이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게
- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.

변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer
ABSTRACT

Overexpression of OsAT-IV, a branched-chain amino acid aminotransferase gene, confers drought stress tolerance in rice

Hye In Jeong

Department of International Agricultural Technology
Graduate School of International Agricultural Technology
Seoul National University

Branched-chain amino acid aminotransferase (BCAT) proteins play crucial roles in the metabolism of branched-chain amino acids (BCAAs) leucine (Leu), isoleucine (Ile) and valine (Val). BCAAs have been reported to accumulate in response to stress which can then function as compatible solutes and/or as alternative source of respiratory substrates for the TCA cycle during stress. Though BCATs have been described in higher plants such as tomato, barley and Arabidopsis, information on roles of BCATs in rice remains unknown. Here, we show the roles of OsAT-IV, a BCAT in rice, in response to drought stress. Endogenous expression of OsAT-IV across different developmental stages was more dominant in root compared to shoot tissues. Under drought stress, OsAT-IV was up-regulated both in leaf and root tissues in an ABA-dependent manner. In silico analysis revealed 13 ABA-responsive cis-
element motifs present within the 4-kb promoter region of OsAT-IV which could explain the ABA-dependent response of OsAT-IV. We also found that OsAT-IV was localized in cytosol as revealed through transient expression in rice protoplasts. In addition, overexpression of OsAT-IV in rice caused significant increase in BCAA concentration under normal conditions. Levels of Val, Ile and Leu were higher by 22.7, 35.6 and 29.6-fold in OsAT-IV overexpressing plants over WT, respectively. Upon exposure to drought stress, 100% of OsAT-IV overexpressing plants survived while those of WT plants were only 40%. Taken together, we show that overexpression of a BCAT, OsAT-IV, resulted in accumulation of BCAA leading to drought stress tolerance in rice.

Keywords: Branched-chain amino acid aminotransferases, Branched-chain amino acid, Rice, Drought stress, Compatible solutes, Alternative source

Student Number: 2016-29695
Contents

ABSTRACT ........................................................................................................ i

CONTENTS ..................................................................................................... iii

LIST OF FIGURES ........................................................................................ v

LIST OF TABLES ............................................................................................. vi

LIST OF ABBREVIATIONS ............................................................................. vii

INTRODUCTION ............................................................................................. 1

MATERIALS AND METHODS ....................................................................... 5

1. Plant materials and growth conditions ............................................................. 5

2. Phylogenetic analysis ....................................................................................... 5

3. Vector Construction and rice transformation ..................................................... 6

4. ABA hormone treatment .................................................................................. 7

5. RNA isolation and Quantitative real-time PCR analysis ................................... 8

6. Subcellular localization using rice protoplasts ................................................. 8

7. Drought tolerance evaluation at vegetative stage ............................................. 10
8. Chlorophyll a fluorescence .......................................................... 10

9. Amino acid contents analysis .......................................................... 11

10. Amino acid feeding assay for osmotic stress ........................................ 11

RESULTS .......................................................................................... 13

1. OsAT-IV is a putative target of OsNAC5 transcription factor ....................... 13

2. Phylogenetic tree of aminotransferase in the Oryza sativa .......................... 14

3. OsAT-IV is localized in cytosol .......................................................... 14

4. OsAT-IV expression is up-regulated under drought .................................... 15

5. OsAT-IV expression is induced by ABA hormone ...................................... 16

6. Overexpression of OsAT-IV confers drought tolerance to rice .................... 17

7. Branched chain amino acids (BCAAs) were elevated in OsAT-IV OX plants ... 18

8. Expression of genes involved in BCAA metabolism are altered in OsAT-IV OX plants ........................................................................................................ 19

9. Exogenous branched chain amino acids induce osmotic stress resistance in rice ........................................................................................................ 20
LIST OF FIGURES

Figure 1. Regulation of OsAT-IV by the OsNAC5 transcription factor 22

Figure 2. Phylogenetic tree of aminotransferases in the Oryza sativa 24

Figure 3. Cytosol localization of OsAT-IV 26

Figure 4. Vector construction and expression levels of OsAT-IV in transgenic rice plants 28

Figure 5. Expression patterns of OsAT-IV under different growth stages and in response to drought stress 30

Figure 6. Expression of OsAT-IV in response to ABA treatment 32

Figure 7. Drought tolerance of OsAT-IV transgenic plants at the vegetative stage 34

Figure 8. Chlorophyll a fluorescence using the JIP test on OsAT-IV transgenic plants under drought stressed conditions 36

Figure 9. Branched chain amino acid contents in leaf and root of WT and OX plants 38

Figure 10. The branched-chain amino acid biosynthesis pathway in plastid through the de novo biosynthetic pathway 40

Figure 11. The branched-chain amino acid degradation pathway in mitochondria 42
Figure 12. Osmotic stress resistance induced by branched chain amino ............ 44
LIST OF TABLE

Table 1. Primer information ................................. 46
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>BCAT</td>
<td>Branched-chain amino acid aminotransferases Branched-chain amino acid,</td>
</tr>
<tr>
<td>BCAA</td>
<td>Branched-chain amino acid</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>Dip</td>
<td>Dehydration stress inducible protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>35S</td>
<td>Cauliflower mosaic virus 35S promoter</td>
</tr>
</tbody>
</table>
INTRODUCTION

Plants have evolved in sophisticated ways in order to resist certain stresses in the environment. Examples of these stresses are salt, heat, flooding and most especially drought. Drought has been considered to be one of the most destructive forms of abiotic stress. During drought, plants adjust its whole biological processes such as morphological, physiological, biochemical and molecular changes to adapt or resist stress (Lipiec, Doussan et al. 2013, Fahad, Bajwa et al. 2017, Lamaoui, Jemo et al. 2018). For example, at the transcriptional level, transcription factors MYB, bHLH, bZIP, AP2/EREBP and NAC alters the expression of several genes involved in stress response (Shinozaki and Yamaguchi-Shinozaki 2007). One of these is the changes in metabolic profiles of plants during stress (Ullah, Yüce et al. 2017) thus the metabolites present in plants are also important in determining its tolerance to specific stress.

Sugars such as trehalose and sugar alcohols such as mannitol have shown benefits to plants during drought by altering expression of genes involved in sugar metabolism or by acting as osmoptotectant (Taji, Ohsumi et al. 2002, Bartels and Sunkar 2005, Redillas, Park et al. 2012, Chan, Wirtz et al. 2013). Understanding the production of compatible solutes including proline, asparagine, polyamines, glycine betaine, γ-amino-N-butyric acid (GABA), raffinose, trehalose, sucrose, and polyols has been one of the many studies in fine tuning the metabolism of plants during stress (Soni, Nutan et al. 2015).
Other metabolites such as amino acids have also shown to provide tolerance in plants. For example, accumulation of proline during stress has been widely documented in stress-tolerant plants and have roles in cellular homeostasis, redox balance, energy status and most commonly as osmoprotectant though the actual function is still debated (Al Hakimi, Monneveux et al. 1995, Ito, Katsura et al. 2006, Valliyodan and Nguyen 2006, Ashraf and Foolad 2007, Szabados and Savoure 2010). In addition, a number of studies have reported that drought adapted varieties have higher proline levels suggesting a correlation between proline accumulation and drought resistance (Hassine, Ghanem et al. 2008, Parida, Dagaonkar et al. 2008, Evers, Lefevre et al. 2010).

