Thesis for the Degree of Doctor of Philosophy

# Study on rice *Histone Deacetylase 2* (*HD2*)family in regulating flowering time and abiotic stress tolerance

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by

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**Dedicated to my parents and all my teachers in my life**

**Abstract**

Rice is a facultative short-day (SD) plant in which flowering is induced under SD conditions or by other environmental factors and internal genetic programs. Overexpression of *Histone Deacetylase 701* (*HDT701*)accelerates flowering in hybrid rice. In this study, I report that mutants defective in *HDT701* flowered late under both SD and long-day conditions. Expression levels of florigens *Heading date 3a* (*Hd3a*) and *Rice Flowering Locus T1* (*RFT1*), and their immediate upstream floral activator *Early heading date 1* (*Ehd1*),were significantly decreased in the *hdt701* mutants, indicating that *HDT701* functions upstream of *Ehd1* in controlling flowering time. Transcript levels of *OsINDETERMINATE SPIKELET 1* (*OsIDS1*), an upstream repressor of *Ehd1*, were significantly increased in the mutants while those of *OsGI* and *Hd1* were reduced. Chromatin-immunoprecipitation assays revealed that HDT701 directly binds to the promoter region of *OsIDS1*. These results suggest that HDT701induces flowering by suppressing *OsIDS1*.

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 *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. Here, I report that *hdt701* mutant seedlings displayed increased sensitivity to both salt and osmotic stresses. Expression levels of *Oryza sativaPhytoene Synthase 3* (*OsPY3*)and *9-cis-epoxycarotenoid dioxygenase 4* (*NCED4*), ABA biosynthesis genes induced by salt stress , and *STRESS-RESPONSIVE NAC 1* (*SNAC1*), anabiotic 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*.

**Contents**

# Chapter 1. General introduction

## 1-1. Impact of abiotic stresses on plants

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 a 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).

## 1-2. Abscisic acid (ABA) as a major regulator in abiotic stresses

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 molecules 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 (Kishor 1985; Bohra et al., 1995; Gurmani et al.,2013). 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 ABAsignaling in guard cells (Kwak et al., 2003; Jiang et al., 2012; Kumar et al., 2013; Rejeb et al., 2015). 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).

**1-3. Functional role of histone deacetylases in abiotic stress tolerance**

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).

### 1-4. Photoperiod flowering in rice

Flowering is one of the most crucial biological processes in plants because it is a prerequisite for the development of fruits and grains. Transition from the vegetative phase is the first step toward reproductive success. Therefore, producing flowers at the appropriate time is a key factor. Whereas early flowering shortens the vegetative phase to an insufficient period that often leads to reduced yields, deferred flowering may also contribute to yield losses when plants in temperate regions are exposed to characteristically colder temperatures later in the growing season. For rice (*Oryza sativa*), chilling at the grain ripening stage results in immature grains while high temperatures are associated with heat damage and a reduction in grain quality. Thus, flowering time is highly correlated with total grain yield and quality in rice (Sun et al., 2014; Zhang et al., 2015; Morita et al., 2017).

The timing of floral transition is regulated by many factors, e.g., internal genetic programming, day length, temperature, nutrient availability, and abiotic/biotic stresses (Cho et al., 2017). In Arabidopsis (*Arabidopsisthaliana*), a long-day (LD) plant, flowering time is accelerated by longer photoperiods. *GIGANTEA* (*GI*) merges signals from photoreceptors and a circadian clock to activate *CONSTANS* (*CO*), which in turn promotes the expression of *Flowering Locus T* (*FT*), a major floral activator that is expressed in the vascular tissues of leaves, all of which lead to the induction of floral transition (Fowler et al., 1999; Park et al., 1999; Samach et al., 2000; Yanovsky and Kay, 2002).

*Oryza sativa GIGANTEA* (*OsGI*), *Heading date 1* (*Hd1*), and *Heading date 3a* (*Hd3a*) are the rice homologues of *GI*, *CO*, and *FT*, respectively. This core flowering pathway is conserved in many plant species. Although *CO* enhances flowering in Arabidopsis, *Hd1* has a dual function in rice. Whereas *Hd1* promotes flowering under short-day (SD) conditions by enhancing the expression of *Early heading date 1* (*Ehd1*) (Zhang et al., 2017), the factorsuppresses flowering under LD conditions by inhibiting *Ehd1* and *Hd3a* (Hayama et al., 2003). In addition to this conserved flowering pathway, *Flowering Locus C* (*FLC*) in Arabidopsis and *Grain number, plant height, and heading date7* (*Ghd7*) and *Early heading date 1* (*Ehd1*)in rice are unique floral regulators. In these dedicated flowering pathways, *FLC* and *Ghd7* act as major flowering repressors while *Ehd1* functions as a floral activator (Doi et al., 2004; Sun et al., 2014).

### 1-5. Regulatory genes that modulate flowering time in rice

Rice is a facultative SD plant. Its heading date is advanced under SD conditions (<13 h of light/day) but retarded under LD conditions (>14 h of light/day) (Nishida et al., 2002; Lee et al., 2007; Ishikawa et al., 2011; Kim et al., 2013; Cho et al., 2016). Rice has two florigens, *Hd3a* and *Rice Flowering Locus T1* (*RFT1*),that are induced by *Ehd1* (Doi et al., 2004; Corbesier et al., 2007; Ryu et al., 2009). Several transcription factors activate or repress the expression of *Ehd1*, a genethat is a critical convergence point for various flowering signals in rice.

Several genes, including *Ghd7* and *OsMADS56*, preferentially function as suppressors of flowering under LD. However, some constitutive suppressors inhibit flowering regardless of day length. For example, two AP2-like genes, *OsINDETERMINATE SPIKELET 1* (*OsIDS1*) and *SUPERNUMERARY BRACT* (*SNB*), repress the expression of *Ehd1* and florigens, resulting in delayed flowering under both LD and SD conditions. In this pathway, *microRNA172*(*miR172*) degrades transcripts of *OsIDS1* and *SNB* to induce flowering, whereas *Oryza sativa Phytochrome B* (*OsPhyB*) enhances the expression of *OsIDS1* and *SNB* by repressing *miR172* toinhibit flowering(Lee et al., 2014). *OsCOL4*, a member of the

CONSTANS-like (COL) family in rice, is up-regulated by *OsPhyB*. The former suppresses flowering under both SD and LD by dampening the transcript levels of *Ehd1* and the florigens via upregulation of floral repressors *OsIDS1* and *SNB* (Lee et al., 2010, 2014). *Oryza sativa LEAFY COTYLEDON 2 and FUSCA 3-LIKE 1* (*OsLFL1*) constitutively deters rice flowering by directly attenuating the transcript level of *Ehd1* (Peng et al., 2007, 2008). Furthermore, *OsLF*, which encodes a typical HLH protein, delays flowering regardless of day length by directly repressing *Hd1* and *OsGI* (Zhao et al., 2011).

**1-6. Role of histone deacetylases in flowering time**

Thehistoneacetyltransferases(HATs)andhistonedeacetylases(HDACs) reversibly catalyze acetylationordeacetylationonhistonelysineresidues for the transcriptional activation and repression, respectively, of target genes. PlantHDACscanbeclassifiedintothreemajorfamilies: theRPD3/HDA1superfamily,theSIR2family,andthe plant-specific HD2 family(Pandeyetal.,2002). In Arabidopsis, histone acetylation and deacetylation are involved in various biological processes such as flowering time, leaf development, seed abortion, and abiotic stress responses (Wuetal.,2000, 2008;Dangletal.,2001;Heetal.,2003; SridhaandWu,2006;Uenoetal.,2007;Luoetal., 2012a, 2015). The rice genome contains at least 19 HDAC genes (Hu et al., 2009), including at least two HD2 genes -- *Histone deacetylase 701* (*HDT701*) and *Histone deacetylase 702* (*HDT702*)-- based on phylogenic analysis (Fu et al., 2007). *HDT702* RNAi plants have smaller-diameter stems and much narrower leaves, implying that this gene hasa roleincelldivisionorgrowth(Huetal.,2009). *HDT701* encodes a histone H4 deacetylase that reduces acetylation levels at the $5^{th}$ and $16^{th}$ lysine residues of histone H4. Its overexpression makes rice plants more susceptible to *Magnoporthe oryzae* and *Xanthomonas oryzae* pv. oryzae whereas *HDT701* RNAi plants are resistant to those pathogens. This suggests that *HDT701* functions as a negative regulator in plant innate immunity by modulating histone H4 acetylation of defense-related genes in rice (Ding et al., 2012). Overexpression of *HDT701* alsoleads to late seed germination due to decreasedhistoneH4acetylationandreducedexpressionofGA-biosyntheticgenes. In addition,

*HDT701*-overexpression transgenic plants display enhanced resistance to salt and osmotic stresses during the seedling stage, thereby denoting the role this gene has in seed germination and responses to abiotic stresses (Zhao et al., 2015). Finally, overexpression of *HDT701* accelerates flowering under natural LD conditions by repressing Os*GI* and *Hd1* (Li et al.,2011).

**1-7. Abiotic stress and flowering time**

Abiotic stresses such as drought, salinity, etc. remarkably influence on plant development including flowering. Abiotic stress factors are also key regulators implicated in the floral transition from the vegetative phase. In response to abiotic stress stimuli or factors, flowering time is either accelerated to set seeds for the next generation or deterred by decelerating their metabolism. How plants differently response to the external stresses or stimuli depends on the concentration of the stimuli, its genetic background and developmental stage. Several genes also have a function role as a regulatory element in controlling both flowering time and abiotic stress tolerance (Kazan and Kyons, 2015; Cho et al., 2017).

Drought, one of the major abiotic stresses, poses a brutal impact on many arable land worldwide. Levels of atmospheric moisture and regional precipitation patterns are altered by global warming, resulting in asymmetric water distributions that trigger drought stress in plant ecosystems. Aspects of plant growth and development, including flowering time are affected when they are exposed to drought stress (Cho et al., 2017). Plants tend to promote flowering process during drought conditions to ensure the next-generation progeny via a phenomenon known as drought escape (Kazan and Kyons, 2015). For example, during drought stress, flowering time is accelerated in *Brassica rapa* and *Mimulus guttatus* (Franks et al., 2007; Jordan et al., 2015). In *Arabidopsis*, it is hastened under LD but postponed under SD, implying that drought mediated Flowering time in association with the photoperiodic flowering pathway (Cho et al., 2017). However, it can advance or delay flowering depending on the plant species. Flowering is inhibited by drought in rice (*Oryza*

*sativa* L.), maize (*Zea mays* L.), and quinoa (*Chenopodium quinoa* Wild.), while promoted in wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and soybean (*Glycine max* L) (Park et al., 2016). In Arabidopsis, GIGANTEA (GI) plays as a key regulator in the drought escape response. Under LD, drought stress induces the floral activators *FT* and *TWIN SISTER OF FT* (*TSF*), in a pathway dependent on GI and abscisic acid (ABA). Under SD, it represses *FT* and *TSF* by enhancing floral repressors. In rice, *Grain number, plant height, and heading date7* (*Ghd7*), which is a major floral repressor in photoperiod flowering time, also has a functional role in drought tolerance (Kazan and Kyons, 2015).

