



**ARTICLE**

## ***EoPHR2*, a Phosphate Starvation Response Transcription Factor, Is Involved in Improving Low-Phosphorus Stress Resistance in *Eremochloa ophiuroides***

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### **ABSTRACT**

As a macronutrient, Phosphorus (P) takes many roles in plant growth and development. It should be significant to explore the molecular mechanism of low-phosphorus stress response of plants. Phosphate starvation response (PHR) transcription factors play important roles in response to phosphorus deficiency stress in plants. In this study, we isolated a gene related to the plant phosphorus signaling system from the acid-soil-resistant centipede-grass (*Eremochloa ophiuroides* [Munro] Hack.), termed *EoPHR2*. The subcellular localization of *EoPHR2* protein was observed to be nuclear located. The expression patterns of *EoPHR2* in different tissues and Al/Pi-stress conditions were analyzed by qRT-PCR, they suggested a potential role in response to the multiple-stress under acid soil adversity. Based on the functional identification through transgenic plants, we found that (1) *EoPHR2* is involved in the Pi-signaling pathway, and (2) overexpression of *EoPHR2* mimics Pi-starvation signalling resulting on enhanced roots whether under Pi-deficiency stress or not. In conclusion, *EoPHR2* transcription factor plays a role in response to the multiple stresses under acid soil conditions, improving the low-phosphorus stress resistance of *Eremochloa ophiuroides*.

### **KEYWORDS**

Phosphate starvation response; transcription factor; phosphorus signaling system; acid soil adaptability; *Eremochloa ophiuroides*

## **1 Introduction**

As an essential macronutrient in plants, phosphorus (P) is a structural component of nucleic acid and cell membrane lipids in plant cells. It participates in various metabolic processes and plays an important role in growth and development [1]. Plants acquire P as free inorganic phosphate (Pi) from soils. The total amount of P in most soils seems to be adequate for plant requirements. However, due to low mobility and a high fixation rate, the actual availability of P can be a limiting factor for plant growth, particularly on acid soils with abundant aluminium (Al) which fixes P at a high rate [2,3]. In plants, the uptake and utilization of phosphorus depend on the phosphate transporters (PTs) [4,5]. Previous studies have shown that a series



of PT proteins are involved in the uptake of phosphorus in roots, or the translocation of phosphorus among tissues [5]. Like many phosphate starvation-induced (PSI) genes, the transcription of most PT genes are induced by Pi starvation, some of which are controlled by the phosphate starvation response (PHR) transcription factors (TFs), the key regulators of Pi starvation signaling [6–9]. PHR transcription factors belong to the MYB-CC family, and regulate the expression of PSI genes by binding to the imperfect palindromic sequences (PIBS; GNATATNC) present in their proximal promoter regions [8]. Previous studies in model plants have revealed that, the AtPHR1 gene in Arabidopsis, or its orthologous gene OsPHR2 in rice, are key regulators in the pathway responding to phosphorus signals [3,5,10]. For the plant phosphorus-signaling system-involved AtPHR1/OsPHR2 transcription factor (abbreviated as PHR1 below), three types of downstream pathways have been reported: PHR1-miR399-PHO2 pathway, PHR1-miR827-NLA pathway, or PHR1 directly activates phosphorus related functional proteins [9,11]. In addition, studies on PHR1 homologous genes in several species [e.g., maize (*Zea mays*) [12], soybean (*Glycine max*) [13], also suggested the important role of the PHR1 transcription factor in response to the phosphorus related stress.

Centipedegrass [*Eremochloa ophiuroides* (Munro.) Hack.] is recognized as an excellent warm-season turfgrass species originated in China [14]. It is naturally distributed on P-deficient acid soils. It should be significant to explore the molecular mechanism that allows centipedegrass adaptation to low-phosphorus acid soil conditions. In this study, based on the protein sequence similarity of AtPHR1/OsPHR2, we isolated an orthologous gene in centipedegrass, termed *EoPHR2*. The protein subcellular localization was observed through the Arabidopsis protoplast transient expression system. The expression patterns of *EoPHR2* in different tissues and Al/Pi-stress conditions were analyzed by qRT-PCR. They suggested a potential role in response to the multiple-stresses under acid soil conditions. Further, to verify the function of *EoPHR2* in the Pi-signaling regulation system, we developed transgenic lines with overexpression of *EoPHR2* in *Arabidopsis* for functional identification. The results indicated that (1) *EoPHR2* is involved in the Pi-signaling pathway, and (2) overexpression of *EoPHR2* mimics Pi-starvation signalling resulting on enhanced roots whether under Pi-deficiency stress or not. In conclusion, *EoPHR2* transcription factor plays a role in response to the multiple stresses found under acid soil conditions, improving the low-phosphorus stress resistance of *Eremochloa ophiuroides*.

## 2 Materials and Methods

### 2.1 Plant Material

Centipedegrass seedlings of the acid-soil-resistant strain ‘E041’ were used for RACE (Rapid Amplification of cDNA Ends) and Real-time PCR analysis. The seedlings were collected from the Turfgrass Germplasm Resource nursery at the Institute of Botany, Chinese Academy of Sciences, Jiangsu Province. Potted seedlings of *Arabidopsis thaliana* ‘Col-0’ were used for protoplast transfection and plant transformation.

### 2.2 Extraction and Purification of DNA and RNA

Genomic DNA was extracted from the newly expanded leaves using the DNasecure Plant Kit (Tiangen Biotech, Beijing, China). Total RNA was extracted from various tissues or organs with the RNAprep Pure Plant Kit (Tiangen) with on-column DNaseI digestion. The nucleic acid concentration was quantified using an ND-1000 spectrophotometer (Nanodrop Technologies Inc., Rockland, DE, USA).

### 2.3 Cloning and Sequencing of Full-Length *EoPHR2* Gene

Based on the transcriptome sequencing data of centipedegrass, nested primers of 3'-RACE and 5'-RACE were designed for the *EoPHR2* gene (Supplementary Table). The 3'-Full RACE Core Set Kit and 5'-Full RACE Kit (Takara, Dalian, China) were used to amplify the full-length sequences. The PCR products

were separated on 1% agarose gels, cloned into the pMD19-T vector (Takara), and finally transformed into competent cells of *Escherichia coli* strain Top10. Colonies were checked by PCR, and inserts from positive colonies were sequenced. The full-length cDNA sequence of the *EoPHR2* gene was obtained by aligning and comparing 3'-RACE and 5'-RACE products. The predicted ORFs were subsequently amplified by PCR and verified by sequencing.