In addition to proline, the three essential amino acids isoleucine, leucine and valine collectively called as branched-chain amino acids (BCAA) due to the short branched carbohydrate residues, have also been found to accumulate during stress (Urano, Maruyama et al. 2009, Bowne, Erwin et al. 2012). BCAAs are major constituents of transmembrane proteins due to its very high hydrophobic nature among other proteinogenic amino acids (Brosnan and Brosnan 2006, Binder, Knill et al. 2007). It is synthesized de novo in plants and are important compounds in many aspects such as building blocks of proteins and synthesis of a number of secondary products in plants (Lea and Ireland 1999). BCAA levels in plants have shown to respond to environmental cues. For example, under osmotic stress, high levels of BCAAs accumulate due to increased biosynthesis (Less and Galili 2008, Urano, Maruyama et al. 2009). It also accumulates in Arabidopsis in response to drought, salt, mannitol, polyethylye glycol, herbicide treatment and nitrogen starvation.
(Huang and Jander 2017). However, conflicting reports have been made whether the accumulation of BCAAs are due to enhanced biosynthesis or increased protein degradation. In either way, the increase in BCAA levels during stress have shown to be beneficial to plants.

The biosynthesis and degradation of BCAAs occur in different organelles. In Arabidopsis, synthesis occurs in plastid while degradation occurs in mitochondria (Binder, Knill et al. 2007). The final steps in producing BCAAs and the first step in degrading BCAAs are catalyzed by the branched-chain amino acid transferases (BCATs) (Binder, Knill et al. 2007). However, several members of BCATs may have different preference towards synthesis or degradation (Maloney, Kochevenko et al. 2010). BCAA biosynthesis is said to be unique such that valine and leucine are synthesized in two parallel pathways each with a set of four identical enzymes catalyzing the reactions with different substrates. These enzymes are acetohydroxyacid synthase (AHAS, EC 4.1.3.18), ketolacid reductoisomerase (KARI, EC 1.1.1.86), dihydroxyacid dehydratase (DHAD, EC 4.2.1.9) and branched-chain aminotransferase (BCAT, EC 2.6.1.42). An exception is the threonine deaminase (TD) catalyzing the deamination and dehydration of threonin to form α-ketobutyrate (KB) and ammonia, the initiating step toward the synthesis of Ile (Binder, Knill et al. 2007).

Here, we characterized a putative rice BCAT named OSAT-IV belonging to IV family of aminotransferas. Overexpression (OX) of OsAT-IV resulted in drought tolerance of rice. We also found that OsAT-IV overexpression resulted in the accumulation of BCAAs even under normal growth condition. Under drought, both
OX and WT plants showed similar increasing pattern of BCAAs however, significant amount of BCAAs were significantly high in OX plants. We propose that OsAT-IV is involved in the synthesis of BCAA resulting in the increased levels in OX plants.
MATERIALS AND METHODS

1. Plant materials and growth conditions

Rice seeds (*Oryza sativa* cv. Dongjin) were germinated on a Murashige–Skoog (MS) solid medium and incubated in a dark growth chamber for 4 days at 28 °C. Seedlings were then transferred to a growth chamber with light and dark cycle of 16 h light/8 h dark having light intensity of 200 μmol m$^{-2}$ s$^{-1}$ and relative humidity of 70%. After 1 week, the seedlings were transferred to a Yoshida solution (Yoshida, Forno et al. 1976) and grown for 2 more weeks.

2. Phylogenetic analysis

Phylogenetic analysis was carried out using amino acid sequences from 49 putative aminotransferase in *Oryza sativa* proteins using the annotation from riceXPro public database (http://ricexpro.dna.affrc.go.jp/). Protein sequences from the gene IDs Os01g0178000, Os01g0238500, Os01g0290100, Os01g0290600, Os01g0729600, Os01g0736400, Os01g0760600, Os02g0236000, Os02g0252600, Os02g0273100, Os02g0302400, Os02g0302700, Os02g0709200, Os02g0797500, Os02g0806900, Os03g0106400, Os03g0171900, Os03g0195100, Os03g0231600, Os03g025800, Os03g0299900, Os03g0338000, Os04g0405700, Os04g0559400,
Os04g0614500, Os05g0129100, Os05g0244700, Os05g0475400, Os05g0558400,
Os06g0345200, Os06g0548000, Os07g0106700, Os07g0108300, Os07g0461900,
Os07g0617800, Os08g0245400, Os08g0532200, Os08g0532200, Os09g0433900,
Os10g0189600, Os10g0390500, Os10g0390600, Os10g0484700, Os10g0549500,
Os10g0560900, Os11g0209900, Os11g055200, Os11g0644800, Os12g0131100
were used to construct the phylogenetic tree through the CLC workbench software
version 7.0 (https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/).

3. Vector Construction and rice transformation

To generate overexpression plants, the coding sequence of OsAT-IV (Os05g0244700) was amplified from rice cDNA (Oryza sativa L. ssp. japonica cv. Nipponbare) using a high-fidelity DNA polymerase PrimeStar (TaKaRa, Japan) and inserted into the rice transformation vectors p700-GOS2 (Jeong, Kim et al. 2010) for whole plant body overexpression and p700-RCc3 for root-specific overexpression using the Gateway system (Invitrogen, Carlsbad, CA). The p700-GOS2 vector contains the 2.2-kb upstream sequence of the gene GOS2 (Os07g0529800) (Pater, Mark et al. 1992) while the p700-RCc3 carries the 1.3-kb promoter region of RCc3 (Os02g0662000). The GOS2 (rice eukaryotic translation initiation factor1-like gene) promoter drives constitutive gene expression throughout the whole plant body (Pater, Mark et al. 1992) while the RCc3 (rice lipid transfer protein-like gene) promoter
drives root-specific gene expression (Xu, Buchholz et al. 1995) generating the GOS2:OsAT-IV and RCc3:OsAT-IV lines, respectively. Plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 through triparental mating and embryonic calli from rice seeds (*Oryza sativa* L. *ssp. japonica* cv. Dongjin) were transformed as previously described (Jang, Nahm et al. 1999). Taq-Man PCR was used to determine the copy number in T0 plants (Bang, Park et al. 2015). Single-copy plants were selected and propagated in a rice paddy field at Kyungpook National University, Gunwi (128:34E/36:15N), Korea. Three homozygous, single-copy lines were selected for further analysis.