Higher degree of soil salinity also has a detrimental effect on growth and development of plants through osmotic and ionic stresses. High salinity significantly inhibits the floral transition in many plant species including Arabidopsis, rice, chickpea and iris. In Arabidopsis, BROTHER OF FT AND TFL1 (BET), a member of the FT/ TERMINAL FLOWER1 (TFL1) family, regulates both flowering time and abiotic stress response. This gene inducible by abiotic stresses such as ABA, drought, and osmotic stress functions as a negative regulator in flowering time. GI is also implicated in tolerance to salt stress by interacting with SPY, an O-linked β-N-acetylglucosamine transferase. *miR169*, which is inducible by cold, drought, and salt, also positively modulate flowering time in Arabidopsis. In rice, OsmiR393a advances flowering time while it functions as a negative regulator in salt tolerance (Park et al., 2016).

Elevated and low temperature also have a significant influence on flowering time like several other abiotic stresses. High temperature deters flowering in many plant species such as stiff brome, chrysanthemum, poinsettia, and okra. However, Flowering time of *Oncidium* hybrid orchid is also promoted by High temperature as a consequence of the increased production of ROS and low ascorbate ration mediated by cytosolic ascorbate peroxidase (cytAPX1).In Arabidopsis, flowering is advanced by high temperature (27°C instead of 23°C) under both LD and SD while it is delayed by lower temperature (16°C in contrast to 23°C) in LD conditions. Heat-inducible HEAT SHOCK PROTEIN 101 (HSP101) is reported to regulate flowering time and inflorescence number in addition to functioning in heat tolerance. LONG VEGETATIVE PHASE, NAC (NAM, ATAF1/2, and CUC2)-domain

transcription factor (LOV1) regulates both flowering time and cold tolerance by modulating CO in a GI-independent manner and cold stress response genes such as COR15a and KIN1 (Park et al., 2016).

The excessive or deficient status of certain nutrients in the soil also serve as important factor that regulate flowering time.The effect of external nutrient status on the flowering time of Arabidopsis is ecotype-dependent. ). Flowering of *Landsberg erecta* (Ler) is postponed in deficient nutrient status while that of ecotype Ler and Colombia (Col) is advanced when transferred to poor nutrient conditions from nutrient rich conditions. Flowering time of *Pharbitis nil*, an SD plant, is accelerated at the limited nutrient conditions under LD but not at the sufficient nutrient conditions. Under phosphorus (P) deficient conditions, flowering time is delayed in *Trifolium subterraneum* and Arabidopsis, but high P supply does not induce flowering in Arabidopsis. In Arabidopsis, flowering is also promoted by a nitrate deficiency under neutral (12 h/12 h) or SD (8 h/16 h, day/night) conditions (Cho et al., 2017). Flowering of delayed-flowering mutants in the photoperiod, GA, and autonomous floral signaling pathways can be induced by low nitrate conditions. Flowering is also earlier in plants with low endogenous nitric oxide (NO) level such as nia1nia2 mutants (Park et al., 2016).

**Chapter 2.** *HDT701* induces flowering in rice by repressing expression of *OsIDS1*.

## 2-1. Introduction

Flowering is one of the most crucial biological processes in plants because it is a prerequisite for the development of fruits and grains. Transition from the vegetative phase is the first step toward reproductive success. Therefore, producing flowers at the appropriate time is a key factor. Whereas early flowering shortens the vegetative phase to an insufficient period that often leads to reduced yields, deferred flowering may also contribute to yield losses when plants in temperate regions are exposed to characteristically colder temperatures later in the growing season. For rice (*Oryza sativa*), chilling at the grain ripening stage

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In this study, I observed the role of *HDT701* in determining flowering time by analyzing knockout (KO) mutants. The results demonstrated that this gene controls flowering time in rice mainly by suppressing *OsIDS1*, which is an upstream suppressor of *Ehd1* and florigens.

## 2-2. Materials and Methods

### Plant materials and growth conditions

In this study, I used the T-DNA mutant tagging line of *HDT701* that was screened 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, I accessed the 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). 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). Additional KO alleles of *hdt701* were generated in the 'Nipponbare' rice background through CRISPR/Cas9 techniques (Miao et al., 2013). The plantswere genotyped by sequencing the CRISPR/Cas9 target region using the genomic DNA extracted from leaf blades. 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).

**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.

**Vector construction and plant transformation**

For constructing the CRISPR/ Cas9 vector, the rational CRISPR/Cas9 target sequences with protospacer adjacent motifs were screened with the aid of the CRISPRdirect web server (http://crispr.dbcls.jp; Naito et al., 2015) to find potential target sequences with minimal off-target cleavage. A spacer sequence (AAAGATCATTCCAGCTCCCA) shown in Table 3 was cloned into entry vector pOs-sgRNA for monitoring the expression of sgRNA. The resulting recombinant entry vector, pOs-sgRNA, was further cloned into a destination vector, pH-Ubi-cas9-7, using the GatewayTM system (Miao et al., 2013). This construct was then introduced into *Agrobacterium tumefaciens* LBA4404 by the freeze–thaw method (An et al., 1989).

**Histochemical assay of GUS activity**

The plantswere grown for 6 d in MS media under continuous light. After vacuum-infiltration for 30 min, samples were kept overnight at 37°C in a GUS-staining solution containing 100 mM sodium phosphate, 5 mM potassium ferricyanide, 5 mM potassiumferrocyanide, 0.5% Triton X-100, 10 mM EDTA (pH 8.0), 0.1% X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid/cyclohexylammonium salt), 2% DMSO, and 5% methanol (Yoonet al., 2014). Chlorophylls were removed by sequentially incubating the samples in 70% and 95% ethanol at 60°C. The GUS-stained samples were then soaked for 30 to 60 min at room temperature in VISKOL clearing reagent (Phytosys LLC,New Brunswick, NJ, USA, http://visikol.com/). After resin-sectioning (10 µm thick), GUS activity was visualized with a BX61 microscope (Olympus, Tokyo, Japan).

**Chromatin-immunoprecipitation (ChIP) analysis**

Transgenic plants expressing HDT701-HA were used for ChIP analysis as previously reported (Yoon et al., 2017). Briefly, 2 g of leaf blade sample were incubated in 3% formaldehyde. After nuclei isolation, chromatins were fragmented to approximately 500- to 1,000-bp lengths by sonication. As an input, 1% of the sample was gathered before pre-clearing. Anti-HA monoclonal antibodies (#2367; Cell Signaling) were used for immunoprecipitation. Data were normalized according to the percent-of-input method (Haring et al., 2007). Tested areas were P1; - 1,886 ~ -1,766 bp, P2; - 1,633~ -1,484 bp, P3; - 1,139~ -1,265 bp, P4; - 953~ -808 bp, P5; - 252~ -143bp upstream from ATG on *OsIDS1* genomic region, respectively. P1; - 1,893 ~ -1,766 bp, P2; - 1,725~ -1,613 bp, P3; - 1,517~ -1,412 bp, P4; - 1,108~ -978 bp, P5; - 821 ~ -692 bp, P6; - 555 ~ -425 bp upstream from ATG on *SNB* genomic region, respectively. The PCR primers for ChIP are listed in Table 2. All assays were conducted at least three times, each involving three biological replicates.

## 2-3. Results

### Identification of late-flowering mutants

I identified a late-flowering mutant line, 1B-05907, by screening T-DNA insertion tagging lines in the paddy field. The T-DNA was inserted in the first intron of *HDT701* (Figure. 1A) and the transcript level for that gene was markedly decreased in the mutant (Figure 1B). That line displayed a phenotype of flowering that was delayed by about two weeks in the field (Figure 1C). Because flowering time is regulated by multiple pathways, including day length-preferential routes, I studied the mutant phenotypes under controlled SD and LD conditions. When compared with wild type (WT) controls, flowering of *hdt701-1* mutant plants was delayed by approximately two weeks under SD and three weeks under LD conditions (Figure 1D). This demonstrated that HDT701 is a constitutive activator of flowering regardless of day length.

In the T-DNA tagging line, the *GUS* coding region was inserted into *HDT701* at the

same orientation as the tagged gene. GUS analysis of that line showed a positive response, indicating that *HDT701* was translationally fused to *GUS*. It was previously reported that a translational fusion between a tagged gene and *GUS* can be made even when T-DNA is inserted within an intron (Wei et al., 2017). Analysis of the genomic DNA of the line revealed that only one copy of T-DNA was present in the entire genome, suggesting that GUS expression was likely due to a fusion between HDT701 and GUS. Histochemical GUS analysis of leaf blades showed that GUS signals were ubiquitous in the leaves, including phloem parenchyma cells and mesophyll cells (Figure 1E). This result is consistent with a previous report that *HDT701* is expressed in various organs (Zhao et al., 2015).

To confirm whether the delay in flowering time was indeed due to a mutation in *HDT701*, we generated additional alleles by the CRISPR/Cas9 method, designing a target site in the 5$^{th}$ exon of *HDT701* (Figure 2A) and obtaining five independent transgenic lines.Sequencing the flanking regions of that site revealed that CRISPR line #4 had deletions in both chromosomes and line #5 had a single-bp insertion, whereas line #1 did not carry any mutation. Further analyses of the two null mutant lines in the next generations (line #4 and #5) showed late floweringwhen compared to WT controls, while line #1 which has no mutation flowered at the same time as the WT (Figure 2B,C). These experiments confirmed that defects in *HDT701* delay flowering.

**Expression levels for floral regulators**

To elucidate the functional roles of *HDT701* in controlling flowering time, I monitored expression levels of previously identified genes that play critical roles in that event. I studied the effects of *hdt701* mutations under both SD and LD conditions because some regulatory

factors function differently depending upon day length. For example, *osgi* mutants display a significant delay in flowering under SD but only a slight delay under LD, indicating that *OsGI* controls flowering time preferentially under SD (Lee and An, 2015).

