

# Identification of phytohormone changes and its related genes under abiotic stresses in transgenic rice

MOHAMMAD UBAIDILLAH<sup>1,2</sup>; MISWAR FAPERTA<sup>1,2</sup>; KYUNG-MIN KIM<sup>1</sup>

<sup>1</sup> School of Applied Biosciences, College of Agriculture & Life Science, Kyungpook National University, Daegu, 41566, Korea

<sup>2</sup> Department of Agronomy, Faculty of Agriculture, Jember University, Jl. Kalimantan 37, Jember, 68121, Indonesia

**Key words:** Abiotic stress, CaMsrB2, Drought, Rice, Salinity

**Abstract:** Abiotic stresses, such as drought and salinity, adversely affect plant growth and productivity. Comparison between non transgenic and transgenic rice harboring *CaMsrB2* gene, which induces tolerance to abiotic stress, is important to observe response of gene under abiotic stress. Phytohormone showed a tendency to increase under the drought stress or salinity stress in the transgenic plant. RT-PCR analysis showed that gene expression and phytohormone levels under abiotic stress, to be closely related. The *CaMsrB2* gene is related to the expression of JA and ABA hormones. Therefore, the level of expression of these genes and hormones was observed. The transcription levels of LOX2 and OsWRKY45 were substantially higher in the wild type rice in comparison to the transformants, which suggested that phytohormone are also required for the regulation of leaf and root. Comparison between control and transgenic rice overexpressing a *CaMsrB2* gene, resulted in different pattern of ABA, JA levels under different stress condition. In both drought and salinity stresses, the expression of OsWRKY45 gene was similar in both treatments with time. These results suggest that gene involved in the plant physiology response in mechanism to abiotic stress.

## Introduction

Abiotic stresses such as extreme temperatures, drought, salinity, chemical, toxicity, and oxidative stress are serious threats to agriculture and have a negative impact on the environment. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Boyer, 1982; Bray *et al.*, 2000). This environmental threat leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang *et al.*, 2001). Drought and soil salinity are among the most damaging abiotic stresses affecting agricultural yield in present times due to the unpredictable rainfall (Jianhua *et al.*, 2006). They are significant plant stressors with major impact on plant development and productivity, which cause serious agricultural yield losses (Flowers, 2004; Godfray *et al.*, 2010; Tester and Langridge, 2010). Plants exposed to abiotic stress may experience limited growth and development. Abiotic stress might cause a worldwide yield reduction of approximately 70% (Acquaah, 2007). The stresses of drought and salinity contribute to water deficit, ionic toxicity, imbalances in ion homeostasis, and the occurrence of

oxidative stress (Foolad, 2007). Plants exhibit some adaptive responses to abiotic stresses at the molecular and cellular level, mainly because of changes in the expression patterns of a group of genes (Kumar, 2013). Therefore, to understand the stress response mechanism in plants, it is important to identify the genes that respond to drought and salinity. *CaMsrB2*, a pepper (*Capsicum annuum*) gene, which encodes a methionine sulfoxide reductase B2, has been implicated in the reactive oxygen species (ROS)-generating response in plants against pathogens (Oh *et al.*, 2010). Kim *et al.* (2014) reported that transgenic rice varieties that overexpress *CaMsrB2* perform better against drought stress than their wild-type counterparts. They reported that transgenic plants show an effect on reactive oxygen scavenging that results from drought stress (Xie *et al.*, 2011). Differential responses to general abiotic stress and stress tolerance can involve different phytohormone levels, sets of genes, and gene products. One strategy that can be employed is the investigation of the genetic and hormonal changes involved in complex biological pathways and processes (Vleeschauwer *et al.*, 2013). The responses to environmental stresses are integrated by signaling networks that mediate the sensors and receptor signals leading to specific mechanisms of responses to complex stress conditions and thus minimize damage. In the present study, we observed the correlation of *CaMsrB2* expression to the tolerance of stress through the evaluation

\*Address Correspondence to: Kyung-Min Kim,  
kkm@knu.ac.kr

of phytohormone levels under the drought stress or salinity stress in rice plants. The experimental advantages associated with exploiting the designated pathways of the mechanism mediated by *CaMsrB2* gene when stress exposed of genes that functional with observation of phytohormone level.

## Materials and Methods

### Plant materials

*Oryza sativa* L. Japonica-type Ilmi (control), *CaMsrB2-8*, and *CaMsrB2-23* mutant strains of rice were used in this study. *CaMsrB2-8* and *CaMsrB2-23* are drought-tolerant transgenic lines of Ilmi. The transgenic rice seeds were supplied by the National Academy of Agricultural Science, Rural Development Administration of Korea (Oh *et al.*, 2013). The rice seeds were germinated by soaking in water for 3 days, transferred to soil, and cultivated in a greenhouse at Kyungpook National University, Daegu, Korea.

### Drought tolerance assay

The drought experiment was performed during the vegetative stages of growth (until the 45-day-old stage). The sterilized seeds from non-transgenic rice plants (wild type) and  $T_8$  lines of *CaMsrB2* transgenic rice (by PCR analysis, Supplementary Fig. 1) were planted with two germinated seeds per 5 cm<sup>3</sup> in a plastic tray with 15 holes and supplied with adequate water for growth. The growing plantlets were maintained in the greenhouse under natural light conditions (16-h light/8-h dark cycle) at an ambient temperature of 30 ± 2°C. Drought stress was imposed upon 5-week-old plants by decanting all water from the tray and stopping irrigation for 4 days. The survival rate was measured in 12 plants among 30 plants of each genotype.

### Salinity tolerance assay

To analyze the salinity tolerance, the plants were grown as described above. Salinity stress was induced by the irrigation of the plants with 200 mM sodium chloride (NaCl) solution for 7 and 14 days. Transgenic and wild-type plants were treated for salinity by decanting all the resting water into the plastic box and irrigating the solution twice after 1 day. The survival rate and chlorophyll content of the transgenic plants were observed and compared with those of the control plants, which served as an indication of salinity tolerance. The chlorophyll content in different parts of the leaves was analyzed in triplicate by using a chlorophyll meter, SPAD-502 (Minolta, Japan). The experiment was conducted in five replicates.

### Sample collection

The leaf and root samples were collected after drought treatment for 3 and 4 days after salt treatment for 0, 1, and 2 weeks. The samples were used immediately after collection or frozen in liquid nitrogen and stored at -70°C for later use in gene expression and hormone analysis.

### Extraction of total RNA from leaf and roots tissues

Total RNA was isolated from leaf and root tissues by using RNeasy Plant Mini Kit (QIAGEN, Germany) in

accordance with the manufacturer's protocol. cDNA was synthesized from total RNA using a qPCRBIO cDNA synthesis kit (PCR Biosystem, USA) in accordance with the manufacturer's protocol.