4. **ABA hormone treatment**

Seeds from *Oryza sativa* cv. Dongjin were germinated on Murashige–Skoog (MS) solid medium and incubated in a dark growth chamber 4 days at 28 °C. Seedlings were then transferred to light with the light and dark cycle of 16 h light/8 h dark. After 2 weeks, the seedlings were grown in Yoshida solution (Yoshida, Forno et al. 1976) for 2 days for adaptation. For the ABA concentration-dependent response of OsAT-IV, eight seedlings were put in 50 ml falcon tubes containing 0, 1, 10, and 100 μM ABA solution and incubated in a growth chamber for 6 h until sampling. For the response sensitivity of OsAT-IV to ABA treatment, 2-week-old rice plants were transferred to water containing 100 μM ABA and harvested at the indicated time points (0, 2, 4, and 6 h). The ABA (Sigma-Aldrich, Korea) used in the study was
dissolved in 100% methanol to make 1 M of ABA stock solution.

5. RNA isolation and Quantitative real-time PCR analysis

Gene All Hybrid-R RNA purification kit (GeneAll Biotechnology, Seoul, Korea) was used to isolate total RNA. 2000 ng of total RNA was used as initial template and the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) was used for the synthesis of cDNA. Real-time PCR analysis was performed using the EvaGreen™ Mix (SolGent, Deajeon, Korea) in a Mx3000P Real-Time PCR system (Stratagene, CA, USA). Primers used for qRT-PCR were listed in Table 1. Rice *Ubiquitin1* (Os06g0681400) expression level was used as internal standard, and rice *Dip1* (Os02g0669100) was used as a molecular marker of drought and ABA stress response. *OsAT-IV* gene specific primers were designed in third exon. Values are the means ± SD (standard deviation) of three biological samples and three experimental repeats.

6. Subcellular localization using rice protoplasts

Fifty etiolated rice seedlings were gathered to collect the leaf sheaths. Using a sharp razor blade leaf sheaths were cut into 1- to 2- mm on a glass plate. The cut leaves were quickly placed in a 10 ml digestion solution containing 0.6 M
mannitol, 0.6 mM 4-morpholineethanesulfonic acid (MES), 1.5 % cellulase RS (Yakult, Japan), 0.75 % macerozyme (Yakult, Japan), 1 mM CaCl$_2$, 0.1 % Bovine Serum Albumin (BSA), 5 mM beta-mercaptoethanol, pH 5.7. The samples were incubated at 28 °C for 4-5 h in dark with gentle shaking (40–50 rpm). Intermittent vacuum infiltration was done every hour for 15 min each. After incubation, the protoplasts were collected in a 50 ml tube. Subsequent washing using 10 ml (twice) of W5 solution (154 mM NaCl, 125 mM CaCl$_2$, 5 mM KC1, 5 mM D-glucose 2 mM MES, pH 5.7) was done and the collected protoplasts were pooled. The suspension was passed through 70 µm and 40 µm nylon mesh to remove debris and centrifuged at 320 g for 8 min and washed three times with W5 solution. The pellet was then re-suspended in MMg solution (4 mM MES, pH 5.7, 0.6 M mannitol, and 15 mM MgCl$_2$). Protoplasts were quantified by microscopy using a hemocytometer before transformation. The 35S: OsAT-IV -GFP plasmid were transformed into protoplasts using PEG-mediated transformation. After 12 h incubation at 28 °C, the protoplasts were harvested by centrifugation at 300 g for 2 min and stained with MitoTracker™ Red CMXRos (Invitrogen). The subcellular localization of OsAT-IV was observed by a Leica SP8 STED laser scanning confocal microscope (Leica, Solms, Germany). GFP was excited at 488 nm and the emitted light was detected between 512 and 560 nm. MitoTracker™ Red was excited at 579 and emission was detected between 599 nm.
7. **Drought tolerance evaluation at vegetative stage**

OsAT-IV transgenic and nontransgenic (NT) control plants (O. Sativa cv. Dongjin) were germinated on Murashige–Skoog (MS) solid medium and incubated in a dark growth chamber 4 days at 28 °C. Seedlings were then transferred to light and grown for 1 day before transplanting to soil. Thirty plants from each line were transplanted into ten soil pots (4×4×6 cm) within a container (59×38.5×15 cm; three plants per pot) having enough supply of water and grown for additional 4 weeks in a greenhouse at 28–30°C. For drought simulation at the vegetative stage, pots were transferred out of the container and allowed to grow for several days without irrigation. The moisture content of soil was monitored using the SM150 Soil Moisture Sensor (AT Delta-T Devices) to check uniform treatment of drought between pots. When plants showed signs of wilting such as leaf rolling and chlorosis, plants were transferred back into the container for re-watering and growth recovery.

8. **Chlorophyll a fluorescence**

Chlorophyll a fluorescence transients were measured using the Handy-PEA fluorimeter (Plant Efficiency Analyzer, Hansatech Instruments Ltd., King’s Lynn Norfolk, PE 30 4NE, UK). 6-weeks-old soil-grown plants were adapted in darkness for at least 1 hour ensure sufficient opening of the reaction centers. All leaves of the plant were induced by a red light (650 nm) of 3500 μmol photons m⁻² s⁻¹ provided by the three light-emitting diodes, focused on a spot of 5 mm in diameter.
and recorded for 1s with 12-bit resolution. Normalization of data was performed using the Biolyzer 4HP software program.

9. **Amino acid contents analysis**

To measure the amino acid contents, single T₃ homozygous transgenic line from GOS2::OsAT-IV (#5) and WT (O. Sativa cv. Dongjin) were grown in a growth chamber for 3 weeks in Yoshida solution. Plants were then air-dried to simulate dehydration stress and sampled at 0, 4, 6, and 10 hr after stress treatment. Plants not exposed to stress were used as control. Samples were collected and sent to the National Instrumentation Center for Environmental Management (NICEM) in Seoul National University, Korea. Analysis was done using HPLC Ultimate 3000 equipped with column VD Spher 100 C18—E (4.6 mm x 150 mm, 3.5 um/VDS, Optilab, Germany) and FL detector 1260 FLD (Agilent, USA) according to manufacturer’s manual.

10. **Amino acid feeding assay for osmotic stress**

Three-week old plants were fed with 10 mM L-Valine, Leucine or Isoleucine (Sigma-Aldrich, Korea) by dipping its roots in a solution containing BCAAs for 24 hrs. Plants were then transferred into a falcon tube containing 25 mL
of 25 % PEG 8000 (Sigma-Aldrich, USA) solution to simulate osmotic stress. Water was used in mock treatments. Plants were sampled for total RNA at 0, 16 and 48 hr after re-watering after PEG treatment. cDNA was then synthesized for qRT-PCR analysis of *Dip1* gene which served as molecular markers for osmotic stress response.
RESULTS