An earlier study showed that overexpression of *HDT701* represses the expression of *OsGI* and *Hd1*, and induces flowering under natural LD conditions (Li et al., 2011). To verify that expression of these genes was also affected in the KO mutants, I performed qRT-PCR experiments with plants grown under controlled LD conditions. Expression was examined at 49 days after germination (DAG) because florigens and most upstream regulatory genes are active at that time when plants are grown under LD (Lee et al., 2016).Leaves were sampled nine times (2- to 4-h intervals) the day to observe any diurnal patterns. I first analysed *HDT701* and confirmed that the gene was completely silent throughout the 24-h period in the *hdt7* mutant (Figure 3A). In the WT, the gene was expressed at higher levels in the dark but at reduced levels under illuminated conditions. This diurnal pattern of expression is similar to that previously reported (Li et al., 2011). Expression of *Hd3a* and *RFT1* was significantly lower in the leaves from mutant plants, indicating that the delay in flowering was due to reduced expression of the florigens (Figure 3B, C). *Ehd1*, an immediate upstream regulator of those genes, was also significantly affected by the mutation (Figure 3D). Activities of *OsGI* and *Hd1* were decreased in the *hdt701* mutant, especially during the dark period (Figure 3E, F). It had not been expected to make these observations because overexpression of *HDT701* in 'YS63' hybrid rice also reduces the expression of *OsGI* and *Hd1* (Li et al., 2011). If these genes were the main regulatory elements contributing to the flowering phenotype in the *hdt701* mutant, then the KO mutants should have flowered early because OsGI functions upstream of Hd1, a floral repressor under LD conditions. Therefore, the *OsGI–Hd1* pathway does not seem to be the main downstream route from *HDT701* to the florigens. Because the *hdt701* mutants flowered late under both SD and LD, the HDT701 target gene is likely a constitutive repressor that functions upstream of *Ehd1*. It was previously determined that two AP2 family genes, *OsIDS1* and *SNB*, are constitutive flowering repressors (Lee et al., 2014). Here, expression levels of the former were significantly increased in the mutant (Figure 3G) while those of the latter were not affected by the mutations (Figure 3H). These results

suggested that *OsIDS1* is downstream of HDT701. Expression levels of other constitutive repressors, i.e., *OsCOL4*, *OsLFL1*, *OsLF*, and *OsPhyB*, were not altered in the mutant (Figure 3I-L).

Because flowering by *hdt701* mutants was also delayed under SD, the expression levels of regulatory genes from plants grown under SD conditions were also measured. Mature leaf blades were sampled at 28 DAG, when the florigens started to be expressed in SD-grown plants. As it had been observed from the LD-grown plants, the mutants expressed no detectable levels of *HDT701* transcript (Figure 4A). Expression of the florigens and *Ehd1* was significantly lower in the mutant leaves than in the WT leaves (Figure 4B-4D). Transcript levels of *OsGI* and *Hd1* were also reduced in the mutants, as noted from LD-grown plants, and especially so under SD (Figure 4E, F). Because both *OsGI* and *Hd1* function as positive regulatory elements under SD conditions, their decreased expression should have caused late flowering, consistent with the mutant phenotype. Transcript levels of *OsIDS1* were reduced at all nine sampling times, as observed under LD conditions (Figure 4G). These results suggested that *OsIDS1* is an important regulator that functions downstream of *HDT701*. Expression was not altered for the other constitutive repressors -- *SNB1*, *OsCOL4*, *OsLFL1*, *OsLF*, and *OsPhyB* -- in mutant plants grown under SD (Figure 4H-L).

**HDT701 directly regulates the expression of *OsIDS1*.**

*HDT701* is an active histone H4 deacetylase that suppresses expression of target genes via histone deacetylation (Ding et al., 2012; Li et al., 2011; Zhao et al., 2015). To study how HDT701 might directly regulate *OsIDS1*expression, ChIP assays were performed using transgenic plants that express HA-tagged HDT701as well as transgenics expressing HA alone as a negative control. Four areas (P1, P2, P3, and P4) in the *OsIDS1* promoter region and one area (P5) in the 5′-untranslated region (UTR) were selected for the binding assay (Figure 5A). Results from the experiments with anti-HA antibodies showed that P4 was preferentially enriched in the chromatins expressing the HDT701-HA fusion protein when

compared with the chromatins from transgenic plants expressing HA tag alone (Figure 5B). However, chromatin enrichment in P1, P2, P3, and P5 was similar between the two types of transgenic plants.

As a negative control, ChIP assays on *SNB* chromatins was also performed because this gene encodes a protein that is highly homologous to IDS1. Six areas in the *SNB* promoter region were selected for the analysis using plants expressing HDT701-HA or HA tag alone(Figure 5A). The chromatin enrichment experiments with HA antibodies demonstrated that all six areas were selected equallyin the HDT701-HA and HA plants (Figure 5C). This implied that the promoter region of *OsIDS1* is a potential target of HDT701.

**Regulatory genes that function upstream of *HDT701***

To identify the regulatory genes that function upstream of *HDT701*, I elucidated its expression patterns in various flowering-time mutants. Transcript levels of *HDT701* were not changed in mutants defective in *OsPhyB* and *OsCOL4*, two positive regulators of *OsIDS1* (Figure 6A, B). Likewise, expression was not altered in the *hd1* and *osgi* mutants (Figure 6C, D).

**2-4. Discussion**

I investigated the role of *HDT701* in controlling flowering time using KO mutants generated by T-DNA insertions and CRISPR/Cas9. The mutant plants flowered later than the WT due to reductions in the expression levels of *Hd3a*, *RFT1*, and *Ehd1*. This indicated that *HDT701* is a floral activator that functions upstream of *Ehd1*. This result is consistent with other observations of *HDT701*-overexpression plants, which flower early because of induced expression of the three genes (Li et al., 2011). The previous experiments were conducted under natural LD conditions (Li et al., 2011). In the current study, I observed that the gene is

a constitutive repressor of flowering under both LD and SD. Because *HDT701* encodes histone 4 deacetylase, deacetylation of floral repressors would enhance florigen expression. Several histonedeacetylation (HDA) genes also control floweringtimeinArabidopsis(Heetal.,2003). Constitutive delayed-flowering phenotypes of mutants defective in *HDA5* and *HDA6* under both LD and SD conditions imply that histone deacetylation accelerates floweringtime in Arabidopsis, similar to that observed in my present study (c.f., Wuet al.,2008; Luo et al., 2015).

Histochemical staining of *hdt701* transgenic plants showed that *HDT701* is expressed not only in mesophyll cells but also in phloem parenchyma cells, indicating that the gene has multiple functions. In addition to its role in controlling flowering time, this gene is involved in plant innate immunity, GA biosynthesis, and abiotic stress responses (Ding et al., 2012; Zhao et al., 2015). Florigens as well as upstream regulatory genes such as *Ehd1* and *Ghd7* are preferentially expressed in phloem parenchyma [cells], whereas other regulatory genes such as *OsCOL4*, *Hd1*, *OsGI*, and *OsPhyB* are strongly expressed in mesophyll cells (Tamaki et al., 2007; Komiya et al., 2008; Xue et al., 2008; Lee et al., 2010; Saito et al., 2012). Therefore, these findings suggest that HDT701 may function in multiple pathways to influence flowering time.

In a previous study, Li et al. (2011) proposed that *HDT701* induces flowering by suppressing *OsGI* and *Hd1* under LD; this was based on observations that overexpression of the former caused a reduction in expression for the latter two. However, the decline in expression of *OsGI* in the *HDT701*-overexpression plants should have resulted in delayed flowering because OsGI is a flowering enhancer. That research group also reported that transcript levels of *OsGI* and *Hd1* were not altered under SD conditions. It was found here that transcript levels of the two upstream regulatory genes were reduced in *hdt701* KO mutants regardless of day length. This discrepancy might have been due to the cultivar used for generating the transgenic plants. Alternatively, overexpression of the gene may have caused side effects by forming unusual protein complexes.

I identified *OsIDS1* as being downstream of *HDT701* because expression of the former was significantly enhanced under both SD and LD in the *hdt701* mutant. Direct interaction

of HDT701 on *IDS1* chromatins was indicated by the ChIP assay. IDS1 is a member of the AP2 family, which is involved in various processes (Lee et al., 2014). For example, six Arabidopsis members in this family delay flowering and are suppressed by *miR172* (Lee et al., 2014). Similarly, increasing expression of *Zea mays* GROSSY 15, an AP2 member, delays flowering (Zhu and Helliwell, 2011). It was previously reported that rice AP2 members IDS1 and SNB act as negative regulatory elements in flowering, and their transcripts are targeted by *miR172* (Lee et al., 2014). Although *SNB* is closely related to *IDS1*, its transcript levels were not affected in *hdt701* mutants. This suggests that HDT701 specifically selects *IDS1* chromatin even though the chromatin-remodeling factor appears to target multiple genes.

In *hdt701* mutants, the mRNA levels of *OsGI* were constitutively down-regulated. That gene plays a positive role in enhancing florigen expression and flowering induction under both LD and SD, although the effect is more severe under SD (Lee et al., 2015). Therefore, the delayed flowering phenotype of the mutant could be explained by lower expression of *OsGI*. However, that reduction in expression was not very significant under LD, although the delay in flowering by *hdt701* mutants was equally significant under both LD and SD.

Transcript levels of *Hd1* were also significantly diminished regardless of day length. Because *OsGI* positively controls the expression of *Hd1* (Hayama et al., 2003),the decrease in expression for *Hd1* could have resulted from the downregulation of *OsGI* in the mutants. Although Hd1 advances flowering under SD, the regulatory element inhibits flowering under LD. Therefore, the reduction in *Hd1* expression in the *hdt701* mutant under LD would accelerate flowering rather than suppress that process. Therefore, I conclude that the delay in flowering by the mutants under LD was not due to an alteration of the *OsGI* and *Hd1* pathway. It is probable that *HDT701–IDS1–Ehd1* is the major pathway under LD. However, under SD, both the *HDT701–OsGI–Hd1–Ehd1* and *HDT701–IDS1–Ehd1* pathways appear to modulate florigen expression (Figure 7).

