# *Histone Deacetylase 701* enhances abiotic stress resistance in rice at the seedling stage by suppressing expression of *OsWRKY45.*

**Antt Htet Wai[1,2] and Gynheung An***

## ABSTRACT

Being sessile organisms, plants need to adapt to unfavorable environmental stresses to modulate their optimal growth and development. When plants are exposed to abiotic stresses, a large number of genes are triggered and synchronized to optimize their growth under diverse abiotic stresses. Expression of *Histone Deacetylase 701* (*HDT701*) is regulated by abiotic stress conditions and HDT701 overexpressing transgenic rice shows higher tolerance to osmotic and salt stresses at the seedling stage as previously reported. *hdt701* mutant seedlings displayed increased sensitivity to both salt and osmotic stresses. Expression levels of *Oryza sativa Phytoene Synthase 3* (*OsPY3*) and *9-cis-epoxycarotenoid dioxygenase 4* (*NCED4*), ABA biosynthesis genes induced by salt stress, and *STRESS-RESPONSIVE NAC 1* (*SNAC1*), an abiotic stress inducible gene, were significantly decreased in the mutants, revealing that *HDT701* functions upstream of them in regulating abiotic stresses. The expression of *Oryza sativa respiratory burst oxidase homolog I* (*OsrbohI*), an NADPH oxygenase gene that is responsible for the production of reactive oxygen species (ROS), was also remarkably suppressed in the mutant seedlings while that of *OsWRKY45*, an upstream suppressor of *SNAC1* and *NCED4*, was dramatically induced. These resulting data suggest that *HDT701* might enhance the salt and osmotic stress tolerance of rice by suppressing *OsWRKY45* as well as through ROS pathway by enhancing *OsrbohI.*

Key words: rice, abiotic stress, salt and osmotic stress tolerance, *HDT701*, *OsWRKY45*

[1]Dr, Assistant Lecturer, Department of Botany, University of Mandalay, Ministry of Education, Myanmar.
[2]PhD degree holder from Graduate School of Biotechnology and Crop Biotech Institute, Kyung Hee University, Yongin 17104, Korea.
[*]Corresponding author, genean@khu.ac.kr, Professor, Graduate School of Biotechnology and Crop Biotech Institute, Kyung Hee University, Yongin 17104, Korea.

## INTRODUCTION

As a consequence of a sessile lifestyle, plants are subjected to various abiotic stresses, which contribute to tremendous detrimental impact on crop production worldwide. Among abiotic stresses encountered by crop plants during their growing seasons, drought and soil salinity are one of the most ferocious environmental factors that limit the productivity of crop plants worldwide (Munns and Tester, 2008). Over 80 million hectares of irrigated land throughout the world, which represents 40% of total irrigated land, have already been ruined by salt (Xiong and Zhu, 2001). Cultivated areas under high salinity are increasing all over the world owing to various factors such as climate change, rise in sea levels, excessive irrigation without appropriate drainage system in inlands and underlying rocks rich in deleterious salts and so on (Wang et al., 2003).

High salinity and drought pose serious brutal effects on the survival rate, biomass production and yield of staple food crops (Thakur et al., 2010; Mantri et al., 2012). Salt stress stimulates not only hyperionic but also hyperosmotic stress in plants, inhibiting the overall metabolic activities of plants. Thus, plants attempt for the well adaptation of environmental changes to tolerate unfavorable abiotic stress conditions by synchronizing a large number of abiotic stress-related genes and by modulating various physiological and biochemical changes (Kumar et al., 2013).

Abscisic acid (ABA) is a stress inducible hormone that is famous for its stress-related properties in addition to its many roles in other biological process of plants (Zeevaart and Creelman, 1988). It is also an important signaling molecule that plays a vital role in acclimation to environmental stress processes of plants, (Santner et al., 2009; Cutler et al., 2010). In rice, ABA accumulation during abiotic stress conditions is well correlated with the higher resistance to abiotic stresses (Kao 2014).In many other plant species as well, ABA improves tolerance to abiotic stresses such as drought (Ashraf 2010; Hussain et al., 2013), salt (LaRosa et al., 1987), freezing (Guy 1990), chilling (Lee et al., 1993), etc. by functioning as an endogenous inducer to endure abiotic stresses in plants (Hadiarto and Tran, 2011). Higher level of endogenous ABA is also detected in the abiotic stress tolerant rice cultivar compared to the sensitive one (Jeong et al., 1980). Moreover, the exogenous application of ABA enhances tolerance to salinity in rice (Bohra et al., 1995). ABA also regulates stomatal closure to maintain water balance during the abiotic stress responses of plants (Zeevaart and Creelman 1988 ; Lee et al., 1993).In addition, many genes are modulated by the endogenous

ABA to promote the adaptive response of rice to abiotic stress conditions (Kumar et al., 2013).

Reactive oxygen species (ROS) are versatile signaling molecules in plants. They also play a significant role in abiotic stress acclimation as second messengers in ABA signaling in guard cells (Kwak et al., 2003; Jiang et al., 2012; Kumar et al., 2013). In plants, adaptive responses to unfavorable abiotic stresses are also mediated through ROS signaling (Jasper et al., 2010). In Arabidopsis plants exposed to abiotic stress conditions, ABA is accumulated to induce the expression of NADPH oxygenase genes that function in guard cells and production of ROS, leading to ABA-induced stomatal closure via ROS pathway in Arabidopsis (Kwak et al., 2003). Overexpression of the *9-cis-epoxycarotenoid dioxygenase* gene (*SgNCED1*) in transgenic tobaccos also results in tolerance to drought and salt stresses through the elevated production of ABA induced $H_2O_2$ via NADPH oxidase (Zhang et al., 2009).

Plant histone deacetylases (HDACs) play a critical role in response to abiotic stresses. In Arabidopsis, plant specific *Histone deacetylase* genes *AtHD2C and AtHD2D* are reported to implicated in response to abiotic stresses (Sridha and Wu, 2006; Luo et al., 2012a ; Han et al., 2016). Overexpression of these genes in Arabidopsis results in decreased transpirational water loss and resistance to salt and drought stresses (Sridha and Wu, 2006; Han et al., 2016). In rice, expression of *HDA705* is modulated by ABA and abiotic stresses and overexpression of *HDA705* in rice exhibits improved tolerance to osmotic stress at the seedling stage (Zhao et al., 2016). Expression of *HDT701* and *HDT702* are also altered under abiotic stress treatments and overexpression of *HDT701* promote the salt and osmotic stress resistance at the seedling stage (Zhao et al., 2015).

In this study, the function of *HDT701* in salt and osmotic stress tolerance of rice was observed by using knockout (KO) mutant plants and revealed that *HDT701* might improve salt and osmotic stress tolerance by suppressing *OsWRKY45*, an upstream repressor of *SNAC1*.

## MATERIALS AND METHODS

### Plant materials and growth conditions

In this study, T-DNA mutant tagging line of *HDT701* was screened and used from a pool of rice T-DNA-tagging lines previously generated (Jeon et al., 2000; Jeong et al., 2002).

