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

Antt Htet Wai¹ and Gynheung An²

**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 1 (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.

Keywords: rice, abiotic stress, salt and osmotic stress tolerance, HDT701, OsWRKY45

**Introduction**

As a consequence of a sessile lifestyle, plants are subjected to various abiotic stresses, which contribute to a 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

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total irrigated land, have already been ruined by salt (Xiong and Zhu, 2001). 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 good 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 processes 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. 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$_2$O$_2$ 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). The 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 promotes 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 TAGCTCCGCCTCCCCACCT (F), TGCCCTGGGAGCTGGAATG (R), and AACGCTGATCAATTCCACAG (NGUS1) (Lee and An, 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 ΔΔCT 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.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'→3')</th>
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<tbody>
<tr>
<td>Ubi_RT_F</td>
<td>TGAAGACCCCTGACTGGGAAG</td>
</tr>
<tr>
<td>Ubi_RT_R</td>
<td>CACGGTTCACAACATCCAC</td>
</tr>
<tr>
<td>HDT701_RT_F</td>
<td>TAGCTCCGCTCCACCT</td>
</tr>
<tr>
<td>HDT701_RT_R</td>
<td>CCGGCTGGGAAACTTTGAG</td>
</tr>
<tr>
<td>HDT702_RT_F</td>
<td>CTGGGCAATCCTGTGAGGT</td>
</tr>
<tr>
<td>HDT702_RT_R</td>
<td>AACGTGCAACATCCATACGCAAT</td>
</tr>
<tr>
<td>Osrbohl_RT_F</td>
<td>ACTCAAGGGTTCGGGTGTTACC</td>
</tr>
<tr>
<td>Osrbohl_RT_R</td>
<td>GATGTTGAGCCTGACGTAGT</td>
</tr>
<tr>
<td>OsAFB2_RT_F</td>
<td>CTCAGGATGAAGCGGATGTGT</td>
</tr>
<tr>
<td>OsAFB2_RT_R</td>
<td>TCTCTCCAGTGAACACGATTT</td>
</tr>
<tr>
<td>OsWRKY45_RT_F</td>
<td>CTTGCAGACAGATTCTCC</td>
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<td>OsWRKY45_RT_R</td>
<td>GGTCTTGCAGACACCCGAA</td>
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<tr>
<td>SNAC1_RT_F</td>
<td>GCACGCTTGGGATCAAGAA</td>
</tr>
<tr>
<td>SNAC1_RT_R</td>
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</tr>
<tr>
<td>NCED4_RT_F</td>
<td>TTGCACGGGCACCTTCATTGG</td>
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<td>NCED4_RT_R</td>
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<td>OsABA1_RT_F</td>
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<td>OsABA1_RT_R</td>
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<tr>
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<tr>
<td>OsABA2_RT_R</td>
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</tr>
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</table>

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 salt 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).
patterns of rice *HDT701* under ABA, salt and PEG stresses. Two-week-old rice seedlings were exposed to 100 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 suggest 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 the gene structure of *HDT701* and comparison of flowering time between WT and *hdt*701-1 mutants. (A) Gene structure of *HDT701*. Black boxes indicate exons in the 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 the 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 *hdt*701-1 by measured by RT-PCR.](image)

Overexpression of *HDT701* in rice improved salt and osmotic resistance during the seedling stage as previously reported (Zhao et al., 2015). In this study, *hdt*701 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.
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. 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). 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. 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.

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.

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).

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 OsWRKY45, a negative repressor upstream of SNAC1 and NCED4, is a target gene of HDT701, the transcript level of OsWRKY45 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. 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 Osrbohl in ABA dependent manner. The reduced expression of abiotic stress-related genes SNAC1, NCED4, OsPY3 and Osrbohl highlighted that the increased insensitivity of KO mutants to salt and osmotic stresses were 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), Osbohl (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

The function of HDT701 in abiotic stress tolerance of rice was 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. HD2 overexpressing transgenic Arabidopsis plants displayed enhanced resistance to salt and drought stresses compared to the wild type (Han et al., 2016). In addition, overexpression of HD2C in Arabidopsis also promotes 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 a 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 were 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 stress, 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 a 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 show 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 decreased level of ROS that enhances the abiotic stress resistance. The increased production of H$_2$O$_2$ induced by a higher level of ABA content in sgNCED1 overexpressing transgenic tobacco plants under abiotic stresses increases tolerance to abiotic stress conditions as reported previously (Zhang et al., 2009). In addition, a 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). These previous studies are well related to the current result of the 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 a 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 Osrbohl 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 Osrbohl.

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