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Investigation of HD2 family histone deacetylases relationship in drought stress response and root growth in Arabidopsis thaliana

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Abstract

Despite evidence that certain HD2 family histone deacetylases (HDACs) play an important role in plant growth and stress response, the coordination of HD2-type HDACs in these processes remains largely unknown. Arabidopsis contains four HD2s called HD2A, HD2B, HD2C, and HD2D. I found that HD2A and HD2C coordinate to positively regulate drought stress response. The *hd2a*.*hd2c* double mutant (Mac16) exhibits decreased survival and increased water loss under drought due to modified stomatal closure as compared to the single mutants *hd2a* and *hd2c*. Gene expression analysis showed that the expression of stomatal closure-related genes *ABI1*, *ABI2*, and *SLAC1* was significantly affected in the Mac16 as compared to the *hd2a* and *hd2c*. Conversely, plants overexpressing *HD2A* or *HD2C* showed enhanced survival under drought stress and decreased water loss from leaves. Both HD2A and HD2C also play role in controlling root growth under stress. Furthermore, I demonstrated that HD2A and HD2C positively regulate root growth. Mac16 showed decreased root growth, compared to *hd2a* and *hd2c*. Importantly, the *GA2ox1* and *GA2ox2*, which catabolise bioactive gibberellic acids, were significantly upregulated in the Mac16 as compared to the single mutants, thus caused decreased root growth in the Mac16. Moreover, increased genome-wide H3K9 acetylation was observed in the Mac16 as compared to the single mutants. Additionally, Y2H and BiFC analysis showed that both HD2s can physically interact with each other. Overall, my investigation revealed that HD2A and HD2C coordinate to play a cumulative role in drought stress response and root growth in Arabidopsis.

Keywords

Arabidopsis**,** histone deacetylase, HD2 family, *HD2A*, *HD2C*, drought stress, root growth, gibberellic acid

Summary for Lay Audience

Drought is an important environmental stress affecting the plant growth and production. Drought stress induces several morphological changes in plants at different stages including roots, shoots, leaves, and flowering to limit the plant growth. All growth changes in plants in response to different environmental stresses are controlled by specific DNA sequences which are generally called as genes. Genes are activated only when they are required by the plants to perform their function. Activation or repression of genes at chromatin level is usually controlled by several enzymes. Histone deacetylases (HDACs) are considered gene repressors and play a role in plant growth and stress response. There are three different families of HDACs in plants and I focused on histone deacetylase 2 (HD2) family in the model plant Arabidopsis.

I conducted research to investigate the role of HD2-type HD2A and HD2C in controlling drought stress response and root growth. For this, I obtained HD2 single gene mutant plants and crossed them with each other to develop *hd2a*.*hd2c* double gene mutant plants. I compared these mutant plants with wild-type (WT) plants which were expressing normal levels of these HD2 genes. I also developed transgenic plants expressing higher levels of these HD2 genes and compared them with WT. These studies revealed that HD2A and HD2C coordinate to positively regulate plant drought stress response. These HDACs control the expression of abscisic acid related genes which play role in the opening and closing of stomata, thus help the plant to minimize water loss by enabling the stomatal closure under drought conditions.

I also demonstrated that HD2A and HD2C work together to control root growth under drought stress by regulating the expression of GA2ox genes. GA2ox genes play a role in the degradation of specific bioactive gibberellic acids, which are considered to promote root growth. Overall, my research revealed that HD2A and HD2C coordinate to positively regulate the drought stress response and root growth in Arabidopsis. Knowledge gained on the role of these HD2 genes in drought stress response and root growth can be used as a potential molecular strategy to improve drought tolerance in related crops.

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List of Abbreviations

Abbreviation	Description
%	percent
1/2 MS	half strength of MS medium
ABA	abscisic acid
ABI1	ABA-insensitive 1
ABI2	ABA-insensitive 2
ABI3	ABA-insensitive 3
ABI4	ABA-insensitive 4
ABI5	ABA-insensitive 5
ac	acetylation
AD	active domain
Ade	adenine
BD	binding domain
BiFC	bimolecular florescent complementation
bp	base pair
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
CDS	coding DNA sequence

Col-0	columbia-0
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ddH_2O	double-distilled H_2O
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetertraacetic acid
ERF	ethylene response factor
GA	gibberellic acid
gDNA	genomic DNA
H2A	histone 2 A
H2B	histone 2 B
H3	histone 3
H3K9	lysine residue 9 of histone 3
H3K9ac	acetylation of H3K9
H4	histone 4
HAT	histone acetyltransferase
HCL	hydrochloric acid
HDAC	histone deacetylase
K	lysine
kb	kilo base pair

LB	left border
Leu	leucine
LP	left primer from the 5’ flanking sequence
mRNA	messenger RNA
MS	Murashige and Skoog
NAC	no apical meristem/cup-shaped cotyledon
NAD-	nicotinamide adenine dinucleotide-
NCBI	National Center for Biotechnology Information
OE	overexpression
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween-20
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl
PP2C	type 2C protein phosphatases
PYR/PYL/RCAR	pyrabactin resistance/pyrabactin-like regulatory components of ABA receptor
RNA	ribonucleic acid
RNAi	RNA interference

RP	right primer from the 3’ flanking sequence
RPD3	reduced potassium dependency protein 3
RT-qPCR	real-time quantitative PCR
SDS	sodium dodecyl sulfate
SE	standard error
SIR2	silent information regulator protein 2
SnRK2	sucrose nonfermenting 1-related protein kinase 2
SWI/SNF	SWItch/Sucrose Non-Fermentable
SRT	sirtuin
TEB	triton extraction buffer
T-DNA	transfer DNA
v/v	volume/volume
w/v	weight/volume
WT	wild-type
Y2H	yeast two-hybrid
YFP	yellow fluorescent protein
UTR	untranslated region
β-ME	β-mercaptoethanol

Gene names are written in italics. Capital letters are used when referring to wild-type alleles. Lowercase letters are used when referring to mutant alleles. Protein names are written in non italic capital letters.

Chapter 1: Introduction

1.1 Impact of drought stress on plants

Plants, because of their sessile lifestyle, face various biotic and abiotic stresses that can lead to a negative impact on overall plant productivity. Among the various stresses encountered by plants throughout their life cycle, drought stress is one of the most damaging environmental factors (Rosegrant and Cline, 2003; Lesk et al., 2016). Water accessibility is fundamental to plant growth, survival, and productivity. A lack of water accessibility causes drought stress, which is considered one of the most important threats to agricultural crop productivity throughout the world (Iqbal et al., 2020). Drought-induced yield losses may exceed the losses from all other causes because of its severity and duration. During the 2000s, drought stress caused the reduction of wheat yield in Russia and Ukraine by 32.7% and 19.3%, respectively. Certain regions in the United States, China, and Australia have also suffered long-term drought effects leading to poor crop yields (Sternberg, 2011). Further increasing food security concerns is the fact that drought problems are expected to increase and spread with the global climate change (Sternberg, 2011; Lesk et al., 2016; Iqbal et al., 2020). Meanwhile, it will be a big challenge to feed a population of approximately 10 billion by 2050 (Smith, 2013). For decades to come, the world will see increased pressure on global food security because of the rapidly growing human population and increasing competition for energy, water, and land. Climate change is an additional threat adding to these concerns (Godfray et al., 2010; Godfray and Garnett, 2014). Feeding a population of 10 billion by 2050 will demand a boost in food production, which is possible by adopting innovative scientific techniques. Current systems of crop productivity are largely dependent on the use of large amounts of inputs including water and fertilizers. The development of agricultural crop plants tolerant to drought and other environmental stresses based on the use of traditional breeding coupled with genetic engineering technology is desperately needed (Smith, 2013; Fita et al., 2015; Grafton et al., 2015; Tahir et al., 2021).

Drought stress affects plant growth at every stage of life, including seed germination, root and shoot growth, flowering, and seed development (Farooq et al., 2009). The drought stress response is a complex phenomenon in plants. Plants attempt to withstand drought

stress and tolerate unfavourable conditions by modifying their behaviour and undergoing a series of morphological, physiological, and biochemical changes at different stages of plant growth and development (Tahir and Tian, 2021). Drought stress stimulates the activation of different stress-responsive genes that encode different functional and regulatory proteins that are associated with gene regulatory networks (Fujita et al., 2011; Kim et al., 2015; Sah et al., 2016). Understanding the drought stress response mechanisms can aid in the development of drought-tolerant crops.

1.2 Phytohormones

Plants under stress stimulate the synthesis of stress hormones, also called phytohormones, which initiates an array of processes to induce changes at the molecular, biochemical, and physiological levels (Nakashima et al., 2009; Liu et al., 2014). These hormones induce the expression of stress-responsive genes which are thought to play a role in signal transduction pathways to respond to stress. Plant stress hormones play a crucial role in the stability and survivability of plants under changing environments by regulating plant growth and development (Kim et al., 2015; Sah et al., 2016). Major phytohormones include abscisic acid (ABA), ethylene, gibberellic acids (GAs), auxins (IAA), cytokinins, and jasmonic acid (JAs) (Morkunas et al., 2014). Phytohormones function as signaling molecules and may work independently or coordinate with each other in a synergistic or antagonistic manner to create a complex network of regulation in the control of the plant stress response.

The phytohormone abscisic acid (ABA) is one of the most important plant hormones and is produced in significant quantity upon stimulation. ABA is known for its stress related characteristics in addition to its important role in regulating various developmental processes (Fujita et al., 2011; Finkelstein, 2013). ABA is closely related to cellular dehydration processes during plant vegetative and reproductive growth and is believed to control about 10% of protein coding genes in Arabidopsis. Many dehydration-responsive genes have been shown to be induced under ABA treatment (Bartels and Souer, 2004). The core signalling pathway of ABA has been well reviewed by Umezawa et al. (2010). Evidence supports the existence of both ABA-dependent and ABA-independent regulatory pathways to regulate drought stress responses in plants (Shinozaki et al., 2003;

Yamaguchi-Shinozaki and Shinozaki, 2005). ABA is involved in regulating the expression of genes associated with dehydration response. Expression of ABA-responsive genes is controlled by a number of ABA related transcription factors. Transcription factors directly recognize and bind to cis-elements located in the upstream promoter regions of target genes to regulate their expression in ABA-mediated pathways (Fujita et al., 2011).

1.2.1 ABA-induced stomatal closure

Plants have developed various strategies to survive under drought stress. Of these, stomatal closure is one of the first lines of defence with respect to maintaining water status (Bharath et al., 2021). Stomata are closed to limit water loss by transpiration and establish an equilibrium between water absorbed by roots and water loss by transpiration (Wilkinson and Davies, 2002). Limited water supply to the roots leads to an increase in the pH of the root apoplast, which results in the transportation of ABA from root cells towards the leaf apoplast. This mobilization causes an increase in the ABA biosynthesis in root and shoot cells (Wilkinson and Davies, 2002; Hopkins and Hüner, 2008). Accumulation of ABA in leaves initiates a signalling cascade to induce stomatal closure to minimize transpirational water loss. Accumulation of ABA in guard cells is recognized by ABA receptors of the START protein family, called PYRABACTIN RESISTANCE 1/PYR1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR (PYR/PYL/RCAR). The Ca^{2+}-independent protein kinases SUCROSE NONFERMENTING-1-RELATED KINASE 2 (SnRK2s) are activated by ABA signalling and are considered one of the major regulators of osmotic stress response (Yoshida et al., 2006). Under normal conditions, PROTEIN PHOSPHATASES 2C (PP2Cs) binds to and inhibits SnRK2s activity by dephosphorylating SnRK2s at multiple residues. As shown in **Figure 1**, under stress conditions, ABA and the PYR/PYL/RCAR complex binds to PP2C and inhibits its phosphatase activity. This inhibition releases the SnRK2s for downstream activation of transcription factors via phosphorylation, which regulate multiple downstream target genes (Weiner et al., 2010). In guard cells, ABA activates anion channels of the plasma membrane which causes K^+ outflow through voltage-dependent channels and subsequent plasma membrane depolarization (Roelfsema et al., 2012; Munemasa et al., 2015). The efflux of K^+ decreases the turgor pressure in guard cells, leading to closure of stomata.

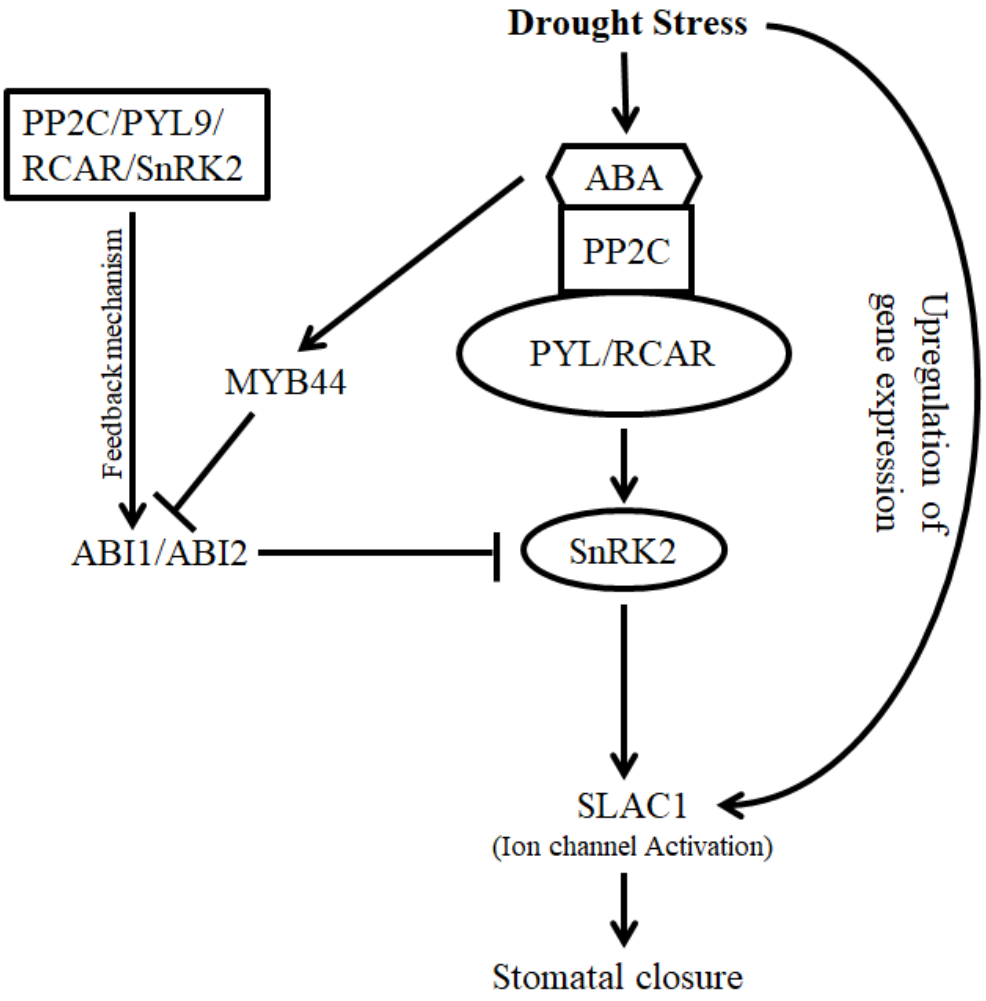
Arabidopsis guard cells contain Slow-sustained (S-type) and Rapid-transient (R-type) anion channels. The S-type anion channels are mainly regulated by SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) (Vahisalu et al., 2008; Zhang et al., 2016). Several studies have reported the ABA-mediated regulation of *SLAC1* gene expression via SnRK2s activity (Daszkowska-Golec and Szarejko, 2013; Imes et al., 2013; Ullah et al., 2017).

ABA-INSENSITIVE 1 and 2 (ABI1 and ABI2) are two PP2C-type protein phosphatases and belong to clade A of the PP2C family. Both ABI1 and ABI2 are considered negative regulators of ABA-mediated signaling and inhibit the phosphorylation activity of SnRK2, thus negatively regulate the stomatal closure in Arabidopsis (Leung et al., 1997; Schweighofer et al., 2004). The loss of function mutants *abi-1* and *abi-2* (recessive mutations) demonstrated an increased sensitivity to ABA in Arabidopsis (Merlot et al., 2001; Umezawa et al., 2010; Fujita et al., 2011). Under low or no ABA, ABI1 dephosphorylates OPEN STOMATA 1 (OST1), a SnRK2-type protein kinase (SnRK2/OST1) to inhibit its phosphorylation activity. Under high ABA levels, ABA, via its receptors, inhibits the binding of ABI1 with SnRK2/OST1. Upon activation, SnRK2/OST1 physically interacts with *SLAC1*, which leads to the activation of the *SLAC1* ion channel through phosphorylation (Yang et al., 2010; Imes et al., 2013).

Figure 1: ABA-mediated stomatal closure under drought stress

Under drought conditions, ABA levels are increased in the guard cells of the leaf. This is perceived by ABA receptors, which interact with and block PP2C phosphatase activity. This activates the SnRK2s to regulate ion channels by upregulating *SLAC1*. ABI1 and ABI2 belong to clade A of the PP2C family and are considered negative regulators of stomatal closure, which inhibit SnRK2's phosphorylation activity. Under increased ABA levels, the *ABI1* and *ABI2* genes are down-regulated via the MYB44 transcription factor, thus leading to stomatal closure under drought stress conditions.

This figure was modified based on the previous reports (Daszkowska-Golec and Szarejko, 2013; Imes et al., 2013; Ullah et al., 2017).


Drought Stress
PP2C/PYL9/
RCAR/SnRK2
ABA
PP2C
PYL/RCAR
MYB44
Feedback mechanism
ABI1/ABI2
SnRK2
Upregulation of
gene expression
SLAC1
(Ion channel Activation)
Stomatal closure

1.2.2 Gibberellic acid

Gibberellic acid (GA) is another important phytohormone that is known for its involvement in plant growth and developmental mechanisms ranging from seed germination and plant elongation to seed and fruit development. Ubeda-Tomás et al. (2009) reported that GAs are involved in the elongation of endodermal cells to promote root growth in Arabidopsis. GA activity is not limited to their site of synthesis. Depending on the requirement, they can be transported to tissues and organs where they are not synthesized (Martins et al., 2018). A number of different GAs have been identified in plants including GA1, GA3, GA4, GA7, GA8, GA9, GA12 GA19, GA20, GA34, GA51, GA53. Only a few are considered bioactive GAs and true phytohormones (e.g., GA1, GA3, GA4), while others are considered as either precursors or inactive forms (Rieu et al., 2008). Three different classes of enzymes are involved in the GA biosynthesis pathway through a series of conversions with the final stage involving GA3-oxidases (GA3ox), GA7-oxidases (GA7ox), GA20-oxidases (GA20ox), and GA2-oxidases (GA2ox). The GA20ox and GA3ox enzymes catalyse the conversion of C-20 GAs to C-19 GAs, leading to the synthesis of C-19 bioactive GAs, GA1 and GA4 from C-20 inactive GAs, GA9 and GA20, respectively (Yamaguchi and Kamiya, 2000; Martins et al., 2018). GA4 is thought to be the major bioactive form of GAs in Arabidopsis, although GA1 is also found in most tissues of Arabidopsis at a relatively lower concentrations (Yamaguchi, 2006).

Maintaining a dynamic homeostasis of bioactive GAs for normal plant growth is an important phenomenon and is carried out by GA2ox enzymes. The GA2ox enzymes play a significant role in limiting the levels of bioactive GAs, GA1 and GA4 to mediate plant growth in response to internal developmental signals and external environmental conditions including salt, drought and cold (Rieu et al., 2008; Colebrook et al., 2014; Chen et al., 2019). Biosynthesis and catabolism of GAs is regulated by various internal and external signals coupled with feedback and feedforward mechanisms (Martins et al., 2018). Higher levels of GAs causes the degradation of DELLA proteins via ubiquitination. Degradation of DELLA proteins causes the upregulation of GA-mediated responsive genes. This GA mediated growth response initiates a feedback response to control GA biosynthesis (Martins et al., 2018). Increased GA activity regulates the biosynthesis of GAs

through a feedback mechanism via DELLA proteins, the central repressors of GA biosynthesis-related transcription factors. Higher GA levels downregulate the expression of biosynthesis genes *GA3ox* and *GA20ox* (Martins et al., 2018). This leads to the upregulation *GA2ox* genes, which are involved in the catabolism of bioactive GAs. In addition, the abiotic stresses in Arabidopsis result in the higher accumulation of DELLA proteins and upregulation of GA2ox genes, which leads to decreased levels of endogenous bioactive GAs, resulting in a retarded root growth (Achard et al., 2006; Achard et al., 2008; Magome et al., 2008). The GA biosynthesis and degradations pathways are further complicated by the interactions of different transcription factors including ABI3/4/5, RGL2, and GATA which involve ABA signalling into the network (Ravindran et al., 2017). ABA and GAs are negatively correlated as higher levels of ABA causes the repression of GA biosynthesis, and vice versa. Furthermore, different abiotic stresses lead to the higher accumulation of ABA and lower levels of GAs (Liu and Hou, 2018). Both hormones are known to play an antagonistic role in plant development (Footitt et al., 2011; Liu and Hou, 2018). ABA is shown to decrease germination and root growth, whereas GAs promote germination, root and stem growth.

1.3 Histone deacetylases and abiotic stress response

Plants, because of their sessile lifestyle, have developed complex and sophisticated mechanisms to adapt and respond to different environmental cues. These environmental stress signals induce changes at the DNA and protein level, along with the modification of enzymatic and metabolic activity (Cutler et al., 2010). Epigenetic regulation plays a key role in different biological processes ranging from developmental scheduling and maintaining genome stability to the regulation of various environmental stress responses (Kapazoglou and Tsaftaris, 2011; Luo et al., 2012; Kim et al., 2015). Epigenetic-mediated regulation of gene expression is governed by nucleosomal core histone protein modifications and DNA methylation (Shinozaki et al., 2003; Urano et al., 2010). The nucleosome is comprised of an octamer of core histone proteins with two molecules of each of H2A, H2B, H3, and H4. The N-terminal lysine residues of histone proteins H3 and H4, also called histone tails, project outward and allow for different post-translation histone modifications, which include acetylation, phosphorylation, methylation,

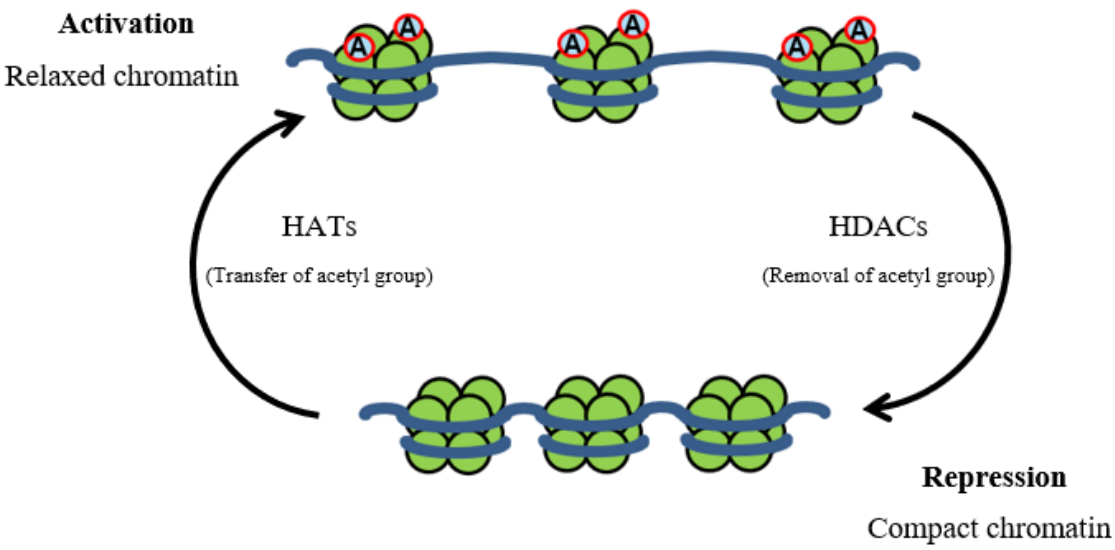
sumoylation and ubiquitination (Kapazoglou and Tsaftaris, 2011; Zhou et al., 2013). These modifications of lysine residues of core histone proteins act as a switch to activate or repress gene expression, and therefore offer a flexible mode of gene expression regulation in developmental programming and in the response to abiotic stresses (Kurdistani et al., 2004; Kouzarides, 2007; Tahir and Tian, 2021).

Histone acetylation and deacetylation is a dynamic reversible process and is considered one of the most important regulations of epigenetic modifications. Histone acetylation and deacetylation is catalysed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. As shown in **Figure 2**, HATs transfer the acetyl group to the core histone lysine tail, thus neutralizing the positive charge, resulting in a transcriptionally active euchromatin. On the other hand, HDACs remove the acetyl group from core histone lysine residues, which results in a compact nucleosome structure. This compact DNA-histone association blocks the availability of DNA to different transcriptional factors, thus leading to transcriptional repression of associated genes (Shahbazian and Grunstein, 2007; Liu et al., 2014). HATs and HDACs are generally considered to play role as components of multiprotein and chromatin remodelling complexes (CRCs). HATs are generally considered gene activators, whereas HDACs are considered gene repressors (Pandey et al., 2002; Wang et al., 2009; Tahir and Tian, 2021). A dynamic equilibrium in the acetylation status of lysine residues of core histone proteins regulated by HATs and HDACs is essential to regulate gene expression in eukaryotes (Pfluger and Wagner, 2007).

Figure 2: Regulation of histone acetylation by HATs and HDACs

Regulation of gene expression via targeted histone acetylation and histone deacetylation carried out by HATS and HDACs, respectively. HATs transfer the acetyl group (red circles) to lysine residues of core histone tails, which relaxes the chromatin structure, leading to gene activation. HDACs remove the acetyl group from lysine residues. The compact chromatin structure results in the repression of gene expression.

This figure is modified from Liu et al. (2016).


Activation
Relaxed chromatin
A
A
A
A
A
A
HATs
(Transfer of acetyl group)
HDACs
(Removal of acetyl group)
Repression
Compact chromatin

1.3.1 Classification of histone deacetylases in plants

Based on their sequence homology, plant HDACs have been classified into three different families: the reduced potassium efficiency 3 (RPD3) family, the silent induced regulator 2 (SIR2) family, and the histone deacetylase 2 (HD2) family (Pandey et al., 2002; Luo et al., 2012; Kim et al., 2015). The RPD3 and SIR2 families of HDACs are homologous to yeast HDAC families and are found throughout eukaryotes. A total of 12 HDACs belonging to the RPD3 family have been identified in Arabidopsis and contain a characteristic histone deacetylase catalytic domain (Pandey et al., 2002). Previous studies have shown the involvement of certain RPD3-type HDACs in ABA and abiotic stress responses. HDA6 and HDA19 are involved in the positive regulation of ABA, salt, and drought stress responses (Chen et al., 2010; Chen and Wu, 2010). The *hda6* and *hda19* mutant plants display hypersensitivity to ABA. HDA9, another member of the RPD3 family, is involved in the negative regulation of salt and drought stresses in Arabidopsis (Zheng et al., 2016). The SIR2-type HDACs are nicotinamide adenine dinucleotide (NAD)-dependent enzymes and have been shown to be involved in the ABA response. Two genes belonging to the SIR2 family, namely *SRT1* and *SRT2* have been identified in Arabidopsis. *SRT1* is involved in the negative regulation of the abiotic stress response in Arabidopsis (Liu et al., 2017). There is very little known with respect to the functional roles of *SIR2* family HDACs in plants.

The HD2 family HDACs share no sequence homology to yeast HDACs and are found only in plants and green algae (Ma et al., 2013; Bourque et al., 2016). The HD2 family, being unique to plants, is emerging as an important regulator of epigenetic modifications in different aspects of plant development and the plant response to different environmental stresses (Tahir and Tian, 2021). HD2-type HDACs were first studied in maize as nucleolar phosphoproteins, where expression of these HD2s was associated with actively dividing embryonic cells (Lusser et al., 1997). Later, HD2s were found in various species, with the number varying in different species. For instance, four HD2s were identified in maize and Arabidopsis, six in tobacco and soybean, three in tomato, two in barley and rice (Dangl et al., 2001; Hollender and Liu, 2008; Demetriou et al., 2009; Zhao et al., 2015; Bourque et al., 2016; Nicolas-Francès et al., 2018; Yang et al., 2018).