### Reverse transcriptase PCR analysis

The expression of the transgene *CaMsrB2* was analyzed with reverse transcriptase (RT)-PCR by using the SuperScript III One-step RT-PCR system with Platinum Taq DNA Polymerase (Invitrogen, USA). RT-PCR was performed by using 2× reaction mixture (25 µL), forward and reverse primer (1 µL at 20 pmol/µL), SuperScript III RT/Platinum TaqMix (25 µL), and total RNA (500 ng). Each gene investigated was amplified from three independent biological samples using specific primers (Supplementary Tab. 1). The cycling conditions for the RT-PCR were: 94°C for 2 min, 94°C for 15 s, 60°C for 30 s, and 72°C for 1 min. Forty cycles of the amplification reaction were performed with a final extension at 72°C for 10 min. The 210 bp product was amplified from *CaMsrB2* by using the gene-specific primers 5'-GTCAGGAGTGCATTAATAGC-3' and 5'-CCTGAGCAGTTCCGTATTCT-3' for the analysis of confirmed transgenic plants (data not shown).

### Quantitative real-time PCR

The quantitative (q) RT-PCR analysis was conducted by using real-time PCR Pre-mix qPCRBIO SyGreen Mix Lo-ROX (PCR BIOSYSTEMS, UK) in accordance with the manufacturer's protocol. cDNA concentration was checked by NanoDrop 2000 (Thermo Scientific, USA). After amplification, the experiment was converted to comparative quantification (calibrator) experiment type and the data were analyzed with the Eco software (Illumina, USA). The rice actin gene *Os11g0247300* was used as an endogenous control.

### Accession numbers

Sequence data from this article can be found in the GenBank/EMBL databases under accession numbers AY026332 (JAMyb), AY838897 (NCED1), AK241395 (LOX2), AK068993 (PAL1), AK120715 (OsNPR1), DQ298181 (OsWRKY45), and AK060893 (OsACTIN).

### Quantitative analysis of major phytohormones

The quantitative analysis of the major phytohormones abscisic acid (ABA), jasmonic acid (JA), and GA<sub>3</sub> was performed as described by Ubaidillah *et al.* (2016).

### Statistical analysis

We sowed seeds in three replicates of 30 each for each experiment and then used 12 plants to test the two abiotic stresses and phytohormone changes in the plants. The results were expressed as means ± standard deviations (SD) of at least three independent experiments performed in duplicate.  $P < 0.05$  was considered statistically significant and was determined using SPSS software (version 14.0, SPSS Science, Chicago, IL, USA). Statistical differences were determined by one-way analysis of variance (ANOVA) and Tukey's post-test.

## Results

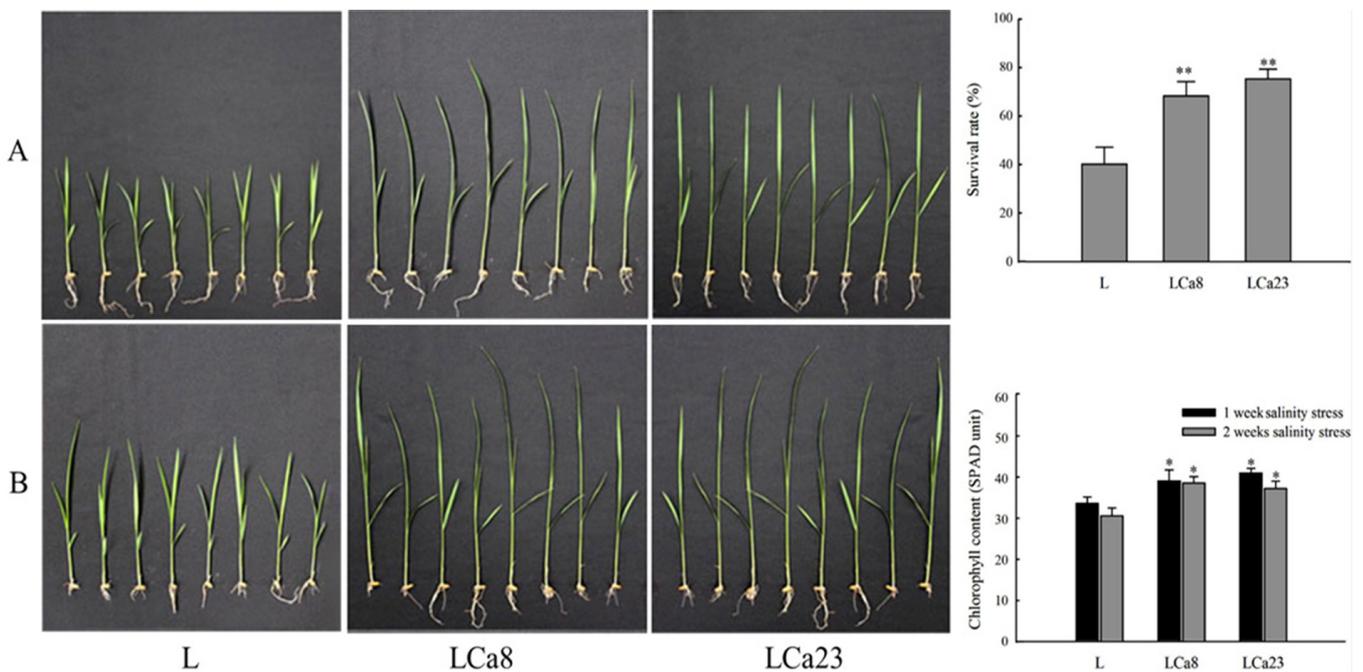
### Plant response during drought and salinity stress

We evaluated the performance of *CaMsrB2*-overexpressing transgenic rice under drought stress and observed it to be better than the control plants. Both lines of transgenic rice were observed to tolerate drought and salinity better than the control plants. The survival rates of all rice lines under both conditions are shown in Fig. 1(A); the survival rates of the *CaMsrB2*-8 and *CaMsrB2*-23 lines were almost 40% higher than those of the control. Under the salinity stress, the chlorophyll content was decreased in all plants by approximately 5 units after 1-2 weeks of treatment (Fig. 1(B)). The two transgenic lines that overexpress *CaMsrB2* demonstrated similar responses, which suggested similarities in the stress tolerance mechanisms of these plants.

