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Identification of AtHD2C as a novel regulator of ABA signaling in Arabidopsis thaliana

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ABSTRACT

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When plants transition from the heterotrophic phase of embryogenesis into the autotrophic phase of sporophytic development, they need to proceed through dormancy and desiccation events. These processes are vital because the commitment to germinate is irreversible, beyond that premature germination would be fatal for the plant. Therefore, maintenance and timely exit from these phases is important. Smooth regulation of this procedure is executed by Abscisic acid (ABA), a plant-specific hormone. In addition, ABA assumes an important role during extreme periods when plants are threatened by abiotic stresses such as drought and high salinity. This report describes *AtHD2C* as a regulator of the ABA controlled events. *AtHD2C* is a member of the HD2-type histone deacetylase family. A GFP (green florescent protein) co-localization assay revealed that AtHD2C localized to the nucleus in *Arabidopsis*. Oligonucleotide-directed mutagenesis, that was utilized to create site specific mutations in AtHD2A, identified that the residues important for repression activity reside at the N-terminal pentapeptide and at H25 of the sequence. A semi-quantitative RT-PCR assay determined the spatial expression profile of the HD2 gene family, with higher transcript accumulation in the reproductive organs. These results indicated an overall physiological significance for the HD2 proteins as well as a possible involvement in embryo development. In continuum with this, seeds overexpressing *AtHD2C* were insensitive to ABA, NaCl and mannitol at germination. This phenotype was supported by ABA-responsive gene expression patterns in the transgenic plants that implicated a negative role for *AtHD2C* in ABA response regulation in this developmental window. Additionally, the *AtHD2C* transcript accumulation was down-regulated by ABA that was reflected by the down-regulation of *AtHD2C* promoter driven *GUS*. In contrast to its function in the germination-post germination phase, AtHD2C seemed to play a positive role in ABA response regulation during the vegetative stage. *AtHD2C* overexpressing mature plants were able to survive extreme osmotic shock and drought conditions. The expression of *AtHD2C* promoter driven *GUS* in all vegetative tissues confirmed the presence of AtHD2C in this stage. The predominantly closed stomata and up-regulated ABA-responsive genes support the stress-tolerant phenotype of the *35S:AtHD2C* transgenic plants. The information obtained from this investigation delineates a dual role for the *AtHD2C* in the regulation of the ABA response signaling in two developmental stages.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

HDAC - Histone deacetylase

HAT- Histone acetyl transferases

ABA - Abscisic acid

GUS- Glucuronidase

GFP- Green fluorescent protein

ATHDA1- Reduced potassium dependancy3

ABI- ABA insensitive

WT- Wild type

At- *Arabidopsis thaliana*

ABRE- ABA responsive element

SKOR- Stelar outward potassium rectifying channel

RD29- Responsive to dehydration

ERA- Enhanced response to ABA

LEA- Late embryogenesis abundant

ICK1- Inhibitor of cyclin dependant kinase

LEC- Leafy cotyledon

INTRODUCTION

Abiotic stress is often the cause of huge losses in the agriculture industry as it is a big hindrance to crop productivity. Therefore, it is very desirable to engineer crops with enhanced stress tolerance to drought, salinity, osmotic shock, cold and other oppressing environmental stresses. This comprises one of the main long-term objectives of agronomic research in addition to other motives such as, increasing crop yield or pathogen resistance. To understand how the study of genetics can tell us about the ultimate physiological responses of an organism, we can refer to the central dogma of molecular biology (Figure 1). DNA codes for all the information that is required for the behavioral responses of the plant. Therefore, it is extremely important that the message encrypted in the DNA be processed efficiently into the functional biocatalysts, proteins. Between DNA and proteins there are messenger molecules, RNAs that keep the information processing intact. Hence, the formation of RNA or *transcription* is crucial for information relay. Consequently, gene expression can be controlled at the very first step of relay, *transcription*. It can also be controlled at later steps of post-transcription, translation or post-translation (Verbsky**,** 2001). Many aspects of development involve epigenetic regulation: mitotically and/or meiotically heritable yet reversible changes in gene expression without changes in DNA sequence (Steimer *et al***,** 2004). Many epigenetic changes depend on the recognition of sequence homology at the DNA or RNA level. This recognition can led to transcriptional gene silencing (TGS), that is associated with DNA methylation and/or chromatin modifications, or to post transcriptional gene silencing (PTGS), either by sequence specific RNA degradation or by

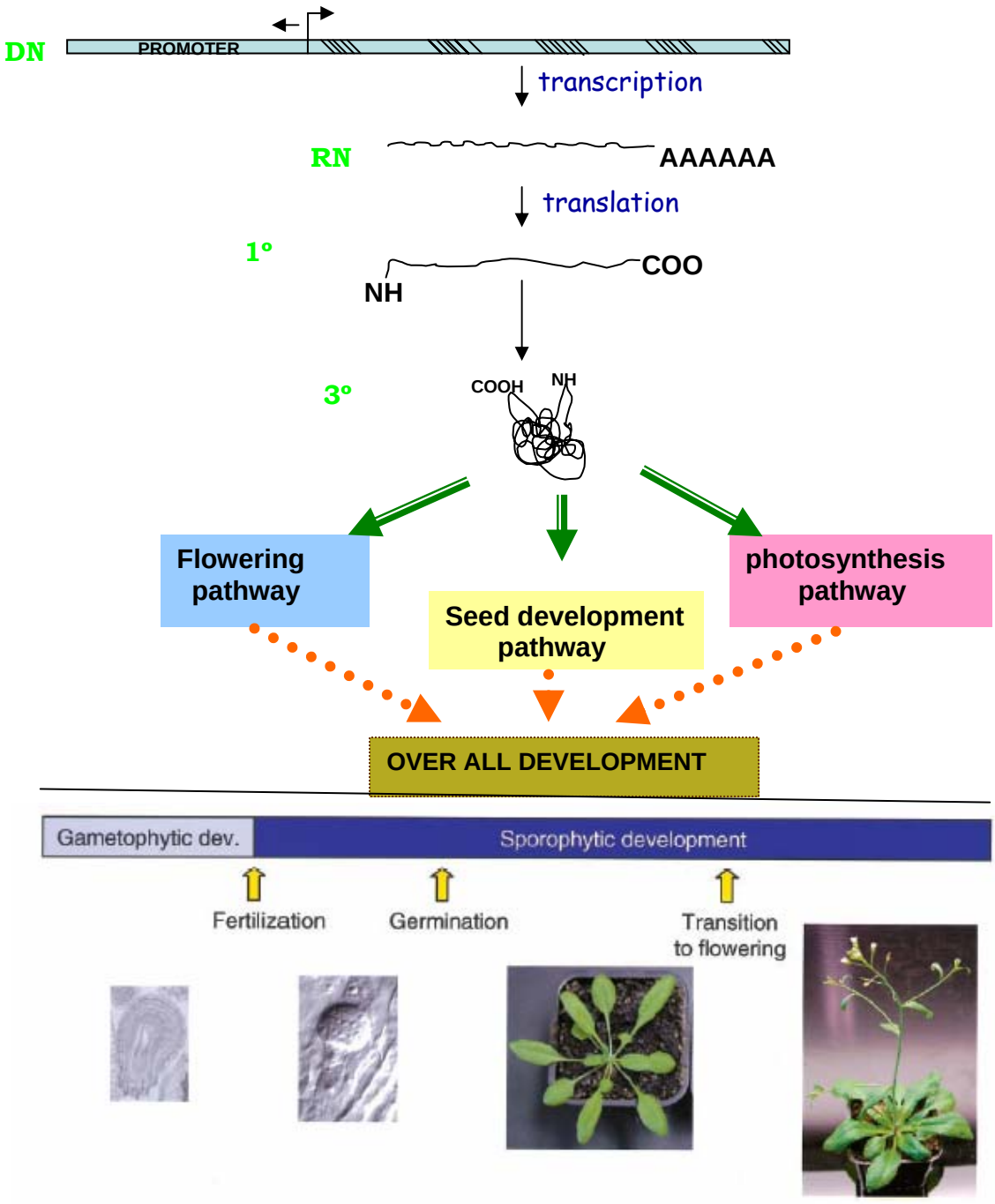


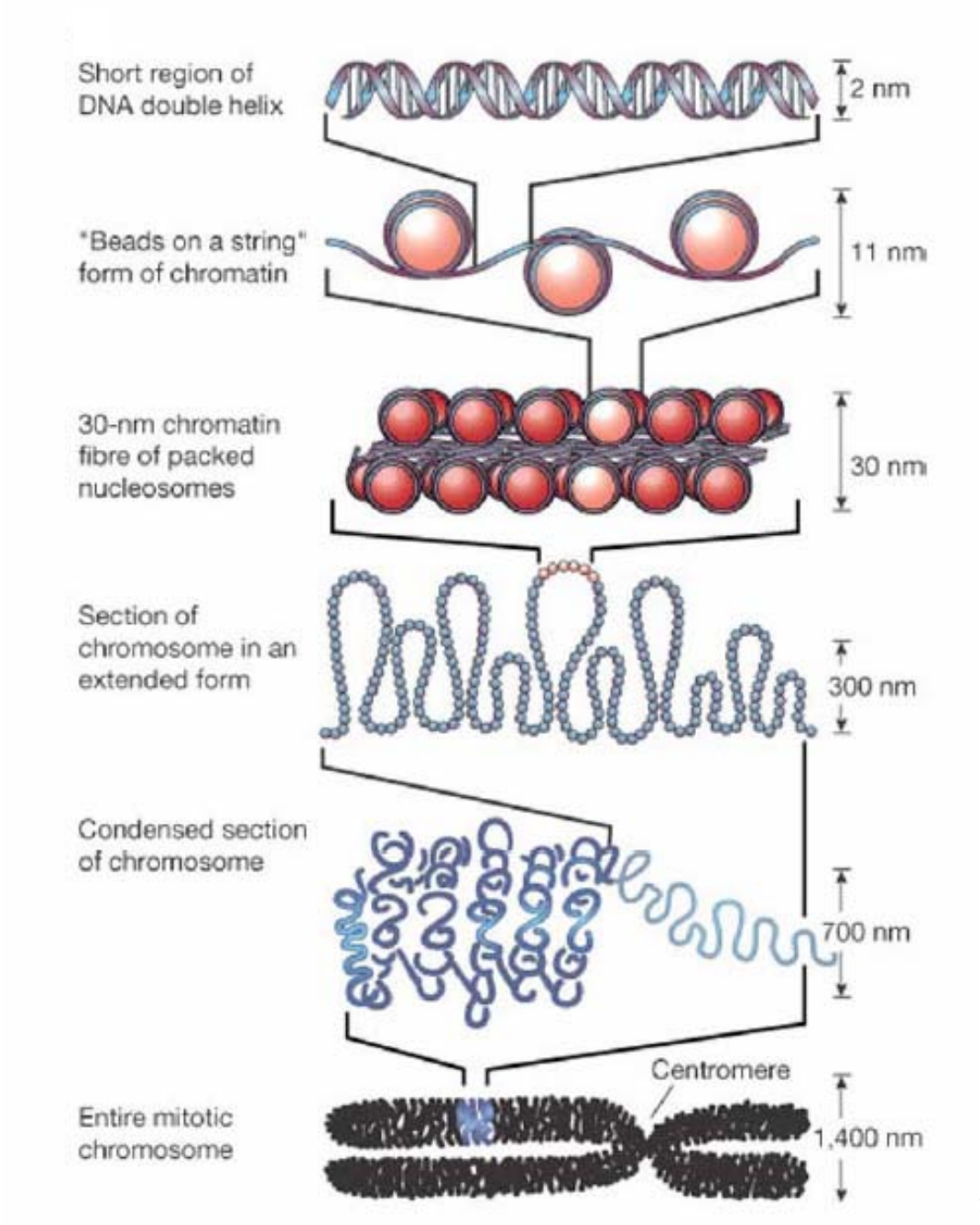
Figure 1 Central Dogma of Molecular Biology

inhibition of translation. The focus of my study is transcriptional gene silencing and its role in the physiology and development of the plant.

(i) The mechanisms to control gene expression at the transcription level

The accurate regulation of gene expression in space and time is fundamental for development. Only certain genes need to be expressed at certain times and specific locations. Other genes need to be turned off at these times and locations in order to maintain normal growth transitions (Finnegan E.J**,** 2001). If all genes in the genome were expressed at the same time, there would be chaos in the biological system as contradicting signals would be generated that would cancel out each other's effect and the net result would be zero development. Therefore, agents that expedite transcriptional repression and activation are essential for maintaining homeostasis in developmental signaling.

To better understand regulation of gene expression, we have to understand that genes are a constituent of nuclear DNA that is compacted into chromatin (Figure 2). Regulation of higher order chromatin structure is directly coupled with regulation of the expression and integrity of the genetic information of plants (Verbsky**,** 2001) and other eukaryotes. In particular, the packaging of DNA into heterochromatin exerts *epigenetic control* over important biological processes (Kadonaga *et al***,** 1998). Chromatin is a complex structure built from repeating units, the nucleosomes (Kornberg and Lorch**,** 1999). These consist of 145 bp of DNA wrapped around an octamer of basic proteins, the core histones. The octamer is formed by histones H2A, H2B, H3 and H4 (Chen and Pikaard**,** 1997) as depicted in Figure 3. At least two different domains can be distinguished in core histones: a globular domain involved in histone-histone interactions



[1]

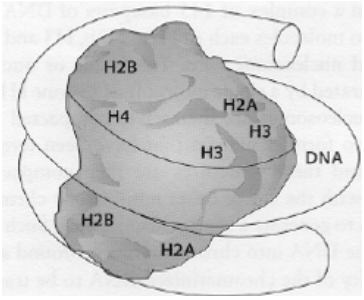
Figure 2 . Compaction of genomic DNA into chromatin and chromosomes.

[1] http://www.path.queensu.ca/present/lillicrap/path425transcription2004march.ppt#256,13,Slide 13

(containing the 'histone fold' motif) and the flexible N-terminal tails of H3 and H4, and N- and C-terminal tails of H2A and H2B (Loidl**,** 2004). A series of consecutive nucleosomes produces a 'beads on a string' structure or 10 nm fiber. A further level of compaction is the 30 nm fiber with six nucleosomes per turn in a solenoid arrangement (Kornberg and Lorch**,** 1999). The traditional picture of eukaryotic chromatin as a static and largely repressive functional state has, over recent years, changed to a more complex view of chromatin as a highly dynamic state that is essential for regulating cellular functions. Epigenetic regulation of developmental patterning and programming is now recognized to play critical roles in plant growth (Steimer *et al*, 2004). The dynamic properties of chromatin are mediated by multiprotein complexes with different functions that set marks overlying the stable information of the DNA (Arhinger**,** 2000; Lusser**,** 2002; Verbsky**,** 2001; Kadonaga**,** 1998). The most prominent factors that influence chromatin structure and function are enzymes that modify the histones and chromatin remodeling machines that utilize ATP (Lusser**,** 2002; Norton *et al***,** 1989; Alfrey *et al***,** 1964; Struhl *et al***,** 1998). Therefore, in addition to changes in the DNA sequence itself, that led to alteration in transcription rates, the epigenetic modifications mediated by affecting histones is a major mechanism regulating transcription (Steimer *et al***,** 2004).

Histones have been conserved during evolution. However, they are dynamically changed by post-translational modifications (Grunstein**,** 1992). These modifications include acetylation, methylation, phosphorylation, ubiquitination, glycosylation, ADP ribosylation, carbonylation, sumoylation and biotinylation (Loidl**,** 2004), that can all cause structural and functional rearrangements in chromatin and are therefore essential elements of the complex 'epigenetic histone code' (Figure 4).

A



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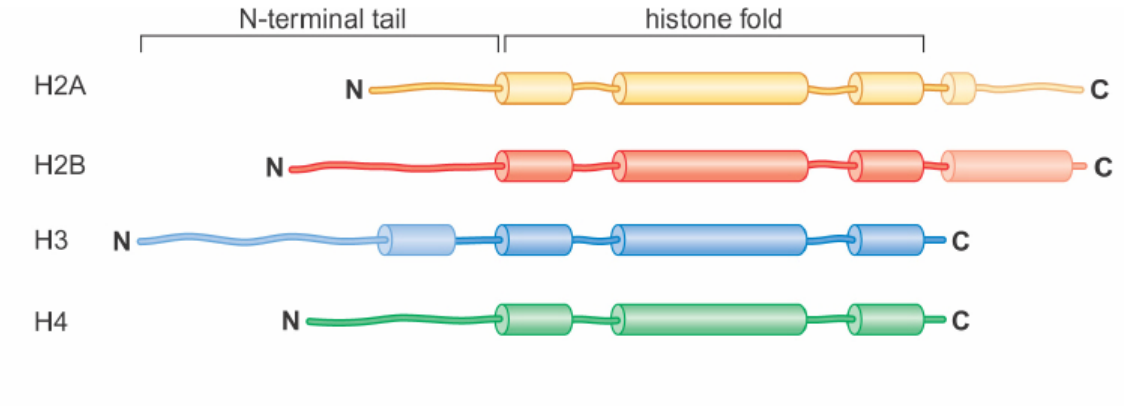


Figure 3. (A) Organization of a single nucleosome: 146bp of DNA wrapped twice around a histone octamer core consisting of H2A, H2B, H3 and H4. Linker DNA is 10~100bp. (B) Structural comparison of the four Histone proteins. The 'Histone fold' is common to all four proteins (Loidl, 2001).

Also, DNA methylation is one of the ways in that transcription can be regulated. There is ample evidence for an interrelation between DNA methylation and histone modifications (Volpe *et al***,** 2002). The relationships and hierarchy between DNA methylation, H3 methylation, H4 acetylation and heterochromatin assembly has been proposed to play a role in nucleolar dominance (Chen and Pikaard**,** 1997). As a result, a tentative model was proposed in that four key players act in a coordinated manner. Following DNA replication, maintenance DNA methyltransferase acts on chromatin with acetylated H4 lysine-16. DNA methylation precedes and governs H3 lysine-9 methylation. The chromatin remodeling factor DDM1 could finally trigger the deacetylation of H4 lysine-16 (Soppe *et al***,** 2002). Also, ATP-dependant chromatin remodeling factors use the energy derived from ATP hydrolysis to catalyze nucleosome mobilization, that is a net change in the position of the histone octamer relative to the DNA (Cairns**,** 1998). All these epigenetic phenomena work in conjunction to expedite transcription regulation.

(ii) Acetylation and deacetylation mechanisms

Acetylation and deacetylation of nucleosomal core histones has been an intensely investigated field in the recent years. Acetylation at the lysine residues on the amino terminal tail of histones neutralizes the charge of the histone tails, thereby reducing their affinity for DNA (Norton *et al***,** 1989). Consequently, histone acetylation alters nucleosomal conformation and makes it more accessible for the transcription regulatory proteins to contact the chromatin templates (Imhof *et al***,** 1997). Hypoacetylation on the other hand, is associated with transcriptionaly silent chromatin (Figure 5). These enzymes can be present and functional in the chromatin context ubiquitously, or may be specifically recruited by special repressor complexes (Ahringer**,** 2000). The enzymes

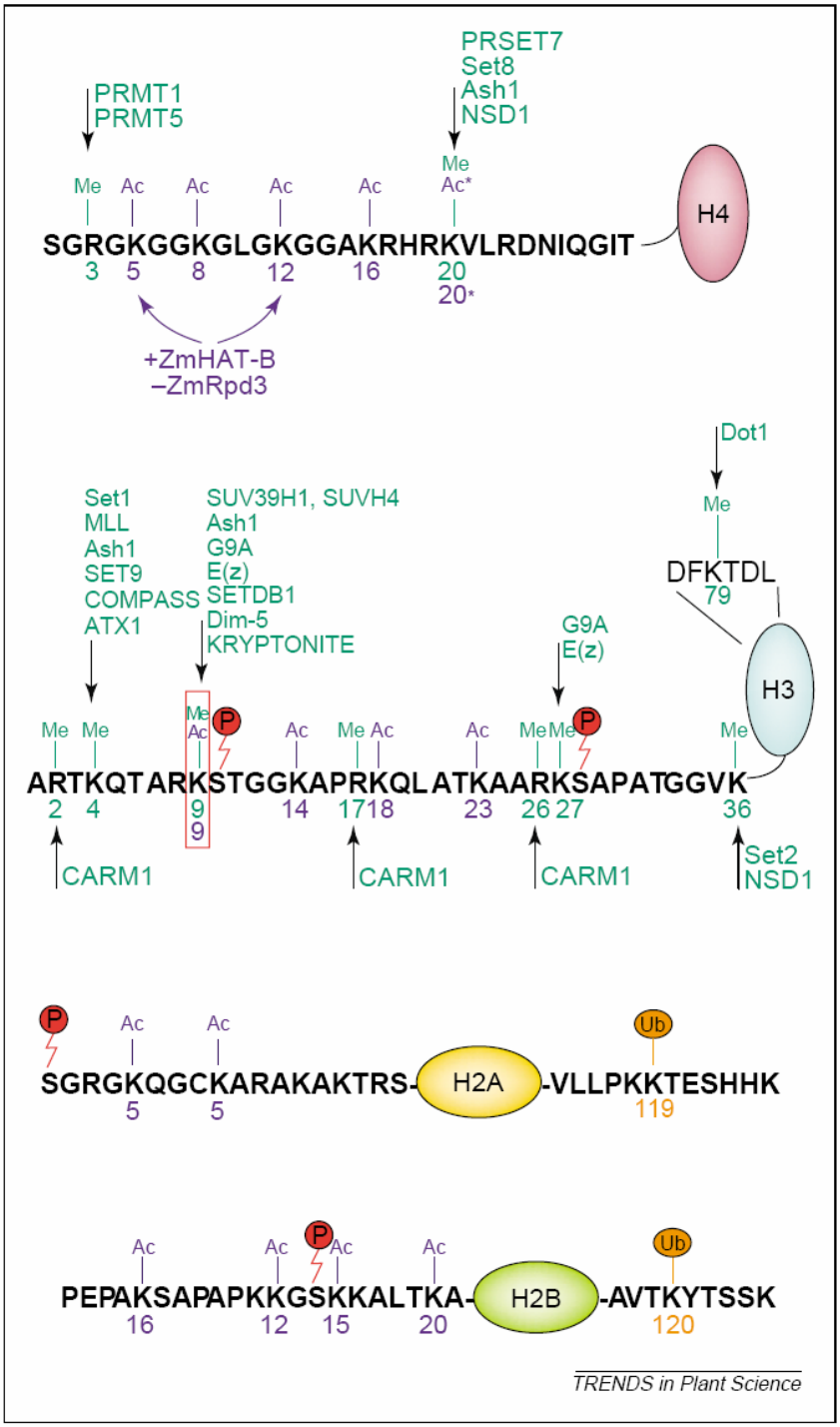

PRSET7
Set8
Ash1
NSD1
PRMT1
PRMT5
Me
Ac*
Me Ac Ac Ac Ac
SGRGKGGKGLGKGGAKRHRKVLRDNIQGIT
3 5 8 12 16 20
20*
H4
+ZmHAT-B
–ZmRpd3
Dot1
Me
DFKTDL
79
Set1
MLL
Ash1
SET9
COMPASS
ATX1
SUV39H1, SUVH4
Ash1
G9A
E(z)
SETDB1
Dim-5
KRYPTONITE
G9A
E(z)
H3
Me Me Me Ac P Ac Me Ac Ac Me Me P Me
ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVK
2 4 9 9 14 17 18 23 26 27 36
CARM1
CARM1
CARM1
Set2
NSD1
P Ac Ac Ub
SGRGKQGCKARAKAKTRS-
5 5
H2A
-VLLPKKTESHHK
119
Ac Ac P Ac Ac Ub
PEPAKSAPAPKKGSKKALTKA-
16 12 15 20
H2B
-AVTKYTSSK
120
TRENDS in Plant Science

Figure 4. Post-translation modification sites of Histone proteins which lead to epigenetic regulation. Core histones are targets for post-translational modifications at distinct amino acid residues. The modifications depicted are acetylation (Ac, purple), methylation (Me, green), phosphorylation (P, red) and ubiquitination (Ub, orange). Methylating enzymes identified to date include PRMT1 , CARM1 , PRMT5 , Set1 , Set2 , SET7/9 , SET8 , PR-Set7 , SUV39H1 , SUVH4 , SETDB1 , G9a , E(z) , KRYPTONITE , dim-5 , Ash1 , MLL , Dot1 , ATX1 and NSD1. In plants, ZmHAT-B has been shown to acetylate specifically lysines (K) 5 and 12 of H4, and the deacetylase ZmRpd3 can specifically deacetylate this distinct acetylation pattern. The plant-specific acetylation of lysine 20 of H4 is marked with an asterisk (p); methylation of H3 lysines 14, 18 and 23 has only been detected in plants and are not included in the cartoon. A red frame marks lysine-9 in H3, which can be modified by acetylation as well as methylation (Loidl, 2001).

that catalyze hyper acetylation are called histone acetyl transferases (HATs) and the ones that catalyze hypoacetylation or removal of acetyl groups from the core histones, are called histone deacetylases (HDACs). Pioneer studies about the classification and function of histone modifying enzymes have been conducted in yeasts, but plants and fungi have also emerged as significant model systems (Graessle**,** 2001; Lusser**,** 2001). The molecular mechanism of action of the HATs and HDACs is illustrated in Figure 3.

(iii) Classification of HATs and HDACs

By sequence analysis, four distinct families of HATs can be distinguished: (i) the GNAT-MYST family; (ii) the p300/CBP coactivator family; (iii) the TAFII250-related family; and (iv) the nuclear receptor coactivator family that is present in vertebrates but not in plants or fungi (Pandey *et al***,** 2002). The HDACs on the other hand, have been classified into two groups in all eukaryotes: (i) members of the ATHDA1/HDA superfamily (Hu *et al***,** 2000; Aravind *et al***,** 1998; Groezinger *et al***,** 1999) and (ii) members of the sirtuin family related to yeast SIR2 (Imai *et al***,** 2000). In contrast to other eukaryotes, plants contain the HD2-type deacetylases, a plant specific class that is unrelated to the other HDAC classes and forms the third HDAC class in plants (Lusser *et al***,** 1997). The HD2 class has arisen from a series of gene duplications and diversions from parent populations. These proteins are thought to have taken over the function of the SIR proteins that are under-represented in plants (Dangl *et al***,** 2001). Due to their unique disposition, this group of proteins forms a very interesting subject for study, with regards to what might be their functional role.

