The HD-ZIP II Transcription Factors Regulate Plant Architecture through the Auxin Pathway

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Received: 12 March 2020; Accepted: 2 May 2020; Published: 4 May 2020

Abstract: The homeodomain-leucine zipper (HD-ZIP) family transcription factors play important roles in plant growth and development. However, the underlying mechanisms remain largely unclear. Here we found that ATHB2, encoding a HD-ZIP transcription factor, is an early auxin responsive gene. Phenotypic analyses show that overexpression of ATHB2 impairs plant architecture, including reduced plant height and small leaves, and also reduces auxin response in leaves when grown in soil. Simultaneously, the seedlings with chemical induction of ATHB2 exhibit abnormal root gravitropism, a typical auxin-related phenotype. We further show that the auxin response pattern is altered in roots of the inducible ATHB2 seedlings. Consistently, the transcript levels of some auxin biosynthetic and transport genes are significantly decreased in these transgenic seedlings. Further, protein and promoter sequence analyses in common wheat showed that the HD-ZIP II subfamily transcription factors have highly conserved motifs and most of these encoding gene promoters contain the canonical auxin-responsive elements. Expression analyses confirm that some of these HD-ZIP II genes are indeed regulated by auxin in wheat. Together, our results suggest that the HD-ZIP II subfamily transcription factors regulate plant development possibly through the auxin pathway in plants.

Keywords: Arabidopsis; wheat; HD-ZIP; ATHB2; auxin

1. Introduction

The plant hormone auxin is important for regulating almost all aspects of plant growth and development [1]. Auxin receptors perceive the auxin concentration changes and then initiate auxin signaling. Nuclear auxin signal perception and consequent alterations in gene expression are carried out by the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) pathway [2,3]. Three major protein families are involved in the TIR1/AFB pathway: auxin-binding TIR1/AFB F-box proteins, AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) repressor proteins, and AUXIN RESPONSE FACTOR (ARF) transcription factors [3,4]. In this pathway, the Aux/IAAs interact with ARFs and inhibit ARF transcription factor activity when the auxin level is low [5]. When the auxin level is increased, the Aux/IAA and TIR1/AFB F-box proteins form a co-receptor complex, which are components of a Skp1-Cullin-F-box (SCF) E3 ubiquitin ligase complex, resulting in ubiquitination and degradation of the Aux/IAA repressor proteins by the 26S proteasome [1,5,6]. Recently, Cao et al. (2019) identified another auxin signaling mechanism, which acts in parallel to the canonical auxin pathway [7]. This signaling mechanism works at the concave side of the apical hook. The auxin-mediated C-terminal cleavage of transmembrane kinase 1 (TMK1) is involved in this signaling [7].

Two distinct pathways are involved in auxin distribution in the plant body: passive diffusion through the plasma membrane (PM) and directional cell-to-cell polar auxin transport (PAT) [8].
PAT is a precise auxin distribution manner that is significantly important for the formation of local auxin maxima, mainly in developing tissues [8,9]. The differential localization of transporters at specific sites on the PM creates a directional auxin flow, which eventually establishes a PAT stream through adjacent cells [9]. AUXIN RESISTANT1 (AUX1)/LIKE-AUX1 (LAX) proteins are auxin influx transporters and PIN-FORMED (PIN) proteins are auxin efflux transporters [10–15]. Other auxin transports, which have nonpolar distribution, consist of the P-GLYCOPROTEIN (PGP), MULTIDRUG RESISTANCE (MDR), or ATP-BINDING CASSETTE SUBFAMILY B (ABCB). AUX/LAX regulates several developmental processes, such as lateral root formation (AUX1 and LAX3) and cotyledon vascular patterning (LAX2) [15,16]. Among the distinct auxin transports, the polar localizations of PIN proteins within cells finely correspond to the directionality of auxin flow, which highlights that PIN proteins that are mainly responsible for the asymmetric distributions of auxin in plants [16–18].

The homeodomain-leucine zipper (HD-ZIP) transcription factors are unique to plants and contribute to various plant physiological processes [19–22]. Based on evolutionary relationship and protein structure, HD-ZIP transcription factors are generally divided into four subfamilies, namely HD-ZIP I, HD-ZIP II, HD-ZIP III and HD-ZIP IV [23]. HD-ZIP II proteins can be stimulated by far red light, and then lead to shade avoidance response [24,25]. Previous studies showed that HD-ZIP II proteins respond to various biotic and abiotic stresses, through regulating hormone signaling pathways and expression of related genes [26–28]. A recent study showed that HD-ZIP II proteins regulate shoot apical meristem (SAM) maintenance and cotyledon polarity through auxin flow, but there is no evidence to suggest how HD-ZIP II proteins regulate auxin perception or auxin response [29,30].