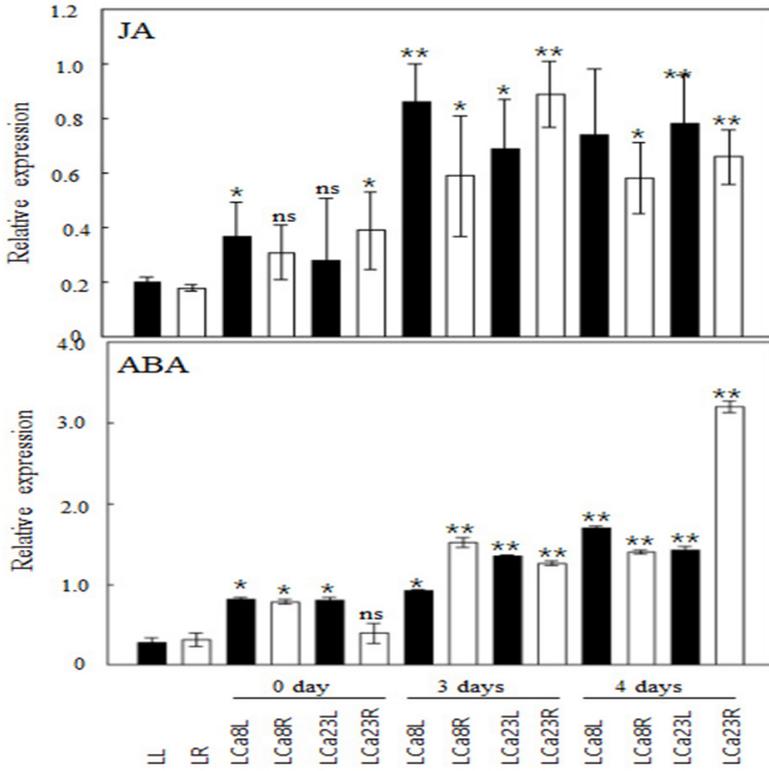
### Gene expression analysis using RT-PCR and qRT-PCR

In the transgenic rice, the *CaMsrB2* transcript level was high during the drought stress period; expression in *CaMsrB2*-8 and *CaMsrB2*-23 was strongly detected in both of the root and leaf. The length of the drought stress period did not affect the transcript level. The transcript levels were high in conditions of salinity stress. The *CaMsrB2* transcript in transgenic rice was highly abundant from the beginning and throughout the prolonged salinity stress. The transcripts were strongly detected in both root and leaf in both *CaMsrB2*-8 and *CaMsrB2*-23 lines. The length of the salinity stress treatment did not affect the transcript levels (Supplementary Fig. 2). *Jsmlyb* (JA) and *NCED1* (ABA) were confirmed by

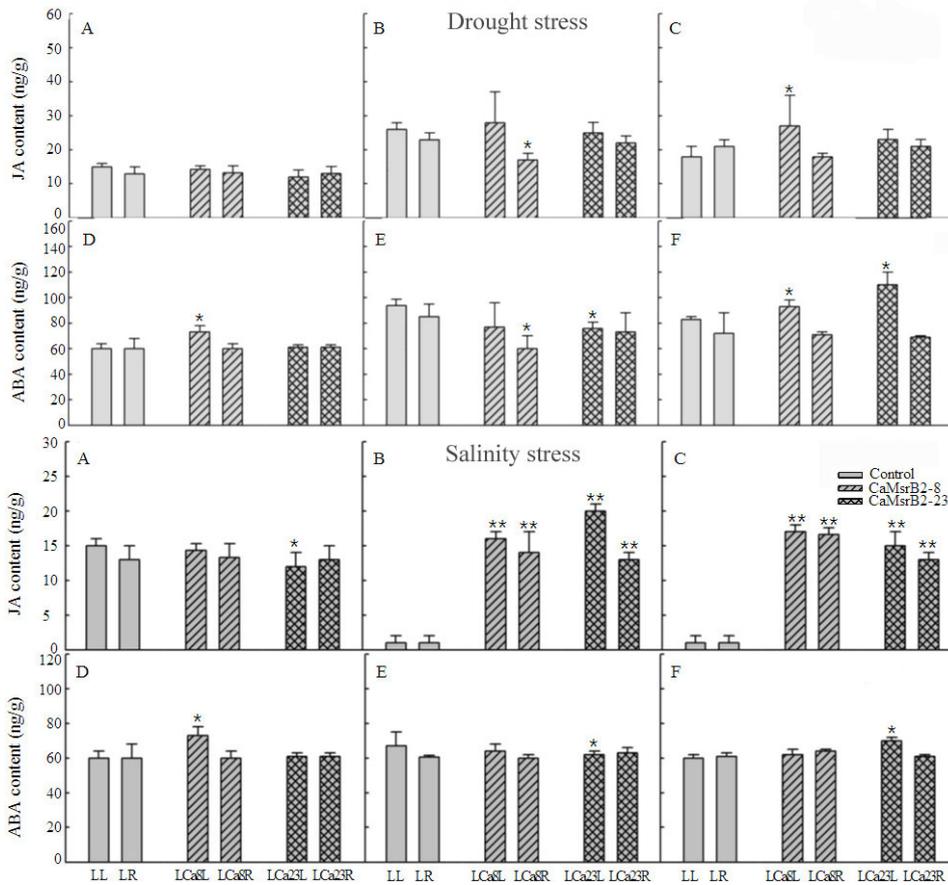
RT-PCR. Expression patterns of the *Jsmlyb* gene related to jasmonic acid were not expressed on the 0th day of the drying treatment and were expressed the same from the 3rd day to the 4th day. The expression of *NCED1* gene associated with ABA was very small on days 0 to 3 and strong on day 4 (Supplementary Fig. 2). The transcript levels in *CaMsrB2*-8 and *CaMsrB2*-23 had similar profiles, but with varying timing during drought and salinity stress conditions (Fig. 2). The levels of gene expression in the transgenic species were evaluated by qRT-PCR. We found that *CaMsrB2* was highly expressed with similar patterns under the drought stress or salinity stress (Fig. 2). In transgenic rice overexpressing *CaMsrB2*, the *CaMsrB2* transcript level was strongly detected during the drought stress period. The transcripts in *CaMsrB2*-8 and *CaMsrB2*-23 were high in all parts of the root and leaf. The length of the drought stress period did not affect the transcript level. The transcript level profiles in *CaMsrB2*-8 and *CaMsrB2*-23 were similar, although they occurred at different times during 0, 3, and 4 days of drought condition. *CaMsrB2* transgenic rice showed high abundance from the beginning throughout the prolonged salinity stress treatment. The transcripts were strongly detected in both the root and leaf in the *CaMsrB2*-8 and *CaMsrB2*-23 lines. The length of stress treatment did not affect the transcript levels. The transcript levels in the *CaMsrB2*-8 and *CaMsrB2*-23 plants had similar profiles, although at different time points, of 0, 1, and 2 weeks of salinity stress. We determined the expression level of *CaMsrB2* when plants were exposed to stress conditions such as drought and salinity and results showed its high level of expression.



**FIGURE 1.** Phenotypes of transgenic plants and control rice after drought treatment and salinity stress condition. (A) Rice phenotype and survival rate under drought stress. (B) Rice phenotype and chlorophyll content under salinity stress. L: Control Ilmi, LCa8: *CaMsrB2*-8 transgenic rice, LCa23: *CaMsrB2*-23 transgenic rice. \*,\*\* Significant at 5% and 1% levels, respectively.