Phylogenetic analysis of at least 1205 HD2 sequences from different species revealed that the first four amino acids of the pentapeptide motif MEFWG in HD2 proteins are fully conserved, with the fifth residue (G) conserved in 96% of the sequences analysed (Bourque et al., 2016). Arabidopsis, maize, rice, tobacco, and soybean contain a fully conserved pentapeptide motif along with histidine at the 25th position, surrounded by hydrophobic amino acids. Existence of these conserved residues and glutamate/aspartate at the 69th position is suggested to be essential for HD2s catalytic activity (Dangl et al., 2001; Zhou et al., 2004; Bourque et al., 2016). Although HD2s are considered gene repressors because of their deacetylation property, the deacetylase domain of HD2 proteins does not show any similarity with any other HDAC family proteins. This characteristic of the HD2 family indicates a special role of HD2s in plant developmental processes and stress responses. Four members of the HD2 family have been identified in Arabidopsis: HD2A/HDT1 (AT3G44750), HD2B/HDT2 (AT5G22650), HD2C/HDT3 (AT5G03740), and HD2D/HDT4 (AT2G27840). The C-terminal domain of HD2A and HD2C proteins contains a putative C_2H_2-type zinc-finger (ZnF) motif, suggesting a unique structural feature of certain members of the plant-specific HD2 family (Dangl et al., 2001; Bourque et al., 2016). The ZnF motifs usually contain a conserved motif, QALGGH and are involved in DNA-protein interactions. However, HD2 proteins do not contain this conserved motif for DNA binding, it is very probable that ZnF in HD2 proteins may be associated with protein-protein interactions in gene regulatory mechanisms (Mackay and Crossley, 1998; Takatsuji, 1998; Dangl et al., 2001). The presence or absence of the ZnF residue in HD2 proteins could possibly play an important role in implicating functional diversity.

1.3.2 Histone acetylation modification during abiotic stress responses

Environmental stresses leads to the modifications in the acetylation status of histone proteins associated with stress responsive genes via ABA-dependent and -independent pathways (Wilkinson and Davies, 2002; Fujita et al., 2011). Genome-wide enrichment of H3 acetylation was observed in Arabidopsis cell-lines when subjected to ABA, salt, and cold stress (Sokol et al., 2007). Many ABA-related transcription factors are known to be associated with HDACs (Glass and Rosenfeld, 2000; Jepsen and Rosenfeld, 2002). Reports show the involvement of different HDACs in regulating abiotic stress responses via ABA-

dependent pathways in different species (Kuang et al., 2012; Song et al., 2019). Drought treatment results in an increase in the acetylation of H3 on ABA response element (ABRE) and dehydration response element (DRE) genes in Arabidopsis, leading to the upregulation of their expression (Kim et al., 2008). Different abiotic stresses, including salt and drought can lead to changes in the acetylation status of lysine 9 and 14 (K9 and K14) at histone H3 and K18 and K27 at histone H4 associated with stress-responsive genes (Chen et al., 2010; Kuang et al., 2012; Luo et al., 2012; Kim et al., 2015). In plants, H3K9ac (acetylated lysine 9 at histone H3) is enriched preferentially at transcriptional start sites (TSS) of genes, indicating that activation of gene transcription is closely associated with H3K9ac during plant stress responses (Zhou et al., 2010; Kim et al., 2015). Drought stress is thought to modify the status of histone acetylation to activate or repress drought-associated genes, as genome-wide H3K9 hyperacetylation was observed in plants under drought stress (Kim et al., 2015; Zheng et al., 2016). Enrichment of H3K9ac at diurnal genes have been observed at the time of day when their expression is required (Baerenfaller et al., 2016), suggests a stimulus-induced gene activation via H3K9 acetylation. This indicates that a large set of functionally related genes are controlled in a coordinated fashion through H3K9 hyperacetylation in different abiotic stress responses in plants. The modification of specific epigenetic marks suggests a dynamic impact on chromatin organization and transcriptional regulation mechanisms.

Several lines of evidence suggest that H3K9 acetylation status is regulated by different HDACs. A significant increase in H3K9 acetylation levels on certain target genes was observed in *hda9* and *hda19* mutants (Chen and Wu, 2010; Zhou et al., 2010; Zheng et al., 2016). Another study showed an increase in H3K9 acetylation levels on ABA receptor genes *PYL4*, *PYL5*, and *PYL6* in *hda19* mutants (Mehdi et al., 2016). Simultaneous knockout of both *HD2A* and *HD2B* resulted in the hyperacetylation of histone H3 at the *GA2ox2* gene locus, which resulted in the shorter root lengths in Arabidopsis (Li et al., 2017). Mitogen-activated protein (MAP) kinase, MPK3 associates with HD2B to modulate H3K9 acetylation status to mediate plant innate immunity in Arabidopsis (Latrasse et al., 2017). Recently, Guo et al. (2020) have shown that HD2C interacts with histone methylation readers MRG1/2 to remove the acetyl group from K9, K23 and K27 of histone H3 at the *FLOWERING LOCUS T* (*FT*) gene to repress its expression. The *FT* gene is a

florigen gene and is responsible for early flowering in Arabidopsis under long-day conditions. Treatment of Arabidopsis seedlings with HDAC inhibitor, trichostatin A (TSA) showed developmental defects and lead to the upregulation of ABRE genes, which are well known to be upregulated in response to diferent abiotic stressess, including salt, drought and cold. (Tai et al., 2005; Tian et al., 2005).

1.4 Role of HD2 family in Arabidopsis

The HD2 family plays a significant role in plant growth and development. The HD2 family genes are predominantly expressed in leaves, flowers, and siliques in Arabidopsis (Wu et al., 2000; Wu et al., 2003; Zhou et al., 2004). HD2A is involved in inflorescence stem development, as knockout of *HD2A* resulted in shorter plants with thinner stems (Zhang et al., 2019). RNA interference (RNAi) based gene silencing of *HD2A* caused the aborted seed development with shorter siliques in Arabidopsis. Overexpression (OE) of *HD2A* caused a delayed flowering in Arabidopsis and affected the expression of several seed developmental-related genes (Wu et al., 2000; Wu et al., 2003; Zhou et al., 2004).

Plant growth and development is considered strongly correlated with the modification of histone acetylation. Two HD2 family proteins, HD2A and HD2B were shown to negatively regulate the expression of *GA2ox2* which is involved in the catabolism of bioactive GAs. Overexpression of *GA2ox2* caused a shorter root phenotype in Arabidopsis (Li et al., 2017). It was shown that both HD2A and HD2B interact with ASYMMETRIC LEAVES 1 and 2 (AS1, AS2) and regulate the expression and distribution of *miRNA165* and *miRNA166*, which are known to control leaf morphology in Arabidopsis (Kidner and Martienssen, 2004; Ueno et al., 2007). The *HD2D* overexpressing plants showed delayed germination, shorter primary roots with denser lateral roots and delayed flowering (Han et al., 2016; Farhi et al., 2017). Several flowering time-related genes were downregulated in HD2D overexpression plants.

Regarding the role of HD2 family HDACs against abiotic stresses, these show differential expression patterns under different abiotic stresses including salt and cold (Wu et al., 2000; Sridha and Wu, 2006; Hollender and Liu, 2008; Kuang et al., 2012). Expression of all HD2 genes was downregulated under ABA and salt treatments and upregulated under

cold stress in Arabidopsis (To et al., 2011; Kuang et al., 2012; Luo et al., 2012). *HD2A* and *HD2C* were shown to be strongly upregulated upon heat stress (Buszewicz et al., 2016). A DDB1-CUL4 Associated factor (DCAF) protein called HOS15 was shown to interact with HD2C with the help of HOS15 binding protein, POWERDRESS (PWR) to carry out HD2C degradation via ubiquitination. Degradation of HD2C at the promoter region of cold-responsive (COR) genes leads to the upregulation of COR genes in Arabidopsis (Zhu et al., 2008; Park et al., 2018; Lim et al., 2020). Overexpression of *HD2C* and *HD2D* resulted in the upregulation of many stress-related genes and transgenic plants showed increased tolerance to different abiotic stresses (Sridha and Wu, 2006; Buszewicz et al., 2016; Han et al., 2016; Farhi et al., 2017). It was shown by Luo et al. (2012) that HD2C associates with the RPD3 family HDAC, HDA6, and mediates ABA and salt stress responses. The double mutant line *hd2c.hda6* showed enrichment of H3K9ac and H3K14ac levels at the locus of *ABI1* and *ABI2*, leading to the upregulation of their expression. The *hd2c* and *hda6* single and double gene mutants demonstrated hypersensitivity to ABA. Later, it was shown that other HD2 proteins can interact, not only with HDA6 but also with HDA19, both of which are well known for their role in abiotic stress tolerance (Kuang et al., 2012; Luo et al., 2012; Luo et al., 2012).

1.5 Hypothesis and objectives

HDACs generally do not function alone and are considered to play a role as a component of multiprotein complexes in a coordinated fashion. These complexes may include multiple HDACs, either belonging to the same family or different families (Chen and Wu, 2010; Luo et al., 2012; Buszewicz et al., 2016; Li et al., 2017; Guo et al., 2020). Among the HD2 family, HD2A and HD2B have been shown to coregulate plant root growth. Many researchers have studied the HD2C and HD2D, for their role in abiotic stress responses (Sridha and Wu, 2006; Buszewicz et al., 2016; Farhi et al., 2017). HD2A, HD2C, and HD2D were shown to interact with HDA6 and HDA19, which are involved in the abiotic stress response (Luo et al., 2012; Luo et al., 2012). HD2A, HD2B, and HD2C were also shown to interact with DNMT2, a methyl transferase responsible for the methylation of DNA for transcriptional repression of target gene (Song et al., 2010). The association of different HD2s with each other during interaction with common interacting partners in response to different internal developmental and external environmental signals cannot be ruled out. Functional association within HD2 family members as part of repression complexes might be of vital importance for regulating gene expression involved in drought stress response and plant growth. I aimed to investigate if any of the HD2-type HDACs coordinate to play a role in drought stress response and root growth in *Arabidopsis thaliana*.

Hypothesis: I hypothesized that certain members of HD2 family histone deacetylases act together to regulate drought stress response and root growth in Arabidopsis.

My overall research to test the hypothesis was based on the following specific objectives:

1. To examine how HD2 family genes respond to drought stress.
2. To study the coordination of HD2s in drought stress response and stomatal regulation.
3. To study the coordination of HD2s in root growth regulation.
4. To evaluate and compare global H3K9ac levels in *hd2* mutants and HD2 overexpression lines.
5. To examine the protein-protein interaction of HD2 proteins.

Chapter 2: Materials and Methods

2.1 Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* wild-type (WT) and transfer DNA (T-DNA) insertional single gene mutants GK355-H03 (*hd2a*: AT3g44750), SAIL1247-A02 (*hd2b*: AT5g22650), SALK129799 (*hd2c*: AT5g03740), and GK279-D04 (*hd2d:* AT2g27840) were used in this study. All these genotypes were in ecotype Columbia (Col-0) background. The T-DNA mutant lines were obtained from the Arabidopsis Biological Resource Center (ABRC). In addition, *Nicotiana benthamiana* seeds were also used in this study for protein-protein interaction analysis. Seeds of WT *Arabidopsis thaliana* and *Nicotiana benthamiana* were obtained from the Agriculture and Agri-Food Canada, London Research and Development Centre (AAFC-LRDC), London, Ontario. Techniques related to Arabidopsis seed handling, growth, transformation, and crossing are based on standard Arabidopsis protocols described by Kim et al. (2006) unless stated otherwise.

For plant growth experiments, all seeds were surface-sterilized in ethanol (70%) for 1 minute followed by bleach (25%) for 10 minutes with gentle shaking at room temperature. The sterilized seeds were rinsed with autoclaved double-distilled water (ddH_2O) and stratified in darkness for 3 days at 4°C before sowing on ProMix-BX soil (Premier Horticulture, Québec) or on growth medium agar plates (containing half-strength (2.15 g/L) Murashige and Skoog (MS) growth medium supplemented with 1% sucrose, 0.8% agar, 0.5 g/L 2-N-morpholino-ethanesulfonic acid (MES), pH 5.7) with or without additives such as antibiotics, plant growth regulators, or chemicals. Plants in the growth room were grown under a constant 16 hours of daylight and 8 hours of dark cycles (long-day conditions) at 22°C with a relative humidity of 60%. Plants incubated in growth chambers were also grown under the same conditions, except dark cycles were at 18°C.

2.2 Phylogenetic analysis

DNA sequence information of the Arabidopsis HD2 family HDACs was obtained from EnsemblPlants (https://plants.ensembl.org/Arabidopsis_thaliana/Info/Index/) and the peptide sequences of HD2-type HDACs in different species for phylogenetic analysis were obtained from the Phytozome database (https://phytozome-next.jgi.doe.gov/). The

phylogenetic tree was generated using MEGA X (Kumar et al., 2018). Amino acid sequence identities and divergences were determined using the National Center for Biotechnology Information (NCBI) tool, Basic local alignment search tool (BLAST) for nucleotide sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3 Plant genomic DNA extraction and PCR-based genotyping of *hd2* mutants

Plant genomic DNA (gDNA) was extracted from plant leaf tissues following the protocol as previously described (Edwards et al., 1991). Briefly, 50 mg of leaf tissues from each of the genotypes was collected in 2.0 ml Eppendorf tube and were ground using disposable plastic grinders for 15 seconds at room temperature without adding buffer. Then, 400 µl of extraction buffer (200 mM Tris HCL pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added and samples were vortexed for 10 seconds. The samples were left at room temperature for 5 minutes. Samples were centrifuged at 13,000 rpm for 1 minute and 300 µl of supernatant from each tube was transferred into fresh sterile 1.5 ml Eppendorf tubes. An equal volume of isopropanol was added to each tube and vortexed again to mix thoroughly. The tubes were incubated at room temperature for 2 minutes before centrifuging them again for 10 minutes at 13,000 rpm to pellet the gDNA. Pelleted gDNA was air-dried and resuspended in 100 µl of 1X TE buffer (1 M Tris HCL, 0.5 M EDTA, pH 8.0). Extracted DNA was stored at -20^{o}C.

All the T-DNA mutant lines obtained from ABRC were genotyped by PCR to identify homozygous plants containing T-DNA insertions in both alleles. To detect WT and/or mutant alleles, three types of primers were used in the genotyping PCR: FP (gene-specific forward primers), RP (gene-specific reverse primers), and LB (left-border T-DNA insertion primers). The FP + RP primer pair amplifies the respective WT allele, whereas the RP + LB primer pair amplifies the respective mutant allele. Primers used for T-DNA mutant genotyping are listed in **Appendix 1** and were designed at http://signal.salk.edu/tdnaprimers.2.html.

For PCR amplification, 2 µl of gDNA extracted from leaves was used as template in a 25 µl PCR reaction which was prepared by adding 5 µl of Phusion High Fidelity Buffer (New England Inc.), 0.5 µl of 10 mM dinucleotide triphosphates (dNTPs), 1 µl of 10 µM of each

primer, 0.2 µl of Phusion DNA polymerase (New England Inc.) and dH_2O to a final volume of 25 µl. The PCR mixture tubes were loaded onto a thermocycler and incubated for 5 minutes at 95°C, followed by 33 cycles of 95°C for 30 seconds, 58-60°C (depending on primers) for 30 seconds and 72°C for 1 minute, followed by a final extension phase of 72 °C for 10 minutes. Gel electrophoresis was carried out to separate and visualize the amplified DNA fragments in the agarose gel.

Seeds obtained from ABRC were from the T4 generation for *hd2a* and *hd2d*, T1 generation for *hd2b*, and T3 generation for *hd2c* T-DNA insertional mutants, as mentioned on ABRC (https://abrc.osu.edu/) and The Arabidopsis Information Resource (TAIR) (https://www.arabidopsis.org/index.jsp) websites. All seeds were allowed to germinate and develop plants normally in growth chambers under the same growth conditions as described above. PCR based genotyping was performed for each of *hd2a*, *hd2b*, *hd2c*, and *hd2d* mutants to identify homozygous T-DNA insertional mutant lines. Appearance of single band in the genotyping PCR with the RP + LB primer pair in the mutant lines confirms the presence of homozygous alleles in the mutant lines. Primers used for T-DNA mutant genotyping are listed in **Appendix 1**. All identified homozygous *hd2* single gene mutants were allowed to grow and develop seeds for use in further experiments.

2.4 Crossing of *hd2* single mutant plants

To generate *hd2* double mutants, the *hd2* single gene mutants (*hd2a*, *hd2b*, *hd2c*, *hd2d*) were crossed with each other in different combinations (**Table 1**). The WT plants were grown in growth chambers under the same conditions described above. About 5 to 6 week old healthy plants were chosen from each of the mutant genotypes for crossing. First, already opened flowers were removed from the plants, and floral buds with barely visible petals were marked to use as pollen recipients. Using a Dumont Arabidopsis crossing tweezer and magnifying glass, the floral parts of the selected plants were carefully removed, leaving behind only intact carpels for crossing. Then, healthy and mature flowers were selected from pollen-donor plants and sepals and petals were removed from the selected flowers using fine forceps. Pollen brimmed anthers were brought in contact with the processed recipient flowers with intact carpels to pollinate the stigma. The process of pollination was repeated twice for every crossing to ensure successful pollination. The

pollinated pistils were covered carefully with small plastic bag and labeled with donor and recipient parent. Plants were kept in separate growth chambers to avoid any cross contamination and were allowed to develop mature siliques containing F1 generation seeds. The F1 seeds collected from the parent plants were grown to obtain F1 plants. PCR genotyping was carried out to confirm the heterozygosity of F1 plants. Seeds collected from F1 plants were regarded as F2 generation seeds which were grown next to develop F2 plant populations. PCR genotyping was carried out in F2 population to identify homozygous double mutant lines.

2.5 Plant RNA extraction and cDNA synthesis

Plant total RNA was isolated from plant tissues (~50 mg) using the Plant/Fungi Total RNA Purification Kit (Norgen) following the manufacturer's instructions. RNA samples were treated with RNase-free DNase (Norgen) to remove gDNA contamination from the RNA samples. Concentration and 260/280 nm ratios of RNA samples were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific). First strand cDNA synthesis was performed using 1 µg of total RNA using iScript Reverse Transcription Supermix (Bio-Rad: cat. 1708840) following the manufacturer's instructions. The reaction mix tubes were loaded onto a thermocycler and incubated at 25°C for 5 minutes followed by 42°C for 30 minutes and finally 85°C for 5 minutes. The cDNA was stored at -20^{o}C.

2.6 Construction of plasmid vectors and generation of stable transgenic Arabidopsis plants

To construct HD2 gene overexpression vectors, the full-length cDNA (except the stop codon) was PCR amplified using gene specific primers containing attB adapter attachment sequences (**Appendix 2**), which were designed by following the guidelines given in the Gateway® technology user-guide (Invitrogen). The PCR reaction mix was loaded onto a thermocycler and incubated for 5 minutes at 95°C, followed by 33 cycles of 95°C for 30 seconds, 63-70°C (depending on primers melting temperature) for 30 seconds and 72°C for 1 minute, followed by a final extension phase of 72 °C for 10 minutes. The amplified products flanked with attB sequences were cloned into the entry vector, pDONR221 (Invitrogen) using BP Clonase reaction mix (Thermo Fisher Scientific cat. 11789020)

according to manufacturer's protocol. The pDONR221 vector contains the kanamycin resistant gene (KanR) for resistance against kanamycin antibiotic which was used as a selection marker in bacteria. The cloned pDONR221 vector was transferred into *Escherichia coli* strain DH5α. Plasmid DNA was isolated from *E. coli* cell culture and sequenced to ensure that no base pair mutation occurred during PCR amplification. The recombinant DNA was then transferred from the entry vector to the destination vector, pEarleyGate101 (Hartley et al., 2000; Earley et al., 2006) using LR Clonase reaction mix (Thermo Fisher Scientific cat. 11791100) following the manufacturer's protocol to generate the HD2 overexpression construct (CaMV35S:HD2-YFP). The pEarleyGate101 vector contains herbicide resistant gene (BlpR) for resistance against phosphinothricin (BASTA) herbicide which was used as a selection marker for transformed plants. HD2 overexpression constructs were transferred into *Agrobacterium tumefaciens* strain GV3101 via electroporation to carry out Agrobacterium-mediated plant transformation (Wise et al., 2006). Primers used for gene cloning and confirmation of DNA insertion are listed in **Appendix 2**.

For Arabidopsis plant transformation, WT plants were grown in a growth chamber under the same conditions mentioned above for about 5 weeks until they started flowering. Healthy plants were selected for Agrobacterium-mediated transformation by floral dip method (Zhang et al., 2006). Already opened flowers and developed siliques were removed from the inflorescences of healthy plants. Briefly, Agrobacterium strain GV3101 containing transformation constructs was grown to a stationary phase (OD_{600} ~ 1.5-2.0) in LB medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 0.5% NaCl) supplemented with gentamicin (10 mg/L) and kanamycin (50 mg/L) at 28°C in a shaker set at 250 rpm. The Agrobacterium culture was centrifuged at 3000 rpm for 15 minutes at room temperature to harvest the cells. The cell pellet was resuspended in 5% sucrose solution to a final OD_{600} of 0.8-1.0. Silwet L-77 (0.02%) was added to the solution prior to the transformation. Inflorescences of selected plants were dipped into the solution for about 10 seconds. Processed plants were covered with plastic bags and laid on their sides for 24 hours to maintain high humidity. These plants were then grown for 3-5 weeks in the growth chamber under control conditions to develop seeds.

Homozygous transgenic plants were obtained based on their response to BASTA herbicide selection. Seeds harvested from the floral dipped plants were regarded as the T0 generation. These seed were grown on selection medium (MS agar media containing BASTA (glufosinate ammonium 25 mg/L) selection). After 10 days of germination, healthy green seedlings from the media plates were transferred to soil, where these plants were sprayed with BASTA (250 mg/L) solution once a week to obtain true transformants and were regarded as T1 plants. Seeds collected from these T1 plants were called as the T2 generation seeds. The T2 seeds were further grown on selection medium and seedlings were either resistant or sensitive to BASTA herbicide. In this test, about 30 T2 seeds from each of the T1 plants were grown on selection medium plates to obtain the T2 seedlings. Vigorous growth and survival of all 30 seedlings under selection was observed in some of the plates, indicating that the seeds collected from the T1 plants were homozygous resistant. These resistant seedlings were transferred to soil, where plants were sprayed with BASTA (250 mg/L) solution once a week and allowed the resistant plants to develop seeds for seed propagation to use for further experiments. Gene expression analysis was performed to analyse and confirm the overexpression of transgenes in HD2 transgenic lines.

2.7 Gene expression analysis

To study the mRNA expression of different genes in *hd2* mutants and HD2 overexpression lines, quantitative real time PCR (RT-qPCR or qPCR) was performed. As explained above, RNA was isolated from leaves or seedlings (~ 50 mg) using the Plant/Fungi Total RNA Purification Kit (Norgen) and cDNA was synthesized from 1 µg of total RNA using iScript Reverse Transcription Supermix (Bio-Rad). RT-qPCR was performed using a SsoFast EvaGreen Supermix kit (Bio-Rad) on CFX96 Real-time PCR detection system (Bio-Rad) following the manufacturer's instructions. Data was analyzed using the Bio-Rad CFX Manager 3.1 software. The expression levels were normalized to housekeeping gene *ACTIN2*. The ΔΔCT method was applied to calculate the fold change in the expression level (Livak and Schmittgen, 2001). All RT-qPCR analysis were performed at least 3 times with three technical replicates for one biological replicate. All primers used for RT-qPCR analysis are listed in **Appendix 4**.

2.8 Western blot analysis

Ten-day old whole seedlings (100 mg) of different genotypes were collected in 2.0 ml Eppendorf tubes in liquid nitrogen and nuclei were isolated from the samples following the histone extraction protocol (abcam). Briefly, ground tissues were fixed in 200 µl of Triton Extraction Buffer (TEB: PBS containing 0.5% Triton X-100, and 2 mM phenylmethylsulfonyl fluoride (PMSF)), followed by incubation of the mixture on ice for 10 minutes. Samples were centrifuged at 2000 rpm for 10 minutes at 4^{o}C and the supernatant was discarded carefully. TEB buffer was again added to the samples and the centrifugation step was repeated. Cell pellets were resuspended in 0.4 µl of 0.2 N HCl and samples were kept at 4^{o}C for 4 hours. Samples were then centrifuged at 2000 rpm for 10 minutes at 4^{o}C and supernatants were transferred into fresh 1.5 ml Eppendorf tubes. Protein concentration was estimated using Bradford assay reagents (Thermo Fisher Scientific) following the standard protocol. Protein samples were stored at -20^{o}C.

To separate proteins by SDS-PAGE, protein samples were denatured by adding 20 mM dithiothreitol (DTT) and incubated at 95^{o}C for about 10 minutes. Samples were cooled down on ice before loading them onto SDS-PAGE gels for separation of proteins at 150 Volts (Bio Rad). Proteins from SDS gels were transferred to PVDF membranes (Bio-Rad) using a Semi-Dry Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The PVDF membrane was incubated in blocking solution containing 5% milk powder in TBS buffer with 0.1% Tween 20 (TBST) for 1 hour with gentle shaking at room temperature. The membrane was then incubated overnight at 4^{o}C in anti-H3K9ac or Anti-H3 antibody solution (Cell Signaling Technology and Millipore). The membranes were then rinsed five times with TBST buffer to remove the excessive primary antibody and then incubated in the secondary antibody (Milipore Sigma) solution for 1 hour. After washing the membrane with TBST buffer, proteins blots were detected using the EZ-ECL Chemiluminescence Detection Kit (Biological Industries) as per manufacturer's instructions with a MicroChemi imager (DNR Bio-Imaging Systems).

2.9 Drought stress treatment

To examine the expression level of the HD2 family genes in WT Arabidopsis under control and drought stress, the soil drying method was employed. Plants were grown in two groups: one group labelled as control and the other group labelled as the drought treatment group (with each group containing six pots). All the plants were grown for 4 weeks under control conditions and were given equal amounts of water. Four-week old plants in the drought group were withheld from watering for 7 days to establish drought stress in the plants. For gene expression analysis, leaf samples were collected daily (from day 1 to day 7) from both the control group and drought treated-group for RNA extraction. Three independent experiments were performed for this study. For each experiment, leaf samples collected from three plants of the control or drought groups were pooled together for RNA extraction.

For the drought stress tolerance assay, *hd2* mutants and HD2 overexpression (HD2-OE) plants were grown in soil in two groups. One group was treated as a control with no drought stress applied. The other group was subjected to drought stress treatment. Two-week old plants of *hd2* mutants and HD2-OE lines in the drought treatment groups were withheld from watering for 11 and 13 days, respectively. After the drought stress period, plants were rewatered for two days to allow them to recover from the drought stress period. Plants with at least two green turgid leaves and regenerated shoots were considered to have survived. The survival rate for each genotype was determined based on the number of plants survived out of the total number of plants grown in each pot. In this assay, three independent experiments were performed for calculating the survival rate of each genotype.

2.10 Leaf relative water content measurement

To determine the water status of plants, leaf relative water content (RWC) was measured from the plants of the control and drought treated groups (Barrs and Weatherley, 1962) described above. Leaves were excised from the shoots using sharp scissors and placed in pre-weighed 2.0 ml Eppendorf tubes. Fresh weight (FW) of all the collected leaves was determined as soon as possible by weighing the tubes containing three leaves and subtracting the respective tube weight. All the leaves were rehydrated by floating them on

water surface for 4 hours and leaf turgid weight (TW) was obtained. All the turgid leaves were oven-dried at 55^{o}C for 24 hours. Oven-dried leaves in the tubes were weighed again to determine the leaf dry weight (DW). Leaf RWC (%) was determined as [(FW – DW) / (TW – FW)] x 100. Three independent experiments were performed and three biological replicates were used for each experiment.

2.11 Fresh leaf water loss measurement

To measure fresh leaf water loss in different genotypes, fully grown rosette leaves were detached from 4 week-old plants grown under control conditions and immediately weighed to measure their fresh weights. Leaves were weighed every 30 minutes for a total of 180-minute period. During each 30-minute interval, leaves were kept open in petri dishes at room temperature and conditions. Results are shown as the percentage water loss compared to the leaf fresh weights at each 30-minute interval. Three independent experiments were performed and three biological replicates were used for each experiment.