In this study, we identified an early auxin responsive gene, ATHB2, which is one of the HD-ZIP II subfamily proteins in Arabidopsis. A previous study showed that the three proteins ATHB2, HAT3 and ATHB4 redundantly regulate embryo development in plants [30]. However, the underlying mechanism remains largely unknown. To investigate whether the biological role of ATHB2 and its homologs (Figure S1) is related to the auxin pathway, the 6 day old wild-type (Col-0) seedlings were treated with 10 μM IAA for different time points. The quantitative reverse transcription polymerase chain reaction (qRT-PCR) results showed that the transcript levels of the ATHB2 gene increased to a maximum level about three-fold at 0.5 h after exogenous IAA treatment (Figure 1A). The auxin-mediated induction of ATHB2 expression was substantially reduced after 1 h of treatment (Figure 1A). Similarly, the transcript levels of HAT1 and HAT2 genes were also obviously elevated after IAA treatment for 0.5 h (Figure 1B, C). By contrast, the transcriptional expression of HAT3 and AHTB4 was downregulated by IAA treatment (Figure 1D, E). The Aux/IAA genes are well known as the early auxin responsive genes and participate in auxin signaling through interacting with ARFs as transcriptional repressors. As a control, the transcriptional expression of IAA19, one representative member of the Aux/IAA family, was rapidly increased at 0.5 h

2. Results

2.1. ATHB2 Is an Early Auxin-Inducible Gene

ATHB2 belongs to a member of the HD-ZIP II subfamily transcription factors in Arabidopsis. A previous study showed that the three proteins ATHB2, HAT3 and ATHB4 redundantly regulate embryo development in plants [30]. However, the underlying mechanism remains largely unknown. To investigate whether the biological role of ATHB2 and its homologs (Figure S1) is related to the auxin pathway, the 6 day old wild-type (Col-0) seedlings were treated with 10 μM IAA for different time points. The quantitative reverse transcription polymerase chain reaction (qRT-PCR) results showed that the transcript levels of the ATHB2 gene increased to a maximum level about three-fold at 0.5 h after exogenous IAA treatment (Figure 1A). The auxin-mediated induction of ATHB2 expression was substantially reduced after 1 h of treatment (Figure 1A). Similarly, the transcript levels of HAT1 and HAT2 genes were also obviously elevated after IAA treatment for 0.5 h (Figure 1B, C). By contrast, the transcriptional expression of HAT3 and AHTB4 was downregulated by IAA treatment (Figure 1D, E). The Aux/IAA genes are well known as the early auxin responsive genes and participate in auxin signaling through interacting with ARFs as transcriptional repressors. As a control, the transcriptional expression of IAA19, one representative member of the Aux/IAA family, was rapidly increased at 0.5 h
after exogenous IAA treatment and reached a maximum at 1 h (Figure 1F). Taken together, like Aux/IAA genes, ATHB2 and its close homologs HAT1 and HAT2 are early auxin responsive genes.