**FIGURE 2.** Quantitative Real Time PCR (qRT-PCR) for *CaMsrB2*. (A) qRT-PCR analysis of *Jsmyb* gene (Top, Jasmonic acid-related gene) and *NCED 1* gene (Bottom, ABA-related gene). M:  $\lambda$ /HindIII size marker, LL: control *Ilmi*-leaf, LR: control *Ilmi*-root, LCa8L: *CaMsrB2-8* transgenic rice-leaf, LCa8R: *CaMsrB2-8* transgenic rice-root, LCa23L: *CaMsrB2-23* transgenic rice-leaf, LCa23R: *CaMsrB2-23* transgenic rice-root. \*,\*\* Significant at 5 and 1% levels, respectively.



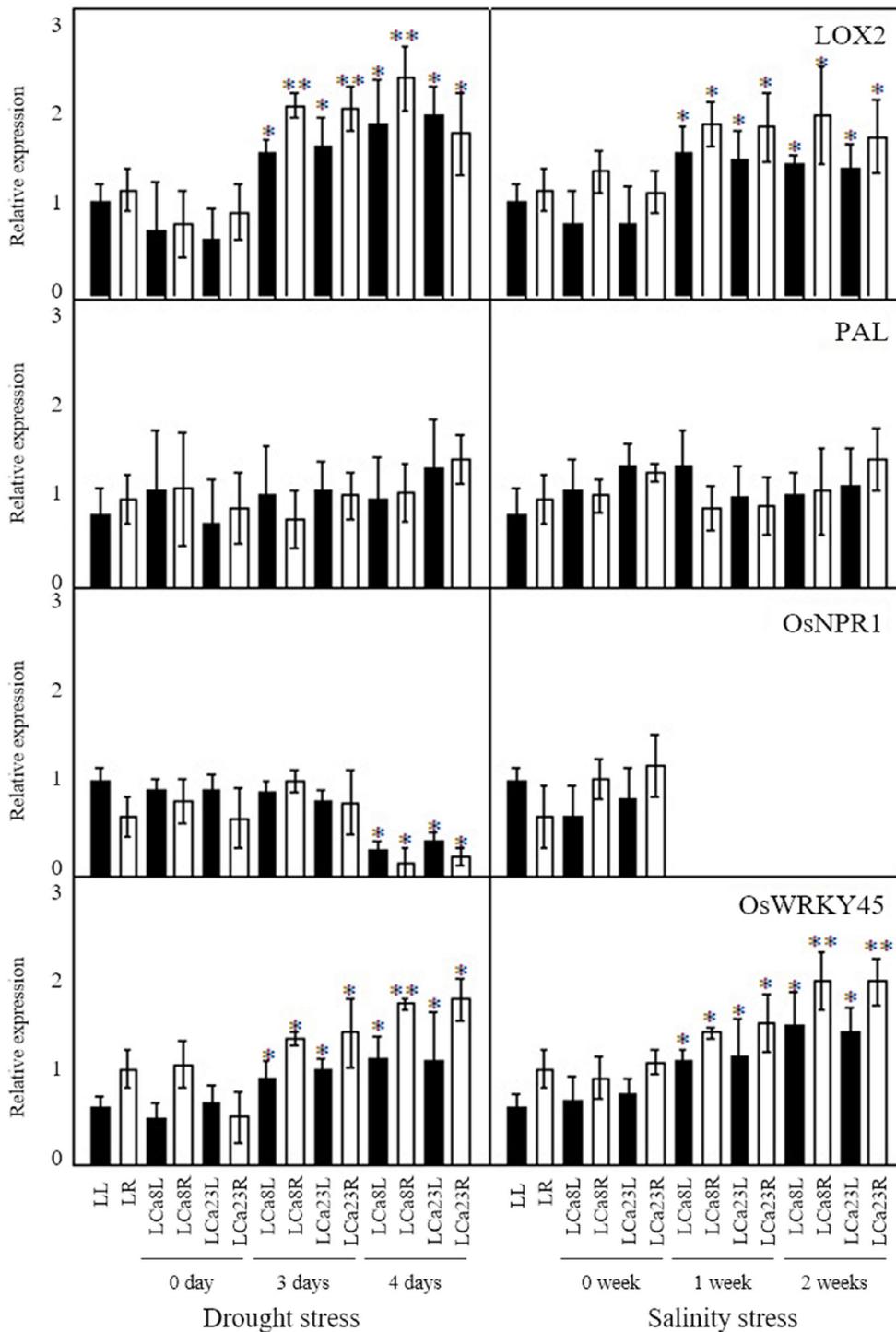
**FIGURE 3.** Jasmonic acid (JA) and abscisic acid (ABA) levels in transgenic and control rice under drought and salinity stress conditions. (A) JA level before starting drought treatment. (B) JA level after 3 days of drought treatment. (C) JA level after 4 days of drought treatment. (D) ABA level before starting drought treatment. (E) ABA level after 3 days of drought treatment. (F) ABA level after 4 days of drought treatment, LL: control leaf, LR: control root, LCa8L: *CaMsrB2-8* transgenic rice leaf, LCa8R: *CaMsrB2-8* transgenic rice root, LCa23L: *CaMsrB2-23* transgenic rice leaf, LCa23R: *CaMsrB2-23* transgenic rice root. \*,\*\*Significant at 5% and 1% levels, respectively.

**Alteration of hormone levels under drought and salinity stress**  
 To investigate the early effects of drought stress on hormone levels, the hormones abscisic acid (ABA) and jasmonic acid (JA) were examined in leaf and root tissues after 0, 3, and 4 days of drought stress (Fig. 3). The ABA levels in control rice increased after 3 days of drought stress but decreased after 4

days of drought stress. In contrast, the ABA levels in *CaMsrB2-8* and *CaMsrB2-23* leaf tissue did not change before 3 days but increased after an additional day of stress. The ABA levels in the root tissue of *CaMsrB2-8* and *CaMsrB2-23* showed no significant changes after 4 days of drought stress compared with the control.

The level of JA in the control rice increased after 3 days of drought in both leaf and root tissues, but it decreased after an additional day of drought stress treatment. JA levels in the transgenic rice *CaMsrB2* were evaluated. In the leaf tissues of *CaMsrB2-8* and *CaMsrB2-23*, the JA level increased after 3 days of drought stress, but it decreased slightly after the fourth day in both leaf and root tissues. The JA levels in control plant and transgenic *CaMsrB2* rice were the same after 3 and 4 days of drought stress. However, the JA levels in leaf tissue were slightly higher in transgenic plants than in control plants after 4 days of treatment.