To download the genomic DNA sequences, Rice Annotation Project Database (RAP-DB; http://rapdb.dna.affrc.go.jp; Tanaka et al., 2008) and the TIGR Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu; Ouyang et al., 2007) were accessed. The *hdt701*-1 mutant (Line number 1B-05907) was identified from the rice T-DNA insertion sequence database (An et al., 2005a; 2005b; Jeong et al., 2006). Homozygous mutants were confirmed by PCR, using genomic DNA extracted from the leaf blade. The primers for genotyping were TAGCTCCGCCTCCCACCT (F), TGCCCTGGGAGCTGGAATG (R), and AACGCTGATCAAT-TCCACAG (NGUS1) (Lee et al., 2015). Seeds were germinated either on an MS medium or in soil, as previously described (Yi and An, 2013). Plants were cultured naturally in the paddy field or else in controlled growth rooms maintained under LD conditions (14 h light, 28°C/10 h dark, 22°C; humidity approximately 60%) or SD conditions (12 h light, 28°C/12 h dark, 22°C; humidity approximately 70%), as previously described (Cho et al., 2016).

**Stress Treatments**

To measure the transcript level of *HDT701* and *HDT702* under various stresses, Dongjin plants were grown in controlled growth rooms maintained under LD conditions (14 h light, 28°C/10 h dark, 22°C. Plants grown in MS (Murashige and Skoog, 2006) medium for 14 days were treated with NaCl, PEG and ABA. For osmotic stress, the seedlings were transferred to MS medium supplemented with 20% PEG and sampled at 0, 1, 3 and 6 h after treatment. For salt stress, the seedlings were transferred to MS medium with 300 mM NaCl solution and sampled at 0, 1, 3 and 6 h after treatment. For ABA hormone treatment, seedlings were transferred to MS medium with 100 μM ABA and sampled at 0, 1, 3 and 6 h after treatment. For the observation of phenotype of *hdt701* mutant plants under osmotic and salt stresses, WT plants and *hdt701* homozygous mutant plants were grown in MS medium for 14 d and then transferred to 20% PEG and 150 mM NaCl for 5 d and 3 d respectively. The surviving plants were counted after recovery in MS medium for 7 days. For the expression analysis of genes related to abiotic stress, WT plants and *hdt701* homozygous mutant plants were grown in MS medium for 14 d and then transferred to MS medium supplemented with 200 mM NaCl and sampled at 12 h after exposure to NaCl.

**RNA isolation and quantitative real-time PCR analyses**

Total RNA was isolated from fully grown uppermost healthy leaves with RNAiso Plus (TaKaRa, Shiga, Japan; http://www.takarabio.com). RNA samples with 260/280 nm ratios of >1.8 (Nano-Drop 2000; Thermo Scientific, Wilmington, DE, USA; http://www.nanodrop.com) were used. First-strand cDNA synthesis was performed with 2 μg of total RNA plus Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA; http://www.promega.com), RNasin® Ribonuclease Inhibitor (Promega), oligo (dT) 18 primer, and dNTP. Afterward, synthesized cDNAs and SYBR Green I Prime Q-Master mix (GENETBIO, Daejeon, Republic of Korea) were utilized to monitor gene expression via quantitative real-time (qRT)-PCR on a Rotor-Gene Q system (QIAGEN, Hilden, Germany) (Ryu et al., 2009; Cho et al., 2016). Rice *Ubi* was used for normalization. All experiments were conducted at least three times and, for each experiment, more than three independent samples were used. To ensure primer specificity, we performed these experiments only when the melting curve displayed a single sharp peak. The $\Delta\Delta$CT method was applied to calculate changes in relative expression. All primers for quantitative real-time PCR are listed in Table 1.

Table 1. List of primers used for qRT-PCR in this study.

| Name | Sequence (5'-3') |
|---|---|
| *Ubi*_RT_F | TGAAGACCCTGACTGGGAAG |
| *Ubi*_RT_R | CACGGTTCAACAACATCCAG |
| *HDT701*_RT_F | TAGCTCCGCCTCCCACCT |
| *HDT701*_RT_R | CCGGCTGGGAAACTTTGTAG |
| *HDT702*_RT_F | CTGGGCAATCCTGTGTAGGT |
| *HDT702*_RT_R | AACGTGCAACATCCATACGCAT |
| *OsrbohI*_RT_F | ACTCAAGGTTGCGGTGTACC |
| *OsrbohI*_RT_R | GATGTGGACGCTGACGTAGT |
| *OsAFB2*_RT_F | CTCAGGATGAAGCGGATGGT |
| *OsAFB2*_RT_R | TCTCTCCAGTGAACCAGCATT |
| *OsWRKY45*_RT_F | CTTCGTCGACCAGATTCTCC |
| *OsWRKY45*_RT_R | GGTTCTTGACGACCACCGAA |
| *SNAC1*_RT_F | GCACGCTTGGGATCAAGAAG |
| *SNAC1*_RT_R | TTGTACAGCCGACACAGCAC |
| *NCED4*_RT_F | TTGCACGGCACCTTCATTGG |
| *NCED4*_RT_R | GCGGTCGTTGTCTGCACTAA |
| *OsABA1*_RT_F | TACAGATCCAGAGCAACGCG |
| *OsABA1*_RT_R | CAACCGCACGAGCAAGAATC |
| *OsABA2*_RT_F | CAAGAGACCTGACGAGACGA |

| | |
|---|---|
| *OsABA2*_RT_R | ACCAGCGCAACCTTGCTTTC |

## RESULTS

### Expression patterns of *HDT701* are altered under abiotic stresses.

Expression patterns of *HDT701* under abiotic stress conditions were analysed. Two-week old WT seedlings were treated with 100 µM ABA, 300 mM sodium chloride (NaCl) for the stimulation of slat stress and 20% polyethylene glycol 6000 (PEG) for the stimulation of osmotic stress, respectively. The expression of *HDT701* was decreased significantly after 1 h treatment with ABA ($P < 0.01$) , but it recovered after 3 and 6 h treatment with ABA (Figure.1A). Likewise, its expression is also attenuated considerably after 1 and 3 h treatment with NaCl ($P < 0.01$) as well as PEG ($P < 0.01$) , but it recovered after 6 h treatment with NaCl (Figure.1B) and PEG (Figure.1C).

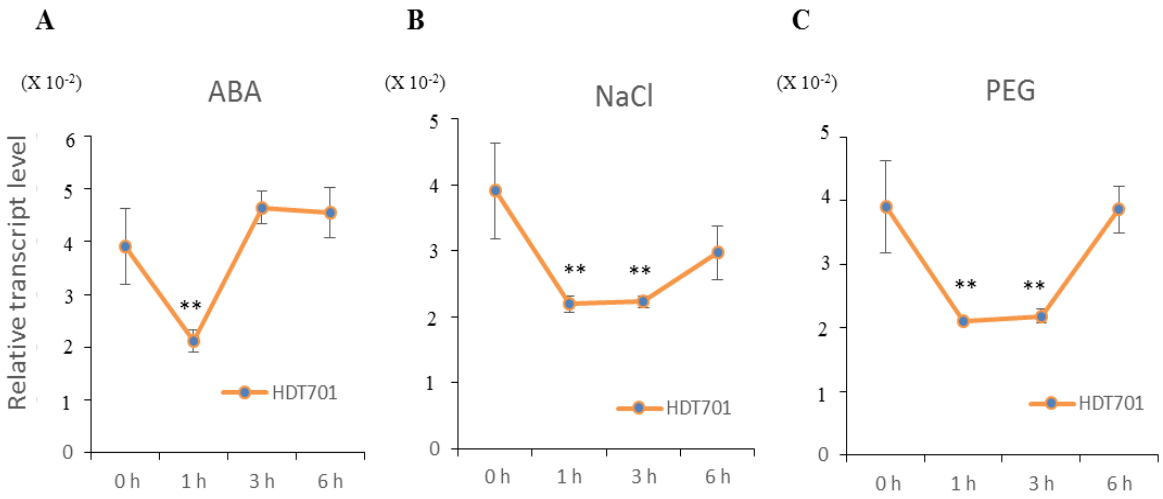


Figure 1**.** Expression patterns of rice *HDT701* under ABA, salt and PEG stresses. Two-week-old rice seedlings were exposed to100 µM ABA (A, D), 300 mM NaCl (B, E), and 20% PEG (C, F) for 0, 1, 3 and 6 h, respectively. Orange line, *HDT701*; relative transcript level of each gene compared with that of rice *Ubi*. Error bars indicate standard deviations; n = 4. Levels of significant difference are indicated by *P < 0.05; **P < 0.01.

