Low-Temperature-Induced Expression of Rice *Ureidoglycolate Amidohydrolase* is Mediated by a C-Repeat/Dehydration-Responsive Element that Specifically Interacts with Rice C-Repeat-Binding Factor 3

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Nitrogen recycling and redistribution are important for the environmental stress response of plants. In non-nitrogen-fixing plants, ureide metabolism is crucial to nitrogen recycling from organic sources. Various studies have suggested that the rate-limiting components of ureide metabolism respond to environmental stresses. However, the underlying regulation mechanism is not well understood. In this report, rice *ureidoglycolate amidohydrolase* (*OsUAH*), which is a recently identified enzyme catalyzing the final step of ureide degradation, was identified as low-temperature- (LT) but not abscisic acid- (ABA) regulated. To elucidate the LT regulatory mechanism at the transcriptional level, we isolated and characterized the promoter region of *OsUAH* (*P* *OsUAH*). Series deletions revealed that a minimal region between –522 and –420 relative to the transcriptional start site was sufficient for the cold induction of *P* *OsUAH*. Detailed analyses of this 103-bp fragment indicated that a C-repeat/dehydration-responsive (CRT/DRE) element localized at position –434 was essential for LT-responsive expression. A rice C-repeat-binding factors/DRE-binding proteins 1 (CBFs/DREB1s) subfamily member, *OsCBF3*, was screened to specifically bind to the CRT/DRE element in the minimal region both in yeast one-hybrid assays and in *in vitro* gel-shift analysis. Moreover, the promoter could be exclusively trans-activated by the interaction between the CRT/DRE element and *OsCBF3* in vivo. These findings may help to elucidate the regulation mechanism of stress-responsive ureide metabolism genes and provide an example of the member-specific manipulation of the CBF/DREB1 subfamily.

Keywords: CRT/DRE element, low temperature stress, OsCBF3, transcriptional regulation, *ureidoglycolate amidohydrolase*
INTRODUCTION

Metabolic adjustments alter the physiological and developmental reactions of plant stress adaptation (Bohnert et al., 1995; Bohnert and Sheveleva, 1998). Nitrogen recycling and redistribution are important for the environmental stress response (Nicolas et al., 1985; Tahir and Nakata, 2005; Zhu et al., 2007; Maruyama et al., 2009; Guttieri et al., 2013; Zhang et al., 2014). The redistributed nitrogen is transported among cells or organs of a plant in the form of amino acids or ureides (Schubert, 1986). In most plant species, ureides are the intermediates of the nitrogen recycling from the purine nucleotides for remobilization into amino acids. The functions of ureides have been well documented in the source-to-sink transportation of nitrogen-fixing plants, whereas accumulating evidence has led to the hypothesis that ureides might also participate in stress adaptation. Ureide catabolism is involved in nitrogen recycling from stressed and senescent tissues in drought-treated legumes (Alamillo et al., 2010; Díaz-Leal et al., 2014). Allantoin and allantoate, the primary types of ureides, are accumulated by stresses such as drought, cold, and salinity (Brychkova et al., 2008; Alamillo et al., 2010; Kanani et al., 2010; Yobi et al., 2013). The accumulation of rice grain allantoic is positively correlated with seedling tolerance to low-temperature (LT) stress (Wang et al., 2012). Allantoin has been wildly used as a biomarker of oxidative stress in mammalian cells (Esen et al., 2011; Chung and Benzie, 2013; Fukuhara et al., 2013). Although lacking antioxidant activity in vitro, allantoin supplementation could effectively mitigate oxidative damage in plants (Gus’kov et al., 2004; Brychkova et al., 2008). Further evidence has indicated that exogenous allantoic could improve plant resistance to various stresses (Watanabe et al., 2010; Wang et al., 2012). In addition, recent studies in Arabidopsis demonstrated that in vivo, allantoin is crucial to determine the cellular abscisic acid (ABA) level by activating de novo ABA biosynthesis and hydrolysis of the ABA-glucose conjugate (Watanabe et al., 2014a,b).