The evaluation  $GA_3$  showed that under drought stress, the accumulation of this hormone in plant tissues was low. The levels were observed in both transgenic and control plants. Under normal conditions,  $GA_3$  accumulates at low levels in leaf and root tissues, but no  $GA_3$  was detected during drought stress (data not shown).



**FIGURE 4.** Signal intensities of genes relative to ACTIN in the wild type and the *CaMsrB2* transformants. The mean  $\pm$  standard error obtained from three individual experiments are shown. M:  $\lambda$ /HindIII marker, LL: Control Ilmi-leaf, LR: Control Ilmi-root, LCa8L: *CaMsrB2-8* transgenic rice-leaf, LCa8R: *CaMsrB2-8* transgenic rice-root, LCa23L: *CaMsrB2-23* transgenic rice-leaf, LCa23R: *CaMsrB2-23* transgenic rice-root. LOX2: *lox2osPil* mRNA for lipoxygenase (AK241395), PAL: phenylalanine ammonia-lyase (AK068993), OsNPR1: Ankyrin-repeat protein (NPR1) mRNA (AK120715), OsWRKY: WRKY transcription factor 45 mRNA (DQ298181). \*,\*\* Significant at 5% and 1% levels, respectively.

*Phytohormone signaling regulates the expression of defense-related genes to JA signaling pathways in affecting age-related transcript levels of four genes*

We selected LOX2, PAL, OsNPR1, and OsWRKY45 genes, which are most closely related to abiotic stress in rice plants. Among these genes, OsWRKY45 showed that the transformant was treated with two stresses clearly expressed in the leaves and roots (Fig. 4, Supplementary Fig. 3). In untreated plants, we observed that the basal levels of LOX2 and OsWRKY45 were higher in control rice than in the *CaMsrB2* mutant. To assess the molecular mechanisms by which phytohormone affect OsWRKY45 gene expression in rice, we compared the expression patterns of four genes known to function in JA-dependent defense signaling pathways in old and new leaves (roots) of the control rice and the *CaMsrB2* mutant. PAL was expressed with lower levels in roots and leaves. LOX2 and OsWRKY45 were expressed at higher levels in the wild-type plants than in the *CaMsrB2* mutant. sNPR1 was expressed at slightly lower levels in the wild-type plants than in the *CaMsrB2* mutant at 4 days and 1-2 weeks, respectively.

### Discussion

To evaluate their responses to various abiotic stresses, the *CaMsrB2* transgenic plants were subjected to the drought stress or salinity stress. The survival rates of transgenic plants after drought stress treatment were higher than those of the control rice plants (Dhungana *et al.*, 2015). Kim *et al.* (2014) reported that several tests after drought and re-watering indicated that the transgenic lines showed fewer oxidative stress symptoms and a strengthened PSII quantum yield under stress conditions, along with an increased survival rate and chlorophyll index. Here, a salt tolerance analysis of *CaMsrB2* transgenic rice was performed through the estimation of the level of chlorophyll breakdown. The estimation of the transcript levels in transgenic plants demonstrated that the transcript levels were high and stable throughout prolonged drought stress. The level of gene expression was observed during the salinity stress. In *CaMsrB2* transgenic rice, the transcript level was high throughout prolonged drought stress. These transcript levels provide evidence of the role of this gene in the induction of tolerance toward the given stresses.

In this study, the changes in hormone levels in *CaMsrB2* transgenic rice, as well as non-transformed control rice, were examined in the drought stress and salinity stress in leaf and root tissues. The changes in hormone levels in rice represent distinct plant responses to abiotic stress that result from different defense mechanisms. *CaMsrB2* has been reported as a novel defense regulator against oxidative stress and pathogen attacks (Acquaah *et al.*, 2007). Plants have several physiological and biochemical mechanisms to survive in soils with a deficiency of water and an excess of salt. Under the drought stress and salinity stress, the first organ to experience the stress is the root system. The roots send various signals to the leaves through the xylem sap; phytohormone are considered to be one of the major root-to-shoot stress signals. In addition, drought stress and salinity stress induce calcium signaling (Knight *et al.*, 1998; Laohavisit *et al.*,

2013), which is involved in the activation of the downstream response mechanisms of the involved genes. Steinhorst and Kudla (Steinhorst and Kudla, 2013) observed that  $Ca^{2+}$  and the reactive oxygen species (ROS) signaling pathway directly enable cell-to-cell communication and thereby long-distance transmission of signals in plants. Under drought stress, ROS production is enhanced owing to stomatal closure and the concomitant limitation on  $CO_2$  fixation (Carvalho, 2008). Osmotic stress during salinity stress impairs the ability of a plant to detoxify ROS (Gupta and Huang, 2014). The synthesis of several phytohormone can be induced by ROS (Bogatek and Gniazdowska, 2007). Phytohormone participate prominently in the important regulatory roles in plant physiology that affect developmental processes during abiotic stresses (Chen *et al.*, 2002; Browse, 2009). Three hormones (ABA, JA, and  $GA_3$ ) that are related to the defense mechanisms during abiotic stress, especially against the drought stress and salinity, were investigated. The roles of ABA, JA, and SA as primary signals in the regulation of plant defenses is well established (Bari and Jones, 2009; Pieterse *et al.*, 2009). These hormones generate a signal transduction network that leads to a cascade of events responsible for the physiological adaptation of plants to stress.

ABA is known for its regulatory role of the integration of environmental adversity with the plant development (Christmann *et al.*, 2005). ABA positively contributes to the adaptations of osmotic stress, a major component of several abiotic stresses (Kissoudis *et al.*, 2014). Its involvement in the regulation of the defense responses has been a topic of recent comprehensive reviews (Asselbergh *et al.*, 2008; Ton *et al.*, 209). The synthesis, degradation, and transport processes dynamically maintain ABA levels in plant cells. Therefore, the plants maintain their developmental programs and stress responses through the modulation of endogenous ABA levels (Schwartz *et al.*, 2003). It has been reported that ABA can stimulate the production of anti-apoptotic proteins and reduce the expression of a number of pro-apoptotic proteins (Scarfi *et al.*, 2009). There was more ABA accumulated in the leaves than the roots of plants in the present study, because the roots are not able to combat stress-induced ABA accumulation. JA is a well-known signaling molecule in plant defense and stress response (Hoeberichts and Woltering, 2003). The participation of JA in response to abiotic stresses, such as drought and salinity, has been reported in several species. Water stress increases endogenous JA content, which is followed by the synthesis of jasmonate-induced proteins (Lehmann *et al.*, 1995). It has been reported that tomato cultivars differ in salt tolerance owing to different basal JA content. Steady-state amounts of JA and related compounds are higher in plants with salt and water stress tolerance (Pedranzani *et al.*, 2003). It has been suggested that JA-related responses are directly associated with a downstream reset of gene expression in the biosynthesis pathway (Thines *et al.*, 2007). Collectively, these studies indicated that JA was an important component of a pathway that positively improves plant defense in *CaMsrB2* transgenic rice. However, the precise mechanisms by which JA signaling improves this defense remain unknown. Plants have developed complex mechanisms to perceive external signals under drought and salinity stresses and allow for an optimal response to