#### 2.4 Sequence Analysis

Online BLAST software of The National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) was used to analyze the DNA and protein sequences. The theoretical isoelectric point (pI) and mass value for the proteins were predicted and calculated using ExPASy ProtParam (<http://web.expasy.org/protparam/>). The functional domains of the protein were predicted using the SMART program (<http://smart.embl-heidelberg.de/>). Secondary structure of amino acid sequences was predicted by the SOPMA program ([https://npsa-prabi.ibcp.fr/cgi-bin/npsa\\_automat.pl?page=npsa\\_sopma.html](https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html)). The tertiary structure of deduced amino acid sequences was predicted using the SWISS MODEL (<https://www.swissmodel.expasy.org/>). The protein affinity and hydrophobicity were predicted using the ProtScale (<https://web.expasy.org/protscal/>). The protein signal peptide was predicted using the SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP-4.1/>). Phosphorylation site was predicted using the NetPhos (<http://www.cbs.dtu.dk/services/NetPhos/>). Protein transmembrane structure was predicted using the TMHMM 2.0. Multiple-sequence alignments of *EoPHR2* proteins were carried out using the ClustalX 2.1 software [15]. The phylogenetic tree was constructed using MEGA6 software with the Neighbor-Joining method and 1000 bootstraps.

#### 2.5 Protoplast Transfection (*EoPHR2* Localization Experiment)

In this study, plasmids were constructed using the Gateway technology (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The *EoPHR2* coding region was cloned into the entry vector, pCR8/GW-TOPO (Invitrogen), via a simple T-A cloning reaction. An LR clonase enzyme mix (Invitrogen) was used to transfer the insert from the entry vector to its destination vector, p2GWF7.0 for C-terminal GFP fusion. The generated GFP fusion vector (35S:: *EoPHR2*-GFP) was a high copy vector driven by a double 35S cauliflower mosaic virus (CaMV) promoter with ampicillin as the bacterial selection marker. The plant protoplast preparation and transformation kit (Real-Times Biotechnology, Beijing, China) was used to isolate and transform *Arabidopsis* protoplasts. The protoplasts were observed using a BX53 microscope (Olympus, Tokyo, Japan).

#### 2.6 RT-PCR

Based on the centipedegrass hydroponic culture system [16], the hydroponic seedlings were pre-cultured for 2 weeks to the best growth state, and then treated using the "long-term Al-P alternate treatment" method [17]. Four treatment conditions, defined as 'Control' (-Al/+Pi), 'P-deficiency' (-Al/-Pi), 'Al-toxicity' (+Al/+Pi) and 'Al-toxicity & P-deficiency' (+Al/-Pi), were set up as follows:

**Control (-Al/+Pi):** A normal solution (1/2 Hoagland, in which the concentration of Pi was 500  $\mu$ M, pH 4.0) and a Mock solution (0.5 mM CaCl<sub>2</sub> solution, pH 4.0) were set up as alternating treatments.

**P-deficiency (-Al/-Pi):** A P-deficient solution (1/2 Hoagland, the concentration of Pi was adjusted to 10  $\mu$ M, pH 4.0) and Mock solution (0.5 mM CaCl<sub>2</sub> solution, pH 4.0) were set up as alternating treatments.

**Al-toxicity (+Al/+Pi):** A normal solution (1/2 Hoagland, in which the concentration of Pi was 500  $\mu$ M, pH 4.0) and an Al-toxicity solution (0.5 mM CaCl<sub>2</sub> solution with 1.5 mM AlCl<sub>3</sub>, pH 4.0) were set up as alternating treatments.

**Al-toxicity & P-deficiency (+Al/-Pi):** A P-deficient solution (1/2 Hoagland substrate, the concentration of Pi was adjusted to 10  $\mu$ M, pH 4.0) and an Al-toxicity solution (0.5 mM CaCl<sub>2</sub> solution with 1.5 mM AlCl<sub>3</sub>, pH 4.0) were set up as alternating treatments.

After 2 weeks of growing in the Normal nutrient solution, the seedlings were exposed to a 0.5 mM CaCl<sub>2</sub> solution either with (+Al) or without (-Al) AlCl<sub>3</sub> for 1 day. Thereafter, they were grown in the Normal (+Pi) or P-deficiency solution (-Pi) on alternate days. After 3 weeks of alternating treatments, the root, stem and leaf tissues were harvested (3 biological replicates). The fresh samples were first cleaned and then immediately frozen in liquid nitrogen.

Total RNA extraction from various tissues was performed as described above. The cDNA was synthesized from the total RNA using Oligo(dT)<sub>18</sub> with the Promega ImPromII Reverse Transcription System (Promega, Madison, WI, USA). For semi-quantitative RT-PCR, specific primers were designed for the *EoPHR2* gene (Supplementary Table). Real-time RT-PCR was carried out in a 20  $\mu$ l reaction mixture using the FastStart Universal SYBR Green Master Mix (Roche Applied Science, Indianapolis, IN, USA). Real-time PCR was performed on an ABI ViiA7 Real-time PCR System (ABI, Foster City, CA, USA). The cycling conditions were: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate. The relative quantitative method [18] was used for data analysis.

## 2.7 Plant Transformation

Plasmids used in this study were constructed by Gateway technology (Invitrogen). The insert was transferred from the entry vector to its destination vector, pBI121-3HA, with a C-terminal HA-tag. The generated GFP fusion vector (35S::*EoPHR2*-HA) was a high copy vector driven by overexpression elements [35S promoter from cauliflower mosaic virus (CaMV)]. Potted seedlings of *Arabidopsis thaliana* 'Col-0' were used for the transgenic experiments. The seedlings were transformed by the *Agrobacterium*-mediated inflorescence infiltration method. Then, the homozygous transgenic seedlings of the T3 generation were screened, and tested for their function with the methods as follows:

**Potted seedlings culture and treatment:** After germinated on the solid MS medium, the wild-type/transgenic seedlings were transplanted to pots. The culture substrate (peat: vermiculite: perlite = 1:1:1) pre-mixed with a base fertilizer of 1:10 diluted MS nutrient solution was no longer fertilized. Potted seedlings were grown under a cycle of 16 h light/8 h dark at 22°C/18°C (day/night) and irrigated with pure water for 3 weeks. Thereafter, the phenotypes were observed.