2.2. Overexpression of ATHB2 Affected Plant Architecture

To observe the morphological phenotypes of ATHB2 overexpression plants at the adult stage, we generated different 35S:ATHB2-FLAG transgenic lines constitutively overexpressing ATHB2. Western blotting analyses demonstrated that the ATHB2-FLAG fusion proteins accumulated in the 35S:ATHB2-FLAG transgenic seedlings (Figure 2A). Phenotypic analyses showed that the 4-week-old 35S:ATHB2-FLAG overexpressing plants under normal growth conditions exhibited auxin-related phenotypes, such as dwarfism and narrow leaf phenotypes (Figure 2B,C). To explore how ATHB2 interacts with auxin to differentially regulate leaf development in the Col-0 and 35S:ATHB2-FLAG transgenic plants, we examined the spatial distribution of the auxin response in Col-0 and 35S:ATHB2-FLAG transgenic plants using the auxin-responsive reporter DR5:GUS [31]. The double transgenic plant DR5:GUS/35S:ATHB2-FLAG was generated through genetic crossing between DR5:GUS and 35S:ATHB2-FLAG plants. DR5:GUS and DR5:GUS/35S:ATHB2-FLAG plants were used for this experiment. As shown in Figure 2D, the expression of DR5:GUS in the 4-week-old leaves of DR5:GUS/35S:ATHB2-FLAG plants was significantly decreased compared with that in the wild type (Figure 2D). These results showed that constitutive overexpression of ATHB2 reduced auxin response and affected leaf development.
were generated. The qRT-PCR results showed that the transcript levels of \textit{ATHB2} were significantly increased in the inducible transgenic lines after \(\beta\)-estradiol treatment for 2 h compared with the Col-0 seedlings (Figure 3A). Notably, the transcript levels of \(\beta\) increased in the inducible transgenic lines after \(\beta\)-estradiol-inducible \textit{ATHB2} overexpressing plants (XVE>>\textit{ATHB2}) were generated. The qRT-PCR results showed that the transcript levels of \textit{ATHB2} were significantly increased in the inducible transgenic lines after \(\beta\)-estradiol treatment for 2 h compared with the Col-0 seedlings (Figure 3A). Notably, the transcript levels of \textit{ATHB2} were greatly upregulated by about 60-fold in the inducible transgenic line (Figure 3A).

To observe the auxin-related phenotype of \(i\text{ATHB2}\) transgenic lines, the 6-day-old \(i\text{ATHB2}\) seedlings were grown on 1/2 Murashige and Skoog (MS) medium with or without 10 \(\mu\)M \(\beta\)-estradiol. In the absence of \(\beta\)-estradiol, the growth phenotype of \(i\text{ATHB2}\) and Col-0 seedlings was comparable. However, two independent \(i\text{ATHB2}\) transgenic lines displayed an agravitropic root phenotype grown on the medium containing 10 \(\mu\)M \(\beta\)-estradiol, whereas the root growth of Col-0 plants was not affected in the presence of 10 \(\mu\)M \(\beta\)-estradiol (Figure 3B). In addition, the primary root length analyses were performed using Col-0 and \(i\text{ATHB2}\) transgenic seedlings grown on the medium containing 10 \(\mu\)M

**Figure 2.** Morphological phenotype of 35S:ATHB2-FLAG transgenic lines. (A) Immunoblotting analysis showing the ATHB2-FLAG protein levels in the 35S:ATHB2-FLAG transgenic plants. The 6 day old seedlings of Col-0 and 35S:ATHB2-FLAG transgenic plants were harvested for immunoblotting analysis. ACTIN was used as a loading control. 1# and 2# represent two independent transgenic lines of 35S:ATHB2-FLAG. The data are representative of three independent experiments. (B and C) Overview of the Col-0 and 35S:ATHB2-FLAG transgenic lines at the adult stage. (B) Scale bars, 4cm. Leaf morphology of the Col-0 and 35S:ATHB2-FLAG transgenic lines at the adult stage. (C) Scale bars, 2 mm. The 4-week-old 35S:ATHB2-FLAG transgenic plants grown under normal growth conditions were used for phenotypic analyses. (B and C) Two independent transgenic lines were used for phenotype observation. The images are representative of three independent experiments. (D) Expression patterns of DR5:GUS in the leaves of DR5:GUS and DR5:GUS/35S:ATHB2-FLAG plants. The leaves of 3-week-old DR5:GUS and DR5:GUS/35S:ATHB2-FLAG plants were used for GUS activity analyses. The images are representative of three independent experiments.

2.3. Inducible-Expression of ATHB2 Led to Auxin-Related Root Phenotypes

In order to determine the biological relevance of \(i\text{ATHB2}\) in auxin-mediated physiological processes, the \(\beta\)-estradiol-inducible ATHB2 overexpressing plants (XVE>>\textit{ATHB2}, simply labeled as \(i\text{ATHB2}\)) were generated. The qRT-PCR results showed that the transcript levels of \textit{ATHB2} were significantly increased in the inducible transgenic lines after \(\beta\)-estradiol treatment for 2 h compared with the Col-0 seedlings (Figure 3A). Notably, the transcript levels of \textit{ATHB2} were greatly upregulated by about 60-fold in the \(i\text{ATHB2}\) 2# transgenic line (Figure 3A).
β-estradiol. As shown in Figure S2, the primary root length of iATHB2 transgenic seedlings was significantly reduced compared with that of Col-0 seedlings. These results suggest that ATHB2 might be involved in the auxin-mediated root development.