In plants other than nitrogen-fixing legumes, ureides are converted via the oxidation of purine. Then, ureides are catabolized in a continuous enzymatic reaction to re-accumulate inorganic nitrogen and are finally converted into glycosylate (Werner et al., 2010, 2013; Werner and Witte, 2011). Many ureide generative or degradative enzymes are involved in environmental stresses. For example, in Arabidopsis, tomato, sugarcane, and ryegrass, environmental stresses, including LT, drought, and salinity, coincidently upregulated the expression of xanthine dehydrogenase (XDH), which is rate-limiting enzyme in purine breakdown (Sagi et al., 1998; Nogueira et al., 2003; Hesberg et al., 2004; Yesbergenova et al., 2005). The repression of Arabidopsis XDH leads to increasing stress sensitivity (Brychkova et al., 2008; Watanabe et al., 2010). A key enzyme gene of ureides catabolism, allantoin amidohydrolase (ALN), is upregulated by drought and ABA treatment in some species of common beans (Alamillo et al., 2010; Coletto et al., 2014). Moreover, mutation of the ALN gene of Arabidopsis greatly enhances the tolerance to water deficit by activating stress-response genes genome-wide (Watanabe et al., 2014b).

Despite several genes of ureide metabolism being associated with stress, the involvement of the remaining components, especially those downstream of allantoate degradation, is largely unknown. More importantly, the regulation mechanisms of stress induction are not well understood. Ureidoglycolate amidohydrolase (UAH) is a recently identified ureide catabolic enzyme in Arabidopsis, rice, and soybeans (Werner et al., 2013) that has the ability in vitro to hydrolyze ureidoglycolate into glyoxylate, carbon dioxide, and two molecules of ammonia (Werner et al., 2010). Here, we report the LT-responsive expression of the rice UAH gene (OsUAH, LOC_Os12g40550), which catalyzes the final step of allantoate degradation (Werner et al., 2010). The molecular mechanism of LT induction of OsUAH is investigated. The results obtained here indicate that C-repeat-binding factors/DRE-binding proteins 1 (CBFs/DREBs1) play a critical role in the LT-responsive expression of OsUAH. Our results may enhance the understanding of the regulation of stress-involved ureide metabolism genes.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Rice plants (Oryza sativa L. ssp. japonica) were used as a source to isolate promoters and genes and for plant transformation. Mature, non-dormant seeds were sterilized and germinated in 1/2 MS medium under a light/dark cycle of 16 h/8 h at 28°C for at least 10 days. Rice seedlings at the trifoliate stage were transferred to plastic buckets with soil at 30°C during the day and 20°C at night in a greenhouse.

Isolation and Sequence Analysis of the Promoter of OsUAH

According to the genomic sequence of OsUAH (LOC_Os12g40550), the region from 2000 bp upstream to 100 bp downstream of the transcription initiation site for this gene was predicted to be the promoter region (PpOsUAH, as showed in Supplementary Sequence). PpOsUAH was PCR-amplified from rice genomic DNA using gene-specific primers. To identify potential functional elements, the full-length sequence of PpOsUAH was analyzed with the PLACE1 and Plant-PAN2 software packages as previously described (Luo et al., 2013).

Promoter-GUS Chimeric Vector Construction and Generation of Transgenic Rice Plants

The 5′ deletions of PpOsUAH at positions −1227, −717, −522, −420, and −137 were generated by PCR amplification using different forward primers and a single downstream primer. A HindIII restriction site was introduced into the forward primers, and an EcoRI restriction site was introduced into the reverse primer. The full-length promoter and five deleted derivatives were
cut by HindIII and EcoRI and then inserted into the plant transforming binary vector PCAMBIA1391 upstream of the GUS coding sequence. The corresponding plasmids were designated as P\text{OsUAH}, P\text{Trn1}, P\text{Trn2}, P\text{Trn3}, P\text{Trn4}, and P\text{Trn5} according to the position at the 5’ end.