**Aseptic seedlings culture and treatment:** Based on the solid MS medium (pH 5.8; added sucrose 20 g/L, agar 9 g/L), the phosphorus concentration was controlled by adjusting the addition amount of KH<sub>2</sub>PO<sub>4</sub> (KCl was added to supplement K to the standard level). Four treatment conditions were set up as follows: 0 mM (Pi-absent), 0.0625 mM (low-Pi), 0.625 mM (half-Pi), 1.25 mM (full-Pi). The seeds of the wild-type and 2 transgenic lines were first sterilized and then seeded on the medium containing any of the four treatments. The phenotypes were observed 3 weeks later: (1) The growth phenotype were scanned by a scanner (WinRHIZO Pro 2017 system); (2) A total of 25 seedlings were weighed to evaluate the fresh weight from each of the wild-type or transgenic lines exposed to the diverse treatment conditions. Meanwhile, the seedlings were frozen in liquid nitrogen and stored at -80°C for RNA extraction and real-time RT-PCR analysis.

## 3 Results

### 3.1 Isolation and Characterization of the *EoPHR2* Gene

The full-length cDNA sequence of the *EoPHR2* gene was cloned from *E. ophiuroides* 'E041' by RACE (Rapid amplification of cDNA ends) procedures. It was 1991 bp in length, containing an open



reading frame (ORF) of 1287 bp, flanked by 246 bp of 5'-untranslated region (UTR) and 458 bp of 3'-UTR. The encoded polypeptide consisted of 429 amino acids, with a corresponding molecular weight (MW) of 46.93 kDa (Fig. 1).

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1   ACATGGGGGCACGCCAAGCCAGGCCAGGCGAGGGGGGGGGGGGGGGGGGGTTCGGTTCACGAGGTTCCCTCCGTTTTCTCGACACCGCCGATCCCGCCACG
109  CGCCGCCGCCGCCGCCGCCGCCAGAGGAGACACAATAATCTAGGTTAGGAGCATACTTCAAAAGTCAAGATAGAAAAGGACATTGAACACTGGAGGAACATTTTGTGTG
217  GTGGTAACTGAGGTTTGTGCTGTTTGTAGATGGAGAGATTAAGCACCAACCAGCTCTACAGTTCGGAGTTCCTGTAACCGTACCAACATCTCTGCCTTCTATACCA
325  M E R L S T N Q L Y S S G V P V T V P T S L P S I P
1   GCGTCTCTGGATGAGAGCTTCCCGAGGCTTGCAGATGCACAGAGTGTGTTGATGGAGAGAGAATTGAGAAGCACTCCACTACCTCCTACCAGACCCTGTTGCCCT
433  A S L D E S F P R L A D A Q S V L M E R E L R S T P L P P H Q T T V A P
27   ATCCGGGGTCAGTTTCATCCAATACTGGATCTGTTGGACCTCTGTGCTCACCTCCGCTGTAAGGTTCTCTCGCTTTCGAACCCCTGAGCAGTACTCCAACCAAT
541  I R G Q F H S N T G S V G P L C S P P S V R F S S L S N P E Q Y S N H N
63   CCATATAATTTCTCAAGCACAAGCACTGCAAGTCTTCAACGCTGAATTACGGGTCAACAATATGGAGGCTTGAACCTTCTATAACCGATTTCCCAAGAGACATTGAA
649  P Y N S Q A P S T A S S S T L N Y G S Q Y G G F E P S I T D F P R D I E
99   CCAACTGGTGCCTGAACCGATGAGAGCATGCTGGATTATCTGGTATGTCCTGCTGGGAATAATTTGACTGGGACCCTTCTATTGGAGCTTCAGATGATCTT
757  P T W C P E P V E S M L G L S G D V P A G N N L T G T T S I G A S D D L
135  ACTAAGCAAATGAATGGTGGACAGAGTTTATGAATGAGGATGGAAAGCACATGGTTGATAACCAACATCCACAGAAATTAACAGGTTGGGCAACCTGTGCAATCA
865  T K Q T E W W T E F M N E D W K D M V D N P T S T E I Q Q V G Q P V Q S
171  TCCATCTCAGTTCACCAACCAGCTACCCAACAAACAGTGTCTCAATCTGTTGAACCGTTGGCAGTTGTGGCACCTCACCATTAGCAGTTGTGGCACCTCACCCT
973  S I S V H Q P A T Q Q T V S Q S V E P L A V V A P S P L A V V A P S P T
207  GCTGGTTCCAATACTGCCAAGGCACGCATGAGATGGACTCCTGAGCTTACGAGCGCTTTTGGATGCTGTCATCAACTTGGTGGCAGTAAAAAGCACTCCTAAG
1081 A G S N T A K A R M R W T P E L H E R F V D A V N Q L G G S E K A T P K
243  GGTGTCCTGAAGCTAATGAAGGCAGACAATTTGACCATTTATCATGTAAAAAGTCATCTTCAGAAATACAGAACGGCTAGATACAGACCAGAGTTATCTGAAGGCTCT
1189 G V L K L M K A D N L T I Y H V K S H L Q K Y R T A R Y R P E L S E G S
279  TCTGAGAAGAAAGTAGCATCAAAGATGATATACCATCTATCGACTTGAAAGGAGTGTGATCTCACTGAAGCACTTCGGCTCCAGTTGGAACCTCAAAGCGGCTT
1297 S E K K V A S K D D I P S I D L K G S F D L T E A L R L Q L E L Q K R L
315  CATGAACAGCTTGAGATACAAAGGAGTTTGCAACTGAGAATTGAGGAACAAGGAAGTGTCTTCAAGATGATGCTAGAGCAGCAGTGCATACCGGGGACAGAAAAGGCAC
1405 H E Q L E I Q R S L Q L R I E E Q G K C L Q M M L E Q Q C I P G T E K A
351  AGGATGCTTCAACCCCTGAAGATGAATTGAAGTTAGCCATCGGAGGTTCCAGAATCTTCTACAGTCAAGGAAGTTCAGAAAAAATCAAATGGATCAACCAAGCAA
1513 Q D A S T P E D E L K L P S E V P E S S T V K E V P E N N Q N G S T K Q
387  ACAGAACTAGCTAACGGCCAATGAAGTTGCAAGCATGAAATGGGTTTCATGATCTGATTGGCTGTTTTTCAGGCTTAAATCAAGTTGTTTTGTCTTTTCGTTGGGAACA
1621 T E S A N G Q *
423  GATGAACACTATTGTTAACTTCGTTGCAAGCCTGGCCAGGTGCATGTAAGTGGGAGTTAGTTGTATAGTACCCACCATCAGGATCACTGAATCCCGGTGTTATG
1729 TGGATGATGATGACCAGAAGCAGCGCAAGCACAATGTCAGGTGTTGACTTGTGTTTTCCCCCTTGTAGGTATCAAGTAGCATCTGCTAGGCATTTGATTATG
1837 GGGCTAATCAAGTATTTACAATAATGTTAGCACCTGTTGTTAGAGCTTGTGATGGTACCAAAATATGATGAAGGATGATTGCCTTTATATGAAGTCTTCGGTTC
1945 GGTCTCCCGACCGTAGCATCTCTCTTCGACGAAAAAATAAAAAA