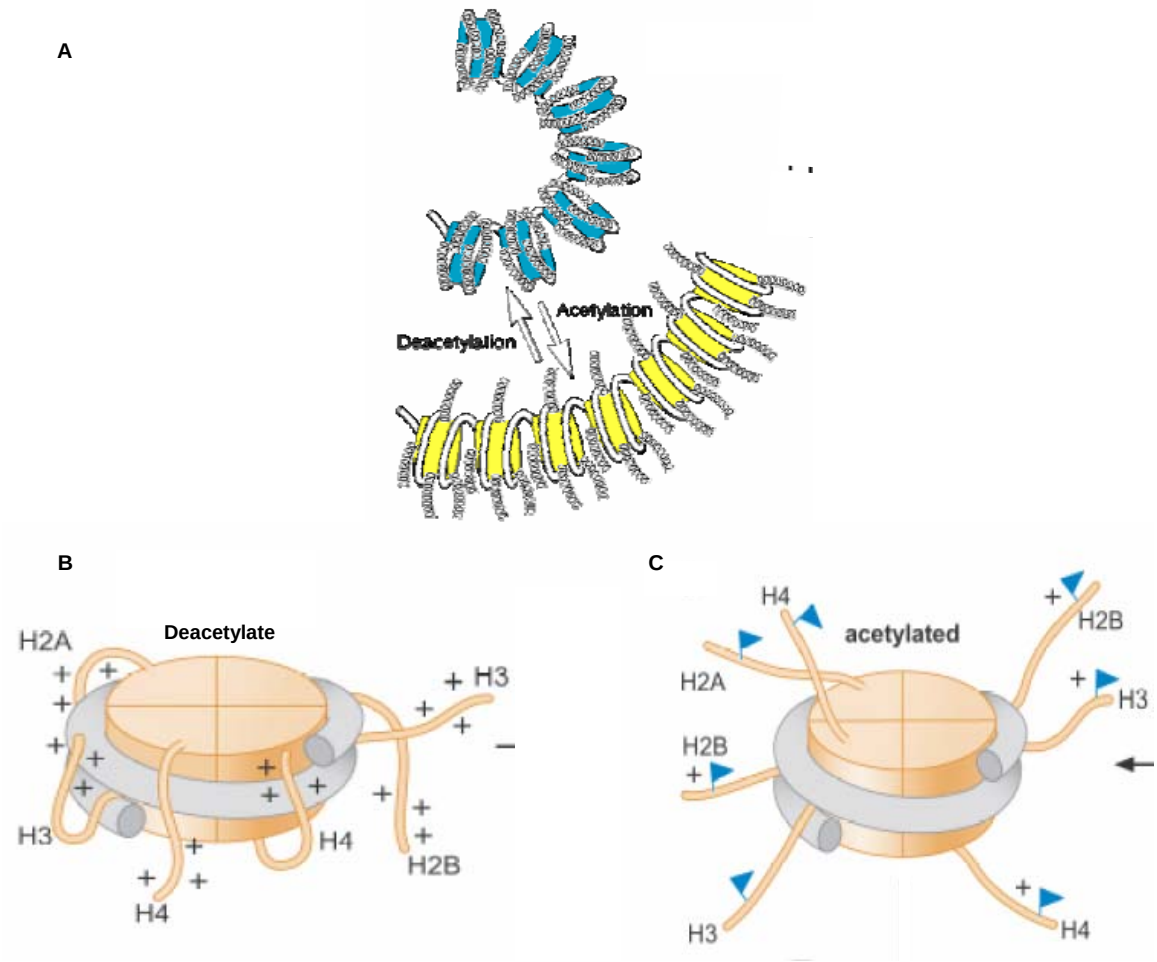


Figure 5. (A) Reversible transition of the chromatin from acetylated to deacetylated state. DNA is negatively charged and histone are positively charged, which leads to the strong histone–DNA interactions. When acetyl groups are added to histone tails, the Histones gain negative charge decreasing their affinity for DNA. Thus the DNA falls of the histone core and is exposed to transcription machinery. (B) Compacted, condensed form or chromatin when DNA is tightly wrapped around the histone octamer, leaving little space for transcriptional activation. (C) Acetylation of N-terminal histone tails leading to transcriptional activation.

(iv) HDACs

Histone deacetylases were have been reported in a different biological systems, including several mammalian cell lines and tissues (Taunton et al., 1996), fungi (Waterborg *et al***,** 1982), plants (Sendra *et al***,** 1988) and yeast (Carmen *et al***,** 1999). Several histone deacetylases have been cloned and sequenced (Yang and Seto**,** 1997; Taunton *et al***,** 1996). Histone deacetylase (HDAC) genes have been isolated and characterised in a number of eukaryotes including humans, mice, chicken (*Gallus gallus*), fruit-fly (*Drosophila melanogaster*) and the yeast *Saccharomyces cerevisae* and *Arabidopsis thaliana* (Yang and Seto**,** 2003). It is evident that in all of these organisms, the HDACs form a highly conserved protein family, that encodes isoforms of the enzyme that differ extensively in substrate specificity, intracellular localization and post-translational modification (Khochbin and Wolffe**,** 1997).

Three mammalian HDAC isoforms (HDAC1-3) and five yeast HDACs had been identified and several of these were biochemically characterized (Rundlett *et al***,** 1996). These HDACs, taken together with the prokaryotic enzymes acetylspermine deacetylase (ASD) and acetoin utilization protein (acuC), constitute a deacetylase superfamily (Leipe and Landsman**,** 1997). In yeast, members of this superfamily can be subdivided into two classes based on size and sequence considerations, as well as the observation that AtHDA1p and Hda1p function in biochemically distinct complexes (Robyr *et al***,** 2002). The first class (I) consists of AtHDA1p, Hos1p, and Hos2p, and the second class contains Hda1p (Fischle *et al***,** 1999). Similarly, in mammals, HDAC1, HDAC2, and HDAC3 conform to class I criteria and three human class II HDAC enzymes, HDAC4, HDAC5, and HDAC6 were found to deacetylate all four core histones *in vitro* (Groezinger *et al***,** 1999; Fischle *et al***,** 2001). HDAC3, that is the smallest mammalian isoform, can be encoded by at least three different splice variants (Yang *et al***,** 1997) and the

human HDAC1/HDAC2 proteins are very similar at the amino acid level and are about 60 residues longer than HDAC3 at the C-termini. This extra domain is quite repetitive and is rich in charged amino acids. An even larger member of the HDAC/AtHDA1 family is the HDA1-p75 component of the HDA histone deacetylase complex in yeast nuclei (Carmen *et al*,1996; Rundlett *et al*, 1996). HDA1 is functionally distinct to yeast AtHDA1, that is less sensitive to TSA and contained in the HDB histone deacetylase complex (Rundlett *et al*, 1996), and HDA1 is not involved in transcriptional regulation of those genes controlled by AtHDA1 (Rundlett et al, 1998). Based on coimmunoprecipitation experiments, these HDACs are not associated with the previously identified NRD and mSin3A complexes that contain HDAC1 and HDAC2, and therefore are likely to be components of distinct complexes that perform alternate functions (Rundlett *et al*, 1996). The picture appears to be less complicated in *Drosophila,* that has only two known orthologues of AtHDA1: d-AtHDA1 (De Rubertis *et al*, 1996), also known as dHDAC1, and dHDAC3 (Johnson *et al*, 2002). In plants, different *HDAC* genes have been identified and classified into three distinct gene families (Pandey *et al*, 2002). The first family, named the *HDA* gene family, contains members related to the yeast sequences AtHDA1 and HDA1 (Rundlett *et al*, 1996; Taunton *et al*, 1996; Rossi *et al*, 1998; Lechner *et al*, 2000). This family is further divided into three classes based on their degree of homology with AtHDA1 (class I), HDA1 (class II), or a third group of sequences phylogenetically distinct from the first two classes. The members of the second family of plant *HDACs*, termed the *SRT* family, are related to yeast Sir2 (Imai *et al*, 2000; Frye, 1999). In contrast to other eukaryotes, plants contain a third family of enzymes, the nucleolar-phosphoproteins HD2 (*HDT* gene family), that appear to be plant-specific (Lusser *et al*, 1997; Dangl *et al*, 2001).

There are three regions of very highly conserved amino acid residues (histidines, aspartates and glycines) that are shared by all members of the HDAC/AtHDA1 family, irrespective of the highly divergent nature of the C-terminal regions (Dangl *et al,* 2001). These regions are presumed to form part of the active site, since the mutation of conserved aspartate and histidine residues to asparganine or alanine could abolish most, if not all, histone deacetylase activity of HDAC1 (Hassig *et al,* 1998). In addition, the interactions with the corepressor proteins mSin3A and RpAp48 were lost in all HDAC1 mutants except histidine 141 (Dangl *et al,* 2001). This suggests that some of the conserved residues are involved in catalysis and others in maintaining the interactions between HDAC1 and other members of the corepressor complex.

(v) HD2-type histone deacetylases

The HD2-type HDAC gene family was first discovered in maize as a heavy molecular weight acidic nucleolar protein that could be activated/deactivated by phosphorylation (Lusser *et al,* 1997). Subsequently, this family was uncovered in *Arabidopsis thaliana* with four genes comprising this group (Wu *et al,* 2000). A subsequent search with the use of the PSI-BLAST program, that incorporated a conservation profile of two plant histone deacetylases, revealed statistically significant (the probability of a random match was below 10^{-3} in each case and was as low as 5×10^{-7} for FKB1_SPOFR) sequence similarity to insect proteins identified as FKBP family peptidyl-prolyl cis-trans isomerases (PPIases) and to a trypanosomal RNA-binding protein (Dangl *et al,* 1997; Altshul *et al,* 1997). The conserved region included an NH_2-terminal domain found in each of these proteins and was distinct from the PPIase domain and the database of expressed sequence tags (ESTs) with these sequences resulted in the characterization of a novel family, that includes proteins from plants, yeast, and two parasitic apicomplexans, *Toxoplasma gondii* and *Cryptosporidium parvum*. Thus, this new protein family, HD2, for that

histone deacetylase activity was predicted, appeared to be widespread among eukaryotes, although the absence of members from vertebrates was conspicuous.

Inspection of the HD2 family alignment shows a number of conserved hydrophobic positions as well as two conserved polar residues (Dangl *et al*, 2001), namely, an invariant aspartic acid and a histidine, that is replaced by an arginine in the trypanosomal RNA-binding protein Nopp44/46 and in the yeast FKBP (Alnemri *et al*, 1994). It appears that the invariant aspartic acid is the nucleophile involved directly in lysine deacetylation, that may be facilitated through a charge relay system with the conserved histidine (arginine). Multiple alignment-based secondary structure prediction indicated an all-beta structure for the histone deacetylase domain, without detectable similarity to any known protein fold. The domain organization of the (predicted) histone deacetylases of the HD2 family is of particular interest. In addition to the deacetylase domain, they all contain acidic stretches of various length, that may be diagnostic of nucleolar localization, or of association with basic tails of histones. Besides HD2, nucleolar localization has been shown for the trypanosomal RNA-binding protein Nopp44/46 and for one of the yeast FKBPs, whereas the *Spodoptera* FKBP46 is a nuclear protein that binds DNA *in vitro*. The presence of a histone deacetylase and a PPIase in one protein as distinct domains makes functional sense, because in order to be targeted to the specific sites of their action on chromatin, histone deacetylases form complexes with a variety of chromatin-associated proteins. The chaperone-like activity of FKBPs may be required for the proper assembly of such complexes. FK506 binding proteins (FKBPs) are a family of distinct proline isomerases that are targets for a number of clinically important immunosuppressive drugs (Martin *et al*, 1995). Members of both families catalyze cis/trans isomerization of peptidyl-prolyl bonds that can be a rate-limiting step during protein folding in vitro and in vivo. These proteins are known to play a

role in stress response pathways in yeast. Alternatively, the FKBP domain of HD2 proteins may be involved in changing the conformation of proline-rich segments in histone COOH-terminal tails, perhaps concomitantly with deacetylation. Additionally, the zinc finger structure at the COOH-terminal of six of the eight HD2 proteins is unique to plant proteins and cannot be observed in the related FKBPs or trapanosomal Nopp44/46 proteins. Because of the nature and spacing of the zinc chelating residues ($CX_2CX_{2-4}FX_5LX_2HX_{3-5}H$), they belong to the TFIIIA-type zinc fingers that are involved directly in DNA binding (Takatsuji**,** 1998). Apart from DNA binding, the zinc fingers can also mediate protein-protein interactions, where individual fingers are sufficient to confer interaction (Mackay and Crossley**,** 1998). This is well in line with previous results demonstrating that *Zm*HD2a was isolated in a 400KDa protein complex.

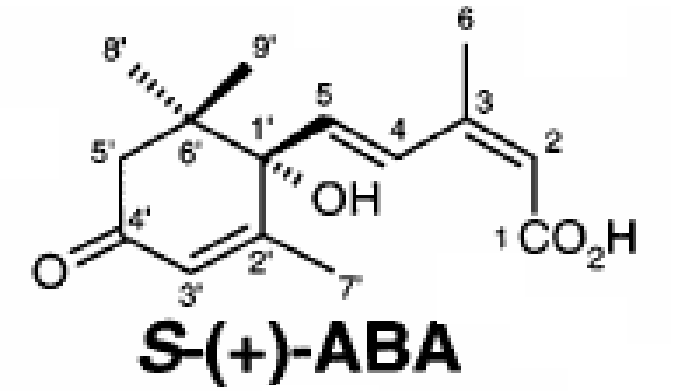
In previous studies conducted on the HD2 gene family, one of the members, AtHD2A, was able to mediate transcriptional repression when targeted to the promoter of a reporter gene (Wu *et al***,** 2000). When *AtHD2A* was knocked out in *Arabidopsis thaliana* using antisense RNA, the seed development was aborted, indicating that these genes might play a role in seed development. Isolation of HD2 proteins from maize embryos by Lusser *et al* (2000). implicates that the HD2 family might play a role in the regulation of gene expression essential in embryo development. Additionally, plant growth and yield potential are significantly influenced by various abiotic stresses such as drought, salinity, and cold. A common feature found in plants grown under water stress is a transient increase in the level of the phytohormone abscisic acid (ABA) (Finklestein and Rock**,** 2002). ABA plays important roles in many aspects of plant growth and development such as embryo maturation, seed dormancy, germination, stomatal aperture, as well as sugar signaling and regulation of expression of stress-responsive genes (Gazzarrini and McCourt**,** 2001). It is quite interesting to investigate the role of HDACs in

hormone signaling because little information is available on the involvement of chromatin modifiers in stress signaling.

(vi) ABA-a seed maturation and stress signaling molecule

When plants transition from heterotrophic to autotrophic growth, the regulation of this phase shift is very tight. Plants have evolved protective mechanisms to ensure their survival under adverse environmental conditions during this transition (Albinsky *et al*, 1999). Also, plants have developed intrinsic defense mechanisms to tolerate oppressive environmental conditions. The plant hormone abscisic acid (ABA) is the forerunner stress responsive agent that plants produce under these extreme situations. ABA regulates many aspects of plant development and physiology, including seed maturation and dormancy, as well as responses to environmental stress conditions, such as drought, salinity, and low temperature (Fedoroff**,** 2002; Finkelstein *et al***,** 2002; Himmelbach *et al***,** 2003). Components of the ABA signal transduction pathways (Giraudat**,** 1995) range from early signaling intermediates such as G proteins and protein kinases/phosphatases, to late stage transcription factors and RNA metabolic proteins (Cowan**,** 2001). Whereas some components appear to be regulators of multiple ABA responses, few are required for all responses, suggesting that ABA responses in different cell types or at various developmental stages may differ to certain extent.

A



B

Name	*Arabidopsis* protein entry code	BAC location	Predicted cell localization
Zeaxanthin epoxidase			
AtZEP	At5g67030	K8A10.10	Chloroplast
9-*cis*-epoxycarotenoid dioxygenase*			
AtNCED1	At3g63520	MAA21.150	Cytoplasm
AtNCED2	At4g18350	F28J12.10	Cytoplasm
AtNCED3	At3g14440	MOA2.4	Chloroplast
AtNCED4	At4g19170	T18B16.140	Chloroplast
AtNCED5a	At1g78390	F3F9.10	Cytoplasm
AtNCED5b	At1g30100	T2H7.10	Chloroplast
AtNCED6	At3g24220	MUJ8.12	Mitochondria
Abscisic aldehyde oxidase			
AAO1	At5g20960	F22D1.130	Cytoplasm
AAO2	At3g43600	F22J12.40	
AAO3	At2g27150	F20F1.2	Cytoplasm
AAO4	At1g04580	T1G11.17	Cytoplasm
Xanthine dehydrogenase	At4g34900	F11I11.140	
Xanthine dehydrogenase-like protein	At4g34890	F11I11.130	

Figure 6. (A) Structure of ABA, a C-15 compound (Finkelstein and Rock, 2002). (B) Table displaying the different enzymes essential during de novo ABA synthesis in plants (Bray, 2002).

(vii) Chemical nature of ABA

ABA is a sesquiterpenoid ($C_{15}H_{20}O_4$) with one asymmetric, optically active carbon atom at C-1' (Finklestein and Rock**,** 2002). The naturally occurring form is *S*-(+)-ABA (Figure 6). ABA biosynthesis branches from the carotenoid biosynthetic pathway. Many of the genes involved in ABA biosynthesis are members of multi-gene families. Members have identical functions the expression of the genes may control ABA biosynthesis in response to different environmental stimuli or developmental cues and permit regulation of ABA biosynthesis (Holdsworth *et al***,** 1999) in different organs or tissues.

(viii) ABA biosynthetic mutants

The first-described ABA-deficient mutant of Arabidopsis emerged out of an allelic series of mutations, *aba* (now designated *aba1*). These mutant forms were isolated from a suppressor screen of the non-germinating gibberellin- deficient *ga1* mutant (Koornneef *et al***,** 1982). The different enzymes involved in ABA biosynthesis are depicted in Figure 6. The transposon-tagged, non-dormant wilty mutant of *Nicotiana plumbaginifolia* (*Npaba2*) was shown to be orthologous to Arabidopsis *aba1* (Marin *et al***,** 1996), from that the *aba1* gene was first identified. The reduction in AtZEP transcript levels was identified as the molecular basis for two *aba1* mutant alleles (Audran *et al***,** 2001). In seeds of Arabidopsis and tobacco, the *ABA1/NpABA2* mRNA level peaks around the middle of development when ABA levels begin to increase. *NpABA2* overexpression in transgenic plants led to increased mRNA levels that increased ABA levels in mature seeds and delayed germination, while antisense *NpABA2* expression resulted in a reduced ABA abundance in transgenic seeds and rapid seed germination (Frey *et al***,** 1999). The rice *OsABA1* gene is an orthologue of *ABA1* since a transposon-tagged *Osaba1* mutant was viviparous, wilty, and ABA-deficient (Agrawal *et al***,** 2001). Two additional

Arabidopsis ABA biosynthetic mutants *aba2, aba3* (Leon-Kloosterziel *et al***,** 1996) were identified. Screens related to hormones, sugar, salt, or stress has led to discovery of more alleles including *aba1, aba2* and *aba3*. Protein extracts from *aba2* and *aba3* plants display a reduced ability to convert xanthoxal to ABA (Schwartz *et al***,** 1997). The *aba2* mutant is blocked in the conversion of xanthoxal to ABA-aldehyde and *aba3* mutant is impaired in the conversion of ABA-aldehyde to ABA (Schwartz et al**,** 1997). The *aao3* mutant is ABA-deficient in leaves and has no detectable AO (Aldehyde Oxidase) activity, but seed dormancy is nearly normal, unlike all other ABA-deficient mutants (Akaba *et al.*, 1998). The epoxy-carotenoid cleavage enzyme (termed NCED, for 9- *cis*-epoxy-carotenoid dioxygenase) is considered to be the key ABA biosynthetic step, in terms of potential regulation by environmental and developmental cues (Chernys et al**,** 2000). Ectopic expression of a tomato *NCED* cDNA causes overproduction of ABA in tomato and tobacco, that suggests that NCED is rate limiting for ABA biosynthesis (Thompson *et al***,** 2000b). Increased seed dormancy has been reported for transgenic tobacco expressing the bean *PvNCED1* (Akaba**,** 2002). By contrast, drought-intolerant phenotype was evidenced as a result of antisense suppression and T-DNA knockout lines of *AtNCED3*. It is evident that the *de novo* production of ABA is essential for proper seed development and germination. Additionally, it is also a requirement for effective stress signaling in plants when they are faced with environmental challenges.

(ix) ABA signaling

Once ABA is produced, how does it expedite its physiological effects? Hormone response mutants have traditionally been defined as individuals that resemble mutants with defects in hormone biosynthesis yet can not be restored to a Wild type phenotype by addition of the relevant hormone (Finklestein and Rock**,** 2002). The genetic screens and selections that have

been used to date include production of non-dormant seeds (Koornneef *et al*, 1982), loss or gain of sensitivity to ABA at germination (Koornneef *et al*, 1984; Finkelstein, 1994; Cutler *et al*, 1996), seedling growth (Lopez-Molina and Chua, 2000), root growth (Himmelbach *et al*, 1998), misexpression of reporter genes (Ishitani *et al*, 1997; Foster and Chua, 1999; Delseny *et al*, 2001) and screens for suppressors or enhancers of GA-deficient non-germinating lines or *ABA-INSENSITIVE* (*ABI*) lines (Steber *et al*, 1998; Beaudoin *et al*, 2000; Ghassemian *et al*, 2000). Additional mutants have been isolated with defects in responses to multiple signals, including ABA, via non-ABA-based screens such as salt-resistant germination (Quesada *et al*, 2000), sugar-resistant seedling growth or gene expression (Arenas-Huertero *et al*, 2000; Huijser *et al*, 2000; Laby *et al*, 2000; Rook *et al*, 2001), or defects in auxin, brassinosteroid or ethylene response (Wilson *et al*, 1990; Alonso *et al*, 1999; Ephritikhine *et al*, 1999; Li *et al*, 2001). Broadly speaking, the ABA response mutants can be categorized into two groups. ABA hypersensitive mutants include mutations in protein farnesylation agent *ERA1,* Inositol signaling agent *FRY1* and RNA processing and turnover agents *ABH1*, *HYL1*, *SAD* (Finklestein and Somerville, 1990). ABA insensitive mutants include phosphatases, *ABI1* and *ABI2* and transcription factors *ABI3*, *ABI4* and *ABI5*. Another set of mutants isolated in a screen for ABA resistant root-growth was the *growth control by ABA* (*gca1-gca8*) mutants (Finklestein *et al*, 2002).

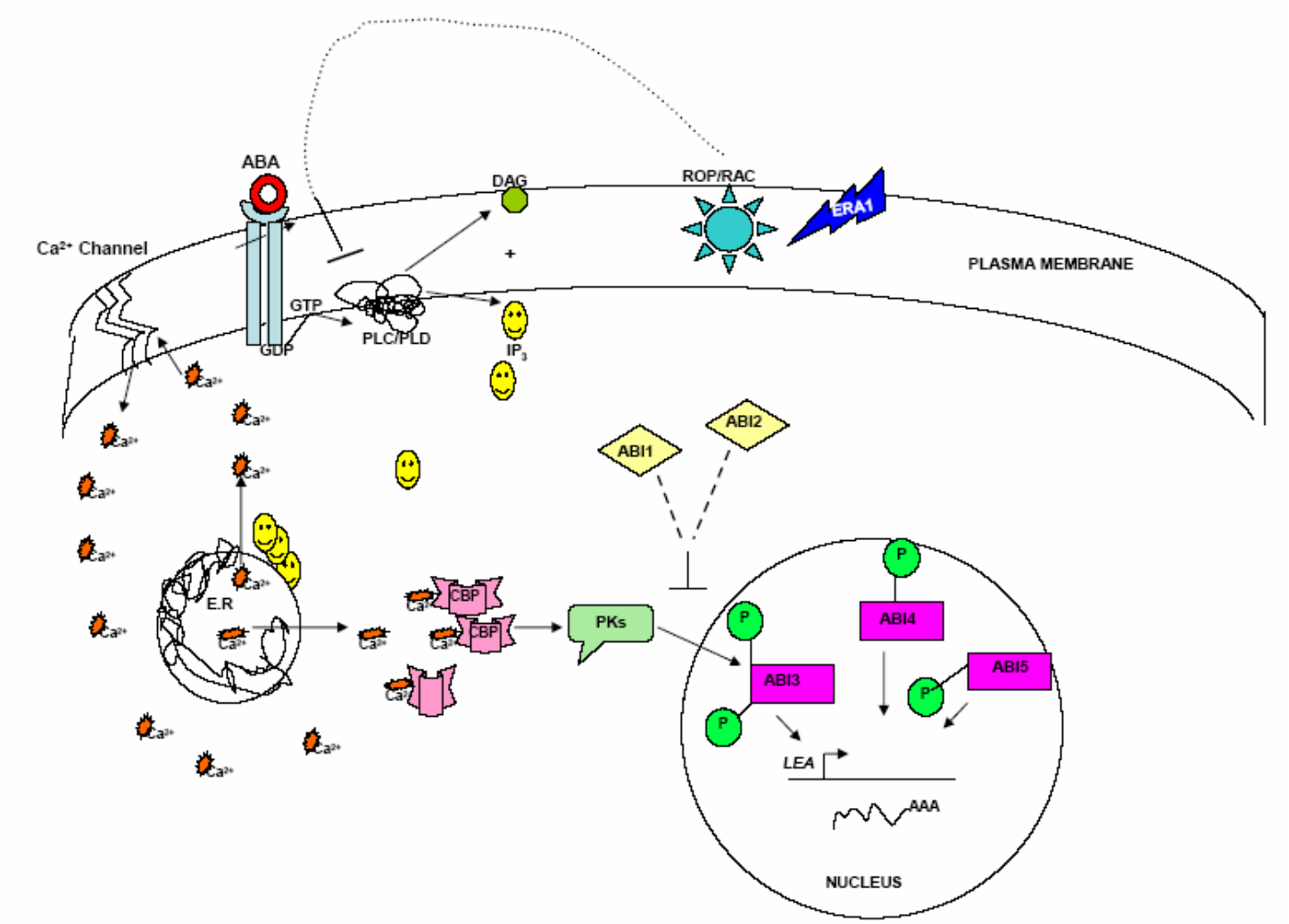

ABA
DAG
ROP/RAC
ERA1
Ca²⁺ Channel
PLASMA MEMBRANE
GTP
GDP
PLC/PLD
IP3
ABI1
ABI2
E.R
CBP
PKs
P
ABI4
ABI3
ABI5
LEA
AAA
NUCLEUS

Figure 7. Model for ABA mediated signal cascade in Arabidopsis. Once ABA binds to its receptor (putative) on the membrane, which is a G-protein coupled receptor, it induces the exchange for a GTP molecule. This event activates the PLD/PLC enzymes which produce DAG and IP3. Subsequently, IP3 travels into the cytosol and activates the ER Ca2+channel. Ca2+release into the cytosol generates more Ca2+channels to open from the plasma membrane. This intracellular Ca2+is bound by Calcium binding proteins (CBPs), which can activate the protein kinases (PKs). The activated PKs can travel to the nucleus and phosphorylate the ABI transcription factors, thus activating them, leading to LEA synthesis. ERA1 inhibits this cascade by recruiting the Rop/Rac GTPases to the membrane. Also, the ABI1/ABI2 proteins de-phosphorylate the phosphoproteins (PKs?) to inhibit activation of nuclear ABI transcription factors. The arrows indicate activation an the perpendicular bars indicate repression.