Figure 3. Root gravitropic phenotype of β-estradiol-inducible ATHB2-overexpression transgenic lines. (A) qRT-PCR analysis showing the β-estradiol-induced expression pattern of ATHB2 in the iATHB2 lines. The ACTIN gene was used as an internal reference. 1# and 2# represent two independent transgenic lines of iATHB2. The qRT-PCR results were performed for three biological replications and similar results were observed. Representative qRT-PCR results with three technical replicates were shown. Error bars denote ± SD. (B) Root gravitropic phenotypes of the iATHB2 transgenic lines. Seedlings of the Col-0 and inducible ATHB2 overexpression plants (iATHB2) were grown on 1/2 MS medium with or without 10 µM β-estradiol for six days. Two independent transgenic lines were used for phenotypic observation. The images are representative of three independent experiments.

2.4. Inducible-Expression of ATHB2 Altered Auxin Distribution in Roots

Considering that root gravitropic bending is triggered by the asymmetric auxin distribution in the root tip [32,33], we examined the spatial expression pattern of the auxin responsive reporter DR5rev:green fluorescent protein (GFP) [34] in the root tips of iATHB2 seedings. We here generated the
double transgenic plant DR5rev:GFP/iATHB2 through genetic crossing between DR5rev:GFP and iATHB2 plants. After growing on 1/2 MS medium supplemented with or without 10 μM β-estradiol for six days, the fluorescence signals were observed in the root tips of DR5rev:GFP/iATHB2 plants. In the presence of 10 μM β-estradiol, the fluorescence signals of DR5rev:GFP/iATHB2 were asymmetric distribution in root tips as shown in the representative image (15/18) (Figure 4). However, the asymmetric DR5rev:GFP fluorescence signal pattern was not observed in the root tips of DR5rev:GFP/iATHB2 plants in the absence of 10 μM β-estradiol (Figure 4). These findings indicate that the asymmetric auxin response in the root tips of iATHB2 seedlings with β-estradiol treatment is correlated with their agravitropic root phenotypes.

**Figure 4.** Asymmetric auxin response was observed in the root tips of iATHB2 plants. The spatial expression pattern of the DR5rev:GFP auxin responsive reporter in the root tips of 6-day-old DR5rev:GFP/iATHB2 plants grown on the medium with or without 10 μM β-estradiol. The images are representative of three independent experiments. Roots were stained with propidium iodide (red). Arrowheads indicate the asymmetric auxin response in the root tips. Scale bars = 50 μm. Arrowheads indicate the asymmetric auxin response of DR5rev:GFP/iATHB2 with 10 μM β-estradiol treatment.

2.5. Expression of Some Auxin Biosynthetic and Transport Genes Was Reduced in the iATHB2 Seedlings

Auxin levels or distribution are controlled through synthesis and transport [35]. The auxin efflux carrier PIN proteins direct auxin flow in plants [36]. To investigate the underlying mechanism of asymmetric auxin response in the root tips of iATHB2 transgenic lines, we examined the expression of auxin biosynthetic and transport genes in the iATHB2 transgenic lines. The iATHB2 transgenic seedlings were grown on 1/2 MS medium for six days and then treated with 10 μM β-estradiol. As shown in Figure 5A–F, the transcript levels of YUCCA2 (YUC2), YUC8, PIN1, PIN3 and PIN4 were obviously decreased after β-estradiol treatment for 2 h. Previous studies have shown that the auxin-efflux facilitator PIN2 is involved in root gravitropism [12,36,37]. The transcriptional expression of PIN2 has no significant change in the iATHB2 transgenic seedlings after β-estradiol treatment. Taken together, these results indicated that the expression of some auxin biosynthetic and transport genes was reduced in the iATHB2 seedlings after β-estradiol treatment.
2.6. Molecular Characterization of HD-ZIP II Proteins in Common Wheat