**2-5. Figures**

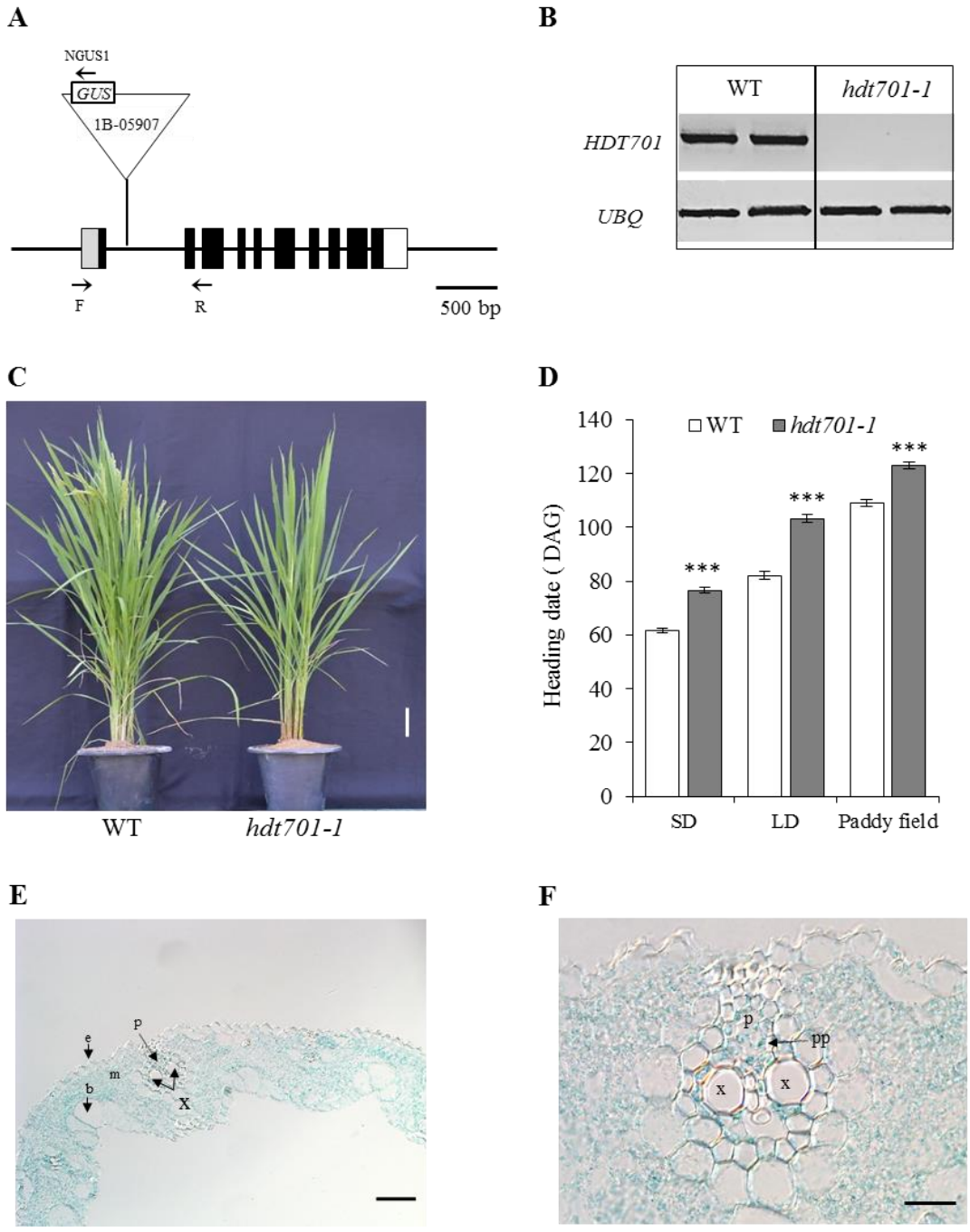
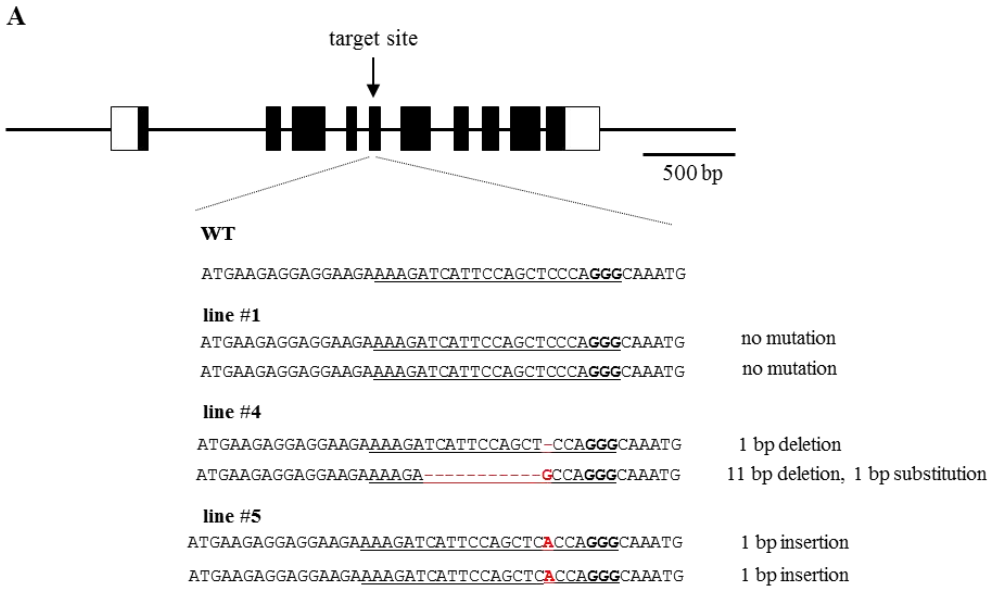
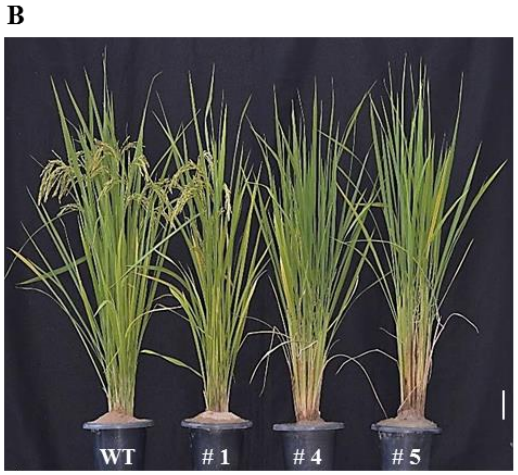
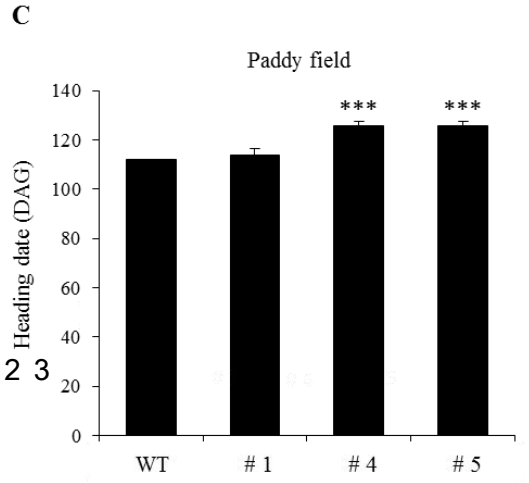



**Figure 1.** Schematic diagram of gene structure of *HDT701* and comparison of flowering time between WT and *hdt701-1* mutants. (A) Gene structure of *HDT701*. Black boxes indicate ex

ons 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. T he 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. (C) Phenotypes of *h dt701-1* and WT at heading stage under paddy field conditions. Scale bar, 10 cm. (D) Days t o heading of WT and *hdt701-1* plants under SD, LD, and natural paddy field conditions. DA G, days after germination. (E) GUS-staining of a cross-section of leaf blade from Line 1B-05 907. (F) A close-up picture of the leaf section at the vasculature region. b, bulliform cells; e, epidermis; m, mesophyll cells; p, phloem; pp. phloem parenchyma; x, xylem. Scale bars , 50 µm(E) and 20 µm (F).




2 3

**Figure 2.** Generation of another *hdt701* alleles by CRISPR/Cas9 method. (A) Schematic diagram of gene structure of *HDT701* and sequence alignment of the sgRNA target region displaying altered bases in the mutant lines. Target region for the vector construction is underlined. Altered DNA sequences are indicated by red color. (B) Phenotypes of the *hdt701* CRISPR/Cas9 KO lines at heading stage. Scale bar, 10 cm. (C) Days to heading of WT, *hdt701* CRISPR/Cas9 KO #1, 4 and 5 under natural paddy field conditions. Days to heading was scored when the first panicle bolted. Error bars indicate standard deviations; n = 10. Levels of significant difference are indicated by **P < 0.01; ***P < 0.005.

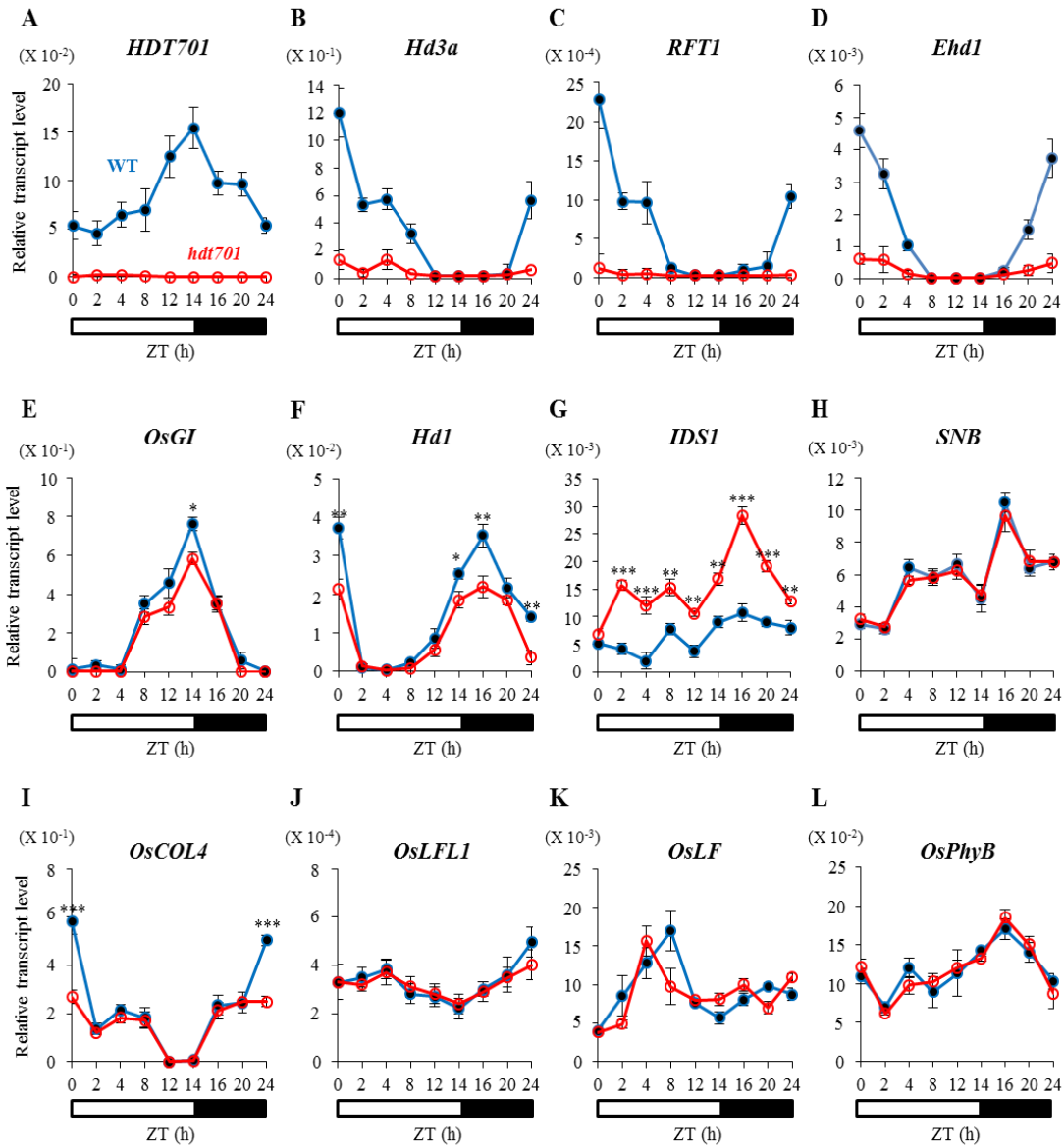



**Figure 3**. Diurnal expression patterns of floral regulators in leaf blades of WT and *hdt701-1* plants at 49 DAG under LD. Quantitative RT-PCR analyses of *HDT701* (A), *Hd3a* (B), *RFT1* (C), *Ehd1* (D), *OsGI* (E), *Hd1* (F), *IDS1* (G), *SNB* (H), *OsCOL4* (I), *OsLFL1* (J), *OsLF* (K) and *OsPhyB* (L). Close circles, WT; open circles, *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), and *** (P <0.005).