1. *OsAT-IV* is a putative target of OsNAC5 transcription factor

Previously, we identified a stress-responsive NAC transcription factor *OsNAC5* involved in drought stress tolerance in rice. Through microarray analysis data, we observed that the *OsAT-IV* gene was significantly upregulated both in whole body and root-specific *OsNAC5*-overexpressing plants (Jeong, Kim et al. 2013) and could potentially be one of the major causative factors on the acquired drought-tolerance of *OsNAC5*-overexpressing plants. Thus, we further analyzed the roles of *OsAT-IV* in rice. Independent from the plants used in microarray analysis, two root-specific *OsNAC5*-overexpressing lines (*RCc3:OsNAC5myc* 6 and 7) were sampled for total RNA and qRT-PCR was performed to confirm the up-regulation of *OsAT-IV* using different overexpression lines. Results showed that the overexpression of *OsNAC5* indeed up-regulated the expression of *OsAT-IV*. Sequence analysis on the 4-kb promoter region of *OsAT-IV* showed 14 NAC binding motif core sequence CACG (Tran, Nakashima et al. 2004) (Figure 1 B). We therefore analyzed the ChIP-seq data reported by Chung et al. 2018 on different *OsNACs*. We found that the NAC binding motif is located 1,807 bp upstream of the ATG translational start site of *OsAT-IV*. These results suggest that overexpression of *OsNAC5* regulates the expression of *OsAT-IV*.
2. Phylogenetic tree of aminotransferase in the *Oryza sativa*

BCATs that are localized in plastids has been studied well in other plants species (Diebold, Schuster et al. 2002). We gathered 49 putative aminotransferase protein sequences from *Oryza sativa* acquired using the annotation from riceXPro public database. We found 5 sequences that was in the same clade with Arabidopsis thaliana plastid branched-chain-amino-acid aminotransferase (AtBCAT3, AtBCAT5) (Binder 2010). In addition, 2 genes (Os01g0238500, Os02g0273100) that were closely related with OsAT-IV (Os05g0244700) were found to be cytosolic branched-chain-amino-acid aminotransferase and belonged to the same group (Figure 2) suggesting that OsAT-IV could also be a cytosol-localized rather than a plastid-localized type of BCAT.

3. OsAT-IV is localized in cytosol

To confirm the subcellular localization of OsAT-IV, we linked the coding sequence of *OsAT-IV* without the stop codon to the green fluorescent protein (GFP). The cassette was driven by a 35s promoter and inserted into the pHBT vector (GeneBank accession number EF090408) producing the plasmid 35s: *OsAT-IV-GFP* (Figure 3 B). Plasmids were then transiently expressed in rice protoplasts and stained with Mito Tracker Red for mitochondrial staining. The GFP signal was abundant in cytosol and showed no GFP signals in mitochondria which is a site of BCAA
degradation (Binder 2010) (Figure 3 B). These results suggest OsAT-IV may be involved in BCAA synthesis rather than degradation.

4. OsAT-IV expression is up-regulated under drought

To confirm whether OsAT-IV expression is affected by drought, we performed qRT-PCR analysis on 21-day-old WT plants exposed to drought through air-drying for 10 hr and sampled at 0, 4, 6, 10 hr. Results showed that after only 4 hr of exposure to stress, OsAT-IV expression was rapidly up-regulated in leaf and root reaching as high as 4-fold and 3-fold, respectively. After 10 hr of exposure to stress, OsAT-IV expression was also up-regulated in leaf reaching as high as 8-fold and roots showed a relatively low up-regulation of 3-fold (Figure 5 B). These results could be due to the differences in the endogenous expression level of OsAT-IV as mentioned in the previous section. In leaves, OsAT-IV was relatively low while in roots the endogenous expression of OsAT-IV was already high thus during drought treatment, OsAT-IV expression in roots was not as high as those observed in leaf tissues. Nevertheless, the expression of OsAT-IV was up-regulated when plants were exposed to drought.
5. *OsAT-IV* expression is induced by ABA hormone

Expression of drought responsive genes has been shown to follow through an ABA-dependent or an ABA-independent pathway (Yoshida, Mogami et al. 2014). In the case of BCAAs, it has been reported that BCAAs concentration accumulate in response to drought and osmotic stress and involves the hormone ABA (Huang and Jander 2017) suggesting that the genes involved in BCAA biosynthesis may follow the ABA-dependent pathway. To investigate the potential role of ABA hormone in regulating *OsAT-IV* expression, we first analyzed the promoter region of *OsAT-IV* (4 kb upstream of the start codon) *in silico* to determine whether ABA-responsive elements (ABREs) are present. Results showed that within the 4-kb region, the sequence contained 13 ABREs composed of the ACGT core sequence (Yoon, Lee et al. 2017) (Figure 6 A). We therefore performed qRT-PCR on *OsAT-IV* in response to ABA treatments. In terms of concentration-dependent response, 14-day-old rice seedlings were treated with ABA at concentrations 1, 10, and 100 μM for 6 hr. The *DEHYDRATION-INDUCIBLE PROTEIN1* (*OsDIP1*) of which expression increases in response to ABA was used as a positive control for ABA treatments. The *OsDIP1* expression was up-regulated which was in agreement with the increase of ABA concentration. When treated with 1 μM of ABA, no significant change in transcript levels observed for both *OsDIP1* and *OsAT-IV* genes. At 10 μM, *OsDIP1* showed a 2.4- and 1.4-fold increase in leaf and roots, respectively while *OsAT-IV* showed a higher increase of 3.3- and 3.2-fold increase in leaf and roots, respectively. With 100 μM, the *OsDIP1* transcript levels significantly increased both in leaves (~6.2) and roots (~4.9-fold) while *OsAT-IV* expression reached 10.3-fold in leaves while roots
showed 6.7-fold increase relative to WT (Figure 6 B). Since OsAT-IV expression showed significant response at 100 uM ABA, we measured the changes in OsAT-IV expression level in relation to time. Fourteen-day old seedlings were exposed to 100 uM ABA and sampled every 2 hr for 6 hr. Results showed that OsAT-IV expression increased both in leaf and roots as the treatment progressed indicating that ABA indeed affects the expression of OsAT-IV thus following the ABA-dependent response pathway (Figure 6 C).

6. Overexpression of OsAT-IV confers drought tolerance to rice

To investigate the biological functions of OsAT-IV, we generated two types of overexpression plants i.e. whole-body overexpression driven by the GOS2 promoter (Pater, Mark et al. 1992) and root-specific overexpression driven by the RCc3 promoter (Xu, Buchholz et al. 1995) (Figure 4 A). To perform drought test, 1-month old transgenic and WT plants were exposed to drought by withholding water for a least 1 day until drought-related symptoms such as leaf rolling appeared (Figure 7 A). The soil moisture was closely monitored and showed similar reduction among pots indicating uniform stress-treatment (Figure 7 B). During treatment, WT showed leaf rolling symptoms earlier than both transgenic plants in as early as 1 day. With prolonged treatment to 2 days, WT plants showed severe drying compared to overexpression plants (Figure 7 A). When plants were re-watered, almost all the
transgenic plants survived while WT plants only had around 40% survival rate showing a clear difference between drought tolerance of transgenic and WT plants (Figure 7 C). We also measured the chlorophyll $a$ fluorescence ($F_v/F_M$) of plants exposed to drought in six-week old plants which can show the photochemical efficiency of plants before and after stress-treatment. Three plants were planted in a pot and grown for 3 weeks (Figure 8 A). Drought was initiated when irrigation was stopped. The $F_v/F_M$ values between plants showed wide difference between transgenic and WT. In WT, plants started to decrease after 5 days of drought treatment while transgenic plants showed reduction in $F_v/F_M$ values only showed after 8 days of stress treatment (Figure 8 B). The delayed reduction in $F_v/F_M$ values of transgenic plants clearly showed that overexpression of OsAT-IV either through whole-body or root-specific overexpression preserved the photochemical efficiency of plants during resulting in drought tolerance of overexpression plants (Figure 8).