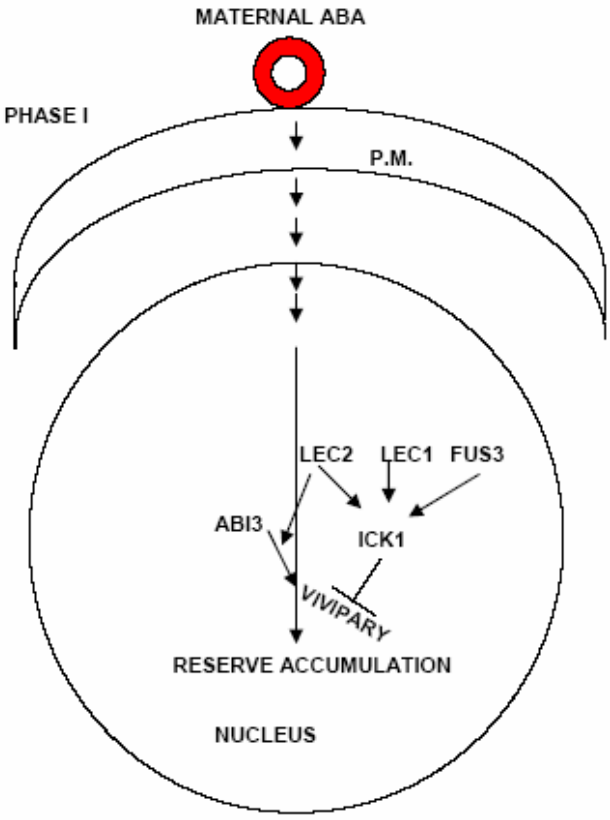
(x) Known signaling cascade

Biochemical and pharmacological studies have shown that early events in ABA signaling involve participation of GTP binding proteins, phospholipases (Hirayama *et al*, 1995), protein kinases (Cadenas *et al*, 1999) and phosphatases (Gosti *et al*, 1999). Downstream signaling includes transcription factors that expedite signals at the development level. Figure 7 illustrates is a schematic of how the signaling might be regulated at various steps. In the plasma membrane, a putative ABA receptor has been shown to induce PLD (Phospholipase D) activity (Gampala *et al*, 2001; Fan *et al*, 1997; Katagiri *et al*, 2001). Also, transgenic studies have shown that the small GTPase *Rop6/AtRac1* can inhibit ABA effects on actin cytoskeleton reorganization in guard cells (Hwang *et al*, 2001), that might indicate G-protein nature for this putative receptor (Hong *et al*, 1997). After contact with ABA, this receptor will activate PLD/PLC (Phospholipase C) activity to produce DAG (Diacyl glycerol) and IP_3 (Inositol triphosphate). IP_3 will induce Ca^{2+} release from intracellular stores and this will led to Ca^{2+} induced Ca^{2+} release from the plasma membrane channels (Hamilton *et al*, 2000). This Ca^{2+} elevation will led to the activation of protein kinases that can travel into the nucleus and phosphorylate transcription factors such as ABI4 and ABI5 (Knetsch *et al*, 1996). Both the ABI4 and ABI5 gene products contain Ser/Thr-rich domains that could be sites of phosphorylation (Finkelstein *et al*, 1998; Finkelstein and Lynch, 2000) and recent studies have demonstrated that ABI5 protein is stabilized by ABA induced phosphorylation (Lopez-Molina *et al*, 2001). This could further led to the activation of these transcription factors, thus allowing development signals to proceed. The ERA1 protein acts as a negative regulator of this process, by adding farnesyl groups to the GTPases that tether them to the membrane, thus recruiting them to the G protein coupled receptors (Cutler *et al*, 1996). Additionally, phosphatases such as ABI1 (Gosti *et al*, 1999; Hagenbeek *et al*, 2000) and ABI2

might dephosphorylate protein kinases thereby inactivating them, leding to inactivation of ABI4 and ABI5. Although either ABI4 or ABI5 could be a substrate for dephosphorylation by the ABI PP2Cs, consistent with a negative regulatory role for the PP2Cs, neither interacts with ABI1 in a two-hybrid assay (Nakamura et al**,** 2001) despite showing strong genetic interactions as digenic mutants (Finkelstein**,** 1994).

(xi) ABA signaling in maturing seed germination and growth

When developing embryos undergo a transition into the maturation phase (Holdsworth *et al***,** 1999), they arrest growth by inhibiting cell division and inducing cell enlargement and begin to accumulate storage reserves (Figure 8). This growth phase transition is correlated with a subsequent increase in seed ABA content that appears to be required for cell cycle arrest at the G1/S transition (Levi *et al***,** 1993; Liu *et al***,** 1994). *LEC* gene function is required to prevent premature germination at the end of the cell division phase of embryogenesis (Raz *et al***,** 2001). *LEC* works in conjunction with cyclin-dependent kinase inhibitors such as ICK1. This level of control is achieved by the maternal peak of ABA in the first phase of maturation (Karssen *et al***,** 1983). In the second phase of maturation, the embryonic peak of ABA allows the LEA (late embryogenesis abundant) proteins to accumulate. These signals induce accumulation of protectants such as small hydrophilic proteins, sugars, proline, and glycine-betaine, that serve as storage reserves and prepare the seed for desiccation (Bray, 2002). ABI3, ABI4 and ABI5 are essential for maintaining the dormancy of the seed and LEA synthesis. Ectopic expression of either *ABI3* or *ABI4* results in ABA hypersensitivity of vegetative tissues, including ABA-inducible vegetative expression of several "seed-specific" genes, that is partly dependent on increased *ABI5* expression (Parcy *et al***,** 1994; Söderman *et al***,** 20 00). Additionally, ABI3 and


MATERNAL ABA
PHASE I
P.M.
LEC2
LEC1
FUS3
ABI3
ICK1
VIVIPARY
RESERVE ACCUMULATION
NUCLEUS

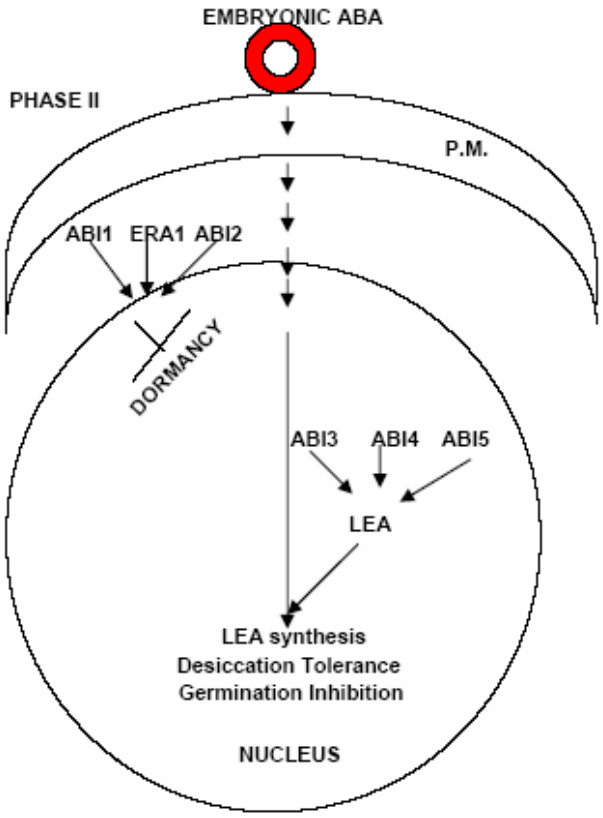

EMBRYONIC ABA
PHASE II
P.M.
ABI1
ERA1
ABI2
DORMANCY
ABI3
ABI4
ABI5
LEA
LEA synthesis
Desiccation Tolerance
Germination Inhibition
NUCLEUS

Figure 8. Model for ABA signaling during embryo-development. Left; Phase I of seed maturation when ABA is of maternal origin. ABA signals travel through the plasma membrane and cytosol and reach the nucleus probably via a MAP Kinase like pathway. Once in the nucleus, essential transcription factors like LEC, which control cell cycle are activated. Also, reserve accumulation promoting actors like ABI3 are activated. Right; Phase II of seed maturation is initiated by a second peak of ABA contributed by the embryo. This time, nuclear transcription factors ABI4 and ABI5 are activated in addition to LEC1 and ABI3. These factors promote LEA synthesis and desiccation tolerance. This dormancy property is down-regulated by ABI1/ABI2 and ERA1 as they disrupt the signals from reaching the nucleus. The arrows indicate activation an the perpendicular bars indicate repression.

ABI5 display direct and synergistic interactions in two-hybrid analyses in yeast and transient reporter activation assays in rice protoplasts (Hobo *et al***,** 1999; Gampala *et al***,** 2001; Nakamura *et al***,** 2001).

The commitment to germinate is also controlled by antagonistic interactions between ABA and gibberellins, ethylene, and brassinosteroids (BR) (Finklestein *et al***,** 2002). The ability to induce ABI5 accumulation is strongly correlated with maintenance of desiccation tolerance in these seedlings. ABA, the induced ABI5, and potentially other interacting factors may prevent the loss of desiccation tolerance by delaying escape from phase two of germination under conditions of low moisture (Lopez-Molina *et al***,** 2001). Although the precise roles of the *ABIs* in regulating lateral root growth are not understood, it is noteworthy that *ABI5* is specifically expressed in root tips from emergence onward (Brocard *et al***,** 2002). A putative model for the ABA signal cascade during this phase is presented in Figure 9.

ABI3 expression is localized to the meristem and appears to regulate vegetative quiescence processes, plastid differentiation, and floral determination (Rohde *et al***,** 1999; Kurup *et al***,** 2000). During stress imposition upon plants, there is a concomitant increase in ABA and its signaling molecules. Many of these encode proteins that are structurally similar to some of the LEA proteins that accumulate during the acquisition of desiccation tolerance in seeds, while others encode proteases, presumed chaperonins, enzymes of sugar or other compatible solute metabolism, ion and water-channel proteins, and enzymes that detoxify active oxygen species (Bray**,** 2004). Drought signals and osmotic shock induce ABA synthesis in the plant system and this has direct effects on stomatal aperture in addition to cell development ABA activates rise in intracellular Ca^{2+} that led to activation of S-type anion channel activity that ultimately led to the de-polarization of the guard cells (Hwang *et al***,** 2001). The series of chemical events that take place upon ABA activation are diagramed in Figure 10.

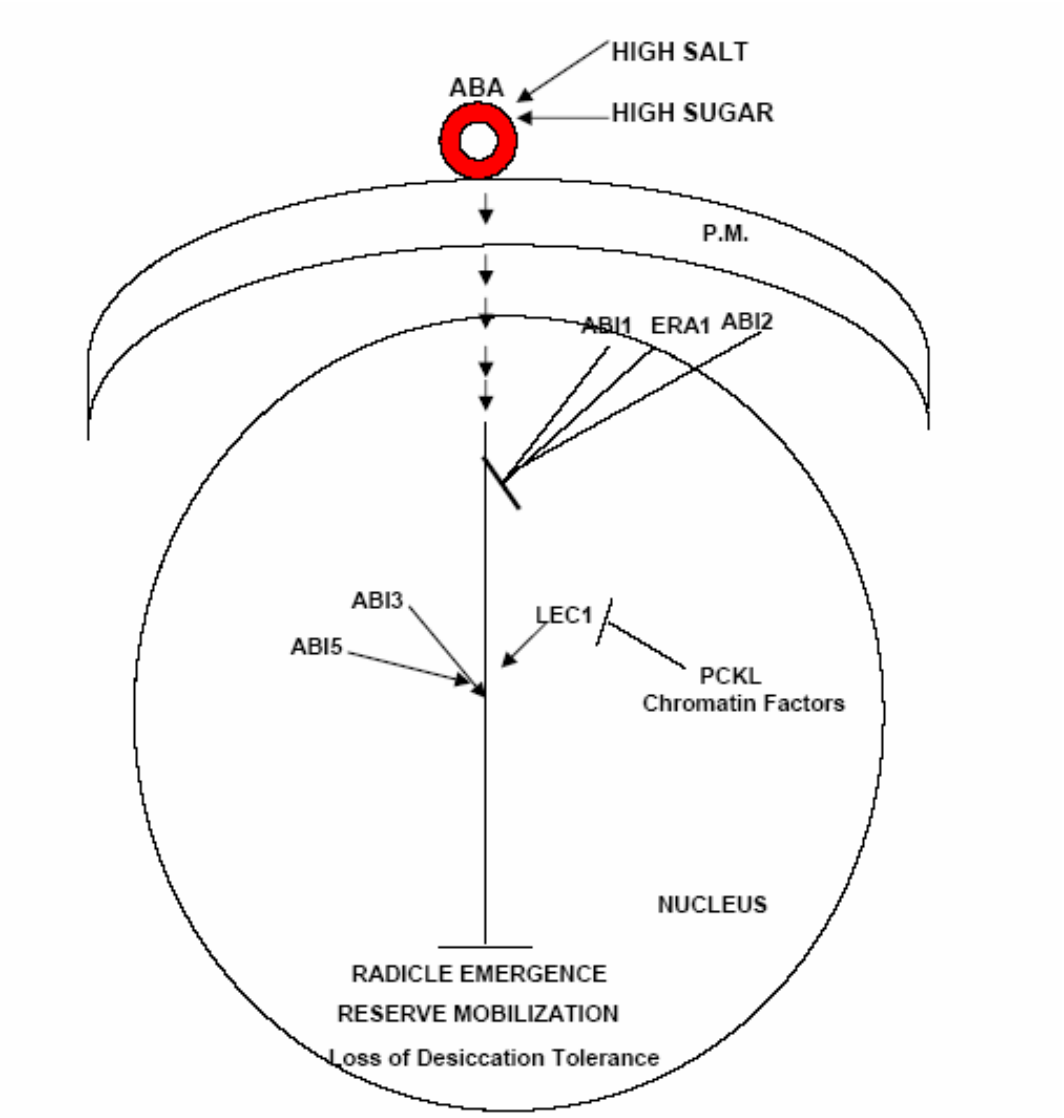

HIGH SALT
ABA
HIGH SUGAR
P.M.
ABI1
ERA1
ABI2
ABI3
LEC1
ABI5
PCKL
Chromatin Factors
NUCLEUS
RADICLE EMERGENCE
RESERVE MOBILIZATION
Loss of Desiccation Tolerance

Figure 9. Model for ABA signaling during germination-post germination phase. During this phase transition, ABA signaling has to be down-regulated to allow germination, the PCKL (PcG proteins) are known to repress LEC1 in non-seed maturation growth stages. The arrows indicate activation and the perpendicular bars indicate repression.

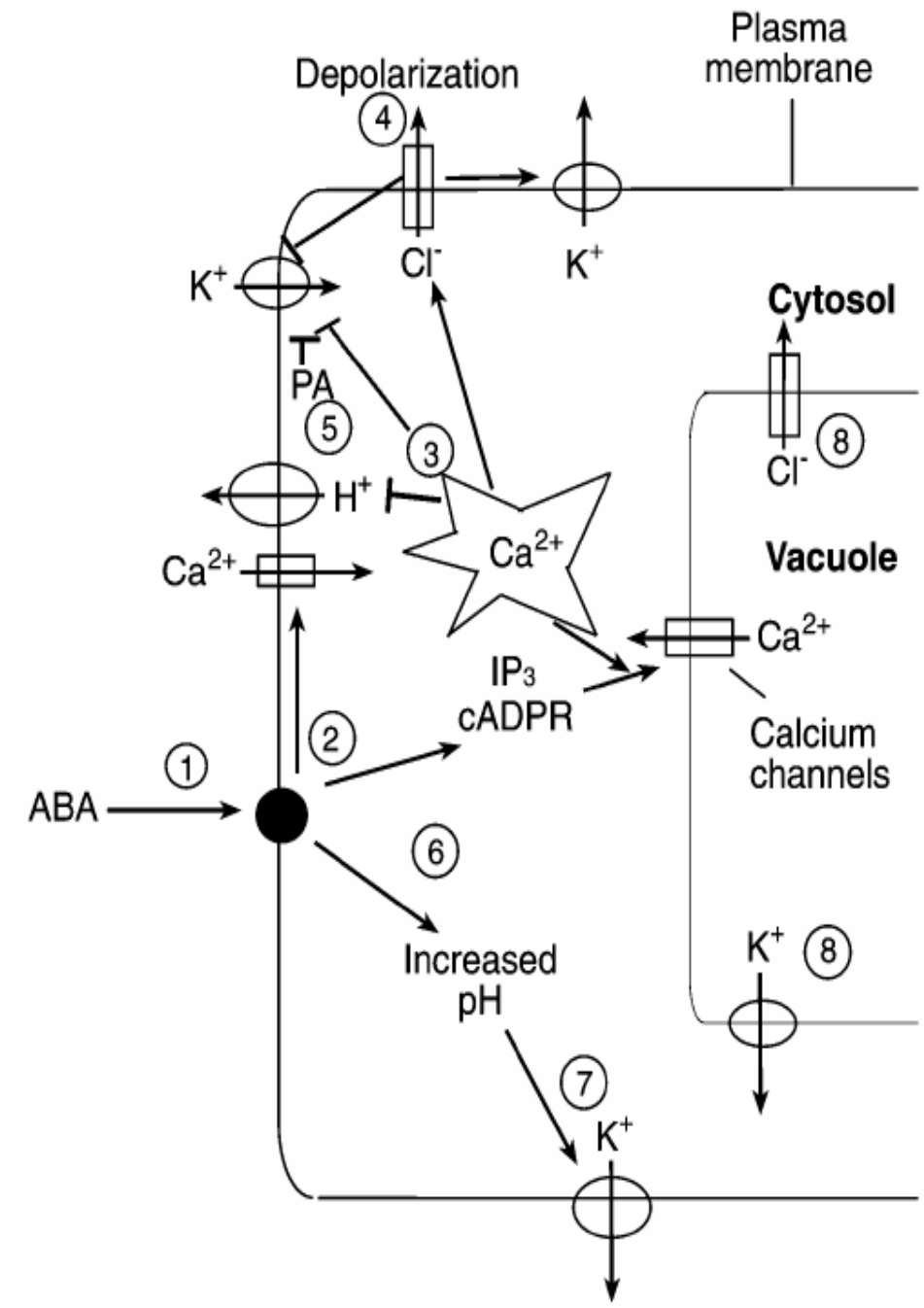

Plasma membrane
Depolarization
4
Cl-
K+
K+
Cytosol
PA
5
3
8
Cl-
H+
Ca2+
Ca2+
Vacuole
Ca2+
IP3
cADPR
2
Calcium channels
1
ABA
6
Increased pH
K+
8
7
K+

Figure 10. Model for ABA signaling in stomatal guard cells.

(1) ABA binds to as yet uncharacterized receptor(s). Although shown here on the plasma membrane, there is evidence for both intra- and extra-cellular perception. (2) ABA induces oscillating increases in cytosolic via production of reactive oxygen species that contribute to opening of plasma membrane Ca^{2+}in channels -release from internal stores through three types of Ca^{2+}channels regulated by IP3 (produced by phospholipase C), cyclic ADPribose (cADPR), and Ca^{2+}itself. (3) The increased Ca^{2+} inhibits plasma membrane H^+ pumps -inhibits K^+ in channels, and -activates Clout (anion) channels, resulting in depolarization of the membrane. (4) Depolarization activates K^+ out and further inhibits K^+ in channels. (5) ABA induces PLD-mediated production of phosphatidic acid (PA), which inactivates K^+ in channels. (6) ABA causes an increase in cytosolic pH which (7) activates K^+ out channels and inhibits H^+ pump activity by depleting the substrate) (8) K^+ and anions to be released across the plasma membrane are first released into the cytosol from guard cell vacuoles. The net result is that K^+ and anions leave the guard cell, guard cell turgor decreases, and the stomata close. These electrophysiological and volume changes are accompanied by, and require, reorganization of the actin cytoskeleton and at least a two-fold change in plasma membrane surface area (Finkelstein and Rock, 2002).

METHODS AND MATERIALS

(i) DNA and protein sequence analysis

DNA and protein sequence analysis was carried out using blast searches (Altschult et al 1990) and the vector NTI suite program (InforMax Inc., Bethesda, MD, USA).

(ii) RT-PCR analysis

One microgram of total RNA was used for the first-strand cDNA synthesis after incubation at 65°C for 10 min as described by Weigel and Glazebrook, (2002). cDNA was synthesized in a volume of 20 µl that contained MoMLV RT buffer (Promega, Madison, WI, USA), 10 mm dithiothreitol, 1.5 µm poly (dT) primer, 0.5 mm dNTPs, 2 U of MoMLV RT at 37°C for 1 h. All PCR reactions were performed with 0.5 U of Taq polymerase (PGC Scientific, Gaithersburg, MD, USA), the buffer provided by the supplier, 0.2 µM dNTPs, and a pair of primers (0.1 µM each) in a final volume of 20 µl. PCR parameters differed for each gene: thermocycling conditions were 94°C for 2 min followed by 25-40 cycles of 94°C for 1 min, 62-65°C for 1 min, and 72°C for 2 min, with a final polymerization step at 72°C for 10 min. The primers used for RT-PCR are listed in Table VI. The primers used for the HD2 family spatial profiling are listed in Table V (21N2pr1/21N2pr4, HD2Bpr3/HD2Bpr4, HD2Cpr3/HD2Cpr4, and HD2Dpr1/HD2Dpr2). The primers used for subcloning the HD2 genes and primers used for their RT-PCR profiling are the same.

(iii) GFP localization

Protoplasts were isolated from *Arabidopsis* seedlings as described by Wigel and Glazebrook (2002). Transgenic seedlings germinated in the dark to reduce chlorophyll content) were

macerated and incubated in enzyme solution (Maceroenzyme R10 and Cellulase R10) for 10 minutes. Subsequently, the tissue-enzyme mixture was subjected to vacuum for thirty minutes. The solution was then incubated at room-temperature shaking for 90 minutes at 40 rpm, after that the solution was filtered through a 70-µm nylon mesh and ready for use. The fluorescence photographs of protoplasts were taken using an Olympus florescent microscope fitted with fluorescein isothiocyanate filters (excitation filter, 450-490 nm; emission filter, 520 nm; and dichroic mirror, 510 nm).

(iv) Particle gun-delivery assays

Tobacco (SR1) plants were grown *in vitro* in half-strength MS medium (Murashige and Skoog**,** 1962) in Magenta boxes (Magenta Corp., Chicago, USA) and kept in a growth chamber at 25°C. After transfer to fresh medium for 2-3 weeks, uniform-sized leaves (about 3 cm in width) were cut off from the plants and placed on a medium consisting of MS salts, B5 vitamins (Gamborg *et al***,** 1968), 1 mg l^{-1} 6-benzyladenine, 0.1 mg l^{-1} naphthalene acetic acid, 3% sucrose, and 0.25% Gelrite in a 20 mm × 15 mm Petri dish. The leaves were pre-conditioned on this medium for 1 day prior to gene delivery. Plasmid DNA was isolated using the Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA, USA). The reporter plasmid was mixed with an effector plasmid at a 1: 1 ratio (weight). In the control, the reporter plasmid was mixed with an equal amount of the control plasmid pUC19. A modified particle inflow gun (Brown *et al***,** 1994) was used for DNA delivery to the tobacco leaves. Twenty-four hours after bombardment, *GUS* gene expression was determined by recording the number of *GUS* reporter gene expression events as indicated by the number of blue foci per explant (Jefferson *et al***,** 1987).

(v) Promoter Motif Analysis

To identify potential stimulus responsive *cis*-elements in the *AtHD2B* and *AtHD2C* promoter, sequence 3000 bp upstream to the transcription start site of the genomic *AtHD2B* and *AtHD2C* was submitted to PlantCARE database at the TAIR cis-element resource website (http://oberon.fvms.ugent.be:8080/PlantCARE/index.html). The motifs of interest were grouped appropriately.

(vi) Histochemical GUS staining

Transgenic tissue expressing promoter driven *GUS* was harvested and incubated in β-glucuronide solution for a period of 12 hours at 37°C. Whole plants were immersed in 1 mM 5-bromo-4-chloro-3-indolyl-glucuronic acid solution in 100 mM sodium phosphate, pH 7.0, 0.1 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, and 0.1% Triton X-100.

(vii) ABA and salt treatment of the GUS transgenic lines

The *AtHD2C promoter:GUS* transgenic seedlings were germinated in MS medium free of ABA or salt. Two to six day old seedlings were transferred onto media containing 100 μM of ABA for 6 hours. Also, these seedlings were transferred to medium containing 200 mM NaCl for 6-12 hours. Subsequent to the treatment, the seedlings were incubated in the X-gluc staining solution at 37ºC for 12 hours.

(viii) Plasmid constructions

For each of the plasmid constructs, the general procedure (Sambrook and Russell**,** 2001) was followed. The sequences to be cloned were amplified by *PCR* (Table I) from either cDNA templates or from genomic DNA sequences prepared from plant extracts. The primers used to amplify these sequences contained restriction sites in their 5’ ends. Simultaneously, the vector

into that the sequence was to be inserted was digested (Table II) with the same restriction enzymes. Subsequently, the amplified DNA sequences and the digested vector (excised band from gel) were purified using the *Qiagen DNA purification* kit. The DNA fragments were digested with restriction enzymes and purified once again after digestion. Then the purified vector as well as the cDNA fragments were mixed together in a ligation (Table III) reaction. The ligation reaction was transformed into *E. coli*. The bacteria were plated on LB medium containing either *ampicillin* or k*anamycin* and colonies resistant to antibiotics were selected. The transformants were then used to isolate plasmid DNA. This plasmid DNA was digested with restriction enzymes specific to each clone and electrophoresed to identify vector and insert. This would confirm that the colony carried the desired construct. The list of enzymes and primer sequences used for different constructs is provided in Table IV.

(a) *E. coli* transformation

Electro-competent cells (Top 10 or DH5-α) that are pre-prepared were retrieved from their -80ºC storage and were thawed on ice. At the same time cuvettes for the transformation are UV-sterilized. Subsequently, 1-2 µl of the ligation reaction mix was added into the melted competent cells and mixed. The mixture was then transferred into the cuvette cavity and the cuvette was then placed in the electroporater (Bio-Rad MicroPulser). The cuvette was pulsed with an electric shock for a millisecond. The cuvette was then removed from the electroporater and 1 ml of sterile LB medium was used to wash the cavity of the cuvette. This medium carrying the transformed cells was then incubated with shaking at 37ºC for 1 hour. Subsequently, the

Table I Standard PCR reaction used for all reactions

Contents of Reaction	Supplier Company	Amount
10X *Taq* Polymerase Buffer	Promega	5µl
25mM $MgCl_2$	Promega	3µl
2.5mM dNTP	Promega	3µl
DNA template	Own	1µl
Primer1	Invitrogen	1µl
Primer2	Invitrogen	1µl
Taq Polymerase	Promega	1µl
Distilled Water	Own	35µl
Total Volume		50µl

Template amount and $MgCl_2$ amount was changed from time to time when the give quantities were not sufficient to give enough PCR product. PCR reaction tubes are set up for required (20-30) amount of cycles at calculated annealing temperature in the automated thermocycler.

Table II Standard double restriction enzyme digestion for all reactions used

Contents of Reaction	Supplier Company	Amount
10X Reaction Buffer	New England Biolabs	2µl
DNA to be digested	Self-prepared	8µl
Restriction Enzyme 1	New England Biolabs	1µl
Restriction Enzyme 2	New England Biolabs	1µl
double distilled Water	Self-prepared	8µl
Total volume		20µl

If the amount of DNA was increased for the reaction (20µl) then the reaction volume was increased to 40µl. Reaction volume should always be 10X of that of the reaction buffer. Therefore for 40µl, 4µl of buffer would be added. The reaction buffer is to be chosen carefully considering maximum enzyme efficiency for both enzymes being used. Usually, the Multicore buffer is an all-purpose buffer. Restriction digestion reaction tubes are set at 37ºC overnight for complete digestion.

Table III. Standard Ligation reaction used for all reactions

Contents of Reaction	Supplier Company	Amount
10X Ligation Buffer	Promega	1µl
Vector DNA (digested)	Own	2µl
Insert DNA (digested)	New England Biolabs	2µl
T4 DNA Ligase	New England Biolabs	1µl
Distilled Water	Own	4µl
Total Volume		10µl

Ligation reactions are set up at room temperature for 1 hour or at 4ºC overnight.

