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HD2D is a Regulator of Abscisic Acid Responses in Arabidopsis

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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(Thesis format: Monograph)

by

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Abstract

Histone deacetylases have important roles in development and stress response in plants. To further investigate their function, the *HD2D* gene, of the plant specific HD2 family, was studied. An *hd2d-1* mutant and two *HD2D* overexpression lines were used in this study. Germination was delayed in *hd2d-1* and *HD2D* overexpression seeds only in the presence of ABA. *HD2D* was found to positively regulate the expression of members of the ABA-response pathway (*ABI1*, *ABI5*, and *RD29A*) leading to increased resistance to drought and salinity treatments. Furthermore, *HD2D* expression delayed flowering by positively regulating *FLC* expression. Using bimolecular fluorescence complementation, the HD2D protein was found to interact with the ABA pathway members ABI1, ABI2, and ABI5. Taken together, the results of this study suggest that *HD2D* is a regulator of ABA responses in Arabidopsis. By expanding the knowledge of plant stress response, this research will help lead to long-term improvements of drought tolerance.

Keywords

Histone deacetylation, ABA-stress response, flowering time, ABA, ABI5, ABI1, drought

Co-Authorship Statement

All work in this thesis was conducted by Joshua Farhi with the exception of selection of *hd2d-1* mutant line, generation of *HD2D* overexpression lines, and generation of BiFC constructs. Collection of F_1 and F_2 seeds from the *hd2d-1* mutant line (GABI-Kat_379G06) and the construction of the associated figure (Fig. 3.1A and B) were conducted by Dr. Gary Tian. Furthermore, generation of the *HD2D* overexpression vector and transformation of Arabidopsis was conducted by Dr. Gary Tian, with the associated figure (Fig. 3.1C and D). The 1-1 and 4-1 homozygous overexpression lines were selected by Joshua Farhi. Vectors used in bimolecular fluorescence complementation experiments were generated by Dr. Yinjie Luo and Dr. Gary Tian.

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List of Abbreviations

ABA	abscisic acid
ABI	abscisic acid insensitive
ABRE	abscisic acid response element
ANOVA	analysis of variance
Arabidopsis	*Arabidopsis thaliana*
BiFC	Bimolecular fluorescence complementation
bZIP	basic leucine zipper
CaMV 35s	cauliflower mosaic virus 35s constitutive promoter
ChIP	chromatin immunoprecipitation
co-IP	co-immunopreciptation
ddH_2O	double-distilled H_2O
DNA	deoxyribonucleic acid
HAT	histone acetyltransferase
HDAC	histone deacetylase
K	lysine (amino acid)
PCR	polymerase chain reaction
PP2C	protein phosphatase 2C
qPCR	quantitative polymerase chain reaction
RCAR	regulator component of abscisic acid receptor
SnRK	SNF1-related protein kinase
T-DNA	transfer DNA
TSA	trichostatin A
YFP	yellow fluorescent protein

Chapter 1: Introduction

1.1 Eukaryotic chromatin

The highly compact form of eukaryotic genomic DNA is, in part, due to its association with histone and non-histone proteins to form chromatin (Sanchez et al., 2008). The basic unit that makes up chromatin is a repeating nucleosome core wrapped by 146 base pairs of DNA that fold 1.65 times around the nucleosome in 7.6 super-helical turns of DNA. The highly conserved nucleosome is made up of a histone octamer, consisting of two of H2A, H2B, H3, and H4 histone proteins (Kornberg, 1974; Workman and Kingston, 1998; Verreault, 2000). This "beads on a string" model is stabilized by an additional histone H1 protein present on the linker DNA between nucleosomes (McGhee and Felsenfeld, 1980). The N-terminal tails of H2B and H3 histones have been shown to directly interact with the DNA, aiding in compaction. Furthermore, the N-terminal tails of both H3 and H4 histones are subject to post-translational modifications that can affect chromatin compaction in adjacent regions (Workman and Kingston, 1998).

1.2 Chromatin compaction affects transcription

The level of chromatin compaction has very important implications for DNA function, specifically transcription. Areas of lower chromatin compaction are referred to as euchromatin and tend to be transcriptionally active. In contrast, regions that are more compact are referred to as heterochromatin and tend to be transcriptionally inactive (Sanchez et al., 2008). The influence of chromatin compaction on transcription is associated with the ability of protein complexes and transcriptional machinery to access the DNA (Margueron and Reinberg, 2010).

The ability to regulate transcription by controlling the extent of chromatin compaction is a powerful mechanism of controlling gene expression, playing an important role in multicellular organisms in the development of different cell types (Margueron and Reinberg, 2010). Chromatin is subject to many different modifications that can affect transcription, some by changing chromatin conformation. The most well studied of these modifications are the reversible processes of: DNA methylation, histone methylation, and histone acetylation. These chromatin modifications are often referred to as epigenetic modifications. Epigenetics is the study of heritable changes in gene expression that do not result from changes in DNA sequence, most commonly referring to inheritance of chromatin modifications (Gendrel and Colot, 2005).

1.3 Histone acetylation is a major mechanism of gene regulation

The control of chromatin compaction through histone acetylation is a rapid and reversible process facilitated by two groups of enzymes: histone acetyltransferases (HATs) that add acetyl groups to histones and histone deacetylases (HDACs) that remove the acetyl groups. HATs and HDACs modify lysine (K) residues on the N-terminus of H3 (K9, K14, K18, K27) and H4 (K5, K8, K12, K16, K20) histone tails (Hollender and Liu, 2008). Hyper-acetylation of those H3 and H4 lysine residues is associated with euchromatic regions and therefore with transcriptional activation (Fig. 1.1).

The presence of acetyl groups on K residues reduces chromatin compaction in a number of ways. First, when bound to the histone tails, acetyl groups neutralize the positive charge of the histone octamer, reducing its affinity for the negatively charged DNA. Second, acetyl groups shape the binding surface for chromatin remodeling factors that

maintain euchromatic conditions. Finally, acetyl groups physically disrupt higher order chromatin folding, reducing compaction (Lusser et al., 2001). The activity of HDACs in turn, by removing acetyl groups from the aforementioned K residues, would counter the effects of HATs resulting in increased chromatin compaction (Fig. 1.1; Hollender and Liu, 2008). It is clear that the regulation of HAT and HDAC expression and activity is a major mechanism in the broad scale regulation of transcription.

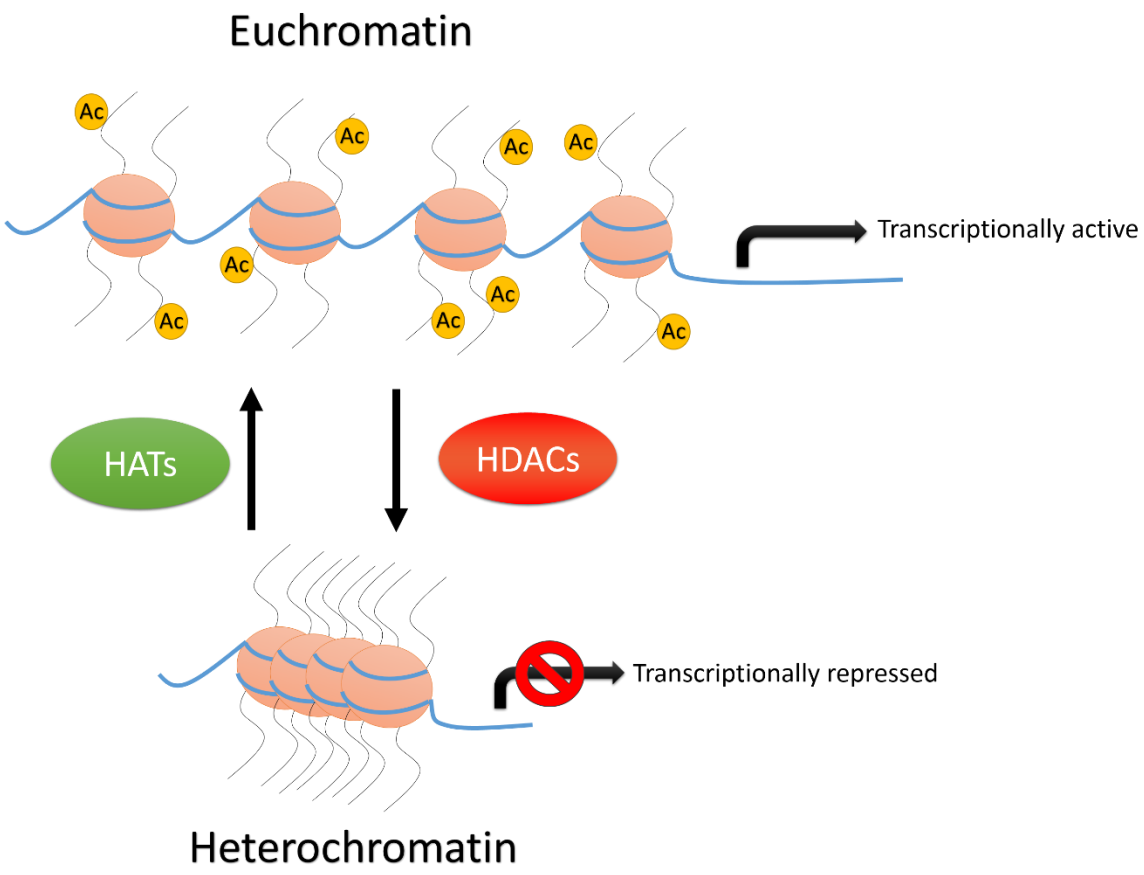

Figure 1.1 Histone acetylation affects DNA accessibility and transcription. The addition of acetyl groups by histone acetyltransferases (HATs) reduces chromatin compaction resulting in formation of euchromatic regions and transcriptional activation. Conversely, the removal of acetyl groups by histone deacetylases (HDACs) results in increased chromatin compaction, the formation of heterochromatic regions, and transcriptional repression.

1.4 Plant histone deacetylases

A number of epigenetic factors, particularly HDACs, have been the subject of increased study in plants due to their involvement in many fundamental processes, particularly: growth, development, and defense responses (Hollender and Liu, 2008). In *Arabidopsis thaliana* (Arabidopsis), three HDAC families, with eighteen HDAC genes, have been identified (Table 1.1). First, the RPD3-like family has been identified based on its sequence homology to the yeast HDAC family RPD3 (reduced potassium deficiency 3). The RPD3-like family has twelve members in Arabidopsis and it is the most extensively HDAC family studied (Hollender and Liu, 2008). Second, the SIR2 family has been identified based on its sequence homology to the yeast HDAC family SIR2 (sirtuin 2). The SIR2 family has two members in Arabidopsis which have a conserved SIR2 domain that makes their activity NAD (nicotinamide adenine dinucleotide)-dependent and is important for binding both K residues and acetyl groups (Hollender and Liu, 2008). Lastly, the plant-specific HD2 (histone deacetylase 2) family was identified in corn due to its histone deacetylase activity (Lopez-Rodas et al., 1991). The HD2 family and has four members in Arabidopsis which have a conserved EFWG domain essential for histone deacetylase activity (Lusser et al., 1997; Wu et al., 2000).

The expression of specific HDAC genes in plants varies spatially and temporally, even within the same HDAC families, suggesting a broad range of functional diversity (Hollender and Liu, 2008). HDAC knockout and overexpression studies have shown that HDACs can have widespread effects on gene expression. For example, when the globally expressed RPD3-like member *HDA19* is knocked out, 7% of the entire Arabidopsis transcriptome shows altered expression (either up- or down-regulated) (Tian et al., 2005).

Knockout and overexpression studies have also been used to establish roles for HDACs in the regulation of gene expression in a variety of plant processes related to plant growth and development and both abiotic and biotic stress responses (Tian and Chen 2001; Zhou et al, 2005; Sridha and Wu, 2006; Chen et al., 2010; Chen and Wu, 2010; Colville et al., 2011; Luo et al., 2012).

HDACs do not act alone, rather they interact with chromatin as part of multi-protein complexes that are recruited to specific loci. The recruitment of HDACs occurs by direct or indirect interaction with DNA-binding proteins (Reyes et al., 2002). For example, HDA19 is recruited to the *AGAMOUS* (*AG*) locus by binding to the transcriptional corepressor LEUNIG (LUG), which binds to DNA-binding proteins through the adapter protein SEUSS (SEU) (Sridhar et al., 2004; Gonzalez et al., 2007). This complex is responsible for the inhibition of *AG* transcription due to HDA19 deacetylase function (Sridhar et al., 2004). It has become evident that HDAC repression complexes are involved in a number of different processes and can contain multiple HDACs from different families (Lu et al., 2012; Luo et al., 2012b; Luo et al., 2015). Furthermore, HDACs display some degree of functional redundancy as some HDACs are involved in the regulation of the same genes (Tanaka et al., 2008). In addition to interacting with each other, HDACs interact with other epigenetic factors such as DNA and histone methyltranferases and demethylases. These interactions have been shown to affect DNA methylation status, histone methylation status, and histone acetylation status, affecting transcription (Yu et al., 2011; Song et al., 2010; Luo et al., 2012).

Table 1.1 HDAC families in Arabidopsis

RPD3-like	**SIR2**	**HD2**
HDA2	SRT1	HD2A (HDT1)
HDA5	SRT2	HD2B (HDT2)
HDA6		HD2C (HDT3)
HDA7		HD2D (HDT4)
HDA8		
HDA9		
HDA10		
HDA14		
HDA15		
HDA16		
HDA17		
HDA19		

1.4.1 HDACs involvement in plant development

Members of the RPD3-like and HD2 HDAC families in Arabidopsis have been as implicated having a regulatory role in developmental pathways since development-related phenotypes are exhibited when their function is disrupted (Tian and Chen, 2001). This became evident after treatment of Arabidopsis with the HDAC inhibitor trichostatin A (TSA) that resulted in developmental defects, including: abnormal germination patterns, early senescence, expression of silenced genes, floral defects, and sterility (Tian and Chen, 2001).

1.4.1.1 HDAC involvement in germination and post-germination growth

Histone deacetylases have been implicated in having a role during embryogenesis, germination, and post-germination growth (Tai et al., 2005). For example, WT Arabidopsis seeds sown on media with TSA displayed reduced germination success and post-germination growth (Tanaka et al., 2008; van Zanten et al., 2014). Additionally, TSA treatment resulted in the expression of embryogenesis-related genes that are normally repressed after embryogenesis (Tai et al., 2005; Tanaka et al., 2008). Furthermore, Tai et al. (2005) found that one day after imbibition there was a transient increase in HDAC activity as well as decreased acetylation levels of H4 histones in the promoter and coding regions of some of the same embryogenesis-related genes repressed during TSA treatment. By using HDAC knockout studies, Tanaka et al. (2008) identified that the RPD3-like family members *HDA6* and *HDA19* redundantly repress some embryogenesis-related genes that were upregulated during treatment with TSA in a study by Tai et al. (2005). The expression of these embryogenesis-related genes resulted in post-germination growth

arrest and development of embryo-like features in *hda6* and *hda19* mutants (Tanaka et al., 2008).

Although *HDA6* and *HDA19* promote seedling development and promote germination by repressing embryogenesis-related genes, a related family member *HDA9* represses seedling development and negatively regulates germination (van Zanten et al., 2014). As such, WT plants have an upregulation of *HDA6* and *HDA19* transcripts during germination and downregulation of the *HDA9* transcript (Alinsug et al., 2009). Similarly to *HDA9*, the HD-family member *HD2A* is a negative regulator of germination, as *hd2a* mutants were found to have increased germination rates (Colville et al., 2011).

In addition to regulating germination and embryogenesis under control (untreated) conditions, HDACs also affect germination when seeds are treated with the phytohormone abscisic acid (ABA) (Sridha and Wu, 2006; Chen et al., 2010; Chen and Wu, 2010; Colville et al., 2011). Arabidopsis lines mutated for *hda6*, *hda19,* or *hd2c* germinated normally in untreated conditions but had significantly reduced germination rates after treatment with ABA (Sridha and Wu, 2006; Chen et al., 2010; Luo et al., 2012). *hda6* and *hd2c* mutants had higher levels of H3K14 acetylation at the loci of a number of ABA response genes, suggesting these HDACs operate directly at those loci (Chen et al., 2010; Luo et al., 2012). In contrast, the *hd2a* mutant had increased germination rates during ABA treatment (Colville et al., 2011). The fact that the effect on germination in *hd2a* mutants was opposite to that of *hd2c* mutants, suggests functional diversity within the HD2 family (Colville et al., 2011). These experiments show that HDACs are involved in the process of germination in both ABA-independent and ABA-dependent manners.