Site-specific mutation was performed using the Quick Change Site-Directed Mutagenesis Kit (Transgene, China). The pEASY-T plasmid containing the P\text{Trn1} fragment was used as the PCR template. The obtained mutated construct was cut by HindIII and EcoRI and ligated into PCAMBIA1391. The corresponding plasmid was designated as P\text{Trn1-M}.

To construct potential gain-of-function vectors, the sequence of the CaMV 35S promoter from –46 to +1 (mini 35S) was amplified and inserted into PCAMBIA1391 upstream of the GUS coding sequence. The obtained construct was named P\text{mini} and used as a control. A 103-bp fragment that was located in the region from –522 to –420 of P\text{OsUAH} was obtained by PCR using sequence-specific primers with a HindIII site and an EcoRI site. After digestion, the fragment was inserted into P\text{mini} to obtain the recombinant plasmid P\text{103bp-mini}. Full-length P\text{OsUAH} was also fused to P\text{mini} as a positive control (construct P\text{OsUAH-mini}). All primer sequences that were used are listed in Supplementary Tables S1–S10.

The binary constructs were introduced into the Agrobacterium tumefaciens strain EHA105. The rice transformation vectors that were used contained the HPT gene under the control of the 35S promoter to enable hygromycin-based plant selection. Embryonic calli from the mature rice seeds (Oryza sativa L. ssp. Japonica) were transformed by co-cultivation, selected with 50 mg/l hygromycin, and used to regenerate transgenic plants as previously described (Duan et al., 2012). The single-copy transgenic lines were screened using the real-time PCR method as described (Yang et al., 2005), and at least four independent T2 lines were selected for further analysis.

**Stress Treatments**

To assess the expression levels of the OsUAH gene under temperature stress, 10-days-after-germination (DAG) seedlings on agar plates were placed in a growth chamber at constant temperatures of 4 or 42°C under a light/dark cycle of 16 h/8 h. The seedlings were incubated in 1/2 MS solution containing 250 mM NaCl for salt treatment and 100 μM ABA for ABA treatment. For drought stress, the seedlings were dried at temperatures, 10-DAG seedlings on agar plates were placed in a growth chamber at 4, 10, and 15°C. The control seedlings were grown under the same conditions but at 30°C. The samples were harvested at 0, 4, 8, 12, and 24 h and frozen in liquid nitrogen for RNA extraction.

To analyze the response of P\text{OsUAH} to LT stress at different temperatures, 10-DAG seedlings on agar plates were placed in growth chambers at 4, 10, and 15°C. The control seedlings were grown under the same conditions but at 30°C. The samples were harvested at 0, 4, 8, 12, and 24 h. Mature plants at 60 DAG were treated for 24 h at 4°C, after which the roots, stems and leaves were collected. To analyze the response to LT stress, transgenic plants of truncation and mutation constructs were treated for 24 h at 4°C as above.

**RNA Isolation and qRT-PCR Analysis**

The total RNA was extracted from rice using the RNAprep Pure Plant Kit (TIANGEN, China) in accordance with the manufacturer’s instructions. To amplify the corresponding genes, cDNAs were synthesized with random primers using the FastQuant RT Kit (TIANGEN, China) as the template for the qRT-PCR. Real-time quantitative PCR was performed using an ABI PRISM 7500 real-time PCR system (Applied Biosystems, USA) with SYBR Green (TIANGEN, China). The real-time PCR conditions were 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The qRT-PCR reactions were performed in triplicate for each cDNA sample. The ACTIN gene was used as an internal control, and the relative expression levels were determined in accordance with standard protocols (Livak and Schmittgen, 2001). The expression difference were statistically determined by a one-side paired t-test.