Table IV. **Primers, restriction enzymes, selective media and vectors used in subcloning**

CONSTRUCTS	**VECTOR**	**SELECTION** Bact/Plants	**RESTRICTION** Digest	**PRIMER** Pairs
35S:HD2B	pCAMBIA1302	Kan/Hyg	5'NcoI/3'BglII	HD2Bpr3 HD2Bpr4
35S:HD2C	pCAMBIA1302	Kan/Hyg	5'NcoI/3'BglII	HD2Cpr3 HD2Cpr4
HD2Bpro:GUS	pCAMBIA1381	Kan/Hyg	5'SalI/3'NcoI	HD2Bpropr1 HD2Cpropr2
HD2Cpro:GUS	pCAMBIA1381	Kan/Hyg	5'EcoRI/3'HindIII	HD2Cprpr3s HD2Cpropr2
pG4BDHD2B	pGBD21-C3	Ampicillin	5'EcoRI/3'BglII	HD2Bpr7 HD2Bpr4
pG4BDHD2C	pGBD21-C3	Ampicillin	5'XmaI/3'BglII	HD2Cpr3 HD2Cpr4
35S:GAL4-HD2A(Δ)	pCAMBIA2300	Kanamycin	5'XmaI/3'SacI	PRM1s PR4
35S:GAL4-HD2A(H25A)	pCAMBIA2300	Kanamycin	5'XmaI/3'SacI	FM1a/PR1 RM1S/PR4
35S:GAL4-HD2A(D69A)	pCAMBIA2300	Kanamycin	5'XmaI/3'SacI	FM2a/PR1 RM2S/PR4

Table V Primers and their sequences used for subcloning

PRIMERS	SEQUENCE
$21N_2$pr1	5'atggagttctggggaatttg3'
$21N_2$pr4	5'cgtgcttggccttgttgtgag3'
HD2Bpr3	5'atggagttctggggtgttgaag3'
HD2Bpr4	5'tcaagcagctgcactgtgtttg-3'
HD2Bpr7	5’aattgaattcatggagttctggggagttg3’
HD2Cpr3	5'atggagttctggggtgttgaag3'
HD2Cpr4	5'tcaagcagctgcactgtgtttg3'
HD2Dpr1	5'atggagttttggggtatcg3'
HD2Dpr2	5'ctacttttgcaagagggac3'
UBQpr1	5'gatctttgccggaaaacaattggaggatggt3'
UBQpr2	5'gacttgtcattagaaagaaagagataacagg3'
HD2Bpropr1	5’aattgtcgacatgcagtgattagggaagag3’
HD2Cpropr2	5’atatccatggttgttgaacgaggaagagag3’
HD2Cprpr3s	5’aattgaattcgcaaagctaaaagaggaac’
HD2Cpropr2	5’atataagcttgtgcgaggtagtgtgatc3’
HD2Cpr3	5'atggagttctggggtgttgaag3'
HD2Cpr4	5'tcaagcagctgcactgtgtttg3'
PRM1s	5'aattcccgggaattgaagttaaatcaggaaagc3'
PR4	5'acgtgagctcagaaaccacttcacttggc3'
FM1a	5'ctgagaaacggcgataagaatgccttcttcag3'
RM1S	5'cattcttatcgccgtttctcaggcatcgcttg3'
FM2a	5'tccccaagtggcagaaagctcaaactccttg3
RM2S	5'gagctttctgccacttggggaaaaggaagtg3

Table VI Primers sequences used for RT-PCR

	SEQUENCES
LEC 5’	5’ctgataatgcccttctgagc3’
LEC 3’	5’tctttaagaggcaagaccc3’
RD29B 5’	5’gataccttccgaccagatagc3’
RD29B 3’	5’cgaaaaccccatagtcccaac3’
RAB18 5’	5’cagctctagctcggaggatg3’
RAB18 3’	5’ccgggaagcttttccttgatc3’
ICK1 5’	5’gaggaaaacgatggaggagac3’
ICK1 3’	5’ctaatggcttctccttctcg3’
ADH1 5’	5’aactgcagacggattagaagccgccgagcgggtgacagccctccgagcttgcatgcaacttcttttc3’
ADH1 3’	5’ttgcaagcttcatggagttgattgtatgcttgg3’
ABI1 5’	5’gccatgtcgagatccattgg3’
ABI1 3’	5’aacgatgcatccccagccac3’
ABI2 5’	5’caagatccattggcgatagatacc3’
ABI2 3’	5’cctcttttctccgcccggaag3’
AREB 5’	5’gaagccagatgtcacctgat3’
AREB 3’	5’caatgtccttcgcaagcatt3’
KAT1 5’	5’cttcatgaaacttagagggcaac3’
KAT1 3’	5’ccactttggctctctctatc3’
KAT2 5’	5’aggttgctctgatgcagatc3’
KAT2 3’	5’ccgcattcgtgacctttgtg3’
SKOR 5’	5’gtggctccaaagagcctaaag3’
SKOR 3'	5’acacaagaagcctccggaac3’
GFPpr1	5’actgtctagaccatggtagatctgact3’
GFPpr3	5’aattgggcccctagctttgtatagttcatcc3’

transformed cells are plated onto ampicillin / kanamycin containing LB medium plate (Sambrook and Russell, 2001)

(b) *Plasmid DNA isolation*

Transformed colonies were inoculated into 2 ml LB medium containing selective antibiotics (amp/kan) overnight. Next day, 1.5 ml of culture was spun in eppendorf tubes for 1 minute. Supernatant was poured off and the 200 µl of cell resuspension solution was added to the pellet. After dissolving the pellet, 200 µl of cell-lysis solution was added. After mixing the solution, neutralization buffer was added. Subsequent to mixing, the tubes were spun for 5 minutes. Supernatant was retrieved and mixed with 0.5 ml of 100% ethanol. DNA pellet was retrieved and washed with 75% ethanol (Sambrook and Russell**,** 2001). Subsequently, the pellet was air-dried and dissolved in 1X TE buffer and stored in -20ºC.

(ix) Plant transformation and growth

(a) *Transformation*

Arabidopsis thaliana (ecotype Columbia) plants were grown in a growth chamber (16 h of light and 8 h of darkness at 23°C) after a 2-4-day vernalization period for the seeds sown. These plants were grown for a period of 35 days, until the plants bolted and floral buds opened. Plant transformation plasmids were electroporated into *Agrobacterium tumefaciens* GV3101 as described by Shaw (1995) and plated on LB medium containing rifampicin/kanamycin/hygromycin and incubated at 30°C. The *Agrobacterium*-mediated transformation of *A. thaliana* was performed as described by Clough and Bent (1998). T_1 seeds were harvested from the fully grown mature transformed plants and dried at 25°C.

Seeds were germinated on sterile medium containing 40 μg ml^{-1} kanamycin or hygromycin to select the transformants. Surviving T_1 plantlets were transferred to soil to set seeds (T_2).

(b) *Seed germination*

For growth under sterile conditions, seeds were surface sterilized (10 min incubation in 5% (v/v) sodium hypochlorite, and rinsed thrice with sterile distilled water) and sown on half-strength Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) supplemented with 1% sucrose, pH 5.7, and 0.8% (w/v) agar in Petri dishes (MS media preparation). For direct germination in soil, the seed sterilization is not necessary and seeds can be directly sown on soil after 2-4 day vernalization period (Weigel and Glazebrook**,** 2002).

(x) Plant DNA isolation

For DNA extraction from *Arabidopsis*, plant tissue was ground with liquid nitrogen, 3-4 times. One ml of plant DNAzol® (Invitrogen, Carlsbad, CA, USA) was added and the plant tissues were further ground. Once the tissues melted, they were collected in eppendorf tubes and incubated at room temperature for 5 minutes. Subsequently, 600 μl of 100% chloroform (Fisher Scientific, Fairlawn, NJ, USA) was added to the plant extract and mixed. This mixture was incubated for 10 minutes at room temperature and subsequently spun for 10 minutes. The supernatant was retrieved and mixed with 1 ml of 100% ethanol (Fisher Scientific, Fairlawn, NJ, USA) and incubated for 5 minutes at room temperature. The mixture was spun for 10 minutes to pellet the DNA. The pellet was then washed with Plant DNAzol Wash (0.75 ml 100% ethanol + 1 ml of DNAzol) , then with 70% ethanol and air-dried. The DNA was dissolved in sterile distilled water.

(xi) Plant RNA isolation

For RNA extraction from *Arabidopsis*, plant tissue was ground with liquid nitrogen. One ml of TRIzol® (Invitrogen) reagent was added and the tissue was further ground. Once the tissue melted, it was collected in eppendorf tubes and was incubated at room temperature for 5 minutes. Subsequently, 200 µl of 100% chloroform (Fisher Scientific, Fairlawn, NJ, USA) was added to the plant extract and mixed. This mixture was incubated for 10 minutes at room temperature and subsequently spun for 15 minutes at 4ºC. The supernatant was retrieved and mixed with 0.5 ml of Isopropanol (Fisher Scientific, Fairlawn, NJ, USA) and incubated for 10 minutes on at room temperature. The mixture was spun for 10 minutes to pellet the RNA at 4ºC. The pellet was then washed with 70% ethanol and air-dried. The RNA was dissolved in sterile DEPC treated water.

(xii) Dose response media preparation

Half-strength Murashige and Skoog (MS) salts (Murashige and Skoog**,**1962) medium supplemented with 1% sucrose, pH 5.7, and 0.8% (w/v) agar was prepared and, after autoclaving , was cooled to 55°C. Subsequently, sterile ABA, Nacl and Mannitol preparations of required concentrations were inoculated into the medium in the sterile hood environment. ABA was dissolved in 75% ethanol to prepare specified concentrations. The medium was mixed thoroughly (120 rotations) before plating.

(xiii) T-DNA insertion mutants

The desired gene knock-out lines were obtained from the ABRC (http://signal.salk.edu/cgi-bin/tdnaexpress). T1 seeds were procured initially and subsequently grown for three more

generations on kanamycin to confirm transgenic lines and homozygosity of these lines. (Concentration of kanamycin used for selection of these mutants is to be kept low (20 µg/ml) as these plants have low resistance initially.)

(xiv) Yeast transformation

HF7c cells were inoculated into 5 ml YPD medium supplemented with 0.2% adenine and cultured overnight. The next morning, the HF7c culture was transferred into 500 ml YPDA medium and grown to mid-log phase for about 3-4 hours in 30ºC with shaking. The OD of the culture was measured until it reached 0.8-1 (~2×. 10^7 cells/ml). Subsequently, the culture was spun at 3500-5000 rpm to retrieve cells. The cells were then washed with sterile water with subsequent rounds of spinning. Subsequently, cells were suspended in 1 ml of 100 mM LiAc and then transferred into 1.5 ml eppendorf tubes. In the meantime, the co-transformation mix was prepared by mixing 1 µl of Plasmid DNA with 36 µl 1M LiAc, 25 µl of boiled and chilled salmon sperm DNA and 50 µl of water.

Subsequently, 240 µl of 50% w/v PEG was layered on top of the competent HF7c cells, after that the transformation mix was added to the tubes. These contents of the tubes were mixed by gentle pipetting and incubated in a 42ºC water bath for 15 minutes. Subsequently, cells were mixed with 500 µl of sterile water and plated onto SD selective medium (Leu^-Trp^-). This protocol was adapted from Sambrook and Russell (2001).

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SPECIFIC AIMS

(i) Analysis of the expression patterns of HD2-type histone deacetylases

(a) Characterization of the spatial expression profile of the HD2-type histone deacetylase gene family in Arabidopsis thaliana. (b) Identification of sub-cellular localization of HD2 proteins, AtHD2B and AtHD2C. (c) Identification of essential residues in AtHD2A protein sequence.

It was unknown whether all the four members of the HD2 gene family would have similar spatial profiles. The HD2 proteins were expected to localize to the nucleus as they are putative transcription factors. In addition, certain conserved histidine and aspartate residues and the N-terminal motif were expected to be essential for catalysis.

(ii) *AtHD2C* promoter analysis

(a) Promoter motif prediction to identify putative cis-elements in the AtHD2B and AtHD2C promoter. (b) Generation of AtHD2B promoter:GUS and AtHD2Cpromoter:GUS transgenic lines and examination of the spatial expression profile of the GUS reporter driven by the AtHD2B and AtHD2C promoters in different developmental windows. (c) Analyze the effect of ABA on AtHD2C promoter regulation of GUS expression.

We expected to find ABA-response elements and seed regulation elements in the *AtHD2B* and *AtHD2C* promoter sequences that would support their involvement in embryo development. The *GUS* gene spatial expression profile was expected to be similar to the HD2 gene family expression profile analyzed in the first set of experiments.

(iii) Analysis of the functional role of HD2-type histone deacetylases in the development of *Arabidopsis thaliana*, using the reverse genetics approach .

(a) Examination of the developmental defects in the AtHD2B and AtHD2C overexpression lines. (b) Analyze ABA, salt and mannitol sensitivity of the AtHD2C overexpression and T-DNA insertion lines in different developmental stages. (c) Analyze the ABA-inducible gene expression patterns in the AtHD2C overexpression and T-DNA insertion lines in respective developmental stages.

Since the HD2 proteins were found to accumulate in the embryos and *AtHD2A* overexpression led to down-regulation of 'seed-specific' genes, it was expected that the *AtHD2B* and *AtHD2C* ectopic expression might led to seed-specific effects. Consequently, we expected to observe changed ABA response phenotypes in the overexpression lines.

(iv) Investigate physical interaction between *AtHD2B*/*AtHD2C* proteins and RPD3 (HDA1) type histone deacetylase proteins

Mammalian systems demonstrated interaction amongst different members of HDAC classes. Based on this report, we expected to find interaction between HD2-type histone deacetylases and class I HDAC (AtHDA1) proteins.

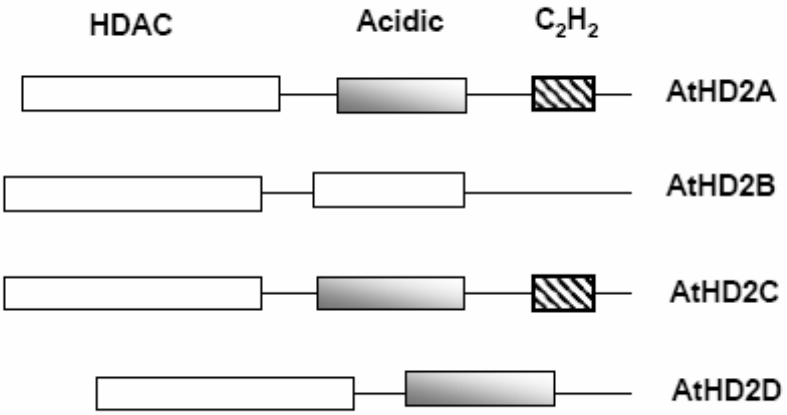
RESULTS

(i) Expression Patterns of the HD2-Type Histone Deacetylases in *Arabidopsis*

(a) Spatial expression profile of the HD2-type histone deacetylase gene family

Little information is available with regards to the expression and function of the HD2-type HDACs in *Arabidopsis*. Antisense knockout of *AtHD2A* led to aborted seed development, implicating the HD2-proteins to be involved in seed maturation (Wu *et al*, 2000). Protein sequence alignment of the four HD2 family members revealed that all of them contain similar structural organization (Figure 11A) and have an N-terminal domain where catalytic activity is predicted to reside (Aravind and Koonin, 2001). They have an acidic stretch in the central domain that is responsible for phosphorylation-regulation of the protein and possibly for nuclear localization. AtHD2A and AtHD2C contain Zn fingers in their C-terminal regions whereas AtHD2B and AtHD2D do not contain Zn finger domains. We analyzed the spatial expression profile of the HD2 gene family in *Arabidopsis*. Different tissues including seedlings (1 week old), mature leaves, stems, flowers and siliques were collected for analysis. Total RNA was isolated from these tissues and gene expression was analyzed using RT-PCR (Weigel and Glazebrook, 2002). It was observed that *HD2A*, *HD2B* and *HD2C* accumulated in all tissues examined with varying quantities in the different tissues (Figure 11B). However, *HD2D* formed the anomaly in this family by exhibiting flower-specific expression with weaker expression in stems. This suggests that some of the HD2 family genes may be differentially expressed in *Arabidopsis*. These results supported the idea that the HD2-type histone deacetylases may play differential functional roles owing to their different expression patterns.

A


HDAC
Acidic
C_2H_2
AtHD2A
AtHD2B
AtHD2C
AtHD2D

B

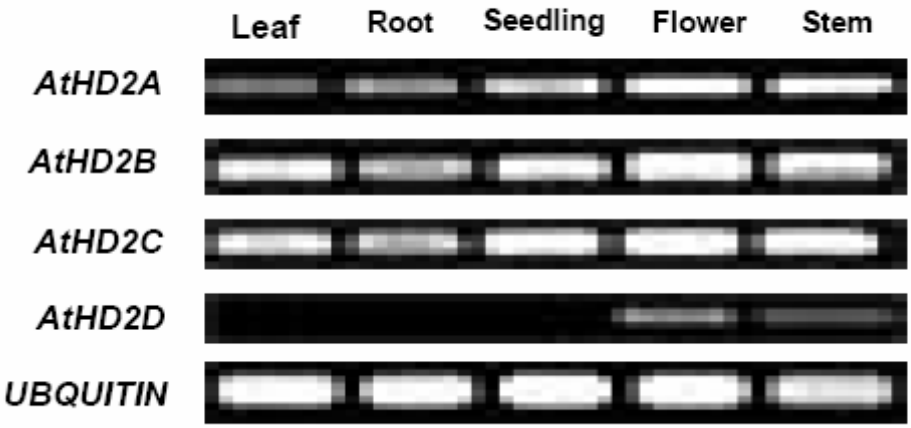

Leaf
Root
Seedling
Flower
Stem
AtHD2A
AtHD2B
AtHD2C
AtHD2D
UBQUITIN

Figure 11. The HD2 gene family of Arabidopsis thaliana. (A) Sequence comparison of the four HD2 proteins in *Arabidopsis thaliana*. The N-terminal, HDAC box represents the catalytic domain of the HD2 proteins. The central box represents the acidic domain rich in E and D residues. The C-terminal boxes in AtHD2A and AtHD2C represent Zn2+ finger domains, which might have a role in recruiting the protein by forming protein-DNA or protein-protein contacts. The figure underscores the fact that the four proteins are very similar in their alignment with differences only due to possession of the Zn finger motifs by AtHD2A and AtHD2C. (B) An RT-PCR assay to examine the spatial expression profile of the HD2 gene family. RNA was isolated from different plant tissues: leaf, root, stem, flower and seedling. cDNA was prepared from RNA and gene expression was analyzed by PCR using gene specific primers. The figure depicts ubiquitous expression of the HD2 gene family with *AtHD2D* being the exception due to flower and stem specific expression.

(b) **HD2C localizes to the nucleus in *Arabidopsis***

The HD2 proteins were first isolated as nucleolar proteins in maize (Lusser *et al,* 1997). Subsequently, they were identified in *Arabidopsis thaliana* (Wu *et al,* 2000). The nuclear/nucleolar localization of the HD2 proteins had not yet been demonstrated in *Arabidopsis*. The nuclear localization of a protein confirms its status as a transcription factor. Therefore, I decided to test the sub-cellular localization of HD2C. *35S:AtHD2C-GFP* and *35S:AtHD2B-GFP* constructs were generated and transformed into *Arabidopsis* to create transgenic plants expressing HD2C-GFP and HD2B-GFP fusion proteins (Table IV). The cDNA sequence of *HD2C* and *HD2B* was fused to the *GFP* reporter gene driven by the *35S* promoter of the *cauliflower mosaic virus* (Figure 12A and 12D). The constructs were used to transform *Arabidopsis* (ecotype Columbia) plants as described by Clough and Bent (1998). Four independent *AtHD2C-GFP* lines (*35S: AtHD2C1, 35S:AtHD2C2, 35S:AtHD2C3* and *35S:AtHD2C4*) and three independent *AtHD2B-GFP* lines (*35S: AtHD2B1, 35S:AtHD2B2, 35S:AtHD2B3)* were further assayed for presence and expression of transgene. Lines expressing higher levels of *HD2C* (*35S:AtHD2C1* and *35S:AtHD2C2*) were selected for detailed studies. The transgenic lines were confirmed for transgene expression using PCR (Figure 12B and 12E) and RT-PCR (12C and 12F) before functional assays were conducted. Genomic DNA was extracted from Wild-type, *35S:AtHD2C-GFP* (1-4) and *35S:AtHD2B-GFP* (1-3) transgenic lines and used as a template for amplification rounds with three different sets of primers: X- HD2Bpr3/GFPpr3 (~1.35 kb), Y-HD2Bpr3/HD2Cpr4 (~800 bp), Z-GFPpr3/GFPpr4 (~500 bp) and P-HD2Cpr3/HD2Cpr4 (~800 bp), Q- HD2Cpr3/GFPpr3 (~1.35 kb). RT-PCR analysis was performed by using RNA samples extracted from 1-week old seedlings that were reverse transcribed to cDNA copies. The cDNA was used as a

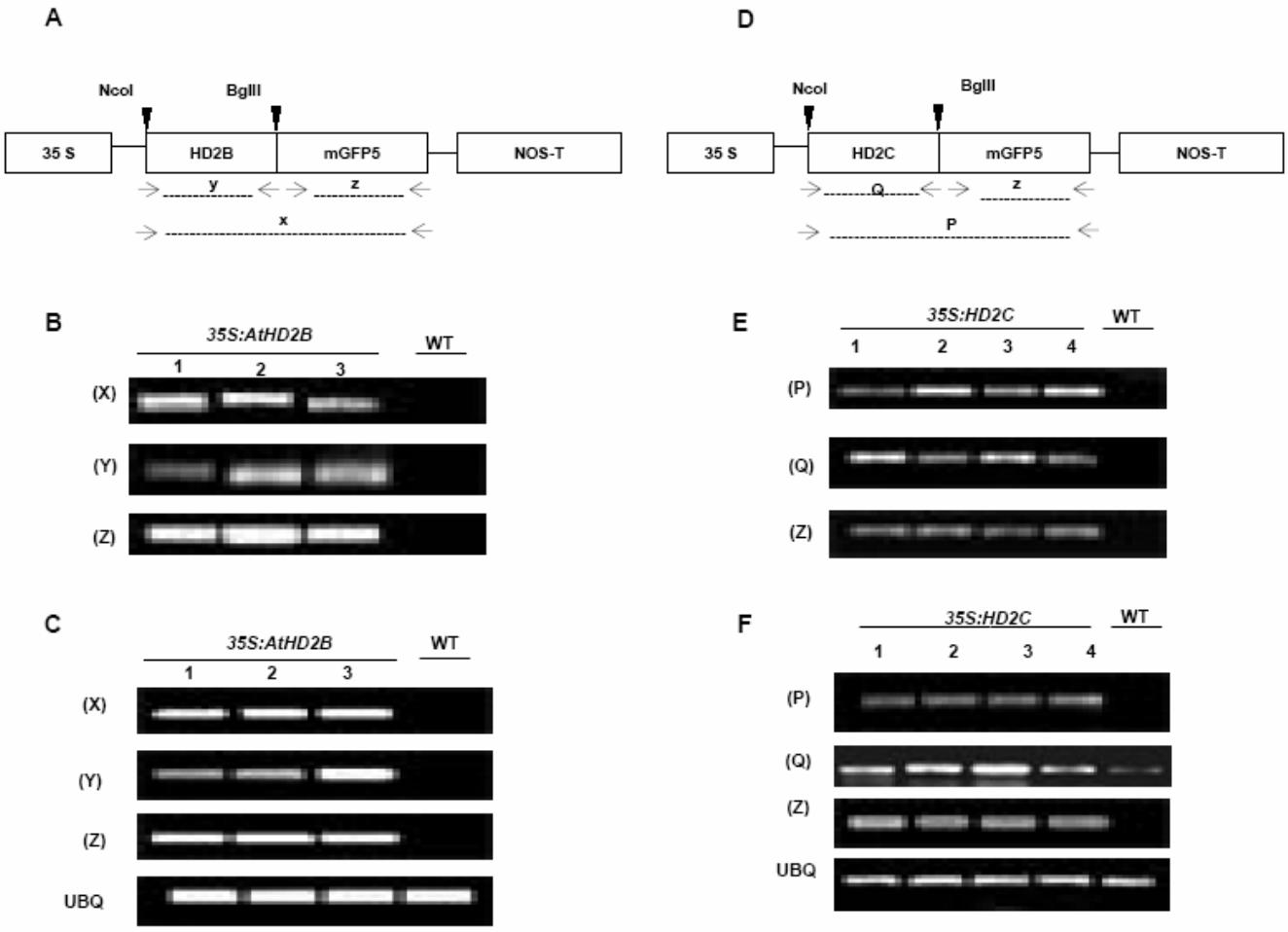

A
NcoI
BglII
35 S
HD2B
mGFP5
NOS-T
y
z
x
D
NcoI
BglII
35 S
HD2C
mGFP5
NOS-T
Q
z
P
B
35S:AtHD2B
WT
1
2
3
(X)
(Y)
(Z)
E
35S:HD2C
WT
1
2
3
4
(P)
(Q)
(Z)
C
35S:AtHD2B
WT
1
2
3
(X)
(Y)
(Z)
UBQ
F
35S:HD2C
WT
1
2
3
4
(P)
(Q)
(Z)
UBQ

Figure 12. (A) Map of the *35S:AtHD2B* construct, *AtHD2B* was subcloned upstream to GFP driven by the CaMV 35S promoter in the pCAMBIA1302 vector. (B) PCR analysis of Arabidopsis transgenic lines expressing *35S:AtHD2B* transgene; Genomic DNA was extracted from wild-type (WT) and *35S:AtHD2B* transgenic lines (1-3) and used as a template for PCR amplification with three different sets of primers: X-HD2Bpr3 and GFPpr3 (~1.35kb), Y-HD2Bpr3 and HD2Bpr4 (~800bp), Z-GFPpr3 and GFPpr4 (~500bp). (C) RT-PCR analysis to assay levels of expression of the *35S:AtHD2B* transgene; 1µg of RNA samples extracted from 1-week old seedlings was reverse transcribed to cDNA. The cDNA was used as a template for amplification with three different sets of primers: X, Y and Z. UBIQUITIN served as internal control. (D) Map of the *35S:AtHD2C* construct: *AtHD2C* was subcloned upstream to GFP driven by the CaMv 35S promoter, into the pCAMBIA1302 vector. (E) PCR analysis of Arabidopsis transgenic lines expressing *35S:AtHD2C* transgene: Genomic DNA was extracted from wild-type (WT) and *35S:AtHD2C* transgenic lines (1-4) and used as a template for PCR amplification with three different sets of primers; P-HD2Cpr3 and GFPpr3 (~1.3kb), Q-HD2Cpr3 and HD2Cpr4 (~800bp), Z-GFPpr3 and GFPpr4 (~500bp). (F) RT-PCR analysis to assay levels of expression of the 35S:AtHD2C transgene; 1 µg of RNA samples extracted from 1-week old seedlings were reverse transcribed to cDNA. The cDNA was used as a template for amplification with three different sets of primers; P, Q and Z. UBIQUITIN served as internal control.