1.4.1.2 The role of HDACs in controlling flowering time

The control of flowering time is composed of four floral induction pathways: photoperiod, autonomous, vernalization, and gibberellic acid (GA) pathways (Corbesier and Coupland, 2006). Each one of these pathways incorporates different internal or external stimuli that affect flowering time. The floral induction pathways control gene expression to promote or delay flowering and they converge at different points (Corbesier and Coupland, 2006).

Histone deacetylation has been implicated in the switch from vegetative growth to reproductive growth (bolting and flowering; He et al., 2003) and have been found to be involved in multiple floral induction pathways. *HDA6* has been found to be a positive regulator of flowering time when Arabidopsis was grown under both long day (LD) and short day (SD) conditions (Wu et al., 2008) by repressing the expression of *FLOWERING LOCUS C* (*FLC)* (Yu et al., 2011). Since *FLC* is known to be a negative regulator of flowering, its repression through the action of HDA6 resulted a delay in the transition to flowering (Michaels and Amasino, 1999). Furthermore, HDA6 was found to control flowering time as a part of a multi-protein repression complex (Yu et al., 2011; Luo et al., 2015). HDA6 interacted with the histone demethylase FLOWERING LOCUS D (FLD) at the locus of *FLC*, repressing its transcription (Yu et al., 2011). Arabidopsis plants mutated at the *fld* gene were hyperacetylated at H4 at the *FLC* locus, highlighting the importance of the interaction between FLD and HDA6 for deacetylation at the *FLC* locus. Recently, the RPD3-like family member HDA5 has been identified as a part of the HDA6-FLD repressor complex, as HDA5 interacted with both HDA6 and FLD to repress *FLC* transcription (Luo et al., 2015). Similarly to *hda6* mutants, *hda5* mutants flowered later

(Luo et al., 2015). Control of *FLC* expression is the convergence point of the vernalization and autonomous pathways (Corbesier and Coupland, 2006). However, *FLD* is a component of autonomous floral induction pathway, suggesting that the HDA6-FLD-HDA5 repression complex is controlled by the autonomous pathway (Liu et al., 2015).

Some HDACs also affect flowering in an entirely different manner. In contrast to the roles of *HDA6* and *HDA5*, their RPD3-like family member *HDA9* represses flowering under SD but not LD conditions (Kim et al., 2013). *hda9* mutants flowered earlier under SD conditions, without affecting *FLC* expression. However, *hda9* mutants did have higher expression of the flowering activator *AGAMOUS-LIKE 19* (*AGL19*), which activates expression of genes downstream of *FLC*. HDA9 was found to associate with the *AGL19* locus and *hda9* mutants were found to have higher H3K9K27 acetylation levels at the *AGL19* promoter, promoting its expression (Kim et al., 2013; Kang et al., 2015). The fact that *HDA9* affected flowering only under SD conditions suggests that *HDA9* controls flowering time through the photoperiod floral induction pathway, as opposed to *HDA5* and *HDA6* that operate through the autonomous pathway (Kim et al., 2013; Kang et al., 2015; Liu et al., 2015).

There is evidence that members of the HD2 family are also involved in regulation of flowering time. Zhou et al. (2004) observed that transgenic Arabidopsis overexpressing the *HD2A* gene exhibited delayed flowering under LD conditions. Although no studies have followed up on this observation, the HD2A protein has been found to interact with HDA6 (Luo et al., 2012b), possibly working in a complex together that regulates flowering time. These findings indicate that HDACs control flowering at multiple points of different floral induction pathways by affecting gene expression.

1.4.2 HDACs in plant stress responses

Epigenetic control of gene expression allows for rapid changes in gene expression in response to changing environmental conditions, including many stress conditions that can adversely affect plant health. HDACs have been implicated in the regulation of plant stress responses to both abiotic (drought, salt, and cold) and biotic stresses (pathogen infection).

Sokol et al. (2007) noted a rapid global increase in the acetylation of H3K14 and H4 when subjecting Arabidopsis and *Nicotiana tabacum* cell lines to cold and salt treatments. Furthermore, treatment with TSA resulted in the upregulation of the ABA response element (ABRE) genes that have previously been shown to be upregulated in drought, high salinity, and cold treatments (Tai et al., 2005). Furthermore, drought treatment resulted in increased H3 acetylation of ABRE and dehydration response element (DRE) genes resulting in an increase in their expression (Kim et al., 2008).

Sridha and Wu (2006) were the first to identify a specific role for an HDAC in abiotic stress responses. Arabidopsis lines overexpressing the HD2 family member *HD2C* were shown to have greater germination success and seedling survival rates under salinity, ABA, and mannitol treatments. Consistent with that finding, Luo et al. (2012) found that *hd2c* mutant Arabidopsis had decreased germination and survival rates when treated with ABA and salt, along with decreased expression levels of a number of ABA-response genes (Luo et al., 2012). Under control conditions, *HD2C* overexpression lines had increased expression of a number of ABA response genes, indicative of an enhanced ABA response (Sridha and Wu, 2006).

HDACs from the RPD3-like family have also been implicated in ABA-response (Chen et al., 2010; Chen and Wu, 2010). After treatment with ABA or salt, *hda6* and *hda19* mutant lines had decreased germination rates along with reduced expression of some ABA- and salt-responsive genes (Chen et al., 2010; Chen and Wu, 2010; Luo et al., 2012). Furthermore, the HD2C and HDA6 proteins interact to regulate expression of some ABA-response genes (Luo et al., 2012) and HD2C was found to interact with HDA19 *in vivo* (Luo et al., 2012b). Taken together, these studies suggest that HDA6, HDA19, and HD2C may form a repressive complex that is required for full induction of ABA- and salt-responsive genes (Sridha and Wu, 2006; Chen et al., 2010; Chen and Wu, 2010; Luo et al., 2012).

1.5 Abscisic acid response

Plants respond to changing environmental conditions in a rapid and specific manner. Environmental stresses are perceived quickly and the stress signal is rapidly transmitted within the plant, leading to a quick and specific response. Plant hormones (phytohormones) are central to the plants' ability to rapidly respond to changing environmental conditions, namely ABA. ABA acts as an endogenous chemical signal during various developmental processes including seed maturation, seed dormancy, germination, cell division, and floral induction. ABA also plays a predominant role in both biotic and abiotic stress responses, including: drought, salinity, cold, and pathogen attacks. Thus, ABA integrates developmental programs with stress responses (Finkelstein et al., 2002; Hopkins and Huner, 2008; Finkelstein, 2013).

1.5.1 ABA in plant development

ABA has been shown to play a fundamental role in many growth and developmental programs, including germination and flowering time. ABA accumulation in the seed leads to growth arrest, accumulation of storage proteins and lipids, and seed dormancy, thus ABA negatively regulates germination (Finkelstein et al., 2002). ABA signaling is used to properly time germination under the favourable environmental conditions (Finkelstein et al., 2002). The application of exogenous ABA during imbibition results in delayed seed germination. The ABA-dependent delay in germination has been used to identify genes involved in the ABA pathway, by screening for mutants with an ABA-insensitive (ABI) phenotype which does not result in a delay in germination, indicative of an altered ABA response (Finkelstein and Sommerville, 1990).

Flowering time is affected by a number of internal cues (ie. plant size, age, and vegetative nodes), external cues (ie. vernalization, photoperiod, and water availability), and external stresses (ie. water deficit, nutrient deficiency, and overcrowding) (Levy and Dean, 1998). Exogenous ABA application has been shown to delay flowering in Arabidopsis and a number of other species, however, how ABA signaling integrates with the other flowering pathways remain poorly understood (Conti et al., 2015)

On the molecular level, a number of genes involved in ABA signaling are also involved in regulation of flowering time. For example, constitutive expression of the *CmMYB2* transcription factor increases sensitivity to exogenous ABA treatment while also resulting in delayed flowering (Shan et al., 2011). In addition, constitutive expression of

the ABA response gene *ABR17* resulted in early flowering as well as increased germination rates under salt stress conditions (Srivistava et al., 2006; Dunfield et al., 2007).

A number of transcription factors known to be upregulated by ABA and which are involved in ABA dependent gene expression have been shown to affect flowering time (Conti et al., 2015). For example, the transcription factors ABI3, ABI4, ABI5, which belong to different gene families, positively regulate ABA dependent gene expression and have been found to delay flowering when constitutively expressed (Kurup et al., 2000; Wang et al., 2013; Shu et al., 2015). Specifically, constitutive expression of either ABI4 or ABI5 has been found to cause an upregulation of the *FLC* transcript – known to negatively regulate flowering (Wang et al., 2013; Shu et al., 2015).

1.5.2 ABA involvement in abiotic stress response

Although ABA signaling is involved in both biotic and abiotic stress responses, much of the research has focused on abiotic stress response, specifically in response to water deficit. Plants respond to water deficit by regulation of stomatal aperture, decreased cell growth and photosynthesis, increased respiration, accumulation of osmolytes and proteins, and induction root growth coupled with repression of shoot growth – all processes ABA is involved in (Shinozaki and Yamaguchi-Shinozaki, 2007).

Lack of water uptake results in an increase in root apoplast pH, promoting ABA mobilization from root cells into the xylem and eventually into the leaf apoplast. Mobilization of ABA is followed by increased levels of ABA biosynthesis and decreased ABA catabolism, in root and shoot tissues alike (Wilkinson and Davies, 2002; Hopkins and Huner, 2008). Once mobilized, ABA is perceived intracellularly in the cytosol and

nucleus and extracellularly at the cell membrane (Cutler et al., 2010). There are a number of ABA receptors, the best characterized being the RCAR/PYR/PYL (RCAR) receptors. These receptors physically bind ABA intracellularly, an interaction that is promoted by binding of protein phosphatase 2C (PP2Cs) co-receptors ABA INSENSITIVE (ABI)1 and ABI2 to RCARs (Fig. 1.2; Cutler et al., 2010; Raghavendra et al., 2010). The ABI1 and ABI2 proteins negatively regulate ABA response by dephosphorylating and inactivating the SNF1-related protein kinases (SnRKs; Raghavendra et al., 2010). In the presence of ABA, RCAR receptors bind ABI1 and ABI2, inhibiting their phosphatase activity and allowing SnRKs to remain phosphorylated and active, inducing the ABA-response (Fig. 1.2; Raghavendra et al., 2010).

The ABA-induced stress response can be broken down to two levels involving the signal transduction pathway described above. The first of wave responses immediately limit water loss, while the second wave of responses deal with chronic water stress conditions by inducing changes in gene expression (Cutler et al., 2010). The first wave involves regulation of stomatal aperture to limit water loss and establish an equilibrium between water supplied by the roots and water lost to transpiration at the leaf surface (Wikinson and Davies, 2002). Stomatal closure is promoted by the loss of guard cell turgor pressure, due to the phosphorylation of ion channels by active SnRKs, leading to an ionic efflux (Raghavendra et al., 2010).

In addition to being the primary chemical signal to induce stomatal closure, ABA induces large scale changes in gene expression (Cutler et al., 2010; Lee and Luan 2012). There are a number of regulatory elements that are induced by ABA, of which ABA-responsive element (ABRE) activation is the best characterized. Similarly to the signal

transduction pathway described above, activated SnRKs phosphorylate transcription factors such as ABA-INSENSITIVE 5 (ABI5), a basic leucine zipper (bZIP) transcription factor, in the nucleus (Fig. 1.2). In its phosphorylated form, ABI5 becomes active and binds to ABREs, resulting in transcription of genes involved in ABA response (Cutler et al., 2010; Raghavendra et al., 2010). For transcription factors to bind to ABREs and other regulatory elements, chromatin must be in a less condensed state. Studies have shown that ABA affects chromatin status (including histone acetylation), allowing for increased DNA accessibility (Sokol et al., 2007). Although exogenous ABA treatment resulted in approximately half of the genes affected being repressed, the mechanism of gene repression is not as well studied as gene activation (Raghavendra et al., 2010).

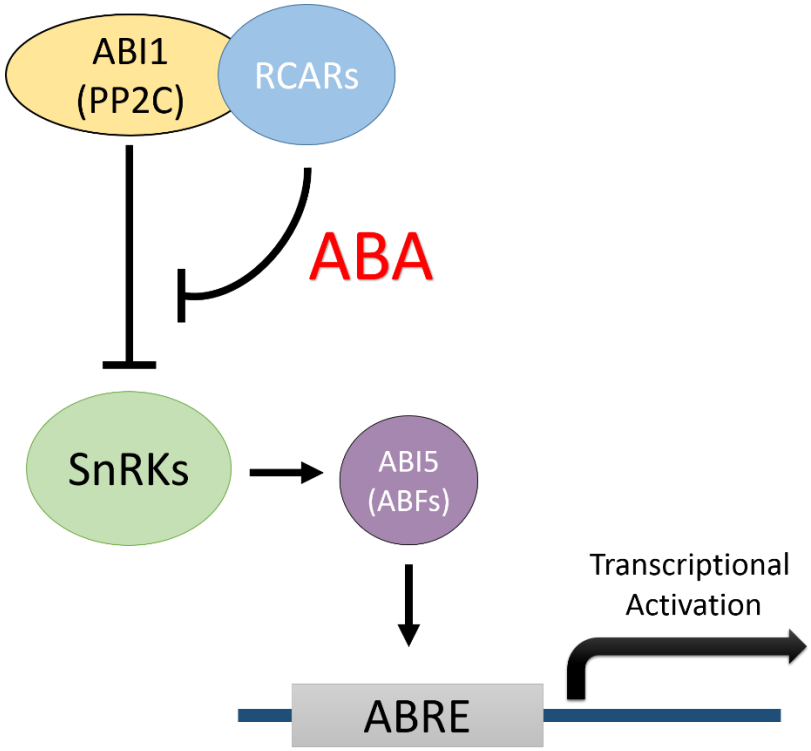

Figure 1.2 ABA signaling in the nucelus resluting in transcriptional activation of ABREs. The binding of ABA to RCAR (regulator component of ABA receptor) receptors inhibits PP2Cs (protein phosphatase 2C) dephosphorylase activity and therefore allows for the phosphorylation and activation of SnRKs (SNF1-related protein kinase). Once active, SnRKs activate ABF (ABRE-binding factors) transcription factors by phosphorylation. The phosphorylation of ABFs results in their stabilization and their binding to ABRE (ABA response elements)-containing genes, induce their transcription and ABA-dependent gene expression. Adaped from Raghavendra et al. (2010).

1.6 Research objectives

HDAC genes have been identified as important epigenetic regulators of ABA responses during plant development and stress responses. Much research has focused on the RPD3-like family of HDACs, despite increasing evidence for the involvement of the HD2 family in development and stress response pathways (Zhou et al., 2004; Sridha and Wu, 2006; Colville et al., 2011; Luo et al., 2012; Luo et al., 2012b). *HD2D* has been suggested to have an important regulatory role in the ABA-dependent processes of seed germination and seedling growth (Colville et al., 2011). Furthermore, *HD2D* transcript levels are affected by exogenous ABA application (Sridha and Wu, 2006). Although evidence supporting a role for HD2D in ABA regulated pathways has emerged, it remains under-studied. Based on these findings, I chose to investigate the effects of *HD2D* expression on ABA-related processes, specifically involving development and response to water deficit. I hypothesize that *HD2D* regulates ABA-dependent developmental programs and water deficit responses in Arabidopsis.

The objectives of the research were:

1. To demonstrate that *HD2D* affects the ABA-related processes of germination, flowering, and water stress response

2. Investigate the effects of *HD2D* expression on ABA-related gene expression

3. Implicate a mode of action for the HD2D protein

These objectives will be accomplished by testing differences in germination, flowering time, and water deficit response in *HD2D* knockout and overexpression plants.

Furthermore, these lines will be tested for differences in ABA-related gene expression and histone acetylation. Finally, I will test the HD2D protein's *in vivo* interaction with components of the ABA signal transduction pathway.