2.12 Stomatal aperture measurement

To examine the stomatal closure under control and stress conditions, stomatal aperture measurement assays were performed in *hd2* mutants and HD2-OE lines following the established protocols (Eisele et al., 2016; Scarpeci et al., 2017) with minor modifications. Healthy leaves were excised from 4 week old plants and floated (with abaxial side down) in petri dishes containing MES/KOH stomatal opening buffer (10 mM MES, 5 mM KCl, 50 μM CaCl2, pH 6.15) for two hours. This group was regarded as the control (no stress treatment given). In the experimental group (stress treatment applied), the same steps were followed, except, after two hours, 10 μM of ABA was added to the petri dishes containing MES/KOH buffer. After two hours of ABA treatment, leaves were taken out from the solution and quickly pat dried with napkins and prepared for microscopy. All experiments were performed at room temperature and conditions.

Preparation of leaf imprints to visualize the stomata using microscopy was performed by following the protocol described by Scarpeci et al. (2017) with minor modifications. Briefly, leaf imprints were obtained by applying a thin layer of transparent nail varnish on the abaxial leaf surface. The applied nail varnish layer was dried for about 30 minutes. The

thin layer of nail varnish from leaf was transferred to a glass slide by gently pressing the leaf onto the slide. Stomata imprints were observed under a 40x objective lens using the EVOS™ XL Core Imaging System (AMEX1000). Stomatal pore widths and lengths were measured using the image processing software ImageJ (http://rsb.info.nih.gov/ij/). Results for stomatal aperture were shown as the ratio of widths to lengths. Three independent experiments were performed for this test. At least 50 stomata were scored from 3 plants of each genotype in each experiment.

2.13 Root growth phenotype analysis

Root growth phenotype analysis was performed to study the role of HD2s in root growth under drought stress. For this, primary root lengths were measured and secondary roots were counted in *hd2* mutants and HD2-OE lines under control and osmotic stress conditions. To establish osmotic stress for seedlings in MS media, Polyethylene glycol (PEG-6000) was used. PEG does not enter the cell wall and larger molecules of PEG (> 3000 g/mol) are apparently not absorbed by plants (Jiang et al., 1995; Landjeva et al., 2008), so it is frequently used to mimic drought stress in plants and study the drought stress-related responses. Seeds of *hd2* mutants and HD2-OE lines were sterilized and stratified as described above and grown on agar plates containing only MS media or MS media supplemented with 10% PEG-6000. Primary root lengths and number of secondary roots were measured on day 10 of germination for all the experiments. In this test, three independent experiments were performed and 20 seedlings were used for measurements in each independent experiment.

For phytohormone gibberellic acid treatment, GA3 and GA4 were used to examine the primary root lengths in *hd2* mutants under gibberellic acids treatment. Seeds of *hd2* mutants were sterilized and stratified before they were grown on MS media supplemented with GA3 or GA4 at 1 µM and 10 µM concentrations. Primary root lengths were measured on day 10 of germination for all the experiments. In this test, three independent experiments were performed and 20 seedlings were used for measurements in each independent experiment.

2.14 Yeast-two hybrid assay

The yeast two-hybrid (Y2H) assay is a robust and popular technique that allows the detection of protein interactions in live yeast cells (Brückner et al., 2009). To study protein-protein interactions of HD2s, Y2H assays were performed following the instructions given in the Matchmaker Gold Yeast Two-Hybrid System user manual (Clontech). As described earlier, amplified HD2 gene products were cloned into pDONR22 following the BP reaction protocol. Using LR Clonase reaction mix, the cloned DNA from pDONR221 was then transferred to destination vectors pGBKT7-BK (bait vector) and pGADT7-AD (prey vector) to generate plasmid constructs for the Y2H assays. The Y2HGold strain was co-transformed with 5 µg of each of bait and prey vectors containing HD2 genes in different combinations (**Appendix 5**). For negative controls, the yeast strain was transformed with bait vector and empty prey vector (containing no gene of interest). The pGBKT7-53 and pGADT7-T plasmids provided with the kit (encode murine p53 and SV40 large T-antigen) were used as positive controls. The transformed Y2HGold cells were plated on minimal selective medium SD-Leu/Trp double dropout (DDO) and incubated at 30°C until colonies appeared on the medium plates (3 - 5 days). Colonies from DDO medium were sub-cultured to stringent selective medium SD-Leu/Trp/Ade/His quadruple dropout (QDO) and QDO supplemented with 40 mg/L X-Alpha Gal (5-Bromo-4-Chloro-3-indolyl a-D-galactopyranoside) and 200 µg/L Aureobasidin-A (QDO/X/A) to examine the protein-protein interactions.

2.15 Bimolecular fluorescence complementation assay

Bimolecular fluorescence complementation (BiFC) assays were performed to confirm the protein-protein interactions (Tian et al., 2011) in plant host. As described earlier, amplified HD2 gene products were cloned into pDONR221 following the BP reaction protocol. Using LR Clonase reaction mix, the cloned DNA from pDONR221 was then transferred to destination vectors, pEarleyGate201-YN and pEarleyGate202-YC to generate plasmid constructs for the BiFC assay. The -YN and -YC represent N-terminal and C-terminal domains of tagged YFP (yellow fluorescent protein) protein in both vectors, respectively. Both vectors were transferred to *Agrobacterium tumefaciens* strain GV3101 via electroporation.

To infiltrate *N. benthamiana* leaf epidermal cells, Agrobacterium containing plasmid constructs for BiFC were grown under gentamicin (10 mg/L) and kanamycin (50 mg/L) antibiotics selection at 28^{o}C in LB medium to an OD_{600} ~ 0.8-1.0. Cell culture was centrifuged at 10,000 rpm for 1 minute and the supernatant was removed. Washing of the pelleted cells was performed three times by using 1 ml of infiltration medium (5 g/L glucose, 2 mM Na_3PO_4, 50 mM MES, and 0.1 mM acetosyringone) (Sparkes et al., 2006; Tian et al., 2011). Cells were finally resuspended in 500 µl of infiltration medium for infiltrating *N. benthamiana* leaf epidermal cells. An equal volume of each of the Agrobacterium cultures of pEarleyGate201-HD2-YN and pEarleyGate202-HD2-YC constructs were mixed in different combinations (**Appendix 5**) for protein-protein interaction studies. For negative controls, cultures containing the pEarleyGate201-HD2-YN construct were co-infiltrated with empty pEarleyGate202-YC vector containing cultures. The *N. benthamiana* plants were grown in a growth chamber under the same conditions as described above. Leaves of 4-week old plants were infiltrated on abaxial sides with 200 µl of Agrobacterium suspension mixture containing two plasmid constructs. Each combination was infiltrated into at least two leaves. Infiltrated plants were returned to the growth chamber. After 48-72 hours, the YFP signal was observed using an Olympus Confocal Laser Scanning Microscope FV3000 located at AAFC-LRDC. The argon excitation laser wavelength was set at 514 nm to visualize the YFP signal. Three independent experiments were performed for this study.

2.16 Statistical analysis

Microsoft Excel 2016 (Microsoft Corp., Redmond, Washington) was used to calculate simple univariate statistics, such as means, standard deviations, and standard errors. The Student's t-test was used to determine the significance of difference between two independent datasets. One-way ANOVA followed by post-hoc Tukey's HSD test was used to perform multiple comparisons to determine the significance of differences between multiple independent datasets. The statistical analyses were performed using IBM SPSS Statistics version 25.0 (IBM Corp. Armonk, New York). A p-value of 0.05 (*) or 0.01 (**) was used to show a statistically significant difference.

2.17 Primer design

All primers used in this study were designed using Primer3Plus (https://primer3plus.com) unless stated otherwise. The primer parameters were set as follows: melting temperatures (57-63°C); GC content (40-60%).

Chapter 3: Results

3.1 Characterization of the HD2 family HDACs

To initiate the study, HDACs belonging to the HD2 family in different species were identified and a phylogenetic tree was constructed using peptide sequences to identify homologous HD2-type HDACs (**Figure 3**) (Tahir and Tian, 2021). Phylogenetic analysis of HD2s in Arabidopsis and other species shows the largest genetic distance between HD2D and other HD2 family proteins. Thus, HD2D is a distantly related member of the HD2 family. Furthermore, sequence analysis of Arabidopsis HD2s shows that the HD2 proteins possess conserved sequence motifs in the N-terminal domain (often regarded as the histone deacetylase domain), which are considered crucial for their histone deacetylation activity (**Figure 4A-B**). The central region of HD2-type HDACs is called acidic domain, enriched with aspartic acid and glutamic acid. The C-terminal domain of HD2A and HD2C possess a C_2H_2-type Zinc finger (ZnF) motif, which is thought to be involved in DNA-protein or protein-protein interactions (Luo et al., 2022). HD2A and HD2B share a common ancestor and are paralogue. Although HD2B differs from HD2A in not containing C_2H_2 signature motif, the HD2A (AT3G44750) and HD2B (AT5G22650) share the maximum sequence similarity (50.5%) and the least divergence (0.493) with each other at the protein level (**Figure 4C**) in the HD2 family. HD2A shares the second maximum similarity (40.5%) and the second least divergence (0.802) with HD2C (AT5G03740). HD2D (AT2G27840) is a distantly related HD2 family member and shares the least similarity and maximum divergence with HD2A, HD2B and HD2C.

Figure 3: Phylogenetic analysis of HD2-type HDACs in different plant species

A phylogenetic tree was generated with bootstrap analysis of 1000 replicates and partial deletion parameters by the maximum likelihood method using protein sequences of the HD2 family HDACs from *A. thaliana* (Arabidopsis), *Zea mays* (maize), *Glycine max* (soybean), *Solanum lycopersicum* (tomato), *Hordeum vulgare* (barley), and *Oriza sativa* (rice). The number at each node indicates the bootstrap value. Each name includes a gene locus number followed by a gene name. Green box includes the HD2s belonging to Arabidopsis.

(This figure has been recently published by our lab in Plant Cell Report journal (doi.org/10.1007/s00299-021-02688-3).

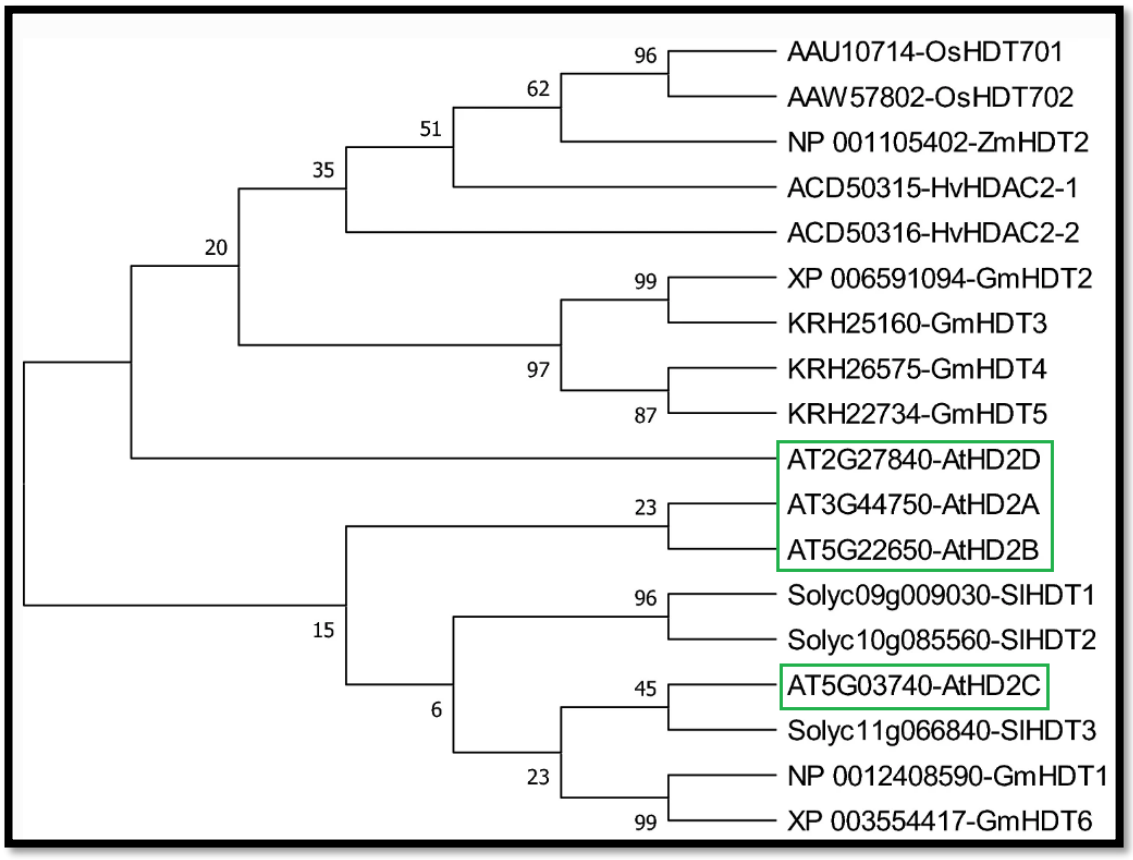

96
62
51
35
20
99
97
87
23
96
15
45
6
23
99
AAU10714-OsHDT701
AAW57802-OsHDT702
NP 001105402-ZmHDT2
ACD50315-HvHDAC2-1
ACD50316-HvHDAC2-2
XP 006591094-GmHDT2
KRH25160-GmHDT3
KRH26575-GmHDT4
KRH22734-GmHDT5
AT2G27840-AtHD2D
AT3G44750-AtHD2A
AT5G22650-AtHD2B
Solyc09g009030-SlHDT1
Solyc10g085560-SlHDT2
AT5G03740-AtHD2C
Solyc11g066840-SlHDT3
NP 0012408590-GmHDT1
XP 003554417-GmHDT6

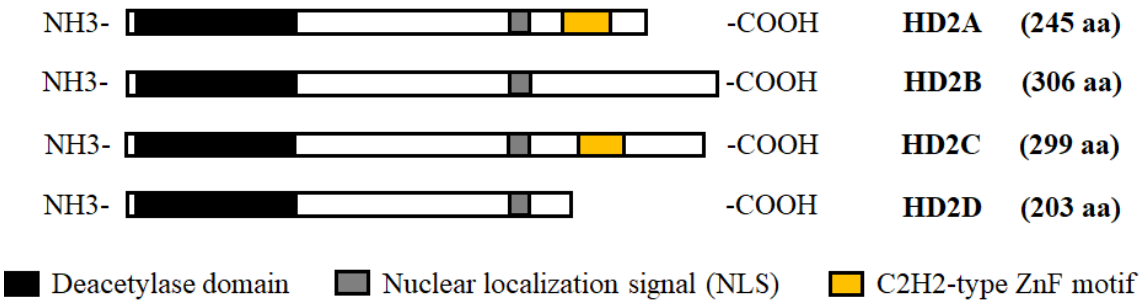
Figure 4: Domain organization and sequence analysis of HD2-type HDACs in Arabidopsis

(A) Schematic representation of the HD2 family HDACs HD2A, HD2B, HD2C, and HD2D in Arabidopsis.

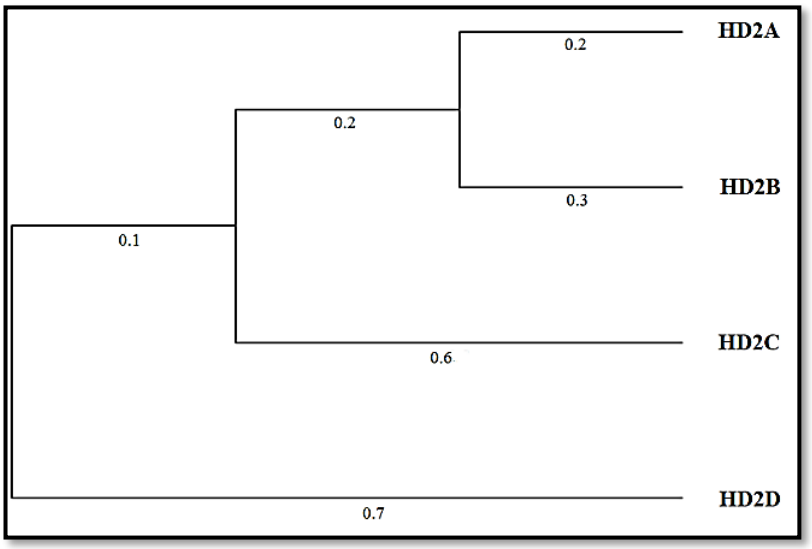
(B) Phylogenetic tree constructed based on protein sequences of HD2 proteins HD2A, HD2B, HD2C, and HD2D in Arabidopsis. The number at each branch indicates the divergence value.

(C) Protein similarities and divergencies among HD2 proteins HD2A, HD2B, HD2C, and HD2D.

A



B



C

Percent identity (%)

AA divergence	HD2A	HD2B	HD2C	HD2D
HD2A		50.5	40.5	36.8
HD2B	0.493		39.5	30.8
HD2C	0.802	0.965		31.3
HD2D	0.981	1.059	1.161	

3.2 Arabidopsis HD2s respond to drought stress

To investigate how HD2 family genes respond to drought stress, the expression levels of the HD2 family genes *HD2A*, *HD2B, HD2C,* and *HD2D* were examined under drought conditions. As described in Section 2.10, 4-week old Arabidopsis WT plants were exposed to drought stress by withholding water for drought treatment for 7 days. Representative images of WT Arabidopsis plants under control and drought stress conditions (day 5) are shown in **Figure 5A**. Leaf samples were collected daily from both the control group and drought-treated group for leaf relative water content (RWC) measurements to determine the water status of plants to ensure the drought treatment effect. As shown in **Figure 5B**, leaf RWC decreased from day 2 and dropped to 15% by day 6, as compared to control.

To examine gene expression, leaf samples were collected daily from both the control group and drought-treated group for RNA extraction. The *RESPONSIVE TO DESICCATION 29A* (*RD29A*) gene is induced in response to different abiotic stresses and is frequently used as a drought responsive marker (Msanne et al., 2011; Bihmidine et al., 2013). To confirm the efficiency of the drought treatment at the molecular level, the relative expression of the *RD29A* was analysed (**Figure 5C**). *RD29A* showed significant change in expression starting on day 3 of the drought treatment and was elevated to 65-fold by day 5. When an effective drought stress initiating from day 3 was confirmed, I then examined the expression of the HD2 family genes *HD2A*, *HD2B*, *HD2C*, and *HD2D* using samples from day 3 of the drought treatment. The qPCR analysis showed that all HD2 genes were significantly upregulated on day 3 in response to drought (**Figure 6**).

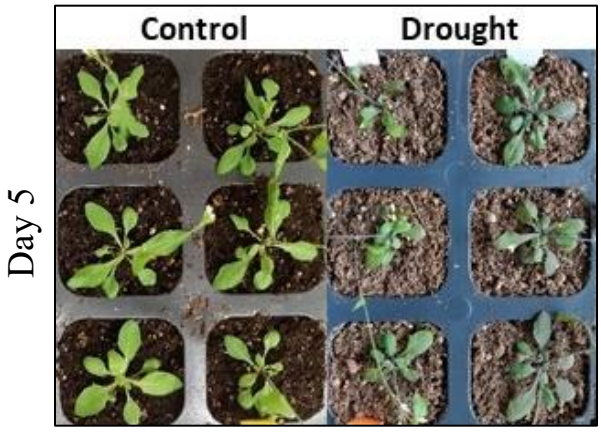
Figure 5: Soil drought stress induced water loss and marker gene upregulation in Arabidopsis plants

(A) Images of the WT Arabidopsis plants under control and drought stress conditions at day 5.

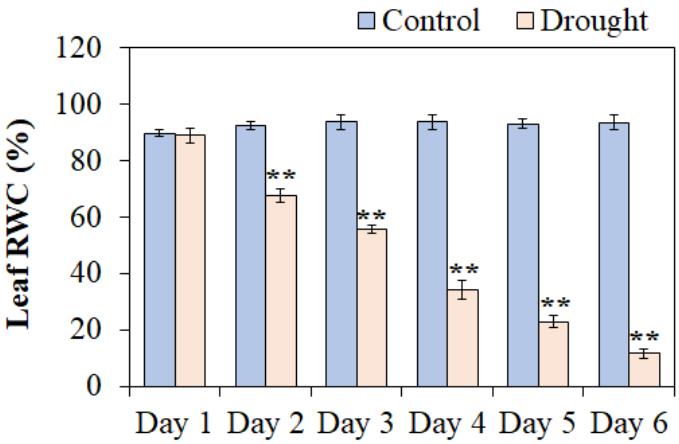
(B) Measurements of leaf relative water content. Leaf RWC of the plants from the control and drought-treated groups was measured from day 1 to day 6. Data shown are means ± standard errors (n = 9). Three independent experiments were performed. In each experiment, measurements were taken from three plants (total three leaves per plant weighed). The significance of the difference was determined by Student's t test (**p < 0.01).

(C) Relative expression (fold change) of *RD29A* under control and drought conditions. Data are shown as the expression level relative to the control conditions, normalized to the *ACTIN2* gene. Data shown are means ± standard errors (n = 3). For each independent experiment, leaf samples collected from three plants from the control or drought-treated groups were pooled together for RNA extraction. The significance of the difference was determined by Student's t test (**p < 0.01).

A


Control
Drought
Day 5

B


Control
Drought
Leaf RWC (%)
120
100
80
60
40
20
0
**
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6

C

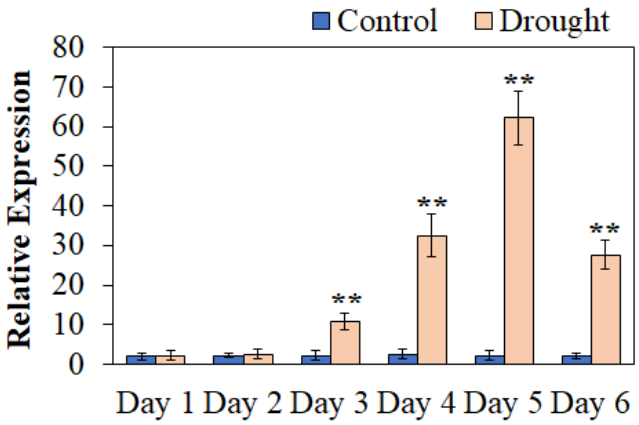
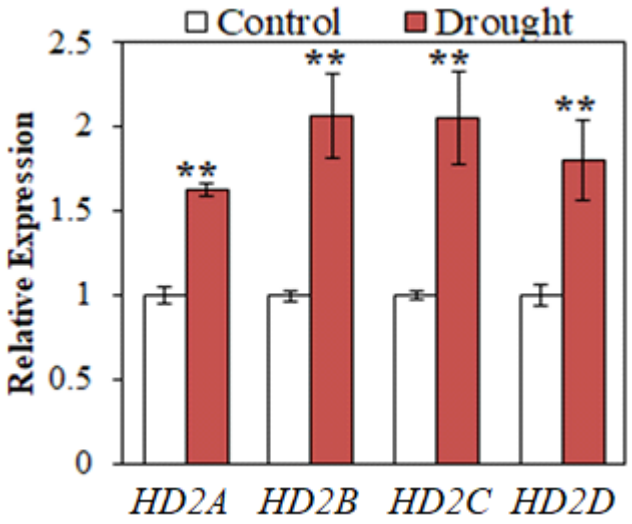

Control
Drought
Relative Expression
80
70
60
50
40
30
20
10
0
**
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6

Figure 6: Expression analysis of the HD2 family genes in Arabidopsis

Relative expression (fold change) of the HD2 family genes *HD2A*, *HD2B*, *HD2C*, and *HD2D* under control and day 3 of drought treatment in WT Arabidopsis. Data are shown as the expression level relative to the control conditions, normalized to the *ACTIN2* gene. Data shown are means ± standard errors (n = 3). For each independent experiment, leaf samples collected from three plants from the control or drought-treated groups were pooled together for RNA extraction. The significance of the difference was determined by Student's t test (**$p < 0.01$).


Control
Drought
Relative Expression
2.5
2
1.5
1
0.5
0
**
**
**
**
HD2A
HD2B
HD2C
HD2D

3.3 Confirmation of *hd2* single mutants and generation of *hd2* double mutant lines

I obtained *hd2* single mutants GK355-H03 (*hd2a*), SAIL1247-A02 (*hd2b*), SALK-129799C (*hd2c*), and GK279-D04 (*hd2d*) from ABRC. The position of the T-DNA insertion in the HD2 genes was determined from the ABRC website and genotyping primers were designed for each of the *hd2* mutant lines according to the T-DNA insertion map of the HD2 genes for all the mutant lines (**Figure 7A**). The *hd2a*, *hd2c*, and *hd2d* mutants had the T-DNA insertions in the 4th, 6th, and 1st exon of genomic DNA sequences, respectively. Whereas the *hd2b* mutant had the T-DNA insertion in the promotor region.

The seeds obtained for *hd2b* were maintained as T1 stock by ABRC. I grew the *hd2b* T1 seeds to obtain the T2 generation seeds which were grown again into next generation. PCR genotyping was performed in the T3 generation of *hd2b* mutant to identify homozygous lines (**Figure 7B**). The seeds for *hd2a* and *hd2d* mutant lines obtained from ABRC were from T4 generation (collected from T3 bulk population), whereas the seeds of *hd2c* mutant were from T3 generation. I grew these seeds into the next generation and carried out genotyping to identify the homozygous T-DNA insertional mutant lines for each of the *hd2* mutants (**Figure 7B**) using T-DNA genotyping primers. Three primers FP, RP, and LB were used in the PCR genotyping. Primer pair FP + RP amplifies only WT allele (Col-0) with a single band appearing in the gel electrophoresis, whereas primer pair LB + RP does not amplify the WT (Col-0) and produces no band in the gel. For homozygous T-DNA mutants, primer pair LB + RP amplifies the mutant allele and shows a single band in the gel electrophoresis, whereas primer pair FP + RP reaction does not. All the uncropped gel images pertaining to the images in **Figure 7B** are given in **Appendix 6**.

To confirm the knockout of HD2 gene expression at the mRNA level, I performed qPCR analysis (**Figure 8**). The *HD2A* and *HD2C* show complete knockout of their expression in the *hd2a* and *hd2c* mutant lines, respectively. *HD2B* and *HD2D* retain their expression at 21% and 10% of the WT levels in the *hd2b* and *hd2d* mutant lines, respectively. This suggests that the *hd2b* mutant is a knockdown line as it retained some of its expression, possibly due to the location of T-DNA insertion in the promoter region.

Next, *hd2* single mutants *hd2a*, *hd2b*, *hd2c*, and *hd2d* were crossed with each other in different combinations (**Table 1**) to generate *hd2* double mutant lines. PCR genotyping of

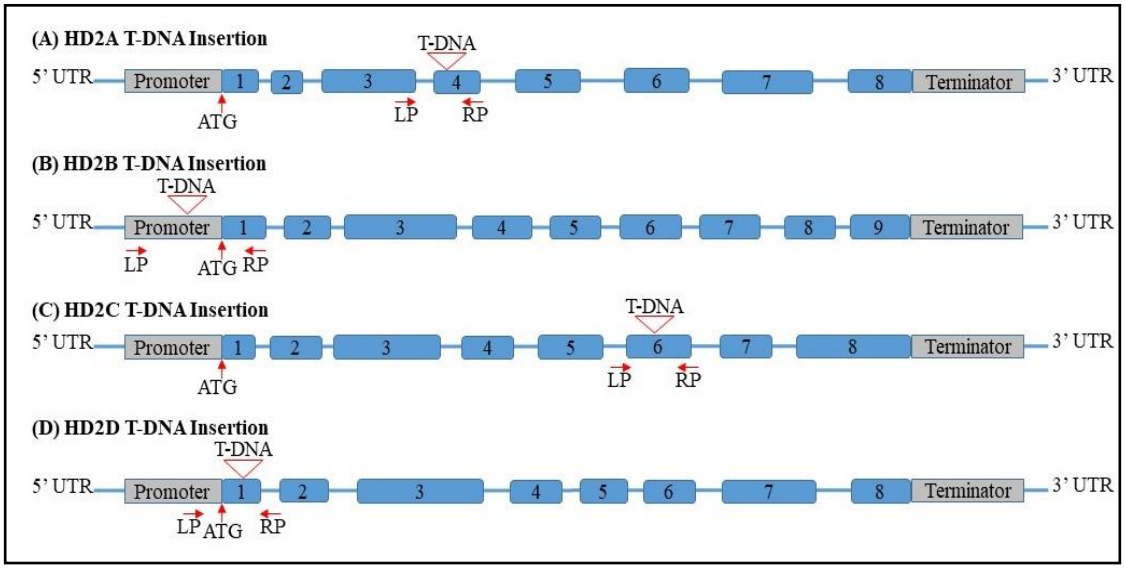
F2 generation plants of each cross was carried out to identify homozygous double mutant lines. From the cross of *hd2a* and *hd2c* mutant lines (dihybrid cross), I did the genotyping of 24 plants in the F2 generation and was able to identify a homozygous double mutant line *hd2a*.*hd2c*, named as Mac16. The homozygous double mutant line Mac16 was further crossed with *hd2b* (trihybrid cross). Total 76 plants were analysed in F2 generation which resulted in two more double mutant lines *hd2a*.*hd2b* (named as Mab4) and *hd2b*.*hd2c* (named as Mbc68). From another successful cross of *hd2c* and *hd2d*, I obtained the double mutant line *hd2c*.*hd2d*, named as Mcd20, after genotyping 28 plants in F2 generation. Previously, *HD2C* and *HD2D* have been studied for their role in ABA and abiotic stress responses (Sridha and Wu, 2006; Luo et al., 2012; Han et al., 2016). Chen et al. (2018) reported that HD2C interacts with HD2B and simultaneous knockout of both HD2s (*hd2b*.*hd2c*) resulted in narrow and pointed leaves as well as shorter roots as compared to the single mutants, *hd2b* and *hd2c*. The role of HD2A and its relationship with HD2C in response to abiotic stresses including drought remains largely unknown. Due to the relative paucity of information on *hd2a* and *hd2c* single and double mutants against drought stress response, I focussed my attention on the Mac16 (*hd2a*.*hd2c*) double mutant line for further detailed study. The knockout of *HD2A* and *HD2C* in Mac16 line was confirmed by genotyping and qPCR analysis (**Figure 9**). All the uncropped gel images pertaining to the images in **Figure 9A** are given in **Appendix 7**. The qPCR analysis showed the knockout of both HD2A and HD2C expression in the double mutant line Mac16 (**Figure 9B**). It also showed that the *HD2C* is upregulated in the *hd2a*. Similarly, *HD2A* is upregulated in the *hd2c* mutant line.

