent experiments. *Asterisks* denote significant differences between the treatments and control at $P < 0.05$ according to Student's *t*-test.

To examine the expression profile of *Arabidopsis HY1*, the transgenic plants of pHY1 harboring the *GUS* reporter gene were constructed. Homozygotic T3 transgenic lines harvested from T2 transgenic plants, which were segregated with the 3:1 Mendelian ratio, were used in the present study. Similar to previous studies, histochemical staining analysis supports that *HY1* was widely and notably expressed throughout various tissues in different development stages (Table 1 Suppl.; Emborg *et al*. 2006, Li *et al*. 2013).

The transgenic seedlings carrying pHY1 were subsequently exposed to a variety of conditions or chemicals to investigate the role of pHY1 in response to environmental stresses and hormones. Quantification of GUS activity reveals that GUS activity of the transgenic seedlings carrying pHY1 treated with NaCl, mannitol, PEG, and cold stress for 6 h increased 2.24-, 1.60-, 2.20-, and 2.12-fold as compared with the untreated seedlings (Fig. 2). The heat stress exhibited only about 44 % GUS activity of the untreated seedlings. Meanwhile, there were 4.50-, 1.76-, 2.03-, 1.43-, and 1.84-fold increases of GUS activity after iron deficiency and addition of Cd, Hg, Pb, and Cu, but no significant changes after addition of Zn and Co.

Hormones participate in stress response. Here, we monitored GUS activity of the transgenic seedlings

treated with 2,4-D, NAA, and SA (Fig. 2). Activity of GUS increased by 2,4-D and SA but not by NAA treatment. In order to examine the role of ROS, the transgenic seedlings were exposed to 0.5 mM H_2O_2 for