Chapter 2: Materials and Methods

2.1 Generation of knockout and overexpression lines

To test the effect of *HD2D* expression on ABA-related processes *Arabidopsis thaliana* (Arabidopsis) of the Columbia-0 (col-0) ecotype *HD2D* knockout and *HD2D* overexpression plants were used. An *hd2d* mutant (GABI-Kat_379G06), containing a disruption in the second intron of the *HD2D* gene, was identified and seeds were ordered from the Arabidopsis Biological Research Center (Columbus, OH). This mutant line was generated using T-DNA (transfer-DNA) mutagenesis, where *Agrobacterium tumefaciens* was used to transform Arabidopsis using T-DNA by the floral dip method (Kleinboelting et al., 2012). The T-DNA insertions were then mapped and mutant lines were made available to the public (Kleinboelting et al., 2012).

To screen for plants containing a homozygous mutation at the *HD2D* gene, DNA was extracted from 4 week old seedlings and a PCR was employed to detect the T-DNA insertion using two primers complementary to either side of the genomic DNA flanking the T-DNA insertion site and another primer on the T-DNA sequence itself (Fig. 3.1A). Once homozygous plants were identified, homozygous F_3 seeds were generated and used in all subsequent experiments. Plants with a homozygous insertion in the *HD2D* locus were designated as *hd2d-1*.

In order to generate plants constitutively expressing *HD2D*, Gateway® cloning (Hartley et al., 2000) was used according to the product's instructions. Briefly, the *HD2D* coding sequence was cloned using the HD2DattF and HD2DattR primers (Fig. 3.1B; Table 2.1), which contain the attB recombination sites, that serve as attachment sites for

recombination proteins. The *HD2D* coding sequence flanked by the attB recombination sites was transferred into the pDONR™221 plasmid using the Gateway® BP Clonase® (Thermo Fisher Scientific cat. 11789-020) reaction mix. This reaction mix contains integrate (Int) and integration host factor (IHF) proteins that recognize attB recombination sites flanking the *HD2D* coding sequence and attP recombination sites on pDONR™221, mediating the transfer of *HD2D* in pDONR™221 (Hartley et al., 2000). The pDONR™221-HD2D vector was electroporated into *Escherichia coli* cells of the DH5α strain. pDONR™221-HD2D plasmids were then isolated and sequenced. The *HD2D* coding sequence was subcloned into the pEarleyGate101 destination vector (Earley et al., 2006) using Gateway® LR Clonase® (Thermo Fisher Scientific cat. 11791-100) reaction mix to form the pEarleyGate101-HD2D expression construct. The LR Clonase® reaction mix contains Int, IHF, and excisionase, allowing for the cloning of HD2D coding sequence into pEarleygate101, as described above (Hartley et al., 2000).

Vectors used in the bimolecular fluorescence complementation (BiFC) test were generated using Gateway® technologies as described above using the *ABI1*, *ABI2*, *ABI5*, *HD2A*, *HD2B*, and *HD2C* coding sequences. After the cloning of these coding sequences into pDONR™221, Gateway® LR Clonase® mix was used to recombine each one of these coding sequences (including *HD2D*) into pEarleyGate202-YC or pEarleyGate202-YN (Tian et al., 2011; Luo et al., 2014).

2.2 Arabidopsis transformation

The pEarleygate101-HD2D construct described in 2.1 was electroporated into *Agrobacterium tumefaciens* GV3101 and used to transform Arabidopsis (col-0) plants via

the floral dip transformation method according to Zhang et al. (2006). To select Arabidopsis seeds containing the transgene, seeds were plated on media containing half strength Murashige and Skoog (MS) salts, 1% sucrose, 0.8% plant agar, brought to a pH of 5.7 using KOH and with 10 µg/mL glufosinate ammonium antibiotic. Surviving seedlings were transferred to soil and DNA was extracted to confirm the presence of the transgene. F_2 seeds were collected and plated on selection media (see above) to select homozygous lines based on their segregation. Two *HD2D* homozygous overexpression lines were selected and F_2 seeds were used in all subsequent experiments, designated 1-1 and 4-1.

2.3 Measurement of germination success

An experiment was designed to determine the rates of germination in WT and transgenic seeds. Seeds were surface sterilized with a 70% ethanol solution for 5 min, followed by a solution of 20% bleach and 0.1% SDS for 15 min, shaking continuously, the seeds were then rinsed 5 times with sterile ddH_2O. Seeds were plated on control media containing half strength Murashige and Skoog (MS) salts, 1% sucrose, 0.8% plant agar, brought to a pH of 5.7 using KOH. Abscisic acid (ABA) treatment plates were identical to control plates except that ABA was added to a final concentration of 1 µM. Petri dishes containing the seeds were incubated at 4ºC for two days, to synchronize germination. Germination was tracked once plates were removed from 4ºC (day zero) and incubated at 20ºC under long day conditions (16/8 hr light/dark cycle). To quantify germination success, the number of seeds of each genotype that germinated was quantified every 24 hour for five days. Each group (control, ABA) was made up of three petri dishes (N=3), each containing 36 seeds from each of the WT, *hd2d-1*, 1-1, and 4-1 lines.

2.4 Measurement of flowering time and rosette leaves

For the flowering time experiments WT, *hd2d*-1, 1-1, and 4-1 seeds were sown in equal amounts of PRO-MIX® BX MYCORRHIZAE™ soil containing equal amount of water to maintain moist soil. Seeds were sown in separate pots and placed at 4ºC for two days, to synchronize germination. The pots were then placed in either long day (LD) or short day (SD) conditions. For the LD treatment, the light conditions were 16 hours light and 8 hours dark, each pot contained 9 plants from WT, *hd2d-1*, 1-1, or 4-1 with a total of six pots per genotype (N=6). For the SD treatment, the light conditions were 8 hours of light and 16 hours of dark, each pot contained 5 plants with a total of six pots per genotype (N=6). In both the LD and SD treatments, the temperature was set to 23ºC in the light and 18ºC in the dark, at a constant humidity of 40%. A clear plastic cover was kept on the trays for two weeks to reduce water loss at the soil surface while the seedlings were still young. Each pot was rotated within the tray every two days and trays were moved within the growth chamber on a daily basis, to prevent positional effects. Additionally, all pots were watered equally to keep soil moist, using a serological pipette. Bolting was defined as inflorescence emergence greater than 5 mm in length.

Rosette leaves were collected on bolting days from the experiment outlined above. The rosette leaves were separated, counted, and photos of the individual leaves were taken. Using the image processing program ImageJ, the total rosette surface area was measured.

2.5 Drought and salinity treatments

The drought and salt treatments had similar soil preparation, sowing conditions, and growth chamber conditions to the LD flowering time experiments. For these

experiments, seeds of different genotypes were sown in the same pot so that each pot contained three seeds of the WT, *hd2d-1*, and either 1-1 or 4-1 genotypes. This was done to control for any potential differences in water loss between pots. For the drought treatment, all plants were watered with equal amounts of water for thirty-five days in order to keep soil moist. At day thirty-five, drought treatment groups had water withheld for fourteen days and control groups continued to receive water regularly. Drought treatment plants were then re-watered and were allowed to recover for two days, at which the percent survival was scored. To ensure correct determination of plant death, all pots were regularly watered and plants monitored for ten days after mortality. Susceptibility to drought treatment was determined from 3 replicates for a total of 18-36 plants per genotype.

To examine salt tolerance, all plants were watered equally for thirty-five days to keep soil moist. At day thirty-five, treatment pots started receiving 20 mL of a 200 mM NaCl solution every 24 hours. To quantify the effects of the salt treatment on the plants, leaf death was recorded. A leaf was considered dead when it exhibited greater than 50% chlorosis. Measurements were taken on day fourteen and day twenty-one of the 200 mM NaCl treatment. Susceptibility to salinity treatment was determined from 3 replicates for a total of 18-36 plants per genotype.

2.6 RNA extraction, reverse transcription, and qPCR

RNA was extracted using TRIzol® Reagent (ambion®, cat.15596-026) from complete rosettes (all leaves in the rosette) from thirty day old WT, *hd2d-1*¸ 1-1, and 4-1 plants. Between 50-100 µg of material per sample was flash frozen using liquid N_2 and

homogenized using a mortar and pestle. In an RNase-free microfuge tube, 1mL of TRIzol® was added to each sample, followed by 200µL of chloroform. Samples were centrifuged at 12000g for 15 min, at 4ºC, and the aqueous phase was transferred into new tubes and saved. In order to precipitate the RNA, 0.5mL of 100% isopropanol was added to each sample and kept at room temperature for 10 min. After centrifuging for 12000g for 15 min, at 4ºC, the supernatant was discarded and the pellet (containing the RNA) was saved. The pellet was washed three times with 1 mL of 70% ethanol per sample. Once ethanol was removed, the pellet was re-suspended in 50µL RNase-free ddH_2O. RNA concentration was quantified using the NanoDrop™ 1000 Spectrophotometer. Samples were diluted to 100ng/µL and were treated with DNase I (ambion®, cat. AM2222) as per product instructions, using the heat-inactivation of DNase I method to inactivate DNase I. RNA samples were then converted to cDNA using iScript™ reverse transcription supermix (BIO-RAD, cat. 170-8841), as per product instructions. The qPCR reaction mix used was SsoFast™ EvaGreen® supermix (BIO-RAD, cat. 172-5200) and analysis was carried out using the CX96™ Real Time System- C1000 Touch Termal Cycler. The Actin 2 gene was used as an internal control in all qPCR experiments.

Table 2.1 Primers used in RT-qPCR analysis and cloning experiments

Primer name	**Primer Sequence (5'→3')**
HD2DattF	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCATGGAGTTTT GGGGTATCGA
HD2DattR	GGGGACCACTTTGTACAAGAAAGCTGGGTACTTTTTGCAAGA GGGACC
HD2DRTF	TGATCTCTACTTAGGGCACG
HD2DRTR	CTACTTTTTGCAAGAGGGAC
Actin2RTF	GTGCTGGATTCTGGTGATGGT
Actin2RTR	GTCAAGACGGAGGATGGCAT
RD29ARTF	GGAAGTGAAAGGAGGAGGAGGAA
RD29ARTR	CACCACCAAACCAGCCAGATG
ABI1RTF	AGAGTGTGCCTTTGTATGGTTTTA
ABI1RTR	CATCCTCTCTCTACAATAGTTCGCT
ABI2RTF	GATGGAAGATTCTGTCTCAACGATT
ABI2RTR	GTTTCTCCTTCACTATCTCCTCCG
ABI5RTF	ATGATCAAGAACCGCGAGTCTGC
ABI5RTR	CGGTTGTGCCCTTGACTTCAAAC
FLCF	CCGAACTCATGTTGAAGCTTGTTGAG
FLCR	CGGAGATTTGTCCAGCAGGTG
SOC1F	GGATCGAGTCAGCACCAAACC
SOC1R	CCCAATGAACAATTGCGTCTC
FULF	TCACAACAATTCGCTTCTCAA
FULR	TTGGACTAATTGTCCTTCTTGCT
SEP3F	GAAAGCTGTACGAGTTTTGCAG
SEP3R	TTGAAGGCACATTGGGTTCT
379G06F	TCTTCTCAAGCAGCCACATCTT
379G06R	AATCAATCTCCTCATCCGTGAGC
GKpAC161LB	ATATTGACCATCATACTCATTGC

2.7 Western blot analysis

Acid-soluble proteins were extracted from the rosettes leaves of thirty-five day old WT, *hd2d*-1, 1-1, and 4-1 plants. Leaves were flash frozen with liquid N_2 and ground using a mortar and pestle. Lysis buffer (0.25M HCl, 10mM Tris-HCl, 2mM EDTA, 20mM β-mercaptoethanol, and 0.2M phenylmethylsulphanyl fluoride) was added to each sample on ice and left for 10 min. Tissue was sonicated using the Fisher Scientific Sonic Dismembrator Model 100 for five intervals of 10 seconds on ice. Sonicated tissue was centrifuged twice at 12g for 15 min, saving the supernatant after each centrifuge step. Protein was quantified using a Bradford assay with the Bio-Rad iMark microplate reader.

Samples were diluted so that each protein sample contained equal amounts of protein. Samples were mixed with 10x Laemmli buffer (100mM Tris-HCl, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 50% (v/v) glycerol, 200mM β-mercaptoethanol) on ice. Samples were separated in a 10% polyacrylamide SDS-PAGE gel. Separated proteins were transferred onto a methanol-activated Immu-Blot™ polyvinylidene fluoride (PVDF) membrane (Bio-Rad, cat. 162-0177) using the Bio-Rad Trans-Blot® SD Cell for semi-dry transfer. Membranes were blocked overnight using 5% (w/v) milk powder. Membranes were incubated overnight with either the anti-histone H3, anti-histone H4, or the anti-H3 controls in 1% (w/v) milk (Table 2.2). Membranes were washed 5 times with tris-buffered saline solution (5-10 minutes). Protein was visualized after membranes were treated with EZ-ECL Chemiluminescence Detection Kit for horseradish peroxidase (Biological Industries, cat. 20-500-500) as per the product's instructions. ECL-treated membranes were exposed to Mandel Bioflex MSI Film (Mandel Scientific, cat. MED-CLMS810). Film was developed using the AGFA CP1000 automatic film processor.

Table 2.2 Antibodies used for Western blot experiments

Antibody name	**Purification**	**Host**	**Supplier**	**Catalog number**	**Dilution**
Anti-histone H3	Monoclonal	Rabbit	Millipore	05-928	1:7500
Anti-acetyl-Histone H3	Polyclonal	Rabbit	Millipore	06-599	1:1000
Anti-acetyl-Histone H4	Polyclonal	Rabbit	Millipore	06-866	1:10000
Anti-rabbit IgG (2º**)**		Goat	Cell Signaling	7074	1:10000

2.8 Bimolecular fluorescence complementation

Bimolecular fluorescence complementation (BiFC) experiments were carried out according to Tian et al. (2011). Briefly, coding regions of *ABI1*, *ABI2*, *ABI5*, *HD2A*, *HD2B*, and *HD2C* were cloned into pEarleygate202-YC while the coding region for *HD2D* was cloned into pEarleygate202-YN, according to section 2.1. Each of these vectors was separately transformed into *Agrobacterium tumefaciens* (GV3101). Fresh colonies were prepared by picking single colonies to inoculate 5 mL of LB, containing 50µg/mL kanamycin, 50 µg/mL gentamicin and 25 µg/mL rifampicin, and grown overnight at 28ºC. Cells were pelleted at 1000g for 10 min and supernatant was discarded. Same volume infiltration media (5 g/L glucose, 50 mM MES hydrate, 2 mM Na_3PO_4, and 0.1 mM acetosyringone) was added to the pelleted cells and then centrifuged again. This step was repeated two more times, each time breaking the pellet apart. Cells were finally re-suspended in half volume infiltration media to concentrate the agrobacteria. A mixture of HD2D-YN and each of the pEarleygate202-YC combinations was created at 50:50 ratios.

Nicotiana benthamiana plants were grown at long day (LD) conditions (16/8 light/dark) at 23ºC. Four week-old plant leaves were used for infiltration. 100µL of the Agrobacterium mixture was used to infiltrate the abaxial side of the leaf. Infiltrated plants were returned to the LD conditions for two days and YFP signal was observed every 24 hours after that using a Leica TCS SP2 confocal microscope.

2.9 Statistical Analysis

One-way analysis of variance (ANOVA) was used for all data analyses testing the effect of *HD2D* expression in WT, *hd2d-1*¸1-1, and 4-1 genotypes on specific phenotypes.

The one-way ANOVA was followed by a post-hoc Tukey test to compare the significance of the differences between individual genotypes. All statistical analyses were performed using the statistics program "R" version 3.1.3 Copyright© 2015 (The R Foundation for Statistical Computing).

Chapter 3: Results

3.1 Selection of *HD2D* knockout lines and *HD2D* overexpression lines

To evaluate the effect of *HD2D* expression on ABA-related processes, *HD2D* knockout and overexpression lines were utilized. An Arabidopsis *HD2D* knockout line (*hd2d-1*) of the Columbia-0 (col-0) ecotype was identified using an online database (www.arabdiopsis.org) and subsequently ordered from the Arabidopsis Biological Research Center (Columbus, OH). Figure 3.1A shows a schematic of the *HD2D* gene with the T-DNA insertion in the second intron of the gene as mapped by Kleinboelting et al. 2012).