Taken together, the resulting data suggests that expression patterns of *HDT701* might be controlled by abiotic stresses.

### Mutation in *HDT701* reduces tolerance to salt and osmotic stress in rice at the seedling stage.

**Identification of abiotic stress sensitive mutants**

Abiotic stress sensitive mutant line 1B-05907 was identified by screening T-DNA insertion tagging lines treated with abiotic stress conditions. The T-DNA was inserted in the first intron of *HDT701* (Figure. 2A) and the transcript level for that gene was markedly decreased in the mutant (Figure. 2B).

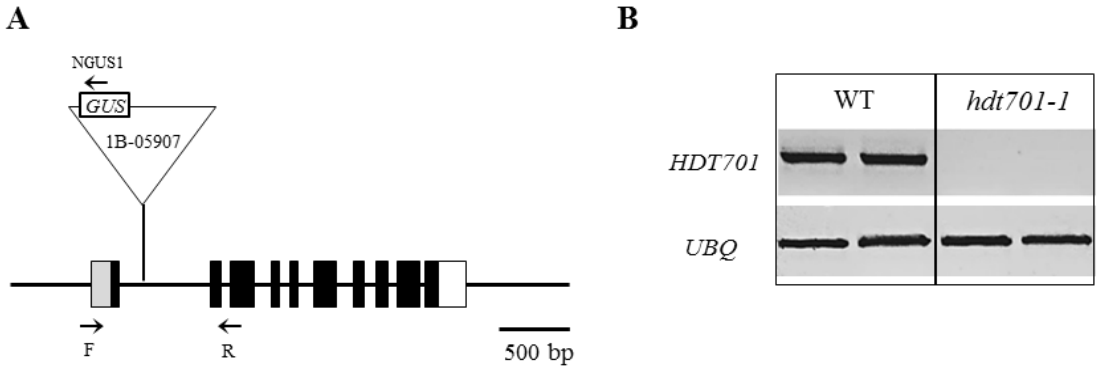


Figure 2. Schematic diagram of gene structure of *HDT701* and comparison of flowering time between WT and *hdt701-1* mutants. (A) Gene structure of *HDT701*. Black boxes indicate exons in coding region; lines connecting boxes indicate introns; gray box, 5'-UTR region; open box, 3'-UTR region. T-DNA is inserted into the first intron of *HDT701* in Line 1B-05907. The direction of promoterless *GUS* reporter gene is indicated within T-DNA (triangle). Primers F, R and NGUS1 were used for genotyping and marked with arrows. Scale bar, 500 bp. (B) *HDT701* transcript level in WT and *hdt701-1* by measured by RT-PCR.

Overexpression of *HDT701* in rice improved salt and osmotic resistance during the seedling stage as previously reported (Zhao et al., 2015). In this study, *hdt701 KO* seedlings were used to investigate the role of *HDT701* in abiotic stress response of rice. The plants were exposed to 150 mM NaCl for 3 days and 20% PEG for 5 days and then recovered in MS medium. The mutant seedlings exhibited increased sensitivity to both salt and osmotic stress at the recovery stage in comparison with the wild type seedlings (Figure. 3A). The survival rate of the mutants was significantly lower than WT seedlings about 30% in the salt stress and about 40% in the osmotic stress (Figure. 3B).The resulting data implies that *HDT701* has an important role in the abiotic stress resistance of rice at the seedling stage.

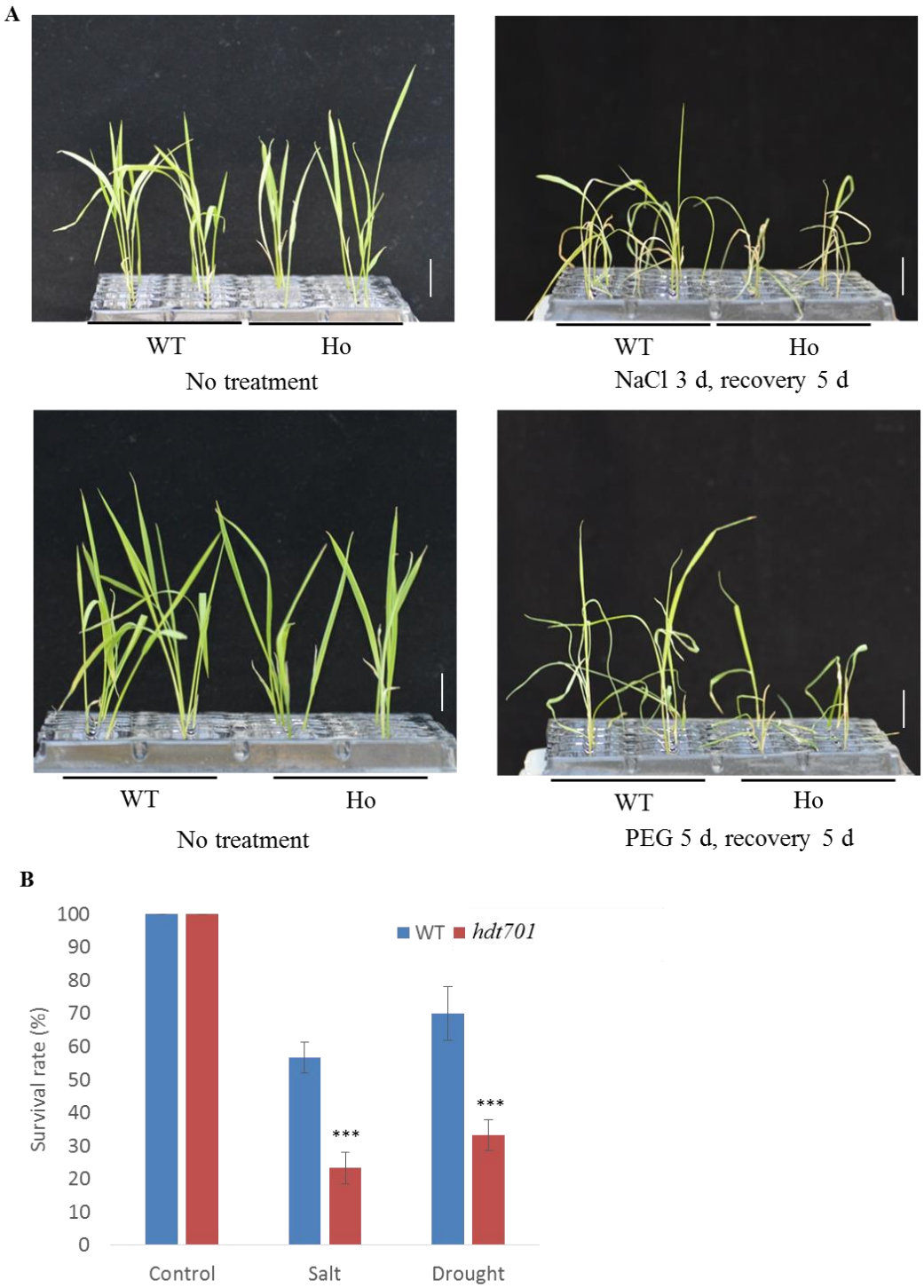


Figure 3. Mutation in *HDT701* attenuates tolerance to salt and osmotic stresses (A) Phenotype of *hdt701* mutant seedlings under salt and osmotic stresses. Scale bar: 5 cm. Mutant seedling and the wild type seedling were exposed to 150mM NaCl or 20% PEG for indicated days and recovered in MS medium (B) Survival rates of *hdt701* mutant seedlings after NaCl and PEG treatment. n = 10. Levels of significant difference are indicated by *P < 0.05; **P < 0.01.