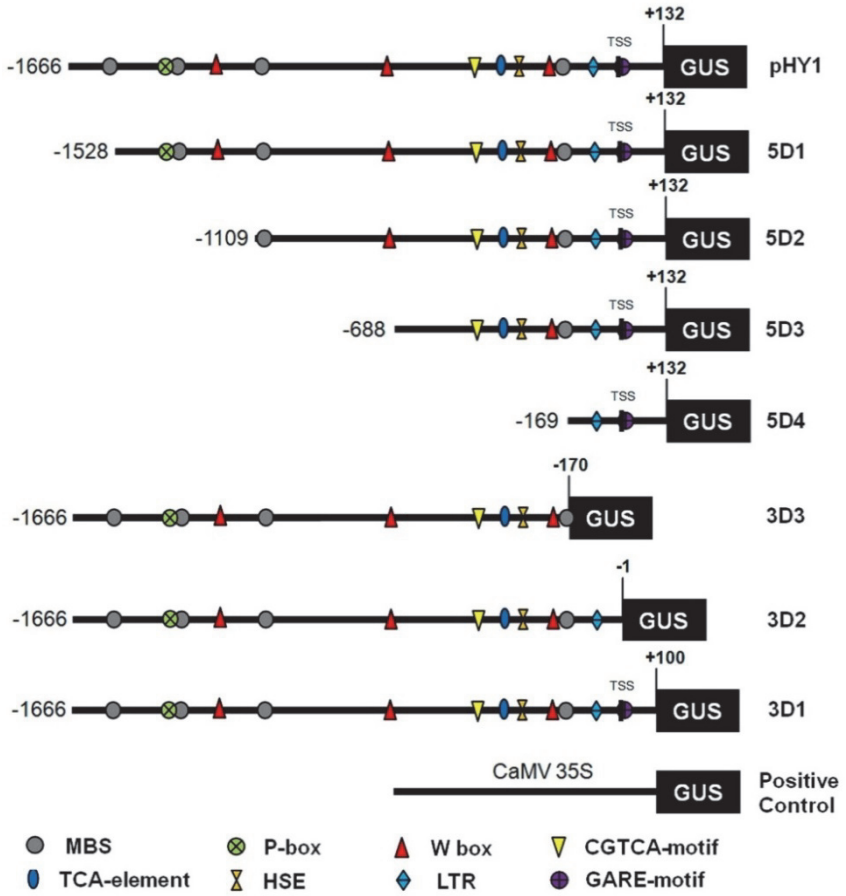

Fig. 3. A schematic representation of *heme oxygenase 1* (*HY1*) promoter constructs fused to the *β-glucuronidase* (*GUS*) reporter gene. A series of 5'- and 3'-deletion *HY1* promoter constructs (pHY1; 5' deletion constructs 5D1, 5D2, 5D3, and 5D4; and 3' deletion constructs 3D1, 3D2, and 3D3) were cloned and fused to the *GUS* reporter gene in pCAMBIA1301. The symbols on the promoter fragments represent predicted *cis*-acting elements that are annotated below the schematic representation. The original pCAMBIA1301 plasmid carrying CaMV35S was used as positive control. The transcription start site was defined as +1.

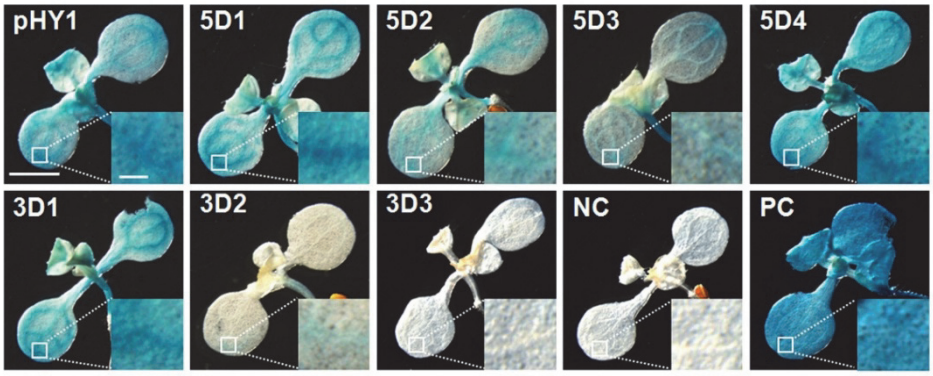

Fig. 4. β-Glucuronidase (GUS) staining assays of 10-d-old seedlings transformed with *heme oxygenase 1* promoter or corresponding deletion constructs. The histochemical staining analysis was carried out by using 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide for 12 h and destained several times with ethanol. Representative photos of eight plants for each promoter construct are shown (*bar* = 2 mm), and the close-up images of leaf tissue are displayed in the small windows (*bar* = 0.2 mm). Wild-type and transgenic plants carrying the CaMV35S promoter fused to the *GUS* reporter gene were used as negative control (NC) and positive control (PC), respectively.

3 h and as expected, a 2.57-fold increase of GUS activity was detected. Moreover, *HY1* expression under the indicated treatments (Fig. 2 Suppl.) was similar to the profile of GUS activity. Similar to the *cis*-acting regulatory motifs of pHY1 (Fig. 1), these results demonstrate that the functional pHY1 was intricately regulated by the environmental stimuli as well as by hormones. For the further experiments, salinity, iron deficiency, and Hg exposure were selected.

To further monitor the active region of the *HY1* promoter, we carried out the promoter deletion experiment by constructing a series of pHY1 5'- and 3'-deletion constructs which were linked with the *GUS* reporter gene (Fig. 3). Activities of GUS were analyzed in the 10-d-old transgenic *Arabidopsis* seedlings by histochemical staining, real-time PCR, and GUS fluorometric assay (Figs. 4 and 5). The transgenic seedlings carrying pHY1 displayed a remarkable but lesser GUS activity in comparison with the CaMV35S transformed seedlings. The 5'-deletion constructs 5D1 (-1528 to +132), 5D2 (-1109 to +132), and 5D3 (-688 to +132) resulted in progressively decreased GUS activities along with a promoter fragment deletion. However, 5D4 (-169 to +132) revealed a mildly stronger GUS activity than pHY1. For the 3'-deletion constructs, GUS activity was similar to pHY1 when the sequence was deleted from