Figure 7: Genotyping of *hd2* single gene mutants by PCR

(A) T-DNA insertion maps of HD2 genes for *hd2* mutant lines. Blue blocks indicate exons. Red triangles indicate the position of T-DNA insertions in the HD2 genomic sequences, whereas horizontal red arrows show primer positions in the sequences.

(B) Genotyping of *hd2* single mutant lines for *HD2A*, *HD2B*, *HD2C,* and *HD2D*. Gene-specific left (LP) and right (RP) primers along with T-DNA primers (LB) were used in genotyping by PCR. LB primers used in the genotyping are GK8474 (for *HD2A* and *HD2D*), LB3 (for *HD2B*), and LBa1 (for *HD2C*).

A


(A) HD2A T-DNA Insertion
T-DNA
5' UTR
Promoter
1
2
3
4
5
6
7
8
Terminator
3' UTR
ATG
LP
RP
(B) HD2B T-DNA Insertion
T-DNA
5' UTR
Promoter
1
2
3
4
5
6
7
8
9
Terminator
3' UTR
LP
ATG
RP
(C) HD2C T-DNA Insertion
T-DNA
5' UTR
Promoter
1
2
3
4
5
6
7
8
Terminator
3' UTR
ATG
LP
RP
(D) HD2D T-DNA Insertion
T-DNA
5' UTR
Promoter
1
2
3
4
5
6
7
8
Terminator
3' UTR
LP
ATG
RP

B

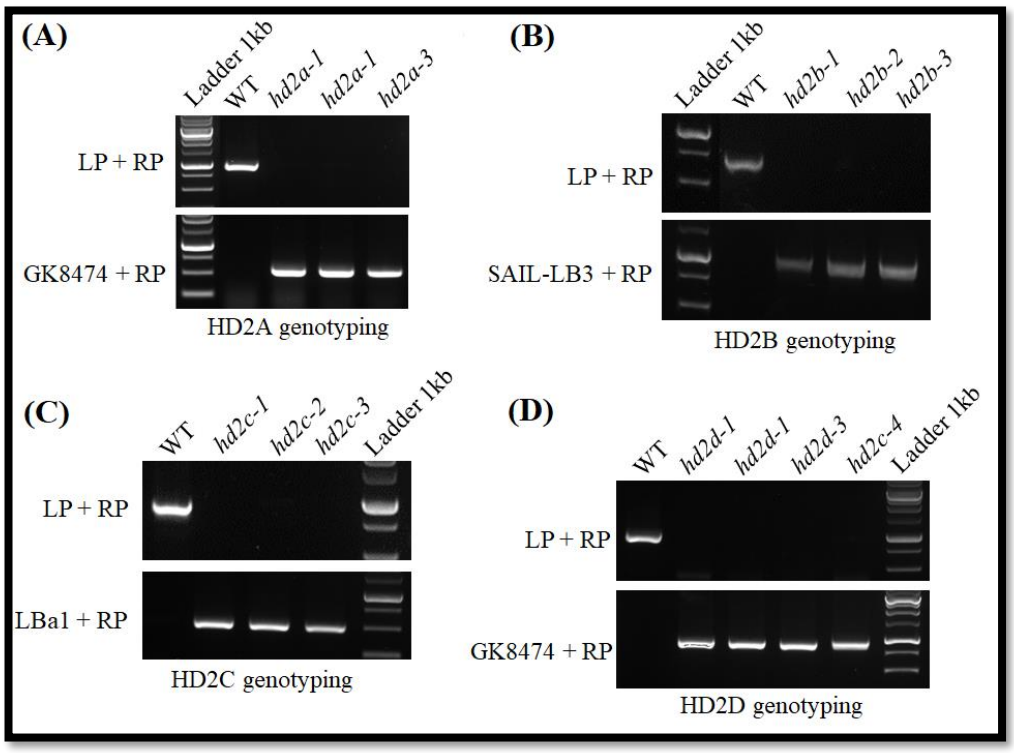

(A)
Ladder 1kb
WT
hd2a-1
hd2a-1
hd2a-3
LP + RP
GK8474 + RP
HD2A genotyping
(B)
Ladder 1kb
WT
hd2b-1
hd2b-2
hd2b-3
LP + RP
SAIL-LB3 + RP
HD2B genotyping
(C)
WT
hd2c-1
hd2c-2
hd2c-3
Ladder 1kb
LP + RP
LBa1 + RP
HD2C genotyping
(D)
WT
hd2d-1
hd2d-1
hd2d-3
hd2c-4
Ladder 1kb
LP + RP
GK8474 + RP
HD2D genotyping

Figure 8: mRNA expression analysis of mutated HD2 genes in *hd2* mutant lines

Relative expression of mutated HD2 family genes *HD2A, HD2B, HD2C*, and *HD2D* in *hd2a, hd2b, hd2c*, and *hd2d* mutant lines, respectively. Data shown are means ± standard errors (n = 3). For each independent experiment, leaf samples collected from three plants were pooled together for RNA extraction.

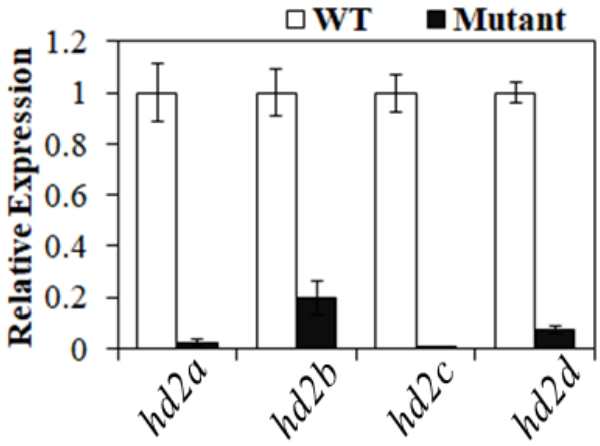

WT
Mutant
Relative Expression
1.2
1
0.8
0.6
0.4
0.2
0
hd2a
hd2b
hd2c
hd2d

Table 1: *hd2* single gene mutants crossed with each other in different combinations

Different *hd2* single mutants crossed with each other. The first three crosses resulted in the successful generation of *hd2* double mutant lines Mac16, Mcd20, Mbc68 and Mab4. Cross of *hd2a* and *hd2b* with *hd2d* did not produce any seeds. So, no *hd2a.hd2d* or *hd2b.hd2d* double mutant lines were obtained after several attempts.

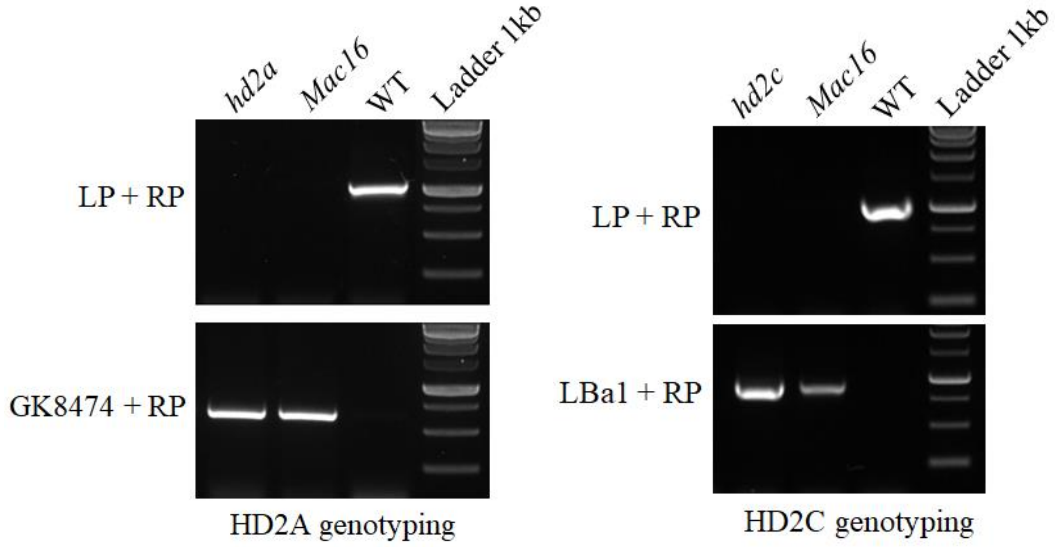
No.	Cross	Double mutant line	Named as
1	*hd2a X hd2c*	*hd2a.hd2c*	Mac16
2	*hd2c X hd2d*	*hd2c.hd2d*	Mcd20
3	*Mac16 X hd2b*	*hd2a.hd2b*	Mab4
	(Triple gene cross)	*hd2b.hd2c*	Mbc68
4	*hd2a X hd2d*	*hd2a.hd2d*	Not successful
5	*hd2b X hd2d*	*hd2b.hd2d*	Not successful

Figure 9: Genotyping and RT-qPCR analysis of the Mac16 double mutant line

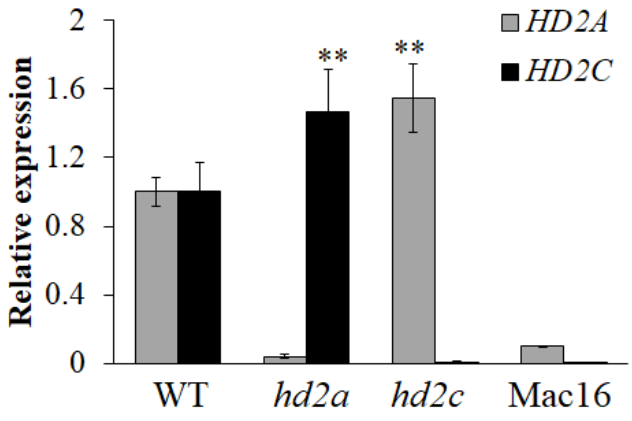
(A) Genotyping of the Mac16 double mutant line for *HD2A* and *HD2C*. Gene-specific left (LP) and right (RP) primers along with T-DNA primers, GK8474 and LBa1, were used in genotyping.

(B) Relative expression of *HD2A* and *HD2C* in WT, single mutants *hd2a* and *hd2c* and the double mutant line Mac16. Data shown are means ± standard errors (n = 3). For each independent experiment, leaf samples collected from three plants were pooled together for RNA extraction. The significance of the differences was determined by Student's t test (**$p < 0.01$).

A


hd2a
Mac16
WT
Ladder 1kb
LP + RP
GK8474 + RP
HD2A genotyping
hd2c
Mac16
WT
Ladder 1kb
LP + RP
LBa1 + RP
HD2C genotyping

B


Relative expression
2
1.6
1.2
0.8
0.4
0
**
**
HD2A
HD2C
WT
hd2a
hd2c
Mac16

3.4 Development of HD2 overexpression lines

Along with the Mac16 double mutant line, I also generated *HD2A* and *HD2C* overexpression lines. The segment of pEarleyGate101 plasmid construct used for generating HD2-OE lines is shown in **Figure 10A**, where the expression of HD2 genes was driven by the CaMV35S promoter. Regenerated putative transgenic plants were selected under BASTA herbicide. In total, I obtained 29 HD2A-OE and 35 HD2C-OE plants in the T1 generation after selection with BASTA. In T2 generation, the transfer-DNA (35S-HD2-YFP) was identified in 11 HD2A-OE and 13 HD2C-OE plants by performing PCR using primers shown in **Figure 10A**. The presence of 35S-HD2A-YFP and 35S-HD2C-YFP transfer-DNA fragment in 5 lines of each of HD2A and HD2C-OE plants, respectively, is shown in **Figure 10B**. The two highly expressing homozygous transgenic lines, OEA6 and OEA10 for HD2A-OE, and OEC13 and OEC25 for HD2C-OE were selected based on PCR analysis. For convenience, the selected lines were renamed to use numbers 1 and 2 instead of their original numbers. The HD2A-OE lines, OEA6 and OEA10 were renamed as OEA1 and OEA2, respectively. The HD2C-OE lines, OEC13 and OEC25 were renamed as OEC1 and OEC2, respectively. qPCR analysis was performed to analyse the expression of *HD2A* and *HD2C* in HD2A-OE and HD2C-OE lines. The results showed that *HD2A* overexpression lines OEA1 and OEA2 exhibited 7.4- and 14.7-fold higher expression, respectively, whereas *HD2C* overexpression lines OEC1 and OEC2 exhibited 19.3- and 12.4-fold higher expression, respectively, compared to WT (**Figure 10C**).

Figure 10: Generation of HD2 overexpression lines

(A) Schematic representation of the region of pEarleyGate101 plasmid construct used for transformation to generate the HD2-OE lines. The green box represents the position of the coding region of HD2 genes, driven by the 35S promoter. The yellow box represents the sequence of the reporter gene, yellow fluorescent protein (*YFP*). RB and LB indicate the right and the left border of the T-DNA region, respectively. Black arrows represent the positions of primers used for PCR confirmation.

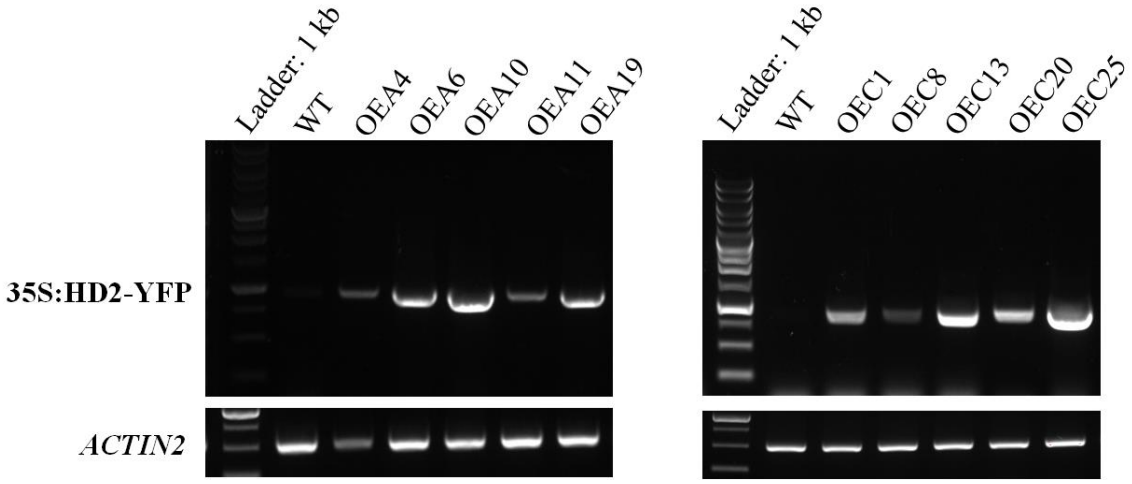
(B) Confirmation of the presence of 35S-HD2A-YFP in the HD2A-OE lines, OEA4, OEA6, OEA10, OEA11, OEA19 (left) and 35S-HD2C-YFP in the HD2C-OE lines, OEC1, OEC8, OEC13, OEC20, OEC25 (right). The genomic DNA of WT Arabidopsis was used as a negative control. A fragment of 854 bp of 35S-HD2A-YFP construct in HD2A-OE lines and 896 bp fragment of 35S-HD2C-YFP in HD2C-OE lines was amplified using the primer pair indicated by black arrows in (A). *ACTIN2* gene primers were used to amplify the gene to indicate the genomic DNA quality.

(C) Left: Relative expression (fold change) of *HD2A* (Left) and *HD2C* (Right) in WT and two OE lines named as OEA1 and OEA2, and OEC1 and OEC2, respectively. Data shown are means ± standard errors (n = 3). For each independent experiment, leaf samples collected from three plants were pooled together for RNA extraction. The numbers on each bar represent fold change in the expression level.

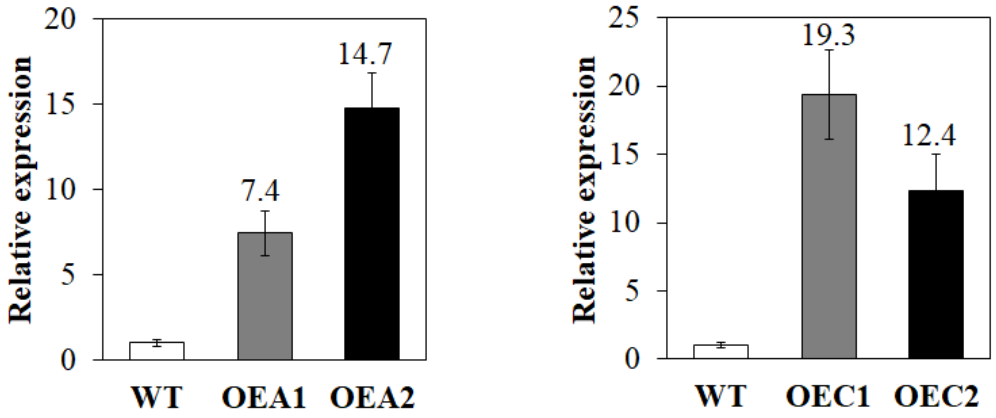
A


RB
BAR
CaMV35S
attR1
AtHD2 gene
attR2
YFP
OCS Ter.
LB

B


Ladder: 1 kb
WT
OEA4
OEA6
OEA10
OEA11
OEA19
Ladder: 1 kb
WT
OEC1
OEC8
OEC13
OEC20
OEC25
35S:HD2-YFP
ACTIN2

C


Relative expression
20
15
10
5
0
14.7
7.4
WT
OEA1
OEA2
Relative expression
25
20
15
10
5
0
19.3
12.4
WT
OEC1
OEC2

3.5 *HD2A* and *HD2C* overexpression or knockout affects plant survivability under drought stress

I first investigated if *HD2A* or *HD2C* overexpression or knockout affects the survivability of plants under drought stress. I performed drought treatment assays as described in Section 2.10 (**Figure 11**). Two-week old plants in drought treatment groups were withheld from watering. After the drought treatment period, plants were rewatered to allow them to recover from the effects of drought stress. Plants with green turgid rosette leaves were considered to have survived and the survival rate for each genotype was determined by measuring the number of plants survived out of total number of plants in each pot. Drought treatment assays of HD2-OE lines showed that the HD2A-OE lines, OEA1 and OEA2 had 52% and 56% survival, respectively, whereas HD2C-OE lines, OEC1 and OEC2 had 84% and 77% survival, respectively, compared to WT plants which showed 34% survival. Next, I compared the drought survival of *hd2* single mutants, *hd2a* and *hd2c* and double mutant Mac16 with WT (**Figure 12**). The single mutants, *hd2a* and *hd2c* showed 79% and 67% survival, respectively, relative to WT (normalized to 100%), whereas the double mutant line Mac16 showed only 47% survival relative to WT.

After examining plant survival under drought stress, I measured the fresh leaf water loss in *hd2* mutants and HD2-OE lines as described in Section 2.11. Fresh leaves were collected from the plants growing under control conditions and the gradual decrease in the leaf weight was measured every 30 minutes over a period of 180 minutes. Results are shown as the percentage water loss at each time point as compared to the leaf fresh weights. The single mutant *hd2c* showed a significantly increased water loss, which was further increased significantly in the double mutant Mac16 as compared to the single mutants and WT (**Figure 13A**). Conversely, both HD2A-OE and HD2C-OE lines demonstrated a decreased water loss from the leaves, especially in HD2C-OE lines, compared to WT plants (**Figure 13B**).

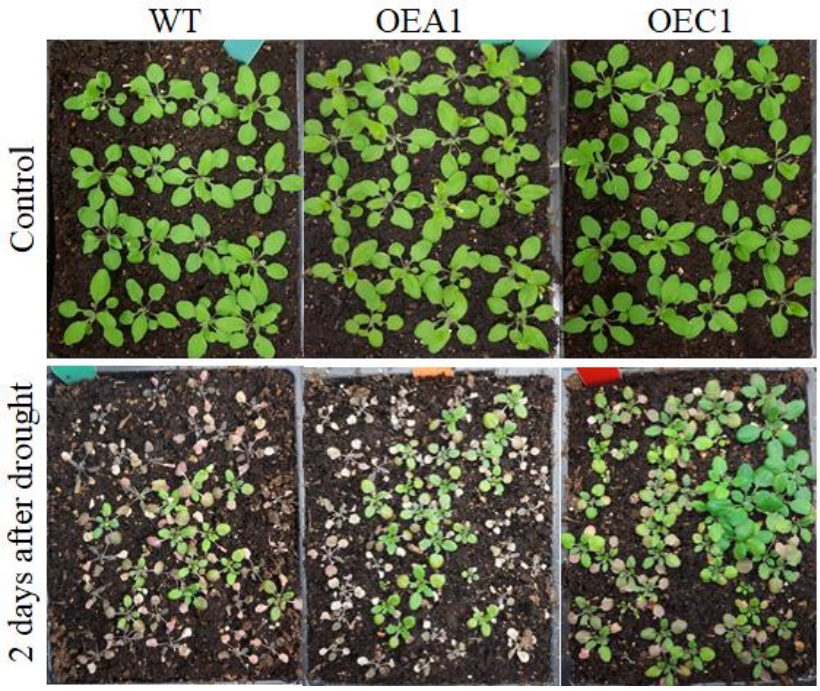
These results indicate that when both *HD2A* and *HD2C* genes are knocked out together, the double mutant plants show a cumulative effect of decreased survival and rapid water loss under drought stress. Analysis of *hd2* mutants and HD2 overexpression lines confirm

the hypothesis that *HD2A* and *HD2C* positively regulate drought stress response and both HDACs might work together to play a role in this regard.

Figure 11: Drought survival of HD2 overexpression lines

(A) Images of WT and HD2-OE plants under control conditions and 2 days after rewatering following 13 days of drought stress.
(B) Survival rates of WT, *HD2A* and *HD2C* overexpression lines after drought stress. Data shown are means ± standard errors (n = 3). In each independent experiment, 30 plants of each genotype were used to calculate the survival. The significance of the differences between different genotypes was determined by one-way ANOVA followed by post-hoc Tukey's HSD tests. Lowercase letters indicate significant differences ($p < 0.01$).

A


WT
OEA1
OEC1
Control
2 days after drought

B

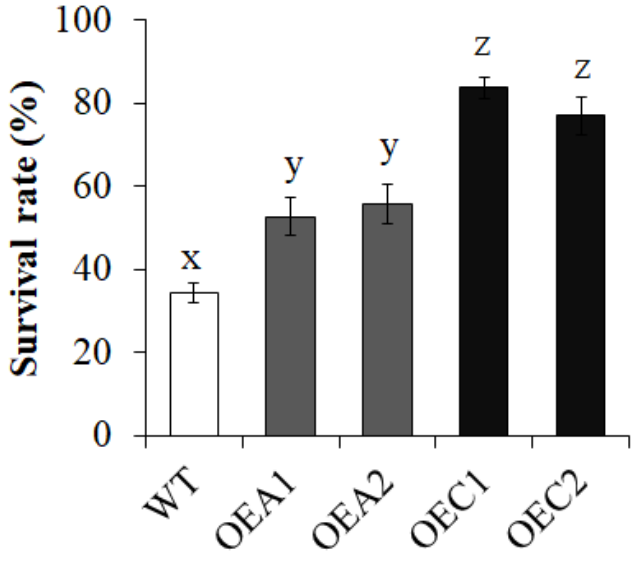
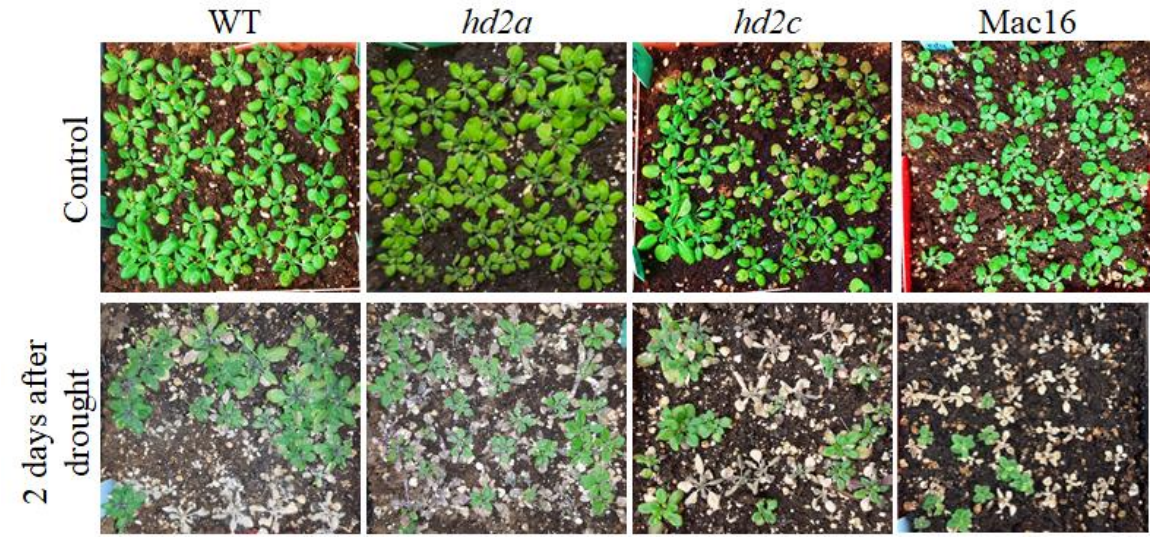

Survival rate (%)
100
80
60
40
20
0
x
y
y
z
z
WT
OEA1
OEA2
OEC1
OEC2

Figure 12: Drought survival of *hd2* mutant lines

(A) Images of WT, the single mutants *hd2a* and *hd2c*, and the double mutant Mac16 under control conditions and 2 days after rewatering following 11 days of drought stress.
(B) Survival rates of *hd2a*, *hd2c*, and Mac16 relative to WT plants after drought stress. Data shown are means ± standard errors (n = 3). In each independent experiment, 30 plants of each genotype were used to calculate the survival. The significance of the differences between different genotypes was determined by one-way ANOVA followed by post-hoc Tukey's HSD tests. Lowercase letters indicate significant differences ($p < 0.01$).