the environment. In this study, the accumulation of JA in transgenic lines was higher than that in the non-transgenic plants under both stresses. Based on RT-PCR data, it appeared that the transgene is expressed even before the onset of stress. If it is involved in the regulation of hormonal metabolism, then the transgenic plants should have elevated levels of hormones when compared to non-transgenic plants before onset of stress. But this is not reflected in the data. However, significant increases in JA were present under the salinity stress. JA and ABA might be involved in different stages of the response, which drive an acclimation process during growth through extensive genetic reprogramming to reach a new homeostasis (Harb *et al.*, 2010). In *Arabidopsis*, both JA and ABA act in response to moderate drought (30% of field capacity) (Aimar *et al.*, 2011). The overexpression of *CaMsrb2* can improve the endogenous levels of ABA and JA during the early stages of moderate drought. We have suggested that *CaMsrb2* is involved in an increase in the endogenous ABA and JA levels and can sufficiently stimulate the preparation of the response needed for drought acclimation.

Rice PAL transcripts have a higher level of accumulation in stems, and observed in lower levels in roots and leaves (Zhu *et al.*, 1995). NH1 (*Arabidopsis* NPR1 homolog 1) has been reported to play an important role in the SA signaling pathway downstream of SA (Chern *et al.*, 2005) and to regulate the expression of PR1a and PR1b genes in rice (Shimono *et al.*, 2007). Xie *et al.* (2011) suggested that these genes are upregulated by phytochrome-mediated signals, which may account for the elevated basal levels of PR1a and PR1b in wild-type plant compared with the phyAphyBphyC mutant. These same genes were developmentally upregulated, as indicated by the higher level of transcripts in the old leaves than in the new leaves. We have proposed a defense model for rice that does not support a dichotomy between the pathogen lifestyle and the effectiveness of the archetypal defense hormones salicylic acid (SA) and jasmonic acid (JA) (Vleesschauwer *et al.*, 2013).

The determination of the changes in the levels of endogenous hormones in transgenic rice subjected to the drought stress and salinity stress will allow a better understanding of the salinity response and assist in the formulation of improved strategies for the generation of salinity-tolerant crops.

## Conclusions

This study examined the relationship between two types of abiotic stress and four genes involved in salinity and drought stress. Especially, salinity and drought resistance of the transgenic plant showed similar patterns of plant hormones. Among the four genes, OsWRKY45 was most related to abiotic stress. We demonstrated that the phytohormone in rice required for age-related resistance to abiotic stress may indirectly increase OsWRKY45 gene expression through the regulation of JA-dependent defense pathways.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of the paper.

## Financial Support

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Acknowledgments

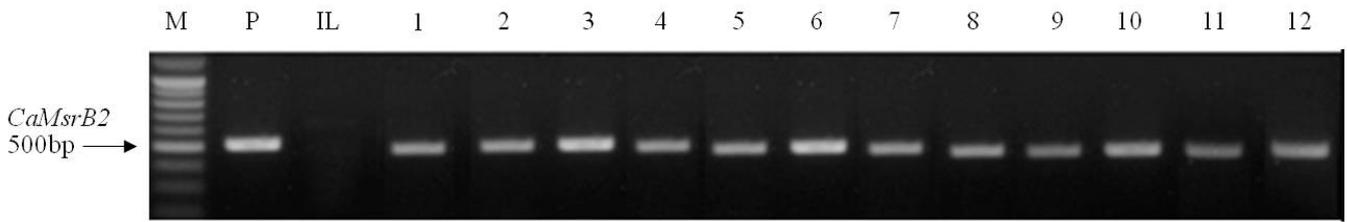
This research was supported by Kyungpook National University Bokhyeon Research Fund, 2017.

## References

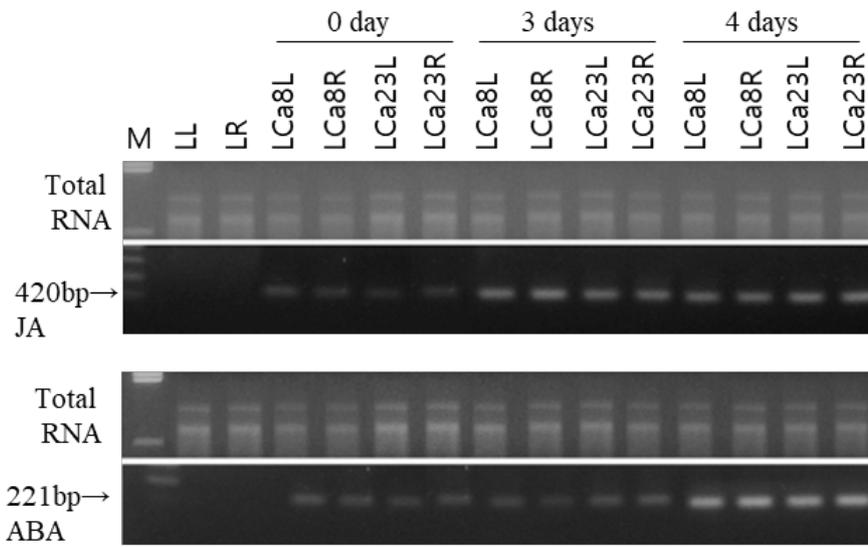
- Acquaah G (2007). Principles of Plant Genetics and Breeding. Blackwell, London.
- Aimar D, Calafat M, Andrade AM, Carassay L, Abdala GI, Molas ML (2011). Drought tolerance and stress hormones: From model organisms to forage crops. *Plants and Environment* **10**: 137-164.
- Asselbergh BD, de Vleesschauwer D, Höfte M (2008). Global switches and fine-tuning-ABA modulates plant pathogen defense. *Molecular Plant-Microbe Interactions* **21**: 709-719.
- Bari R, Jones JDG (2009). Role of plant hormones in plant defence responses. *Plant Molecular Biology* **69**: 473-488.
- Bogatek R, Gniazdowska A (2007). ROS and phytohormones in plant-plant allelopathic interaction. *Plant Signaling & Behavior* **2**: 317-318.
- Boyer JS (1982). Plant productivity and environment. *Science* **218**: 443-448.
- Bray EA, Bailey-Serres J, Weretilnyk E (2000). Responses to abiotic stresses. In: Biochemistry and molecular biology of plants. (W Gruissem, B Buchanan, R Jones, eds.), pp. 1158-1249. American Society of Plant Physiologists, Rockville, MD.
- Browse J (2009). Jasmonate passes muster: a receptor and targets for the defense hormone. *Annual Review of Plant Biology* **60**: 183-205.
- Carvalho MHC (2008). Drought stress and reactive oxygen species. *Plant Signaling & Behavior* **3**: 156-165.
- Chen W, Provar NJ, Glazebrook J, Katagiri F, Chang HS, Eulgem T, Mauch F, Luan S, Zou G, Whitham SA, Budworth PR, Tao Y, Xie Z, Chen X, Steve L, Kreps JA, Harper JE, Ammour SA, Mani BM, Heinlein M, Kobayashi K, Hohn T, Dangl JL, Wang X, Zhu T (2002). Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. *The Plant Cell* **14**: 559-574.
- Chern M, Fitzgerald HA, Canlas PE, Navarre DA, Ronald PC (2005). Overexpression of a rice NPR1 homolog leads to constitutive activation of defense response and hypersensitivity to light. *Molecular Plant-Microbe Interactions* **18**: 511-520.
- Christmann A, Hoffmann T, Teplova I, Grill E, Müller A (2005). Generation of active pools of abscisic acid revealed by in vivo imaging of water-stressed *Arabidopsis*. *Plant Physiology* **137**: 209-219.
- Dhungana SK, Kim BR, Son JH, Kim HR, Shin DH (2015). Comparative study of *CaMsrb2* gene containing drought-tolerant transgenic rice (*Oryza sativa* L.) and non-transgenic counterpart. *Journal of Agronomy and Crop Science* **201**: 10-16.
- Flowers TJ (2004). Improving crop salt tolerance. *Journal of Experimental Botany* **55**: 307-319.
- Foolad MR (2007). Current status of breeding tomatoes for salt