Seedlings were screened to confirm the presence of the T-DNA insertion in the second intron of the *HD2D* gene. To confirm the insertion, a PCR was run on isolated genomic DNA from *hd2d-1* plants and the products were run on an agarose gel and compared to WT genomic DNA (Fig. 3.1B). The data was also used to confirm the homozygosity of the T-DNA insertion at both *HD2D* alleles in *hd2d-1* seedlings. Seedlings containing a homozygous T-DNA insertion had only one 614 base pair band on the agrose gel. However, seedlings containing undisrupted *HD2D* alleles one 1007 base pair band, the same size as WT genomic DNA (Fig. 3.1B). Heterozygous seedlings would have had two bands, one at 614 base pairs and one at 1007 base pairs, however, no heterozygous seedlings were recovered.

To generate the *HD2D* overexpression lines, the coding sequence of the *HD2D* gene was cloned into the Gateway® vector pEarleygate101 (Fig 3.1C) (Earley et al., 2003). Agrobacterium-mediated transformation was used to deliver the pEarleygate101-HD2D

construct into WT Arabidopsis (col-0), using the floral dip method of transformation. Using PCR, five independent transgenic lines containing the transgene were identified (Fig. 3.1D). Two overexpression lines (1-1 and 4-1) were bred to homozygosity and enough seeds were collected to be used in all subsequent experiments.

Using an RT-qPCR, the levels of the *HD2D* transcript were quantified in WT, *hd2d-1*, 1-1, and 4-1 plants (Fig. 3.2). *hd2d-1* knockout plants had limited *HD2D* transcript levels detected, 0.01 ± 0.004 fold lower ($p<0.001$) than WT plants. The 1-1 and 4-1 overexpression lines were shown to have *HD2D* transcript levels that were 3.87 ± 0.46 fold higher ($p<0.01$) and 4.02 ± 0.88 fold higher ($p<0.01$) than the WT, respectively.

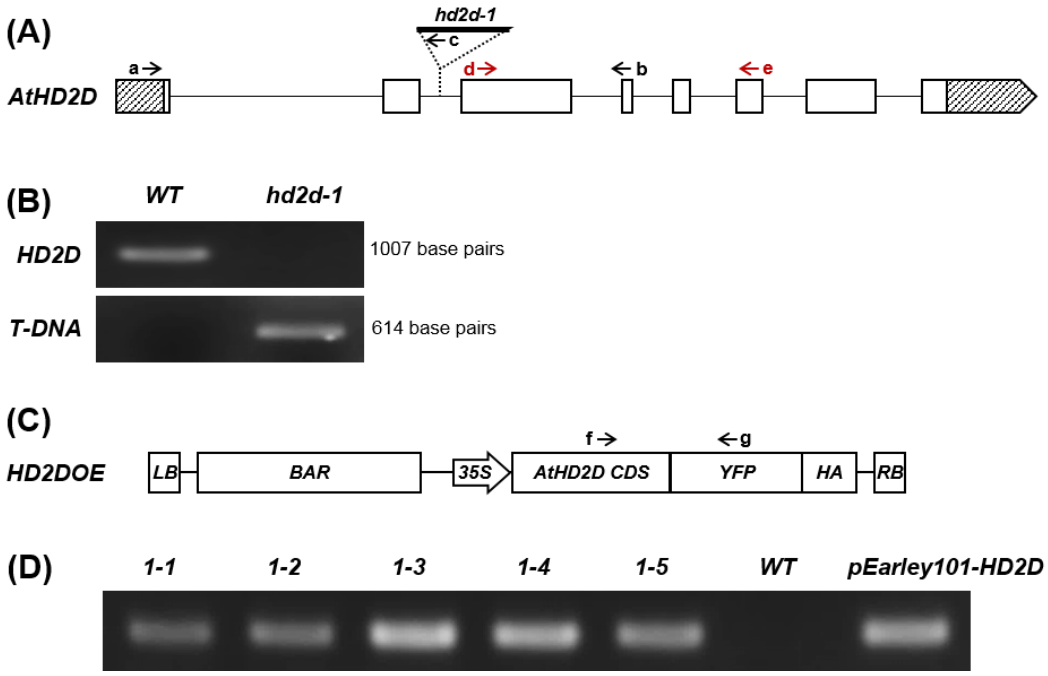

Figure 3.1 Generation of *HD2D* knockout and overexpression lines. (A) Schematic of the *HD2D* gene containing a T-DNA insertion in the second intron (black line), separated by exons (white boxes), and UTRs (shaded boxes) flanking the gene in the mutant line *hd2d-1* (GABI-Kat_379G06) as determined by Kleinboelting et al. (2012). (B) The *HD2D* WT allele was detected in WT but not *hd2d-1* plants using primers a and b, whereas the T-DNA insertion was detected in *hd2d-1* but not WT plants using primers a and c. (C) Schematic of the *HD2D* overexpression construct (pEarleygate101 backbone) inserted into WT Arabidopsis, separated by right and left borders (RB/LB), containing the glufosinate ammonium resistance gene (BAR), Cauliflower mosaic virus constitutive promoter (35S) driving the expression of *HD2D* coding sequence with a yellow fluorescent protein marker (YFP) on its C-terminus followed by an HA tag. (D) The T-DNA region of pEarleygate101-HD2D vector was detected in five transformed lines but not in WT plants using primers f and g. (primers: a:379G06F; b: 379G06R; c: GKpAC161LB; d:HD2DRTF; e: HD2DRTR; f: HD2D-101F; g: HD2D-101R)

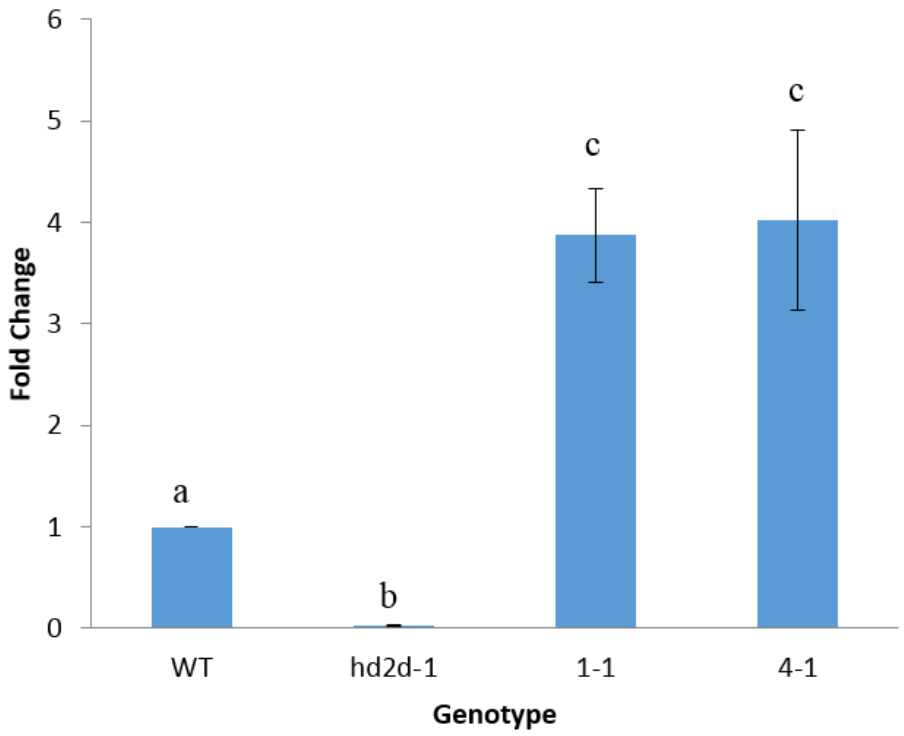

Figure 3.2 *HD2D* transcript levels in *HD2D* knockout and overexpression plants. Quantitative RT-PCR analysis indicated that the *HD2D* transcript was up-regulated in HD2DOE (1-1 and 4-1) plants and down-regulated in *hd2d-1* knockout plants. Transcripts were detected using the primers d and e in fig. 3.1. Different letter indicate significant differences between the genotypes of at least $p<0.01$.

3.2 *HD2D* expression affects germination in the presence of ABA

Germination is a process that is affected by many factors such as water conditions, light availability, temperature as well as proper ABA signaling (Finkelstein et al., 2000). The effect of *HD2D* expression on germination was investigated using *HD2D* knockout (*hd2d-1*) and overexpression lines (1-1 and 4-1). The percentage of germinated seeds of WT, *hd2d-1*, 1-1, and 4-1 was examined in sterile conditions in the presence and absence of 1 µM ABA. As mentioned in the Materials and Methods (section 2.3), germination was defined as complete radicle emergence and scored every 24 hours after plates were removed from 4ºC.

Under control growth conditions, no difference in germination success was observed between the WT, *hd2d-1*, 1-1, or 4-1 lines on any of the days and all lines achieved at least 94% germination by day two and 100% germination by day four (Fig. 3.3A). However, compared to control conditions, exposure to 1 µM ABA resulted in delayed germination in all lines (Fig. 3.3A and B). Interestingly, the extent of the delay in germination was genotype dependent. On day three, 74.1 ± 7.1% of WT seeds had germinated, compared to only 18.5 ± 5.7% of the *hd2d-1* knockout seeds ($p<0.01$), 40.1 ± 6.9% of the 1-1 overexpression seeds ($p<0.05$), and 35.2 ± 5.7% of the 4-1 overexpression seeds ($p<0.05$). However, on day three no significant difference in germination percentage was observed between the *hd2d-1* knockout seeds and the two *HD2D* overexpression seeds 1-1 ($p=0.17$) and 4-1 ($p=0.37$). While ABA treatment resulted in delayed germination in the *hd2d-1*, 1-1, and 4-1 lines, all seeds used in the experiment eventually germinated; WT reached 100% germination by day four while *hd2d-1*, 1-1, and 4-1 had reached 100% germination by day five.

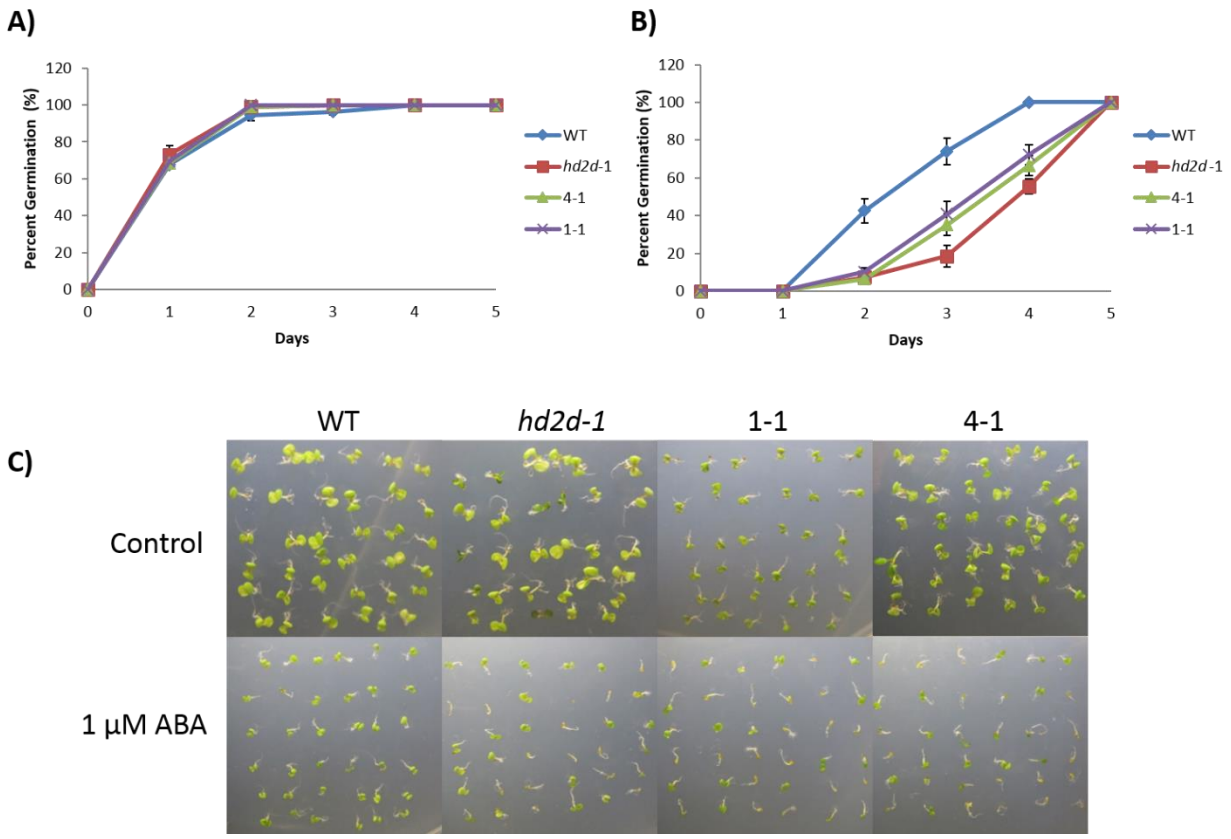

Figure 3.3 *HD2D* expression affects germination percentage in the presence of ABA. Germination rate of WT, *hd2d-1*, 1-1, and 4-1 seeds over five days under control conditions (A) and 1 µM ABA treatment (B). Mean ± SE values were determined from three replicates (N=3) and a total of 108 seeds per genotype. (C) Visual comparison of wild-type, *hd2d-1*, 1-1, and 4-1 seed germination after five days in the absence and presence of 1 µM ABA

3.3 *HD2D* prolongs vegetative growth phase and delays flowering

Proper timing of flowering is a process that is, in part, dependent on proper ABA signaling (Rogler and Hackett, 1975). In order to investigate whether *HD2D* expression affects flowering time, WT, *hd2d-1*, 1-1, and 4-1 plants were tracked under long day (LD) and short day (SD) light conditions and the day when bolting has occurred was recorded. Bolting is an indication of the switch from the vegetative phase to the reproductive phase of plant growth and can be used as an indicator of flowering time. As mentioned in the Materials and Methods (section 2.4), bolting was defined as inflorescence emergence greater than 5 mm in length.

As shown in Figures 3.4A, under SD conditions, *hd2d-1* plants bolted earlier than the WT plants while the 1-1 and 4-1 overexpression plants exhibited a clear delay in bolting. Specifically, on day forty-two, 57.8 ± 5.1% of *hd2d-1* plants had bolted, compared to only 24.0 ± 6.8% of WT that had bolted ($p<0.001$). No plants from the 1-1 and 4-1 overexpression lines had bolted by day forty-two, which was significantly less than the bolting percentage of WT plants ($p<0.05$ for both). As shown in Figure 3.4A, even though WT plants initially exhibited delayed bolting compared to *hd2d-1* plants, the difference in bolting eventually narrowed as *hd2d-1* lines reached 100% on day forty-seven, a day earlier than WT plants (day forty-eight). The 1-1 and 4-1 overexpression plants showed a similar bolting pattern to each other, achieving 100% on day fifty-six, later than both the WT and *hd2d-1* lines.

It is well known that Arabidopsis, an LD plant, flowers earlier when day length is LD conditions (16 hours of day light) than when day length is SD conditions (8 hours of

day light) (Teper-Bamnolker and Samach, 2005). As seen in figure 3.4A and B, my results support this earlier finding as, on average, all plants flowered earlier under LD conditions, regardless of genotype. Similarly to the bolting patterns under SD conditions, on average, under LD conditions *hd2d-1* plants bolted earlier than WT plants, while 1-1 and 4-1 overexpression plants bolted later than the WT (Fig. 3.4B). By day twenty-nine, 89.8 ± 15.6% of *hd2d-1* plants had bolted, compared to only 28.6 ± 11.7% of WT plants that had bolted ($p<0.001$). However by day twenty-nine, only 5.6 ± 2.5% of 1-1 plants had bolted and none of the 4-1 plants had bolted, much less than WT plants ($p<0.05$ and $p<0.01$, respectively). Furthermore, by day thirty-six, 100% of *hd2d-1* had bolted while WT, 1-1, and 4-1 lines reached 100% bolting on days forty, forty-four, and forty-four, respectively. These results demonstrate that increased *HD2D* is capable of delaying bolting under both SD and LD conditions.