A


WT
hd2a
hd2c
Mac16
Control
2 days after drought

B

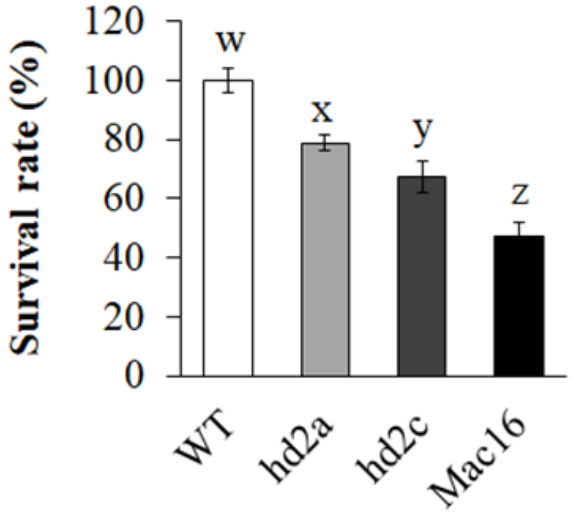
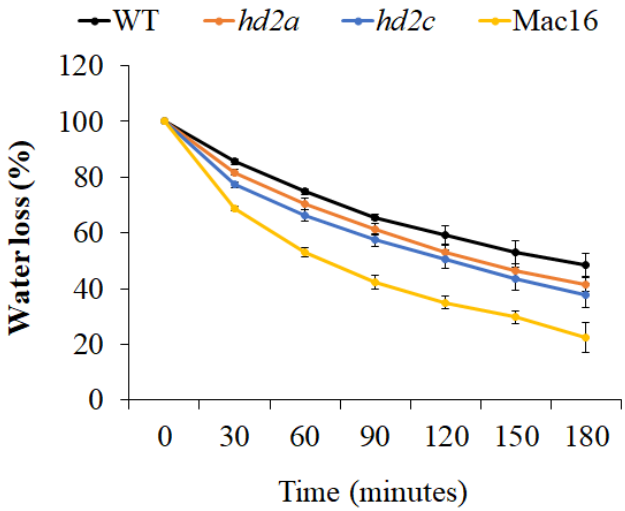

Survival rate (%)
120
100
80
60
40
20
0
w
x
y
z
WT
hd2a
hd2c
Mac16

Figure 13: Fresh leaf water loss in *hd2* mutants and HD2 overexpression lines

(A) Fresh leaf water loss measured as the percentage weight loss from leaf samples of WT, *hd2a*, *hd2c*, and Mac16 after every 30 minutes for a total of 180 minutes. Data shown are mean percentage water loss at each time point ± standard errors (n = 9). Three independent experiments were performed. In each experiment, measurements were taken from three plants (total three leaves per plant collected and weighed). The significance of the differences between different genotypes at each time point was determined by one-way ANOVA followed by post-hoc Tukey's HSD tests (*$p < 0.05$, **$p < 0.01$).

(B) Fresh leaf water loss measured as the percentage weight loss from leaf samples of (left) WT, OEA1, and OEA2 and (right) WT, OEC1, and OEC2 after every 30 minutes for a total of 180 minutes. Data shown are mean percentage water loss at each time point ± standard errors (n = 9). Three independent experiments were performed. In each experiment, measurements were taken from three plants (total three leaves per plant collected and weighed). The significance of the differences between different genotypes at each time was determined by Student's t test (*$p < 0.05$, **$p < 0.01$).

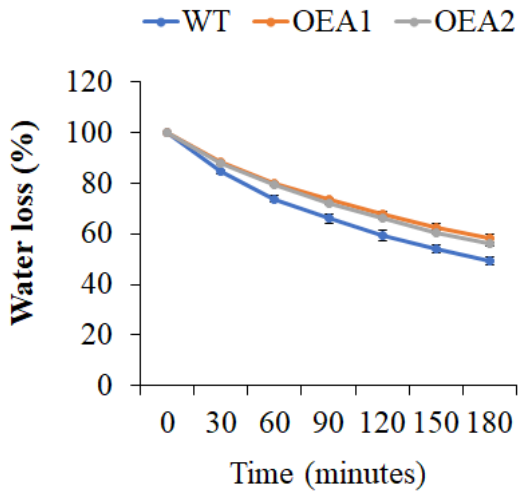
A

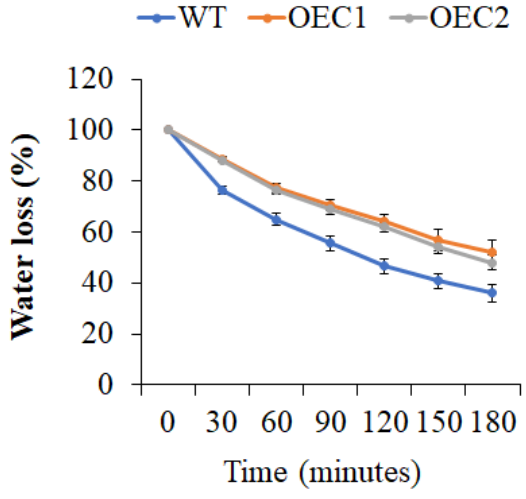


Time	30	60	90	120	150	180
hd2a	*	NS	*	NS	NS	*
hd2c	**	**	**	*	*	**
Mac16	**	**	**	**	**	**

Compared to WT; NS: Not Significant; *p < 0.05, **p < 0.01

B





Time	30	60	90	120	150	180
OEA1	NS	*	*	*	**	**
OEA2	NS	*	*	*	**	**

Compared to WT; NS: Not Significant; *p < 0.05, **p < 0.01

Time	30	60	90	120	150	180
OEC1	**	**	**	**	**	**
OEC2	**	**	**	**	**	**

Compared to WT; NS: Not Significant; *p < 0.05, **p < 0.01

3.6 HD2A and HD2C coregulate stomatal closure under stress

As described in Section 1.2.1 of Chapter 1, stomata are closed in response to drought stress to limit water loss by transpiration. So, I questioned if decreased survival and increased water loss in *hd2* mutants is related to functioning of stomata. I studied the stomatal closure in HD2-OE lines and *hd2* mutants to link the drought stress tolerance with stomatal functioning. First, I measured the stomatal pore widths and lengths in both HD2-OE lines and *hd2* mutants following the protocol described in Section 2.12. Fresh leaves obtained from plants were kept in MES/KOH solution for equilibration and then were treated with ABA to mimic a stress environment to initiate stress-related signalling which causes the stomata to close. After two hours of ABA treatment, imaging was performed to visualize the stomata.

The *HD2A* overexpression lines did not show any change in the stomatal opening under control conditions, whereas the overexpression of *HD2C* caused the stomata to be more open as compared to WT under control conditions. However, under ABA-induced stress, both HD2-OE showed complete closing of stomata as compared to WT (**Figure 14-15**). Among the *hd2* mutants, *hd2a* did not show any change in stomatal aperture under control conditions, whereas the *hd2c* and Mac16 mutants showed reduced stomatal aperture with respect to the WT. However, under ABA-induced stress, the *hd2c* mutant showed larger stomatal aperture compared to the *hd2a* mutant and WT. Knockout of both genes in the Mac16 resulted in a significantly larger stomatal aperture as compared to both single mutants and WT (**Figure 16**). This indicates that the *HD2A* and *HD2C* might coregulate stomatal closure regulation pathway, thus playing a role in drought stress response.

3.6.1 HD2A and HD2C are involved in downregulation of stomatal closure-related gene expression

Several studies have reported the ABA-mediated regulation of *SLAC1* gene expression via *SnRK2/OST1* phosphorylation, leading to stomatal closure. *ABI1* and *ABI2* are considered negative regulators of ABA-mediated stomatal closure and inhibit the expression of the *SLAC1* by dephosphorylating and subsequently down-regulating *SnRK2*/*OST1* (Vahisalu et al., 2008; Roelfsema et al., 2012; Munemasa et al., 2015; Zhang et al., 2016). I thus

analysed the expression of the *ABI1*, *ABI2*, and *SLAC1* genes in *hd2* mutants after imposing drought stress for 5 days (**Figure 17**). Analysis of *ABI1* and *ABI2* gene expression showed that the expression of both *ABI1* and *ABI2* did not change in the *hd2a* and upregulated in the *hd2c* mutant. However, the knockout of both *HD2A* and *HD2C* resulted in significant upregulation of both *ABI1* and *ABI2* expression in Mac16, as compared to the single mutants and WT. Gene expression analysis of *SLAC1* showed that its expression was not affected in *hd2a* and decreased in *hd2c* mutant. The knockout of both *HD2A* and *HD2C* resulted in a drastic decrease of *SLAC1* gene expression in the Mac16 line. The upregulation of *ABI1* and *ABI2* and downregulation of *SLAC1* led to the significant change in stomatal opening and closing pattern in the Mac16 double mutant line under stress.

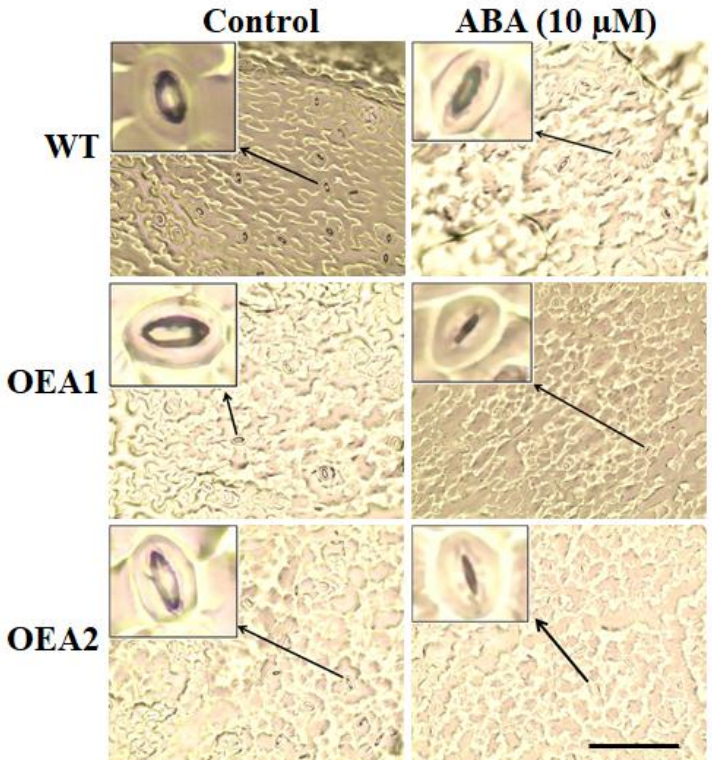
Taken together, It is likely that *HD2A* or *HD2C* does not play an independent role in regulating stomatal opening and closure. Knockout of both genes led to a stronger phenotype in terms of drought tolerance and stomatal closure as compared to the single mutants, suggests an additive effect of both genes in drought stress response.

Figure 14: Stomatal closure under stress in *HD2A* overexpression lines

(A) Images of stomatal pore opening and closure in WT and HD2A-OE lines OEA1 and OEA2 under control conditions and ABA-induced stress. Image J software was used for stomatal apertures analysis (scale bar: 50 µm).

(B) Stomatal aperture shown as width/length of the stomatal pore in HD2A-OE lines OEA1 and OEA2, compared to WT under control conditions and ABA-induced stress. Data shown are means ± standard errors from at least 150 stomata. Three independent experiments were performed in this test. In each experiment, at least 50 stomatal measurements were taken from three plants of each genotype (two leaves per plant). The significance of the differences between different genotypes was determined by Student's t test (**$p < 0.01$).

A


Control
ABA (10 μM)
WT
OEA1
OEA2

B

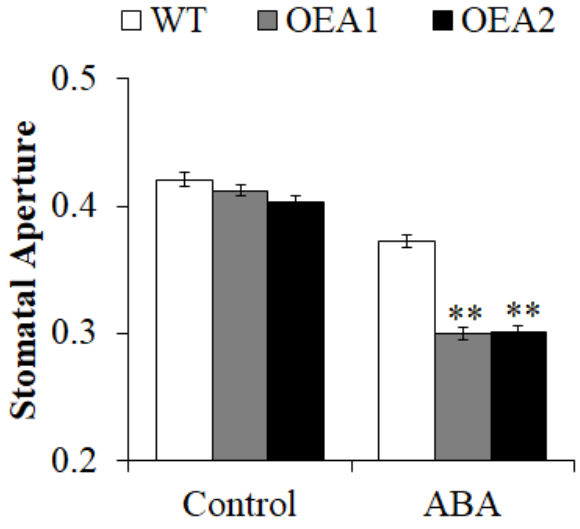
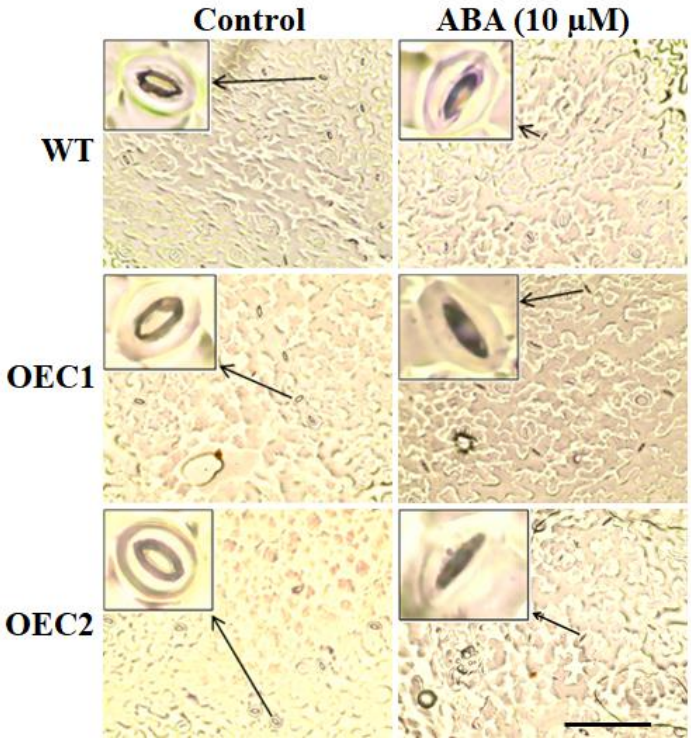

WT
OEA1
OEA2
Stomatal Aperture
0.5
0.4
0.3
0.2
**
**
Control
ABA

Figure 15: Stomatal closure under stress in *HD2C* overexpression lines

(A) Images of stomatal pore opening and closure in WT and HD2C-OE lines OEC1 and OEC2 under control conditions and ABA-induced stress. Image J software was used for stomatal apertures analysis (scale bar: 50 µm).

(B) Stomatal aperture shown as width/length of the stomatal pore in HD2C-OE lines OEC1 and OEC2, compared to WT under control conditions and ABA-induced stress. Data shown are means ± standard errors from at least 150 stomata. Three independent experiments were performed in this test. In each experiment, at least 50 stomatal measurements were taken from three plants of each genotype (two leaves per plant). The significance of the differences between different genotypes was determined by Student's t test (**$p < 0.01$).

A


Control
ABA (10 µM)
WT
OEC1
OEC2

B

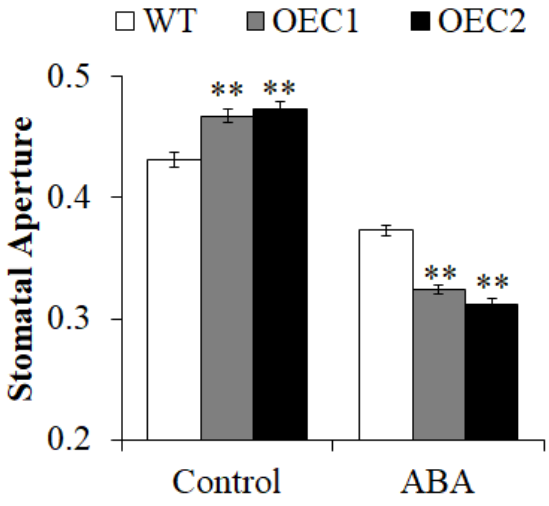
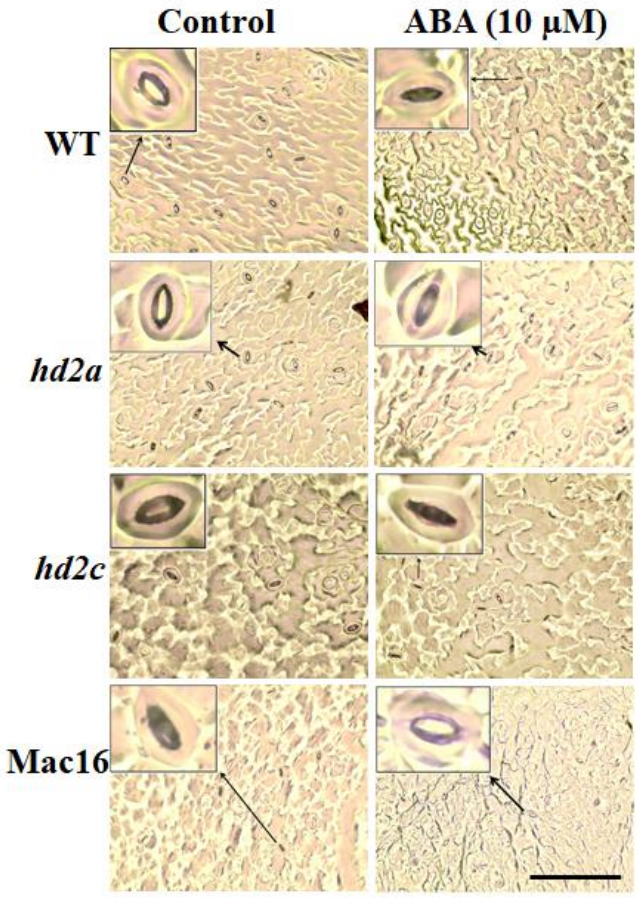

WT
OEC1
OEC2
Stomatal Aperture
0.5
0.4
0.3
0.2
**
**
**
**
Control
ABA

Figure 16: Stomatal closure under stress in *hd2* mutant lines

(A) Images of stomatal pore opening and closure studied in WT, *hd2a*, *hd2c*, and Mac16 under control conditions and ABA-induced stress. Image J software was used for stomatal apertures analysis, (scale bar: 50 µm).

(B) Stomatal aperture shown as width/length of the stomatal pore in *hd2a*, *hd2c*, and Mac16, compared to WT under control conditions and ABA-induced stress. Data shown are means ± standard errors from at least 150 stomata. Three independent experiments were performed in this test. In each experiment, stomatal measurements were taken from three plants (two leaves per plant). The significance of the differences between different genotypes was determined by one-way ANOVA followed by post-hoc Tukey's HSD tests. Lowercase letters indicate significant differences ($p < 0.01$).

A


Control
ABA (10 µM)
WT
hd2a
hd2c
Mac16

B

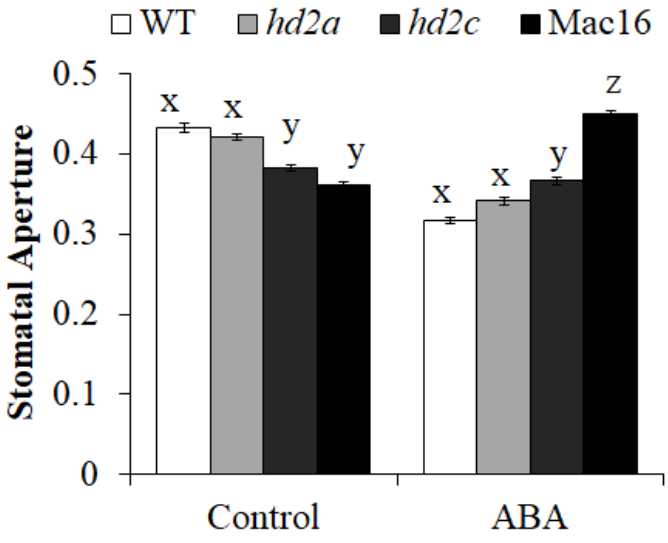
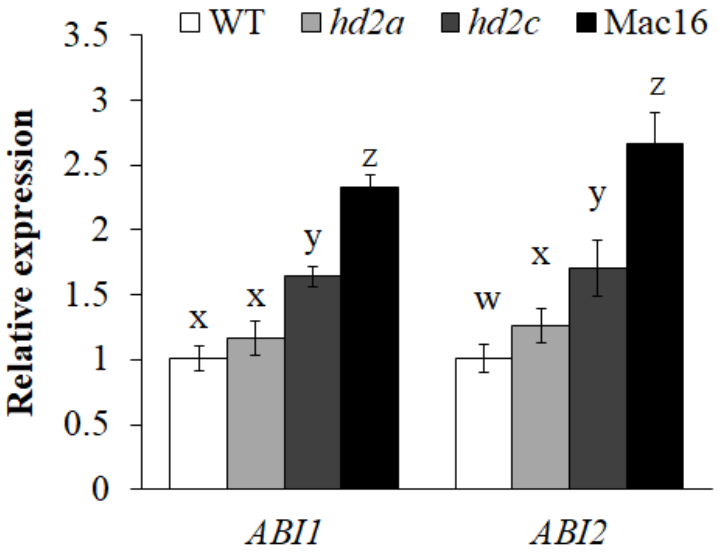

WT
hd2a
hd2c
Mac16
Stomatal Aperture
0.5
0.4
0.3
0.2
0.1
0
x
x
y
y
x
x
y
z
Control
ABA

Figure 17: Expression analysis of stomatal closure-related genes in *hd2* mutant lines

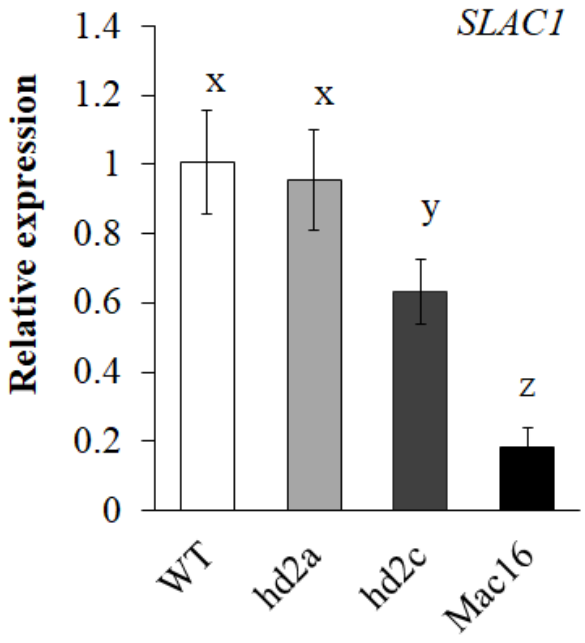
(A) Relative expression of *ABI1* and *ABI2* in WT, *hd2a*, *hd2c*, and Mac16 under drought conditions. Data shown are means ± standard errors (n = 3). Three independent experiments were performed in this test. For each independent experiment, leaf samples collected from three plants were pooled together for RNA extraction. The significance of the differences between different genotypes was determined by one-way ANOVA followed by post-hoc Tukey's HSD tests. Lowercase letters indicate significant differences ($p < 0.01$).

(B) Relative expression of the *SLAC1* in WT, *hd2a*, *hd2c*, and Mac16 under drought conditions. Data shown are means ± standard errors (n = 3). Three independent experiments were performed in this test. For each independent experiment, leaf samples collected from three plants were pooled together for RNA extraction. The significance of the differences between different genotypes was determined by one-way ANOVA followed by post-hoc Tukey's HSD tests. Lowercase letters indicate significant differences ($p < 0.01$).

A


WT
hd2a
hd2c
Mac16
Relative expression
3.5
3
2.5
2
1.5
1
0.5
0
x
x
y
z
w
x
y
z
ABI1
ABI2

B


SLAC1
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
x
x
y
z
WT
hd2a
hd2c
Mac16

3.7 HD2A and HD2C play a role in regulating root growth in Arabidopsis

Roots, which are generally classified as primary and secondary roots, play a vital role in maintaining stable water absorption from the soil and its supply to the aerial parts of the plants. The primary root is the basic but vital part of the plant root system and plays an important role in early seedling growth and its survival under stress. My next objective was to examine whether *HD2A* and *HD2C* play a role in regulating root growth under drought stress. First, I measured the primary root lengths and secondary root numbers in 10-day old seedlings of *hd2* mutants and HD2-OE lines under control and osmotic stress conditions mimicked by using PEG-6000 as described in Section 2.14.

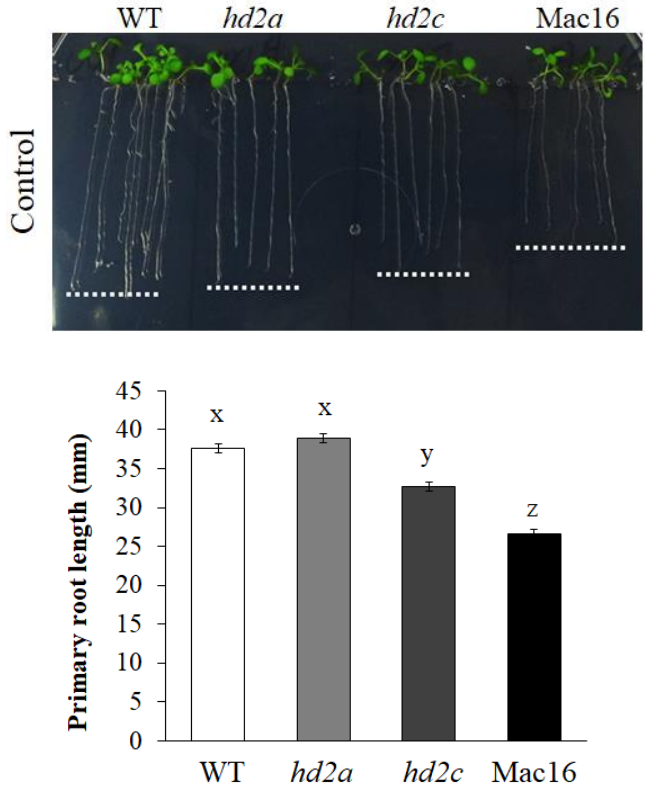
Primary root length was significantly decreased in the double mutant line Mac16 as compared to the *hd2c* mutant under control conditions (**Figure 18**), whereas root length did not change in the *hd2a* mutant line as compared to WT. Application of osmotic stress (PEG) caused a significant decrease in the primary root lengths of both *hd2* single mutants, *hd2a* and *hd2c*, and further decreased in the double mutant line Mac16. For secondary roots, no difference was observed in the *hd2c* mutant in comparison to WT, whereas the *hd2a* mutant line showed fewer secondary roots under control conditions. However, knockout of both HD2 genes resulted in a significant decrease in the number of secondary roots in the Mac16 (**Figure 19**). On the other hand, overexpression of both *HD2A* and *HD2C* resulted in a significant increase in the primary root lengths of HD2 overexpression lines under control and PEG stress conditions (**Figure 20-21**). These overexpression lines also displayed a higher number of secondary roots compared to WT seedlings (**Figure 22**).

Figure 18: Primary root length in *hd2* mutant lines

(A) Top: Root growth shown in 10-day old WT, *hd2a*, *hd2c,* and Mac16 seedlings under control conditions. Bottom: Primary root lengths measured in 10-day old WT, *hd2a*, *hd2c*, and Mac16 seedlings grown under control conditions. Data shown are means ± standard errors (n = 60). Three independent experiments were performed in this test. In each experiment, 20 seedlings of each genotype were used to measure the primary root lengths. The significance of the differences between different genotypes was determined by one-way ANOVA followed by post-hoc Tukey's HSD tests. Lowercase letters indicate significant differences ($p < 0.01$).

(B) Top: Root growth shown in 10-day old WT, *hd2a*, *hd2c,* and Mac16 seedlings under PEG-6000 (10%) treatment. Bottom: Primary root lengths measured in 10-day old WT, *hd2a*, *hd2c*, and Mac16 seedlings grown under PEG-6000 treatment. Data shown are means ± standard errors (n = 60). Three independent experiments were performed in this test. In each experiment, 20 seedlings of each genotype were used to measure the primary root lengths. The significance of the differences between different genotypes was determined by one-way ANOVA followed by post-hoc Tukey's HSD tests. Lowercase letters indicate significant differences ($p < 0.01$).

A


WT
hd2a
hd2c
Mac16
Control
Primary root length (mm)
45
40
35
30
25
20
15
10
5
0
x
x
y
z
WT
hd2a
hd2c
Mac16

B

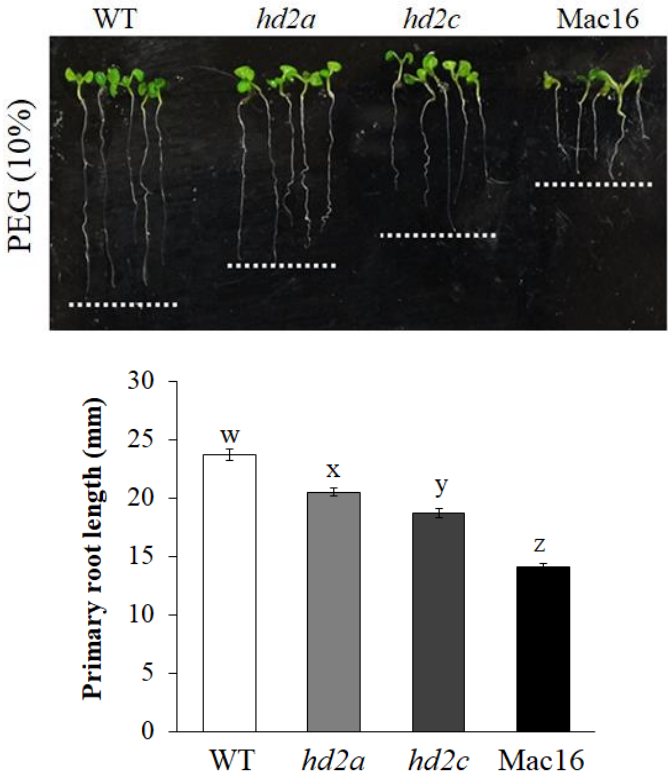

WT
hd2a
hd2c
Mac16
PEG (10%)
Primary root length (mm)
30
25
20
15
10
5
0
w
x
y
z
WT
hd2a
hd2c
Mac16

Figure 19: Secondary root growth in *hd2* mutant lines

Left: Secondary root growth shown in 10-day old WT, *hd2a*, *hd2c*, and Mac16 seedlings under control conditions. Right: Secondary root number measured in 10-day old WT, *hd2a*, *hd2c*, and Mac16 seedlings grown under control conditions. Data shown are means ± standard errors (n = 60). Three independent experiments were performed in this test. In each experiment, 20 seedlings of each genotype were used to count the number of secondary roots. The significance of the differences between different genotypes was determined by one-way ANOVA followed by post-hoc Tukey's HSD tests. Lowercase letters indicate significant differences ($p < 0.01$).