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**Figure 1:** cDNA and amino acid sequence of *EoPHR2*

The physical and chemical properties of the protein encoded by *EoPHR2* were predicted by the ProtParam software. It was shown that the theoretical isoelectric point, fatty acid coefficient, average hydrophilicity and instability parameter were 4.99, 70.44, -0.634 and 55.58, respectively. The protein domain predicted by SMART showed a low complexity domain (222-243), a Myb\_DNA-binding domain (251-302), and a Myb-CC\_LHEQLE domain (334-380) with the conserved LHEQLE motif (Fig. 2A). The secondary structure analysis of the *EoPHR2* protein, identified using the SOPMA program, revealed the following proportions of each component: 26.57% alpha helices, 6.06% extended strands, 2.33% beta turns and 65.03% random coils (Fig. 2B). Furthermore, the three-dimensional model of *EoPHR2* constructed by the SWISS MODEL was consistent with the prediction of a secondary structure (Fig. 2C). In addition, phosphorylation site analysis of the *EoPHR2* protein, predicted using the DISPHOS program, showed that 59.615% of serine sites, 36.364% of threonine sites and 50.000% of tyrosine sites were phosphorylated (Fig. 2D).



According to the protein BLAST software, the predicted EoPHR2 protein belonged to the Myb-CC family with the conserved LHEQLE motif. The various alignments of the amino acid sequences were performed with the homologous proteins from different plant species using the ClustalX 2.1 software (GeneBank: *Eremochloa ophiuroides*, EoPHR2; *Sorghum bicolor*, XP\_021309376.1; *Zea mays*, XP\_008651544.1; *Panicum miliaceum*, RLN35937.1; *Panicum hallii* var. *hallii*, PUZ69446.1; *Setaria italica*, XP\_004956084.1; *Dichanthelium oligosanthes*, OEL30463.1; *Eragrostis curvula*, TVU39510.1; *Aegilops tauschii* subsp. *Tauschii*, XP\_020191223.1; *Triticum turgidum* subsp. *Durum*, VAN57702.1; *Hordeum vulgare*, KAE8802583.1; *Brachypodium distachyon*, XP\_003563187.1; *Oryza meyeriana* var. *Granulata*, KAF0909536.1; *Oryza sativa Japonica Group*, XP\_015647735.1, OsPHR2; *Oryza brachyantha*, XP\_006657651.1). The multiple alignments of the homologous proteins ranged, in percentage identity to EoPHR2 (*Eremochloa ophiuroides*), from 92.09% (*Sorghum bicolor*) to 71.89% (*Oryza brachyantha*), and revealed a conserved Myb-CC\_LHEQLE domain in the C-terminus (Fig. 3).

To understand the evolutionary relationships of the EoPHR2 proteins from different species, the amino acid sequences of 15 EoPHR2 proteins were aligned, and the phylogenetic tree of the homologous proteins was constructed by the Neighbor-Joining method using the MEGA6 software with bootstrap testing on 1000 replicates. The results showed the evolutionary relationships among the 15 proteins from different species (Fig. 4). The shortest evolutionary distance from *E. ophiuroides* was *Sorghum bicolor*, another member of the Panicoideae. The longest evolutionary distance from *E. ophiuroides* was *Oryza brachyantha* in the Oryzoideae.

### 3.2 Subcellular Localization of the EoPHR2 Protein

Subcellular localization information is one of the key features of protein function research. The amino acid sequence of the EoPHR2 protein was predicted and analyzed by SignalP 4.1. The results showed that the average S-score of the EoPHR2 protein was 0.103 and no signal peptide sequence was found. This indicated that the protein was a non-secretory protein. Further, the cellular localization of the EoPHR2 protein was probed using the Arabidopsis protoplast transient expression system. Using fluorescence microscopy, the EoPHR2-GFP fusion protein was detected in the nucleus in protoplasts, while the fluorescence signal of 35::GFP fusion protein had no clear cellular localization as the positive control (Fig. 5). The observed subcellular localization of the EoPHR2 protein was consistent with its functional localization as a transcription factor.

### 3.3 Expression Pattern of EoPHR2 under Multiple Conditions

To verify the expression pattern of *EoPHR2* in *E. ophiuroides*, the expression levels of the *EoPHR2* gene were measured in diverse tissues under different Al-P conditions by real-time PCR. It was found that under the 'Control' condition, EoPHR2 mainly expressed in root (Fig. 6A). Under the P-deficiency stress, the expression of EoPHR2 appeared no significantly differ in root, stem or leaf tissue, relative to the Control condition (Fig. 6B). These results showed that the EoPHR2 gene was mainly expressed in the root and would not be significantly induced by the P-deficiency stress, which confirmed the conclusions drawn from the homologous genes of other plant species [3,5,10].

On the other hand, interestingly, the expression level of EoPHR2 could be significantly up-regulated by Al-toxicity stress or Al-toxicity & P-deficiency multiple-stress in root, stem and leaf tissues, among which the gene expression was induced strongly in root. In addition, in root and leaf tissues, the EoPHR2 gene showed a stronger up-regulation under the multiple-stress of Al-toxicity & P-deficiency than the single stress of Al-toxicity (Fig. 6B). The results suggest a potential role of EoPHR2 in the Al-toxicity stress response of centipedegrass.