7. Branched chaine amino acids (BCAAs) were elevated in OsAT-IV OX plants

BCAAs are known to accumulate when plants are exposed to different types of stress such as drought, salt, herbicide and even during nitrogen starvation (Huang and Jander 2017) and are degraded in mitochondria. To determine whether transgenic plants have altered content of BCAAs compared to WT, we measured the BCAA content of plants under normal and air-dried conditions. Leaves and roots of
three-week old transgenic and WT plants were air-dried for 10 hr and sampled at 0, 4, 6, 10 hr. Samples were then measured for free amino acid contents. Under normal conditions, the BCAA content in transgenic plants were significantly higher than WT indicating that the overexpression of OsAT-IV enhanced the synthesis of BCAA. Under air-dried condition, both transgenic and WT plants showed accumulation of BCAA in response to stress. Transgenic plants, which already contained high BCAA under normal condition, continued to accumulate BCAA during stress treatment (Figure 9). These results suggest that BCAA indeed accumulate during drought and the overexpression of OsAT-IV further enhanced its biosynthesis.

8. Expression of genes involved in BCAA metabolism are altered in OsAT-IV OX plants

Biosynthesis and catabolism of BCAAs occur in different subcellular compartments (Maloney, Kochevenko et al. 2010). Generally, BCAAs are produced in plastids and are degraded in mitochondria (Diebold, Schuster et al. 2002). To determine the expression of genes involved in BCAA biosynthesis under water-stressed conditions, leaves of three-week old transgenic and WT plants were air-dried for 10 hr and sampled at 0, 4, 6, 10 hr. The relative expression of 2 key enzymes in the biosynthesis pathway- Ketol-acid reductoisomerase (KARI) and Dihydroxy-acid dehydratase (DHAD) expression were analyzed in WT and OX plants (Figure
Under normal conditions, the expression of these genes in WT were significantly higher than OX plants. Under air-dried condition, both OX and WT plants showed up-regulated expression in response to stress however, the levels in WT were consistently higher than those of OX plants (Figure 10 B). This result suggests a possible feedback regulation of the biosynthetic genes resulting from the accumulation of BCAA following the overexpression of OsAT-IV. On the other hand, since BCAAs can function as alternative source of respiratory substrates for the TCA cycle during stress when degraded (Taylor, Heazlewood et al. 2004), we analyzed both in WT and OX lines the expression of 2 key enzymes Branched-chain keto acid dehydrogenase (BCKD) and Isovaleryl-CoA dehydrogenase (IVD) of the catabolic pathway through qRT-PCR analysis (Figure 11 A). Under normal conditions, the expression of these two genes in both plants were relatively similar. Under air-dried condition, both OX and WT plants showed an up-regulated expression in response to stress but the levels in OX plants were significantly higher than those of WT plants (Figure 10 B). This result suggests that BCAAs of OX plant were possibly degraded more than WT plants such that the OsAT-IV overexpressing plants can provide higher alternative energy source or substrates that can be passed to the TCA pathway.

9. Exogenous branched chain amino acids induce osmotic stress resistance in rice

To investigate the effects of exogenous source of branched chain amino acid
to plants, roots were first treated with either individual BCAA or as a solution of the three BCAAs before they were transferred in a PEG-containing solution. Incubation lasted until osmotic stress-related symptoms such as leaf rolling appeared (Figure 12). During treatment, mock plants showed leaf rolling earlier than plants pre-treated with BCAAs. Valine- leucine, isoleucine and BCAAs-treated plants showed more resistance than the mock plants. When plants were removed from PEG and re-watered, most plants that were pre-treated with BCAAs survived compared to mock-treated plants (figure 12).
OsAT-IV was previously reported to be up-regulated when the transcription factor OsNAC5 was overexpressed in rice (Jeong, Kim et al. 2013). (A) The 4 kb promoter region of OsAT-IV contained NAC cis-elements where OsNAC5 transcription factor can putatively bind. (B) Expression of OsAT-IV in roots of two-week old RCc3:OsNAC5myc-6 and RCc3:OsNAC5myc-7 lines relative to WT. Ubiquitin1 (Ubi1) expression was used as an internal control. Data are shown as ±SD of three technical replicates.
(A) NAC binding motif

4kb promoter of OsAT-IV

-4kb  -2kb  +1

ATG

(B) Relative expression

<table>
<thead>
<tr>
<th></th>
<th>NT</th>
<th>Nac5-#6</th>
<th>Nac5-#7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>

0  2  4  6  8

NT  Nac5-#6  Nac5-#7
Phylogenetic analysis was carried out using amino acid sequences from 49 putative aminotransferase in Oryza sativa proteins using the annotation from riceXPro public database (http://ricexpro.dna.affrc.go.jp/). Phylogenetic tree was made using the CLC workbench software version 7.0, (https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/) based on full-length amino acid sequences. The 5 sequences of indicated as group 1 were similar to Arabidopsis thaliana branched-chain-amino-acid aminotransferase (AtBCAT3, AtBCAT5) while OsAT-IV (Os05g0244700) belonged to a different group (designated as Group 2) with 2 other similar genes (Os01g0238500, Os02g0273100).
(A) The vector used for subcellular localization contained the OsAT-IV coding region without the stop codon and was linked to a GFP marker protein. The linked genes were driven by a 35S CamV promoter. (B) Subcellular localization assay was performed through transient expression in rice protoplasts. After a 12-hr overnight incubation, protoplasts were stained with MitoTracker™ Red CMXRos to stain the mitochondria red (a common location of BCATs). The GFP and RFP fluorescence were observed using a Leica SP8 STED laser scanning confocal microscope.
Figure 4. Vector construction and expression levels of OsAT-IV in transgenic rice plants

(A) Two overexpression systems were developed to produce whole-body constitutive overexpression of OSAT-IV using the GOS2 promoter and root-specific overexpression using the RCc3 promoter. For transgenic rice plants selection, the sGFP was linked to the drought-inducible WSi18 promoter which expresses GFP in dried grains as well as the bacterial phosphinothricin acetyltransferase gene (bar) expression cassette for antibiotic resistance. (B) qRT-PCR analysis was done on two-week old selected lines of GOS2::OsAT-IV (line 1, 5, 13 and 60), RCc3:: OsAT-IV (line 4, 24, 50 and 54) and NT plants for expression levels of OsAT-IV. All values were relative to the NT controls. Ubiquitin1 expression was used an internal control. Values are the means ± SD (standard deviation) from three biological samples and three experimental repeats.
(A) 

(B) 

\[ \text{GOS2::OsAT-IV} \]

\begin{align*}
\text{Relative expression} & \\
\text{NT} & \#1 & \#5 & \#12 & \#40
\end{align*}

\begin{align*}
\text{RCC3::OsAT-IV} \quad \text{Relative expression} & \\
\text{NT} & \#4 & \#24 & \#50 & \#54
\end{align*}
Figure 5. Expression patterns of OsAT-IV under different growth stages and in response to drought stress