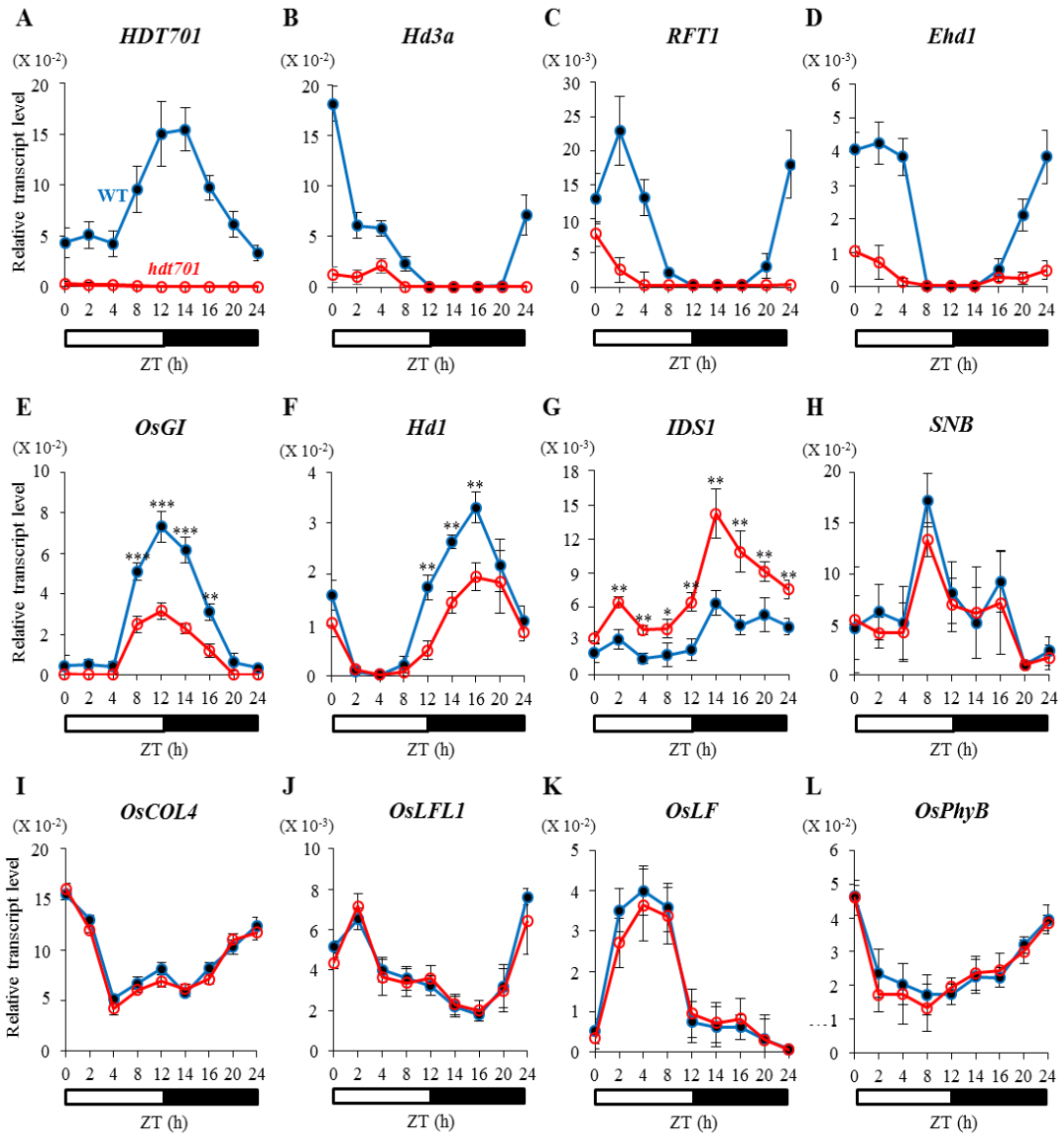



**Figure 4**. Diurnal expression patterns of floral regulators in leaf blades of WT and *hdt701-1* plants at 28 DAG under SD. Quantitative RT-PCR analyses of *HDT701* (A), *Hd3a* (B), *RFT1* (C), *Ehd1* (D), *OsGI* (E), *Hd1* (F), *IDS1* (G), *SNB* (H), *OsCOL4* (I), *OsLFL1* (J), *OsLF* (K) and *OsPhyB* (L). Close circles, WT; open circles, *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), and *** (P <0.005).

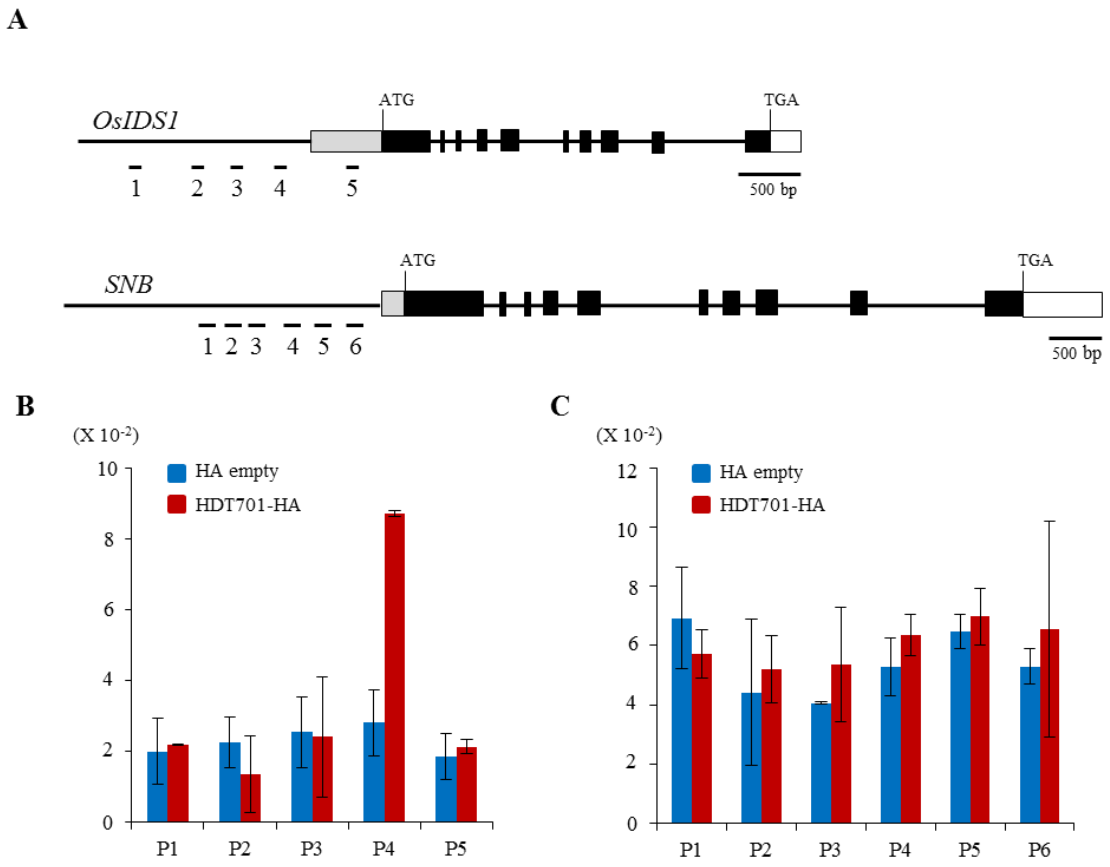



**Figure 5.** Chromatin immunoprecpitation (ChIP) analyses of *OsIDS1* chromatin and SNB chromatin (A) Genomic structures of *OsIDS1* and *SNB*. Tested areas were P1; - 1,886 ~ -1,766 bp, P2; - 1,633~ -1,484 bp, P3; - 1,139~ -1,265 bp, P4; - 953~ -808 bp, P5; - 252~ -143bp upstream from ATG on *OsIDS1* genomic region, respectively. P1; - 1,893 ~ -1,766 bp, P2; - 1,725~ -1,613 bp, P3; - 1,517~ -1,412 bp, P4; - 1,108~ -978 bp, P5; - 821 ~ -692 bp, P6; - 555 ~ -425 bp upstream from ATG on *SNB* genomic region, respectively. . (B) ChIP analysis of HDT701 enrichment on *OsIDS1* chromatin. HDT701-HA-tagged transgenic plants were used to detect enrichment. Transgenic plants expressing HA tag alone were used as a control. Leaf blades were harvested at 30 DAG from the transgenic plants. The percent of input method was used for normalization. (C) ChIP assay of HDT701-HA enrichment on *SNB* chromatin as described in Panel B.

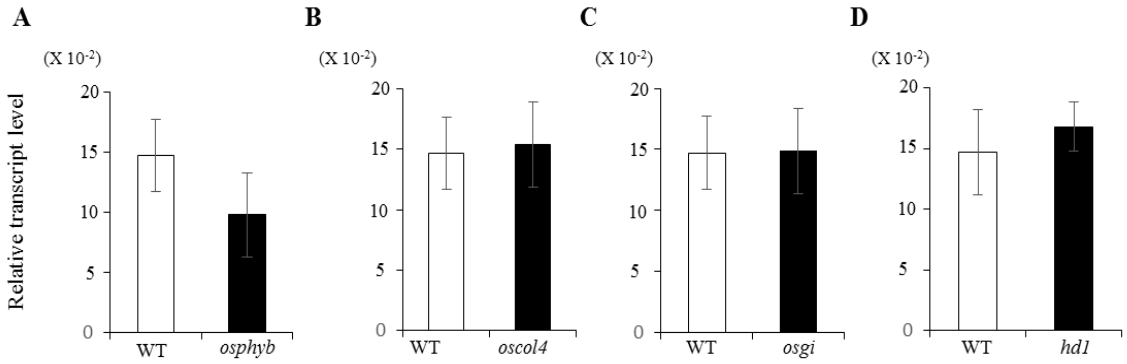



**Figure 6.** Expression levels of *HDT701* in *osphyb* (A), *oscol4* (B), *osgi* (C),and *hd1* (D). Total RNAs were prepared from leaf blades at 42 DAG under LD. Error bars display standard deviations; n = 4.