**Histochemical GUS Staining**

The histochemical localization of GUS activity in transgenic plants was performed as previously described (Wu et al., 2003). The samples were incubated in GUS staining solution (50 mM sodium phosphate at pH 7.0, 10 mM Na2-EDTA, 0.1% Triton X-100, 1 mg/ml X-Gluc) at 37°C for 24 h after 15 min of vacuum filtration. After staining, the samples were fixed in 70% ethanol, and photographs were taken under a dissecting microscope.

**Generation of Yeast Reporter Strains for One-Hybrid Screening**

For the one-hybrid assay, three tandem copies of the fragment from –443 to –418 of P\text{OsUAH} was synthesized as a bait. In addition, a two-base-substitution fragment and a five-base-substitution fragment at the CRT/DRE element was used as controls. The sequences are shown in Supplementary Table S7. The above three fragments were separately digested with HindIII and SalI and inserted into the plasmid pAbAi. The obtained recombinant bait plasmids were recognized as pAbAi-E1, pAbAi-E1m2, and pAbAi-E1m5. After digestion with BsuI, the linearized bait plasmids were transformed into yeast strains according to the method that is described in the Matchmaker™ Gold Yeast One-Hybrid Library Screening System Kit (Clontech, USA).

To analyze the CRT/DRE-element-binding activity of OsCBFs, the ORFs of the five rice OsCBF transcription factors OsCBF1, OsCBF2, OsCBF3, OsCBF4, and OsDREB1B were cloned from the cDNA of Japonica rice and were inserted into a GAL4 AD backbone. The pGAD-OsCBF plasmids were then transformed into yeast strains that were integrated with a reporter vector via the LiAc yeast transformation method. The empty AD vector was used as a negative control.

**Purification of Bacterially Expressed Proteins and Electrophoretic Mobility Shift Assay (EMSA)**

The pGEX-4T-1 bacterial expression vector system was used to produce a fusion protein with glutathione S-transferase (GST). To obtain the fused GST-OsCBF3 protein, the recombinant plasmid was transformed into Escherichia coli (Rosetta 2 (DE3)
pLysS strain. The recombinant GST-OsCBF3 protein was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 20 h at 20°C. Protein purification of the GST fusion protein from bacterial extracts was achieved by affinity chromatography with Glutathione Sepharose 4B Resin (GE Healthcare) following the instruction of the manufacturer. Cells carrying the pGEM-4T-1 empty vector were processed as negative controls in an identical manner.

For Electrophoretic Mobility Shift Assay (EMSA), complementary single-stranded oligonucleotides of the E2 probe and its mutants were synthesized, labeled with biotin, and annealed to make probes (Supplementary Table S10). EMSAs were performed using biotin-labeled double-stranded DNA probes with the Light-Shift Chemiluminescent EMSA Kit (Thermo-Fisher Scientific, USA) according to the manufacturer’s instructions.

Trans-activation Experiment with Transgenic Rice
Effector plasmids were constructed with DNA fragments containing OsCBF1, OsCBF2, OsCBF3, OsCBF4, and OsDREB1B coding regions that were under the control of the maize UBI promoter. The effector constructs used PMI as a selected marker gene to enable mannose-based plant selection. The transgenic plants were generated, and the expression of the corresponding CBF genes was examined using qRT-PCR assays. The overexpressing lines were crossed with three independent single-copy PTrml and PTrml-M reporter lines containing the HPT marker gene as described above. The crossed plants were obtained by hygromycin and mannose double selection.

RESULTS

Identification the LT Induction of OsUAH
To identify the stress response of OsUAH, the transcript levels were monitored in time-course treatments of drought, LT, high temperature (HT), salinity, and ABA. As shown in Figure 1, OsUAH was greatly induced by the cold treatment. The OsUAH transcript began to accumulate after 4 h of cold stress treatment and increased in a time-dependent manner. After 24 h of incubation at 4°C, the mRNA level of OsUAH was 9.14-fold relative to that of the untreated control. Cool stress (10–15°C) is the most frequently abiotic stress during the early growth stage of rice. Similar with the response to cold stress, OsUAH transcripts were also accumulated by the 10 and 15°C incubations (Supplementary Figure S1). In contrast, neither HT, salinity stress nor ABA treatment upregulated the expression of OsUAH at any tested time point (Figure 1). Under water deficit, OsUAH expression did not transcriptionally respond to drought stress in a short period (4 and 8 h air-dry treatment); however, 3.4-fold and 4.2-fold inductions were observed after 12 and 24 h of stress incubation, respectively (Figure 1).