To investigate whether the delay in flowering was due to growth retardation or a longer vegetative growth phase, the average number of rosette leaves was recorded at bolting for WT, *hd2d-1*¸ 1-1, and 4-1 plants. Rosette leaf number is an indication of the length of the vegetative phase. Plants with longer vegetative phases will exhibit a greater number of rosette leaves at flowering, while plants exhibiting retarded growth will have the same number of rosette leaves at flowering but will still flower later (Koornneef et al., 1991). As shown in figure 3.5A and D, the level of *HD2D* expression had a significant effect on the average number of rosette leaves at bolting ($p<0.001$). WT plants had an average of 15.9 ± 1.05 leaves in their rosettes, less than the 1-1 overexpression plants ($p<0.001$) which had an average of 25.0 ± 1.5 leaves in its rosette and less than the 4-1 overexpression plants ($p<0.01$) had an average of 22.5 ± 0.9 leaves in its rosette (Fig, 3.4A

and B), indicating that *HD2D* overexpression plants have longer vegetative phases than WT plants. Interestingly, *hd2d-1* plants had an average of 16.0 ± 0.61 leaves in their rosette at bolting which did not differ from WT plants ($p=0.70$). However, when comparing the total surface area of the rosette between the different lines, WT plants had a significantly greater ($p<0.05$) total rosette leaf surface area than *hd2d-1* plants (Fig. 3.5B and C), indicating that *hd2d-1* plants may have a shorter vegetative phase than WT plants. In addition, 1-1 and 4-1 plants had significantly greater total rosette leaf surface area than WT plants ($p<0.001$ and $p<0.001$, respectively), supporting the rosette leaf number results which indicated that 1-1 and 4-1 overexpression plants had longer vegetative growth phases.

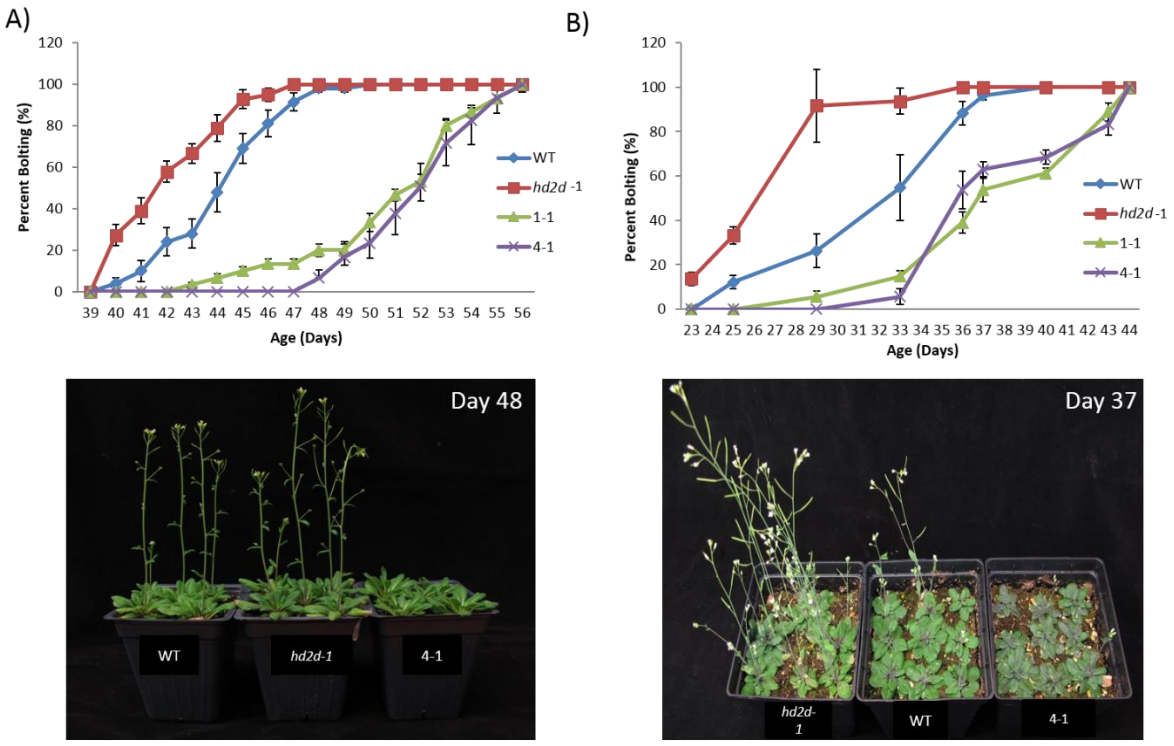

Figure 3.4 *HD2D* expression affects bolting time under long day and short day conditions. (A) Percent bolting of WT, *hd2d-1*, 1-1, and 4-1 plants under short day conditions over the course of seventeen days. Mean ± SE values were determined from greater than six replicates (total of 30-60 plants per genotype). (B) Percent bolting under long day conditions of WT, *hd2d-1*, 1-1, and 4-1 plants over the course of twenty-one days. Mean ± SE values were determined from six replicates (54 plants per genotype).

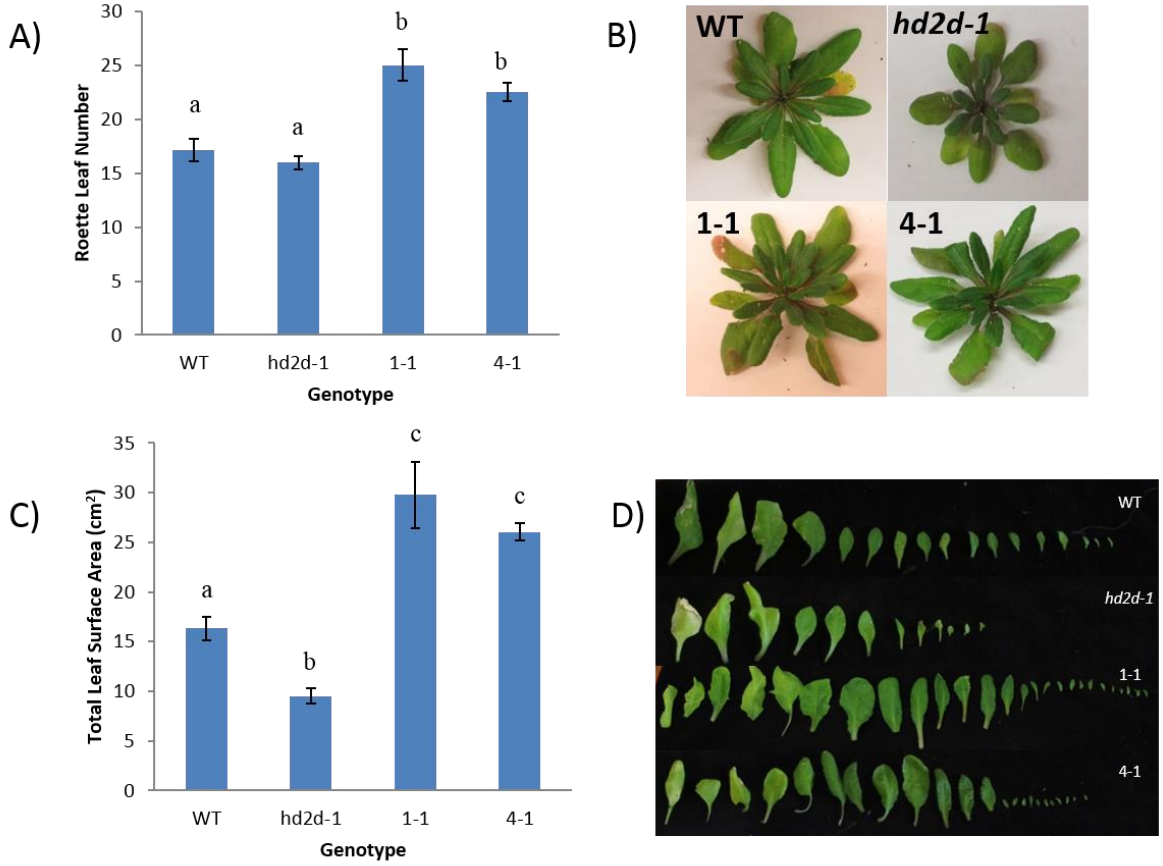

Figure 3.5 *HD2D* expression affects rosette leaf number and rosette leaf surface area. (A) Mean rosette leaf number at bolting for WT, *hd2d-1*, 1-1, and 4-1 plants. Mean ± SE values were determined from 4-7 replicates. (B) Visual representation of rosettes at bolting. (C) Mean total rosette leaf surface area at bolting for WT, *hd2d-1*, 1-1, and 4-1 plants. Mean ± SE values were determined from 4-5. (D) Leaf number at bolting, beginning with true leaves. Letters denote statistical differences between genotypes.

3.4 *HD2D* expression affects plant susceptibility to drought stress and salinity stress

It is well established that ABA is a key player in the activation of stress responses plants exhibit to both drought and high salinity conditions (Raghavendra et al., 2010). Moreover, in recent years it has become evident that HDACs play a key role in ABA signaling (Sridha and Wu, 2006; Chen et al., 2010; Luo et al., 2012). To examine the effect of *HD2D* expression on drought tolerance plants of all four genotypes (WT, *hd2d-1*, 1-1, and 4-1) were subjected to a desiccation regime (outlined in section 2.5). Interestingly, the abundance of *HD2D* transcript did affect the survival of different genotypes during the drought treatment ($p<0.001$; Fig. 3.6A and B). The *hd2d-1* knockout plants were the least resistant to the drought treatment with only 13.9 ± 5.01% of plants surviving, less than the WT plants ($p<0.001$) of which 47.2 ± 5.01% had survived. The *HD2D* overexpression plants were most resistant to the drought treatment with 83.33 ± 6.8% of 1-1 plants and 88.9 ± 3.93% of 4-1 plants surviving the treatment, both more resistant than the WT plants ($p<0.05$ and $p<0.001$, respectively). To ensure estimates of plant mortality were accurate, all plants were monitored for another ten days, all estimates were correct.

To examine the effect of *HD2D* expression on salt tolerance, all four genotypes (WT, *hd2d-1*, 1-1, and 4-1) were subjected to the salt treatment. As outlined in section 2.5, percent leaf death was measured and a leaf was considered dead when it exhibited greater than 50% chlorosis. By day fourteen of the salt treatment, *HD2D* expression had a significant effect on percent leaf death ($p<0.001$). However, the differences between the genotypes were not completely evident until day twenty-one of treatment ($p<0.001$; Fig. 3.7A and B). After twenty-one days of treatment, *HD2D* overexpression plants were found

to be the most resistant to the salt treatment as 16.8 ± 1.6% of 1-1 leaves and 14.6 ± 2.2% of 4-1 leaves had died, significantly lower ($p<0.001$ for both) than the WT plants of which 30.2 ± 1.9% of leaves had died. Interestingly, in response to the salt treatment only 36.2 ± 2.2% of the *hd2d-1* leaves had died, not significantly different than WT leaf death ($p=0.13$). The results of the drought and salt experiments demonstrated that *HD2D* is involved in plant response to drought and salt conditions and that increased *HD2D* transcript levels results in increased resistance to these abiotic stresses.

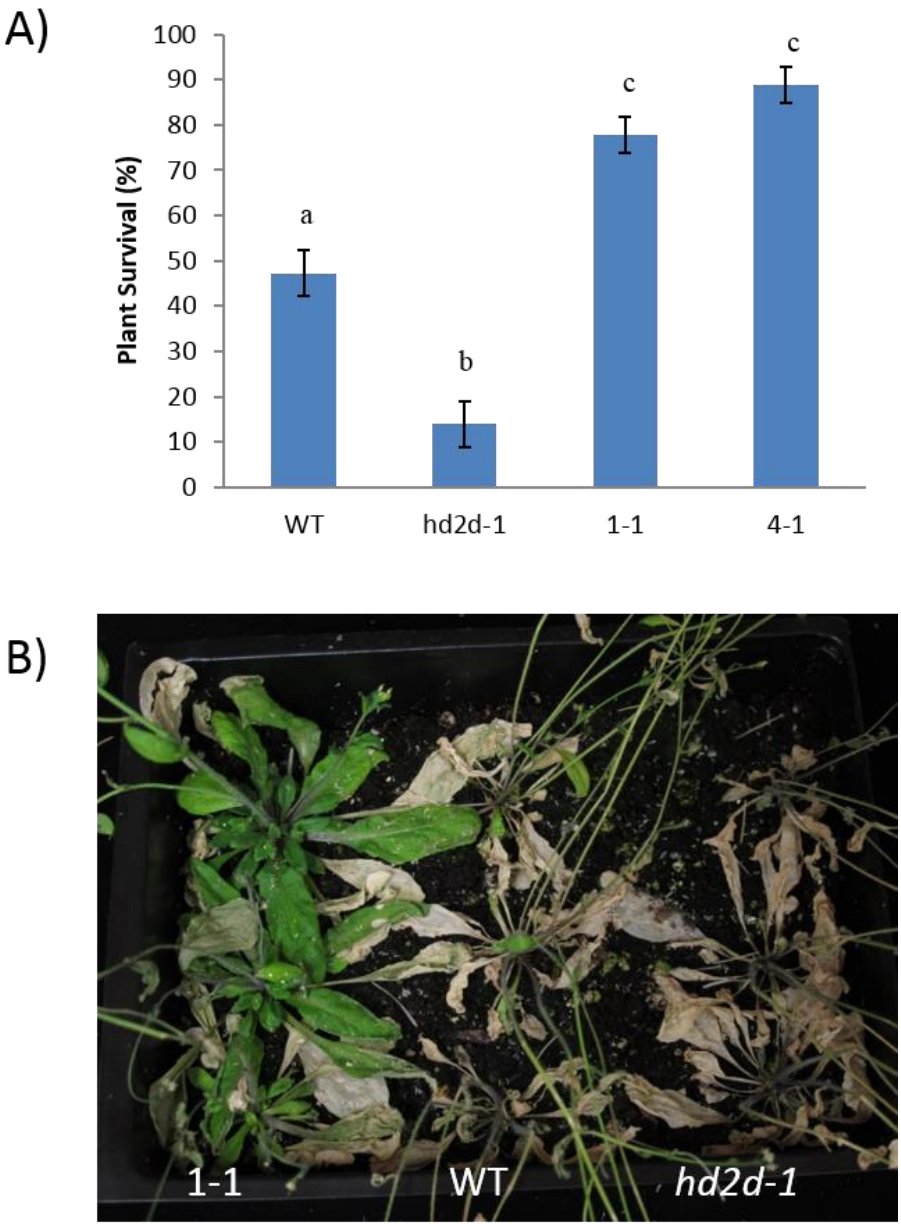

Figure 3.6 *HD2D* expression affects plant survival under drought stress. (A) Plant recovery after a fourteen day desiccation treatment, two days after being re-watered and allowed to recover. (B) Photograph of plant recovery after the desiccation treatment in A. Mean ± SE values were determined from three replicates (total of 18-36 plants). Different letters indicate significant differences between the genotypes ($p<0.05$)