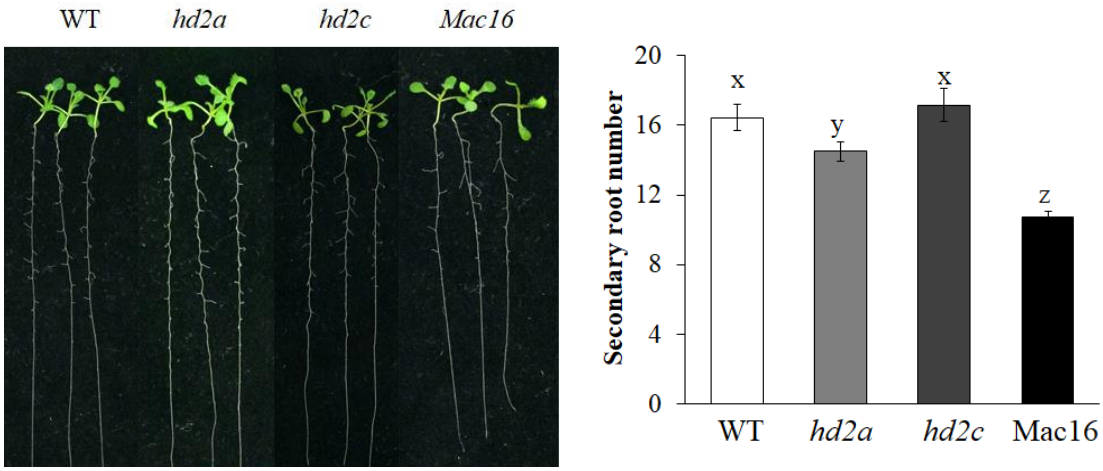

WT
hd2a
hd2c
Mac16
Secondary root number
20
16
12
8
4
0
x
y
x
z
WT
hd2a
hd2c
Mac16

Figure 20: Primary root length in *HD2A* overexpression lines

(A) Root growth shown in 10-day old WT and HD2A-OE line OEA1 seedlings under control conditions and PEG-6000 (10%) treatment.

(B) Primary root lengths measured in 10-day old WT, OEA1, and OEA2 seedlings under control conditions and PEG-6000 treatment. Data shown are means ± standard errors (n = 60). Three independent experiments were performed in this test. In each experiment, 20 seedlings of each genotype were used to measure the primary root lengths. The significance of the differences between different genotypes was determined by Student's t test (**p < 0.01).

A


Control
WT
OEA1
PEG 10%
WT
OEA1

B

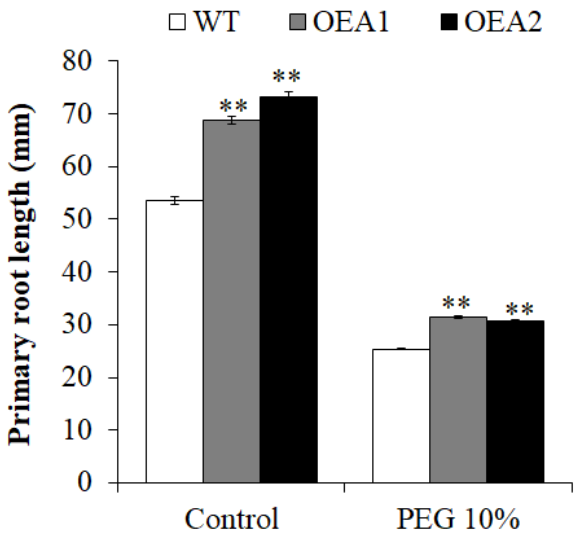
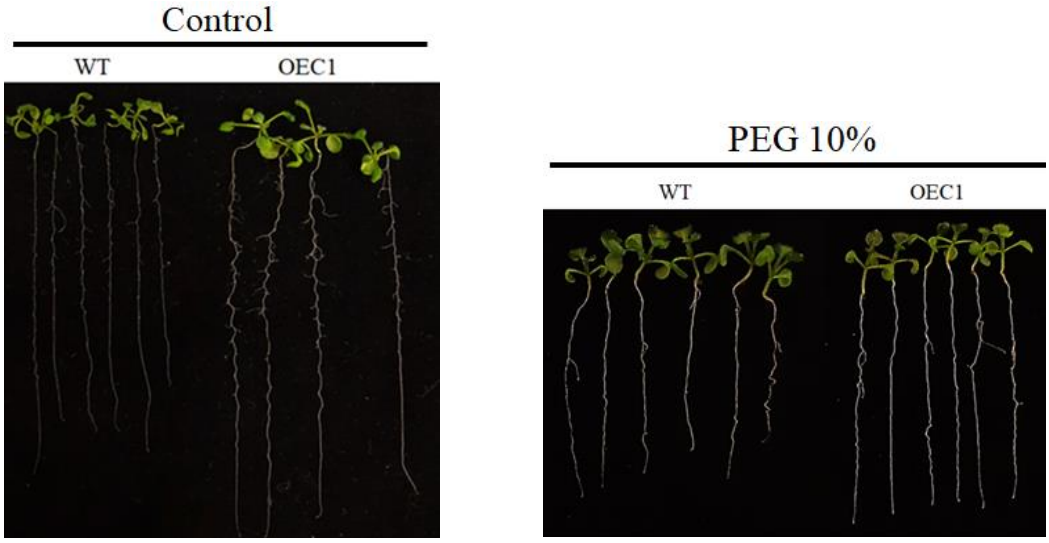

WT
OEA1
OEA2
Primary root length (mm)
80
70
60
50
40
30
20
10
0
**
**
**
**
Control
PEG 10%

Figure 21: Primary root length in *HD2C* overexpression lines

(A) Root growth shown in 10-day old WT and HD2C-OE line OEC1 seedlings under control conditions and PEG-6000 (10%) treatment.

(B) Primary root lengths measured in 10-day old WT, OEC1, and OEC2 seedlings under control conditions and PEG-6000 treatment. Data shown are means ± standard errors (n = 60). Three independent experiments were performed in this test. In each experiment, 20 seedlings of each genotype were used to measure the primary root lengths. The significance of the differences between different genotypes was determined by Student's t test (**p < 0.01).

A


Control
WT
OEC1
PEG 10%
WT
OEC1

B

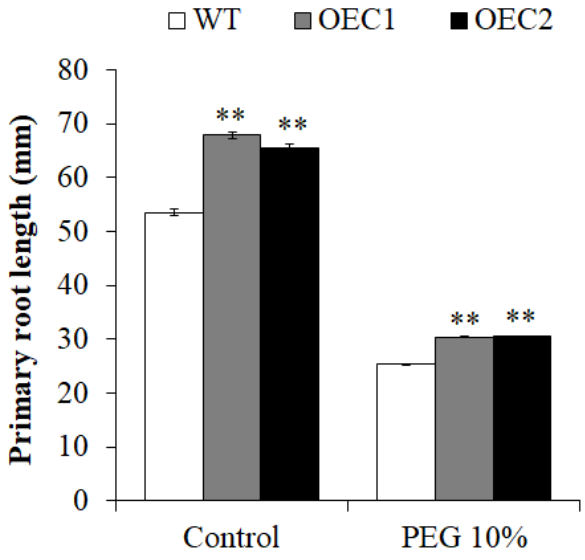
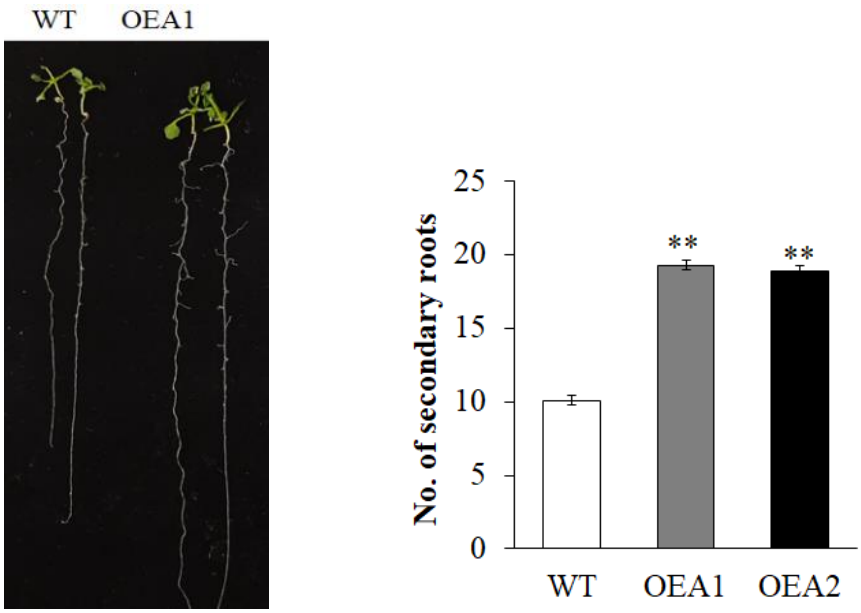

WT
OEC1
OEC2
Primary root length (mm)
80
70
60
50
40
30
20
10
0
**
**
**
**
Control
PEG 10%

Figure 22: Secondary root growth in *HD2A and HD2C* overexpression lines

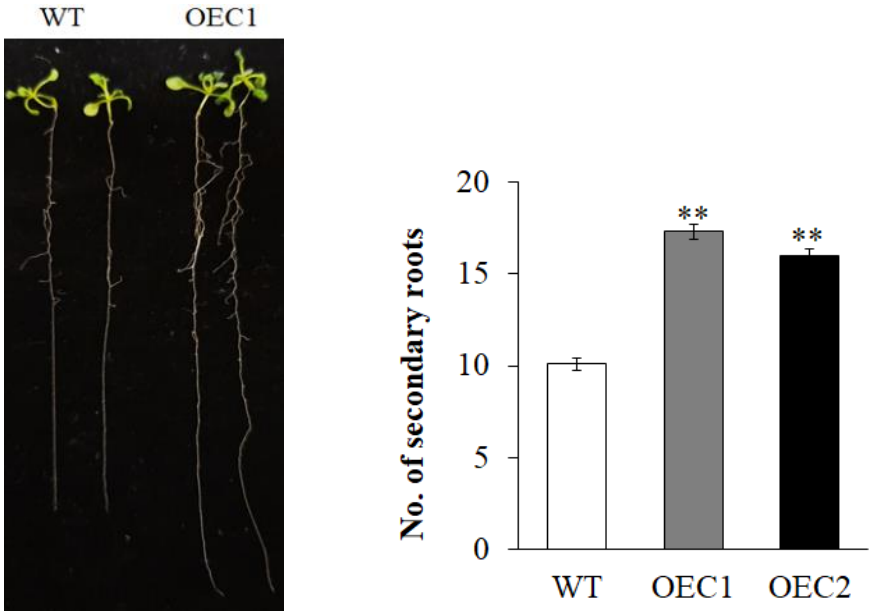
(A) Left: Secondary root growth shown in 10-day old WT and HD2A-OE line OEA1 seedlings under control conditions. Right: Secondary root number measured in 10-day old WT, OEA1, and OEA2 seedlings under control conditions. Data shown are means ± standard errors (n = 60). Three independent experiments were performed in this test. In each experiment, 20 seedlings of each genotype were used to count the number of secondary roots. The significance of the differences between different genotypes was determined by Student's t test (**p < 0.01).

(B) Left: Secondary root growth shown in 10-day old WT and HD2C-OE line OEC1 seedlings under control conditions. Right: Secondary root number measured in 10-day old WT, OEC1, and OEC2 seedlings under control conditions. Data shown are means ± standard errors (n = 60). Three independent experiments were performed in this test. In each experiment, 20 seedlings of each genotype were used to count the number of secondary roots. The significance of the differences between different genotypes was determined by Student's t test (**p < 0.01).

A


WT
OEA1
No. of secondary roots
25
20
15
10
5
0
**
**
WT
OEA1
OEA2

B


WT
OEC1
No. of secondary roots
20
15
10
5
0
**
**
WT
OEC1
OEC2

3.8 HD2A and HD2C coregulate root growth via GA2ox-mediated GA catabolism

Gibberellic acids (GAs) are known to induce germination and root growth at low concentrations and strongly promote shoot growth. It is reported that GAs play a role in the elongation of endodermal cells to promote root growth (Ubeda-Tomás et al., 2009). Maintaining a dynamic homeostasis of bioactive GAs for normal root growth is an important phenomenon, carried out by GA2ox enzymes that play a significant role in limiting the levels of bioactive GAs. Transcriptional activation of GA2ox genes is associated with histone acetylation status, activated by ABA-mediated signalling in response to developmental signals and external environmental conditions including salt, drought and cold (Rieu et al., 2008; Colebrook et al., 2014; Chen et al., 2019). HD2A and HD2B have been shown to repress the *GA2ox2* gene by modifying the histone acetylation status at the *GA2ox2* gene locus (Li et al., 2017). I thus questioned if *HD2A* and *HD2C* are also involved in regulating the expression of the GA2ox family genes. I analysed the expression of GA2ox genes in the *hd2* mutants and HD2-OE lines under control conditions (**Figure 23**). Expression of *GA2ox1* and *GA2ox2* was upregulated many fold in the double mutant line Mac16 as compared to the single mutants *hd2a* and *hd2c*, and WT. However, the expression of these genes was not affected in the *hd2a* as compared to WT. Conversely, overexpression of *HD2A* and *HD2C* resulted in the downregulation of both *GA2ox1* and *GA2ox2* genes. qPCR analysis of other GA2ox genes, *GA2ox3*, *GA2ox4*, *GA2ox6* did not show changes in their expression pattern.

As GA1 and GA4 serve as the substrates for GA2ox-mediated GA catabolism, and GA3 is not considered as its substrate (Yamauchi et al., 2007; Rieu et al., 2008; Li et al., 2017), I examined the primary root lengths in *hd2* mutants under GA4 and GA3 treatments (**Figure 24**). Although, the primary root growth of all the genotypes was increased significantly with the application of 1 µM of GA4 as compared to control (untreated) conditions (**Figure 18A),** the *hd2* mutant lines did not show any significant differences in root growth as compared to WT under 1 µM of GA4 treatment. However, the double mutant line showed a slight increase in the primary root growth as compared to WT under 10 µM of GA4. Application of GA3 treatments at a lower concentration (1 µM) in *hd2* mutants led to a significant increase in root lengths in the *hd2* double mutant Mac16 as

compared to the *hd2* single mutants, *hd2a* and *hd2c*, and WT. Increase in the GA3 concentration (10 µM) caused a further increase in the primary root lengths of *hd2* single and double mutants as compared to WT seedlings.

Taken together, these data suggests that *HD2A* and *HD2C* play a role in regulating the expression of GA2ox genes. It is likely that the upregulation of *GA2ox1* and *GA2ox2* in the *hd2* double mutant enhanced the degradation of the bioactive GA4 gibberellins, which led to the decreased root growth in the *hd2* mutants. GA3 is not considered as a substrate of GA2ox enzymes, thus it restored the root growth in the mutant lines.

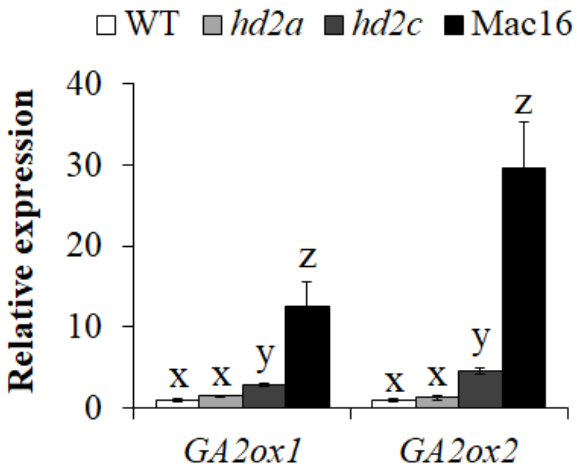
Figure 23: Expression analysis of *GA2ox1* and *GA2ox2* in *hd2* mutants and HD2-OE lines

(A) Relative expression of *GA2ox1* and *GA2ox2* in 10-day old WT, *hd2a*, *hd2c*, and Mac16 seedlings under control conditions. Data shown are means ± standard errors (n = 3). Three independent experiments were performed in this test. For each experiment, 5 seedlings were pooled together for RNA extraction for each genotype. The significance of the differences between different genotypes was determined by one-way ANOVA followed by post-hoc Tukey's HSD tests. Lowercase letters indicate significant differences ($p < 0.01$).

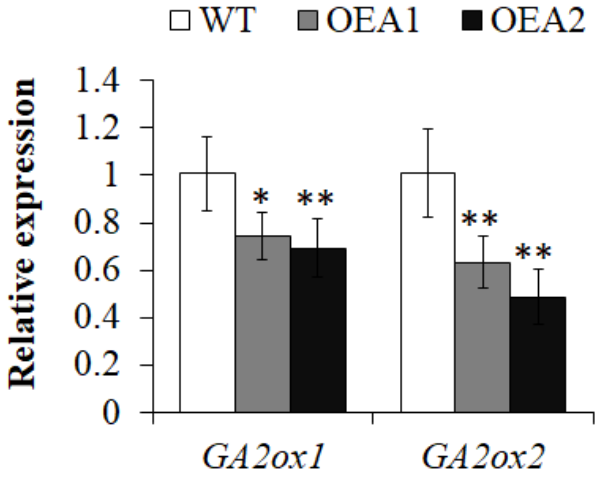
(B) Relative expression of *GA2ox1* and *GA2ox2* in 10-day old WT and HD2A-OE (OEA1 and OEA2) seedlings under control conditions. Data shown are means ± standard errors (n = 3). Three independent experiments were performed in this test. For each experiment, 5 seedlings were pooled together for RNA extraction for each genotype. The significance of the differences between different genotypes was determined by Student's t test (*$p < 0.05$, **$p < 0.01$).

(C) Relative expression of *GA2ox1* and *GA2ox2* in 10-day old WT and HD2C-OE (OEC1 and OEC2) seedlings under control conditions. Data shown are means ± standard errors (n = 3). Three independent experiments were performed in this test. For each experiment, 5 seedlings were pooled together for RNA extraction for each genotype. The significance of the differences between different genotypes was determined by Student's t test (*$p < 0.05$, **$p < 0.01$).

A


WT
hd2a
hd2c
Mac16
Relative expression
40
30
20
10
0
x
x
y
z
x
x
y
z
GA2ox1
GA2ox2

B


WT
OEA1
OEA2
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
**
**
GA2ox1
GA2ox2

C

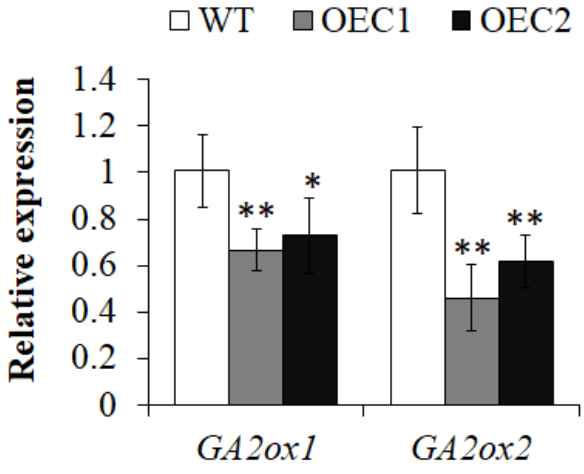

WT
OEC1
OEC2
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
*
**
**
GA2ox1
GA2ox2

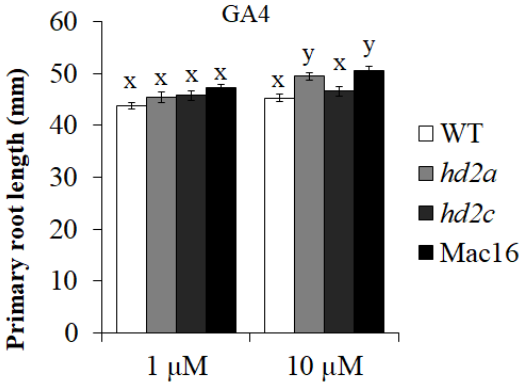
Figure 24: Primary root length in *hd2* mutant lines under GAs treatments

(A) GA4 treatment - Left: Root growth shown in 10-day old WT, *hd2a*, *hd2c* and Mac16 seedlings under GA4 treatment (10 µM). Right: Primary root lengths measured in 10-day old WT, *hd2a*, *hd2c* and Mac16 seedlings under GA4 treatments (1 µM and 10 µM). Data shown are means ± standard errors (n = 60). Three independent experiments were performed in this test. In each experiment, 20 seedlings of each genotype were used to measure the primary root lengths. The significance of the differences between different genotypes was determined by two-way ANOVA followed by post-hoc Tukey's HSD tests. Lowercase letters indicate significant differences ($p < 0.05$).

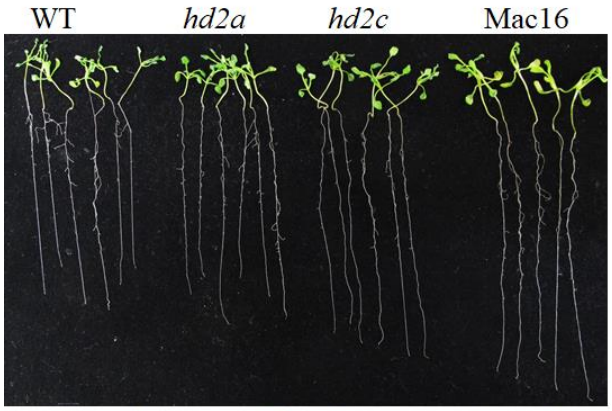
(B) GA3 treatment - Left: Root growth shown in 10-day old WT, *hd2a*, *hd2c* and Mac16 seedlings under GA3 treatment (10 µM). Right: Primary root lengths measured in 10-day old WT, *hd2a*, *hd2c* and Mac16 seedlings under GA3 treatments (1 µM and 10 µM). Data shown are means ± standard errors (n = 60). Three independent experiments were performed in this test. In each experiment, 20 seedlings of each genotype were used to measure the primary root lengths. The significance of the differences between different genotypes was determined by two-way ANOVA followed by post-hoc Tukey's HSD tests. Lowercase letters indicate significant differences ($p < 0.05$).

A

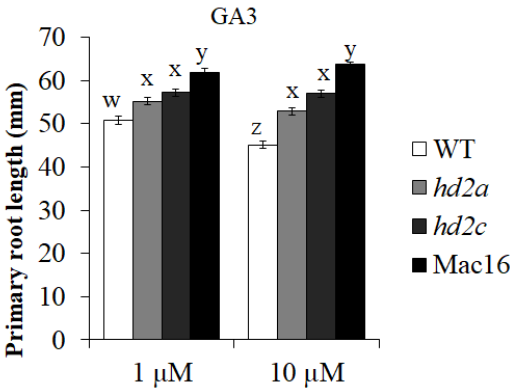
Root phenotype under GA4 treatment (10 µM)



B



Root phenotype under GA3 treatment (10 µM)



3.9 HD2A and HD2C decrease H3K9 acetylation levels

HD2C has been shown to decrease H3K9 acetylation levels at the *FT* gene locus in Arabidopsis (Guo et al., 2020). Increased H3K9/K14 acetylation was observed at the locus of *ABI1* and *ABI2* genes in the single mutants *hd2c* and *hda6*, and the double mutant line *hd2c.hda6*, which resulted in increased expression of *ABI1* and *ABI2* genes (Luo et al., 2012). Similarly, knockout of both the *HD2A* and *HD2B* together in the *hd2a.hd2b* double mutant resulted in the hyperacetylation of histone H3 at the *GA2ox2* gene locus, which resulted in shorter root length in Arabidopsis (Li et al., 2017). I questioned if knockout of *HD2A* and *HD2C* affects the global H3K9 acetylation levels. By performing western blot, I measured the H3K9ac levels in *hd2* mutants and HD2-OE lines and compared it with WT (**Figure 25**). I measured the signal intensities of H3K9ac and H3 in the blots and relative intensities were calculated as H3K9ac/H3. The fold change of H3K9ac/H3 levels was found higher in the double mutant line Mac16 (1.41) as compared to the single mutants, *hd2a* (1.14) and *hd2c* (1.18) and WT (normalized to 1). In HD2-OE lines, the HD2A-OE lines, OEA1 and OEA2 showed 0.84- and 0.80-fold change in the relative intensities of H3K9ac/H3, respectively (**Figure 25B**), whereas the HD2C-OE lines, OEC1 and OEC2 showed 0.73- and 0.82-fold change in the relative intensities of H3K9ac/H3, respectively (**Figure 25C**).

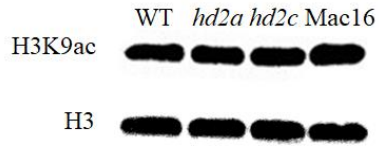
Figure 25: *HD2A* and *HD2C* knockout leads to an increase in genome-wide H3K9 acetylation levels

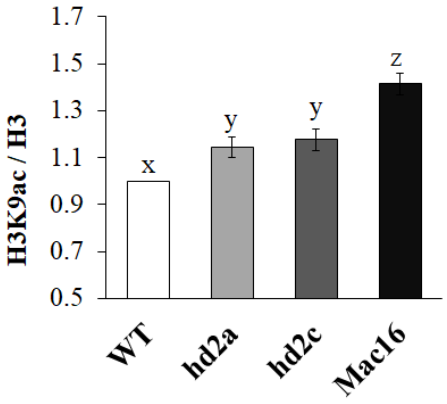
(A) Left: Images of western blot analysis of protein extracts from 10-day old seedlings of WT, *hd2a*, *hd2c*, and the double mutant line Mac16, using anti-H3K9ac and anti-H3 antibodies. Right: Relative intensities of H3K9ac/H3 in WT, *hd2a*, *hd2c*, and the double mutant line Mac16. The fold change in the relative intensities of H3K9ac/H3 in *hd2* mutants were normalized to WT. The signal intensities of blots were quantified using Image J software. Data shown are means ± standard errors (n = 3). For each independent experiment, about 8-10 seedlings (100 mg) of each genotype were pooled together for protein extraction. The significance of the differences between different genotypes was determined using one-way ANOVA followed by post-hoc Tukey's HSD tests. Lowercase letters indicate significant differences ($p < 0.01$).

(B) Left: Images of western blot analysis of protein extracts from 10-day old seedlings of WT and HD2A-OE lines OEA1 and OEA2 using anti-H3K9ac and anti-H3 antibodies. Right: Relative intensities of H3K9ac/H3 in WT and HD2A-OE lines OEA1 and OEA2. The fold change in the relative intensities of H3K9ac/H3 were normalized to WT. The signal intensities of blots were quantified using Image J software. Data shown are means ± standard errors (n = 3). For each independent experiment, about 8-10 seedlings (100 mg) of each genotype were pooled together for protein extraction. The significance of the differences between different genotypes were determined using Student's t tests (**$p < 0.01$).

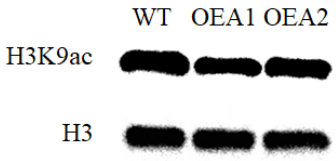
(C) Left: Images of western blot analysis of protein extracts from 10-day old seedlings of WT and HD2C-OE lines OEC1 and OEC2 using anti-H3K9ac and anti-H3 antibodies. Right: Relative intensities of H3K9ac/H3 in WT and HD2C-OE lines OEC1 and OEC2. The fold change in the relative intensities of H3K9ac/H3 were normalized to WT. The signal intensities of blots were quantified using Image J software. Data shown are means ± standard errors (n = 3). For each independent experiment, about 8-10 seedlings (100 mg) of each genotype were pooled together for protein extraction. The significance of the differences between different genotypes were determined using Student's t tests (**$p < 0.01$).