*Lines: 1-*35S:AtHD2B/C1*, 2-*35S:AtHD2B/C2*, 3-*35S:AtHD2B/C3*, 4-*35S:AtHD2C4*

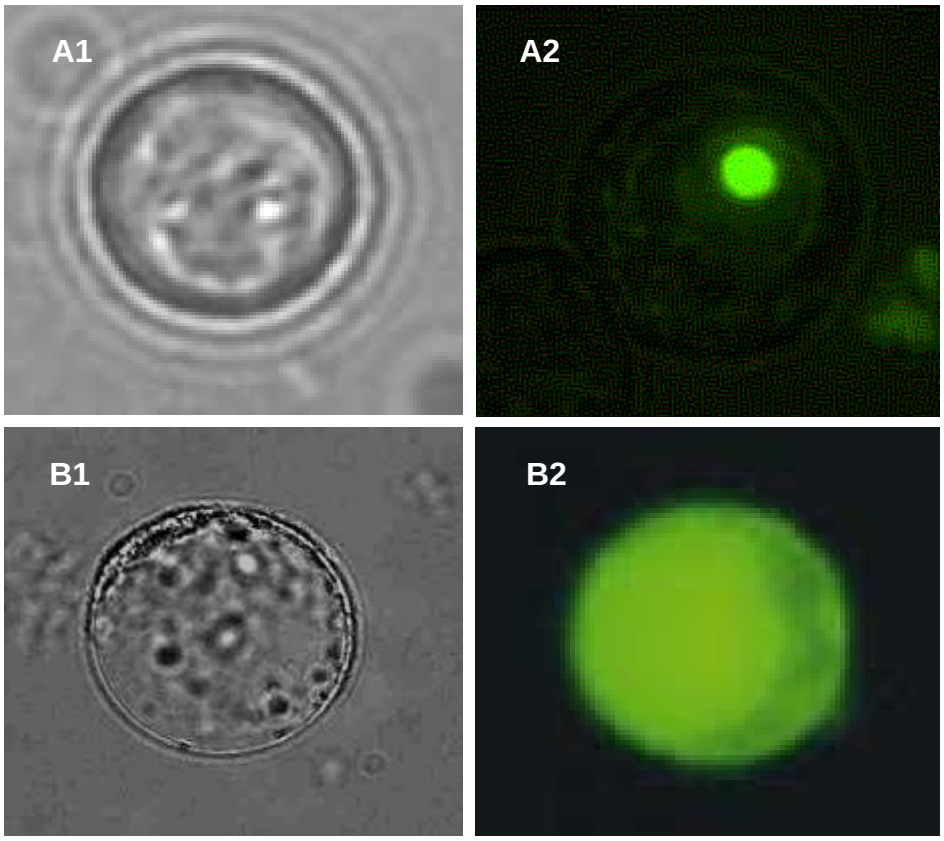


Figure 13. Subcellular localization of AtHD2C, *The 35S:AtHD2C-GFP* overexpression line was used to isolate transgenic protoplasts and GFP fluorescence was examined by fluorescence microscopy. (A1) Single *35S:AtHD2C-GFP* transgenic protoplast under bright light. (A2) nuclear localization of the HD2C protein imaged by GFP fluorescence under UV light. (B1) Single *35S:GFP* transgenic protoplast under bright light. (B2) Cytoplasmic localization of the GFP protein from the control line imaged by GFP fluorescence under UV light.

template for PCR amplification rounds with three different sets of primers: X, Y and Z or P, Q and Z. Both assays confirmed the presence and expression of *AtHD2C* transgene in the plants. Subsequently, *35S:AtHD2C* T_3 homozygous seedlings were selected after subsequent rounds of generation. In the first two generations (T_1 and T_2), transgenic seeds grown in Hygromycin selective medium germinated in 3:1 (germinated: non-germinated) ratios. The hygromycin resistant seedlings were selected for selfing and subsequent harvest. In the next set of germination (T_3) on hygromycin selective medium, all seeds germinated giving 100% hygromycin resistant phenotype. This was used as an indicator for possible homozygosity amongst transgenic plants. *AtHD2C1* T_2 plants germinated in a ratio of 62:21 (~3:1) and *AtHD2C2* T_2 plants germinated in a ratio of 77:15 (~3:1). These two lines were further selected for T_3 generation seeds that were 100% resistant to hygromycin. Protoplasts were isolated from these transgenic lines and were examined for GFP florescence signal within the cells. Distinct bright green spots were observed under florescence light in the boundary confining the nuclear space within the cells for both the *HD2C-GFP* lines (Figure 13A2). This confirmed the nuclear localization of the proteins. Additionally, the bright green spots had sharp boundaries and were localized to a particular region within the nucleus and not diffused throughout. This implicated nucleolar localization, but further analysis is required to confirm this. *35S:GFP* transformed plants served as a control as the GFP from these protoplasts was localized throughout the cytoplasm.

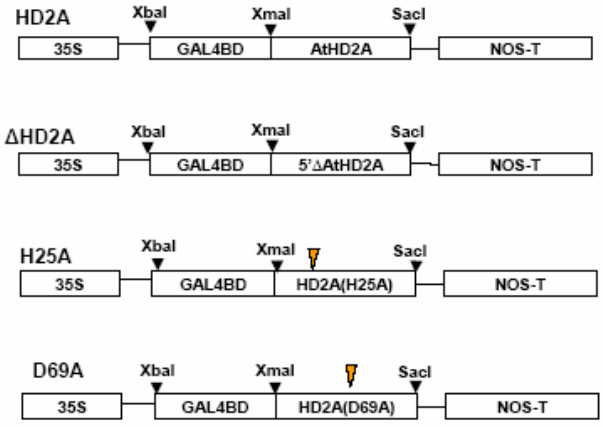
(c) **The N-terminal motif and H25 are essential for AtHD2A mediated Repression**

It had been previously demonstrated that HD2A, HD2B, and HD2C repressed transcription as GAL4 fusion proteins when directed to a promoter containing GAL4-binding sites (Wu *et al*, 2003). We wanted to investigate residues that are essential for mediating this repression.

Therefore, we used site-directed mutagenesis to identify residues in HD2A that are required for gene repression activity. Sequence alignments of all known HD2 proteins revealed that the N-terminal region of HD2 proteins contain an MEFWG motif as well as 18 conserved amino acid residues (Dangl *et al*, 2000). It was proposed that the invariant aspartic acid is the nucleophile involved directly in lysine deacetylation that may be facilitated through a charge relay system with the conserved histidine (Dangl *et al*, 2000). To determine the relevance of each of these residues, three mutation constructs were generated: a deletion of the N-terminal EFWG motif (ΔAtHD2A) (Figure 14A), a substitution of the Histidine 25 to Alanine (H25A) (Figure14A) and a substitution of Aspartate 69 to Alanine (D69A) (Figure 14A). These mutation constructs were co-transformed with a reporter construct in a transient expression assay and the activity of gene repression was assessed by the level of expression of the *GUS* reporter. As shown in Figure 14C, deletion of the N-terminal EFWG motif resulted in loss of gene repression activity, whereas the H25A mutant yielded decreased gene repression activity compared with Wild-type. The D69A mutant, however, showed little change in gene repression activity compared with Wild-type protein. The experiment was repeated thrice with similar results. These results indicated that the N-terminal EFWG motif is essential for the gene repression activity, and that the amino acid residue H25 may also be important, but to a lesser extent.

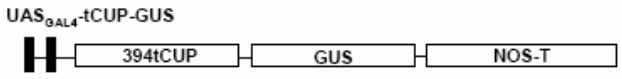
A

Effector Plasmids



B

Reporter Plasmid



C

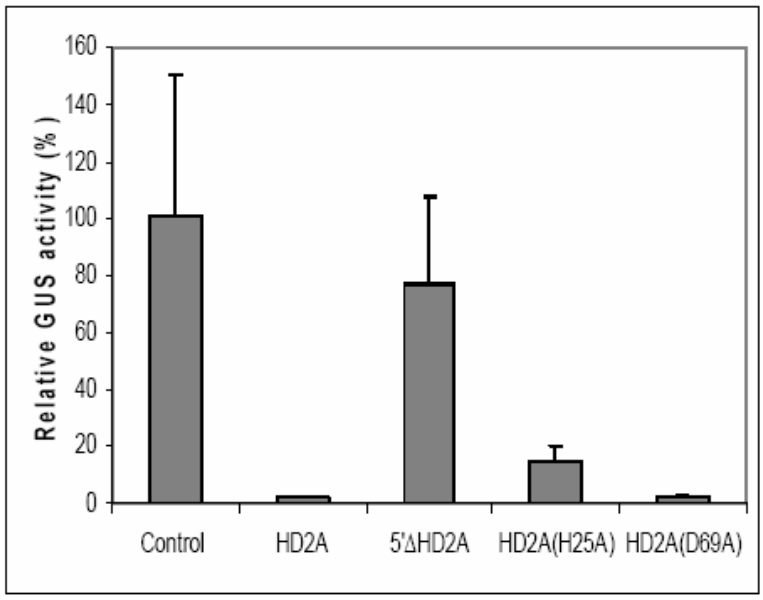


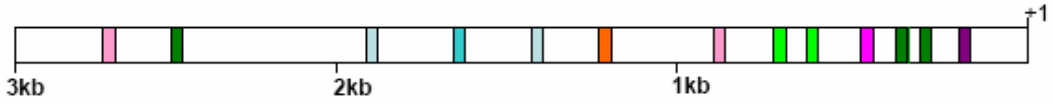
Figure 14. Mutation analysis of the essential catalytic residues in the AtHD2A protein. (A) and (B) Schematic diagram of effector and reporter constructs, respectively, used in a co-bombardment assay used to investigate the extent of gene repression caused by the HD2A protein. The effector constructs contain the GAL4 DNA binding domain (GAL4BD) which will bind to the UAS sequence in the UASGAL4-tCUP-GUS reporter and recruit the fused HD2A protein to the promoter. ΔHD2A was a deletion in the N-terminal EFWG motif of HD2A protein: H25A was a substitution of the conserved histidine motif at position 25 to an alanine residue, D69A was a substitution of the conserved aspartate residue at position 69 to an alanine residue. The orange signs indicate relative positions of the substitutions on the HD2A protein. (C) Repression of the GUS reporter gene expression. D69A displays approximately the same activity as the full length protein. pUC19 served as the control plasmid. Each of the effector plasmids were co-bombarded with the reporter plasmid and GUS activity was measured in a transient expression assay. Bars indicate the SE of three replicates.

(ii) Analysis of *AtHD2B* and *AtHD2C* promoter Activities

(a) *AtHD2B* and *AtHD2C* promoter motif analysis

A 3000 bp sequence 5' upstream to the transcription start site of the genomic sequences of *AtHD2B* and *AtHD2C* was submitted to PlantCARE database (http://oberon.fvms.ugent.be:8080/PlantCARE/index.html) was submitted to PlantCARE (cis acting regulatory elements) in the TAIR database for motif prediction. The *AtHD2B* and *AtHD2C* promoter contained motifs for ABA, seed-specific regulation and cell cycle inhibition (Figure 15 and 16). The ABA response motifs found in these promoters are ABREs that are G-box, elements (Finklestein and Rock**,** 2002). The ACGT core element that is essential to the ABRE G-box can be found in the *AtHD2B* and *AtHD2C* promoter sequences. Another *cis*-acting sequence required for ABA-inducibility, RY-element, was found in these two promoters. RY elements are bound by B3 domain proteins such as ABI3 **(**Ezcurra *et al***,** 2000). The MRE (Myc regulation element) motif was also found within these promoters. This element is bound by the Myc class transcription factors, that have the b-HLH-ZIP domain structure and are ABA-inducible (Abe *et al***,** 1997). The presence of the MSA regulator in these promoters suggested that these genes might be targets of cell-cycle mediating proteins (Ito *et al***,** 2001). The positions and sequences of these different elements in the *AtHD2B* and At*HD2C* promoters is outlined in Figure 15A and 15C Subsequent to this preliminary study, we subcloned 1.7 kb of the *AtHD2B*

A

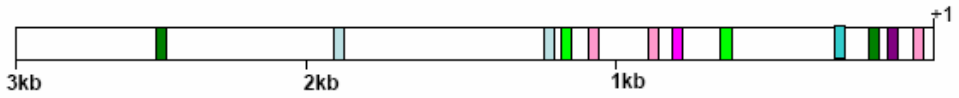


B

MOTIF NAME	POSITION	SEQUENCE	FUNCTION
ABRE	1447 1980	TCTACGTAGT, AGGGCGTCG	ABA responsive element
MYB	1246	CAActg	MYB binding in drought induction
MRE	531, 546	AACCaaa, AACCtac	Light regulation element
c-repeat DRE	203, 217, 2568	gtgCCGAc, cctCCGAc, tccCGAC	Dehydration response
MSA	128	TGaAACGgt	Cell cycle regulation
GCN4	720, 2764	ttaGTCA, tcaGTCA	Cis-regulation element in
Prolamin	485	tgCAAGg	Box associated with GCN4
RY-element	1670	CATGatG	Seed-specific regulator

Figure 15. Motif prediction in the *AtHD2C* promoter sequence 3000bp 5' upstream to the transcription start site was submitted to PlantCARE database for identification of putative cis-elements. (A) Map of the *AtHD2C* promoter with the different putative elements depicted in different color codes in their respective positions in the sequence. (B) Table describing the color codes for the cis-elements, indicating their upstream distance relative to the transcription start site (+1), sequence and known function.

A



B

MOTIF NAME	POSITION	SEQUENCE	FUNCTION
ABRE	1143	cctACGTagg	ABA-responsive element
MRE	703, 1141	AACCaaa, AACCtac	Light regulation element
C-repeat DRE	205	TACCgacct	Draught inducible motif
MSA	31	ttgAACGgt	Cell cycle regulation
GCN4	9, 1047	taaGCCA, tggGTCA	Cis-regulation element in endosperm expression
Prolamin	870, 1474	tgtAAAGt, tgaAAAGc	Box associated with GCN4
RY-element	244	CATGcatt	Seed-specific regulator

Figure 16. Motif prediction in the *AtHD2B* promoter sequence 3000bp 5' upstream to the transcription start site was submitted to PlantCARE database for identification of putative cis-elements. (A) Map of the *AtHD2B* promoter with the different putative elements depicted in different color codes in their respective positions in the sequence. (B) Table describing the color codes for the cis-elements, indicating their upstream distance relative to the transcription start site (+1), sequence and known function.

and *AtHD2C* promoter sequences into the *pCAMBIA1381* vector to generate transgenic promoter lines expressing *GUS* driven by the *AtHD2B* and *AtHD2C* promoter. These transgenic promoter lines were used for subsequent assays.

(b) ***AtHD2B* promoter and *AtHD2C* promoter driven *GUS* is expressed in all mature vegetative tissues**

The *AtHD2B* and *AtHD2C* promoter *GUS* fusion constructs are depicted in Figure 16. From the previous RT-PCR data, *AtHD2B* and *AtHD2C* expression was detected in all tissue types, e.g., leaves, stems, flowers, siliques and seedlings. Therefore, we examined the expression of the *GUS* reporter in the *AtHD2B* promoter:*GUS* (Figure 17B) and *AtHD2C* promoter:*GUS* (Figure 17A) transformed tissues. Figure 17 demonstrates that *GUS* was strongly expressed in all tissues examined in both *AtHD2B* and *AtHD2C* promoter lines including mature rosette leaves, floral meristems, stems, flowers (anthers, pollen, carpel, sepals) and silique (funiculus and mature seeds), the only exception being petals where there was a conspicuous absence of *GUS*.

(c) ***AtHD2C* promoter:*GUS* expression is down-regulated by ABA and NaCl in the post-germination stage**

To investigate the regulation of *AtHD2C* expression in response to ABA at the post-germination stage, *AtHD2C* promoter:GUS expression was assayed by histochemical β-glucuronidase staining of the transgenic plants. *GUS* reporter gene was expressed in all parts of the seedling with weaker staining in the RAM (root apical meristem) (Figure 18A).

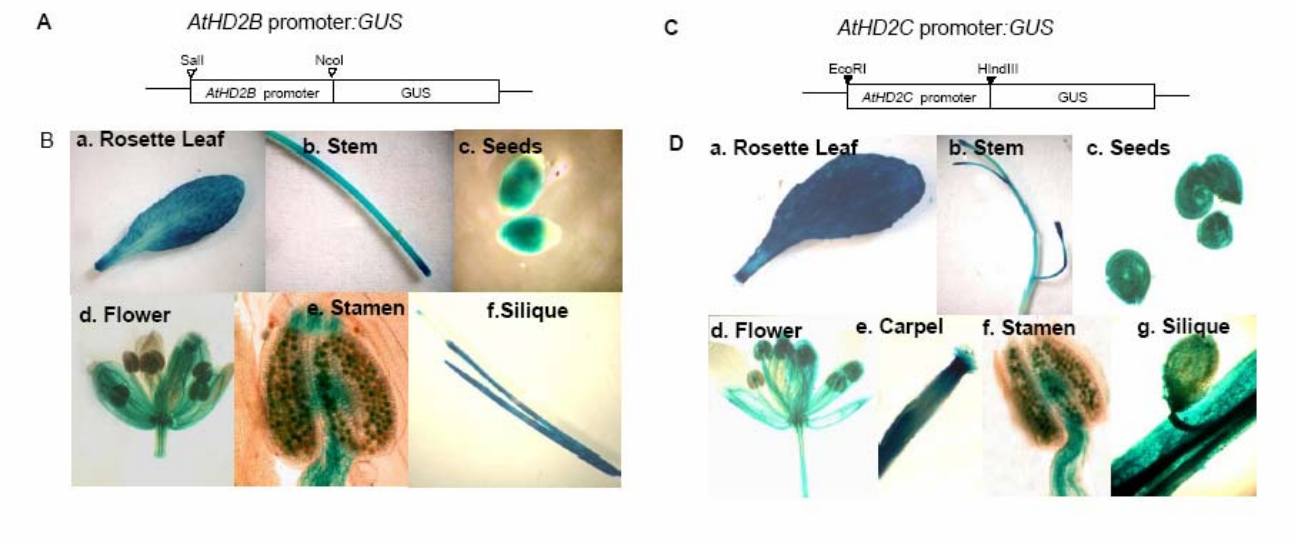


Figure 17 (A) Diagram of *AtHD2B* promoter:*GUS* construct. (B) Histochemical GUS staining of the *AtHD2B* promoter activity in vegetative tissues. T3 plants were stained with 5-bromo-4-chloro-3-indolyl--glucuronic acid for 12 hrs; Staining of (a) mature rosette leaf, (b) stem, c. post-imbibition seeds-:stained after 2-days stratification, (d) mature flower, (e) stamen and (f) siliques. (C) Diagram of *AtHD2C* promoter:*GUS* construct. (D) Histochemical GUS staining of the *AtHD2C* promoter activity in vegetative tissues. Plants were stained with 5-bromo-4-chloro-3-indolyl--glucuronic acid for 12 hrs. Staining of (a) mature rosette leaf, (b) stem, (c) post-imbibition seeds, stained after 2-days stratification, (d) mature flower, (e) carpel, (f) stamen and (g) siliques (arrow indicates stained funiculus).

Interestingly, the *AtHD2C* promoter was induced in the RAM after ABA treatment. Without ABA application there was very little or no *GUS* accumulation in the RAM. Additionally, *AtHD2C* promoter activity was quite strong in the shoot apical meristem, both before and after ABA application, as opposed to other seedling parts that showed a slight reduction in staining after ABA application. Thus *GUS* accumulation at the meristems seems to be an ABA induced phenomenon implicating a synergistic interaction between ABA signal mediators and *AtHD2C* promoter expression at specific locations. The *AtHD2C* promoter:*GUS* bearing plants, demonstrated downregulation of the *GUS* accumulation in the seedlings upon NaCl application (Figure 18B). Also, *GUS* accumulation in the SAM was maintained after NaCl treatment (Figure 18B) and was induced in the RAM post-treatment (Figure 18B) that is similar to the response observed for the ABA treated plants. These results indicate that AtHD2C down-regulates ABA dependant salt stress signaling during germination and post-germination growth stages.

(iii) Reverse Genetics Approach to Study the HD2–Type HDACs

(a) Growth phenotypes of *35S:AtHD2C* Overexpression Lines

To investigate the function of *AtHD2C* in *planta,* transgenic plants overexpressing *HD2C* were generated and T3 plants were selected as described in section I. Compared to wild-type plants, the *35S:AtHD2C-GFP* lines exhibited some abnormal phenotypes. The *35S:AtHD2C1* and *35S:AtHD2C2* seeds germinated 6 hours before the wild-type seeds (Figure 19A) and the

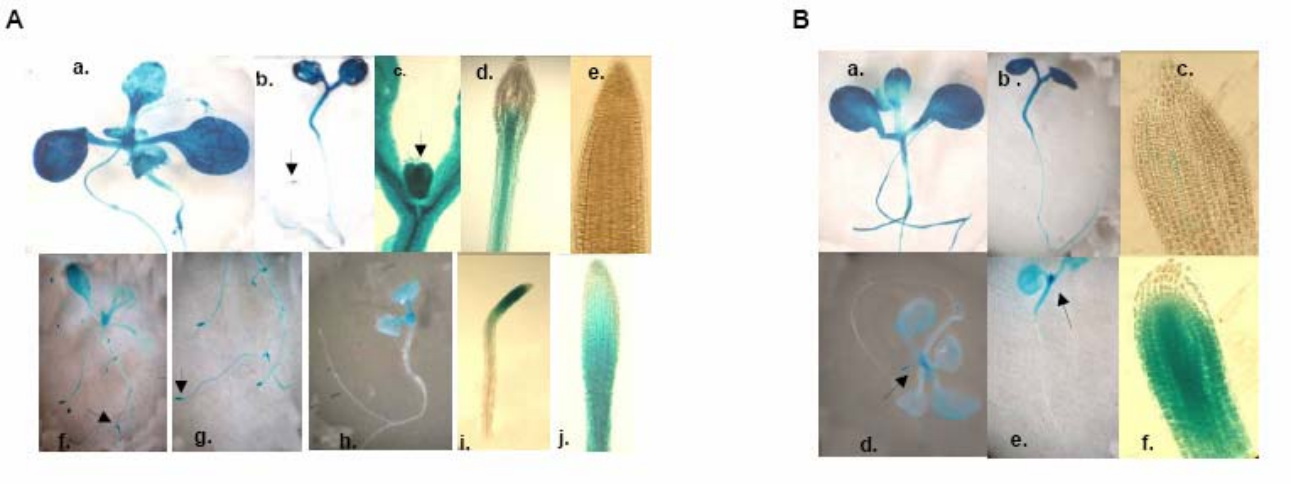


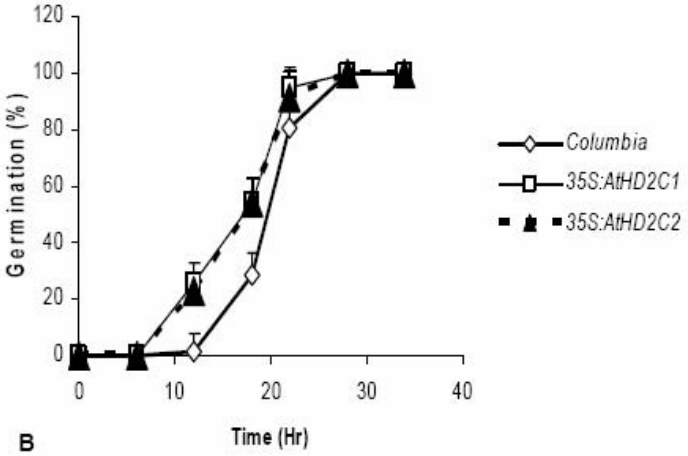
Figure 18. (A) Effect of ABA on the *AtHD2C* promoter:*GUS*. T3 *AtHD2C* promoter:*GUS* transgenic seedlings grown on ABA-free MS medium were transferred to medium containing 100 µM ABA for 6 hours and were subsequently collected for staining with 5-bromo-4-chloro-3-indolyl--glucuronic acid. (a) 6-day-old seedling (post-germination), (b) 2-day-old seedling, (c) shoot apical meristem (SAM-arrowhead), (d) Em (embryonic) root, (e) root apical meristem. (f)-(j) tissues incubated on 100 µM ABA. (f) 6-day-old seedling (arrowhead points to GUS accumulation in RAM), (g) GUS accumulation in RAM after ABA treatment, (h) 2-day-old seedling, (i) Em (embryonic) root, (j) root apical meristem. (B) Effect of NaCl on the *AtHD2C* promoter driven *GUS*. *AtHD2C* promoter:*GUS* transgenic seedlings grown on NaCl-free MS medium were transferred to medium containing 400 mM NaCl for 12 hours and were subsequently collected for staining with 5-bromo-4-chloro-3-indolyl--glucuronic acid. (a)-(c) tissues grown on NaCl free medium. (a) 6-day-old seedling (post-germination). (b) 2-day-old seedling. (c) Root apical meristem (RAM). (d)-(f) tissues incubated on 400 mM NaCl. (d) 6-day old-seedling; (e) 2-day-old seedling; (f) Root apical meristem (RAM).

35S:AtHD2C1 and *35S:AtHD2C2* seedlings demonstrated robust growth and larger sized plants than their wild-type counterparts (Figure 19C). The transgenic plants flowered much earlier than the wild-type plants (Figure 19B) and one prominent abnormality observed in the *35S:AtHD2C* transformed plants was the improper development of mature rosette leaves into a 'squeezed' leaf phenotype (Figure 19B). The relative number of the 'squeezed' leaf found in the different lines is listed in Table VI. A large population of siliques in the *35S:AtHD2C1* and *35S:AtHD2C2* transgenic plants were shorter than the normal wild-type siliques (Figure 19, Table IX). Additionally, the seed count was reduced in the transgenic siliques as compared with the wild-type siliques (Table VIII). The empty vector transformed lines (*35S:GFP)* demonstrated same behavioral patterns as wild-type (data not shown) and served as control. However, these results do not identify a definitive role for HD2C in *Arabidopsis* development as ectopic expression may cause abnormal hierarchical cascades leading to evident phenotypes.

(b) ***AtHD2C* T-DNA insertion line analysis**

We identified a T-DNA insertion mutant of *HD2C* in the Salk collection of T-DNA lines, *SALK_039784* (http://signal.salk.edu/cgi-bin/tdnaexpress). T-DNA insertion lines were selected over subsequent generations by their ability to germinate in the presence of kanamycin. Homozygosity was confirmed by using PCR for three plants of the same *SALK_039784* insertion lines (Figure 20B). The T-DNA insertion lies in the sixth exon of *AtHD2C* (Figure 20A) and is localized at the nucleotide 2116 of the *HD2C* gene (numbering refers to relative nucleotide position to the ATG start codon). This insertion may lead to the disruption of *AtHD2C* expression. RT-PCR (Figure 20C) failed to detect *HD2C* transcript accumulation in the insertion lines, confirming the knockout of *HD2C* in these lines.

A


120
100
80
60
40
20
0
Germination (%)
0
10
20
30
40
Time (Hr)
Columbia
35S:AtHD2C1
35S:AtHD2C2

B


a.Normal leaf
b.Squeezed leaf
c.35S:AtHD2C2 24 day
d. Wild type 24 day
e.35S:AtHD2C2 24 day
f. Wild type 24 day

C


WILD-TYPE
1 week old seedlings
35S:AtHD2C1
1 week old seedlings
35S:AtHD2C2
1 week old seedlings

D

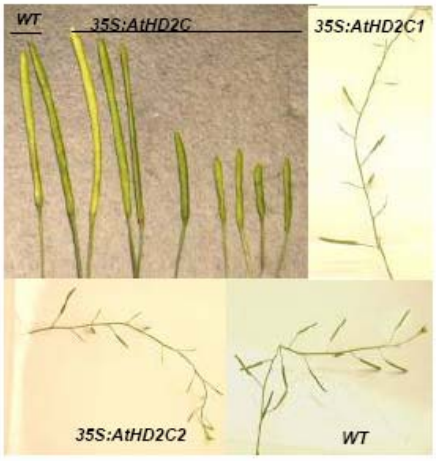

WT
35S:AtHD2C
35S:AtHD2C1
35S:AtHD2C2
WT

Figure 19. Growth phenotypes of the *AtHD2C* overexpression lines (A) Germination rates of the WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic lines on ABA-free medium. 8-week old seeds were plated on ABA-free medium after 2 days of cold treatment and germination (full radicle emergence and cotyledon formation) was scored starting at 0 hours of incubation up to 34 hours post-incubation, counting germination at 6 days intervals. Standard error is plotted for three replicate assays with ~100 seeds (n=100) in each plate. (B) Abnormalities associated with the transgenic plants: 'Squeezed leaf' in the *35S:AtHD2C1* and *35S:AtHD2C2* transgenic lines. Early flowering phenotype at 24 days after germination (DAG). (C) Growth of the transgenic seedlings on ABA-free medium. Seeds were plated on ABA-free medium after 2 days of cold treatment. Post-germination growth (cotyledon greening/expansion and embryonic root elongation) was monitored and seedling sizes compared. (D) Fully mature siliques in WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic lines.