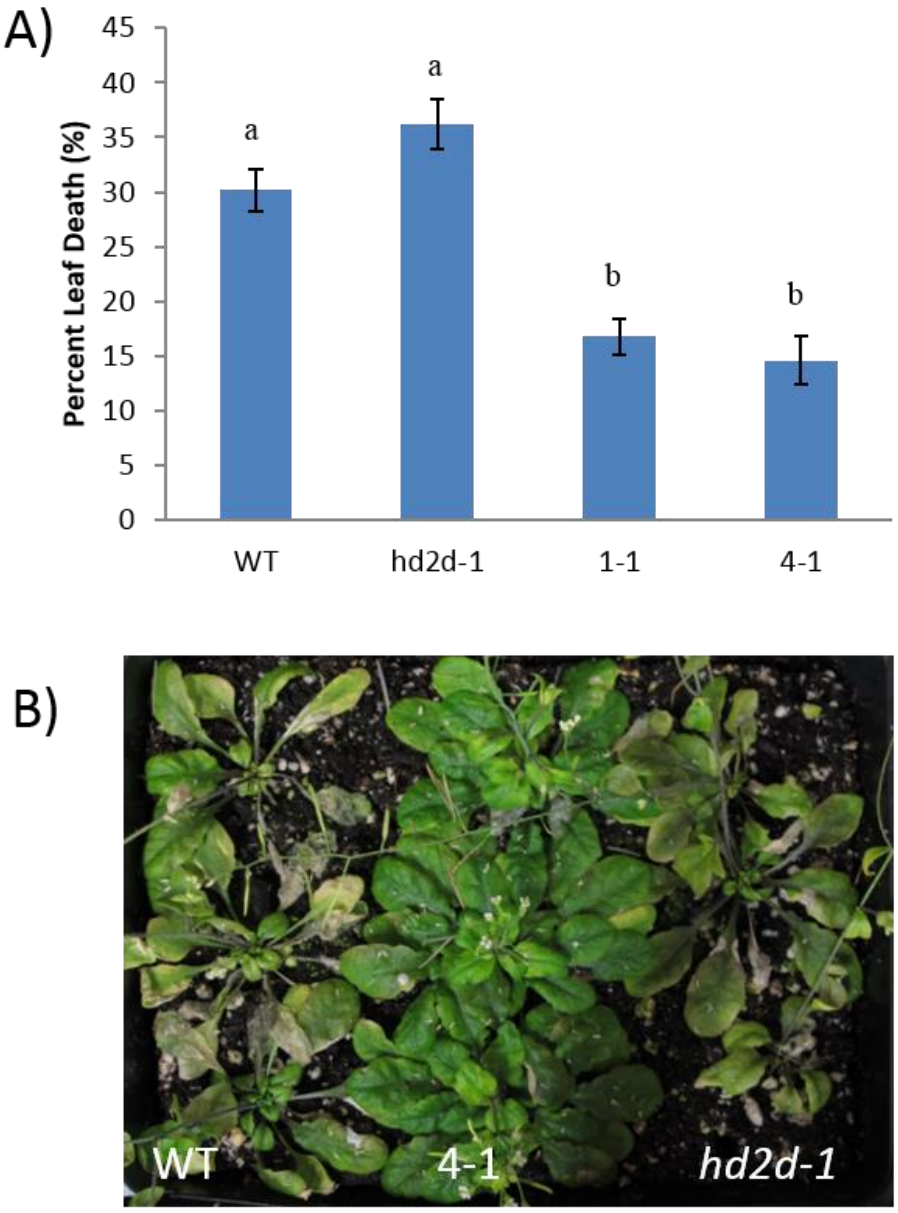

Figure 3.7 *HD2D* expression affects plant survival under salinity stress. (A) Percent leaf survival after plants were treated with 200mM NaCl for twenty-one days. (B) Photograph of plant recovery taken on day twenty-one of the 200mM NaCl treatment in (A). Mean ± SE values were determined from three replicates (total of 18-36 plants per genotype). Different letter indicate significant differences between the genotypes ($p<0.05$)

3.5 *HD2D* affects transcription of development- and drought-related genes

Once *HD2D* expression was found to affect development and stress response in Arabidopsis, I decided to investigate the effect of *HD2D* expression at the molecular level, specifically gene expression. To quantify changes in expression of drought and development-related genes, RNA was extracted from entire rosettes of thirty day old WT, *hd2d-1*, 1-1, and 4-1 plants under normal conditions.

Since my earlier findings suggested that *HD2D* affects flowering time (section 3.3), I evaluated the potential role of *HD2D* on the transcript levels of key genes known to be involved in the timing of flowering. These genes included: *FLC* (*Flowering Locus C*), *SOC1* (*Suppressor of Overexpression of Constans 1*), *FUL* (*Fruitfull*), and *SEP3* (*Sepallata 3*). *FLC* is involved in regulating germination and in the repression of flowering (Michaels and Amasino, 1999; Chiang et al., 2009). As shown in Figure 3.8A, compared to WT plants the transcript levels of *FLC* were 2.80 ± 0.37 fold greater in 1-1 plants ($p<0.01$) and 2.68 ± 0.43 fold greater in 4-1 plants ($p<0.05$). In comparison, transcript levels of *FLC* in *hd2d-1* plants were 0.28 ± 0.05 fold lower ($p<0.01$) than in WT plants. High transcript levels of the *SOC1* transcription factor was found to promote early flowering (Samach et al., 2000). As shown in Figure 3.8A, compared to WT plants the transcript levels of *SOC1* were 0.04 ± 0.02 fold lower in 1-1 plants ($p<0.01$) and 0.13 ± 0.07 fold lower in 4-1 plants ($p<0.05$). However, in *hd2d-1* plants, transcript levels of *SOC1* were 1.55 ± 0.11 fold higher than that of WT plants, which was not significant ($p=0.92$). *FUL* is a transcription factor that has been shown to be essential for correct timing of flowering during development, as the *ful-1* mutant displays a delayed flowering phenotype (Banmolker and Samach, 2001). As

shown in Figure 3.8A, compared to the WT, transcript levels of *FUL* were found to be 0.33 ± 0.07 fold lower ($p<0.01$) in 1-1 plants. However in 4-1 plants, the transcript levels of *FUL* were only 0.52 ± 0.03 fold lower than that of WT, which not significantly different from WT plants ($p=0.13$) or 1-1 plants ($p=0.22$). Transcript levels of *FUL* in *hd2d-1* plants were found to be 2.71 ± 0.26 fold higher than WT plants ($p<0.05$). *SEP3* is a transcription factor that is a positive regulator of flowering (Samach et al., 2000). Compared to WT plants, transcript levels of *SEP3* were 0.18 ± 0.04 fold lower ($p<0.01$) in 1-1 plants and 0.29 ± 0.12 fold lower ($p<0.05$) in 4-1 plants (Fig. 3.8A). In *hd2d-1* plants, *SEP3* transcript levels were found to be 1.7 ± 0.08 fold higher than expression in WT plants but not significant (Fig. 3.8A; $p=0.53$).

Since my earlier finding suggested that *HD2D* affects plant resistance to drought and salt treatments (section 3.4), I evaluated the potential role of *HD2D* on the transcript levels of key genes involved in the ABA-response to water stress. These genes included: *RD29A* (*Responsive to Desiccation 29A*), *ABI1* (*ABA-insensitive 1*), and *ABI5* (*ABA-insensitive 5*). The *RD29A* gene contains an ABA-responsive element (ABRE) and its transcript levels were shown to be increased in response to water stress in ABA-dependent and ABA-independent pathways (Narusaka et al., 2003). Compared to WT plants, transcript levels of *RD29A* were found to be 3.78 ± 0.11 fold higher ($p<0.001$) in 1-1 plants and 3.16 ± 0.77 fold higher ($p<0.01$) in 4-1 plants (Fig. 3.8B). The transcript levels of *RD29A* in *hd2d-1* plants were 0.38 ± 0.02 fold lower than in WT plants ($p<0.01$). *ABI1* is a phosphatase that regulates the ABA response and is required for proper ABA response and stomatal closure (Leung et al., 1997). Compared to WT plants, *ABI1* transcript levels were 4.34 ± 0.72 fold higher ($p<0.001$) in 1-1 plants and 2.61 ± 0.04 fold higher ($p<0.01$)

in 4-1 plants (Fig. 3.8B). In contrast, *ABI1* transcript levels in *hd2d-1* plants was 0.44 ± 0.13 fold lower ($p<0.01$) than WT plants (Fig. 3.8B). *ABI5* is a transcription factor that is upregulated in response to drought or salt treatments and it regulates the expression of a number of ABA-responsive genes (Lopez-Molina et al., 2001). Compared to WT plants, *ABI5* transcript levels were 5.88 ± 1.27 fold higher ($p<0.001$) in 1-1 plants and 6.41 ± 0.69 fold higher ($p<0.001$) in 4-1 plants (Fig 3.8B). In contrast, compared to WT plants *ABI5* transcript levels were 0.24 ± 0.02 fold lower ($p<0.01$) in *hd2d-1* plants (Fig 3.8B). These results demonstrate that the expression of *HD2D* does affect the transcript levels of drought and development-related genes.

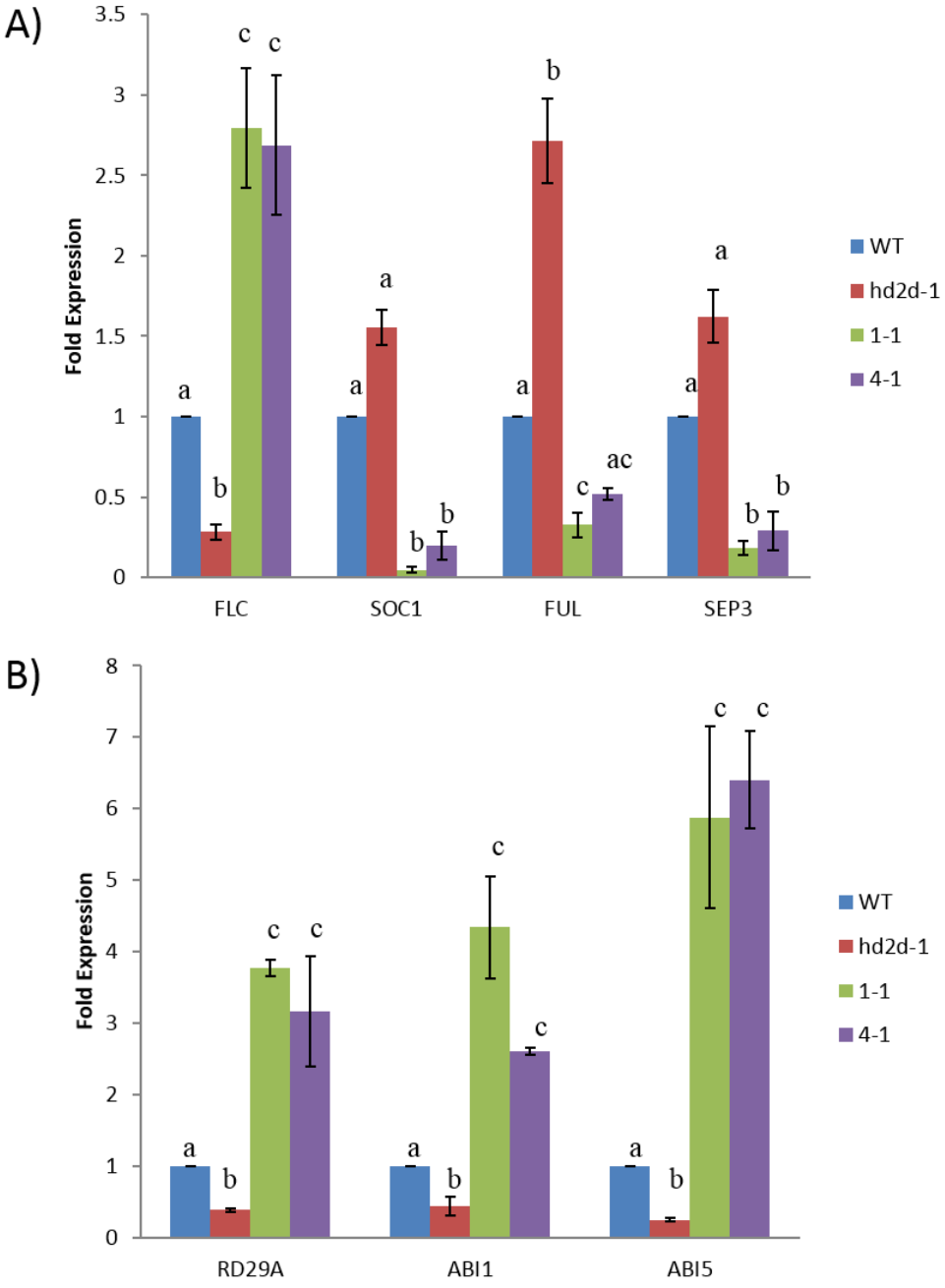

Figure 3.8 *HD2D* affects the expression of development- and drought-related genes. Gene expression analysis of development-related (A) and drought-related (B) RNA samples were extracted from thirty day old plants' rosette leaves growing under short-day conditions. Mean ± SE values were determined from 3-4 biological replicates. Different letters indicate significant differences in expression of a specific gene between genotypes of at least $p<0.05$.

3.6 *HD2D* expression does not affect global H3 and H4 acetylation

HDACs can affect gene transcription by removing acetyl groups from K residues of H3 and H4 histone tails. To investigate whether the level of *HD2D* affected global acetylation of H3 and H4, Western blot analysis was used. Acid-soluble proteins (containing histones) were extracted from the rosette leaves of 4 week old WT, *hd2d-1*, 1-1, and 4-1 seedlings, grown under optimal conditions, and separated using gel electrophoresis (see section 2.7). Proteins were probed for with anti-acetyl H3, anti-acetyl H4, or anti-H3 as a loading control (Table 2.2). Western blot analysis revealed that *HD2D* does not affect global H3 and H4 acetylation levels (Fig. 3.9).

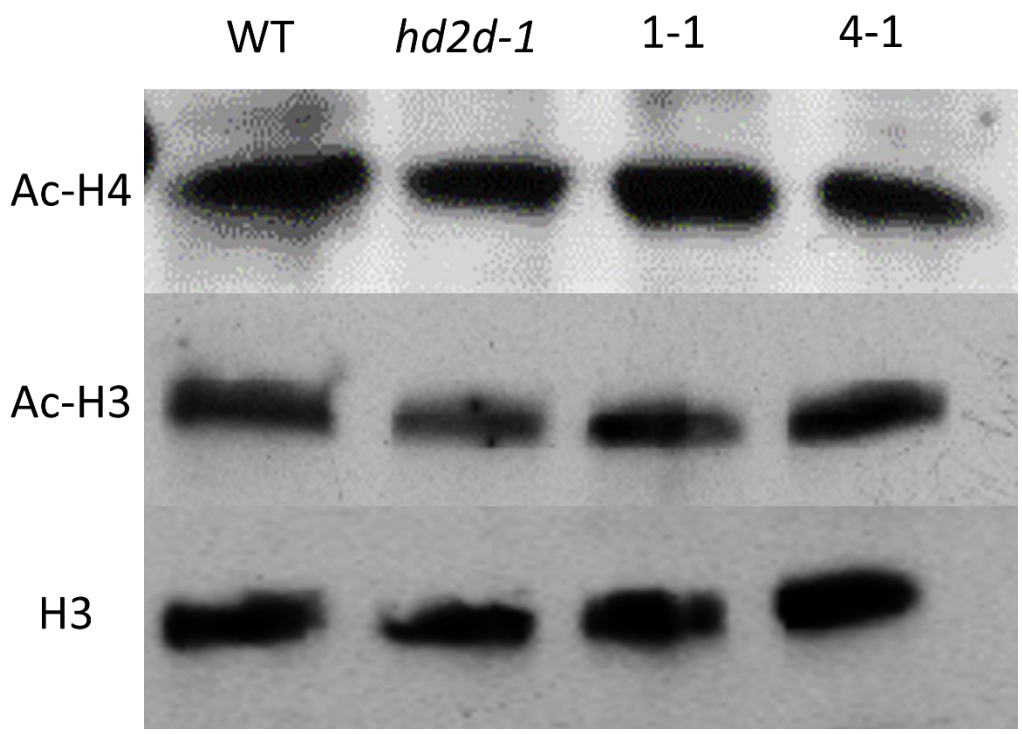

Figure 3.9 *HD2D* does not affect global acetylation levels of H3 and H4. Acid-soluble proteins were extracted from 4 week old WT, *hd2d-1*, 1-1, and 4-1 rosettes and global acetylation of H3 and H4 were measured using Western blot analysis, no difference in acetylation was observed between genotypes. Histone H3 was used as a loading control.

3.7 HD2D interacts with proteins involved in ABA signaling and other HD2 family members

Biomolecular fluorescence complementation (BiFC) is a technology that can be used to show protein-protein interactions *in vivo*. Yellow fluorescence protein (YFP) is used an indication of interaction between two proteins of interest, by fusing the N-terminus end of YFP to one protein and the C-terminus end of YFP to the other. BiFC was used to investigate whether the HD2D protein interacts with proteins involved in ABA signaling and other HD2 family members, *in vivo*. Specifically, the ABI1, ABI2, and ABI5 proteins that are key regulators of the ABA stress response (Raghavendra et al., 2010) and the HD2A, HD2B, and HD2C proteins that are members of HD2D's own HD2 family. The HD2D coding region was fused to the N-terminal amino acid portion of the YFP in the pEarleygate202-YN vector. The *ABI1*, *ABI2*, *ABI5*, *HD2A*, *HD2B*, and *HD2C* genes' coding regions were fused separately to the C-terminal amino acid portion of YFP in the pEarleygate202-YC vector (see section 2.1).

To visualize the interaction *in vivo* between HD2D and these proteins, HD2D-YN was co-delivered with each one of the other six vectors into leaves of 4 week old *Nicotiana benthamiana* plants, and visualized using confocal microscopy. To visualize YFP expression, fluorescence at YFP's excitation wavelength (527 nm) was observed. As a control for these experiments, the auto-fluorescence in untreated *N. benthamiana* leaves was measured.