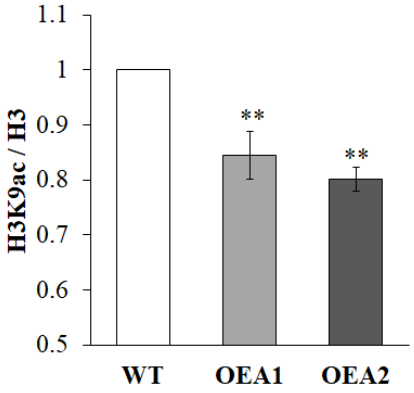
A


WT hd2a hd2c Mac16
H3K9ac
H3

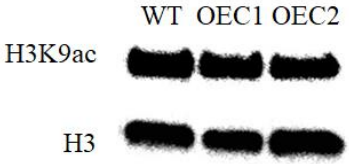

H3K9ac / H3
1.7
1.5
1.3
1.1
0.9
0.7
0.5
x
y
y
z
WT
hd2a
hd2c
Mac16

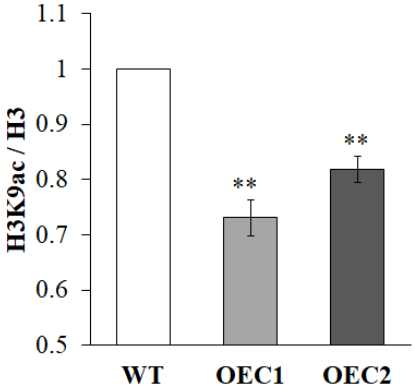
B


WT OEA1 OEA2
H3K9ac
H3


H3K9ac / H3
1.1
1
0.9
0.8
0.7
0.6
0.5
**
**
WT
OEA1
OEA2

C


WT OEC1 OEC2
H3K9ac
H3


H3K9ac / H3
1.1
1
0.9
0.8
0.7
0.6
0.5
**
**
WT
OEC1
OEC2

3.10 HD2A and HD2C physically interact with each other

The additive effect of HD2A and HD2C in drought stress response and root growth led me to investigate if HD2A and HD2C can physically interact with each other. To study this protein-protein interaction, I performed Y2H and BiFC assays as described in Sections 2.15 and 2.16. For the Y2H assay, HD2 gene products were transferred to destination vectors, pGBKT7-BK (bait vector), and the pGADT7-AD (prey vector) to generate plasmid constructs for the Y2H assay. The Y2HGold strain was co-transformed with bait and prey vectors containing *HD2A* and *HD2C* coding regions, respectively and vice versa. For negative controls, yeast cells were co-transformed with prey vector containing HD2A-AD or HD2C-BK and empty bait vector pGBKT7 (containing no gene of interest). The Y2HGold cells were platted on minimal selective medium DDO and sub-cultured to stringent selective medium QDO and QDO/X/A (**Figure 26A**). The yeast strains expressing HD2A-AD and HD2C-BK independently did not grow on selective medium QDO and QDO/X/A. However, when co-transformed with constructs expressing HD2A and HD2C, yeast cells developed colonies on QDO and QDO/X/A medium, indicating a physical interaction of HD2A and HD2C (see **Appendix 5** for all the protein interaction results).

BiFC assays were performed to further confirm the protein-protein interaction results obtained from Y2H in the plant host. For BiFC assays, HD2 genes were transferred to destination vectors, pEarleyGate201-YN and pEarleyGate201-YC to generate plasmid constructs for BiFC. Agrobacterium-mediated transformation was carried out by infiltrating *N. benthamiana* leaf epidermal cells with mixtures containing equal volumes of each of Agrobacterium cultures of pEarleyGate201-*HD2A-YN* and pEarleyGate202-*HD2C-YC* (or pEarleyGate201-*HD2C-YN* and pEarleyGate202-*HD2A-YC*) constructs for transient expression of genes of interests. For negative controls, the pEarleyGate201-YN constructs containing HD2A or HD2C were co-infiltrated with an empty pEarleyGate202-YC vector. After 48-72 hours, the YFP signal was observed with a confocal microscope at 514 nm wavelength (**Figure 26B**). The *HD2A-YN* or *HD2C-YN* construct did not show any YFP signal (yellow fluorescence) when fused with an empty pEarleyGate202-YC vector. The YFP signal in *N. benthamiana* treated leaves was observed when both constructs

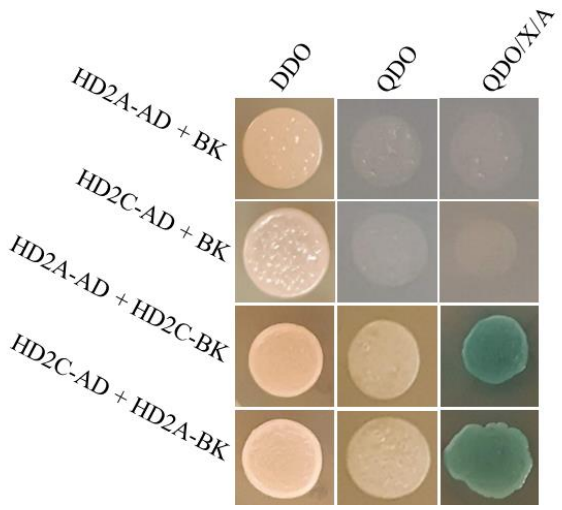
HD2A-YN and *HD2C-YC* (or *HD2A-YC* and *HD2C-YN*) were infiltrated together, again indicating a physical interaction of HD2A and HD2C (see **Appendix 5** for all the protein interaction results).

Figure 26: HD2A and HD2C physically interact with each other

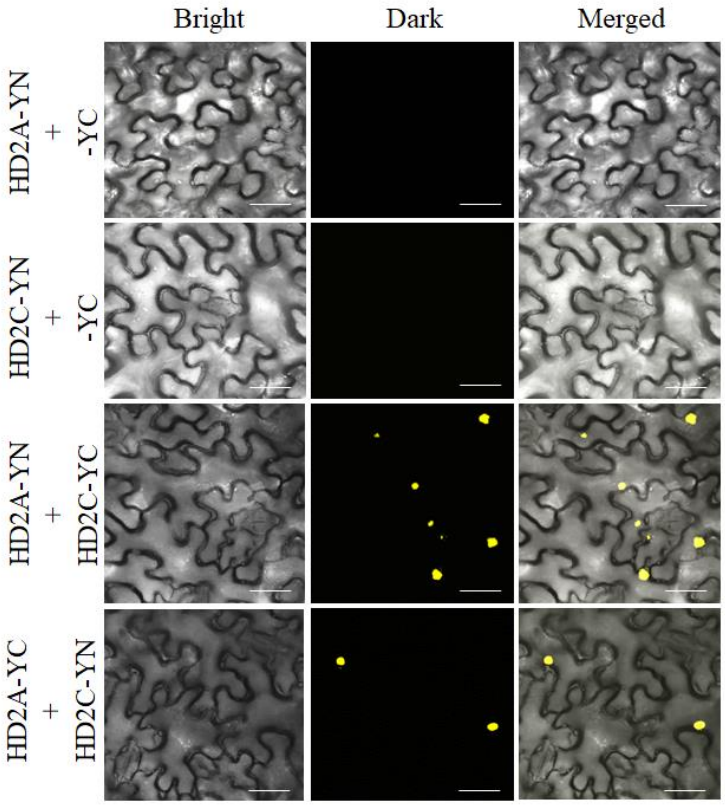
(A) Y2H assay showing interaction of HD2A and HD2C. Transformed yeast cells were plated on minimal selective medium DDO and sub-cultured to QDO and QDO/X/A medium to determine the ability to grow and identify protein interactions. Empty vector pGBKT7 was co-transformed with either HD2A-AD or HD2C-AD as a negative control. Three independent experiments were performed in this test.

(B) BiFC assay showing interaction of HD2A and HD2C. The constructs HD2A-YN and HD2C-YC were co-infiltrated into leaf epidermal cells of *N. benthamiana* via Agrobacterium-mediated transformation for transient expression of *HD2A* and *HD2C* and YFP signal was detected after 48-72 hours of infiltration. Empty vector pEarleyGate202-YC was co-transformed with either HD2A-YN or HD2C-YN as a negative control. Three independent experiments were performed in this test (scale bar 50 μm).

A


DDO
QDO
QDO/X/A
HD2A-AD + BK
HD2C-AD + BK
HD2A-AD + HD2C-BK
HD2C-AD + HD2A-BK

B


Bright
Dark
Merged
HD2A-YN + -YC
HD2C-YN + -YC
HD2A-YN + HD2C-YC
HD2A-YC + HD2C-YN

Chapter 4: Discussion

Previous reports have demonstrated the involvement of many HDACs such as *HDA6*, *HDA9*, and *HDA19* from the RPD3 family, as well as *HD2C* and *HD2D* from the HD2 family in abiotic stress response in Arabidopsis (Chen and Wu, 2010; Luo et al., 2012; Zheng et al., 2016; Farhi et al., 2017). HDACs have previously been shown to regulate the expression of many stress-related genes involved in transcriptional regulatory networks (Kim et al., 2015; Tahir and Tian, 2021). HDACs generally do not function alone and are considered to play a role as a component of multiprotein complexes in a coordinated fashion. These complexes may include multiple HDACs, either belonging to same family or different families (Chen and Wu, 2010; Luo et al., 2012; Buszewicz et al., 2016; Li et al., 2017; Guo et al., 2020). Many researchers have studied the HD2 family HDACs, HD2C and HD2D for their role in abiotic stress responses (Sridha and Wu, 2006; Buszewicz et al., 2016; Farhi et al., 2017). Previously, HD2C was shown to interact with HDA6 and HDA19, all of which are involved in the abiotic stress response. Another two members of the HD2 family, HD2A and HD2B, have also been shown to coregulate plant root growth. Phylogenetic analysis of DNA and protein sequences of HD2s suggests that a single ancestral HD2 gene in the monocots and dicots led to the formation of all other HD2s in different species (Pandey et al., 2002). Arabidopsis contain four HD2s which appeared from three successive rounds of genome duplication events (Bourque et al., 2016). Sequence analysis of Arabidopsis HD2s shows that all HD2s possess a conserved pentapeptide MEFWG sequence motif and histidine 25 (H25) in the N-terminal domain, which are considered crucial for their histone deacetylase activity (Tahir and Tian, 2021). The C-terminal domain of HD2A and HD2C possess a C_2H_2-type ZnF motif, which is thought to be involved in DNA-protein or protein-protein interactions. HD2D contains only C2 part of C_2H_2 motif, which probably occurred due to the partial loss of C-terminal domain sequence during evolution (Luo et al., 2022). Although HD2B differs from HD2A in not containing C_2H_2 signature motif, the HD2A and HD2B share the maximum sequence similarity (50.5%) and the least divergence (0.493) with each other at the protein level. HD2A shares the second maximum similarity (40.5%) and the least divergence (0.802) with HD2C, following the HD2A with HD2B (**Figure 4**). HD2C shares least similarity and maximum divergence with the HD2D, a distantly related HD2 family member. The

phylogenetic analysis alone does not suggest whether a certain HD2 protein may interact or correlate with other members of the family. Further research was carried out to understand the relationship of the HD2 family HDACs in regulating drought stress response and root growth in Arabidopsis.

My overall aim was to investigate if any of the HD2s coordinate with each other in regulating plant response and root growth under drought stress. For this purpose, I obtained *hd2* single gene mutants and generated different *hd2* double mutants by crossing *hd2* single gene mutants in different combinations to investigate the relationship of different HD2s (**Table 1**). Along with these, I also developed HD2 overexpression lines. These *hd2* mutants and HD2-OE lines are important plant resources and can be used to study HD2-mediated plant developmental processes and stress response pathways in Arabidopsis. In the sections below, I will highlight and discuss several interesting findings of this study that will aid in understanding the role of HD2s in drought stress response and root growth under drought stress in Arabidopsis.

4.1 HD2 family genes respond to drought stress in Arabidopsis

HD2 family genes show differential expression profiles in response to different abiotic stresses. Arabidopsis plants treated with cold temperature resulted in the upregulation of *HD2A, HD2B* and *HD2C* (To et al., 2011). Arabidopsis plants exposed to heat stress for 3 hours resulted in the upregulation of *HD2A* and *HD2C* (Buszewicz et al., 2016). However, salt and ABA treatment resulted in the downregulation of HD2 genes in Arabidopsis (Luo et al., 2012). To study how HD2 family genes respond to drought stress, I measured the expression levels of *HD2A, HD2B, HD2C,* and *HD2D* in WT Arabidopsis under soil drought stress treatment. Analysis showed that all the HD2 genes are significantly upregulated in response to drought stress (**Figure 6**). The upregulation of the HD2 family genes indicates that these may be involved in the drought stress response, which is considered one of the major abiotic stresses.

4.2 HD2A and HD2C associate to positively regulate drought stress response in Arabidopsis

Previously, overexpression of *HD2C* reported to show enhanced tolerance to ABA, salt, and cold stresses, whereas *hd2c* mutants showed decreased tolerance to salt and ABA (Sridha and Wu, 2006; Luo et al., 2012). Chen et al. (2018) reported that the HD2C interacts with HD2B and knockout of both HD2s together (*hd2b*.*hd2c*) resulted in narrow and pointed leaves as well as shorter roots as compared to the single mutants *hd2b* and *hd2c*. Transgenic plants overexpressing *HD2D* showed increased tolerance to salt, drought, and cold stresses (Han et al., 2016). To study the relationship of HD2s in drought stress response and root growth, I obtained *hd2* single mutants, *hd2a*, *hd2b*, *hd2c*, and *hd2d* from ABRC and performed PCR-based genotyping to confirm the homozygosity of T-DNA insertions in each of the HD2 gene mutant line (**Figure 7B**). RT-qPCR analysis was performed to confirm the knockout of HD2 gene expression at the mRNA level (**Figure 8**). Analysis showed that the mutant lines *hd2a* and *hd2c* were complete knockout lines, whereas *hd2b* and *hd2d* retained their expression at 21% and 10% of the WT level, respectively. The double mutant lines Mac16 (*hd2a*.*hd2c*), Mbc68 (*hd2b*.*hd2c*), and Mcd20 (*hd2c*.*hd2d*) were generated by crossing *hd2* single mutants (**Table 1**). The two double mutants Mcd20 and Mbc68 did not show compelling results in some of the experiments performed in this research (see **Appendix 8**). Due to the relative paucity of information on *hd2a* and *hd2c* single and double mutants against drought stress response, I focussed my attention on the Mac16 (*hd2a*.*hd2c*) double mutant line only for further detailed study. Gene expression analysis of *hd2* mutants showed that when the *HD2A* is knocked out in *hd2a*, expression of *HD2C* is upregulated in *hd2a*. Similarly, when *HD2C* is knocked out in *hd2c*, *HD2A* is upregulated (**Figure 9**), suggesting that *HD2A* and *HD2C* may partially complement the function of the other when one of the genes is knocked out.

Use of mutants and overexpression lines to define the roles of HDACs in genes expression regulation in a variety of plant growth-related processes and abiotic stress responses is considered a very important strategy and has been established in many reports (Sridha and Wu, 2006; Chen et al., 2010; Colville et al., 2011; Luo et al., 2012; Farhi et al., 2017). I also generated *HD2A* and *HD2C* overexpression lines (**Figure 10**). RT-qPCR analysis of selected overexpression lines confirmed that the *HD2A* and *HD2C* exhibit many fold

higher expression in their respective overexpression lines, OEA1/OEA2 and OEC1/OEC2, compared to WT Arabidopsis.

To study the role of *HD2A* and *HD2C* in drought stress response, I first measured the survival rates in the HD2-OE lines and *hd2* mutants after imposing drought stress treatment (**Figures 11-12**). Both *HD2A* and *HD2C* overexpression lines showed enhanced survival under drought stress as compared to WT. On the other hand, the single mutants, *hd2a* and *hd2c* had 79% and 67% survival relative to WT, whereas the *hd2* double mutant line Mac16 showed only 47% survival relative to WT, suggesting a combined knockout effect of both HD2 genes. Previously, overexpression of *HD2C* resulted in an increased tolerance against ABA and salt stress response, whereas the single mutants, *hd2c* and *hda6* and the double mutant *hd2c.hda6* were reported to show decreased tolerance under ABA and salt stresses (Sridha and Wu, 2006; Luo et al., 2012).

Under restricted water supply, the stomata are closed to limit water loss by transpiration and establish an equilibrium between water absorbed by roots and water loss by transpiration. Sridha and Wu (2006) demonstrated that *HD2C* overexpression lines had slower water loss under stress as compared to WT plants. It is probable that the decreased survival in the *hd2* double mutant line Mac16 under drought stress might be related to increased water loss from leaves associated with modified stomatal functioning pattern. I first measured the fresh leaf water loss in *hd2* mutants and HD2-OE lines (**Figure 13**). The double mutant line Mac16 showed significantly increased water loss as compared to both single mutants, *hd2a* and *hd2c* and WT plants, whereas *HD2A* and *HD2C* overexpression lines showed decreased water loss from the fresh leaves as compared to WT plants. These results indicate that the individual HD2s knockouts (*hd2a*, *hd2c*) result in lower survival and increased water loss. However, when both genes are knocked out together (Mac16), plants show additive effects in terms of decreased survival and rapid water loss under drought stress. Next, I questioned if decreased survival and increased water loss in *hd2* double mutants is associated with stomatal functioning. I measured the stomatal aperture in both HD2-OE and *hd2* mutant lines. The *HD2A* overexpression lines did not show any differences in the stomatal opening under control conditions, however, stomata closed under ABA-induced stress as compared to WT plants. Overexpression of *HD2C* caused

the stomata to be more open under high water availability and fully close in response to ABA-induced stress as compared to WT (**Figure 14-15**). Knockout of double HD2 genes (Mac16) did not show any significant change in the stomatal aperture as compared to *hd2c* under high water availability. However, the Mac16 double mutant line exhibited significantly larger stomatal aperture (i.e., stomata did not close fully) under ABA-induced stress as compared to both the single mutants *hd2a* and *hd2c*, and WT (**Figure 16**), suggesting that *HD2A* and *HD2C* might correlate to play a role in stomatal closure regulation pathway and thereby play a role in drought stress response.

Limited water supply to the roots promotes the biosynthesis of ABA in the roots and its transportation toward leaves. Accumulation of ABA in leaves under drought stress stimulates a core signalling pathway to induce stomatal closure to minimize water loss through transpiration. The ABA in guard cells activates the S-type anion channel of plasma membrane, mainly regulated by *SLAC1* gene, which causes the K^+ outflow and subsequently plasma membrane depolarization, leading to closure of stomata under drought stress (Vahisalu et al., 2008; Roelfsema et al., 2012; Munemasa et al., 2015; Zhang et al., 2016). Several studies have demonstrated the ABA-mediated regulation of *SLAC1* gene expression via SnRK2/OST1 phosphorylation, leading to stomatal closure (Daszkowska-Golec and Szarejko, 2013; Imes et al., 2013; Ullah et al., 2017). Zhang et al. (2016) reported the upregulation of *SLAC1* in guard cells of Arabidopsis in response to drought stress, whereas *slac1* mutant plants displayed a strongly impaired stomatal functioning (Vahisalu et al., 2008). ABI1 and ABI2 are considered negative regulators of ABA-mediated stomatal closure and inhibit the expression of the *SLAC1* gene by dephosphorylating and subsequently down-regulating SnRK2/OST1. So, I analysed the expression of *ABI1*, *ABI2* and *SLAC1* genes in *hd2* mutants under drought stress (**Figure 17**). Analysis showed that the ABA negative regulator genes, *ABI1* and *ABI2* were upregulated significantly in the Mac16 line as compared to the single mutants and WT. In contrast, expression of the *SLAC1* gene was decreased significantly in the Mac16 as compared to the single mutant *hd2c*. However, its expression was not affected in the *hd2a* mutant line as compared to WT. The significant increase in the expression of *ABI1* and *ABI2* and decrease in the expression of *SLAC1* led to the change in normal pattern of stomatal opening and closing in the Mac16 double mutant line under stress. These results

show that the knockout of both *HD2A* and *HD2C* (Mac16) results in a stronger phenotype in terms of drought tolerance and stomatal closure regulation as compared to the single mutants, suggests a cumulative effect of *HD2A* and *HD2C* in regulating the drought response and stomatal closure.

Previously, *HD2C* has been shown to be involved in ABA and abiotic stress responses. The *HD2C* overexpression lines exhibited an insensitive phenotype to ABA and increased tolerance to salt, heat and cold stresses, whereas the *hd2c* mutant plants exhibited decreased tolerance to these stresses (Luo et al., 2012; Buszewicz et al., 2016; Lim et al., 2020). Overexpression of *HD2C* caused the upregulation of LEA class, *RD29B* and *RAB18* genes and the downregulation of the *ABI2* gene (Sridha and Wu, 2006). Luo et al. (2012) reported that the HD2C interacts with HDA6 and can mediate plant abiotic stress response. The double mutant line *hd2c.hda6* showed stronger effects on germination pattern under ABA and salt stress as compared to the single mutants *hd2c* and *hda6*. Expression of *ABI1* and *ABI2* genes was significantly upregulated in the double mutant line *hd2c.hda6* as compared to the single mutants *hd2c* and *hda6* (Luo et al., 2012). It is evident that *HD2C* is involved in ABA-related pathways and plays a role in enhancing the expression of ABA-responsive downstream target genes and inhibiting the expression of negative regulators of ABA pathways (*ABI1* and *ABI2*). Based on the results obtained from the analysis of drought stress response, stomatal closure and related genes expression profiling in this thesis, it is very likely that the HD2C also coordinates with the HD2A to function together in response to drought stress to downregulate the expression of *ABI1* and *ABI2* genes possibly through ABA-mediated signalling pathway to regulate the expression of the *SLAC1* gene, thus leading to ABA-mediated stomatal closure under drought stress. The analysis of *HD2A* and *HD2C* single and double gene mutants as well as overexpression lines in this thesis confirmed the hypothesis that the *HD2A* and *HD2C* are involved in drought stress response and both HD2s might work together to play role in this regard.

4.3 HD2A and HD2C coregulate root growth in Arabidopsis

Plants deal with the drought stress mainly in two organs: roots (to absorb water) and leaves (to retain water). Stomatal closure is one of the initial responses of plants to drought stress to maintain water status (Bharath et al., 2021). Persistent drought conditions cause the

plants to modify root growth to enhance their capability to absorb soil water (Rosales et al., 2019). Roots, which play a vital role in maintaining stable water absorption from the soil and its supply to the aerial parts of the plants, are generally classified as primary and secondary roots. A primary root is the fundamental and vital part of the plant root system and plays an important role in early seedling growth and its survival under stress. The primary root length is maintained by cell division in the root apical meristem and cell elongation in the elongation zone. Correct root growth maintenance is the result of a cross-talk of different phytohormones such as ABA, auxins, gibberellic acids, and cytokinins, which are known to play an essential role in regulating root growth in response to internal developmental signals and external stress conditions (Pacifici et al., 2015; Qin et al., 2019). My next objective was to study if HD2A and HD2C also play a role in regulating root growth. First, I examined the primary root lengths and secondary root numbers in *hd2* mutants and HD2-OE lines under control and osmotic stress conditions mimicked by using PEG-6000. Under control conditions, root length in the *hd2a* mutant did not change as compared to WT, whereas *hd2c* mutant showed shorter root length. However, primary root length was significantly decreased in the double mutant line Mac16 as compared to the single mutants and WT (**Figure 18**). Application of osmotic stress (PEG) resulted in a significant decrease in the primary root lengths of both *hd2a* and *hd2c* and a further significant decrease in the double mutant line Mac16. For secondary roots, no difference was observed in the *hd2c* mutant, whereas the *hd2a* mutant line had fewer secondary roots as compared to WT. Knockout of both *HD2A* and *HD2C* resulted in a significant decrease in the number of secondary roots in Mac16 under control conditions (**Figure 19**). Conversely, increased primary root length was observed in both HD2A-OE and HD2C-OE lines under control and osmotic stress conditions, in comparison to WT (**Figure 20-21**). These overexpression lines also displayed more secondary roots (**Figure 22**). Previously, it was reported that the double mutant line *hd2a.hd2b* showed significantly decreased primary root length as compared to the single mutant *hd2b*, suggesting a cumulative effect of both HD2A and HD2B in controlling root length in Arabidopsis (Li et al., 2017). Moreover, overexpression of *HD2C* resulted in an increased root length under ABA and salt, whereas the *hd2c* mutant line displayed shorter root lengths, indicating that HD2C also mediates root growth under abiotic stresses (Sridha and Wu, 2006; Luo et al.,

2012). Recently, it was shown by Chen et al. (2018) that the double mutant line *hd2b.hd2c* showed less root length as compared to *hd2c* and *hd2b* single mutants, indicating a coordination of HD2C and HD2B in root growth regulation. By examining root growth in *hd2a* and *hd2c* single and double mutants and HD2-OE lines in this thesis, I showed that HD2C associates with HD2A to control root growth in Arabidopsis.

Gibberellic acids (GAs) are known to induce germination and root growth at low concentrations and strongly promote shoot growth. It is suggested that the GAs play a role in the elongation of endodermal cells to promote root growth in Arabidopsis (Ubeda-Tomás et al., 2009). Maintaining a dynamic homeostasis of bioactive GAs for normal plant root growth is an important phenomenon and is carried out by GA2ox enzymes. The GA2ox enzymes play a significant role in lowering the levels of certain bioactive GAs including GA1 and GA4, thus mediate plant root growth. Previously, it has been shown that the transcriptional activation of GA2ox genes is associated with the modification of histone acetylation status. Studies show that the GA2ox genes are activated in response to internal developmental signals (including ABA signalling) and external environmental conditions including salt, drought, and cold (Rieu et al., 2008; Colebrook et al., 2014; Chen et al., 2019). GA2ox enzyme family is considered a major regulator of C19-GA-inactivation pathway in Arabidopsis and contains 5 members namely, GA2ox1, GA2ox2, GA2ox3, GA2ox4, GA2ox6 (Rieu et al., 2008). It was shown that the HD2A and HD2B repress the *GA2ox2* by modifying histone H3 acetylation status at the specific *GA2ox2* gene locus (Li et al., 2017). Overexpression of *HD2B* caused an increase in the endogenous gibberellin levels in HD2B-OE lines, whereas *HD2B* transgenic seeds treated with an HDAC inhibitor, trichostatin A (TSA), showed a decreased accumulation of endogenous GAs in the imbibed seeds (Colville et al., 2011; Yano et al., 2013). I thus examined the expression of GA2ox genes in HD2-OE lines and *hd2* mutants (**Figure 23**). Overexpression of both *HD2A* and *HD2C* caused the downregulation of *GA2ox1* and *GA2ox2* genes. On the other hand, the *hd2a* mutant did not show any change in the expression levels of both *GA2ox1* and *GA2ox2* genes as compared to WT (**Figure 23A**), thus showed normal root growth as WT (**Figure 18A**). However, knockout of *HD2A* along with *HD2C* led to a significant elevation in the expression levels of both Ga2ox genes in the double mutant line Mac16 as compared to the single mutants and WT. The significant

elevation of *GA2ox1* and *GA2ox2* genes in the *hd2* double mutant corresponds with the significant decrease in the primary root growth of double mutant line Mac16 under control conditions. Cumulative increase in the enrichment of H3K9 acetylation (**Figure 25**) in the double mutant line Mac16 might be associated with the significant elevation of *GA2ox1* and *GA2ox2* genes, which resulted in inhibiting the primary root growth in the double mutant line. Other GA2ox genes *GA2ox3*, *GA2ox4*, and *GA2ox6* did not show any significant changes in their expression levels in the *hd2* mutants and HD2-OE lines.