In this study, we have shown that ATHB2, one member of the HD-ZIP II subfamily transcription factor in *Arabidopsis*, is involved in the regulation of plant architecture potentially through the auxin pathway. To further understand the roles of HD-ZIP II proteins in monocot crops, we analyzed the protein structure and motif composition of HD-ZIP II subfamily proteins in common wheat. In a recent study, a total of 32 HD-ZIP II subfamily genes were identified in common wheat [38]. Protein structure analysis was conducted via the SMART software using the 32 HD-ZIP II proteins in common wheat, indicating that all the 32 HD-ZIP II proteins contain a homeobox domain (HD) and an adjacent leucine zipper (LZ) motif (Figure 6). The evolutionary relationships among the HD-ZIP II subfamily proteins of both *Arabidopsis* and wheat were analyzed via the MEGA7.0 software using the neighbor-joining method (Figures S1 and S3).
To comprehensively identify the potential conserved domains of wheat HD-ZIP II transcription factors, the full-length amino acid sequences were analyzed by the MEME online server. The results showed that eight predicted conserved motifs were identified, named as Motif 1 to 8 (Figure 7). As shown in Figure 7, each member of wheat HD-ZIP II subfamily proteins contains four common predicted motifs, including Motifs 1, 2, 3 and 5. Motif 1, a putative HD domain, was comprised of 78 amino acids (EDDGDGGGGARKKLRLSKEQSALLEESFKEHSTLSPKQKAALARQLGLRPRQVEVWFQNRRARTKLKQTEVDCEYLKR) (Figure S4). Motif 2, a putative LZ domain, was comprised of 19 amino acids (CCETLTEENRRLQRELAEL) (Figure S4). Motif 3 and Motif 5, whose functions remain unknown, were comprised of 19 amino acids (YYMPLPATTLTMCPSCERV) and 21 amino acids (EAEEDLGLALGLSLGAGSRPS), respectively (Figure S4). In addition to these conserved motifs contained in all the HD-ZIP II subfamily members, there are four predicted motifs which are specific to some members of the HD-ZIP II proteins.
Figure 7. Putative motifs of the wheat HD-ZIP II subfamily proteins using the MEME program. The different conserved motifs are marked by different colors.

2.7. Auxin-Responsive Elements in the Promoters of Wheat HD-ZIP II Genes

Auxin-responsive promoter elements (AuxREs) presented in the upstream region of genes play an important role in the auxin pathway. The canonical AuxRE “TGTCTC” shows a strong association with the auxin responsive expression pattern [39,40]. To understand the association of wheat HD-ZIP II subfamily proteins with the auxin pathway, we scanned the “TGTCTC” element in the 3 Kb genomic regions upstream of their coding regions. Interestingly, we found that among the 32 gene promoters, only five gene promoters did not contain the canonical AuxRE “TGTCTC” (Figure 8). Notably, the TaHDZ13-6A/6B/6D and TaHDZ16-4A/6B/6D homologous genes shared almost similar “TGTCTC” distribution patterns in their promoters (Figure 8). These findings indicated that the HD-ZIP II subfamily genes might be regulated by auxin in wheat. To further confirm this idea, the 4-day-old wheat seedlings were treated with 10 μM IAA for 4 h for qRT-PCR analysis. The results showed that the transcript levels of TaHDZ19-3A/3B/3D, TaHDZ20-1A/1B/1D and TaHDZ21-2A/2B/2D were up-regulated in wheat roots.
with auxin treatment, whereas the transcript levels of TaHDZ23-7A/7D was downregulated (Figure 9). Taken together, some wheat HD-ZIP II subfamily genes are regulated by auxin.

Figure 8. The “TGTCTC” elements in the 3 Kb genomic regions upstream of coding regions are indicated by blue box.

Figure 9. Several wheat HD-ZIP II subfamily genes were regulated by auxin. (A–D) Auxin-induced expression pattern of TaHDZ19-3A/3B/3D, TaHDZ20-1A/1B/1D, TaHDZ21-2A/2B/2D and TaHDZ23-7A/7D by qRT-PCR. The 4-day-old wheat seedlings were treated with 10 μM IAA for 4 h. The TaGAPDH gene was used as an internal reference. The qRT-PCR results were performed for three biological replications and similar results were observed. Representative qRT-PCR results with three technical replicates are shown. Error bars denote ± SD. ** p < 0.01, Student’s t test. No significant difference is shown by n.s.
3. Discussion

Auxin regulates auxin-responsive gene expression which relies on an elegantly short signal transduction pathway (TIR1/AFB pathway), which has been extensively reviewed [5,41]. The Aux/IAA, which are well-known as the early auxin responsive genes, act as transcriptional repressors in this signaling [31]. Aux/IAA proteins recruit corepressors of the TOPLESS (TPL) family through a conserved EAR domain to silence ARF target genes [42,43]. The Aux/IAAs do not themselves bind DNA, but they can dimerize with the ARF family transcription factors [5]. The ARF family transcription factors regulate auxin-responsive gene expression.