## Expression analysis of abiotic stress- related genes

To elucidate the regulatory pathway controlled by *HDT701* in abiotic stresses, the expression levels of previously identified genes that are important in the stress tolerance of

rice were analysed. Expression patterns of the genes related to ABA biosynthesis were observed because ABA functions as a major regulator in the signaling of abiotic stress responses in plants. Under high salinity-induced osmotic stress conditions, ABA biosynthesis is accelerated to enhance the tolerance of rice in response to abiotic stress conditions (Kumar et al., 2013).

Several genes are implicated in ABA biosynthesis through terpenoid pathway that begins with isopentenyl pyrophosphate (IPP) (Ye et al. 2012). Among them, *OsPSY3* and *OsNCED4*, are well known to be induced one hour after salt stress. *OsSPY3* catalyzes the conversion of GGPP, geranylgeranyl diphosphate into phytoene through chain-elongating condensation in the biosynthesis of ABA (Welsch et al., 2008). *NCED4* catalyze the oxidative cleavage of the major epoxycarotenoid 9-*cis*-neoxanthin into xanthoxin in the ABA biosynthesis pathway (Schwartz et al., 1997). Their expression levels are well concomitant with the level of ABA in rice (Welsch et al., 2008). Therefore, the expression of *OsPSY3* and *OsNCED4* was analysed and found that their transcript levels are significantly decreased ($P < 0.05$) in the mutants compared to the WT (Figure. 4D,E). The reduced expression levels of these genes might contribute to the low level of ABA in the mutants and the increased susceptibility of the mutant plants to salt and osmotic stresses.

Transcript levels of *ABA1* and *ABA2*, the genes that are critical in the ABA biosynthesis, were also analysed to verify if other ABA biosynthesis genes are also modulated by *HDT701* during the abiotic responses of rice. *ABA1* is induced by abiotic stress conditions and catalyze the conversion zeaxanthin to violaxanthin via antheraxanthin (Oliver et al., 2007; Teng et al., 2014). *ABA2* catalyze the conversion of xanthoxin into ABA-aldehyde in the ABA biosynthesis pathway (Cheng et al., 2002). However, expression levels of both genes remained unchanged (Figure. 4G,H), implying that *HDT701* might regulate only the expression of *OsPSY3* and *OsNCED4* in ABA biosynthesis pathway to enhance abiotic stress tolerance of rice.

Many regulatory genes also play a crucial role in the abiotic stress tolerance of rice via the ABA dependent pathway (Kumar et al., 2013). Among them, *STRESS-RESPONSIVE NAC 1* (*SNAC1*) is one of the renowned genes which is induced by various types of abiotic stresses and involved in the abiotic stress response and tolerance of rice (Hu et al. 2006). Overexpression of *SNAC1* significantly enhances abiotic stress tolerance of rice and several stress-related genes were up-regulated in the *SNAC1*-overexpressing plants. Thus, the

expression of that gene was investigated and observed that its transcript level was significantly downregulated ($P < 0.01$) in the mutants (Figure. 4B). This result suggests that *HDT701* might be an upstream activator of *SNAC1* in the abiotic stress tolerance of rice.

MicroRNAs (miRNAs), ubiquitous regulators of gene expression in eukaryotic organisms, also play an important role as an endogenous regulators in abiotic stress tolerance in plants. In rice, *MIR393a* functions negatively in the salt and alkali stress tolerance. Overexpression of *MIR393a* in rice and Arabidopsis lead to increased susceptibility to salt and alkali treatment compared to the WT. In addition, its expression level is altered under salinity and alkaline stress conditions ( Gao et al., 2010, 2011).The reduced expression of *OsAFB2* (AUXIN SIGNALING F-BOX), one of the target gene of *miR393a*, in the *OsmiR393*-overexpressing plants resulted in reduced tolerance to abiotic stresses in rice ( Xia et al., 2012). In order to examine if *HDT701* regulate abiotic stress tolerance of rice through this microRNA pathway, the expression level of *OsAFB2*, the downstream gene of *miR393a* was analysed. However, its expression was unaffected by mutation in *HDT701* (Figure. 4F).

It was previously reported that *OsWRKY45* alleles plays an important role in abiotic stress tolerance of rice. Both *OsWRKY45* alleles are regulated by various abiotic stress conditions and show higher tolerance to drought and cold stresses when they are overexpressed. Overexpression of *OsWRKY45* in rice showed reduced tolerance to cold and drought stresses while Os*WRKY45* RNAi plants are more tolerant. In addition, many genes related to ABA biosynthesis and stress tolerance including *NCED4* and *SNAC1* are altered in *OsWRKY45* transgenic plants. The expression levels of both *NCED4* and *SNAC1* are repressed in the *OsWRKY45*-overexpressing plants but increased in Os*WRKY45* RNAi plants, suggesting that *OsWRKY45* might regulate the abiotic resistance of rice by suppressing *SNAC1* and *NCED4* through ABA dependent pathway (Tao et al., 2011). In *hdt701* mutant plants as well, the expression of *SNAC1* ($P < 0.01$) and *NCED4* ($P < 0.05$) are significantly downregulated. Because *HDT701* functions positively in abiotic stress tolerance of rice and suppresses the expression of target genes, the putative target gene of HDT701 should function negatively in abiotic stress tolerance of rice and show increased expression in the mutant plants. In order to investigate if Os*WRKY45* is a target gene of HDT701, the transcript level of Os*WRKY45* was observed and detected to be increased significantly ($P < 0.01$) in the mutant plants (Figure. 4A). This result suggests that *HDT701* might enhance abiotic stress tolerance of rice by suppressing *OsWRKY45*.

An NADPH oxidase gene, *OsrbohI*, enhances the production of reactive oxygen species (ROS) during stress conditions. ROS induced by ABA, biotic and abiotic stresses function as signal transduction molecules in stress responses of plants (Apel and Hirt, 2004; Foyer and Noctor, 2005; Torres and Dangl, 2005; Miller et al., 2009, 2010). Increased accumulation of ROS results in ABA induced stomatal closing in abiotic stress responses in plants (Kwak et al., 2003).To examine if *HDT701* also modulates the abiotic stress tolerance of rice through ROS pathway, expression of *HDT701* was analysed. The decrease transcript level of the gene in *hdt701* mutants (Figure. 4C) implies that *HDT701* might also regulate salt tolerance of rice through ROS pathway via *OsrbohI* in ABA dependent manner. The reduced expression of abiotic stress-related genes *SNAC1*, *NCED4*, *OsPY3* and *OsrbohI* highlighted that the increased insensitivity of KO mutants to salt and osmotic stresses was due to reduced expression of these genes.