To precisely investigate the LT induction of OsUAH, the sequence of the predicted promoter (P_{OsUAH}) with a length of 2100 base pairs (bp) was isolated from the rice (Oryza sativa L. cv. Nipponbare) genome and contains 2000 bp immediately upstream and 100 bp downstream (−2000/+100) of the transcription initiation site. The DNA fragment was inserted into a PCAMBIA 1391 vector, generating a rice transformation construct with P_{OsUAH} driving the GUS reporter gene. A total of 28 independent transgenic lines were generated. Among these lines, six single-copy transgenic lines harboring the P_{OsUAH}-GUS construct were screened and selected to determine the expression pattern of P_{OsUAH}.

The activity of P_{OsUAH} was first examined by GUS histochemical staining. Under normal growth conditions, the GUS staining could not be detected in any of transgenic plants, regardless of tissues or developmental stages (Figure 2A). However, after LT treatment, obvious blue staining was observed not only in 5-DAG seedlings, but also in tissues of plants at the booting stage (60 DAG). The LT response of P_{OsUAH} was also determined by quantitative reverse real-time PCR (qRT-PCR). The GUS transcript was significantly induced after 4 h of incubation at 4°C (p < 0.05). The expression gradually increased in a time-dependent manner, reaching 8.08-fold at 24 h. The LT responses of the promoter were also examined by the incubation at 10 and 15°C. As shown in Figure 2B, the activities of the promoter could be significantly induced after 4 h of incubations (p < 0.05), after which the activities slowly increased or remained relatively constant (Figure 2B). To further investigate the expression pattern of P_{OsUAH}, GUS levels were individually measured in different tissues of 60-DAG plants. The transcripts were markedly increased by LT stress (4°C incubation for 24 h), while the fold-induction level in the roots (11.01-fold) was relatively higher than that in the leaves (5.76-fold) and stems (3.94-fold), (Figure 2C).

Identification of the Minimal Promoter Region for LT-Inducible Expression
In an attempt to define the specific regions of P_{OsUAH} that are involved in LT-inducible expression, a series of 5’ deletions...
OsCBF3 specifically binds to the CRT/DRE element in the minimal region of \(P_{OsUAH}\)

The interaction between the CRT/DRE elements with the C-repeat/dehydration-responsive (CRT/DRE) element, was located at position –434, to activate the LT-induced expression and should contain cis-elements, which are responsible for the LT stress response.

To further identify the element(s) that are responsible for LT induction, a sequence analysis was performed on the 103-bp fragment between positions –522 and –420. A CCGAC element, which is the core sequence of the C-repeat/dehydration-responsive (CRT/DRE) element, was located at position –434, closely associated with the expression of LT or drought stresses. To determine whether the CRT/DRE element is involved in LT induction, the CCGAC sequence was substituted \(\text{in situ}\) in the construct \(P_{Tru1}\) with an irrelevant 5-bp sequence, TGGCA, generating the construct \(P_{Tru1}-M\). Under normal conditions, the activity of \(P_{Tru1}-M\) remained the same as that of the non-mutated \(P_{Tru1}\) in the corresponding transgenic plants. However, the \(GUS\) mRNA accumulations by LT treatment were fully abolished in all of the tested \(P_{103bp-\text{mini}}\) (4.89-fold). In addition, the expression of \(P_{\text{mini}}\) was not altered by LT stress. These results indicate that the 103-bp minimal region is sufficient to activate the LT-induced expression and should contain cis-elements, which are responsible for the LT stress response.