- and drought tolerance. In: *Advances in molecular breeding toward drought and salt tolerant crops* (MA Jenks, PM Hasegawa, SM Jain, eds.), pp. 660-700. Springer, New York.
- Godfray HC, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Pretty J, Robinson S, Thomas SM, Toulmin C (2010). Food security: the challenge of feeding 9 billion people. *Science* **327**: 812-818.
- Gupta B, Huang B (2014). Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. *International Journal of Genomics* **2014**: 701596.
- Harb A, Krishnan A, Ambavaram MM, Pereira A (2010). Molecular and physiological analysis of drought stress in *Arabidopsis* reveals early responses leading to acclimation in plant growth. *Plant Physiology* **154**: 1254-1271.
- Hoeberichts FA, Woltering EJ (2003). Multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators. *BioEssays* **25**: 47-57.
- Jianhua Z, WensuoJia, Jianchang Y, Abdelbagi, Ismail M (2006). Role of ABA in integrating plant responses to drought and salt stresses. *Field Crops Research* **97**: 111-119.
- Kim JS, Park HM, Chae S, Lee TH, Hwang DJ, Oh SD, Park JS, Song DG, Pan CH, Choi D, Kim YH, Nahm BH, Kim YK (2014). A pepper *MSRB2* gene confers drought tolerance in rice through the protection of chloroplast-targeted genes. *PLoS One* **9**: e90588.
- Kissoudis C, van de Wiel C, Visser RG, van der Linden G (2014). Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk. *Frontiers in Plant Science* **5**: 207.
- Knight H, Brandt S, Knight MR (1998). A history of stress alters drought calcium signaling pathways in *Arabidopsis*. *Plant Journal* **16**: 681-687.
- Kumar M (2013). Crop plants and abiotic stresses. *Journal of Biomolecular Research & Therapeutics* **3**: e125.
- Laohavisit A, Richards SL, Shabala L, Chen C, Colaço DDR, Swarbreck SM, Shaw E, Dark A, Shabala S, Shang Z, Davies JM (2013). Salinity-induced calcium signaling and root adaptation in *Arabidopsis* require the calcium regulatory protein annexin1. *Plant Physiology* **163**: 253-262.
- Lehmann J, Atzorn R, Brückner C, Reinbothe S, Leopold J, Wasternack C, Parthier B (1995). Accumulation of jasmonate, abscisic acid, specific transcripts and proteins in osmotically stressed barley leaf segments. *Planta* **197**: 156-162.
- Oh SK, Baek KH, Seong ES, Joung YH, Choi GJ, Park JM, Cho HS, Kim EA, Lee S, Choi D (2010). *CaMsrb2*, pepper methionine sulfoxide reductase B2, is a novel defense regulator against oxidative stress and pathogen attack. *Plant Physiology* **154**: 245-261.
- Pedranzani H, Racagni G, Alemanno S, Miersch O, Ramírez I, Peña-Cortés H, Taleisnik E, Machado-Domenech E, Abdala G (2003). Salt tolerant tomato plants show increased levels of jasmonic acid. *Plant Growth Regulation* **41**: 149-158.
- Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM (2009). Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology* **5**: 308-316.
- Oh SD, Lee K, Park SK, Park HM, Lee SM, Ahn HI, Sohn SI, Cho HS, Ryu TH (2013). Biosafety assessment for drought-tolerant transgenic rice (*CaMsrb2-8*): Progeny stability study for three generations. *Korean Journal of Breeding Science* **45**: 109-118.
- Scarfi S, Fresia C, Ferraris C, Bruzzone S, Fruscione F, Usai C, Benvenuto F, Magnone M, Podestà M, Sturla L, Guida L, Albanesi E, Damonte G, Salis A, De Flora A, Zocchi E (2009). The plant hormone abscisic acid stimulates the proliferation of human hemopoietic progenitors through the second messenger cyclic ADP-ribose. *Stem Cells* **10**: 2469-2477.
- Schwartz SH, Qin X, Zeevaart JA (2003). Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes. *Plant Physiology* **131**: 1591-1601.
- Shimono M, Sugano S, Nakayama A, Jiang CJ, Ono K, Toki S, Takatsuji H (2007). Rice *WRKY45* plays a crucial role in benzothiadiazole-inducible blast resistance. *Plant Cell* **19**: 2064-2076.
- Steinhorst L, Kudla J (2013). Calcium and reactive oxygen species rule the waves of signaling. *Plant Physiology* **163**: 471-485.
- Tamaoki M (2008). The role of phytohormone signaling in ozone-induced cell death in plants. *Plant Signaling & Behavior* **3**: 166-174.
- Tester M, Langridge P (2010). Breeding technologies to increase crop production in a changing world. *Science* **327**: 818-822.
- Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007). JAZ repressor proteins are targets of the SCF<sup>COI1</sup> complex during jasmonate signalling. *Nature* **448**: 661-665.
- Ton J, Flors V, Mauch-Mani B (2009). The multifaceted role of ABA in disease resistance. *Trends in Plant Science* **14**: 310-317.
- Ubaiddillah M, Safitri FA, Jo JH, Lee SK, Hussain A, Mun BG, Chung IK, Yun BW, Kim KM (2016). Roles of plant hormones and anti-apoptosis genes during drought stress in rice (*Oryza sativa* L.). *3 Biotech* **6**: 247.
- Vleesschauwer DD, Gheysen G, Höfte M (2013). Hormone defense networking in rice: tales from a different world. *Trends in Plant Science* **18**: 555-565.
- Wang WX, Vinocur B, Shoseyov O, Altman A (2001). Biotechnology of plant osmotic stress tolerance: Physiological and molecular considerations. *Acta Horticulturae* **560**: 285-292.
- Xie XZ, Xue YJ, Zhou JJ, Zhang B, Chang H, Takano M (2011). Phytochromes regulate SA and JA signaling pathways in rice and are required for developmentally controlled resistance to *Magnaporthe grisea*. *Molecular Plant* **4**: 688-696.
- Zhu Q, Dabi T, Beeche A, Yamamoto R, Lawton MA, Lamb C (1995). Cloning and properties of a rice gene encoding phenylalanine ammonia-lyase. *Plant Molecular Biology* **29**: 535-550.