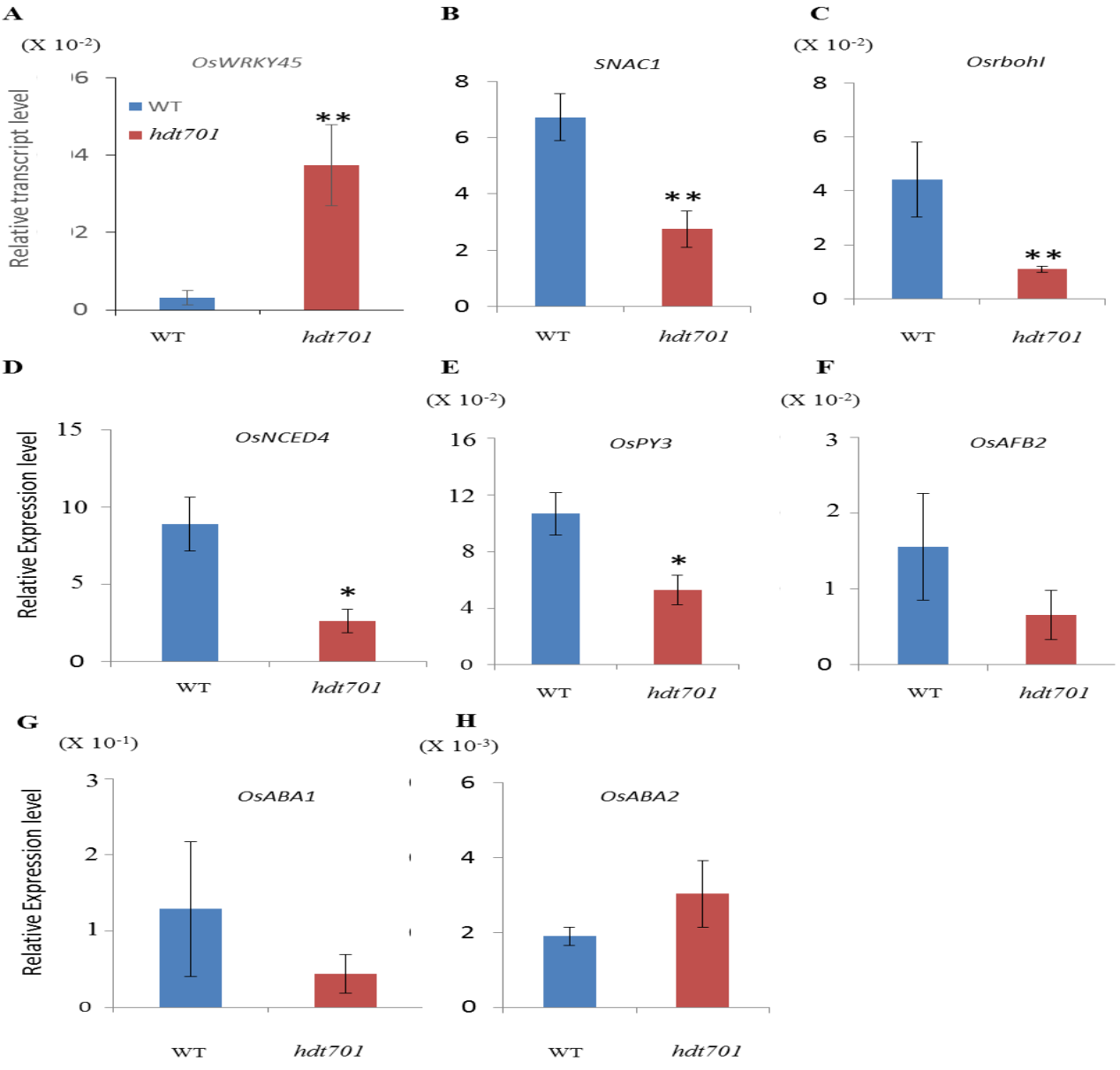

Figure 4. Expression patterns of abiotic stress-related genes in leaf blades of WT and *hdt701-1* plants at 14 DAG under salt stress. Quantitative RT-PCR analyses of *OsWRkY45* (A), *SNAC1* (B), *OsrbohI* (C), *OsNCED4* (D), *OsPY3* (E), *OsABF2* (F), *OsABA1* (G) and *OsABA2* (H),. Blue bar, WT; red bar, *hdt701-1*. y-axis, relative transcript level of each gene compared with that of rice *Ubi*. Error bars indicate standard deviations; n = 4. Levels of significant difference are indicated by *P < 0.05; **P < 0.01.

## DISCUSSION

Function of *HDT701* in abiotic stress tolerance of rice were analysed using KO mutant plants raised by T-DNA insertion and showed that the mutant plants are more sensitive to salt and osmotic stresses compared to the wild type. The number of surviving plants is remarkably reduced in the mutant seedlings under both abiotic stress treatments. This observation is in good agreement with a previous report that overexpression of *HDT701* in rice increases resistance to salt and osmotic treatments at the seedling stage (Zhao et al., 2015).

Plant specific *Histone Deacetylase 2* (*HD2*) genes in Arabidopsis also exhibit increased endurance to abiotic stresses when they are overexpressed. HD2D overexpressing transgenic Arabidopsis plants displayed enhance resistance to salt and drought stresses compared to the wild type (Han et al., 2016). In addition, overexpression of HD2C in Arabidopsis also promote salt and drought tolerance by regulating ABA-responsive genes (Sridha and Wu, 2006). These previous findings are well consistent with my observations and support that plant specific *Histone Deacetylase 2* (*HD2*) *genes* have an important function in abiotic stress responses of plants.

Expression patterns of *HDT701* and *HDT702* are responsive to abiotic stresses in rice. The expression levels of *HDT701* and *HDT702* were altered under abiotic stress treatments in the present study, which is consistent with that previously reported (Zhao et al., 2015). Moreover, the expression of Arabidopsis homologous genes *HD2A*, *HD2B*, *HD2C*, and *HD2D* is also altered under ABA and high salt treatment (Luo et al., 2012b), suggesting that expression of plant specific *Histone Deacetylase 2* genes might be modulated by abiotic stresses and have similar role in abiotic stress tolerance.

To verify the regulatory pathway governed by *HDT701* in the abiotic stress response of rice, expression patterns of the previously reported genes that contribute to the abiotic stress tolerance in rice was analysed and revealed that the expression of *SNAC1*, *NCED4*, *OsPY3* and *OsrbohI* was significantly decreased while *WRKY45* was greatly upregulated in

the mutant plants in comparison with the wild type. However, the transcript levels of *ABA1*, *ABA2* and *OsAFB2* was unchanged in the mutants.

The reduced expression of abiotic stress-related genes *SNAC1*, *NCED4*, *OsPY3* and *OsrbohI* highlighted that the increased insensitivity of KO mutants to salt and osmotic stresses was due to reduced expression of these genes. *SNAC1* is reported to positively control the abiotic stress tolerance of rice. Its expression was induced by various abiotic stress treatments and overexpression of the gene increase abiotic stress resistance in rice (Hu et al. 2006). This previous study is well correlated with the current results of reduced expression of *SNAC1* in *hdt701* mutants and their increased susceptibility to the drought and salt stresses. The decreased transcript level of *SNAC1* in the mutants also suggested that *HDT701* is a positive regulator that functions upstream of *SNAC1* in the abiotic stress tolerance of rice.