To confirm that the region between positions –522 and –420 plays a crucial role in \(P_{OsUAH}\), the 103-bp fragment was separated and fused to a mini35S promoter (construct \(P_{103bp-\text{mini}}\)) and then linked to the binary vector to drive \(GUS\). Meanwhile, full-length \(P_{OsUAH}\) was also fused to the mini35S promoter as a positive control (construct \(P_{OsUAH-\text{mini}}\)). The constructs, as well as the empty mini35S::\(GUS\) vector (\(P_{\text{mini}}\)), were stably transformed into rice. As shown in Figure 3B, both \(P_{OsUAH-\text{mini}}\) and the \(P_{103bp-\text{mini}}\) were still able to be induced by LT treatment, although the fold-induction level of \(P_{OsUAH-\text{mini}}\) (8.1-fold) was relatively higher than the level of \(P_{103bp-\text{mini}}\) (4.89-fold). In addition, the expression of \(P_{\text{mini}}\) was not altered by LT stress. These results indicate that the 103-bp minimal region is sufficient to activate the LT-induced expression and should contain cis-elements, which are responsible for the LT stress response.

OsCBF3 specifically binds to the CRT/DRE element in the minimal region of \(P_{OsUAH}\).
putative CBF/DREB homologs (OsDREB1A – OsDREB1J) have been identified. Five of these homologs, OsDREB1C/OsCBF1 (LOC_Os06g03670), OsDREB1F/OsCBF2 (LOC_Os01g73770), OsDREB1A/OsCBF3 (LOC_Os09g35030), OsDREB1D/OsCBF4 (LOC_Os06g06970), and OsDREB1B (LOC_Os09g35010), are induced by LT stress (Dubouzet et al., 2003; Liu et al., 2007;
Wang et al., 2008). To determine the specific rice CBF factor that could directly target the core CRT/DRE element in the minimal region of P$_{OsUAH}$, a yeast one-hybrid assay was used. Three tandem copies of the 25-bp sequence surrounding the CRT/DRE element were synthesized as E1 bait. E1 was fused in front of the reporter gene AUR1-C, an antibiotic resistance gene that confers Aureobasidin A (AbA) resistance in yeast. Meanwhile, five OsCBF/DREB1 genes were separately cloned and fused to a GAL4 activation domain (AD) as preys. After being co-transformed with the promoter and individual CBF, only the yeast cells harboring OsCBF3 and E1 could grow on the 100 ng/ml SD/-Leu/AbA medium, while the co-transformant with other rice CBFs could not survive under AbA selection. These observations indicate that OsCBF3 is the only candidate among the tested rice CBF members in yeast (Figure 4A).

To explore whether the element is the binding site of OsCBF3 in the promoter, two site-directed mutations were performed on the core sequence of E1, generating a two-base substitution (E1m2: CGGa) and a five-base substitution (E1m5: gaacta). The AD-OsCBF3 yeast cells harboring baits with either E1m2 or E1m5 could not grow on the leucine dropout medium that was supplemented with 100 ng/ml AbA. In contrast, the growth of yeast cells with OsCBF3 and wild-type E1 was not inhibited by AbA (Figure 4B). These results suggest that the core CRT/DRE element is the specific binding site of OsCBF3 in the P$_{OsUAH}$ fragment.

To further confirm that OsCBF3 binds to P$_{OsUAH}$ at the CRT/DRE element, purified full-length OsCBF3 protein was obtained using a GST-fusion purification system and used to perform Electrophoretic Mobility Shift Assay (EMSA). As shown in Figure 4C, the GST-OsCBF3 protein bound to a 59-bp E2 probe from the minimal region of P$_{OsUAH}$ that contained the CRT/DRE element. A competition EMSA was performed in parallel with wild-type and mutant unlabeled E2 probes. Figure 4C showed that excessive wild-type E2 probe could compete with the labeled probe, but the same amount of unlabeled mutant E2 probe with the two-base substitution could not.
OsCBF3 Trans-activates the Expression of P<sub>OsUAH</sub> in a CRT/DRE Element-Dependent Manner