Previous studies have shown that several HD-ZIP II proteins are well known for their role in shade avoidance [44], carpel margin development [45] and leaf polarity [46]. Moreover, members of the HD-ZIP II subfamily also control embryonic apical patterning and SAM function [30]. In this study, we found that ATHB2, encoding a transcription factor of the HD-ZIP II subfamily, is an early auxin-responsive gene (Figure 1). Phenotypic analyses showed that overexpression of ATHB2 impaired plant architecture, including reduced plant height and small leaves, which decreased auxin response in leaves when grown in soil (Figure 2). Meanwhile, the seedlings with chemical induction of ATHB2 exhibited abnormal root gravitropism, a typical auxin-related phenotype (Figure 3). We further showed that asymmetric auxin response occurred in the root tips of inducible ATHB2 plants (Figure 4). Therefore, both Aux/IAA and ATHB2 are early auxin-responsive genes and act as repressors of the auxin pathway, but possibly through distinct mechanisms.

The asymmetrical localization of PIN transporters (PIN1–PIN4 and PIN7) on PM contributes to the directionality of the auxin flow [36,47,48]. The differential expression and polar localization of PIN proteins constitutes the backbone of a transport network for directional auxin distribution in different tissues of the plant [49]. Directional auxin distribution leads to the formation of cellular auxin maxima and minima, which provides an essential cue for plant growth and differentiation at the level of individual cell and tissue [48,50]. PIN1, localizing to the basal (rootward) plasma membrane in root stele cells, directly transports auxin toward the root tip [12]. PIN2, PIN3 and PIN4, which also act in the root tip, mediate the auxin maximum and auxin redistribution for root gravitropism [11,37,51]. To investigate the underlying mechanism of the ATHB2-regulated auxin-related phenotype, we examined the expression of some PIN genes in the iATHB2 transgenic lines. qRT-PCR results showed that the transcript levels of PIN1, PIN3 and PIN4 were obviously downregulated by inducible overexpression of ATHB2 (Figure 5). Meanwhile, the auxin biosynthetic genes YUC2 and YUC8 were also downregulated by ATHB2 overexpression (Figure 5). Taken together, we demonstrate that ATHB2 regulates plant development possibly through modulating the auxin pathway in Arabidopsis.

In common wheat, a total of 113 HD-ZIP members were identified in recent studies [38,52]. However, the relationship between wheat HD-ZIP II transcription factors and the auxin pathway remains unclear. In this study, we analyzed the canonical AuxRE distribution in the promoters of wheat HD-ZIP II subfamily genes (Figure 8). A number of AuxREs were recognized in the promoters of wheat HD-ZIP II subfamily genes. Indeed, some wheat HD-ZIP II subfamily genes are regulated by auxin. Such knowledge may be useful to understand the regulation of wheat HD-ZIP II transcription factor in the auxin-mediated plant developmental processes.

4. Methods and Materials

4.1. Plant Materials and Growth Conditions

All the plants described in this study were in the Col-0 background. The full-length ATHB2 coding sequence was cloned into pMDC7 vector (iATHB2) [53,54] and p35S:FLAG vector (p35S:ATHB2-FLAG) [55], respectively. Col-0 was transformed with iATHB2 and p35S:ATHB2-FLAG by the floral dip transformation method, respectively [56]. Two independent transgenic lines of iATHB2 and p35S:ATHB2-FLAG were used for the experiments in this study. The DR5rev:GFP/iATHB2 and
DR5:GUS/35S:ATHB2-FLAG plants were prepared by genetic crossing. *Arabidopsis thaliana* and wheat (*Triticum aestivum*) were grown under LD (16 h light/8 h dark) condition at 22 °C.

### 4.2. DNA Constructs

DNA constructs used in this study were generated based on construction methods. The construction methods were carried out with the classic molecular biology protocols and Gateway technology (Invitrogen). For ligase-independent ligation assays, the Ligation-Free MasterMix (abm) was used according to the application handbook. For Gateway cloning, pQBV3 vector (Gateway) was used as the entry vector and subsequently specific destination vectors were introduced into the Gateway system (Invitrogen). The primers used for generation in this study are shown in Table S1.