*NCED4* and *OsPY3* are ABA biosynthesis genes inducible by abiotic stresses (Kumar et al., 2013). The reduced transcript levels of these ABA biosynthesis genes in the mutants might contribute to the low level of ABA under stresses, resulting in decreased resistance to abiotic stresses. This hypothesis is also supported by the previous studies in which overexpression of *NCED* genes in transgenic plants leads to ABA accumulation and enhanced resistance to abiotic stresses (Thompson et al., 2000; Iuchi et al., 2001; Qin and Zeevaart, 2002; Aswath et al., 2005; Wan and Li, 2006). However, the unaltered expression levels of *ABA1* and *ABA2*, other *ABA* biosynthesis genes, in the mutant plants implies that *HDT701* might enhance abiotic stress resistance by modulating only the expression of *NCED4* and *OsPY3* in ABA dependent manner.

*OsAFB2* is a target gene of *OsmiR393* and reduced expression of this gene in *OsmiR393*-overexpressing plants shows higher level of sensitivity to abiotic stresses in rice (Xia et al., 2012). Nevertheless, the expression of this gene was not affected in the mutants, indicating that *HDT701* may not regulate abiotic tolerance through *OsmiR393* pathway.

*OsWRKY45* is an abiotic stress responsive gene that is implicated in ABA signaling and abiotic stress tolerance of rice. It negatively functions in the abiotic tolerance of rice by repressing *SNAC1* and *NCEDC4* and overexpression of this gene shows enhanced susceptibility to abiotic stresses (Tao et al., 2011).This observation is well concomitant with the current result in which expression of *SNAC1* and *NCEDC4* was decreased while that of *OsWRKY45* is up-regulated in the mutants and *hdt701* mutant plants are more sensitive to salt

and osmotic stresses. Thus, *OsWRKY45* was identified as a putative target of HDT701 because only the expression of the former was significantly enhanced under salt stress in the *hdt701* mutants.

*OsrbohI* , an NADPH oxidase gene, contributes to the production of ROS (Wong et al., 2007). The expression of *OsrbohI* is found to be significantly decreased in the mutants compared to the wild type. The reduced transcript level of the gene may lead to the decrease level of ROS that enhances the abiotic stress resistance. The increased production of $H_2O_2$ induced by higher level of ABA content in *sgNCED1* overexpressing transgenic tobacco plants under abiotic stresses increase tolerance to abiotic stress conditions as reported previously (Zhang et al., 2009).In addition, mutation in NADPH oxidases *AtrbohD* and *AtrbohF* decreases ABA-induced stomatal closing and ABA promotion of ROS production, leading to reduced tolerance to soil salinity in Arabidopsis (Kwak et al., 2003; Jiang et al., 2012). This previous studies are well related to the current result of decreased expression level of *OsrbohI* and reduced tolerance of the mutant plants. Thus, this observation suggests that *HDT701* might also mediate the abiotic stress response through ROS pathway by enhancing *OsrbohI* in addition to reducing the expression of *OsWRKY45* ( Figure.5 ). However, further investigation is necessary to evaluate if *HDT701*enhances tolerance of rice to abiotic stresses by directly repressing *OsWRKY45*.

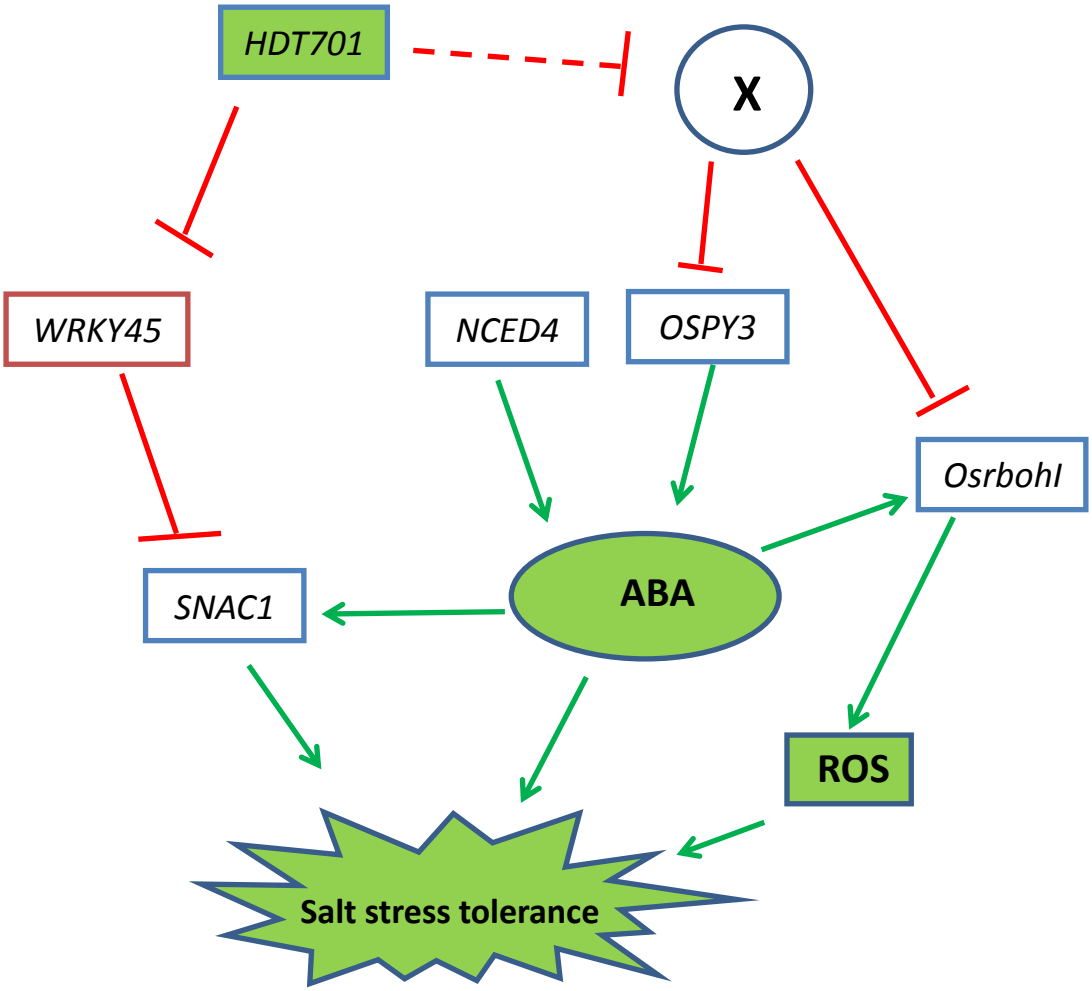


Figure 5. A model for regulatory pathway mediated by *HDT701* in the salt stress tolerance in rice

## CONCLUSION

*hdt701* mutant seedling plants were more sensitive to salt and osmotic stresses in comparison with WT controls. HDT701 overexpressing transgenic seedlings also show higher tolerance to osmotic and salt stresses as previously reported, suggesting that *HDT701* has an important role in the abiotic stress tolerance of rice. The expression of abiotic stress related genes *SNAC1*, *NCED4*, *OsPY3* and *OsrbohI* was significantly decreased while *WRKY45*, an upstream suppressor of *SNAC1* and *NCED4*, was greatly upregulated in the mutant plants in comparison with the wild type, indicating that *HDT701* might enhance the

salt and osmotic stress tolerance of rice by repressing *OsWRKY45* as well as through ROS pathway by enhancing *OsrbohI*.