To detect the in vivo interactions between CBFs and P<sub>OsUAH</sub>, promoter trans-activation assays were performed. The constructs are schematically represented in Figure 5A. Because the truncation fragment at position -1227 (P<sub>Tru</sub>) has a similar expression pattern as that of the full-length promoter P<sub>OsUAH</sub>, P<sub>Tru</sub> and the above-described P<sub>Tru</sub>-M containing the mutated CRT/DRE element were used as reporters. Five LT-responsive rice CBF members were overexpressed by a maize ubiquitin promoter as effectors. The transgenic plants containing the effector were regenerated and crossed with the single-copy reporter plant lines. The crossed plants harboring P<sub>Tru</sub> and OsCBF3 resulted in an 11.47-fold increase in GUS mRNA accumulation compared to the background level of the cross of P<sub>Tru</sub> and an empty-effector vector, whereas the co-expression of other CBF members exhibited the same GUS levels as that of the empty-effector vector (Figure 5B). These results indicate that P<sub>Tru</sub> only could be activated by OsCBF3, which is consistent with the results of the previous binding activity assay in yeast. None of the effectors could induce the expression of the mutated P<sub>Tru</sub>-M reporter, suggesting that the trans-activation of P<sub>OsUAH</sub> by OsCBF3 depends on the CRT/DRE element.

DISCUSSION

Nitrogen supplementation plays a critical role in the utilization of absorbed light energy and photosynthetic carbon metabolism (Kato et al., 2003; Huang et al., 2004). Adequate nitrogen supplementation mitigates the damages of abiotic stresses (Huang et al., 2004; Waraich et al., 2011), while a high risk of photo-oxidative damage is expected in the nitrogen-deficient leaves under stress (Verhoeven et al., 1997). Purine catabolism is important for nitrogen recycling. In this study, we identified OsUAH, the catalyzer of the final step of the ureide-degrading reactions of purine ring catabolism (Werner et al., 2010), as an LT-responsive gene. This result has been confirmed by independent transcriptome analyses (Maruyama et al., 2011; Zhang et al., 2012; Shaik and Ramakrishna, 2013). In addition to previous evidences of the LT induction of other catabolic genes, our results suggest that ureide degradation might be critical for nitrogen redistribution in the LT adaption of plants. Furthermore, our results indicate the OsUAH has a relatively higher induction level in the roots than in photosynthetic tissues, suggesting a potential nitrogen redistribution pattern in response to stress. Various studies have emphasized that nitrogen metabolism genes are regulated by LT stress (Cui et al., 2005; Pageau et al., 2006; Zhu et al., 2007; Robinson and Parkin, 2008; Maruyama et al., 2009; Zhang et al., 2012), while the underlying mechanisms have seldom been reported. The hydrolase genes of purine catabolism, e.g., XDH and ALN, are regulated by ABA (Hesberg et al., 2004; Alamillo et al., 2010). However, we found that the expression of OsUAH was not induced by exogenous ABA (Figure 1), and our data indicated the LT-induction of OsUAH was associated with a CRT/DRE element of P<sub>OsUAH</sub>. The evidence from yeast assays and the in vitro interaction analysis demonstrate that this element physically binds to the CBF transcriptional factor OsCBF3. Further assays demonstrated that P<sub>OsUAH</sub> activity could be upregulated by overexpressing OsCBF3 in vivo. These data suggest that the regulation of OsUAH is involved in a CBF-related LT-response pathway. In addition to LT stress, OsUAH is also induced by drought stress, which is consistent with previous transcriptional profile studies (Degenkolbe et al., 2009; Gao et al., 2009). Because OsCBF3...
is a transcription activator of cold- and drought-responsive gene expression, the induction of OsUAH could be explained by the interaction between the CRT/DRE element of P_{OsUAH} and OsCBF3. In most plants, the CBF pathway of the drought and LT stress responses is ABA-independent (Shinozaki et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006). Therefore, our results suggest the OsUAH might respond to environmental stress in a distinct way compared to other ureide metabolism genes.