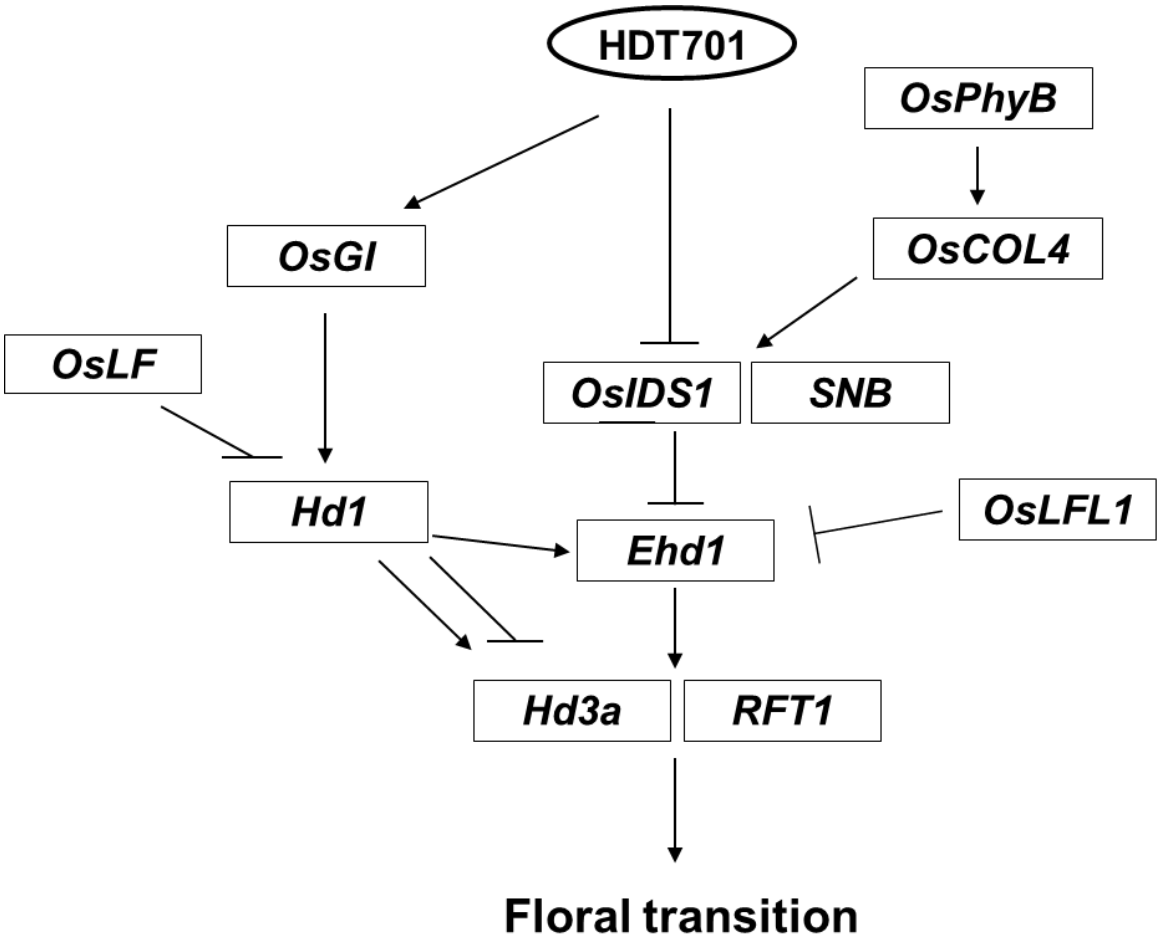



**Figure 7.** A model for regulatory pathway governed by HDT701 in the control of flowering time.

**2-6. Tables**

**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 |
| *OsGI*_RT_F | TGGAGAAAGGTTGTGGATGC |
| *OsGI*_RT_R | GATAGACGGCACTTCAGCAGAT |
| *Hd1*_RT_F | AACCAAGATCGGCAGTATGG |
| *Hd1*_RT_R | GATTGATTGCTCCAGCAGGT |
| *Ehd1*_RT_F | GTTGCCAGTCATCTGCAGAA |
| *Ehd1*_RT_R | GGATGTGGATCATGAGACAT |
| *Hd3a*_RT_F | AGCCCAAGTGACCCTAACCT |
| *Hd3a*_RT_R | GTTGTAGAGCTCGGCGAAGT |
| *RFT1*_RT_F | TGACCTAGATTCAAAGTCTAATCCTT |
| *RFT1*_RT_R | TGCCGGCCATGTCAAATTAATAAC |
| *OsCOL4*_RT_F | ATCCACTCGGCGAACCCGCT |
| *OsCOL4*_RT_R | CGCTTCTCCCTGTACCGCAT |
| *OsPhyB*_RT_F | ATGGAACAGACACAATGCTT |
| *OsPhyB*_RT_R | AGCATACACCATATCAGCTT |
| *OsIDS1*_RT_F | CTGGCCTCCAGTTAACTTGT |
| *OsIDS1*_RT_R | GGCGCCGGCAGAGAATCCT |
| *OsLF*_RT_F | AACCCTAGGGAATGGCAATG |
| *OsLF*_RT_R | CGCCCAAATGCAAGTACAGT |
| *OsLFL1*_RT_F | CAAAATGCACAACTCTGGACC |
| *OsLFL1*_RT_R | ACCACTTCCCTGTCAGTCTCAC |
| *SNB*_RT_F | ATGGAAGGGAAGCTGTTAC |
| *SNB*_RT_R | AATGTGGATGCTGGGACATC |

**Table 2.** List of primers used for ChIP assay in this study.

| Name | Sequence (5'-3') |
|---|---|
| *OsIDS1*_P1_F | CACGATTTCCTCCCTAACTA |
| *OsIDS1*_P1_R | GCCCTGTTTAGTTCCCAAAT |
| *OsIDS1*_P2_F | ACACATCCTAAAACGGCTGC |
| *OsIDS1*_P2_R | CCATTGCCCTCCACTTCAAC |
| *OsIDS1*_P3_F | ACTATCCAACAAGAGGGTAC |
| *OsIDS1*_P3_R | GACACATGGCCATTCATATC |
| *OsIDS1*_P4_F | CGGAAGCTCTAAAGAACGTT |
| *OsIDS1*_P4_R | GACGTTGTCAAGGTGGTTAT |
| *OsIDS1*_P5_F | CCTCTTCTTCTTCATCCAAC |
| *OsIDS1*_P5_R | AGTGAGTCGTCGTCAGTCGA |
| *SNB*_P1_F | GAAACTACACCGGTGGATAT |
| *SNB*_P1_R | TGACATGATGTATCTGCAGG |
| *SNB*_P2_F | GTTTGCTCCTTTGATATTTATA |
| *SNB*_P2_R | TGAAGTCTAACTCAGCTTCTG |
| *SNB*_P3_F | GGAATATTATGGAATGGTGGAA |
| *SNB*_P3_R | TAAGCTAACGGGCAAACGAT |
| *SNB*_P4_F | AGCCAACAATGCTAGCTTAG |
| *SNB*_P4_R | TCGACTTATAACACGGTTGG |
| *SNB*_P5_F | GCAATGTCGAGTGGAAAATAC |
| *SNB*_P5_R | CTTGAAAGAGTTTGATTTTGACC |
| *SNB*_P6_F | ACCTGAAGCAGTTTAACTTTGAT |
| *SNB*_P6_R | GAGTGTGCTATGCTTTGTTTG |

**Table 3.** List of primers used for raising transgenic plants in this study.

| Name | Sequence (5'-3') |
|---|---|
| *HDT701*_OX_HindIII_F | AAGCTTTAGCTCCGCCTCCCACCT |
| *HDT701*_OX_SpeI_R | ACTAGTCTTGGCGGGGTGCTTGGC |
| *HDT701*_CRISPR_F | GGCAAAAGATCATTCCAGCTCCCA |
| *HDT701*_CRISPR_R | AAACTGGGAGCTGGAATGATCTTT |

## Chapter 3. *Histone Deacetylase 701* enhances salt and osmotic stress resistance in rice by suppressing expression of *OsWRKY45.*

### 3-1. 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 a 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 molecules 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 (Kishor 1985; Bohra et al., 1995; Gurmani et al.,2013). 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 ABAsignaling in guard cells (Kwak et al., 2003; Jiang et al., 2012; Kumar et al., 2013; Rejeb et al., 2015). 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 I revealed that *HDT701* might improve salt and osmotic stress tolerance by suppressing *OsWRKY45*, an upstream repressor of *SNAC1*.

## 3-2. Materials and methods

### Growth conditions and 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 (MurashigeandSkoog,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 together with control plants at 1, 3 and 6

h after treatment. For salt stress, the seedlings were transferred to MS medium with 300 mM NaCl solution and sampled together with control plants at 1, 3 and 6 h after treatment. For ABA hormone treatment, seedlings were transferred to MS medium with 100 μM ABA and sampled together with control plants at 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.

**Statistical analysis**

Student's t-test was performed using the online tool available at http://www.physics.csbsju.edu/stats/ttest bulk form.html to analyse the significant differences between the control and treatment of thesamples or between control and transgenic plants.

**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 *ΔΔ*CT method was applied to calculate changes in relative expression.All primers for quantitative real-time PCR are listed in Table 4.

**3-3. Results**

I investigated the expression patterns of *HDT701* and *HDT702* under abiotic stress conditions in which plants 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 after 1 h treatment with ABA, but it recovered after 3 and 6 h treatment with ABA (Figure 8A). Likewise, its expression is also attenuated considerably after 1 h treatment with NaCl as well as PEG, but it recovered after 3 and 6 h treatment with NaCl and PEG (Figure 8A). The mRNA levels of *HDT702* were reduced after 1h ABA exposure, but they were increased after 3 and 6 h ABA exposure (Figure 8B). Its expression levels are also detected to be increased after 3 and 6 h NaCl and PEG exposure (Figure 8B).I also analysed their expression

patterns under various abiotic stress treatments using data series downloaded from NCBI GEO Series and found that they are regulated by the abiotic stress treatments (Figure 8C).Taken together, the resulting data suggests that expression patterns of *HDT701* and *HDT702* might be controlled by abiotic stresses.

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

Overexpression of *HDT701* in rice improved salt and osmotic resistance during the seedling stage as previously reported (Zhao et al., 2015). In this study, I used *hdt701* KO seedlings 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 higher level of sensitivity to both salt and osmotic stresses at the recovery stage in comparison with the control seedlings (Figure 9A). Thesurvivalrateofthe mutantswassignificantly lowerthanWTseedlingsabout 30% in the salt stress and about 40% in the osmotic stress (Figure 9B).This result impliesthat*HDT701*has an important role in the abiotic stress endurance of riceattheseedlingstage.