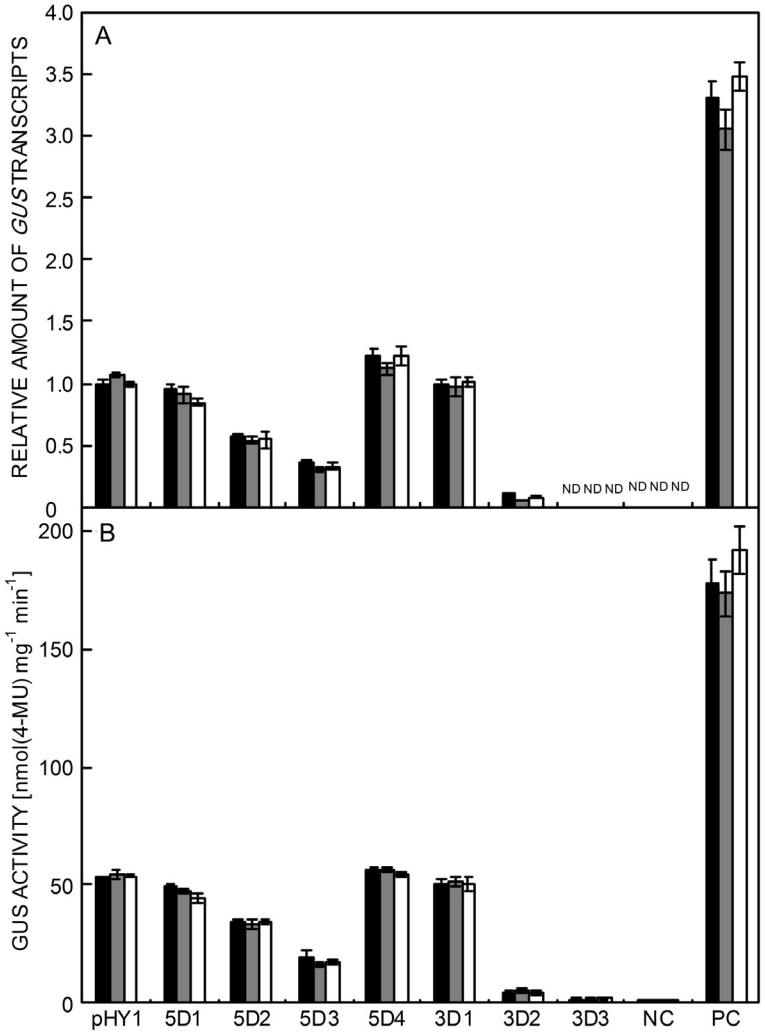

Fig. 5. β-Glucuronidase (GUS) expression analysis of 10-d-old seedlings transformed with *heme oxygenase 1* (*HY1*) promoter or corresponding deletion constructs. For real-time PCR (*A*), each column represents the mean *GUS* transcription from four seedlings and three replicate measurements. *Actin2* was used as internal control. ND means none detected. For GUS fluorometric assay (*B*), 4-methylumbelliferyl-β-D-glucuronide (4-MU) was used as substrate, and the product of 4-MU was measured from 10 plants and 3 replicate measurements. Three independent transgenic lines for each promoter construct were performed. Wild-type and transgenic plants carrying the CaMV 35S promoter fused to the *GUS* reporter gene were used as negative control (NC) and positive control (PC), respectively. Means ± SDs of three independent measurements.