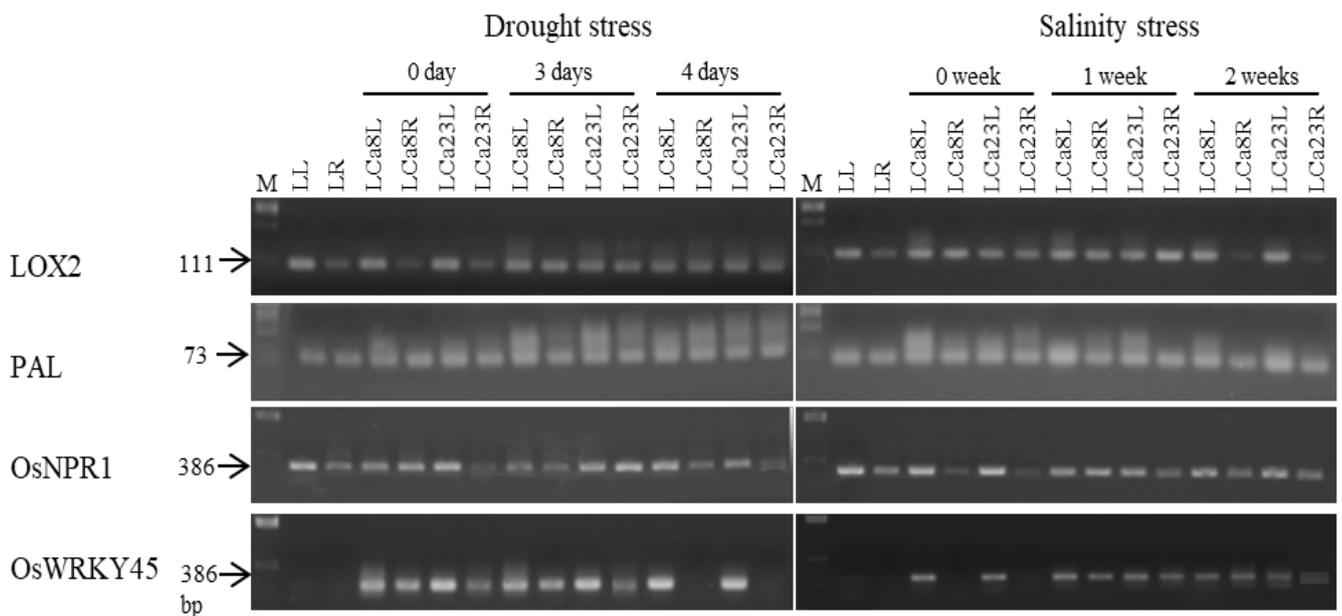
Supplementary File



**SUPPLEMENTARY FIGURE S1.** Amplification of PCR result to confirm *CaMsrB2* insertion in transgenic plants *CaMsrB2-8* and *CaMsrB2-23*. DNA was isolated and PCR reactions were performed using *CaMsrB2* primers. Lane M: Lambda DNA/*Hind* III marker, Lane P: Plasmid DNA, Lane IL: Ilmi, Lane 1-6: *CaMsrB2-8* lines, Lane 7-12: *CaMsrB2-23* lines.



**SUPPLEMENTARY FIGURE S2.** Reverse Transcriptase-PCR (RT-PCR) for *CaMsrB2*. RT-PCR assay for determining relative mRNA expression levels of *Jmyb* gene (Top, Jasmonic acid-related gene) and *NCED 1* gene (Bottom, ABA related gene). M:  $\lambda$ /*Hind*III size marker, LL: control Ilmi-leaf, LR: control Ilmi-root, LCa8L: *CaMsrB2-8* transgenic rice-leaf, LCa8R: *CaMsrB2-8* transgenic rice-root, LCa23L: *CaMsrB2-23* transgenic rice-leaf, LCa23R: *CaMsrB2-23* transgenic rice-root. \*,\*\* Significant at 5 and 1% levels, respectively.



**SUPPLEMENTARY FIGURE S3.** Transcript levels of genes in response to stresses of drought and salinity stress as analyzed by RT-PCR in the wild type and the *CaMsrB2* transformed mutants. M:  $\lambda$ /*Hind*III marker, LL: Control Ilmi-leaf, LR: Control Ilmi-root, LCa8L: *CaMsrB2-8* transgenic rice-leaf, LCa8R: *CaMsrB2-8* transgenic rice-root, LCa23L: *CaMsrB2-23* transgenic rice-leaf, LCa23R: *CaMsrB2-23* transgenic rice-root. LOX2: *lox2osPil* mRNA for lipoxygenase (AK241395), PAL: phenylalanine ammonia-lyase (AK068993), OsNPR1: Ankyrin-repeat protein (NPR1) mRNA (AK120715), OsWRKY45: WRKY transcription factor 45 mRNA (DQ298181).

## SUPPLEMENTARY TABLE 1

## Primer pairs used for RT-PCR and qRT-PCR

Gene name	Forward primer (5' -3' )	Reverse primer (5' -3' )
JAMyb	AGGACCTCACCCATCAATTA	CTTCTCATCATCACCATGCAC
NCED1	CTCACCATGAAGTCCATGAGGCTT	GTTCTCGTAGTCTTGGTCTTGGCT
LOX2	AGATGAGGCGCGTGATGAC	CATGGAAGTCGAGCATGAAC
PAL	GGTGTCTGCGAGGTGAT	AGGGTGGTGCTTCAGCTT
OsNPR1	CTGATCCGGTTTCCCTCGGA	GACCTGTCATTCTCCTCCTTG
OsWRKY45	GGACCAGGGCGATGTCACGT	TGTCCATCCATGATTCTTCGGTGA
OsACTIN	TCCATCTTGGCATCTCTCAG	GTACCCGCATCAGGCATCTG