TABLE VII. Average percentage of 'squeezed' leaves for 20 mature rosettes in wild-type and transgenic plants.

Average percentage (%)of abnormal leaves			
Wild type	*35S:AtHD2C1*	*35S:AtHD2C2*	*1303*
0.78±1.34	8.765±2.677	6.55±2.464	0.95±1.5667

* Individual percentages were calculated as the number of abnormal leaves from the total number of leaves in each rosette. (±) values indicate standard error for n=20

TABLE VIII. Depicts average number of seeds for 40 mature siliques from wild-type and transgenic plants.

Average number of seeds		
Wild type	*35S:AtHD2C1*	*35S:AtHD2C2*
101±3.47	62±5.2	60± 5.365

(±) values indicate standard error for n=40

TABLE IX. Depicts average percentage* of mature stunted siliques for 20 wild-type and transgenic plants.

Average percentage (%) of stunted siliques		
Wild type	*35S:AtHD2C1*	*35S:AtHD2C2*
6.2051±1.0507	20.282± 1.458	21.128±1.3820

* Individual percentages were calculated as the number of stunted siliques from the total number of siliques on each plant. (±) values indicate standard error for n=20

This insertion line was subsequently used for ABA, salt and mannitol sensitivity assays in the seedling stage. The T-DNA insertion line demonstrated similar phenotypic sensitivity as the wild-type plants to treatment with ABA, salt or mannitol (Figure 20D-G). The germination rates and root length elongation efficiency was the same as wild-type. Also, the T-DNA insertion plants were as sensitive to salt and drought in the vegetative stage as the wild-type plants (Figure 20E).

(c) *35S:AtHD2C* seeds are insensitive to ABA, NaCl and Mannitol during germination and post- germination development phase

ABA response

ABA is instrumental for embryo maturation and maintaining smooth transitions between developmental windows, such as radicle emergence and seedling growth **(**Hoecker *et al***,** 1995). Based on previous reports describing accumulation of *HD2B* and *HD2C* mRNA in geminating embryos of *Arabidopsis* and down-regulation of 'seed specific' genes in *AtHD2A* mutant (Wu *et al***,** 2004), we tested ABA sensitivity of the *35S:AtHD2C* plants. The *35S:AtHD2C* seeds were not viviparous and did not demonstrate defects in chlorophyll loss or attaining desiccation tolerance as evidenced by the normal maturation and hardening of the seeds. The loss of green color and hardening of seed-coat in normal time-span was the indicator of ABA production in the transgenic seeds. We further examined the sensitivity of the transgenic lines to ABA, during the germination post-germination events of radicle emergence, seedling greening and expansion. *35S:AtHD2C* transgenic seeds were

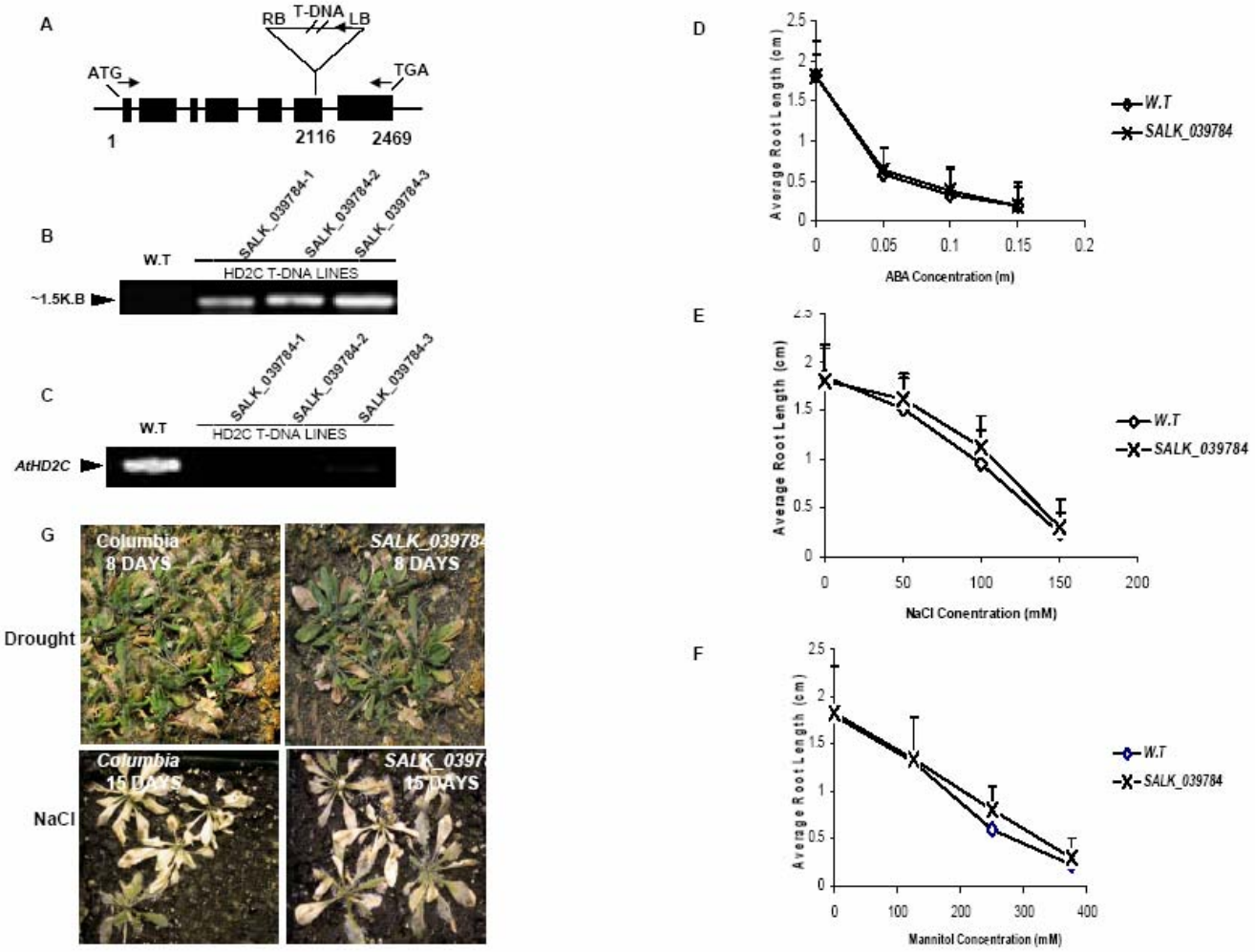

A
RB
T-DNA
LB
ATG
TGA
1
2116
2469
B
W.T
SALK_039784-1
SALK_039784-2
SALK_039784-3
HD2C T-DNA LINES
~1.5K.B
C
W.T
SALK_039784-1
SALK_039784-2
SALK_039784-3
HD2C T-DNA LINES
AtHD2C
D
Average Root Length (cm)
ABA Concentration (m)
W.T
SALK_039784
E
Average Root Length (cm)
NaCl Conentration (mM)
W.T
SALK_039784
F
Average Root Length (cm)
Mannitol Concentration (mM)
W.T
SALK_039784
G
Drought
NaCl
Columbia
8 DAYS
SALK_039784
8 DAYS
Columbia
15 DAYS
SALK_039784
15 DAYS

Figure 20. *SALK_039784 (*T-DNA insertion Line) plants are sensitive to ABA and stress in germination/post-germination and vegetative stages. (A) Map of the AtHD2C gene, black boxes indicating position of exons. The open triangle indicates position of insertion of the T-DNA. The arrow inside the triangle indicates T-DNA specific primer used in the PCR analysis. (B) PCR analysis to confirm homozygosity of the insertion lines. Genomic DNA was extracted from T3 plants selected on kanamycin and was used as a template for PCR using the primer pairs a 5' *AtHD2C* specific primer and a 3' T-DNA (Indicated by an arrow inside the triangle) LB primer. Three plants of the same insertion line were examined, i.e., *SALK_039784-1*, *SALK_039784-2*, *SALK_039784-3*. (C) RT-PCR analysis to confirm decrease in *AtHD2C* transcript accumulation. RNA levels of *AtHD2C* were determined by RT-PCR using total RNA isolated from 4-week old plant grown in soil in the 16 hour light photoperiod. (D) Root growth of WT and *SALK_039784* (T-DNA insertion Line) seedlings on ABA medium. Seeds were germinated on MS medium containing varying concentrations of ABA ranging from 0-0.2 μM. Root elongation was measured 5 days after incubation. The experiments were performed more than three times, sometimes and the results were consistent. The bars represent standard errors (n=40). (E) Root growth of WT, *SALK_039784* (T-DNA insertion Line) seedlings on NaCl medium. Root elongation was measured 5 days after incubation. The small bars represent standard errors (n=40). (F) Root growth of WT, *SALK_039784* (T-DNA insertion Line) seedlings on mannitol medium. (G) Draught and Salt sensitivity of the *SALK_039784* (T-DNA insertion Line) plants.

incubated on medium with ABA concentrations ranging from 0.05 μM-0.2 μM, 2 days post-stratification. The wild-type plants were able to germinate and form cotyledons on medium with 0.05 μM of ABA, but they displayed acute sensitivity to 0.1 μM ABA (Figure 21A). Subsequent to seed-coat breakage and radicle emergence, there was growth arrest with inhibition of cotyledon formation and further development on medium with 0.1 μM ABA. On the other hand, the *35S:AtHD2C* transgenic seeds were able to germinate and grow unrestricted at this ABA concentration and successfully developed healthy cotyledons and true leaves. Like the study conducted by Kang *et al***,** (2002), we wanted to determine the stage specificity of the ABA response. Therefore, we analyzed the ability of the *35S:AtHD2C* seeds to germinate and develop embryonic root in the presence of ABA. At 0.1 μM concentration of ABA, 63% retardation in the germination rate of the wild-type plants was observed. However, there was only 28% retardation in the germination rate of the two *35S:AtHD2C* transgenic lines (Figure 21C). A 0.05 μM concentration of ABA was able to restrict root growth in the wild-type seedlings by a margin of 97.3%. At the same time, the *35S:AtHD2C* transgenic seedlings were able to elongate root primordia with a minimal inhibition of 6.7% compared to control rates. Additionally, the development of the aerial parts from the shoot apical meristem was severely limited in wild-type seedlings at the concentration of 0.05 μM ABA, whereas the *35S:AtHD2C* transgenic seedlings continued to develop cotyledons and true leaves at a concentration of 0.1 μM ABA (Figure 21D and 21B). Upon further increasing ABA concentrations to 0.15 μM, the transgenic cotyledons displayed growth arrest, thus succumbing to retardation. These results reflect the ability of

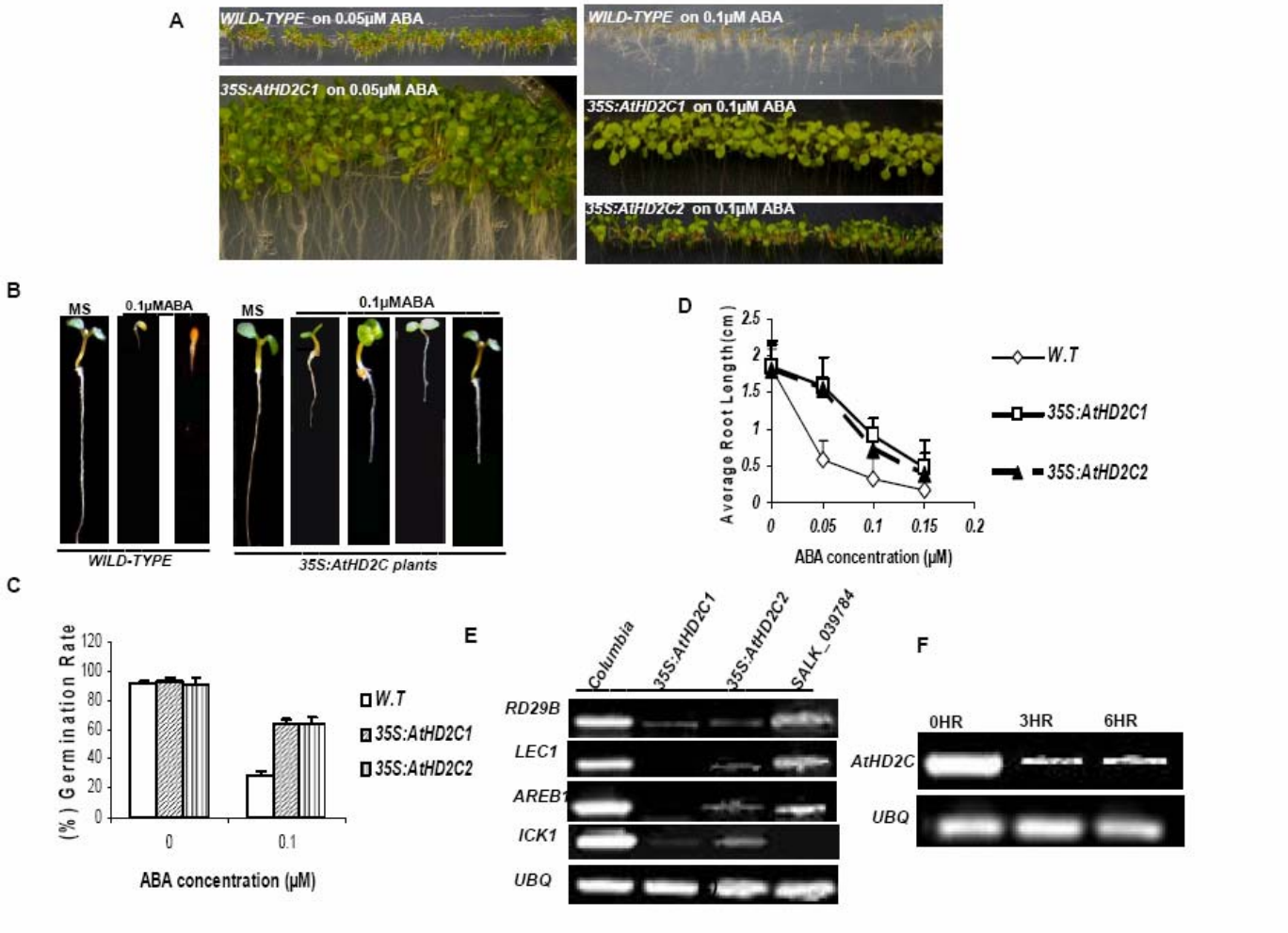
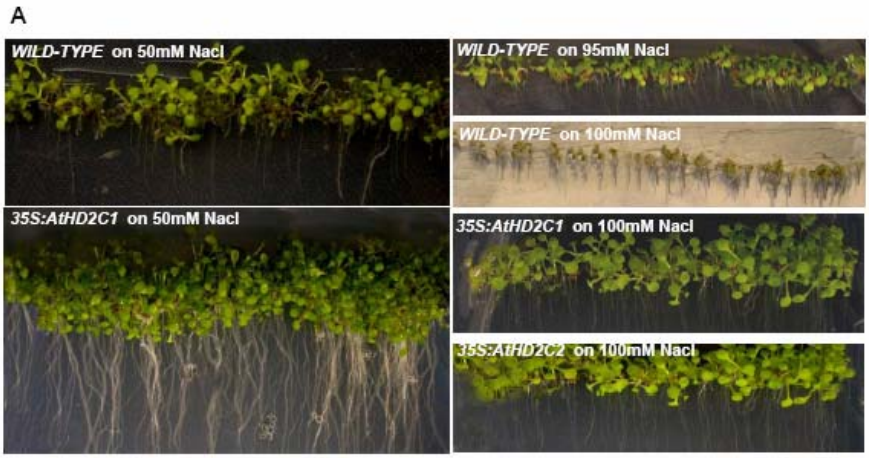
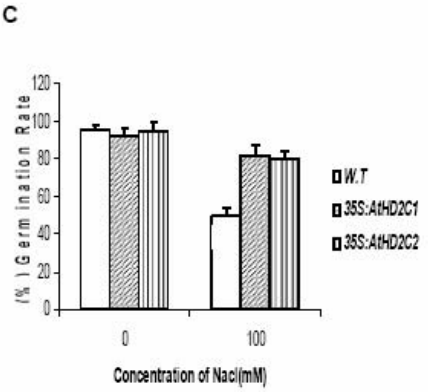

A
WILD-TYPE on 0.05μM ABA
WILD-TYPE on 0.1μM ABA
35S:AtHD2C1 on 0.05μM ABA
35S:AtHD2C1 on 0.1μM ABA
35S:AtHD2C2 on 0.1μM ABA
B
MS
0.1μMABA
MS
0.1μMABA
WILD-TYPE
35S:AtHD2C plants
C
(%) Germination Rate
120
100
80
60
40
20
0
0
0.1
ABA concentration (μM)
W.T
35S:AtHD2C1
35S:AtHD2C2
D
Average Root Length(cm)
2.5
2
1.5
1
0.5
0
0
0.05
0.1
0.15
0.2
ABA concentration (μM)
W.T
35S:AtHD2C1
35S:AtHD2C2
E
Columbia
35S:AtHD2C1
35S:AtHD2C2
SALK_039784
RD29B
LEC1
AREB1
ICK1
UBQ
F
0HR
3HR
6HR
AtHD2C
UBQ

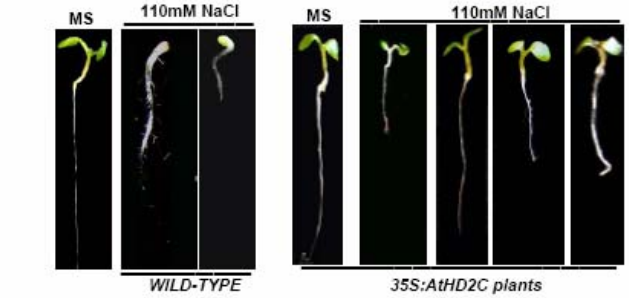
Figure 21. ABA sensitivity of the *35S:AtHD2C1* and *35S:AtHD2C2* lines in germination and post-germination stage. (A) Growth of WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic seedlings on MS medium containing 0.05 μM and 0.1 μM of ABA respectively. Seeds were germinated and grown for 12 days. (B) Growth of WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic seedlings on MS medium containing 0.1μM ABA. Seeds were germinated on the medium for 4 days, and representative plants (out of forty examined) were shown. (C) Germination rate of WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic seeds on ABA. 8-week old T3 seeds were pre-chilled for 2 days at 4ºC and were germinated on MS medium containing 0.1μM of ABA. Seedlings with fully emerged radicles and cotyledons were scored to obtain percent germination. Experiments were performed in triplicate (n=100 each), and the bars show standard errors. (D) Root growth of WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic seedlings on ABA. 8-week old T3 seeds were germinated on MS medium containing varying concentrations of ABA ranging from 0-0.2μM. Root elongation was measured 5 days after incubation. The experiments were performed more than three times. The bars represent standard errors (n=40). (E) Expression of ABA-regulated genes in the germination/post-germination phase in WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic lines, and *SALK_039784* (T-DNA Line), ABA-responsive genes were determined by RT-PCR using total RNAs isolated from 1-week-old plants grown on MS plates. Ubiquitin served as the internal control. (F) Expression of *AtHD2C* in WT treated with ABA; RNA levels of *AtHD2C* were determined by RT-PCR using total 1μg RNAs isolated from 1-week-old wild-type seedlings.

AtHD2C to interfere with ABA-mediated growth retardation at the germination and post-germination growth stages.

LEC1, *RD29B* and *AREB1* genes are typical markers of the different phases of embryonic maturation (Finklestein and Rock**,** 2002). Therefore, we examined the alteration in expression patterns of these genes in the *35S:AtHD2C* transgenic seedlings (Figure 21E). *35S:AtHD2C* transgenic seedlings were collected immediately after cotyledon formation and embryonic root establishment (4 days post-germination) and were assayed for ABA-inducible gene expression patterns. *LEC1* functions at the terminal stage of the cell division phase of embryogenesis to prevent further cell division and precocious germination (Holdsworth *et al***,** 1999). The expression of this gene was down-regulated in the *35S:AtHD2C* transgenic seedlings. Corresponding to this observation, the expression of the *AREB1* was also down- regulated. AREB proteins are bZIP class transcription factors that work synergistically during embryonic growth (reviewed in Holdsworth *et al***,** 1999, 2001). The *ICK1* gene works hand-in-hand with *LEC1* in the process of cell division termination as it is an inhibitor of cyclin dependant kinases that propagate cell division (Finklestein and Rock, 2002). The expression of LEA (Late embryogenesis abundant) gene, RD29B that is essential for maintaining dormancy (Delseny *et al*, 2001) was also decreased. Transcript accumulation of all these genes was also examined in the SALK_039784 (T-DNA insertion line) line (characterized in Figure 20). Expression of these genes was weaker in the *SALK_039784* line compared with wild-type. There is speculation about role of post-germination down-regulation of embryogenesis promoting regulators such as *LEC1* by epigenetic modification mechanisms (Ogas et al., 1999). To analyze the response of *AtHD2C* to ectopic application of ABA in the seedling stage, the transcript accumulation of *AtHD2C* in response to 6 hours ABA (100 μM) treatment was analyzed.


A
WILD-TYPE on 50mM Nacl
WILD-TYPE on 95mM Nacl
WILD-TYPE on 100mM Nacl
35S:AtHD2C1 on 50mM Nacl
35S:AtHD2C1 on 100mM Nacl
35S:AtHD2C2 on 100mM Nacl


C
(%) Germination Rate
120
100
80
60
40
20
0
0
100
Concentration of Nacl(mM)
W.T
35S:AtHD2C1
35S:AtHD2C2


B
MS
110mM NaCl
MS
110mM NaCl
WILD-TYPE
35S:AtHD2C plants

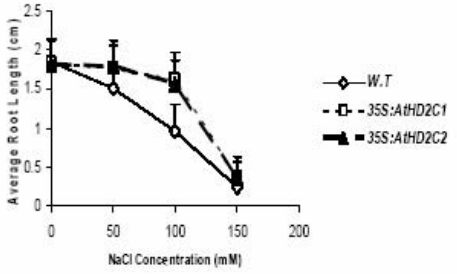
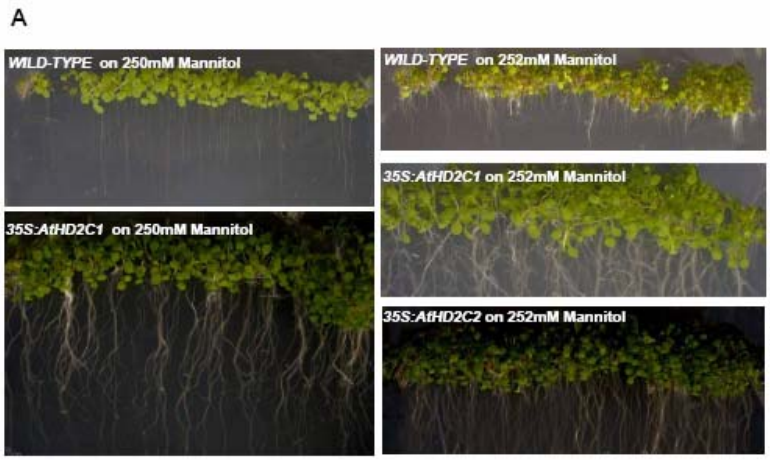

D
Average Root Length (cm)
2.5
2
1.5
1
0.5
0
0
50
100
150
200
NaCl Concentration (mM)
W.T
35S:AtHD2C1
35S:AtHD2C2

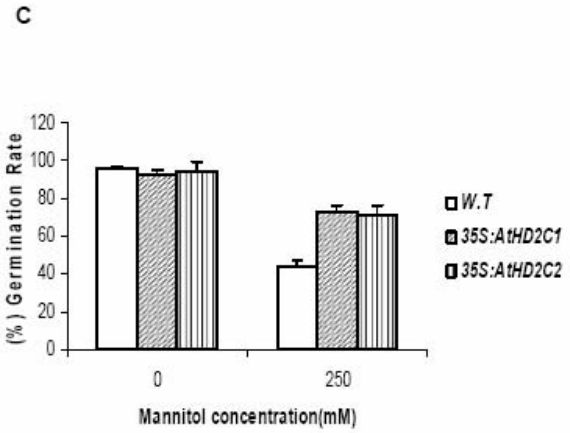
Figure 22. NaCl sensitivity of the *35S:AtHD2C1* and *35S:AtHD2C2* lines in germination and post-germination stage . (A) Growth of WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic seedlings on MS medium containing 50 mM and 100 mM of Nacl, respectively. Seeds were germinated and grown for 12 days. (B) Growth of WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic seedlings on MS medium containing 100 mM Nacl. Seed were germinated on the medium for 4 days, and representative plants (out of forty examined) are shown. (C) Germination rate of WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic seeds on NaCl. 8-week old T3 seeds were pre-chilled for 2 days at 4ºC and were germinated on MS medium containing 100 mM of NaCl. Seedlings with fully emerged radicles and cotyledons were scored to obtain percent germination. Experiments were performed in triplicate (n=100 each), and the bars represent standard errors. (D) Root growth of WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic seedlings on NaCl media. Seeds were germinated on MS medium containing varying concentrations of NaCl from 0-200 mM. Root elongation was measured 5 days after incubation. The bars represent standard errors (n=40).

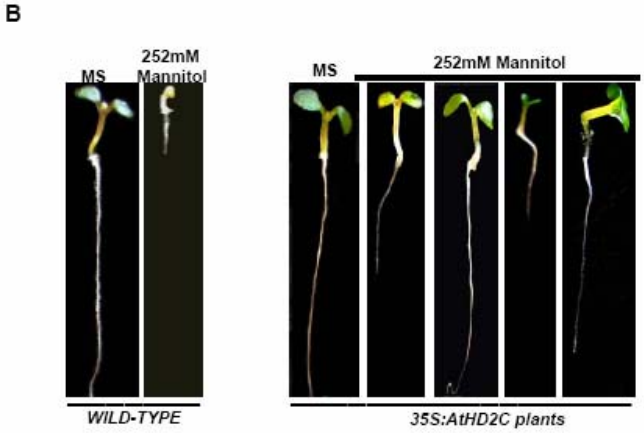
It was observed that the *AtHD2C* transcript was partially reduced in the seedlings by ABA treatment (Figure 21F). This observation supports the idea that ABA regulates *AtHD2C* expression negatively at certain steps in the signaling cascade, probably to de-repress essential signal transducers. Both germination and post-germination growth stages are insensitive to ABA and this is supported by the down-regulation of the ABA-inducible genes in the *35S:AtHD2C* seedlings.

NaCl response

There now is substantial evidence for cross talk between signaling pathways regulating response to ABA and assorted stresses (e.g., drought, salinity, and cold) (Ishitani *et al***,** 1997), sugars (Arenas-Huertero *et al***,** 2000; Finkelstein and Lynch, 2000; Huijser *et al***,** 2000; Laby *et al***,** 2000), and even meristem function (Ziegelhoffer *et al***,** 2000). Therefore, we tested the salt sensitivity of the *AtHD2C* overexpression line based on its resistance to ABA. All ABA-deficient (*aba*) and ABA-insensitive (*abi*) mutants tend to exhibit salt insensitivity during germination (Leon-Kloosterziel et al., 1996). Figure 22C depicts that 100 mM of NaCl was able to reduce germination of the wild-type plants by a margin of 45.9% whereas the germination efficiencies of the *35S:AtHD2C1* and *35S:AtHD2C2* lines were reduced by mere margins of 11.2% and 14.17% respectively. Additionally, the *35S:AtHD2C* overexpression lines demonstrated healthy germination, radicle emergence, cotyledon expansion and true leaf formation when grown on increasing concentrations of NaCl ranging from 50-110 mM (Figure 22A), after that root elongation was curbed and growth was halted.