C-repeat-binding factor transcriptional factors are involved in the activation of LT-responsive genes by interacting with CRT/DRE elements in the promoter. Most LT-responsive genes have multiple CRT/DRE elements in their promoters. However, the element that is located at −434 bp is an unique copy in the P_{OsUAH} sequence. Our assays identified a synthetic promoter with a 103-bp fragment containing an element that exhibited similar LT-induction activity as that of P_{OsUAH}, whereas the deletion or mutation of this element thoroughly abolished this induction, indicating that this element is sufficient to determine the LT response of P_{OsUAH}. In addition, the deletion between −1227 and −717 of P_{OsUAH} significantly decreased LT induction level. However, the bioinformatic screening did not detect any potential stress response element in this region. Therefore, it is likely that an enhancer exists in the area that facilitates the intensity of the promoter.

C-repeat-binding factor/DREB1 is a small subfamily of the APETALA2/Ethylene response factor (AP2/EREBP) family of transcription factors. In Arabidopsis, this subfamily contains six members (Nakano et al., 2006; Lata and Prasad, 2011). Extensive studies have demonstrated that three tandem-distributing CBF genes, CBF1, CBF2, and CBF3 (also known as DREB1B, DREB1C, and DREB1A, respectively), play a central role in the transcriptional regulation of LT responsive genes. Five rice CBFs were identified as induced by cold stress, and overexpressing any of them can result in the enhancement of plant cold tolerance (Dubouzet et al., 2003; Ito et al., 2006; Liu et al., 2007; Qin et al., 2007; Wang et al., 2008). CBFs have highly conserved functional domains. In most cases, the cold-induced CBFs have redundant activities in plant development and stress adaption as identified in gain-of-function experiments by activating the same gene clusters (Gilmour et al., 2004; Zhou et al., 2010; Lata and Prasad, 2011). However, little evidence had suggested that each individual CBF might regulate different targets and thus separately contribute to transcriptome alteration in response to cold. In Arabidopsis, CBF1 and CBF3 RNAi transgenic plants impair the induction of cold-responsive genes; in contrast, the cold tolerance of the loss-of-function mutant of CBF2, cbf2, is enhanced by upregulating CBF1 and CBF3 (Novillo et al., 2004), indicating that the functional differentiation despite the gain-of-function modification leads to exactly same phenotype (Gilmour et al., 2004). In this study, we first demonstrated that P_{OsUAH} exclusively bound to OsCBF3 in yeast. Then, we found that OsCBF3 could activate P_{OsUAH} by interacting with the CRT/DRE element. Meanwhile, the other four rice CBFs did not affect the promoter activity, although their expression was successfully enhanced similar to OsCBF3 (Supplementary Figure S2). These results suggest that rice CBFs also regulate different objectives, similar to Arabidopsis homologs, and that their function does not fully overlap. Although the overexpression of rice CBFs exhibited a similar stress adaption phenotype (Dubouzet et al., 2003; Ito et al., 2006; Wang et al., 2008), it was reasonable to expect a diversity of function when individual CBF mutant of rice was generated. Furthermore, the rice CBF family has a more complicated structure than that of Arabidopsis or other identified dicot plants. In this study, the activity of P_{OsUAH} was not detected before or after LT induction in a transient expression system of tobacco leaves. However, P_{OsUAH} could be activated via the co-agroinjection of OsCBF3 in tobacco (Supplementary Figure S3). These results indicate that tobacco may lack functional homologs of OsCBF3, suggesting that the OsCBF3-specific trans-activated regulation might be unique between species.

**FUNDING**

This work was supported by the National Natural Science Foundation of China [No. 31501239 to R-YQ, No. 31401454 to HL]; and the Creative Foundation of The Anhui Agricultural Academy of Sciences [13C0101 and 14A0101 to P-CW, 14B0113 to R-YQ].

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.01011

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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