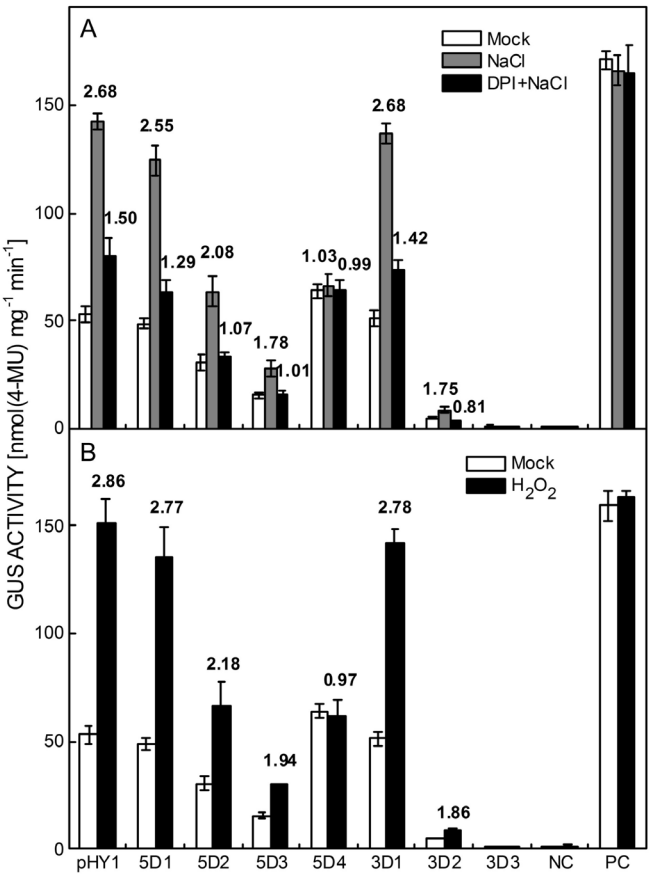

Fig. 6. β-Glucuronidase (GUS) activities in 10-d-old seedlings transformed with *heme oxygenase 1* (*HY1*) promoter or corresponding deletion constructs in response to mild salinity for 6 h with or without pre-incubation with 20 μM diphenylene iodonium (DPI, an inhibitor of reactive oxygen species production (*A*) and 0.5 mM H_2O_2 for 3 h (*B*); 4-MU - 4-methylumbelliferone. The numbers over the *black bars* represent the fold changes of GUS activity after the indicated treatment of the transgenic seedlings compared to the corresponding control (mock) treatment. Means ± SDs of three independent experiments.

+132 to +100 (3D1). However, a further reduction in length of the promoter fragment to -1 (3D2) led to a marked decrease in GUS activity. When the sequence was deleted to -170 (3D3), no or a very weak GUS expression was detected. Interestingly, these results of GUS activity determined by histochemical staining, real-time PCR, and GUS fluorometric assay show approximately similar tendencies. Moreover, GUS staining the 15-d-old seedlings (Fig. 3 Suppl.) was approximately consistent with the results in the 10-d-old seedlings (Fig. 4). Combined with the similar tendencies in the above quantitative and qualitative results, we therefore deduced that different *HY1* promoter activities in the deletion constructs might be mediated by various *cis*-acting elements (Fig. 1).

After 10 mM NaCl treatment for 6 h, we observed that the transgenic seedlings carrying pHY1 showed a 2.68-fold increase in GUS activities compared to the untreated seedlings (Fig. 6*A*). Along with the 5' deletion from -1666 to -688 of the promoter fragments (5D1, 5D2, and 5D3), the increased GUS activity were gradually weakened but remarkably to different degrees. When the promoter fragment was 5' deleted to -169 (5D4), no obvious induction was observed. For the 3' deletion constructs, GUS activity of the 3D1 (-1666 to +100) transgenic plants exhibited an ~2.68-fold increase. A very low but induced GUS activity of ~1.75-fold was observed in 3D2 (-1666 to -1). Moreover, very little GUS activity was measured in the 3D3 construct (-1666 to -170). Interestingly, the transgenic seedlings which were treated with 0.5 mM H_2O_2 for 3 h displayed a similar expression pattern (Fig. 6*B*). Additionally, pre-incubation with DPI, an inhibitor of ROS production, obviously decreased GUS activity induced by NaCl (Fig. 6*A*). These results clearly indicate that the salinity- and H_2O_2-induced GUS activity may be regulated by the same *cis*-acting element. Alternatively, these observations support the former conclusion that salinity-induced *HY1* expression activity is related to ROS signaling (especially H_2O_2; Xie *et al.* 2011).

To investigate the detailed expression profile of *HY1* promoter, the transgenic *Arabidopsis* seedlings harboring the pHY1 deletion constructs were exposed to the iron deficiency medium for 24 h (Fig. 7). Activity of GUS

increased approximately 4.02-fold in the plants carrying the pHY1 construct, and 4.13-, 4.06-, 3.78-, 2.61-, and 4.07-fold in those carrying the 5D1, 5D2, 5D3, 5D4, and 3D1 constructs, respectively. Activity of GUS showed a 3.34-fold increase in the 3D2 construct with a very low basal GUS activity in the untreated transformed plants. However, very little GUS activity was observed both in the 3D3 construct and in the mock treatment.