A
WILD-TYPE on 250mM Mannitol
WILD-TYPE on 252mM Mannitol
35S:AtHD2C1 on 250mM Mannitol
35S:AtHD2C1 on 252mM Mannitol
35S:AtHD2C2 on 252mM Mannitol


C
(%) Germination Rate
120
100
80
60
40
20
0
0
250
Mannitol concentration(mM)
W.T
35S:AtHD2C1
35S:AtHD2C2


B
MS
252mM Mannitol
MS
252mM Mannitol
WILD-TYPE
35S:AtHD2C plants

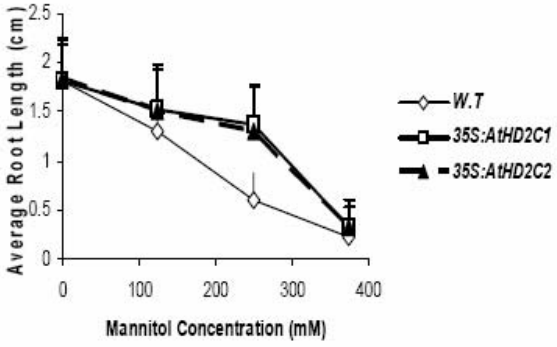

D
Average Root Length (cm)
2.5
2
1.5
1
0.5
0
0
100
200
300
400
Mannitol Concentration (mM)
W.T
35S:AtHD2C1
35S:AtHD2C2

Figure 23. Mannitol sensitivity of the *35S:AtHD2C1* and *35S:AtHD2C2* lines in germination and post-germination stage. (A) Growth of WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic seedlings on MS medium containing 50 mM and 250 mM of mannitol respectively. Seeds were germinated and grown for 12 days. (B) Growth of WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic seedlings on MS medium containing 250 mM mannitol. Seeds were germinated on the medium for 4 days, and representative plants (out of forty examined) are shown. (C) Germination rate of WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic seeds on a mannitol medium. Seeds were pre-chilled for 2 days at 4ºC and were germinated on MS medium containing 250 mM concentration of mannitol. Seedlings with fully emerged radicles and cotyledons were scored to obtain percent germination. Experiments were performed in triplicate (n=100 each), and the bars represent standard errors. (D) Root growth of WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic seedlings on mannitol. 8-week old T3 seeds were germinated on MS medium containing varying concentrations of mannitol ranging from 0-400 mM. Root elongation was measured 5 days after incubation. The bars represent standard errors (n=40).

At 50 mM of NaCl, the wild-type root elongation dropped by 18%, whereas the *35S:AtHD2C* transgenic lines lost 3.9% and 3% of their root elongation efficacy at the same concentration for *35S:AtHD2C1* and *35S:AtHD2C2* lines respectively (Figure 22D). At a 100 mM concentration of NaCl could we observed a distinct difference in the behavior of the wild-type and transgenic lines; wild-type plants showed a decrease in root length by 48% as compared to the 11.9% and 14.4% drop of the *35S:AtHD2C1* and *35S:AtHD2C2* lines, respectively (Figure 22B). Further increase in NaCl concentration proved to be toxic and growth inhibitory for the transgenic plants. The root elongation pattern differences can be evidenced in Figure 20B. Down-regulation of *LEC* and *RD29B* in the *35S:AtHD2C* lines (Figure 22E) was regarded as a marker for reduction in ABA mediated NaCl signaling in these lines as salt inhibits cell division and enhances desiccation tolerance via ABA signaling.

Mannitol response

Mannitol accumulation increases when plants are exposed to low water potential (Patonnier *et al***,** 1999), and accumulation is regulated by inhibition of competing pathways and decreased mannitol consumption and catabolism (Pharr *et al***,** 1995; Stoop *et al***,** 1996). Mannitol is used to asses the response of plants to osmotic stress. *35S:AtHD2C* transgenic seedlings were germinated 2 days post-stratification on MS medium containing 0-400 mM concentrations of mannitol; Columbia wild-type seedlings could grow and develop on medium containing 225-240 mM of mannitol. A 250 mM concentration of mannitol arrested their post-germination development. However, the wild-type seedlings could germinate and develop true-leaves, but the seedlings were unhealthy (yellow in color). Whereas, the *35S:AtHD2C* transgenic plants demonstrated insensitivity to the same concentrations of

mannitol as germination was robust and root elongation was expansive (Figure 23A). Germination was reduced by 52.2% in wild-type seeds at 252 mM of mannitol, whereas the *35S:AtHD2C1* and *35S:AtHD2C2* lines lost only 19.8% and 22.8% of their germination efficiency, respectively, at the same concentration of mannitol (Figure 23C).

Additionally, root development was monitored for embryonic root extension and cotyledon expansion. Wild type seedlings did not develop healthy green cotyledons subsequent to germination and radicle establishment on medium containing 250 mM concentration of mannitol. Rather, they incurred a massive reduction of 67.3% in root growth that declined further on higher concentrations. The *35S:AtHD2C1* and *35S:AtHD2C2* lines however were able to establish embryonic root and form healthy green cotyledons and suffered a relatively minor loss of 28.5% and 18.2% in their root growth, respectively (Figure 23B and 23D). This data indicates a role for AtHD2C not only in ionic stress response, but also in regulation of osmotic stress networks.

(d) ***35S:AtHD2C* plants demonstrate vegetative stress tolerance in response to NaCl and drought**

Apart from its critical function in maintaining seed dormancy regulating germination and seedling growth, ABA helps in optimizing vegetative growth during environmental stress conditions by maintaining osmotic homeostasis. At the cellular level, ABA is known to promote tolerance to abiotic stresses such as cold, drought and salinity (reviewed in Rock**,** 2000; Shinozaki and Yamaguchi-Shinozaki**,** 2000; Xiong and Zhu**,** 2001; Larkindale and Knight**,** 2002). Our results indicated a function for AtHD2C in the ABA mediated regulation of germination and post-germinative programming. We sought to investigate the response of the *35S:AtHD2C* transgenic plants to abiotic stress imposition by exposing them to physiological concentrations of salt and low-water stress.

Wild-type plants and *35S:AtHD2C* transgenic, 30-day old plants were watered with 300 mM of NaCl for a period of 25 days. Differences between wild-type and transgenic plants in their ability to tolerate ion toxicity and osmotic stress became evident 5th day onwards. The wild-type leaves demonstrated yellowing and manifestation of senescence, whereas the transgenic leaves maintained a healthy green appearance and very little cellular decay. This survival efficiency was measured as the percentage of green leaves over the time-span. On the 5th day after treatment (DAT), 63% of the wild-type leaves were green whereas 93.9% and 90.9% of the *35S:AtHD2C1* and *35S:AtHD2C2* leaves survived, respectively. The *35S:AtHD2C1* and *35S:AtHD2C2* leaves stayed green and the plants bolted, with senescence setting in at 20 days after treatment (60.4% and 56.5%), at that point onwards there was a sharp decline to 20.4% and 15.6% survival rates respectively. This was in significant contrast to the wild-type counterparts, that senesced to 34.3% by the 10th day and further perished by the 20th day, when their survival rate was just 5.2%. The wild- type plants never bolted and consequently never transited to flowering (Figure 24) ABA has been shown to be involved in the regulation of many stress-induced genes, and in some instances has been shown to be required for changes in gene expression in response to water-deficit stress (Bray, 1997). Twenty-two day old wild-type and transgenic plants were grown in pots that were left unwatered for a period of 25 days. Significant difference between wild-type and transgenic plants was observed 10 days after the plants were left unwatered.

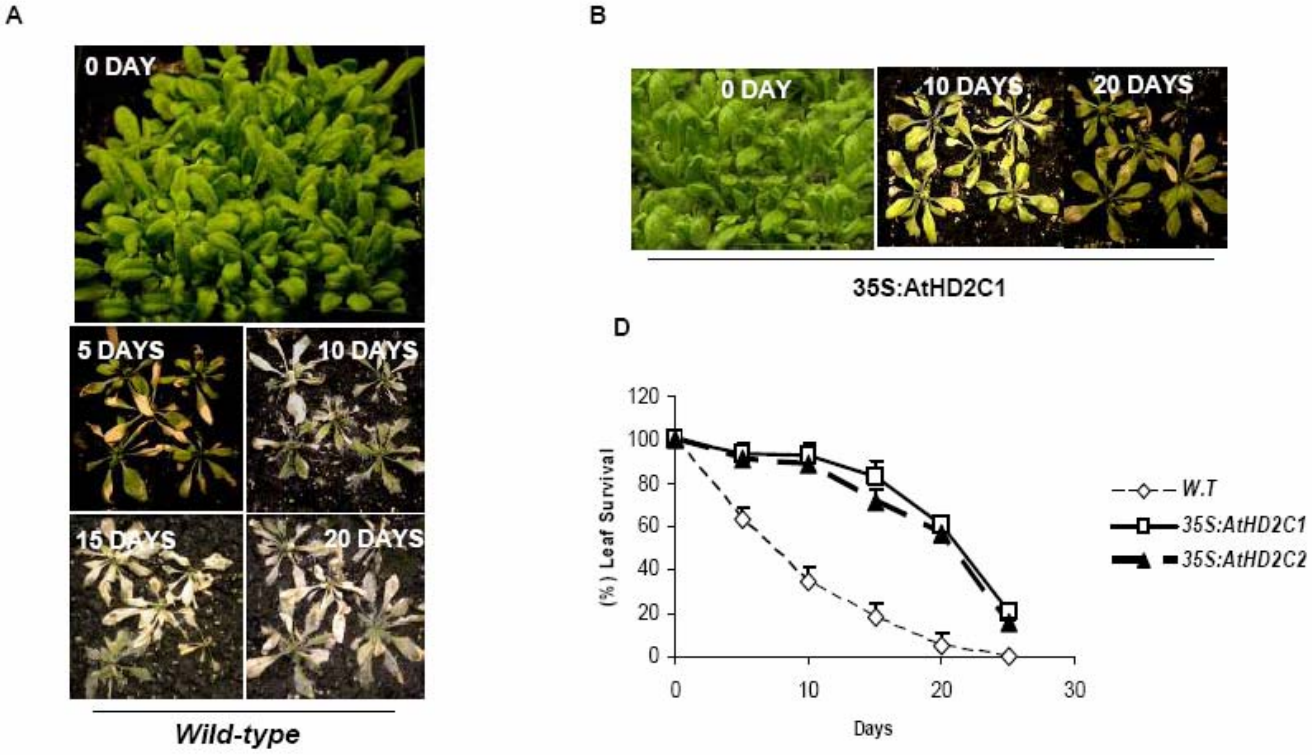

A
0 DAY
5 DAYS
10 DAYS
15 DAYS
20 DAYS
Wild-type
B
0 DAY
10 DAYS
20 DAYS
35S:AtHD2C1
D
120
100
80
60
40
20
0
(%) Leaf Survival
0
10
20
30
Days
W.T
35S:AtHD2C1
35S:AtHD2C2

Figure 24. Salt tolerant phenotype of the *35S:AtHD2C* plants in the vegetative stage. (A) Salt sensitivity of WT plants. Plants were germinated and grown on soil for 22 days and the mature rosettes were subsequently supplemented with water containing 300 mM of NaCl for 25 days. The photographs were taken at every five day intervals. (B) Salt tolerance of *35S:AtHD2C1* and transgenic plants. Transgenic plants were germinated and grown on soil for 22 days and the mature rosettes were subsequently supplemented with water containing 300 mM NaCl for 25 days. The photographs were taken at very five day intervals. (C) Salt tolerance of *35S:AtHD2C2* and transgenic plants. Transgenic plants were germinated and grown on soil for 22 days and the mature rosettes were subsequently supplemented with water containing 300 mM of Nacl for 25 days. The photographs were taken at very five day intervals. (D) Leaf survival rate of the WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic plants in salt stress conditions. Plants were germinated and grown on soil for 30 days and the mature rosettes were subsequently supplemented with water containing 300mM of NaCl for 25 days. Number of green leaves in a rosette were counted at five day intervals for 40 plants to obtain the survival curve. Bars depict standard error (n=40).

The marker for survival here also, was the percentage of leaves sustaining chlorophyll after treatment. On the 10th day, the survival count dropped to 44.4% for the wild-type plants, whereas it was relatively stable for the two transgenic lines at 88.6% and 80%, respectively. From this point onwards both wild-type and transgenic lines demonstrated sharp declines in their respective survival rates, but the transgenic plant decayed at much slower rate when compared with wild-types. On the 15th day, 6.7% of the wild-type leaves survived whereas *35S:AtHD2C1* and *35S:AtHD2C2* plants maintained 47.6% and 45.1% leaf survival, respectively (Figure25A, 25C and 25D).

We also measured the fresh weights of the wild-type and transgenic plants with these treatment conditions. As shown in Figure 25B, the *35S:AtHD2C1* and *35S:AtHD2C2* plants maintained a slow decline from their pre-treatment weights as compared with the wild-type plants that experienced a drastic drop in their weight when exposed to drought. Additionally, the stomatal aperture was investigated in the wild-type and transgenic plants in the middle of the dehydration treatment. It was evident that, the stomata in the *AtHD2C1* and *AtHD2C2* leaves were either partially or completely open as opposed to the wild-type stomata that were closed in dehydrating conditions (Figure 26). Water-deficit or high-salt conditions induce dehydration of plant cells, that may trigger physiological and biochemical responses against such stresses. Most of the genes that respond to dehydration, salinity and low temperature are inducible by ABA (Nordin and Palva**,** 1992). Many genes that respond to ABA are also expressed at the late stages of embryogenesis during the development of seeds and are thought to function in the protection of cell dehydration. Therefore, we checked the expression levels (Figure 27) of LEA class genes, *RD29B* and *RAB18* that function

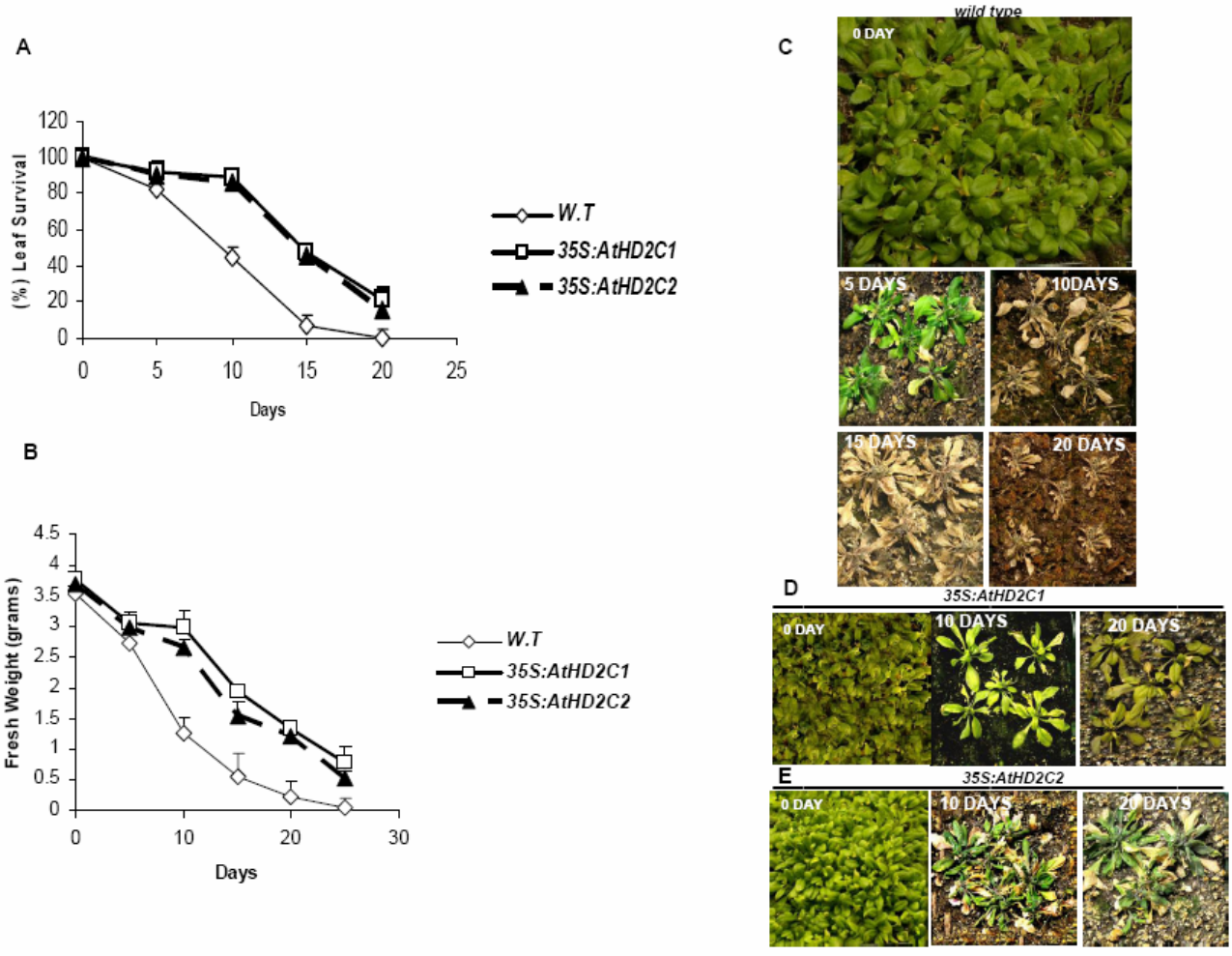

A
(%) Leaf Survival
120
100
80
60
40
20
0
0
5
10
15
20
25
Days
W.T
35S:AtHD2C1
35S:AtHD2C2
B
Fresh Weight (grams)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
10
20
30
Days
W.T
35S:AtHD2C1
35S:AtHD2C2
C
wild type
0 DAY
5 DAYS
10DAYS
15 DAYS
20 DAYS
D
35S:AtHD2C1
0 DAY
10 DAYS
20 DAYS
E
35S:AtHD2C2
0 DAY
10 DAYS
20 DAYS

Figure 25. Drought tolerance of the *35S:AtHD2C* plants; (A) Leaf survival rate of the WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic plants in draught stress conditions. Plants were germinated and grown on soil for 30 days and the mature rosettes were subsequently withheld from water for 25 days. Number of green leaves in a rosette were counted at five day intervals for 40 plants to obtain the survival curve. Bars represent standard error (n=40). (B) Fresh weight loss of the WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic plants in draught stress conditions. Plants were germinated and grown on soil for 22 days and the mature rosettes were subsequently withheld from water for 25 days. The fresh weight of detached rosettes was measured at every five day intervals for 20 plants. Bars represent standard error (n=20). (C) Draught sensitivity of wild-type plants. Plants were germinated and grown on soil for 22 days and the mature rosettes were subsequently withheld from water for 25 days. The photographs were taken at every five day intervals. (D) Drought tolerance of *35S:AtHD2C1* and transgenic plants. Transgenic plants were germinated and grown on soil for 22 days and the mature rosettes were subsequently withheld from water for 25 days. The photographs were taken at very five day intervals.

during the late phase of seed maturation to confer desiccation tolerance to the seed and are known to accumulate during dehydration stress in the vegetative stage.A distinct up-regulation of these genes was observed in *35S:AtHD2C1* and *35S:AtHD2C2* transgenic tissue, collected from mature rosette leaves (30 days after germination). Genetic studies have established that *ABI1* and *ABI2* are negative regulators of ABA transduction signals (Gosti *et al***,** 1999; Leung *et al***,** 2001). The concomitant increases of *ABI1* and *ABI2* mRNA levels and corresponding protein phosphatase activities induced by ABA have led to a model (Merlot *et al***,** 1997) in that *ABI1* and *ABI2* take part in a negative feedback regulatory loop that continuously resets the ABA signaling cascade to adjust the response to the ABA level (Cherel *et al***,** 2002). These two genes are positioned upstream to Ca^{2+} induced S-type anion channel activation whereby, K^+ efflux is initiated in the guard cells leading to stomatal closure (Hamilton *et al***,** 2000). *ABI1* accumulation was unaffected in the *35S:AtHD2C* transgenic lines, but *ABI2* levels were severely reduced in the transgenic lines. These results were complemented by a weak rescue of *ABI2* expression in the *S28601* line, indicating specific targeting of the *ABI2* dependant signal processing. This led us to examine expression of *ABI2* inducible gene *ADH1,* as de Bruxelles *et al***,** (1996) demonstrated a reduction in *ADH1* accumulation in the *abi2* mutants. Accordingly, a down-regulation of *ADH1* was observed in the *35S:AtHD2C* transgenic lines. KAT1 and KAT2 are K+ inward rectifying channels that are regulated by their phosphorylation status and are inhibited by ABA production during dehydration stress (Gaymard *et al***,** 1998). This mediates the closing of stomata due to turgor loss and membrane depolarization that permits retention of water during deficit. The expression of both of these genes was reduced in the *35S:AtHD2C*

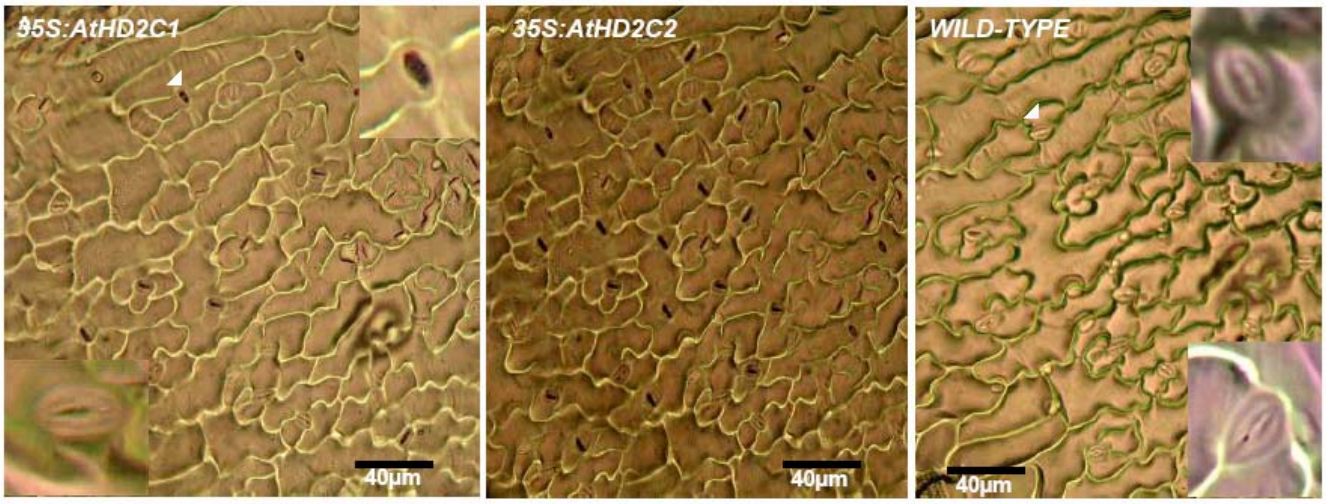


TABLE IX. Depicts Average percentage of closed stomata (adaxial surface) counted for 10 wild-type and transgenic leaves respectively.

Average percentage (%) of open stomata*		
Wild type	*35S:AtHD2C1*	*35S:AtHD2C2*
83.3±**1.667**	42.4±0.645	35.05±**1.656**

* Individual percentages were calculated as the number of number of closed stomata from the total number of 400 stomata on the adaxial surface of each leaf. (±) values indicate standard error for n=10

Figure 26. Stomatal aperture of the WT, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic plants; Stomatal guard cells were observed in the middle of the dry period (15 days after dewatering). Arrows indicate guard cells, and the insets show representative stomata. The bars at the bottom of the pictures represent the magnification under which these bright field pictures were taken.

transgenic lines and rescued in the *SALK_039784* lines, further supporting a positive role for AtHD2C in vegetative ABA mediated stress signaling Xylem K^+ content is regulated by activity of a stelar K^+ outward rectifier (SKOR) (Gaymard *et al***,** 1998) that is repressed by ABA. Repression of its expression has been suggested to be part of adaptive water stress response mediated by ABA. Expression of this gene was significantly reduced in the *35S:AtHD2C* transgenic lines indicating a positive role for AtHD2C in ABA signaling. The ABA-regulated genes, expression patterns in the *35S:AtHD2C* vegetative tissues delineated a positive regulatory role for AtHD2C in ABA mediated stress signaling. As the results appear, ABA signaling mediators and AtHD2C seem to share a positive as well as a negative relationship at distinct spatial windows of germination.

(iv) Interaction between HD2-Type HDACs and RPD3-Type HDACs

(a) AtHD2B/AtHD2C and AtHDA1 do not interact

To better understand the molecular mechanism of the HD2 family, we thought it important to determine, proteins they form complexes with. HDACs are recruited in complexes with other HDACs and co-repressors (Pandey *et al***,** 2002). Therefore, we tested if HD2 genes were indeed HDACs, associate with other HDACs. Hence, a yeast two hybrid screen was conducted to examine if AtHD2B and AtHD2C could interact with AtHDA1 that is a class I HDAC. *AtHD2B* and *AtHD2C* were subcloned downstream to the DNA binding domain of the yeast GAL4 gene (Figure 28A and 28C). *HDA1* was subcloned downstream to the activation domain of the *GAL4* gene. The *AtHD2B/AtHD2C-GAL4BD* constructs and the *AtHDA1-GAL4AD* construct pairs were transformed sequentially into a yeast host strain (HF7C).

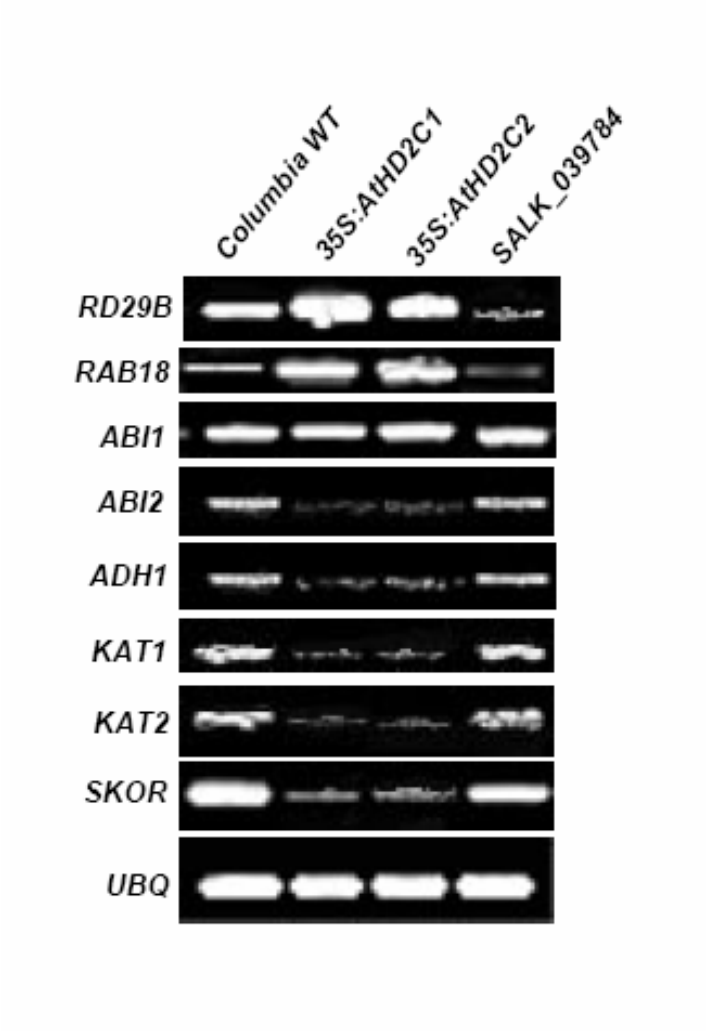


Figure 27. Expression of ABA-regulated genes in the vegetative phase in wild-type, *35S:AtHD2C1* and *35S:AtHD2C2* transgenic lines and *SALK_039784* (T-DNA Line); RNA levels of ABA-responsive genes were determined by RT-PCR using total RNAs isolated from 4-week old plants grown in soil in 16 hour light photoperiod.