### 4.3. Root Phenotype Analyses

Seeds were sterilized by 75% (v/v) ethanol for seven minutes and then 100% (v/v) ethanol for three minutes (v/v). Seeds were stratified at 4 °C for three days. The seeds were then grown on 1/2 MS medium with or without 10 μM β-estradiol. For the phenotypic observation, the seedlings were grown vertically on 1/2 MS containing 10 μM β-estradiol for six days. The primary root lengths were measured by using ImageJ software (http://rsb.info.nih.gov/ij).

### 4.4. RNA Extraction and Gene Expression Analysis

The 6-day-old seedlings were collected as described. Total RNA was extracted using Trizol (Invitrogen) reagent. About 2 μg total RNA was applied to synthesize cDNA using the 5× All-In-One RT MasterMix system (Applied Biological Materials). The cDNA was diluted to 100 μL with water in a 1:5 ratio, and 2 μL of the diluted cDNA was used as a template. SYBR® Premix ExTaq Kit (TaKaRa) was used for qPCR reactions. qRT-PCR was performed using LightCycler 96 (Roche). Expression levels of target genes were normalized by *ACTIN7*. All the experiments were repeated independently three times. All the primers used for qRT-PCR are shown in Table S1.

### 4.5. Confocal Microscopy

Fluorescent samples were inspected by confocal microscopy (Carl Zeiss, LSM880, Germany). For imaging GFP and propidium iodide (PI) observation the 488 nm laser was used for excitation. Emission between 500 and 550 nm band-pass was detected for GFP, between 560 and 610 nm band-pass for PI.

### 4.6. Protein Extraction and Immunoblotting

The extracted buffer (125 mM Tris-HCl at pH 6.8, 4% SDS, 20% glycerol, 0.001% bromophenol blue) with freshly added 2% β-mercaptoethanol was used for total protein extraction. Immunoblots were performed as described [57]. To detect a FLAG-tagged protein, we used anti-FLAG (1:5000; MBL, Japan) and anti-mouse IgG (1:75000) antibodies. Actins (1:5000; CWBIO) were used as the loading controls. Three independent biological replicates were performed with similar results.

### 4.7. Analysis of GUS Activity

For GUS activity analysis, 4-week-old *Arabidopsis* leaves were transferred into staining solution (1 mM 5-bromo-4-chloro-3-indolyl-beta-glucuronic acid solution in 100 mM sodium phosphate, pH 7.0, 0.1 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide and 0.1% Triton X-100) [58]. Leaves were then applied to vacuum for 20 min and incubated at 37 °C overnight. To clear chlorophyll from plant tissues, 100% ethanol was used. Individual representative seedlings were photographed.
4.8. Phylogenetic Analysis

A neighbor-joining phylogenetic tree was constructed based on 1000 bootstrap replicates by comparing full-length protein sequences aligned with the Clustal W algorithm within MEGA7.0.

4.9. Protein Structure and Motif Composition Analyses

Predicted protein domains were identified by the SMART tool (http://smart.embl-heidelberg.de/). The MEME online program (Bailey et al., 2009) was used to identify conserved motifs.

4.10. Accession Numbers

Sequence data of Arabidopsis from this article can be found in the Arabidopsis Genome initiative data library under the following accession numbers: ATHB2 (AT4G16780), IAA19 (AT3G15540), YUC2 (AT4G13260), YUC8 (AT4G28720), PIN1 (AT1G73590), PIN2 (AT5G57090), PIN3 (AT1G70940), PIN4 (AT2G01420), ATHB4 (AT1G73590), ATHB17 (AT2G01430), AT3G15540, AT1G70920), HAT1 (AT4G17460), HAT2 (AT5G47370), HAT9 (AT1G70920), HAT14 (AT5G06710) and HAT22 (AT4G37790).

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/9/3250/s1.

Author Contributions: J.S. designed the research project; G.H. and P.L. performed the experiments; G.H., J.S. and H.Z. analyzed the data and wrote the article. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Institute of Crop Sciences, Chinese Academy of Agricultural Sciences (CAAS), the Agricultural Science and Technology Innovation Program of CAAS, and Youth Talent Plan of CAAS.

Acknowledgments: The authors thank Klaus Palme for providing some research materials.

Conflicts of Interest: The authors declare no conflict of interest.

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