Mercury is one of the major heavy metal contaminants that results in growth stunt and oxidative damage to plants. In order to verify the mercury-responsive promoter region, we tested inducibility of GUS expression activity directed by the pHY1 deletion constructs in the transgenic seedlings treated with 50 μM $HgCl_2$ (Fig. 8). The transgenic seedlings carrying pHY1, 5D1, 5D2, 5D3, and 3D1 had different GUS activities: a 2.79-fold increase in the plants carrying the 3D2 construct and only a 0.76-fold increase in those carrying the 5D4 compared to the untreated seedlings. The inducibility of GUS activity directed by the pHY1 deletion constructs suggests that the mercury-responsive *cis*-acting element of *HY1* promoter might be mainly present in the region from -688 to -169.

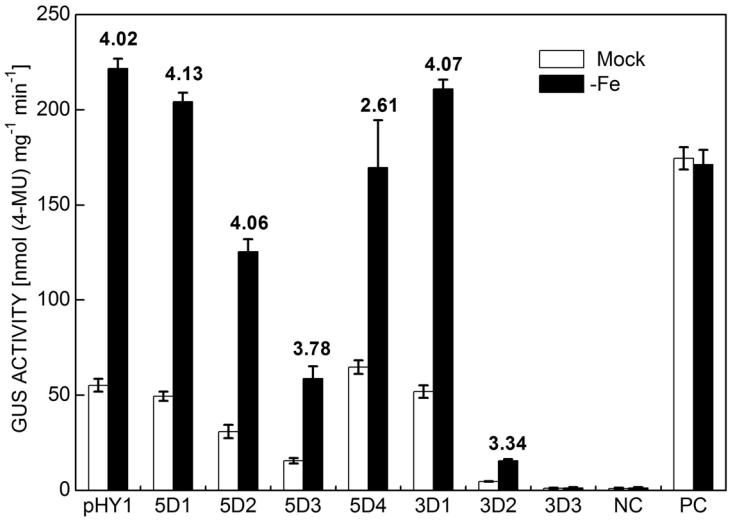

Fig. 7. β-Glucuronidase (GUS) activity in 10-d-old transgenic seedlings carrying pHY1 or corresponding deletion constructs in response to iron deficiency for 24 h; 4-MU - 4-methylumbelliferone. The numbers over the *black bars* represent the fold changes of GUS activity after iron deficiency compared to the mock treatment. Means ± SDs of three independent experiments.

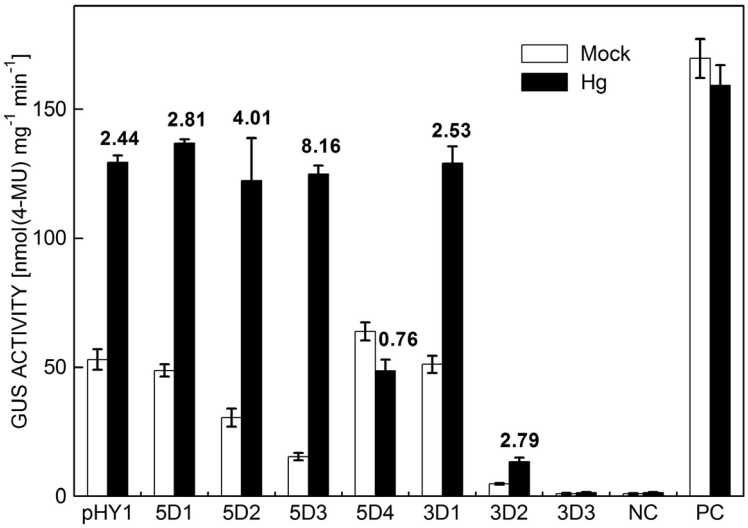

Fig. 8. β-Glucuronidase (GUS) activity in 10-d-old transgenic seedlings carrying pHY1 or corresponding deletion constructs in response to $HgCl_2$ for 6 h; 4-MU - 4-methylumbelliferone. The numbers over the *black bars* represent the fold change of GUS activity after Hg exposure compared to the mock treatment. Means ± SDs of three independent experiments.