## ACKNOWLEDGEMENTS

First of all, I would like to express my heartfelt appreciation to my PhD advisor, Prof. Dr. Gynheung An, who kindly gives me the golden opportunity to extend my further and advance studies at Kyung Hee University in Korea, for kindly providing the all the necessary research facilities and his brilliant supervision on this research. I am also grateful to all my lab members for their invaluable help during this research work in Korea. I am indebted to Prof. Dr. San San Aye, the Prorector of Mawlamyine University, for her kind support for this research work in Korea. I also would like to express my sincere appreciation to Prof. Dr. Myat Myat Moe, the Head of Botany Department at Dagon University, for kindly inviting me to attend the 2nd Myanmar-Korean conference. Last but not least, I am also thankful to Prof. Dr. Nu Nu Yee, the Head of Botany Department at the University of Mandalay, for kindly allowing me to attend this conference.


## REFERENCES


An, G., Jeong, D.H., Jung, K.H., and Lee, S. (2005a). Reverse genetic approaches for functional genomics of rice. Plant Mol. Biol. *59*, 111-123.

An, G., Lee, S., Kim, S.H., and Kim, S.R. (2005b). Molecular genetics using T-DNA in rice. Plant Cell Physiol. *46*, 14-22.

Apel, K., and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. *55*, 373-399.

Ashraf, M. (2010). Inducing drought tolerance in plants: recent advances. Biotechnol. Adv. *28*,169-183.

Aswath C.R., Kim S.H., Mo S.Y., and Kim D.H. (2005). Transgenic plants of creeping bent grass harboring the stress inducible gene, 9-*cis*-epoxycarotenoid dioxygenase, are highly tolerant to drought and NaCl stress. Plant Growth Regul. *47*, 129-139.

Bohra, J.S., Dörffling, H., and Dörffling, K. (1995). Salinity tolerance of rice (*Oryza sativa* L.) with reference to endogenous and exogenous abscisic acid. J. Agron. Crop Sci. *174*, 79-86.

Cheng, W.H., Endo, A., Zhou, L., Penney, J., Chen, H.C., Arroyo, A., Leon, P., Nambara, E., Asami, T., Seo, M., and Koshiba, T. (2002). A unique short-chain dehydrogenase/reductase in Arabidopsis glucose signaling and abscisic acid biosynthesis and functions. Plant Cell *14*, 2723–2743

Cho, L.H., Yoon, J., Pasriga, R., and An, G. (2016). Homodimerization of Ehd1 is required to induce flowering in rice. Plant Physiol. *170*, 2159-2171.

Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R., and Abrams, S.R. (2010). Abscisic acid: Emergence of a core signaling network. Annu. Rev. Plant Biol. *61*,651-679.

Foyer, C.H., and Noctor, G. (2005). Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ. *28*, 1056 -1071.

Gao, P., Bai, X., Yang, L., Lv, D., Li, Y., Cai, H., Ji, W., Guo, D., and Zhu, Y. (2010). Overexpression of osa-MIR396c decreases salt and alkali stress tolerance. Planta *231*,991-1001.

Gao, P., Bai, X., Yang, L., Lv, D., Pan, X., Li, Y., Cai, H., Ji, W., Chen, Q., and Zhu, Y. (2011). Osa-MIR393: a salinity and alkaline stress-related microRNA gene. Mol. Biol. Rep. *38*:237-242.

Gurmani, A.R., Bano, A., Ullah, N., Khan, H., Jahangir, M., and Flowers, T.J. (2013). Exogenous abscisic acid (ABA) and silicon (Si) promote salinity tolerance by reducing sodium (Na+) transport and by pass flow in rice (*Oryza sativa indica*). Aust. J. Crop Sci. *7*, 1219-1226.

Guy, C.L. (1990). Cold acclimation and freezing stress tolerance: Role of protein metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. *41*,187-223.

Hadiarto, T., and Tran, L.S.P. (2011). Progress studies of drought-responsive genes in rice. Plant Cell Rep. *30*,297-310.

Han, Z., Yu, H., Zhao, Z., Hunter, D., Luo, X., Duan, J., and Tian, L. (2016). AtHD2D gene plays a role in plant growth, development, and response to abiotic stresses in Arabidopsis thaliana. Front. Plant Sci. *7*,310.

Hu, H., Dai, M., Yao, J., Xiao, B., Li, X., Zhang, Q., and Xiong, L. (2006). Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proc. Natl. Acad. Sci. *103*, 12987-12992.

Hussain, S., Saleem, M.F., Ashraf, M.Y., Cheema, M.A., and Haq, M.A. (2013). Abscisic acid, a stress hormone helps in improving water relation and yield of sunflower (*Helliantuus annuu*s L.) hybrids under drought. Pak. J. Bot. *2*, 2177-2189.

Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T., Tabata, S., Kakubari, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2001). Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. Plant J. *27*, 325-333.

Jaspers, P. and Kangasjärvi, J. (2010). Reactive oxygen species in abiotic stress signaling. Physiol. Plant. *138*, 405-413.

Jeon, J.S., Lee, S., Jung, K.H., Jun, S.H., Jeong, D.H., Lee, J., Kim, C., Jang, S., Lee, S., Yang, K., et al. (2000). T-DNA insertional mutagenesis for functional genomics in rice. Plant J. *22*, 561-570.

Jeong, Y.H., Nakamura, H., and Ota, Y. (1980). Physiological studies on photochemical oxidants injury in rice plants: 1. Varietal difference of abscisic acid content and its relation to the resistance to ozone. Jpn. J. Crop Sci. *49*, 456-460.

Jeong, D.H., An, S., Kang, H.G., Moon, S., Han, J.J., Park, S., Lee, H.S., An, K., and An, G. (2002). T-DNA insertional mutagenesis for activation tagging in rice. Plant Physiol. *130*, 1636-1644.

Jeong, D.H., An, S., Park, S., Kang, H.G., Park, G.G., Kim, S.R., Sim, J., Kim, Y.O., Kim, M.K., Kim, S.R., et al. (2006). Generation of a flanking sequence-tag database for activation-tagging lines in japonica rice. Plant J. *45*, 123-132.

Kao, C.H. (2014). Role of abscisic acid in abiotic stress tolerance in rice. *Crop Environ. Bioinform. 11*, 57-64.

Kumar, K., Kumar, M., Kim, S.R., Ryu, H., and Cho, Y.G. (2013). Insights into genomics of salt stress response in rice. *Rice 6*, 27.

Kwak, J.M., Mori, I.C., Pei, Z.M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D., and Schroeder, J.I. (2003). NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. EMBO J. *22*, 2623-2633.

LaRosa, P.C., Hasegawa, P.M., Rhodes, D., Clithero, J.M., Watad, A.E.A., and Bressan, R.A., (1987) Abscsic acid stimulated osmotic adjustment and its involvement in adaptation of tobacco cells to NaCl. Plant Physiol. *85*,174-181.

Lee, T.M., Lur, H.S., and Chu, C. (1993). Role of abscisic acid in chilling tolerance of rice (*Orysa staiva* L.) seedlings. I. Endogenous abscisic acid levels. Plant Cell Environ. *16*,481-490.

Lee, Y.S., and An, G. (2015). *OsGI* controls flowering time by modulating rhythmic flowering time regulators preferentially under short day in rice. J. Plant Biol. *58*, 137-145.

Luo, M., Liu, X., Singh, P., Cui, Y., Zimmerli, L., and Wu, K. (2012a). Chromatin modifications and remodeling in plant abiotic stress responses. Biochim. Biophys. Acta. *1819*, 129-136.