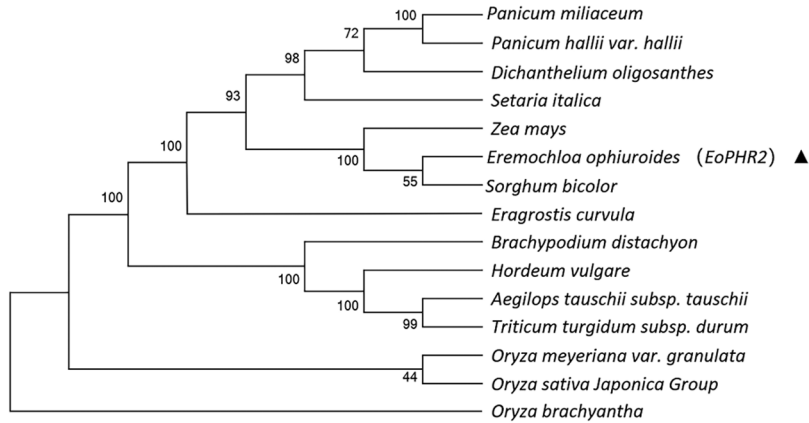
**Figure 3:** Alignment of the amino acid sequences on EoPHR2 proteins from various species

### 3.4 EoPHR2 is Involved in Regulating Plant Growth under Pi Deficiency

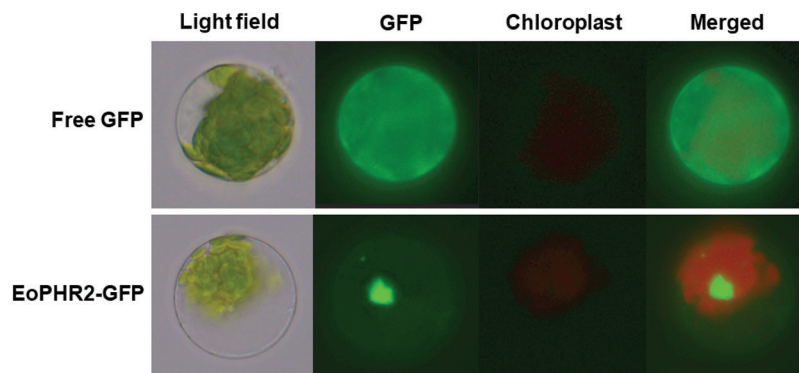
Transgenic *Arabidopsis* plants overexpressing the *EoPHR2* gene were generated to analyze the function of *EoPHR2* *in vivo*. The full-length coding sequence (CDS) region of the *EoPHR2* gene was amplified from cDNAs, subcloned into a modified pBI121-HA overexpression vector, and transferred into *Arabidopsis* using an *Agrobacterium*-mediated transformation method. After a period of iteratively screen, two



homozygous transgenic lines were confirmed for the further experiments, termed EoPHR2-OV1 and EoPHR2-OV2 (Supplementary Figure).