Discussion

It has been well established that *A. thaliana HY1* widely expresses in various tissues and participates in hormonal response and abiotic stresses (for review, see Shekhawat and Verma 2010, He and He 2014). In this report, we highlight the functional characterization of the *HY1* promoter, and its response to the hormones and abiotic stresses, especially to a mild salinity, iron deficiency, and Hg exposure.

By *in silico* analysis firstly, we found several stress- or hormone-associated *cis*-acting elements, such as MBS (involved in dehydration inducibility) at -1542, -1333, -1078, and -177; heat shock element (HSE) at -302; low temperature response (LTR) element at -79; and TCA-element at -353, were present in the cloned ~1.8 kb promoter fragment of *HY1* (pHY1) (Fig. 1; Bienz and Pelham 1986, Urao *et al*. 1993, Merkouropoulos *et al*. 1999, Abe *et al*. 2003, Alcázar *et al*. 2006). This finding might provide part of evidence that the GUS activities of the transgenic seedlings were modulated by mannitol, PEG, NaCl, SA, heat, and cold treatment (Fig. 2). Consistently, up-regulation of expression of *HO1* has been previously discovered under salinity and osmotic stress (Xie *et al*. 2008, 2011, Cao *et al*. 2011, Bose *et al*. 2013). Although no corresponding *cis*-acting elements were presented in the *HY1* promoter, the GUS activity was induced by iron deficiency, Cd, Hg, Pb, Cu, and 2,4-D treatment (Fig. 2). These results were partly consistent with the gene expression or promoter activity of *HO1* in *Arabidopsis* (Fig. 2 Suppl.; Han *et al*. 2014), as well as in soybean (Balestrasse *et al*. 2008), *Medicago sativa* (Cui *et al*. 2011), and *Brassica napus* (Shen *et al*. 2011).

By deletion analysis, we found that the promoter region from +1 to +100 in the 5'-UTR was essential for basal promoter activity of *HY1*. In higher plants, the TATA-box, which is usually located 25~30 bp upstream of the transcription initiation site, is a conserved core motif for gene expression in eukaryotes (Joshi 1987). Interestingly, *in silico* analysis show that two TATA-boxes (+7 and +23) were present in the *HY1* promoter (Fig. 1); this finding might interpret the phenomenon of 5'-UTR-driven *HY1* expression. Consistently, the important roles of the 5'-UTR in influencing gene expression have been elucidated in many plant genes (Fiume *et al*. 2004, Liu *et al*. 2010, Wu *et al*. 2014, De la Torre and Finer 2015).

In *Arabidopsis*, *HY1* and ROS participate in salt acclimation signaling (Xie *et al*. 2011). Activity of GUS directed by the pHY1 construct consistently increased after the 0.5 mM H_2O_2 treatment for 3 h and after the 10 mM NaCl treatment for 6 h (Fig. 6). A number of pHY1 deletion constructs were used for further investigation of possible salinity- and H_2O_2-responsive regulatory regions (Fig. 3). Along with the 5' deletion to -688, the induced GUS activities were gradually weakened in the NaCl and H_2O_2 treated samples (Fig. 6). Meanwhile, four MBS elements, being MYB transcription factor binding sites, were predicted in -1542, -1333, -1078 and -177 of the *HY1* promoter (Figs. 1 and 3; Yamaguchi-Shinozaki and Shinozaki 1993). We also notice that the decreased GUS activities by progressive 5' deletion were correlated with the reduced numbers of MBS elements. Since the MYB transcription factor is a key factor controlling abiotic stress (Dubos *et al*. 2010), we deduce that MBS elements might be responsible for the above induced responses upon NaCl and H_2O_2. This deduction is further confirmed by the results (Fig. 6) showing that the MBS-free promoter construct 5D4 (-169 to +132) lost inducibility of promoter activity upon the salinity and H_2O_2 treatments. Combined with a previous result (Xie *et al*. 2011), we therefore propose that the MBS elements present here might be essential for NaCl-induced *HY1* up-regulation in an H_2O_2-dependent fashion.