**Expression analysis of abiotic stress- related genes**

To ascertain the molecular pathway controlled by *HDT701* in abiotic stresses, I also analysed the expression levels of previously identified genes that are important in the adaptive stress response of rice. 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 promote 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 were significantly decreased ($P < 0.05$) in the mutants compared to the WT (Figure 10D,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 *OsABA1* and *OsABA2*, the genes that are critical in the ABA biosynthesis, were also examined to verify if other ABA biosynthesis genes are also modulated by *HDT701* during the abiotic responses of rice. *OsABA1* 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 10G,H), implying that *HDT701* might regulate the expression of *OsPSY3* and *OsNCED4* in ABA biosynthesis pathway to improve salt and osmotic stress tolerance.

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 abiotic stress adaption responses of rice. Overexpression of *SNAC1* significantly promote tolerance to drought and salt stresses and several stress-related genes were up-regulated in the *SNAC1*-overexpressing plants (Hu et al. 2006). Thus, the expression of that gene was investigated and observed that its transcript level was significantly downregulated ($P < 0.01$) in the mutants (Figure10B). 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. 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 salt and drought 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*(Figure10F).

It was previously reported that *OsWRKY45* alleles plays an important role in abiotic stress tolerance of rice. Expression of both *OsWRKY45* alleles, *OsWRKY45-1* in IRAT109 cultivar and *OsWRKY45-2* in Zhenshan 97 cultivar, are regulated by several abiotic stress conditions. Overexpression of both *OsWRKY45* alleles in rice shows reduced tolerance to cold and drought stresses while both OsWRKY45-suppressinglines are more tolerant. *OsWRKY45-2*-overexpression plants also

displays higher level of sensitivity to salt stress compared to the corresponding controls while the RNAi plants exhibits higher level of tolerance. 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* arerepressed 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 10A). This result suggests that *HDT701* might enhance abiotic stress resistance of rice by suppressing *OsWRKY45*.

An NADPH oxidase gene, *OsrbohI*, accelerates 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 investigated. The decrease transcript level of the gene in *hdt701* mutants (Figure 10C) implies that *HDT701* might also regulate salt tolerance of rice through ROS pathway via *OsrbohI* in ABA dependent manner.

## 3-4. Discussion

I also scrutinized the function of *HDT701* in tolerance of rice to salt and osmotic stresses by using KO mutant plants raised by T-DNA insertion and showed that the mutant plants are more sensitive to both stresses compared to the corresponding controls. The number of surviving plants are remarkably reduced in the mutant seedlings under treatments with both osmotic stressors. This observation is in good agreement with aprevious 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 higher 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 (Fu et al., 2007; Zhao et al., 2015). The expression of *HDT701* was decreased 1 h after exposure to ABA as well as 1 h exposure to NaCl and PEG, but it recovered at 3 and 6 h after exposure to ABA, NaCl and PEG. *HDT72* was repressed at one after treatment with ABA, but increased at 3 and 6 h treatment with ABA, NaCl and PEG. These results indicated that the expression levels of *HDT701* and *HDT702* were altered under abiotic stress treatments, which is consistent with that previously reported (Fu et al., 2007; Zhao et al., 2015). Moreover, the expression of

Arabidopsis orthologs *AtHD2A*, *AtHD2B*, *AtHD2C*, and *AtHD2D* 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 resistance of rice, I also analysed the expression patterns of the previously reported genes responsible for the adaptive response to abiotic stress and revealed that the expression of *SNAC1*, *NCED4*, *OsPY3* and *OsrbohI* was significantly decreased while *WRKY45* was greatly induced in the mutant plants in comparison with the control wild type plants. However, the transcript levels of *OsABA1*, *OsABA2* 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 suggests that *HDT701* is a positive regulator that functions upstream of *SNAC1* in the rice response to abiotic stresses.

*NCED4* and *OsPY3* are ABA biosynthesis genes inducible by salt 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 lower 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 *OsABA1* and *OsABA2*,other *ABA* biosynthesis genes, in the mutant plants implies that *HDT701* might improve abiotic stress response by modulating 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 response of rice. It negatively functions in the abiotic resistance of rice by repressing *SNAC1* and *NCEDC4* and overexpression of this gene shows enhanced susceptibility to salt, drought and cold stresses (Tao et al., 2011).This observation is well concomitant with the current result in which expression of *SNAC1* and *NCEDC4* was reduced while that of *OsWRKY45* is up-regulated in the mutants and *hdt701* mutant plants are more sensitive to salt and osmotic stresses. Thus, I identified *OsWRKY45* as a putative target of HDT701 because only the expression of the former was significantly enhanced in the *hdt701* mutants under salt stress.

*OsrbohI*, an NADPH oxidase gene, contributes to the production of ROS (Wong et al., 2007). The expression of *OsrbohI* was found to be significantly suppressed in the mutants. The reduced transcript level of the gene may lead to the lower 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 endurance 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 lower resistance to soil salinity in Arabidopsis (Kwak et al., 2003; Jiang et al., 2012; Rejeb et al., 2015). This previous studies are well related to the current result of lower expression level of *OsrbohI* and reduced tolerance of the mutant plants. Together, this observation further suggests that *HDT701* might also mediate the abiotic stress response through ROS pathway by enhancing *OsrbohI* in addition to suppressing the expression of *OsWRKY45* (Figure 11). However, further investigation is necessary to evaluate if *HDT701*enhances tolerance of rice to osmotic stressors by directly repressing *OsWRKY45.*

## 3-5. Figures

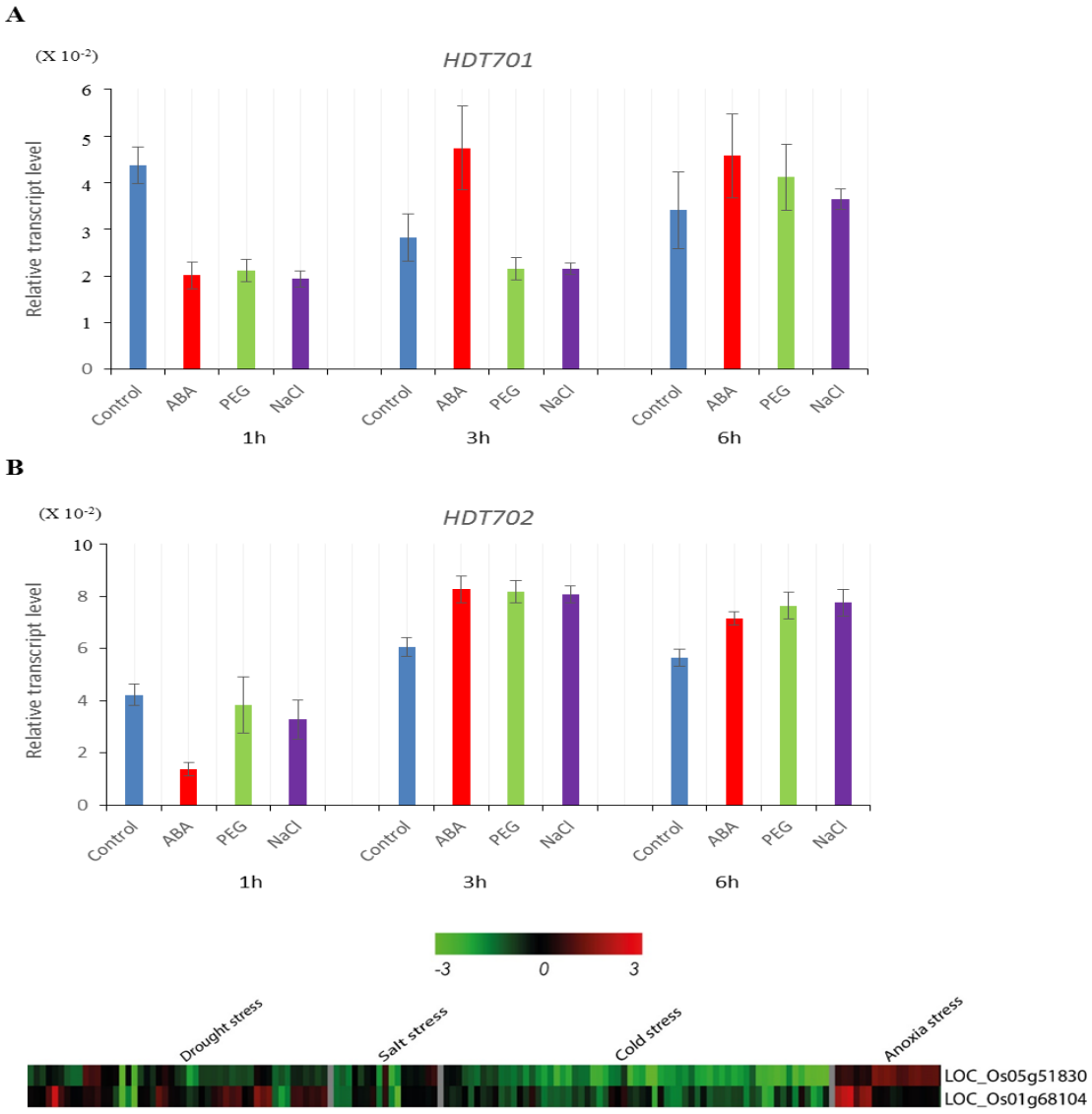



**Figure 8.** Expression patterns of rice *HDT701* (A) and *HDT702* (B)under ABA, salt and PEG stresses. Two-week-old rice seedlings were exposed to no treatment (blue bar), or 100 μM ABA (red bar), 300 mM NaCl (purple bar), and 20% PEG (light green bar) for 1, 3 and 6 h, respectively. y-axis, relative transcript level of each gene compared with that of rice *Ubi*. Error bars indicate standard deviations; n = 4.(C) Expression analysisof *HDT701* and *HDT702*. Dataseries GSE6901, GSE16108 and GSE21651 were downloaded from NCBI GEO Series and normalized using affy package. Heatmaps were visualized using MeV Software.