(A) qRT-PCR analysis was performed on leaf and root tissues of WT plants under different growth stages showing higher expression in roots compared to those in leaves. 1-month leaf was considered as control as it showed the lowest expression level. D, Dark, L, Light. (B) To confirm whether OsAT-IV expression is affected by drought, WT plants were grown for 21-days and exposed to air-drying for 10 hr and sampled at 0, 4, 6, 10 hr. All values were relative to the 0 hr. Ubiquitin1 (Ubi1) gene expression was used as internal control. Data are shown as the means ± SD of three replicates.
Figure 6. Expression of *OsAT-IV* in response to ABA treatment

(A) To determine whether *OsAT-IV* contains ABA-responsive elements (ABREs), a 4-kb upstream sequence of the *OsAT-IV* transcription start site (TSS) was uploaded to PLANTPAN 2.0 public database (http://plantpan2.itps.ncku.edu.tw/) for motif scanning and prediction. (B) qRT-PCR analysis of OsAT-IV expression in leaf and root in response to ABA treatment. WT plants (*Oryza sativa* cv. Dongjin) were grown in soil for 2 weeks and exposed to different concentrations of ABA i.e. 0 (mock), 1, 10, and 100 μM ABA and sampled after 6 hr. (C) Plants were also grown in soil for 2 weeks and treated with 100 μM ABA. Leaf and root samples were collected every 2 hr for 6 hr. *OsDIP1* (Os02g0669100) was used as an ABA-responsive marker. *Ubiquitin1* expression was used as an internal control. Data are shown as ±SD of three technical replicates.
(A) ABA-responsive elements (ABREs) motif

4kb promoter of OsAT-IV

-4kb -2kb +1

ATG

(B) Leaf

<table>
<thead>
<tr>
<th></th>
<th>OsDIP1</th>
<th>OsAT-IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1μM</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>10μM</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>100μM</td>
<td>3</td>
<td>10</td>
</tr>
</tbody>
</table>

Root

<table>
<thead>
<tr>
<th></th>
<th>OsDIP1</th>
<th>OsAT-IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1μM</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>10μM</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>100μM</td>
<td>3</td>
<td>10</td>
</tr>
</tbody>
</table>

(C) Leaf Root

<table>
<thead>
<tr>
<th></th>
<th>Leaf</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 7. Drought tolerance of *OsAT-IV* transgenic plants at the vegetative stage

(A) Drought tolerance of transgenic and WT plants. Four-week old plants grown in soil under sufficient supply of water were exposed to drought for 2 days, followed by re-watering for 7 days in a glass house. Images were taken accordingly. (B) Soil moisture in pots was measured at the indicated time points to show uniform treatment of drought across all pots. (C) Survival rate 7 days after re-watering scoring 30 plants for transgenic lines and 120 plants for NT.
Figure 8. Chlorophyll $a$ fluorescence using the JIP test on $OsAT-IV$ transgenic plants under drought stressed conditions

(A) Drought tolerance of transgenic and NT plants. All plants were grown in soil for 6 weeks receiving ample supply of water and exposed to drought stress for 9 days. (B) JIP test for Chlorophyll $a$ fluorescence to measure the $Fv/Fm$ of plants when exposed to drought using the Handy-PEA fluorimeter. Each value measured using 30 points of leaves in 3 independents transgenic and NT plants.
(A) 

0 day 0 day

9 day 9 day

NT #1 #5 #48 #61

GOS2::OsAT-IV

NT #4 #24 #50 #54

RCc3::OsAT-IV

(B) 

GOS2::OsAT-IV

RCc3::OsAT-IV

Fv/F0 vs. 9 (day)

WT #1 #5 #48 #61

Fv/F0 vs. 9 (day)

WT #4 #24 #50 #54
Figure 9. Branched chain amino acid contents in leaf and root of WT and OX plants.

Three-week old plants were exposed to dehydration through air-drying for 10 hr and sampled at 0, 4, 6, 10 hr. Leaf and root samples were collected for amino acid analysis specifically valine, isoleucine and leucine both in WT and OX plants (n=2).
Figure 10. The branched-chain amino acid biosynthesis pathway in plastid through the de novo biosynthetic pathway

(A) Enzymes in boxes are common to parallel pathways of isoleucine, leucine, and valine synthesis. Image screen-grabbed from Vijay Joshi et al. (2010). (B) Relative expression of 2 key enzymes of the BCAA biosynthesis pathway. qRT-PCR analysis of *Ketol-acid reductoisomerase (KARI)* and *Dihydroxy-acid dehydratase (DHAD)* in 21-day-old WT and OX plants that were exposed to dehydration. *Ubiquitin1* expression was used as an internal control. Data are shown as ± SD of three technical replicates.
(A) 2-oxobutanoate → pyruvate
   ↓
   Acetolactate synthase → acetolactate
   ↓
   Ketol-acid reductoisomerase → 2,3-dihydroxy-3-methylvalerate
   ↓
   2,3-dihydroxy-lisovalerate
   ↓
   Dihydroxy-acid dehydratase → 2-keto-3-methylvalerate
   ↓
   2-keto-lisovalerate
   ↓
   Branched-chain amino acid aminotransferase → isoleucine
   ↓
   valine
   ↓
   leucine

(B) 

![Graphs showing relative expression of KARI and DHAD over time (0-10 hours) for WT and ox conditions.](image)
Figure 11. The branched-chain amino acid degradation pathway in mitochondria

(A) Key enzymes (blue) and metabolites (black) of branched-chain amino acid degradation. Image adapted from Binder, et al. (2010). (B) Relative expression of 2 key enzymes of the BCAA degradation pathway. qRT-PCR analysis of branched-chain keto acid dehydrogenase (BCAD) and Isovaleryl-CoA dehydrogenase (IVD) expression in 21-day-old WT and OX plants that were exposed to dehydration. Ubiquitin1 expression was used as internal control. Data are shown as means ± SD of three replicates.
(A) 

\[(\text{L-Valine}) \xrightarrow{} \text{L-Leucine} \xrightarrow{} (\text{L-Isoleucine})\]  

\[\text{Branched-Chain Aminotransferase}\]  

\[\text{Branched-chain 2-oxo acids}\]  

\[\text{Acyl-CoA}\]  

\[\text{Respiratory chain}\]  

\[\text{FAD} \xrightarrow{} \text{FADH}_2\]  

\[\text{Enoyl-CoA}\]  

\[\text{Methylcrotonyl-CoA Carboxylase}\]  

\[\text{Methylglutaconyl-CoA}\]  

\[\text{Enoyl-CoA Dehydratase}\]  

\[\text{Hydroxyacyl-CoA}\]  

\[\text{TCA cycle}\]

(B) 

\begin{align*}
\text{BCKD} & \quad \square \, \text{WT} \quad \square \, \text{OX} \\
0 & \quad 4 & \quad 6 & \quad 10 \\
\end{align*}

\begin{align*}
\text{IVD} & \quad \square \, \text{WT} \quad \square \, \text{OX} \\
0 & \quad 4 & \quad 6 & \quad 10 \\
\end{align*}
Figure 12. Osmotic stress resistance induced by branched chain amino

(A) Osmotic stress resistance of mock and amino acid-treated plants. Roots were dipped in a solution of 10 mM L-amino acid for 12 and 24 hr before being transferred to 25 % PEG solution for osmotic stress treatments.