We also found that *RD22* transcripts exhibited a significant inducible response upon 10 mM NaCl and 0.5 mM H_2O_2 treatments (data not shown). It is well-known that *RD22* is a classical osmotic- and salt-inducible gene (Yamaguchi-Shinozaki and Shinozaki 1993, 2006, Abe *et al*. 2003). In the *RD22* promoter, a MBS element, which is recognized by AtMYB2, is confirmed to direct gene up-regulation driven by salt, drought, and abscisic acid (Abe *et al*. 2003). Production of H_2O_2 is thought to be increased under various abiotic stresses, and H_2O_2-induced *HY1* gene expression may play an important role in signal transduction for abiotic stress tolerance (Chen *et al*. 2009, Xie *et al*. 2011, 2014). Above findings further suggest that MBS might be a regulatory *cis*-acting element involved in H_2O_2-dependent HY1-mediated salinity tolerance. Certainly, the key roles of MBS elements existing in the *HY1* promoter need to be confirmed by more experiments (including sequence mutation, yeast one-hybrid analysis, electrophoretic mobility shift assay, *etc*).

Iron is one of the accompanying products of heme oxidative conversion by HO1 (Shekhawat and Verma 2010). Iron deficiency signaling significantly increases expression of *HO1* and releases free iron (Li *et al*. 2013). Over-expression of *HY1* improves iron accumulation in Cd^{2+}-induced iron deficiency in *Arabidopsis* seedlings, whereas an *HY1*-knock-out mutant (*hy1-100*) shows the contrary phenomenon (Han *et al*. 2014). Here, after the iron deficiency treatment for 24 h, GUS activity of pHY1 exhibited a 4.02-fold increase (Fig. 7) approximately similar to the previous research (Li *et al*. 2013). Although in different levels, the significant inducibility of pHY1 activity caused by iron deficiency was found in all the 5' deletion constructs (from -1666 to -169), 3D1 (-1666 to +100), and 3D2 (+1666 to -1). Therefore, we further deduce that in the region from -169 to -1 iron deficiency responsive elements might exist. Certainly, this possibility

remains to be further investigated.

Mercury is one of the major toxic heavy metals that can replace other nutrient metals and generate excess free radicals resulting in growth stunt. An *HO1* inducer hematin exhibits protective effect against Hg-induced oxidative damage in roots of alfalfa (Han *et al*. 2007). A genetic approach with over-expression of *HO1* exhibits a remarkably lower Hg accumulation and higher biomass under Hg exposure in *Brassica napus* (Shen *et al*. 2011). In this report, a relationship between *HY1* and Hg was assessed by a promoter analysis strategy. The promoter constructs with pHY1, 5D1, 5D2, and 5D3 (in particular) differentially induced GUS activity after Hg exposure, although they had different basal activities (Fig. 8). Besides, the 5D4 did not have any inducibility. These results clearly demonstrate that the promoter region from -688 to -169 was necessary for Hg responsibility. Since no reported element involved in Hg responsibility was found in the cloned *HY1* promoter by *in silico* analysis, novel *cis*-acting elements responsible for Hg response might be present in the promoter region from -688 to -169. Additionally, the full loss of GUS activity in 3D3 upon the mild salinity, iron deficiency, and Hg exposure (Figs. 6 to 8) might be related to the absent core region in the promoter.

In summary, the *Arabidopsis HY1* promoter-driven *GUS* reporter gene was regulated by a number of environmental stimuli and hormones. The deletion analysis reveals that the region from +1 to +100 in the 5'-UTR, containing two TATA-boxes (+7 and +23), was essential for *HY1* promoter basal activity. The comprehensive analyses of the *HY1* promoter indicates that the MBS elements in the *HY1* promoter might be critical for salinity-induced *HY1* up-regulation in an H_2O_2-dependent fashion. Moreover, the regions from -169 to -1 and from -688 to -169 were also deduced as regulatory regions of the *HY1* promoter in response to iron deficiency and mercury exposure, respectively. Our study therefore strengthens understanding the basal activity and regulatory characters of the *HY1* promoter. However, in the *HY1* promoter, the genuine roles of MBS under salinity and precise regulatory elements in response to iron deficiency and Hg exposure remain to be investigated further.

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