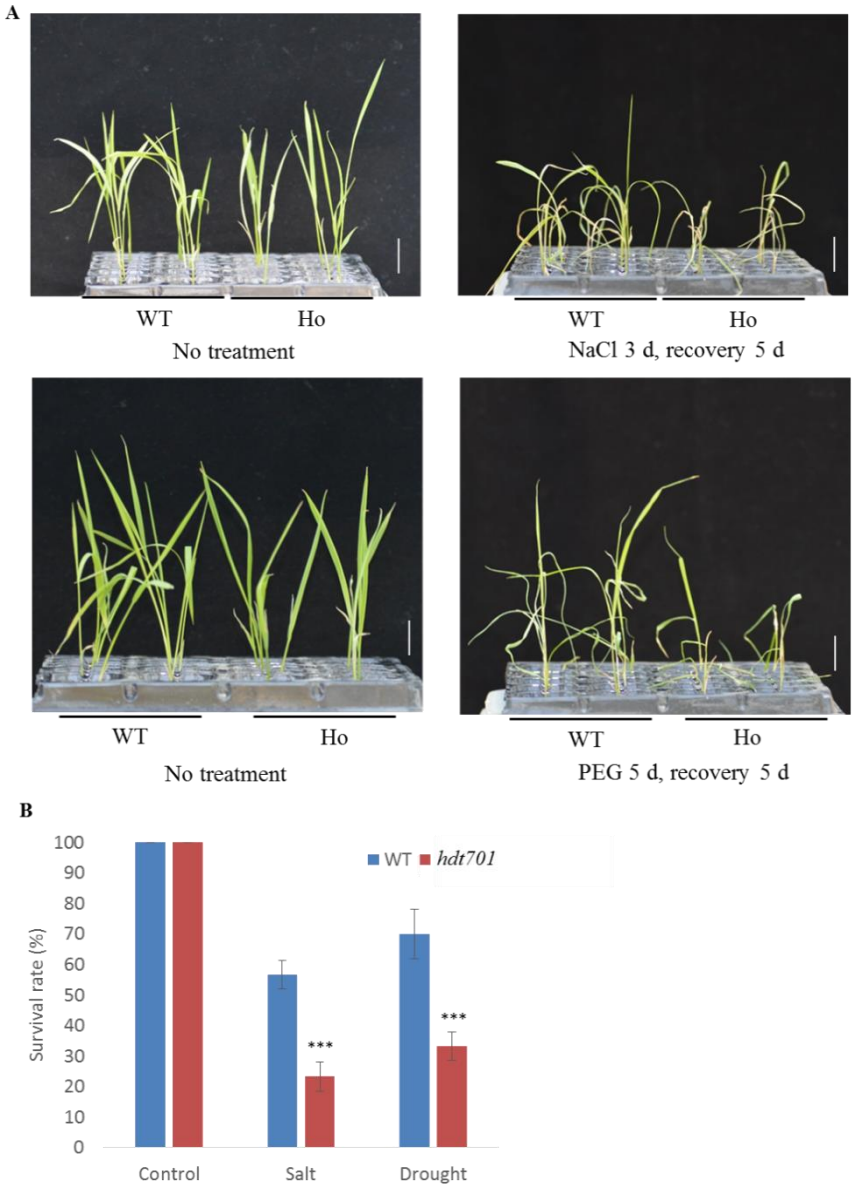



**Figure 9.** Mutation in *HDT701* attenuates tolerance to salt and osmotic stresses (A) Phenotype of *hdt701* mutant seedlings under salt and osmotic stresses. Scale bar: 5 cm. Mutant seedling and the wild type seedling were exposed to 150mM NaCl or 20% PEG for indicated days and recovered in MS medium (B) Survival rates of *hdt701* mutantseedlings after NaCl and PEG treatment.n = 10. Levels of significant difference are indicated by *P < 0.05; **P < 0.01.

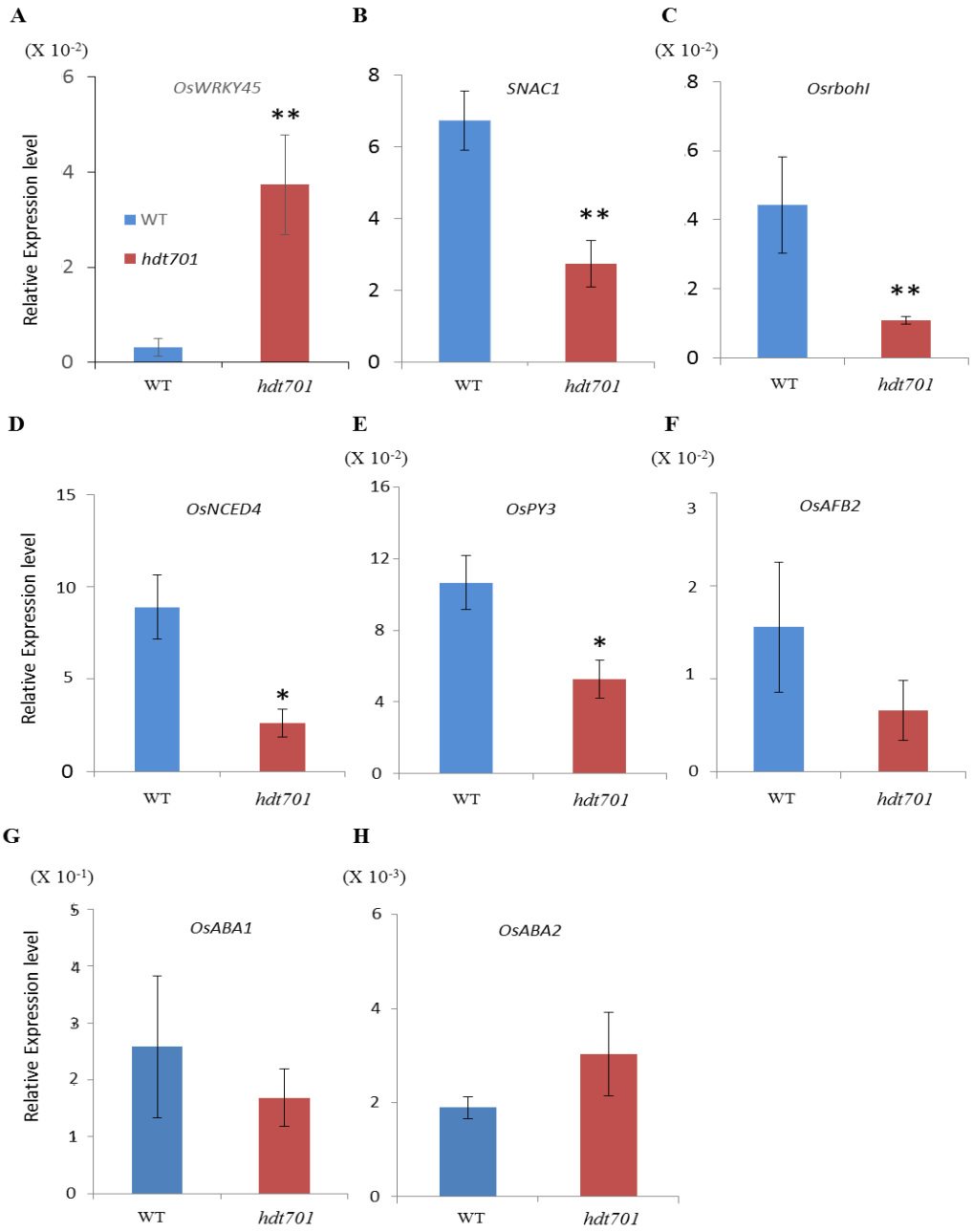



**Figure 10**. Expression patterns of abiotic stress-related genes in leaf blades of WT and *hdt701-1* plants at 14 DAG under salt stress. Quantitative RT-PCR analyses of *OsWRkY45* (A), *SNAC1* (B), *OsrbohI* (C), *OsNCED4* (D), *OsPY3* (E), *OsABF2* (F), *OsABA1* (G) and *OsABA2* (H),. Blue bar, WT; 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.

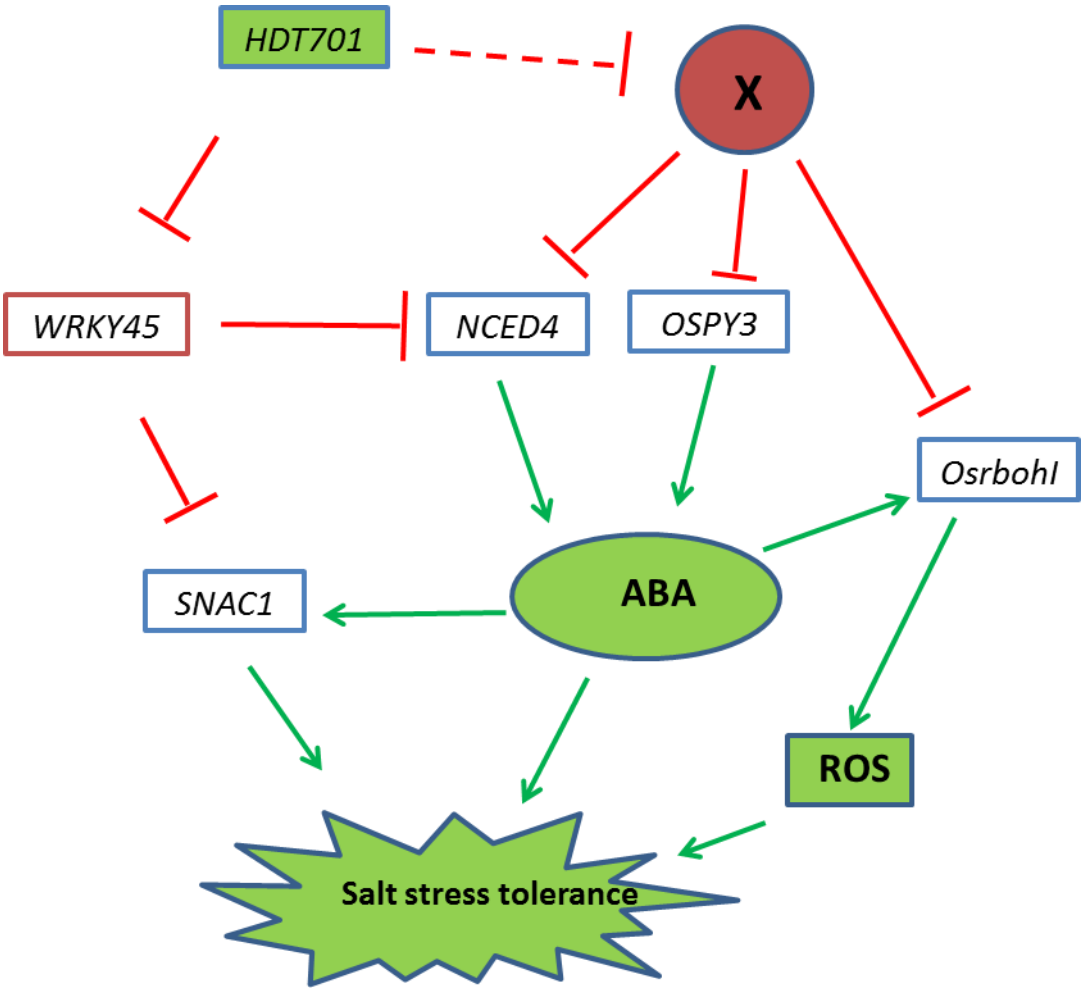



**Figure 11**. A model for regulatory pathway mediated by *HDT701* in the salt stress tolerance in rice

### 3-6. Tables

**Table 4. 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 |

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