(B) Temporal expression patterns of stress-related genes. Roots were treated with water (mock) or 10 mM each amino acid for the indicated periods of time. Total RNA was extracted from the roots and leaves, and the expression of the genes of interest was analyzed by qRT-PCR. Ubiquitin1 (Ubi1) expression was used as an internal control. Data are shown as mean ± SD of three technical replicates.
Table 1. Primer information
<table>
<thead>
<tr>
<th>Oligo name</th>
<th>5’ to 3’ sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>qRT-PCR</td>
<td></td>
</tr>
<tr>
<td>OsAT-IV-qRT-F</td>
<td>GAG CTG GAT TCG CAC TTG GA</td>
</tr>
<tr>
<td>OsAT-IV-qRT-R</td>
<td>TCG AGC CAT TCC TGC ACT TG</td>
</tr>
<tr>
<td>Ubi1(Os06g06814010)</td>
<td></td>
</tr>
<tr>
<td>Ubi-F</td>
<td>ATG GAG CTG CTG CTG TCC TA</td>
</tr>
<tr>
<td>Ubi-R</td>
<td>TTC TTC CAT GCT GCT CTA CC</td>
</tr>
<tr>
<td>Dip1(Os12g0274700)</td>
<td></td>
</tr>
<tr>
<td>Dip1-F</td>
<td>GAG CTT GTC ACC GGC ATG GA</td>
</tr>
<tr>
<td>Dip2-R</td>
<td>AGC TGG AGC TGG AGC TGG AT</td>
</tr>
<tr>
<td>RbcS1(Os02g0669100)</td>
<td></td>
</tr>
<tr>
<td>RbcS1-F</td>
<td>GGC AGG TAC TGG ATG TG</td>
</tr>
<tr>
<td>RbcS1-R</td>
<td>TTG TCG AAG CCG ATG ATA CG</td>
</tr>
<tr>
<td>pHBT-OsAT-IV-GFP</td>
<td></td>
</tr>
<tr>
<td>pHBT-OsAT-IV-infusion-R</td>
<td>AAA GCG GCC GCA AAT GCT CGC TTAG ATG AAG AAC AAG</td>
</tr>
<tr>
<td>pHBT-OsAT-IV-infusion-F</td>
<td>TTG TCG AAG CCG ATG ATG</td>
</tr>
<tr>
<td>Promoter-isolation</td>
<td></td>
</tr>
<tr>
<td>Promoter-F</td>
<td>CAC CCA CCA GTG GAT GAC GCC G</td>
</tr>
<tr>
<td>Promoter-R</td>
<td>GAT TCT CAC CTC GGT GCC TG</td>
</tr>
<tr>
<td>CRISPR/Cas9 system</td>
<td></td>
</tr>
<tr>
<td>CRISPR/Cas9-F</td>
<td>GGA TCT CCG CCG CCG ACG CGG TTG TAG ATG AAA TAG C</td>
</tr>
<tr>
<td>CRISPR/Cas9-R</td>
<td>CCC CAG TCT AAT AAG CTA GAG</td>
</tr>
<tr>
<td>OsU3 pro(HindIII)-F</td>
<td>CCC AAG CTT AAG GAA TCT TTA AAC ATA CGA</td>
</tr>
<tr>
<td>gRNAter(XbaI)-R</td>
<td>TGG TCT AGA AAA ACA AAA AAG CAC CGA CTC GGT GC</td>
</tr>
<tr>
<td>IVD (Os05t0125500)</td>
<td></td>
</tr>
<tr>
<td>IVD-F</td>
<td>TTT GCT GCT GAA AGG GCA AC</td>
</tr>
<tr>
<td>IVD-R</td>
<td>ATC TCT CAG CAA TCG GCC AG</td>
</tr>
<tr>
<td>BCKD (Os07t0170100)</td>
<td></td>
</tr>
<tr>
<td>BCKD-F</td>
<td>TCCAATCACCGGAGGGTTTG</td>
</tr>
<tr>
<td>BCKD-R</td>
<td>GCGTCCAGGACTTGTCTTT</td>
</tr>
<tr>
<td>KARI (Os02g0151600)</td>
<td></td>
</tr>
<tr>
<td>KARI-F</td>
<td>CTC CGA TCC CTG CCA TAG TG</td>
</tr>
<tr>
<td>KARI-R</td>
<td>CGA AGA TTAG TCG CAG CTC TG</td>
</tr>
<tr>
<td>DHAD(Os08g0559600)</td>
<td></td>
</tr>
<tr>
<td>DHAD-F</td>
<td>TGC TGA CCG ATG GCA GAT TT</td>
</tr>
<tr>
<td>DHAD-R</td>
<td>GCG TCC ATT TCC TCC GTC TT</td>
</tr>
</tbody>
</table>
Discussion

Amino acid pools and concentration are influenced by various abiotic stresses in plants. Branched-chain amino acid aminotransferase (BCAT) proteins play crucial roles both in the synthesis and degradation of the branched-chain amino acids (BCAAs) leucine (Leu), isoleucine (Ile) and valine (Val). The metabolism of BCAAs have important roles in plant stress tolerance. For examples, the accumulation of BCAAs could act as compatible solutes (osmolytes) and at the same time function as alternative source of respiratory substrates for the TCA cycle during stress (Taylor, Heazlewood et al. 2004). In addition, BCAAs can act in defence mechanisms by serving as precursors of secondary metabolites involved in pathogen response. Also the level of expression was increased in response to drought stress (Rizhsky et al. 2004).

In this study, we showed that the regulation of OsAT-IV expression was regulated by the transcription factor OsNAC5. The transcription factors from different families such as transcription factors MYB, bHLH, bZIP, AP2/EREBP and NAC have been reported as improving abiotic stress tolerance in rice (Hu, Dai et al. 2006) (Nakashima, Takasaki et al. 2012) (Singh, Foley et al. 2002). OsNAC5 is a NAC transcription factor involved in abiotic stress responses and OsNAC5-overexpressing rice plants exhibit significantly enhanced drought, high-salinity and ABA stress tolerance (Jeong, Kim et al. 2013). Through the microarray analysis, OsAT-IV was up-regulated gene in the OsNAC5-overexpressing plants. In addition, we found that OsNAC5 was found to bind to the promoter region of OsAT-IV through
ChiP-qPCR analysis, explaining the overexpression of OsNAC5 resulted in the up-regulation of OsAT-IV expression. In addition, the expression of OsAT-IV was also responsive to ABA treatment, suggesting an ABA-dependent response pathway. It was reported that a branched-chain amino acid aminotransferase gene was induced by ABA gene (Huang and Jander 2017). Nambara et. al. (1998) reported that the ABA-deficient mutant has a lower accumulation of BCAAs than non-mutant under the same stress conditions. In this study, we addressed that the transcriptional regulation of OsAT-IV was brought about by ABA treatment. Also, the OsAT-IV expression was upregulated in leaves and roots soon after ABA treatment and the expression level was positively correlated with time. Also, we found the 13 ABA-responsive ABRE elements in the OsAT-IV promoter (Figure 6 A), suggesting that OsAT-IV was transcriptionally regulated by drought stress in an ABA-dependent manner.