Luo, M., Wang, Y.Y., Liu, X., Yang, S., Lu, Q., Cui, Y., and Wu, K. (2012b). HD2C interacts with HDA6 and is involved in ABA and salt stress response in Arabidopsis. J. Exp. Bot. *63*, 3297–3306.

Mantri, N., Patade, V., Penna, S., Ford, R., and Pang, E. (2012) Abiotic stress responses in plants: present and future. In *Abiotic stress responses in plants*. Springer, New York, 1-19.

Miller, G., Schlauch, K., Tam, R., Cortes, D., Torres, M.A., Shulaev, V., Dangl, J.L., and Mittler, R. (2009). The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. Sci. Signal. *2*, ra45.

Miller, G.A.D., Suzuki, N., CIFTCI-YILMAZ, S.U.L.T.A.N., and Mittler, R.O.N. (2010). Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ. *33*, 453-467.

Munns, R., and Tester, M. (2008). Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. *59*,651-681.

Murashige, T., and Skoog, F. (2006). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. *15*, 473-497.

Oliver, S.N., Dennis, E.S., and Dolferus, R. (2007). ABA regulates apoplastic sugar transport and is a potential signal for cold-induced pollen sterility in rice. Plant Cell Physiol. *48*, 1319-1330.

Ouyang, S., Zhu, W., Hamilton, J., Lin, H., Campbell, M., Childs, K., Thibaud-Nissen, F., Malek, R.L., Lee, Y., Zheng, L., et al. (2007).The TIGR Rice Genome Annotation Resource: improvements and new features. Nucleic Acids Res. *35*, D883-D887.

Qin, X., and Zeevaart, J.A.D. (2002). Overexpression of a 9-cisepoxycarotenoid dioxygenase gene in *Nicotiana plumbaginifolia* increases abscisic acid and phaseic acid levels and enhances drought tolerance. Plant Physiol. *128*, 544-551.

Ryu, C.H., Lee, S., Cho, L.H., Kim, S.L., Lee, Y.S., Choi, S.C., Jeong, H.J., Yi, J., Park, S.J., Han, C.D., et al. (2009). *OsMADS50* and *OsMADS56* function antagonistically in regulating long day (LD) -dependent flowering in rice. Plant Cell Environ. *32*, 1412-1427.

Santner, A., Calderon-Villalobos, L.I.A., and Estelle, M. (2009) Plant hormones are versatile chemical regulators of plant growth. Nat. Chem. Biol. *5*,301-307.

Schwartz, S.H., Tan, B.C., Gage, D.A., Zeevaart, J.A., and McCarty, D.R. (1997). Specific oxidative cleavage of carotenoid by VP14 of maize. Science *276*, 1872-1874.

Sridha, S., and Wu, K. (2006). Identification of *AtHD2C* as a novel regulator of abscisic acid responses in Arabidopsis. Plant J. *46*, 124-133.

Tanaka, T., Antonio, B.A., Kikuchi, S., Matsumoto, T., Nagamura, Y., Numa, H., Sakai, H,. Wu, J., Itoh, T., Sasaki, T., et al. (2008). The rice annotation project database (RAP-DB): 2008 update. Nucleic Acids Res. 36, D1028-D1033.

Tao, Z., Kou, Y., Liu, H., Li, X., Xiao, J., and Wang, S. (2011). OsWRKY45 alleles play different roles in abscisic acid signalling and salt stress tolerance but similar roles in drought and cold tolerance in rice. J. Exp. Bot. *62*, 4863-4874.

Teng, K., Li, J., Liu, L., Han, Y., Du, Y., Zhang, J., Sun, H., and Zhao, Q. (2014). Exogenous ABA induces drought tolerance in upland rice: the role of chloroplast and ABA biosynthesis-related gene expression on photosystem II during PEG stress. **Acta Physiol. Plant.** *36*, 2219-2227.

Thakur, P., Kumar, S., Malik, J.A., Berger, J.D., and Nayyar, H. (2010). Cold stress effects on reproductive development in grain crops: an overview. Environ. Exp. Bot. *67*,429-443

Thompson, A.J., Jackson, A.C., Symonds, R.C., Mulholland, B.J., Dadswell, A.R., Blake, P.S., Burbidge, A., and Taylor I.B. (2000). Ectopic expression of a tomato 9-cis-epoxycarotenoid dioxygenase gene causes over-production of abscisic acid. Plant J. *23*, 363-374.

Torres, M.A., Jones, J.D., and Dangl, J.L. (2005). Pathogen-induced, NADPH oxidase-derived reactive oxygen intermediates suppress spread of cell death in *Arabidopsis thaliana*. Nat. genet.*37*, 1130-1134.

Wan, X.R., and Li, L. (2006). Regulation of ABA level and water stress tolerance of *Arabidopsis* by ectopic expression of a peanut 9-cis-epoxycarotenoid dioxygenase gene. Biochem. Biophys. Res. Commun. *347*, 1030-1038.

Wang, W., Vinocur, B., and Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta *218*, 1-14.

Welsch, R., Wüst, F., Bär, C., Al-Babili, S., and Beyer, P. (2008). A third phytoene synthase is devoted to abiotic stress-induced abscisic acid formation in rice and defines functional diversification of phytoene synthase genes. Plant Physiol. *147*,367-380.

Wong, H.L., Pinontoan, R., Hayashi, K., Tabata, R., Yaeno, T., Hasegawa, K., Kojima, C., Yoshioka, H., Iba, K., Kawasaki, T., et al. (2007) Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. Plant Cell *19*, 4022-4034.

Xia, K., Wang, R., Ou, X., Fang, Z., Tian, C., Duan, J., Wang, Y., and Zhang, M. (2012). OsTIR1 and OsAFB2 downregulation via OsmiR393 overexpression leads to more tillers, early flowering and less tolerance to salt and drought in rice. PloS one *7*, e30039.

Xiong, L., Zhu, J.K. (2001). Abiotic stress signal transduction in plants: molecular and genetic perspectives. Physiol. Plant. *112*,152-166.

Ye, N., Jia, L., Zhang, J. (2012). ABA signal in rice under stress conditions. Rice *5*, 1-9.

Yi, J., and An, G. (2013). Utilization of T-DNA tagging lines in rice. J. Plant Biol. *56*, 85-90.

Zeevaart, J.A.D., and Creelman, R.A.(1988). Metabolism and physiology of abscisic acid. Annu. Rev. Plant Physiol. Plant Mol. Biol. *39*, 439-473.

Zhang, Y., Tan, J., Guo, Z., Lu, S., He, S., Shu, W., and Zhou, B. (2009). Increased abscisic acid levels in transgenic tobacco over-expressing 9 cis-epoxycarotenoid dioxygenase

influence H2O2 and NO production and antioxidant defences. Plant Cell Environ. *32*, 509-519.

Zhao, J., Zhang, J., Zhang, W., Wu, K., Zheng, F., Tian, L., Liu, X., and Duan, J. (2015). Expression and functional analysis of the plant-specific histone deacetylase *HDT701* in rice. Front. Plant Sci. *5*, 764.

Zhao, J., Li, M., Gu, D., Liu, X., Zhang, J., Wu, K., Zhang, X., da Silva, J.A.T., and Duan, J. (2016). Involvement of rice histone deacetylase HDA705 in seed germination and in response to ABA and abiotic stresses. Biochem. Biophys. Res. Commun. *470*,439-444.