Bioactive GAs, GA1 and GA4 serve as substrates for GA2ox-mediated GA inactivation and are converted into inactive forms, GA8 and GA34, whereas GA3 is not considered as its substrate because of its very low stability and unsaturation properties (Yamauchi et al., 2007; Rieu et al., 2008; Li et al., 2017). Yamauchi et al. (2007) reported that the *ga2ox2* mutant plants had higher levels of GA4, whereas the overexpression of *GA2ox2* in Arabidopsis lowered the GA4 levels in roots and resulted in a significant decrease in primary root lengths (Li et al., 2017). I thus examined primary root lengths in the *hd2* mutants under GA4 and GA3 treatments (**Figure 24**). Although, application of 1 µM of GA4 enhanced the primary root growth of all the genotypes as compared to control (untreated) conditions (**Figure 18A)**, the *hd2* mutant lines did not show any significant differences in the primary root growth as compared to WT under 1 µM of GA4 treatment. However, a slight increase in the primary root lengths of double mutant line Mac16 was observed when the concentration of GA4 treatment was increased to 10 µM. On the other hand, 1 µM of GA3 treatment led to a significant increase in the primary root length of the double mutant Mac16 as compared to the single mutants *hd2a* and *hd2c*. Increase in the GA3 concentration to 10 µM resulted in a further increase in root lengths of *hd2* single and double mutants. It is very likely that the HD2A and HD2C coordinate to regulate the expression of GA2ox genes, which are responsible to catabolise and degrade the bioactive GA4 gibberellins. Upregulation of *GA2ox1* and *GA2ox2* in the *hd2* double mutant enhanced the degradation of the bioactive GA4 gibberellins, which led to the decreased root growth in the *hd2* mutants. Additionally, exogenous GA4 application did not increase the root growth. However, GA3 is not considered as a substrate of GA2ox enzymes, thus it restored the root growth in the mutant lines.

4.4 HD2A and HD2C decrease global H3K9 acetylation levels

Genome-wide histone H3 modifications in plants have been reported in many studies (Zhou et al., 2010; Lu et al., 2015; Baerenfaller et al., 2016). Histone modifications are well correlated with the gene expression regulation. Genome-wide enrichment of H3 acetylation was observed in Arabidopsis cell lines when subjected to ABA, salt and cold stress (Sokol et al., 2007). Drought treatment caused the hyperacetylation of H3 on ABA response element (ABRE) and dehydration response element (DRE) genes in Arabidopsis, leading to the upregulation of their expression (Kim et al., 2008). The H3K9 acetylation is enriched preferentially at transcription start sites of target genes, indicating that activation of gene transcription is closely associated with H3K9 acetylation during plant stress repsonses (Zhou et al., 2010; Kim et al., 2015). This indicates that a large set of functionally related genes are controlled in a coordinated fashion through H3K9 hyperacetylation in different abiotic stress responses in plants. Recently, HD2C was shown to interact with MRG1/2 and a decreased H3K9, H3K23, and H3K27 acetylation levels were observed at the *FT* gene locus, an early flowering gene in Arabidopsis, resulting in a delayed flowering phenotype (Guo et al., 2020). HD2s can also regulate H3K9 acetylation status. Knockout of *HD2A* and *HD2B* together (*hd2a*/*hd2b*) resulted in the hyperacetylation of histone H3 at the *GA2ox2* gene locus, which resulted in the upregulation of the *GA2ox2* in *hd2a*.*hd2b* as compared to the single mutant *hd2b*. The upregulation of *GA2ox2* lead to a significant shorter root lengths in the *hd2a*.*hd2b* double mutant line (Li et al., 2017). In my study, I measured the H3K9 acetylation levels in the HD2A and HD2C single and double mutants to examine if knockout of these HD2s results in an increased global H3K9 acetylation levels (**Figure 25**). Higher H3K9 acetylation levels were observed in the double mutant Mac16 as compared to the single mutants *hd2a* and *hd2c*, and WT. Overexpression of HD2A and HD2C resulted in a decrease in the H3K9 acetylation levels. These results are in accordance with previous reports (Luo et al., 2012; Li et al., 2017; Guo et al., 2020) that showed higher H3K9ac levels at the locus of genes which were upregulated in different *hd2* mutants.

4.5 Root growth: a phenomenon of phytohormones crosstalk under drought stress

As discussed earlier, plants under stress tend to increase the biosynthesis of ABA to trigger the plant stress response. ABA biosynthesis is closely related to the cellular dehydration processes during plant vegetative and reproductive growth (Wilkinson and Davies, 2002; Bartels and Souer, 2004; Fujita et al., 2011). Correct root growth maintenance in Arabidopsis is achieved through a crosstalk between phytohormones such as ABA, GAs, ethylene, and cytokinins, which play an essential role in regulating root growth under control and stress conditions (Pacifici et al., 2015; Qin et al., 2019). ABA is considered a negative regulator of Arabidopsis root growth. Exogenous ABA application resulted in a decreased primary root length with lesser number of secondary roots (Luo et al., 2014; Park et al., 2020). However, GAs play a role in the elongation of endodermal cells to promote root growth (Ubeda-Tomás et al., 2009; Corbineau et al., 2014; Qin et al., 2019). Both ABA and GAs are known to play an antagonistic role in plant growth and development. Levels of ABA and GAs are negatively correlated as higher levels of ABA causes the repression of GA biosynthesis, and vice versa. Reports have shown the higher accumulation of ABA and lower levels of GAs in response to different abiotic stresses (Footitt et al., 2011; Liu and Hou, 2018). It is reported that the higher ABA accumulation under stress causes the repression of GA biosynthesis by downregulating the biosynthesis-related GA20ox genes and upregulating the GA catabolism-related GA2ox genes. Transcriptional activation of GA2ox genes is associated with the modification of histone acetylation status triggered by ABA-mediated signalling during different abiotic stresses such as drought, salt and cold (Rieu et al., 2008; Liu et al., 2010; Colebrook et al., 2014; Lee et al., 2016; Ravindran et al., 2017; Chen et al., 2019). These reports suggest that the catabolism and degradation of bioactive GAs is initiated via ABA-mediated induction of GA2ox genes through histone acetylation modification in response to osmotic stress. In this thesis, I found that the *GA2ox1* and *GA2ox2* genes were upregulated in the double mutant line Mac16 (*hd2a.hd2c*) as compared to the single mutants *hd2a* and *hd2c*, which lead to a decreased primary root length phenotype in the Mac16. Also, higher H3K9 acetylation levels were observed in the Mac16 line. Based on the results obtained, I can speculate that the higher H3K9 acetylation levels in the Mac16 might be associated with the transcriptional activation of GA2ox genes, as activation of these genes is associated

with the higher histone acetylation levels as discussed earlier. Upregulation of GA2ox genes in the double mutant line may enhanced the degradation of GA4, as exogenous GA4 application (1 µM) did not increase root length in the Mac16 line. However, GA3 application increased the primary root length in the double mutant line. These results validate the hypothesis that HD2A and HD2C play a cumulative role in controlling root growth under drought stress by regulating the GA2ox expression through histone acetylation modification.

4.6 HD2A and HD2C physically interact with each other

HDACs are generally considered to function as part of multiprotein complexes. The deacetylase activity of HDACs often depends on interaction and coordination with other enzymes and transcription factors (Yang and Seto, 2008). HD2C was shown to interact with the DNMT2, a methyl transferase responsible for the methylation of DNA to mediate gene repression. Methylation activity of DNMT2 was significantly impaired in the *hd2c* mutant (Song et al., 2010). The HD2A and HD2B were also shown to physically interact with DNMT2. The interdependence of DNMT2 and HD2-type HDACs signifies an important relationship in gene expression regulation in response to abiotic stresses (Song et al., 2010). Buszewicz et al. (2016) studied the interaction of HD2C with chromatin remodeling complexes in heat stress response and precipitated proteins bound to HD2C via GFP-binding affinity chromatography. They found the HD2A bound to HD2C along with BRM-containing SWI/SNF chromatin remodelling complexes. As discussed earlier, HD2A, HD2B, and HD2C also show interactions with HDA6 and HDA19, which are well known for their role in the abiotic stress response. Functional association within HD2 family members as well as with RPD3-type HDACs as part of repression complexes might be of vital importance for regulating gene expression through histone modifications (Tahir and Tian, 2021). Although HD2A and HD2C have been reported to play an opposite role during seed germination (Colville et al., 2011), the occurrence of both HD2A and HD2C in a common gene repression complex in response to different internal developmental and external environmental signals cannot be ruled out, as many studies have shown the interaction of both HD2A and HD2C with common interacting partners such as DNMT2, HDA6, and HDA19. I thus investigated if HD2A and HD2C can physically interact with

each other (**Figure 26**). Y2H and BiFC analysis revealed that both HD2A and HD2C proteins can physically interact each other. The protein-protein interaction of both HD2s validate my hypothesis that HD2A and HD2C can physically interact with each other to play a role in drought stress response and root growth regulation in Arabidopsis.

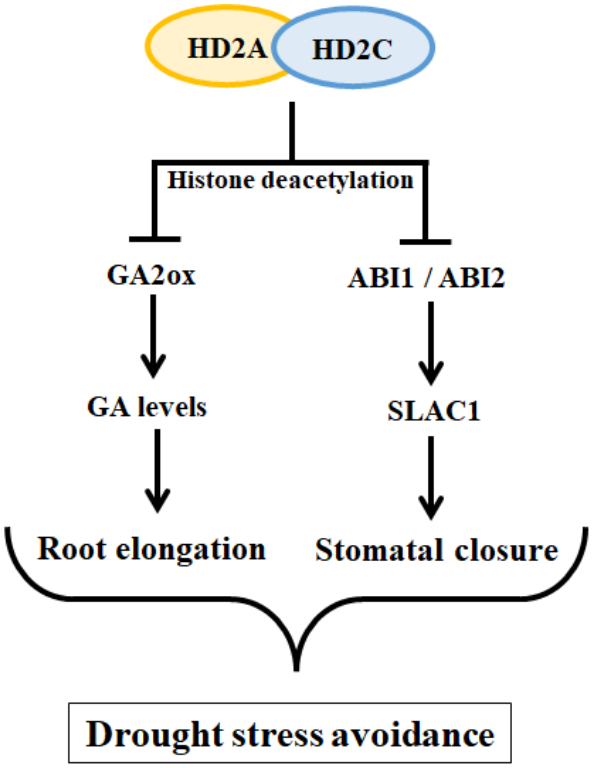
4.7 Concluding remarks and future perspective

In conclusion, my investigations presented in this thesis revealed the relationship of HD2A and HD2C, which positively regulates the drought stress response and root growth in *Arabidopsis thaliana*. The working model of HD2A and HD2C illustrated in **Figure 27** represents the involvement and coordination of both HD2s in drought stress response. HD2A and HD2C adopt a comprehensive strategy at leaf and root levels in responding to drought stress. At leaf level, both HD2s control the water loss from leaves by negatively regulating the expression of *ABI1* and *ABI2* genes, thus playing role in the regulation of SLAC1-mediated stomatal closure via ABA signalling pathway of gene regulation. Whereas at root level, both HD2s coordinate to play an important role in regulating root growth by downregulating the expression of the GA2ox genes via histone acetylation modification, to maintain the bioactive Gibberellin levels to promote root growth, thus helping the plant to grow in response to stress environment.

Previously, HD2C has been studied for its role in ABA and different abiotic stresses during the germination and seedling stage. However, no study was performed to investigate if HD2C coordinates with any other member of the HD2 family. Similarly, the role of HD2A in stress response has not been investigated previously. By investigating the single and double gene mutants of *HD2A* and *HD2C*, I revealed that both HDACs are not only involved in drought stress response, but also coordinate to coregulate the plant response to drought stress, as double mutant lines showed poor survival and increased water loss under stressed conditions due to abnormal stomatal functioning as compared to the single mutants. Moreover, my study also revealed that the overexpression of both *HD2A* and *HD2C* can enhance plant tolerance to drought stress by controlling the water loss from leaves by mediating the expression of genes involved in stomatal closure under drought stress. By examining root growth in the *HD2A* and *HD2C* single and double mutants and overexpression lines, I also demonstrated that the HD2A and HD2C coordinate to control

Figure 27: The working model of HD2A and HD2C in drought stress response in Arabidopsis

The working model of HD2A and HD2C shows that both HD2s coordinate to positively regulate drought stress response at both leaf and root levels, by modulating the expression of *ABI1* and *ABI2*, involved in ABA-mediated stomatal closure, and GA2ox genes via histone acetylation modification. All arrow heads represent positive regulation, while all stop lines represent negative regulation associated with related pathways.



root growth in Arabidopsis by mediating the expression of GA2ox genes, thus leading to stable root growth under optimum conditions. My study also showed that the higher expression of GA2ox genes in *hd2* double mutant (Mac16) might be associated with increased H3K9 acetylation, leading to shorter root growth in Mac16. This study lays out the basics which can be of importance in future studies to understand how exactly HD2A and HD2C correlate for controlling root growth and drought stress response. Knowledge gained on the role of these HD2 genes in drought stress response and root growth can be used as a potential molecular strategy to improve drought tolerance in other related crops.

Further investigation is required to comprehensively understand the exact role of HD2A and HD2C relationship in root growth and drought stress response. One of several strategies would be to explore the target genes at the genome-wide level and study the transcriptional activation of specific target genes associated with H3K9 acetylation modification. RNA-seq expression analysis could also be conducted to reveal differential expression patterns of genes in the *HD2A* and *HD2C* single and double gene mutants under drought stress. Several histone H3 acetylation marks (H3K9, H3K14, H3K18, H3K23, H3K27) have been reported to be modified by different HDACs (Tahir and Tian, 2021). I studied H3K9 acetylation levels in the *hd2* mutants and overexpression lines. It would be interesting to explore if HD2A and HD2C can also co-target other histone activation marks. Double HD2 gene knockout decreases the genome-wide H3K9 acetylation levels which is associated with transcriptional repression of genes. A ChIP-seq assay targeting H3K9ac in *hd2* single and double gene mutants would be an interesting approach to explore the direct co-targets of HD2A and HD2C. This would provide a detailed knowledge of the genes being directly targeted by HD2A and HD2C in Arabidopsis.

The HDACs are generally recruited to function as a part of multiprotein complexes to repress gene expression. The HDACs deacetylation activity often depends on interaction and coordination with transcription factors. Different proteins have been shown which can interact with HD2A and HD2C. It is quite possible that both HD2A and HD2C may interact with the same proteins and exist in a common multiprotein complex to carryout gene repression activity. GFP-binding affinity chromatography and co-immunoprecipitation (Co-IP) can be performed to elute common proteins interacting with both these HD2s. This

approach can help to investigate the relationship of HD2A and HD2C with other HDACs and transcription factors involved in the mechanism of transcriptional regulation of target genes.

Investigating the relationship of HD2A and HD2C in drought stress response and root growth in Arabidopsis will expand the understanding of the epigenetic regulation of drought-responsive gene regulatory networks in plant stress responses. It will contribute to the understanding of how plants utilize plant-specific HD2-type histone deacetylases to deal with drought stress conditions at chromatin level. The homologue of Arabidopsis HD2A and HD2C in other related plant species can be characterized and investigated to study their role in drought stress response. Knowledge gained on the role of these HD2 genes in drought stress response and root growth can be used as a potential molecular strategy to improve drought tolerance in related crops.

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Appendices

Appendix 1: Primers used in PCR genotyping

Genes	Primers	5′- Sequences -3′
HD2A	HD2A_GK355F	CGGCTTCGTATTAAAACCCTC
	HD2A_GK355R	GCCTTTGGTTTAGCTACAGCC
HD2B	HD2B_SL1247F	TCTTCTTCTCCTCCCGATAGC
	HD2B_SL1247R	AACAAGTTTAGCCCCACCAAC
HD2C	HD2C_SALK_F	GATGATCAACTTGAGGCTGCT
	HD2C_SALK_R	CAGAATTCTTACCCGCCTGT
HD2D	HD2D_GK279F	ACCCTACCTGTGAAAAGGAAG
	2D_GK279R	CCCATTCTCATCATCACCAATC
LB primer	GK8474	ATAATAACGCTGCGGACATCTACATT
LB primer	LB3	TAGCATCTGAATTTCATAACCAATCTCGA TACAC
LB primer	LBb1.3	ATTTTGCCGATTTCGGAAC
LB primer	LBa1	TGGTTCACGTAGTGGGCCATCG

Appendix 2: Primers used in Gateway cloning

Genes	Primers	5′- Sequences -3′
HD2A	AtHD2A_GW_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTA CATGGAGTTCTGGGGAATTGAAG
	AtHD2A_GW_R	GGGGACCACTTTGTACAAGAAAGCTGGGTC CTTGGCAGCAGCGTGCTT
HD2B	AtHD2B_GW_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTA CATGGAGTTCTGGGGAGTTG
	AtHD2B_GW_R	GGGGACCACTTTGTACAAGAAAGCTGGGTC AGCTCTACCCTTTCCCTTG
HD2C	AtHD2C_GW_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTA CATGGAGTTCTGGGGTGTTG
	AtHD2C_GW_R	GGGGACCACTTTGTACAAGAAAGCTGGGTC AGCAGCTGCACTGTGTTTG
HD2D	AtHD2D_GW_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTA CATGGAGTTTTGGGGTATCGA
	AtHD2D_GW_R	GGGGACCACTTTGTACAAGAAAGCTGGGTC CTTTTTGCAAGAGGGACCA

Appendix 3: Primers used for 35S-HD2-YFP confirmation in HD2-OE lines

Genes	Primers	5′- Sequences -3′
HD2A	35SHD2A_For	AAGCACCTGTGTCAGCAAAG
HD2C	35SHD2C_For	GATGATCAACTTGAGGCTGCT
YFP	HD2B_SL1247F	GTCTTGTAGTTGCCGTCGTC

Appendix 4: Primers used in RT-qPCR analysis

Genes	Primers	5′- Sequences -3′
HD2A	HD2A_CDS_F3	GTGAAGCCAGCTGTTGATGA
	HD2A_CDS_R3	CTTTCGCCTTCTTTGCTGAC
HD2B	HD2B_CDS_F2	ATCCAAAACACCCGTCTCTG
	HD2B_CDS_R2	CCGGATGATTGACCTCCAG
HD2C	HD2C_CDS_F1	CAGGTTGCTTTGGGAGAGAG
	HD2C_CDS_R1	GTATGAGACAGCGCAAAGTTCC
HD2D	HD2D_CDS_F1	AGATGGGTTTGGACGAGGATG
	HD2D_CDS_R1	GGGCATCTCTTCTTCCCTCC
RD29A	RD29A_qF	AACGACGACAAAGGAAGTGG
	RD29A_qR	AACCAGCCAGATGATTTTGG
SLAC1	SLAC1CT_F	TGGAAACAGAGGACCAAACC
	SLAC1CT_F	TCTGTTTTCCGACCATCTCC
ACTIN2	ACT2_RT_FOR	TATCGCTGACCGTATGAGCA
	ACT2_RT_REV	ATCATACTCGGCCTTGGAGA
GA2ox1	Ga2OX1_F	CAAGAGCGTGAGGCATAGGG
	Ga2OX1_R	AGTCAATGAAGGTCCAGCGAAG
GA2ox2	Ga2OX2_F	CATTCTCTGCGGTTTGTTTGG
	Ga2OX2_R	CGTGAGTCTCAGTGTCTACATAG
GA2ox3	Ga2OX3_F	TGCCTGAGAATGAACCATTACCC
	Ga2OX3_R	TGTTCCATCTTTGACACAGATTTGC
GA2ox4	Ga2OX4_F	GCTCGGCAGTGAATTGTTACATAG
	Ga2OX4_R	CACAGATTGGTCAGAAAGATTGGC
GA2ox6	Ga2OX6_F	ACAGAAGTCTAGCGAAGTGAGTG
	Ga2OX6_R	CGGTGCTGGTGGATAGTGATTC
ABI1	ABI1_F	TGAAGAAGCGTGTGAGATGG
	ABI1_R	CTGTATCGCCAGCTTTGACA
ABI2	ABI2_F	GATGGAAGATTCTGTCTCAACGATT
	ABI2_R	GTTTCTCCTTCACTATCTCCTCCG

Appendix 5: Protein-protein interactions

All interactions of HD2 proteins with each other tested by Y2H

-AD \ -BK	HD2A-BK	HD2B-BK	HD2C-BK	HD2D-BK
HD2A-AD	NA	Yes	Yes	Yes
HD2B-AD	Yes	NA	No	No
HD2C-AD	Yes	No	NA	Yes
HD2D-AD	Yes	No	Yes	NA

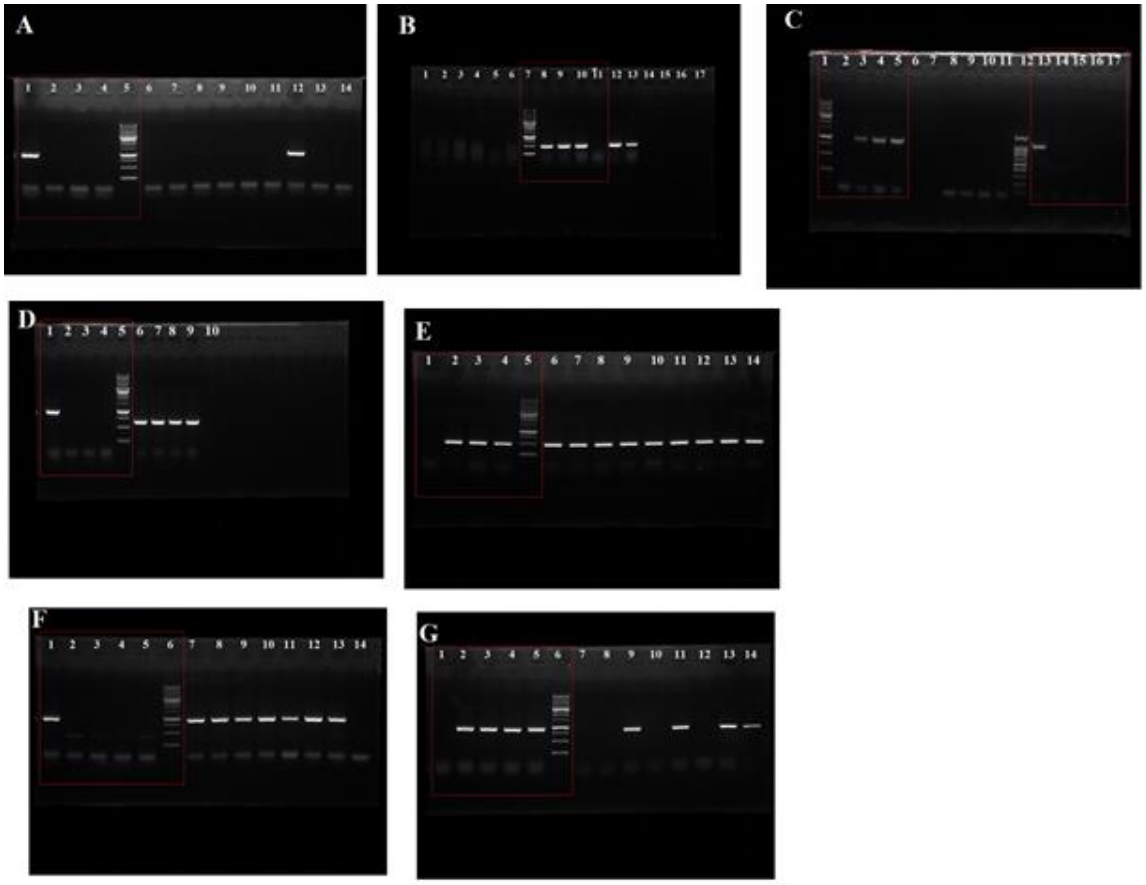
Yes: Interaction; No: No interaction; NA: Not tested (not available)

All interactions of HD2 proteins with each other tested by BiFC

-YC \ -YN	HD2A-YN	HD2B-YN	HD2C-YN	HD2D-YN
HD2A-YC	NA	Yes	Yes	Yes
HD2B-YC	Yes	NA	Yes	No
HD2C-YC	Yes	Yes	NA	Yes
HD2D-YC	Yes	No	Yes	NA

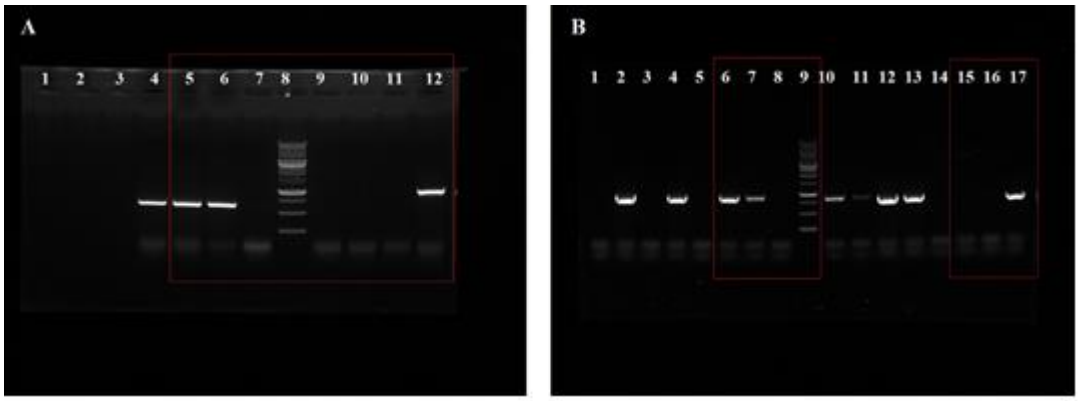
Yes: Interaction; No: No interaction; NA: Not tested (not available)

Appendix 6: Uncropped gel images of Figure 7B



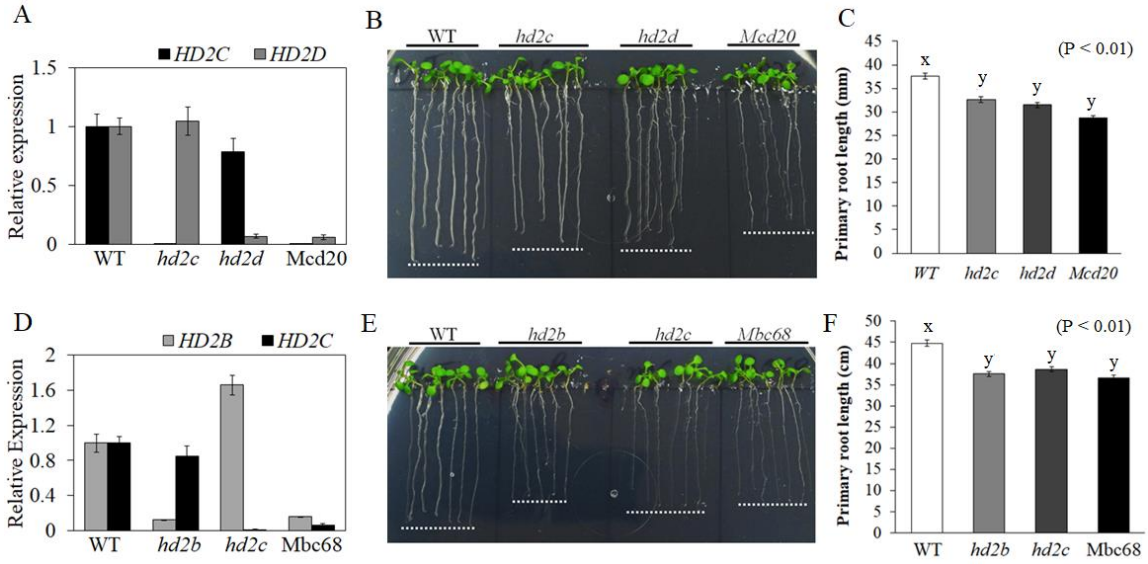
Uncropped gel images of Figure 7B. A-B gel Images correspond to HD2A genotyping, cropped from lanes 1-5 and 7-11 and dragged the ladder to the left. C gel Image corresponds to HD2B genotyping, cropped from lanes 1-5 and 13-17. D-E gel Images correspond to HD2C genotyping, cropped from lanes 1-5. F-G gel Images corresponds to HD2D genotyping, cropped from lanes 1-6.

Appendix 7: Uncropped gel images of Figure 9A



Uncropped gel images of Figure 9A. A gel Image corresponds to HD2A genotyping, cropped from lanes 5-11 and dragged the ladder to the left. B gel image correspond to HD2C genotyping, cropped from lanes 6-9 and 15-17 and dragged the ladder to the left.

Appendix 8: Gene expression analysis and primary root length in double mutants Mcd20 and Mbc68



Gene expression analysis and primary root length in *hd2c*, *hd2d*, and Mcd20 (A-C) and *hd2b*, *hd2c*, and Mbc68 (D-F). (A) *HD2D* expression is not affected in *hd2c* mutant whereas expression of *HD2C* slightly decreased in *hd2d* mutant. (D) Expression of *HD2C* is not affected in hd2b mutant whereas expression of HD2B is upregulated in hd2c mutant. Root length in both double mutants Mcd20 and Mbc68 does not change as compared to their single mutants.

Curriculum Vitae

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Work experience

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Selected Publications

Tahir, Muhammad Sufyan, and Tian, L. (2021). HD2-type histone deacetylases: unique regulators of plant development and stress responses. Plant Cell Rep 40, 1603–1615.

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