In spite of the relatively large increase in BCAA accumulation in response to abiotic stress, not much researches were done on directing toward their role. OsAT-IV was involved in amino acid metabolism, and amino acid analysis showed that the concentrations of Val, Ile and Leu were altered in OsAT-IV –overexpressing plants both under normal and drought stress conditions. In addition, OsAT-IV – overexpressing plants caused a significant increase in BCAA concentration under normal conditions. The expression levels of Val, Ile and Leu were higher by 22.7, 35.6 and 29.6-fold in OsAT-IV overexpressing plants over WT, respectively in leaves. In roots expression levels of these amino acids were similar in both plants, however the contents of BCAAs in OX plants were considerably higher than WT plants under
air-dried condition through time. Under air-dried conditions, both transgenic and WT plants showed accumulation of BCAA in response to stresses (Figure 9). The transgenic plants, which already contained high BCAA under normal condition, continued to accumulate BCAA during stress treatment. These results suggest that the high BCAAs contents may provide tolerance by acting as compatible solutes or substrate source when degraded. Thus the increased BCAAs in transgenic plant can protect plant immediately and effectively when exposed to stress compared to WT. This was supported by the qRT-PCR analysis on the BCAA catabolic enzymes BCKD and IVD (Figure 11 A). Under normal conditions, the expression levels in transgenic plants are similar both in WT and OX plants whereas under air-dried condition, the expression levels were up-regulated through time and the expression levels in OX plants were significantly higher than WT (Figure 11 B). These results suggest that expression of catabolic enzymes in OX plants were induced compared to those of WT plants, which lead to the degradation of BCAA more in OX plant that function as alternative source of respiratory substrates for the TCA cycle during stress.

We hypothesized that the BCAAs could also act as compatible solutes since they were significantly accumulated under abiotic stress. To confirm this, we performed BCAA feeding on plants before exposing them to osmotic stress. The results showed that BCAA pre-treated plants were more resistant to osmotic shock, compared to mock-treated plants in general. The osmotic stress tolerant phenotype was profound in isoleucine and valine-treated plants followed by leucine-treated and BCAA-mix-treated plants. Among the three BCAAs, leucine is the most
hydrophobic which could have limited its uptake in plant roots. In general, exogenous branched chain amino acids induced osmotic stress resistance in rice. In summary, our results suggest that branched chain amino acids can act as compatible solutes which aids the plants in resisting abiotic stress. The overexpression of OsAT-IV showed important roles in the accumulation of BCAA levels which conferred drought tolerance in rice. Thus, OsAT-IV is a very valuable candidate for crop biotechnology in improving the growth of rice under drought-stressed conditions.
Recommendations for further study

BCAT activity through complementation assay

In order to demonstrate the function of OsAT-IV in vivo, complementation and kinetic parameters assay using *Escherichia coli* can be performed. The *E. coli* strain BW25113 with knockouts in ilvE (JW5606-1, encoding Isoleucine-valine aminotransferase) and tyrB (JW4014-2, encoding an aromatic amino acid aminotransferase) were ordered from the Keio Collection (Baba, Ara et al. 2006). Double knockouts will be produced through biparental mating and the resulting double knockout mutant will completely lack BCAT activity and does not grow on medium lacking all BCAAs.

Kinetic parameters assay or enzyme activity of OsAT-IV

In order to determine the kinetic properties of OsAT-IV, the recombinant protein was produced by subcloning into pENTR/SD/D-TOPO (Invitrogen) and into a pMAL-DC-myc-vector (provided by Prof. Jung). The tomato BCAT gene (*SIBCAT6*) from the study of Maloney et al. (2010) will be used as control. The expressed proteins will be expressed in *E. coli* cells and purified. Enzyme assays will be performed with each recombinant OsAT-IV and SIBCAT6 in both the forward (amino acid-forming) and reverse (amino acid-degrading) directions following the instructions in the article published by Maloney et al. (2010).
Evaluation of knockout lines

To determine whether a complete knockout of the OSAT-IV gene results in significant effect in rice, knock-out lines through CRISPR/Cas9 was produced which targets the 4th exon of OsAT-IV.
REFERENCES


5 4


engineering for enhanced drought tolerance in plants.” 


59
ABSTRACT IN KOREAN

분지아미노산 아미노기 전이 효소인
OsAT-IV 과발현 벼의 내건성 향상에 대한 연구

정혜인

서울대학교 국제농업기술대학원 국제농업기술학과

지도교수 김주곤

최근 빈번히 발생하는 기상이변은 곡물 생산성을 감소시키는 요인으로 작용하며, 이는 곡물 가격 상승으로 인한 물가인상 등의 다양한 사회적 문제를 야기한다. 따라서 작물이 스트레스에 대응하는 분자생물학적 메커니즘을 규명하는 것은 중요한 과제이다. 본 연구에서는 벼의 분지 아미노산 아미노기 전이 효소인 OsAT-IV를 통한 분지 아미노산의 증가가 가뭄 저항성을 향상시키는 것을 확인하였다.

분지 아미노산 아미노기 전이 효소는 분지 아미노산인 류신, 아이소류신, 발린의 대사작용에 관여한다. 식물은 가뭄 스트레스를 받으면 분지 아미노산의 축적이 증가한다. 이는 세포 내 샌투 조절에 관여하는 화합성 용질(compatible solute)로 작용되고, 또한 이 증가된 아미노산은 분
해됨에 따라 분해 산물이 TCA cycle에서의 에너지 생성을 위한 대체 기질(alternative energy source)로 이용되어 스트레스 저항성을 높일 수 있는 것으로 알려져 있다. 환경 스트레스에 대한 OsAT-IV의 반응을 알아보기 위해 qRT-PCR 수행하였으며, OsAT-IV의 발현이 가뭄 스트레스 및 ABA에 의해 유도된 것을 확인하였다. 아미노산 함량 분석결과, OsAT-IV 과발현 버가 비 형질전환 버에 비해서 각 분지 아미노산이 22~36배 높은 것을 확인했다. 가뭄 처리를 하였을 때 OsAT-IV 과발현 버에서는 100% 생존율을 보인 반면 비 형질전환버의 생존율은 40%로 현저히 낮았다.

이에 본 결과는 OsAT-IV이 벼의 분지 아미노산 생합성을 증가시켜 가뭄 스트레스에 내성을 부여한다는 것을 제안한다.

키워드: 분지아미노산 아미노기 전이효소, 분지 아미노산, 화합성 용질, 환경스트레스, 가뭄저항성, 형질전환