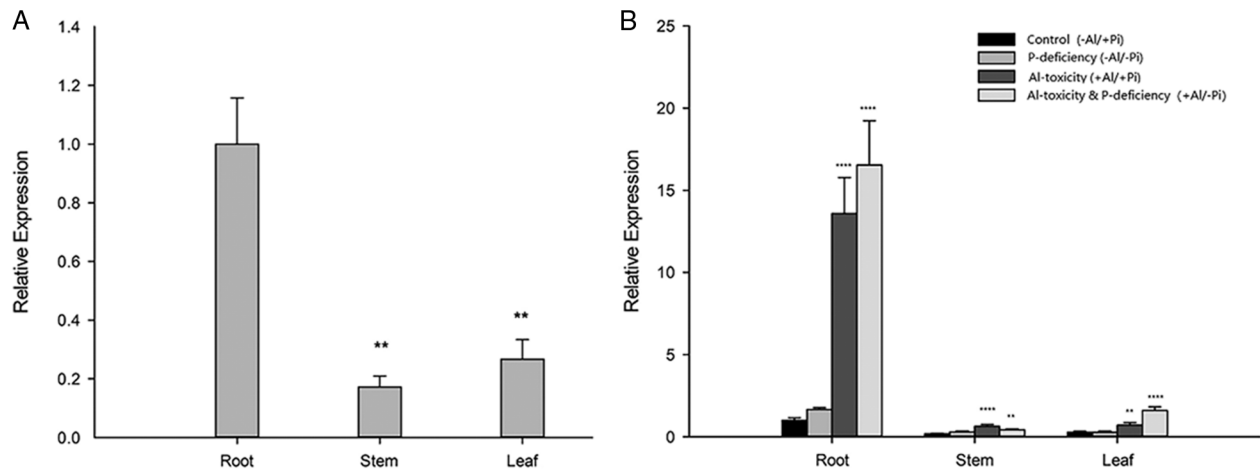


**Figure 4:** Phylogenetic analysis of EoPHR2 proteins from various species

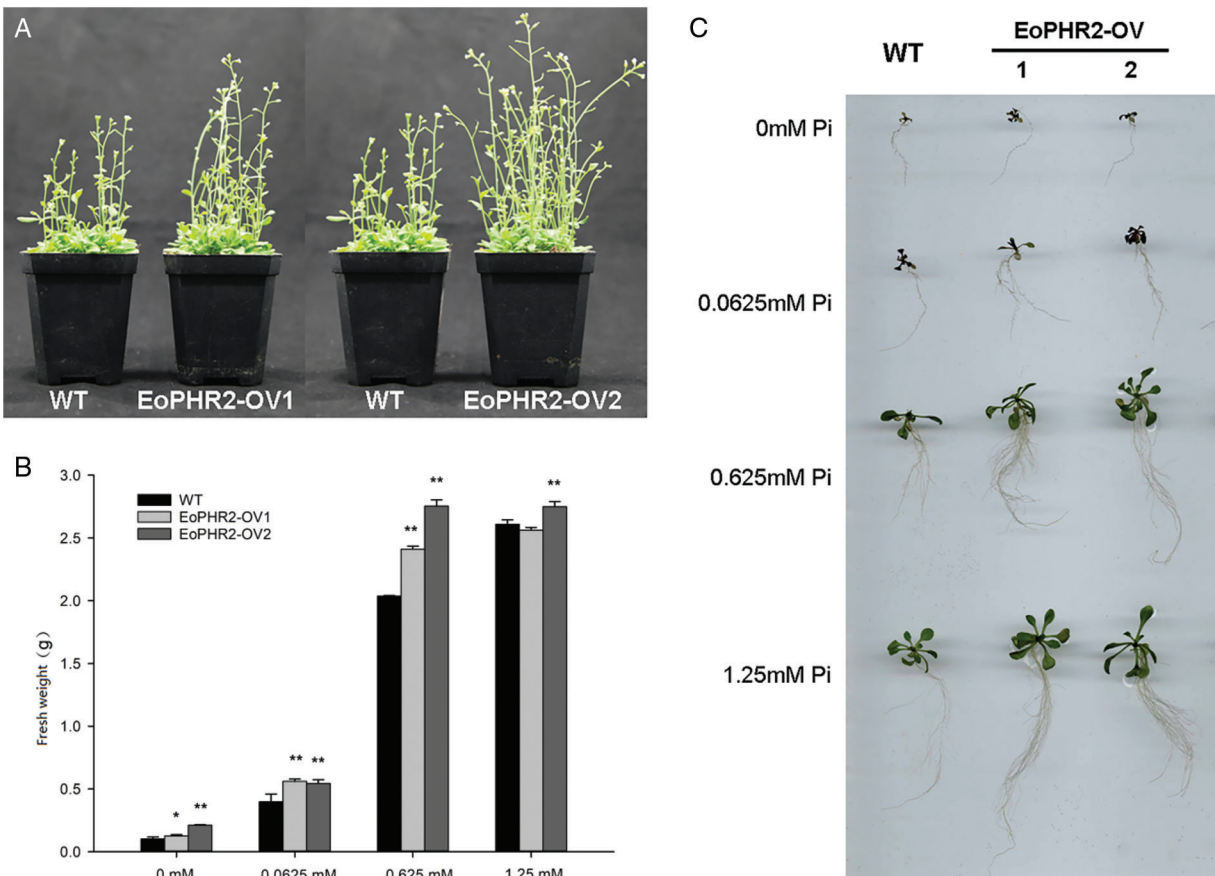


**Figure 5:** Subcellular localization of the EoPHR2 protein. The EoPHR2-GFP fusion protein was detected in the nucleus. The 35::GFP protein was used as a positive control

In the condition of potted culture and treatment, growth of the 2 transgenic *Arabidopsis* lines was observed better than that of the wild-type (Fig. 7A). Then, the seeds of wild-type (WT) and 2 transgenic (EoPHR2-OV1, EoPHR2-OV2) lines were sterilized and sown on the solid medium with inorganic phosphorus concentration of 0 mM (Pi-absent), 0.0625 mM (low-Pi), 0.625 mM (half-Pi) or 1.25 mM (full-Pi), respectively. Three weeks later, the phenotype was observed and the fresh-weight was determined. In the extremely Pi-absent condition (0 mM Pi), the wild-type and transgenic lines showed obvious growth inhibition as a result of absence of the essential macronutrient. Then, in the condition of low-Pi (0.0625 mM Pi), the transgenic plants began to show advantages in vegetative growth compared with the wild-type; the fresh weight of transgenic plants was slightly higher than that of the wild-type. Moreover, the transgenic plants appeared to have more developed lateral roots. Further, in the condition with the half-Pi concentration of 0.625 mM, the transgenic plants showed obvious growth advantages compared with the wild-type; the two transgenic lines exhibited higher fresh weight with better developed root systems than the wild-type. In addition, under the full-Pi condition (1.25 mM Pi), there was no significant difference in plant fresh weight between the transgenic and the wild-type plants. However, the root systems of the transgenic lines were still observed superior to that in the wild-type (Figs. 7B and 7C).

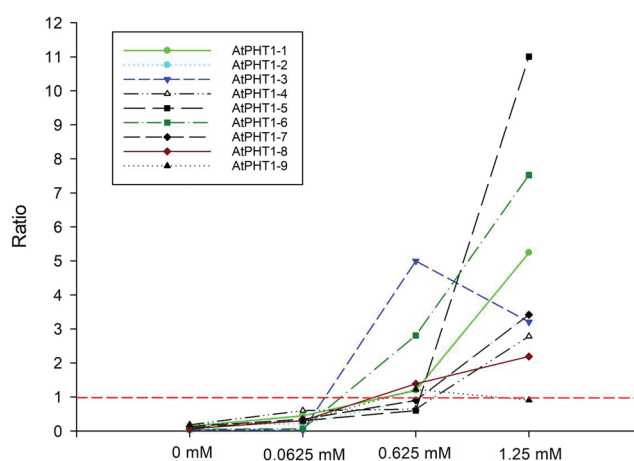


**Figure 6:** Gene expression profile analysis of *EoPHR2* in *E. ophiuroides*. (A) Expression of *EoPHR2* in different plant tissues; the expression levels were calculated relative to the ‘Root’; (B) Expression of *EoPHR2* under different treatments; the expression levels were calculated relative to the ‘Root’ of ‘Control’. Bars indicate mean expression  $\pm$  SD of three replicates. The \*\*, \*\*\*, and \*\*\*\* mean  $P < 0.01$ ,  $P < 0.001$ ,  $P < 0.0001$ , respectively



**Figure 7:** Phenotype identification of *EoPHR2* in transgenic *Arabidopsis* plants. (A) Phenotype of potted seedlings; (B) Fresh weight of seedlings under different phosphorus concentration treatments (each from 25 plants). Bars indicate mean  $\pm$  SD of three replicates. The \*, \*\*, mean  $P < 0.05$ ,  $P < 0.01$ , respectively; (C) Phenotypes of seedlings under different phosphorus concentration treatments

Previous studies in the model plants have revealed that *AtPHR1/OsPHR2* is a key regulator in the pathway responding to phosphorus signals [3,5,10]. Overexpression of *EoPHR2* should be able to mimic Pi-starvation signalling and activate the transcription of the Pi-starvation-induced *PHT1* genes in transgenic *Arabidopsis* plants. The expression levels of *PHT1* genes were measured by real-time RT-PCR to verify the mode of action of *EoPHR2*. According to previous studies [19], nine *Arabidopsis PHT1* genes were found, and primers were designed to quantify the level of expression of each *AtPHT1* gene. Under the full-Pi condition (1.25 mM Pi), the expression levels of most *PHT1* genes were up-regulated in the transgenic plants without Pi-starvation stress, relative to the wild type. Overexpression of *EoPHR2* could induce the expression of eight *AtPHT1* genes apart from the *AtPHT1-9* (Fig. 8, Fig. S2). As members of an important phosphate transporter family, *PHT1* genes have frequently been used to study phosphate starvation responses in plants. The induction of *PHT1* genes in transgenic plants suggests a potential *in vivo* role for *EoPHR2* in the Pi-signaling pathway and low-phosphorus stress resistance.