When the HD2D construct was co-transfected with either the HD2A or HD2C constructs, YFP expression was evident in the nucleus (Fig. 3.10), indicating HD2D

interaction. In contrast, when HD2D was co-transfected with HD2B (Fig. 3.10), no YFP expression was evident, indicating that these two proteins do not interact. Furthermore, when the HD2D construct was co-transfected with the PP2C proteins ABI1 or ABI2 constructs, YFP expression was evident in the nucleus, cytoplasm, and possibly the plasma membrane (Fig. 3.11), indicating that HD2D interacts with these proteins in those regions. When the HD2D construct was co-transfected with the transcription factor ABI5, YFP expression was evident in the nucleus (Fig. 3.11), indicating that HD2D interacts with ABI5 in the nucleus. The localization of HD2D to the nucleus, cytoplasm, and the plasma membrane is in accordance with findings by Dr. Gary Tian (unpublished).

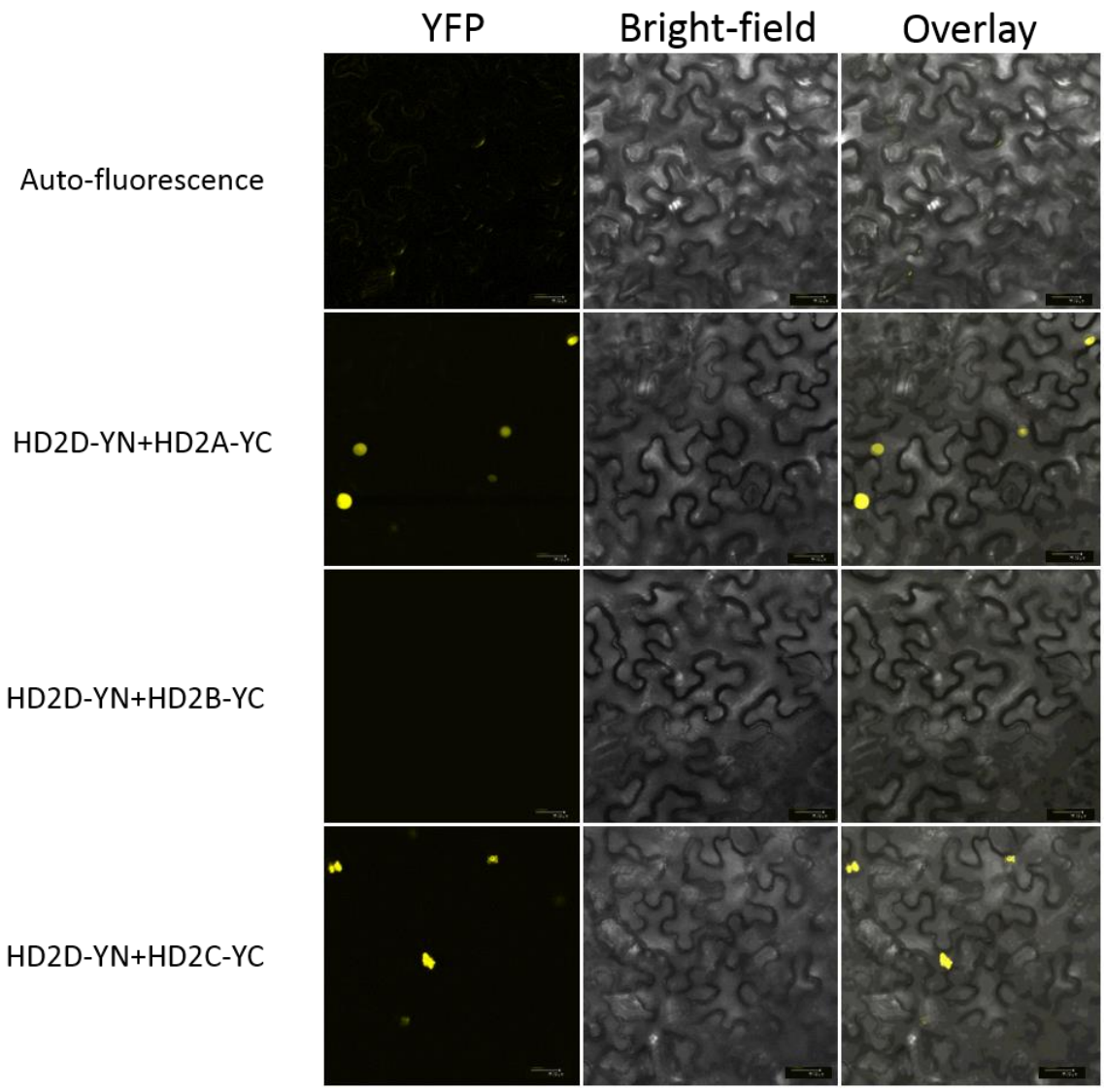

Figure 3.10 HD2D interacts with members of the HD2 family in BiFC assays. HD2D was fused with N-terminal of YFP (YN), while HD2A, HD2B, and HD2C were individually fused with C-terminal of YFP (YC). HD2D-YN together with each -YC combination were co-transfected into *N.benthamiana* leaves using *Agrobacterium tumefaciens* and visualized with a confocal microscope.

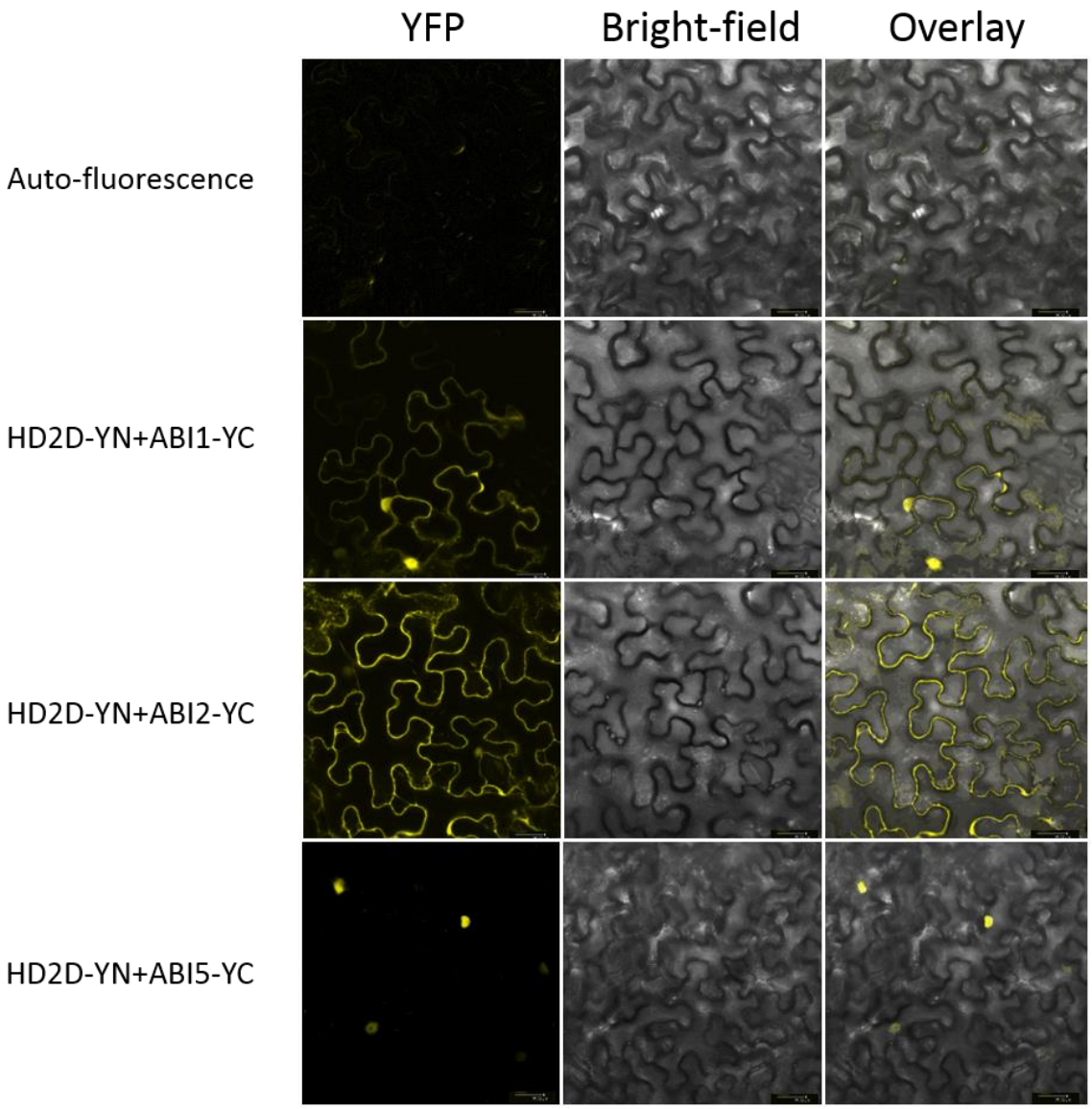

Figure 3.11 HD2D interacts with components of the ABA pathway in BiFC assays. HD2D was fused with N-terminal of YFP (YN), while ABI1, ABI2, and ABI5 were individually fused with C-terminal of YFP (YC). HD2D-YN together with each -YC combination were co-transfected into *N.benthamiana* leaves using *Agrobacterium tumefaciens* and visualized with a confocal microscope.

Chapter 4: Discussion

The overall aim of this study was to investigate the involvement of *HD2D* in ABA-related developmental programs and stress responses in Arabidopsis. This was accomplished by using Arabidopsis genotypes that had altered *HD2D* expression: an *HD2D* knockout line (*hd2d-1)* and two *HD2D* overexpression lines (1-1 and 4-1). I studied the effect of *HD2D* expression on the ABA-related processes of germination, flowering, drought, and salinity stress.

4.1 *HD2D* is a positive regulator of the ABA stress response

The major finding of this study indicated that *HD2D* positively regulates the ABA stress response in Arabidopsis. The overexpression of *HD2D* resulted in increased transcript levels of ABA-response genes (*RD29A*, *ABI1*, and *ABI5*) and increased resistance to salt and drought treatments. Furthermore, the knockout of *HD2D* reduced transcript levels of ABA-response genes (*RD29A*, *ABI1*, and *ABI5*) and reduced resistance to salt or drought treatments.

The promoter of *RD29A* contains both ABRE (ABA-dependent) and dehydration regulatory elements (ABA-independent) that contribute to increased transcription in response to dehydration, high-salinity, and low-temperature in ABA-dependent and ABA-independent manners (Narusaka et al., 2003). The ABA-dependent upregulation of the *RD29A* transcript can be explained by the involvement of the bZIP transcription factor ABI5 (Miura et al., 2009), a major component of the ABA signaling pathway that binds to ABREs, promoting transcription (Lopez-Molina et al., 2002). In addition, *ABI1* encodes a protein phosphatase 2C (PP2C) that is a major component of ABA signaling, negatively

regulating the ABA-response in an ABA-dependent feedback loop (Merlot et al., 2001). However, *ABI1* transcript levels are increased in response to ABA and ABI1 enhances the interaction between ABA and its RCAR receptors (Leung et al., 1997; Cutler et al., 2010). The fact that genetic manipulation of *HD2D* expression affected the transcript levels of these major regulatory genes in the ABA pathway suggests that *HD2D* is a regulator of the ABA stress response.

Through the use of BiFC assays, this study also demonstrated that the HD2D protein interacts with the ABI1, ABI2, and ABI5 proteins – all major components of the ABA signal transduction pathway (Raghavendra et al., 2010), *in vivo*. The interaction of HD2D with the PP2C homologs ABI1 and ABI2 is evident in the nuclear, cytoplasmic, and possibly plasma membrane regions, coinciding with the cellular localization of both ABI1 (Zhang et al., 2004; Moes et al., 2008) and HD2D (Dr. Gary Tian, unpublished results). Moreover, Zhang et al. (2004) found that ABI1 localization is crucial for its regulation of the ABA-response. The ABI1 protein is relocated from the nucleus to the cytoplasm and plasma membrane in an ABA-dependent manner. In addition to the ABA-dependent inhibition of ABI1 activity (see section 1.5.2; Fig. 1.2), the ABA-dependent relocation of ABI1 further reduces its negative regulation of the ABA response (Moes et al., 2008).

The finding that HD2D interacts with ABI1 and ABI2 in multiple compartments within the cell raises a number of interesting possibilities for the interactions of HD2D with ABI1 and ABI2. First, acetylation of non-histone proteins has been shown to occur in plants (Finkelmeier et al., 2011; Wu et al., 2011) and the acetylation of non-histone proteins can affect enzymatic activity and protein localization in other eukaryotic systems (Glozak

et al., 2005). This raises the possibility that HD2D regulates the localization or phosphatase activity of ABI1 and ABI2 by their acetylation, reducing their negative regulation of the ABA response, leading to the enhanced ABA response seen in *HD2D* overexpression lines. Second, the activity of phosphatases and kinases has been shown to affect chromatin acetylation in Arabidopsis by direct modification of epigenetic factors. Specifically, the PP2C enzyme AtPP2C-6-6 was found to directly interact and dephosphorylate GCN5, a major HAT affecting large-scale expression in Arabidopsis (Servet et al., 2008). Taking into consideration that HD2D's maize homolog HD2 has been shown to be phosphorylated (Lusser et al., 1997), these findings suggest that the PP2C enzymes ABI1 and ABI2 may modify HD2D's phosphorylation status. The phosphorylation of HD2D could affect its HDAC function during ABA response, causing changes in gene expression observed in this study. Finally, Sokol et al. (2007) found that in response to water stress or ABA treatments, histone H3 undergoes both phosphorylation and acetylation events. This raises the possibility that HD2D interacts with ABI1 and ABI2 as part of a complex that regulates gene expression directly. Interestingly, Himmelbach et al. (2002) demonstrated that ABI1 forms a complex capable of repressing gene expression by directly interacting with the homeodomain-containing transcription factor AtHB6 – suggesting ABI1 may be directly involved in the repression of gene expression.

The current study found that HD2D interacts with the bZIP transcription factor ABI5 in the nucleus. The fact that ABI5 is a transcription factor means that that ABI5 may be able to recruit HD2D to specific loci. The association of these proteins could form a complex that directly regulates gene expression, suggesting ABI5 may be involved in inhibition of transcription, due to HD2D's HDAC activities. Research by Kim et al. (2008)

has shown that some ABRE-containing genes are subject to epigenetic modifications such as acetylation, raising the possibility that ABI5 and HD2D may be involved in the regulation of these genes, since ABI5 is a regulator of some ABRE-containing genes. Another possibility for the interaction between HD2D and ABI5, is that HD2D regulates ABI5 activity through deacetylation. The ABI5 protein is subject to post-translational modifications such as phosphorylation and sumoylation, which promote ABI5 activation and stability in an ABA-dependent manner (Lopez-Molina et al., 2001; Muira et al., 2009). Phosphorylation of ABI5 by SnRKs results in its activation and stabilization (Lopez-Molina et al., 2001), while its sumoylation by SIZ1 protects it from degradation (Miura et al., 2009). Therefore it is possible that HD2D affects ABI5 stability or activity by post-translational modifications, as it is well known that non-histone proteins (including transcription factors) can be targets of HATs and HDACs (Glozak et al., 2005; Wu et al., 2011).

HDACs have previously been implicated in regulation of the ABA stress response. For example, the RPD3-like family members *HDA6* and *HDA19* and the HD2 family member *HD2C* have all been found to have a regulatory role in the ABA stress response (Sridha and Wu, 2006; Chen et al., 2010; Chen and Wu., 2010; Luo et al., 2012). Similarly to the *HD2D* overexpression plants investigated in this study, Sridha and Wu (2006) found that *HD2C* overexpression resulted in Arabidopsis plants that were more resistant to drought and high salinity. Furthermore, *HD2D* overexpression plants had increased expression of the ABA and drought responsive gene *RD29A*, a homolog of *RD29B* also upregulated in *HD2C* overexpression plants (Yamaguchi-Shinozaki and Shinozaki, 1993; Sridha and Wu, 2006). The *RD29A* and *RD29B* homologs have very similar expression

profiles (Yamaguchi-Shinozaki and Shinozaki, 1993), suggesting that *HD2D* and *HD2C* may regulate these genes in a similar manner. Furthermore, unlike the *HD2D* overexpression seeds that exhibited delayed germination when treated with ABA, indicative of an enhanced ABA response (Lopez-Molina et al., 2001), *HD2C* overexpression seeds germinated early when treated with ABA (Sridha and Wu, 2006). However, seeds overexpressing *HD2C* germinated early under control conditions as well (Sridha and Wu, 2006), suggesting that the early germination was not due to ABA treatment and that *HD2C* may be involved in additional developmental programs compared to *HD2D*.

Similarly to the *hd2d-1* mutant line investigated in this study, *hd2c*, *hda6*, and *hda19* mutant lines have previously been shown to exhibit delayed or reduced germination in response to ABA treatments in addition exhibiting reduced survival in response to drought and salt treatments (Sridha and Wu, 2006; Chen et al., 2010; Chen and Wu, 2010). However, unlike *hd2c* and *hda6* mutant plants in which the *ABI1* transcript was upregulated (Luo et al., 2012), *hd2d-1* mutant plants had decreased levels of the *ABI1* transcript. These results suggest that *HD2C* and *HDA6* may regulate ABA stress response differently than *HD2D*. Furthermore, Luo et al. (2012) found that *hd2c* and *hda6* mutants had increased levels of acetylation at a number of loci involved in the ABA response. This suggests that *HD2C* and *HDA6* may regulate ABA stress response by histone acetylation. The results of this study showed that *HD2D* does not affect global H3 and H4 histone acetylation, however the effects of *HD2D* expression on acetylation at specific loci or specific K residues remains to be investigated. Interestingly, the current study indicated that HD2D can interact with HD2C in the nucleus while a previous study found that HD2D can also

interact with HDA6 in the nucleus (Luo et al., 2012b). Since HD2C and HDA6 have been shown to interact with each other as part of a complex that regulates ABA stress responses (Luo et al., 2012), the fact that HD2D interacts with both HD2C and HDA6, suggests that HD2D could be a part of that complex.

The results of the current study demonstrate that *HD2D* is positive regulator of ABA signaling during stress responses. The fact that *HD2D* affects the expression of major components of the ABA stress response pathway highlights the importance of *HD2D* in this pathway. Interestingly, HD2D also appears to physically interact with a number of major regulators of the ABA signal transduction pathway, suggesting HD2D may play a much larger role in the regulation of the ABA response than previously thought (Fig. 4.1).

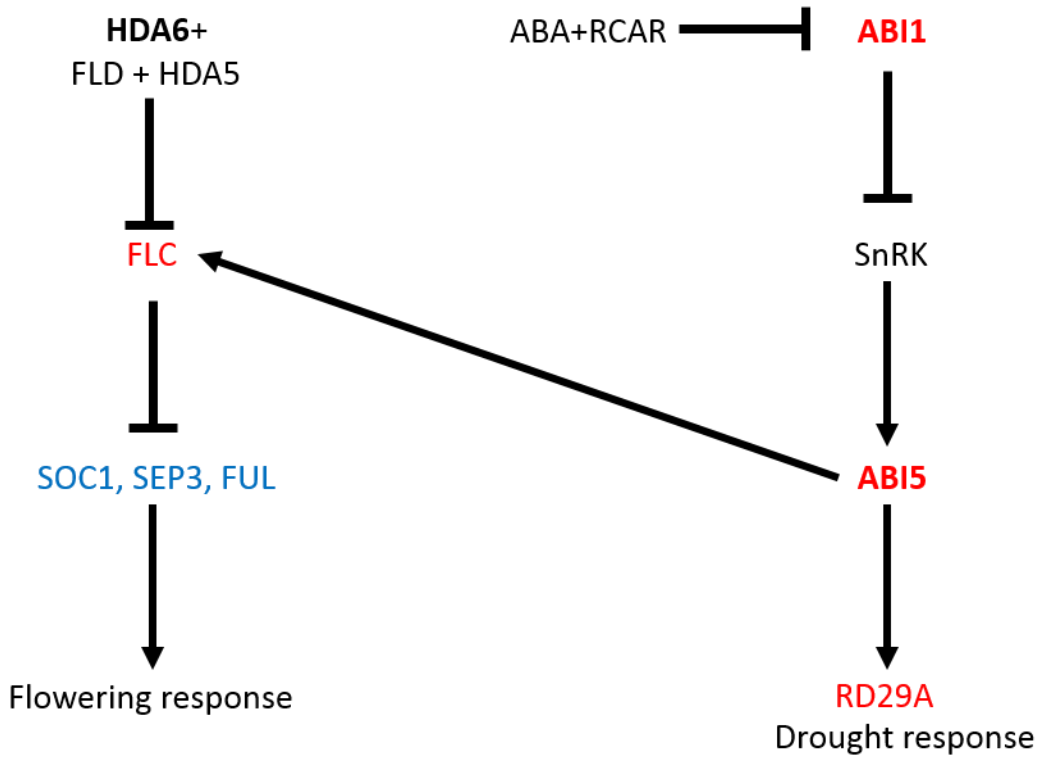

Figure 4.1 *HD2D* promotes drought response and delays flowering. *HD2D* expression results in upregulation of FLC, ABI1, ABI5, and RD29A (red) and downregulation of SOC1, SEP3, and FUL (blue). The HD2D protein appears to interact with the ABI1, ABI5, and HDA6 proteins (bold), although the results of these interactions are not well understood.

4.2 *HD2D* prolongs the vegetative growth phase and delays flowering

In Arabidopsis, the switch from the vegetative phase of growth to the reproductive phase of growth is marked by rapid emergence of inflorescence from the center of the rosette leaves (bolting), followed by flowering. The results of this study found *HD2D* negatively regulates flowering by lengthening the vegetative phase under both short day (SD) and long day (LD) conditions. These results were further supported by gene expression data that showed an increase in transcript levels of a floral inhibitor (*FLC*) and a decrease in floral activators (*SOC1*, *SEP3*, and *FUL*) in *HD2D* overexpression plants prior to bolting. The opposite expression pattern of *FLC*, *SOC1*, *SEP3*, and *FUL* was observed in the *hd2d-1* knockout line.

SOC1 is a MADS box transcription factor that is a "floral integrator" that is regulated by signals from the autonomous, vernalization, GA, and photoperiod floral induction pathways to induce flowering (Samach et al., 2000; Corbesier and Coupland, 2006). *FUL* and *SEP3* are MADS box transcription factors that positively regulate flowering and their expression is affected through the autonomous, vernalization, and photoperiod floral induction pathways (Corbesier and Coupland, 2006). Conversely, the major floral regulator *FLC* is a negative regulator of flowering that has been shown to act upstream of *SOC1*, *SEP3*, and *FUL*, negatively regulating their expression (Corbesier and Coupland, 2006). Furthermore, *FLC* expression is controlled by both the autonomous and vernalization pathways, acting as a convergence point between the two (Cobesier and Coupland, 2006). The fact that *HD2D* expression affects *FLC* expression suggests that *HD2D* may affect flowering through regulation by either the autonomous or vernalization floral induction pathways.

Chiang et al. (2009) found that *FLC* overexpression led to ABA degradation and increased germination under cold conditions. Additionally, Seo et al. (2009) found that during exposure to cold stress, *FLC* is overexpressed to delay flowering and prevent damage to reproductive organs. The results of these studies suggest that FLC may be involved in ABA signaling during cold stress response and possibly flowering. Interestingly, higher *FLC* expression has been linked with increased seed germination under cold conditions (Chaing et al., 2009), suggesting that *HD2D* overexpression lines may be better able to germinate in low temperatures, while *hd2d-1* may be less tolerant, however, this remains to be studied.

Interestingly, upregulation of the *FLC* transcript has been found in plants overexpressing the *ABI5* transcription factor which is intimately involved in the ABA signal transduction pathway (Raghavendra et al., 2010; Wang et al., 2013). Taken together with the fact that both *ABI5* and *FLC* transcripts were upregulated in *HD2D* overexpression plants, these results suggest that the upregulation of the *FLC* transcript seen in HD2D overexpression plants could be due to *ABI5* upregulation (Fig. 4.1).

HDACs have previously been implicated in regulation of flowering in Arabidopsis. The RPD3-like family members *HDA5, HDA6* and *HDA9* and the HD2 family member *HD2A* have all been found to affect flowering time (Zhou et al., 2004; Wu et al., 2008; Kim et al., 2013; Luo et al., 2015). Unlike the negative regulation of flowering by *HD2D* under both SD and LD conditions observed in this study, Kim et al. (2013) observed that *HDA9* expression promoted flowering solely under SD conditions without affecting *FLC* expression. These results demonstrate that unlike *HD2D* which most likely affects

flowering through the autonomic or vernalization pathways, *HDA9* affects flowering through the photoperiod floral induction pathway (Kim et al., 2013).

Additionally, Zhou et al. (2004) observed that plants overexpressing *HD2A* exhibited a delay in flowering under LD conditions, however, to the best of my knowledge, no experiments testing the effects of *HD2A* expression under SD conditions has been reported. Nonetheless, the *HD2A* induced delay of flowering is particularly interesting because similarly to *HD2D*, *HD2A* appears to have a negative regulatory role on flowering. Additionally, both genes are members of the HD2 family and the current study has demonstrated that HD2D and HD2A interact in the nucleus, suggesting that they may operate together as part of a complex that negatively regulates flowering

Unlike the early flowering *hd2d-1* mutants, *hda5* and *hda6* mutants exhibit a delay in flowering (Wu et al., 2008; Luo et al., 2015). Furthermore, plants overexpressing *HDA6* were found to flower early under both SD and LD conditions (Yu et al., 2011), suggesting that *HDA6* is not involved with the photoperiod pathway, similarly to *HD2D*. Moreover, unlike *HD2D* that promotes *FLC* expression, *HDA5* and *HDA6* negatively regulate *FLC* expression (Yu et al., 2011; Luo et al., 2015). Recently, the HDA5 and HDA6 proteins were found to interact and form a complex with the histone demethylase FLD, directly inhibiting *FLC* expression (Yu et al., 2011; Luo et al., 2015). The fact that FLD is a regulator in the autonomous pathway suggests HDA5 and HDA6 regulate flowering through the autonomous floral induction pathway as well (Luo et al., 2015).

The effect of *HDA6* on flowering time is especially interesting since the HD2D protein has been found to interact with the HDA6 protein in the nucleus (Luo et al., 2012b),

even though both affect flowering in opposite ways. A possible explanation for the HD2D-dependent increase in *FLC* expression is that the interaction between HDA6 and HD2D disrupts the interaction or activity of the FLD, HDA6 and HDA5 complex, preventing the complex from inducing flowering in a timely manner. Alternatively, HD2D could reduce gene expression through histone deacetylation of genes within the autonomous or vernalization floral induction pathways, which would cause an increase in *FLC* expression and delay flowering, separately from the HDA5, HDA6, and FLD complex.

The results of the current study demonstrated that *HD2D* is a negative regulator of flowering in Arabidopsis. The fact that *HD2D* affects the expression of major components of the floral induction pathway highlights the importance of *HD2D* in controlling flowering time. HD2D also physically interacts with other HDACs that regulate flowering, supporting that HDACs are heavily involved in regulating flowering time (Fig. 4.1).

Chapter 5: Future Perspectives

The current study implicated an important role for *HD2D* in ABA-related processes of germination, flowering, and abiotic stress response. Additionally, it was discovered that HD2D can interact with important regulators of the ABA signal transduction pathway (ABI1, ABI2, and ABI5). However, much remains unknown regarding HD2D's exact role in the ABA pathway and how it affects gene expression.

It is important to establish HD2D's role in repression of gene expression. Following research previously done by Wu et al. (2003) on other HDACs, the DNA binding domain of a transcription factor would be fused to HD2D and expression of a reporter gene that the transcription factor associates with would be quantified. This would

provide evidence for whether or not *HD2D* is capable of directly repressing gene expression.

This current study evaluated a limited number of histone modifications under control conditions. However, there are four K residues on H3 tails (K9, K14, K18, K27) and five K residues on H4 tails (K5, K8, K12, K16, and K20) that are subject to acetylation and deacetylation. It is important to evaluate whether *HD2D* can affect acetylation status of specific K residues that were not tested in this study under both control and stress conditions. Since *HD2D* was found to be involved in stress responses, it may only affect acetylation status under those specific stress conditions. Examining the acetylation status of these K residues under stress conditions in addition to control conditions may provide more information about *HD2D*'s HDAC activity.

Chromatin immunoprecipitation (ChIP) is a powerful technology that could be used to identify specific loci that HD2D interacts with. To test this, the effect of HD2D expression on chromatin status at specific loci in WT, *hd2d-1* mutants, and *HD2D* overexpression lines could be examined by pulling down chromatin with activation marker antibodies and then quantifying these markers at these loci. Identifying the loci as well as the histone modifications would aid in determining where in the ABA pathway HD2D comes into play and the exact manner it regulates gene expression. Based on the gene expression data generated in this study, the obvious genes to examine are the ones affected by *HD2D* expression, namely, *FLC*, *ABI1* and *ABI5*. This is because *FLC* acts upstream of the other developmental genes that were examined and *ABI5* has been found to act upstream of *RD29A*. In order to identify additional candidates for ChIP analysis, further gene expression data should be generated. The genes to investigate would be ones that act

upstream of *FLC* (from either the autonomous or vernalization pathways), such as: *FVE*, *MGOUN3*, *FLD*, and *HOS15* (Ausin et al., 2004; Guyomarc'h et al., 2006; Zhou et al., 2008; Yu et al., 2011). Alternatively, microarray technology or RNA-sequencing technology could be used to investigate large scale changes in gene expression.

The interactions of other HDAC proteins (HDA5 and HDA6) with the histone demethylase FLD promotes flowering by directly targeting the *FLC* locus and limiting *FLC* expression (Luo et al., 2015). The interaction between HDA6 and HD2D has already been established (Luo et al., 2012b) and the current study demonstrated a role for HD2D in promoting *FLC* expression (Fig. 3.8A). The possibility that HD2D interacts with and possibly regulated the FLD complex is intriguing. The interaction between HD2D and FLD could be investigated using BiFC experiments, similarly to those conducted in the current study (Fig. 3.10 and Fig. 3.11). If an interaction is established, the manner that HD2D affects the FLD complex and FLC expression would need to be investigated. Alternatively, HD2D interaction with HDA6 could disrupt the interaction between FLD and HDA6, limiting the repression of FLC by the HDA6-HDA5-FLD complex. This could be studied by observing whether *HD2D* expression affects the interaction of FLD and HDA6 using co-immunoprecipitation (Co-IP) experiments. This would require the transformation of *HD2D* overexpression and mutant lines with FLD-HA and HDA6-FLAG constructs driven by their native promoters. Then pulling down one of HDA6 or FLD using one of the antibodies (FLAG or HA) and using the remaining antibody to test for the presence of the other protein. Differences in FLD-HDA6 interaction between WT, *hd2d-1*, and HD2D overexpression lines would indicate that HD2D affects the interaction.

Since HD2D has been found to interact with ABI1, ABI2, and ABI5, it is important to determine exactly how HD2D affects these proteins' activity. The activity of ABI1 and ABI2 is affected, in part, due to their transportation out of the nucleus in response to ABA, limiting their negative regulation within the nucleus (Moes et al., 2008). Whether HD2D affects the localization of ABI1 and ABI2 is important to examine as they are fundamental components of the ABA pathway (Raghavendra et al., 2010). In order to determine this, double mutants could be generated by transforming the *hd2d-1* mutant and *HD2D* overexpression lines with a construct containing a native ABI1 or ABI2 promoter driving the expression of an ABI1 or ABI2 coding sequence tagged with a reporter gene such as GFP and a FLAG antigen sequence. Using confocal microscopy to visualize the localization, this experiment would conclude whether *HD2D* expression affects the localization of these proteins.

As discussed in section 4.1, non-histone proteins can be targets of HATs and HDACs (Glozak et al., 2005; Wu et al., 2011). It is important to consider whether ABI1, ABI2, or ABI5 are post-translationally modified by HD2D. Using the double mutants discussed above, the ABI1, ABI2, and ABI5 could be pulled down (using a FLAG antibody) and then separated using liquid chromatography and identified using mass spectroscopy and look for differences in acetylation levels (Parker et al., 2010; Wu et al., 2011).

Identifying a role for HD2D in ABA-related molecular mechanisms will expand the research community's understanding of plant stress response. As such, it will contribute to the understanding of how plants deal with abiotic stress conditions, specifically drought.

This research, along with that of many others, will lead to long-term improvement of tolerance to drought in economically valuable plants.

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