**Figure 8:** Gene expression profile analysis of *AtPHT1* family genes in transgenic *Arabidopsis* plants. Under different phosphorus concentration treatments, all nine *AtPHT1* genes were verified by real-time RT-PCR respectively. The ratio of gene expression level in transgenic *Arabidopsis* plants relative to the wild-type was used for evaluation. Ratio greater than 1: gene up-regulated in transgenic plants; Ratio less than 1: gene down-regulated in transgenic plants; Ratio equal to 1: gene was not induced in transgenic plants

#### 4 Discussion

Acid soils are distributed globally. Approximately 30% of the world's total land area consists of acid soils, and it has been estimated that over 50% of the world's potential arable lands are acidic [20]. Decades ago, acid soils accounted for 22.7% of the total land area in China [21]. As a native species in southern China [14], centipedegrass (*Eremochloa ophiuroides*) is naturally distributed in the low-phosphorus soil areas, particularly on the extremely P-deficient acid soils. It is important to explore the molecular mechanism of low-phosphorus stress responses in plants which naturally adapt to low-phosphorus soil conditions. This is to guide breeding of P-efficient varieties, for improving the utilization rate and ecology of barren land areas. In this study, a low-phosphorus response related gene was first cloned from centipedegrass (*E. ophiuroides*), identified to be an orthologous gene of the *AtPHR1/OsPHR2* gene, termed *EoPHR2*.

According to previous studies in the model species, the *AtPHR1* gene in *Arabidopsis*, or its orthologous gene *OsPHR2* in rice, are key regulators in the pathway responding to phosphorus starvation signal [3,5,10]. Therefore, the root architectural alteration of transgenic plants overexpressing *AtPHR1/OsPHR2*



(abbreviated as *PHR1* below) is considered as the strategy of plants to cope with low-phosphorus stress [6,7]. On the other hand, previous studies of phosphorus transporter genes found that the level of phosphorus content in the roots was not the decisive factor to determine the root architectural alteration in response to low-phosphorus stress [22,23]. It is speculated that *PHR1* regulates root development through an unknown pathway independently of the plant tissue phosphorus level. In this study, *EoPHR2* transgenic plants showed well developed and architecturally alternative roots compared to the wild-type, whether under low-Pi (0.0625 mM Pi), half-Pi (0.625 mM) and full-Pi (1.25 mM Pi) conditions. The results were consistent with previous studies in that *EoPHR2* could regulate root development by a growth regulation pathway unaffected by the level of plant tissue phosphorus. Additionally, in the extremely Pi-absent condition (0 mM Pi), the growth inhibition observed in either wild-type or transgenic lines could be a result of absence of the essential macronutrient.

In previous studies, overexpression of the *PHR1* transcription factor would cause growth inhibition because of toxic physiological effects due to excessive Pi accumulation in shoots, called Pi-toxicity [6,7]. In this study, the fresh weight of transgenic plants did not increase significantly in comparison with the wild-type in the full-Pi condition. This was despite the significantly well-developed roots of the transgenic plants, which suggested that the growth of the aerial parts was inhibited. Nevertheless, the increased fresh weight of transgenic plants was observed under the conditions of low-Pi and half-Pi. It is speculated that the Pi-toxicity and the Pi nutritional limitation act as a pair of competitive factors affecting the growth of the aerial parts. In the case of P-deficiency, the phosphorus efficiency advantage may exceed the negative effect of Pi-toxicity in the transgenic plants, appearing as a better growth phenotype. Considering the actual low P-availability in natural soils [2], as expected, the transgenic plants would grow better than the wild-type in pots.

Based on previous studies in various species, the *PHR1* gene should mainly express in roots, and the gene expression could not be induced by low-phosphorus stress [3,5,10]. In this study, we analysed the expression pattern of the *EoPHR2* gene which responded to low-phosphorus stress (10  $\mu$ M Pi) in different plant tissues. The result showed that the *EoPHR2* constitutively expressed in roots, unrelated to the low-phosphorus stress, which was consistent with the results of previous studies. Considering that the centipedegrass is naturally distributed on acid soils with low-phosphorus coupled with Al-toxicity stress [20], we also analysed the gene expression pattern under 'Al-toxicity' (1.5 mM Al) and 'Al-toxicity & P-deficiency' (10  $\mu$ M Pi, 1.5 mM Al) treatments. Interestingly, as a phosphorus-related gene, the expression of *EoPHR2* could be significantly induced by 'Al-toxicity' stress or 'Al-toxicity & P-deficiency' couple-stress. It suggested that some special regulatory mechanisms respond to the Al-P stress in acid soils and relate to the phosphorus efficiency of centipedegrass. However, this hypothesis should be further studied in the future.

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**Availability of Data and Materials:** The sequence data of the *EoPHR2* gene has been submitted to the GenBank and acquired the accession number MZ227015.

**Author Contributions:** HW designed the research. HW, YC and CL wrote the manuscript. HW, CL, QH, JL, JW, LL, XY, SZ and YC performed the research.

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**Conflicts of Interest:** The authors declare that they have no conflicts of interest to report regarding the present study.

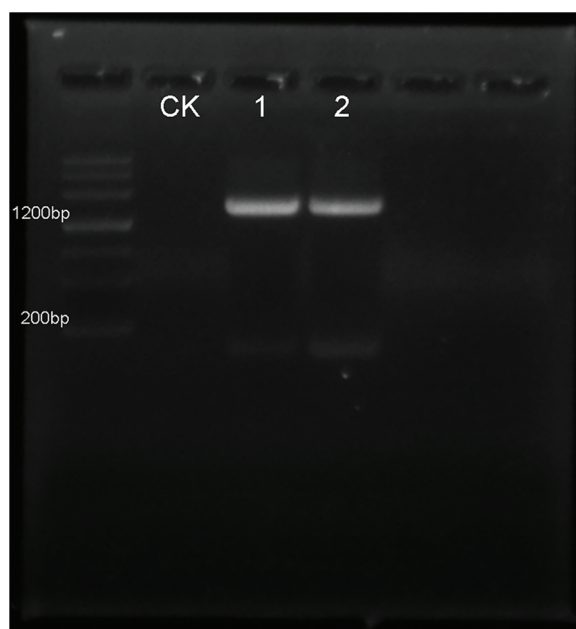
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## Supplementary Figure and Table

### Appendix A



**Figure S1:** Verification of the transgenic *Arabidopsis* seedlings by PCR. The *EoPHR2* gene was amplified from cDNA, subcloned into a modified pBI121-HA overexpression vector, and transferred into *Arabidopsis* using an *Agrobacterium*-mediated transformation method. According to the results of PCR using specific detecting primers, 2 transgenic plants of *EoPHR2* (showed in lanes 1, 2) were confirmed. CK stands for the wild-type plant

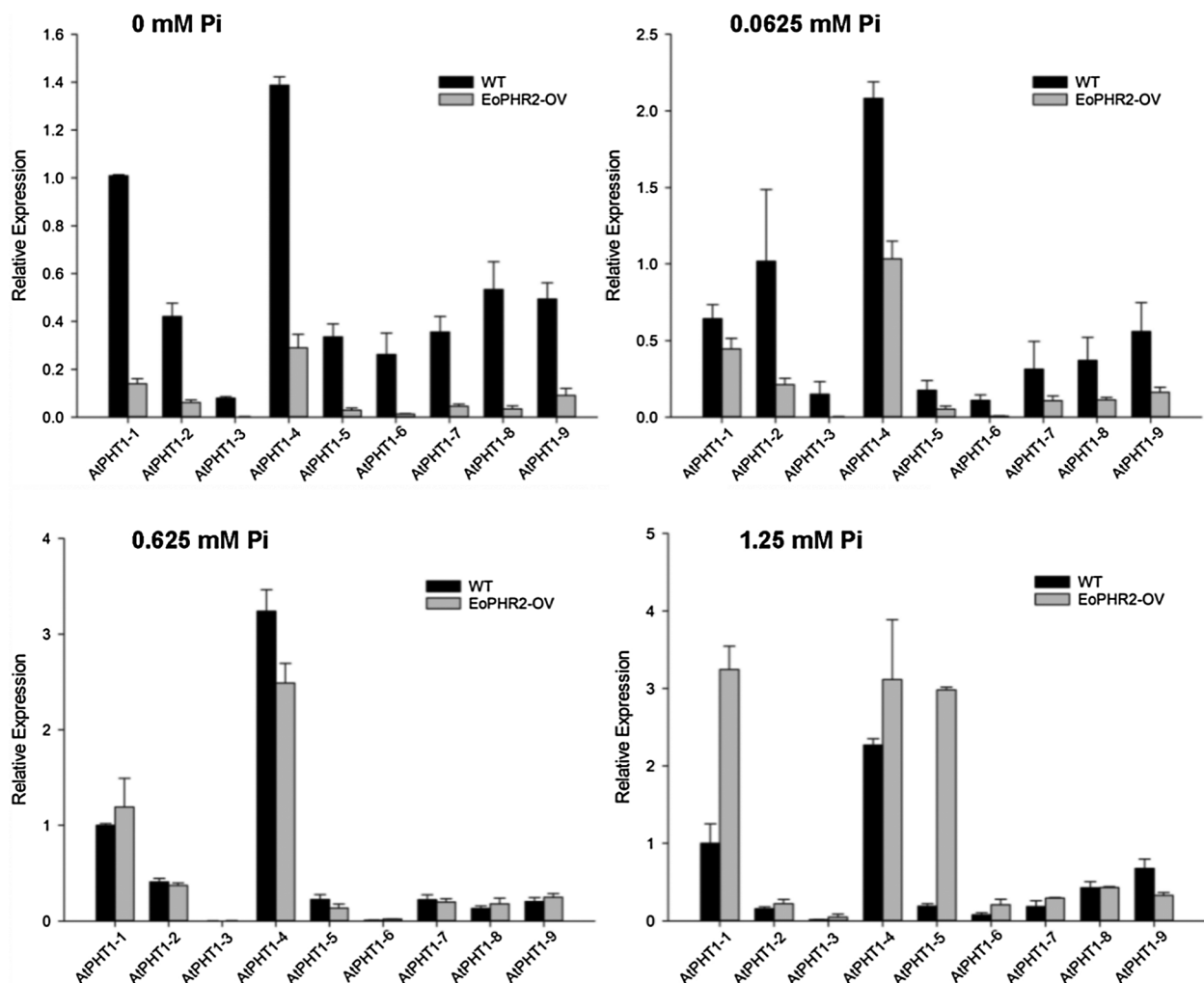


## Appendix B

Table S1: Primers used in experiments

Primer ID	Forward primer (5'-3')	Reverse primer (5'-3')
<i>EoPHR2</i> _Gene Cloning primers		
3'RACE Outer	GCATCGCGCTGGTGACGAGGCTC	Takara Kit matching primer
3'RACE Inner	GCATCTACTACTCCGACCCAGCTCCAAGGATC	Takara Kit matching primer
5'RACE Outer	Takara Kit matching primer	TCTGTGGATGTTGGGTATCAAC
5'RACE Inner	Takara Kit matching primer	TCTGTCCACCATTTCAGTTTGCTTAGTAAGATC
ORF	ATGGAGAGATTAAGCACCAACCAGC	TTGGCCGTTAGCTGATTCTGTTTGC
qRT-PCR primers		
<i>EoPHR2</i>	CTGAATGGTGGACAGAGTTTAT	GACACCCTTAGGAGTTGCTTT
<i>Actin</i>	GCACGGAATCGTCAGCAA	CCCTCGTAGATGGGGACAGT

## Appendix C

Figure S2: Gene expression profile analysis of *AtPHT1* family genes in the wild-type and transgenic *Arabidopsis* plants