No interaction was observed between AtHD2B and AtHDA1 (Figure 28B) and between AtHD2C and AtHDA1 (Figure 28D). This conclusion was based on the disability of the co-transformants to thrive on the histidine free medium as compared to the positive control. Additionally, interaction was not detected between AtHD2B/AtHD2C and AtHDA1 in the LacZ assay as compared to the positive control (data not shown). The HD2 family members might interact with some other transcription factors and this is yet to be uncovered.

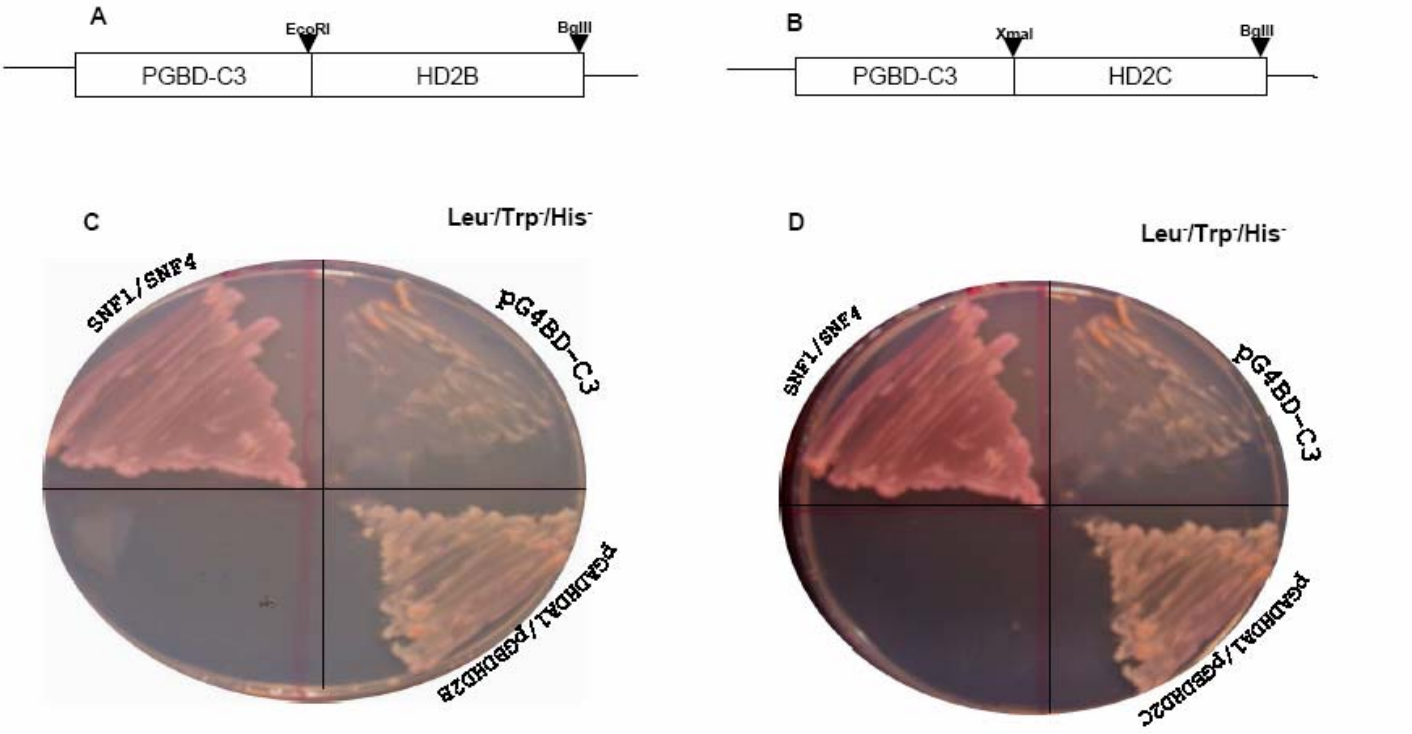


Figure 28. Yeast two hybrid screen for interaction between AtHD2B/AtHD2C and HDA1. (A) Map of PGBDHD2B. (B) Plate assay to test interaction between PGBDHD2B and PGADHDA1 (C) Map of PGBDHD2C. (D) Plate assay to test interaction between PGBDHD2C and PGADHDA1.

DISCUSSION AND CONCLUSIONS

(i) HD2 proteins act as transcription repressors

Studies conducted on the HD2 gene family, have been channeled towards deciphering the functional significance of these genes in the developmental context (Wu *et al,* 2000; Lusser *et al,* 1997). To further characterize this family, we demonstrated that the *AtHD2A*, *AtHD2B*, *AtHD2C* had the same spatial pattern of expression as they accumulated in all the organs examined, but at different levels. Similar spatial patterns of expression have been observed for genes that control embryogenesis, such as *WUSCHEL* and somatic embryogenesis receptor kinase (*SERK*) (Mayer *et al,* 1998) where they are involved in the maintenance of both shoot apical meristems and embryonic stem cells. An *in situ* hybridization performed by Zhou *et al,* (2004) showed the strongest expression of the *AtHD2A*, *AtHD2B*, and *AtHD2C* genes in embryos, implicating that the aborted seed phenotype that was observed in the *AtHD2A* antisense plants (Wu *et al,* 2000) might result from a defect in embryogenesis, resulting from the silencing of *AtHD2A*. Additionally, in this same report, ectopic expression of *BBM* that can induce somatic embryogenesis allowed for the expression of *AtHD2A*, *AtHD2B*, and *AtHD2C*, particularly in pre-embryonic tissues and somatic embryos. This data indicated that the expression of these genes is tightly correlated with both somatic and zygotic embryogenesis and is likely to be essential for embryo development. *AtHD2D* on the other hand, formed the exception in this group by accumulating selectively in the flowers and to a much smaller extent in the stems. It might play a specialized role in flower development that is yet to be investigated. The expansive pattern of expression of this gene family implicates that these genes have some special function in the developmental pathways of the

plants. However, the similarity of *AtHD2A*, *AtHD2B*, and *AtHD2C* expression patterns also raises the possibility of functional redundancy.

The control of intracellular location is an important regulatory mechanism for HDAC proteins in yeast and mammalian cells (Hirschler-Laszkiewicz *et al***,** 2001). It was demonstrated that mammalian HDA1-type HDACs were mobilized from the cytoplasm to the nucleus by phosphorylation. Using GFP fusions, we demonstrated that HD2A, HD2B, and HD2C accumulated in the nuclei of *Arabidopsis* cells. Recently, it was found that *Arabidopsis* HD2A is present in the *Arabidopsis* nuclear matrix using ESI tandem mass spectrometry to study the nucleoprotein complexes (Calikowsk *et al***,** 2003). In addition, maize HD2 was also localized in the nucleolus (Lusser *et al***,** 1997). This study indicated that *Arabidopsis* HD2 proteins may also be localized into nucleolus. The nucleolar localization of other types of HDACs has also been demonstrated in yeast and mammalian cells (Hirschler-Laszkiewicz *et al***,** 2001). The nucleolus is recognized as the site of rRNA transcription, rRNA processing, and ribosome assembly. However, recent studies suggest that the nucleolus functions more broadly in gene expression (Pederson**,** 1998) and may play a crucial role in cellular processes such as the control of cell cycle, aging, and mRNA export. Therefore, the HD2 proteins might play crucial roles in these essential developmental processes.

Most *Zm*HD2a homologs show a characteristic domain structure. A conserved NH_2-terminal region, a central part consisting of two acidic stretches interrupted by a region rich in basic residues and another basic domain adjacent to the second acidic stretch, that contains putative nuclear localization signals (Dangl *et al***,** 2000). The amino terminal of these proteins consists of an invariable pentapeptide motif (MEFWG) as well as two

conserved amino acid residues, that have been postulated to be critical for catalytic activity (Aravind and Koonin**,** *1998*), a histidine at position 25 surrounded by hydrophobic amino acids and an aspartate (glutamate in At-HD2c) at position 72. The presence of these residues in all sequences analyzed strongly suggests that they are indeed HDACs, although enzymatic activity has not yet been determined. Three distinct domains have been identified in the HD2 family of proteins: the predicted N-terminal deacetylase catalytic domain, the middle region consisting of the extended acidic domain, and the C-terminal domain (Aravind and Koonin**,** 1998; Dangl *et al***,** 2001; Lusser *et al***,** 1997; Wu *et al***,** 2000). Both AtHD2A and AtHD2C have a putative zinc-finger domain in their C-terminal, whereas AtHD2B and AtHD2D do not. Deletion of the C-terminal domain from AtHD2A did not affect gene repression activity, indicating that this domain is not required for gene repression (Wu *et al***,** 2000). Deletion of the extended acidic domain and the domain containing predicted catalytic residues of AtHD2A resulted in the loss of gene repression activity, suggesting that both domains may be essential for AtHD2A function. At the very C-terminus, six of the eight proteins show a single, putative zinc-finger motif. The study conducted here, indicated that the N-terminal EFWG motif is essential, and H25 is important for gene repression activity of AtHD2A. H25 has been suggested to be important for the activity in HD2-type HDACs (Aravind and Koonin**,** 1998). It is speculated that the invariant aspartic acid is the nucleophile involved directly in lysine deacetylation, that may be facilitated through a charge relay system with the conserved histidine (arginine), thus making the histidine essential for catalysis.

(ii) *AtHD2C* expression is regulated by ABA

Upon examination of the *AtHD2B* and *AtHD2C* promoters, several motifs involved in seed-development regulation and ABA regulation were found. These elements are grouped into

Tables in figure 15 and 16 where the core consensus sequences and functions of these motifs are outlined. The *cis*-acting sequences essential for ABA responsiveness can be classified into four main groups (reviewed by Finklestein and Rock**,** 2002): the G-box elements designated ABREs (CACGTG) and the functionally equivalent CE3 (coupling element)-like sequences, the RY/Sph (CATGCATG) elements, and recognition sequences for MYB (YAAC (G/T) G) and MYC (CANNTG) class transcription factors (Rock**,** 2000). The *AtHD2C* promoter has ABRE (ABA response element) and RY elements. Both of these elements are involved in ABA response. The bZIP class transcription factors such as ABI5 bind to the ABRE in the target genes by interacting with the B3 domain transcription factor ABI3 and tether them onto their target promoters (Nakamura *et al***,** 2001). On the other hand the ABI3 transcription factors are known to bind the RY motifs (Kim *et al***,** 1997; 2000; Uno *et al***,** 2000). Additionally, Chandrasekharan *et al***,** (2003) demonstrated that there is RY module specific activation of the phaseolin promoter during embryogenesis, further confirming that the presence of the RY elements in the promoter implicates involvement in seed maturation. It could be possible that these transcription factors could be targeting the *AtHD2C* promoter and recruiting other repressors to, to down-regulate its expression. Additionally, the *AtHD2C* promoter also contained the cell-cycle regulation motif MSA (M-specific activator) that suggests that these proteins might be recruited to enhance cell-cycle progression rates and therefore *MSA* needs to be silenced in the early phase of seed maturation when cell-cycle is arrested for developmental transition (Ito *et al***,** 2001).The periodic expression of B-type cyclin genes is regulated at least in part by a periodic change in the activity of their promoters in the cell cycle (Shaul *et al***,** 1996; Ito *et al***,** 1998; Colón-Carmona *et al***,** 1999; Tréhin *et al*., 1999). It was demonstrated that the promoter activation

timing of the cyclin B1 gene, *CycB1,* from *Catharanthus roseus* during the cell cycle, is determined by a single type of *cis* element called MSA (M-specific activator), that is necessary and sufficient for periodic promoter activation (Ito *et al***,** 1998a). MSA-like motifs are found in B-type cyclin promoters from various plant species. Additionally, these elements are bound by MYB class of transcription activators that are ABA-inducible (Ito *et al***,** 2001). Therefore, the presence of this element in the *HD2B* and *HD2C* promoter implicates that these genes may be targeted by ABA-regulated transcription factors that in turn control the timing of cell cycle progression that has an intricate involvement with histone acetylation status (Jasencakova *et al***,** 2001). The MBS or MYB binding site (CAACTG) element found in the *HD2B and HD2C* promoters is drought inducible and is bound by the MYB class transcription factors, that are bHLH- type proteins (Yamaguchi-Shinozaki and Shinozaki**,** 1994; Abe *et al***,** 1997). The presence of this motif might explain the involvement of HD2C in drought response of the *35S:AtHD2C* transgenic plants. The HD2C promoter could be up-regulated by the binding of the MYB transcription factors during drought to de-repress stress response genes. The *HD2C* promoter also contained endosperm–expression motifs Skn-1(GTCAT) and GCN4 (GTCA) that indicate a function for this protein in some aspects of seed development (Hoecker *et al***,** 1995). These elements were previously identified to be essential for endosperm expression in the rice storage protein glutelin GLU-B1HAT. This is quite interesting as the repressor is activated in some situations. We speculate that the expression of HD2 genes expression could be up-regulated by seed maturation factors, either dependant or independent of ABA to regulate endosperm formation.

The *AtHD2B* and *AtHD2C* promoter driven *GUS* expression patterns were also examined in the vegetative stage to determine if the expression patterns were the same as that

observed in the spatial profiling of the HD2 gene family. Additionally, we wanted to confirm if the HD2 proteins are expressed in vegetative and flowering phases of plant development that would indirectly implicate a function for them in these stages. *GUS* was found to be expressed in almost all mature tissues examined including leaves, flowers, siliques and all parts of the flower except the petals. These results suggest that the HD2 protein accumulation in the vegetative phase might facilitate their positive regulatory role during stress impositions. Why the HD2 proteins might act as a developmental switch is very complicated as there are very few examples in nature when proteins exercise different levels of control in different developmental stages. One of the examples where a protein exercises a dual role in stage–specific manner is c-myc protein that was found to be expressed in the embryogenesis stages of development of mice , but down-regulated in the adult stages (Cre'ancier *et al***,** 2000).

We examined *GUS* reporter expression driven by the *AtHD2C* promoters in the germination stage to check if it supports the results observed in the ABA sensitivity assay. Accordingly, the *GUS* expression was down-regulated in seedlings at early (2 days) and late (6 days) post-germination stages. This observation signifies that ABA down-regulates *AtHD2C* expression in the post-germination phase. This can be interpreted in a way that when there is a stressful environment during the time of germination or post-germination, ABA down-regulates post-germinative growth promoting factors such as HD2 proteins to induce transient dormancy to tide over the challenging period. Post-germinative growth involves a range of biochemical processes to function smoothly including lipid catabolism and activation of the glyoxalate cycle (Eastmond *et al***,** 2000). It is possible that the HD2 proteins might be involved in propitiating these processes to help keep the transition smooth.

Although the precise roles of the *ABIs* in regulating lateral root growth are not understood, it is noteworthy that *ABI5* is specifically expressed in root tips from emergence onward (Brocard *et al***,** 2002). We found an accumulation of *GUS* at the root tips after ABA treatment. It has been demonstrated that root meristems and shoot meristems have different responses to hypoxic stress (Ellis *et al***,** 1999). This result led us to speculate if AtHD2C accumulates in the root tips concomitantly with ABI5 to down-regulate any growth inhibition attempts by the ABA signal cascade. Alternatively, HD2 proteins could also be part of the ABA feedback loop mechanism. When stress levels increase, ABI5 and AtHD2C (and other HD2 proteins) might accumulate in the root tips and the ABI5 will enhance growth inhibition and transient dormancy. On the other hand, AtHD2C might just sit in that location 'poised' for action. Once the stressful period is over, HD2 proteins will serve to down-regulate the ABI5 (AREB) proteins, thereby relieving ABA mediated repression. Also, as can be seen the *GUS* accumulation was very high in the post-imbibition seeds, i.e., seeds that are ready to germinate. This indicated that at the time of phase transition to post-germinative growth, the HD2 proteins accumulate in high levels. Therefore, results thus far indicate that ABA and HD2 proteins share an antagonistic relationship that is to be further analyzed.

(iii) *AtHD2C* is involved in ABA response

The *AtHD2C* was ectopically expressed in *Arabidopsis* to asses the range of developmental processes this gene might regulate. To complement this study, an *AtHD2C* T-DNA insertion line was also analyzed. The *AtHD2C* overexpression lines had some obvious phenotypes. Marked early flowering, reduction in seed number, stunted siliques and early germination. Ectopic expression of *AtHD2A* as a fusion protein disrupted normal development and generated many pleiotropic effects in a variety of somatic and reproductive tissues (Zhou *et*

al, 2004). This observation indicated that the components needed for specificity may be absent in non-target tissues and/or that ectopically expressed *GFP–HD2A* may be acting on atypical non-specific target sites. However, the results obtained from overexpressing genes needs to be interpreted carefully. Overexpression may induce direct or secondary effects on gene expression. For example, overexpressing *AtHD2A* may induce silencing of endogenous *HD2* genes. Overexpression might lead to hierarchical cascades that result in the apparent phenotypes rather than gene-specific abnormality (Finklestein *et al*, 2002). However, the early germination phenotype is consistent with the involvement of the HD2 proteins in embryo development. Additionally, the effects on flowering were consistent upon several examinations and were very prominent. This indicates that the HD2 proteins might be involved in flowering time regulation. The role of histone deacetylases and other repressors in regulating transition from vegetative to reproductive phase is gradually coming to light (Kornreef *et al*, 1998). It is a quite well known fact that the polycomb group of proteins control the embryo and floral development by regulating respective homeotic genes (Chanvivattana *et al*, 2004). Additionally, there have been reports about the interaction between PcG proteins and HDACs speculating that the PcGs might recruit the HDACs to achieve repression (Finklestein and Rock, 2002). The HD2 proteins might be a part of this repression complex as it was initially isolated in a large 400KDa complex (Lusser *et al*, 1997). Also, these proteins have Zn-fingers at their C-terminal ends through which they can mediate protein-protein or protein-DNA interactions (Dangl *et al*, 2001). The abnormal leaf phenotypes demonstrated by the overexpression lines might be due to the role of the HD2 proteins in multiple development pathways or it might be an artifact of overexpression. The reduced fruit and seed phenotype may also be due to the involvement of the HD2 gene family

with seed maturation and development and partly due to the exaggerated environment of ectopic expression. It can be observed in Tables VII, VIII and IX that there is a very low frequency of naturally occurring abnormalities in the wild-type plants itself. However, the frequency in the overexpression lines was markedly different from that of wild-type supporting a vital role for the HD2 proteins in the overall developmental context.

Since the HD2 proteins accumulated in the ovules (Zhou *et al***,** 2004), knockout of AtHD2A led to aborted seed development (Wu *et al***,** 2000) and the 'seed-specific' gene expression was found to be down-regulated in the AtHD2A overexpression lines, it was speculated that the HD2 proteins might be involved in a process regulated by another embryo development regulator, Abscisic acid (ABA). The common method for identifying involvement with ABA signaling is the germination and post-germination growth sensitivity assay (Gazzarrini and McCourt**,** 2001). The distinguishing feature between hormone biosynthetic mutants and response mutants is that the response mutants are insensitive even to ectopic application of the hormone, whereas the biosynthesis mutants will have restored phenotype upon hormone application (Finklestein and Rock**,** 2002). The *AtHD2C* and *AtHD2B* overexpression lines had early germination, even on ABA supplemented plates indicating that are deficient in ABA response. Subsequently, these lines were able to establish cotyledons and begin the vegetative development process when their wild-type counter-parts were unable to form the aerial parts on the same or higher concentrations of ABA. At the gene regulation level, the four ABA-inducible or responsive genes were down-regulated in the overexpression lines. LEC1 and ICK1 are essential for embryo transition from first phase of maturation to the second (Finklestein and Rock**,** 2002). Also, AREB, that is a bZIP class transcription factor, will induce the expression of LEA gene RD29B to initiate

desiccation tolerance and dormancy (Bray**,** 2004, 2001). When seeds exit from the dormancy phase to enter the vegetative growth phase, they need to down-regulate the factors that might prevent this process (Delseny *et al***,** 2001), such as ABA signaling factors. It was reported that *PICKLE* (*PKL*), that is a transcription factor belonging to the PcG group of repressors, represses post-germination expression of embryogenesis promoting regulators such as *LEC1* (Ogas *et al***,** 1999). Since the HD2 overexpression led to repression of *LEC1* and three other embryogenesis promoting and maintenance factors, it led us to speculate that HD2 proteins might be involved in the transition from dormancy to germination by down-regulating 'gate keeping ' ABA signals. The down-regulation of *LEC1* and *ICK1* might also explain the early germination, as these two genes ensure termination of cell division to prevent precocious germination.

Salt, drought, and to some extent, cold stress cause an increased biosynthesis and accumulation of ABA, that can be rapidly catabolized following the relief of stress (Shinozaki and Yamaguchi-Shinozaki**,** 2000). The role of ABA in osmotic stress signal transduction was previously addressed by studying the stress induction of several of these genes in the Arabidopsis ABA-deficient mutants (Akaba *et al*., 1998; Assmann *et al***,** 2001; Audran *et al***,** 1998; Audran *et al***,** 2000; Chernys and Zeevaart**,** 2000). In genetic screens, a group of mutants that exhibit diminished expression of *RD29A-LUC* under osmotic stress compared with Wild-type plants was recovered (Lee *et al*, 1998; Zhu, 2000). Two of the loci defined by these mutants, *LOS5* and *LOS6*, have been characterized and the genes isolated. In *los5*, the expression of several stress-responsive genes, such as *RD29A*, *COR15*, *COR47*, *RD22*, and *P5CS*, was severely reduced or even completely blocked during salt stress (Ingram and Bartels**,** 1996). The molecular cloning of *los5* revealed that *LOS5* encodes a

molybdenum cofactor sulfurase (MCSU) and is allelic to *ABA3* and the *los5* plants were defective in drought-induced ABA biosynthesis (Bittner *et al*, 2001). When exogenous ABA was applied, salt induction of *RD29A-LUC* was restored to the wild-type level, demonstrating that the ABA deficiency was responsible for the defect in osmotic stress regulation of gene expression (Finklestein and Rock, 2002). These findings suggested that osmotic stress induction of these stress-responsive genes is almost entirely dependent on ABA. Therefore, we expected that if a mutant is unresponsive to ABA, it would show insensitivity even to osmotic stress such as salt and sugar. Accordingly, the *AtHD2C* and *AtHD2B* overexpression lines demonstrated insensitivity to salt application depicted in the germination assay graphs and root length curve in figure 22. The commitment to germinate is irreversible that is why there are many checkpoints before the seed can enter into this process. The most major checkpoint is the environment-sensitive ABA response. If there is the slightest hint of osmotic stress or environmental shock, the ABA biosynthesis is induced, that results in the accumulation of the ABI3-5 (AREBs) transcription factors and the consequent accumulation of the LEA proteins, such as RD29B that maintain the dormancy and prevent mobilization of reserves (Ingram and Bartels, 1996). Therefore, we perceived the down-regulation of RD29B and AREB in the overexpression lines as a marker for ability to germinate in salt medium. NaCl resistance of the HD2 overexpression lines implicates that they are not only resistant to osmotic stress, but also to ion toxicity. However, the effects of mannitol were less severe on the wild-type as compared with ABA or salt. Mannitol is an important stress management factor that plants accumulate in harsh environments (Abebe *et al*, 2003). Although the wild-type plants were able to germinate and establish true leaves, the plants were extremely unhealthy and very few of them survived to set seed in the mannitol supplemented medium.

On the other hand, the overexpression line flourished on the mannitol supplemented medium displaying healthy transitions and development. These results indicate a definite role for AtHD2C in the regulation of dormancy to post germination growth switch. It is most likely that the other HD2 family members may play the same role during this phase owing to their intra-familial homology interaction potential. Additionally, these HD2 proteins might be working in conjunction with other regulatory factors such as PcG proteins and other HDAC class members, but this remains to be tested.

To complement the overexpression study, we incorporated an *AtHD2C* T-DNA insertion line into the experiment with the expectation that the T-DNA line would most probably give the opposite phenotype to the overexpression thereby, making our results comprehensive. Upon examination of the response of the T-DNA lines to stress treatment, it appeared that the plants had the same phenotype as Wild-type in the post-germination stage and in the vegetative stress environment when exposed to ABA, salt, mannitol and drought. This is not consistent with our expectation but there could be a few reasons, as to why we obtained this result. *AtHD2C* is just one of the members of the HD2 protein family and its loss may be substituted by the presence of other HD2 family members. Therefore, the functional loss of the gene is not evident as a phenotype. We would have to do multiple gene knockouts to examine importance of these proteins function. Alternatively, RNAi lines can be used as they might be able to achieve obliteration of HD2 protein family by using the RNA specific sequence for one member. Nevertheless, the T-DNA line was able to demonstrate some differences at the gene expression level as can be seen in Figure 20. This could be possible although the gene could not affect the plants development at a visually manifested level, it could introduce subtle changes in expression of specific target genes.

However, it can be seen that the rescue of the various genes examined is not very robust owing to the HD2 family gene redundancy in the system.

The down-regulation of *AtHD2C* in response to ABA was confirmed by examining actual *AtHD2C* expression in response to ABA. It was observed that there was a partial reduction in *AtHD2A, AtHD2B, AtHD2C* message levels upon 3-6 hours of ABA treatment. The partial reduction pattern can be explained by that. HD2 proteins may not be the only proteins exercising this level of control, i.e., they may be part of a huge complex (Lusser et al**,** 1997), and therefore, they are not targeted to obliteration, maybe below functional threshold levels. Alternatively, even the proteins may play a crucial role in this process, the applied ABA treatment and time of action were not enough to detect acute reduction levels.

A critical function of ABA during vegetative growth is to optimize growth during environmental stress by maintaining osmotic homeostasis (Finklestein and Rock**,** 2002). At the cellular level, ABA can promote tolerance of some abiotic stresses including drought, salinity, and cold or heat (reviewed in Rock**,** 2000; Shinozaki and Yamaguchi- Shinozaki**,** 2000; Xiong and Zhu**,** 2001; Larkindale and Knight**,** 2002). In addition, it can induce tolerance of hypoxic stress in roots, but not shoots (Ellis et al., 1999). Within the first 3 hours of stress induction, ABA levels are known to peak, inducing the MAPK pathway to subsequently led to activation of transcription factors that can induce LEA protein accumulation within the next 10 hours when ABA levels peak again (reviewed in Shinozaki and Yamaguchi-Shinozaki., 2000; Xiong and Zhu., 2001). Therefore, ABA is not only a germination checkpoint regulator, it is also an important stress-relief agent in the later stages of the plant life. We wanted to examine if the HD2 proteins had some functional role at this

developmental stage owing to their involvement in the germination stage. Therefore, mature *AtHD2C* overexpression plants were exposed to artificial salt stress and drought stress. To our surprise, the overexpression lines were resistant to both these conditions (Figure 24 & 25). Interestingly, the number of closed stomata in the overexpression lines was much reduced in comparison to wild-type stomata (Table X) under drought conditions. This observation was different from our expectations as the partially open stomata in the transgenic lines did not support their drought resistant phenotype. The accumulation of inorganic ions, organic acids, sugars, and other compounds is required to maintain the internal water balance (Leigh, 1997). In principle, increased vacuolar solute accumulation could confer salt and drought tolerance. The sequestration of ions such as sodium could increase the osmotic pressure of the plant and at the same time reduce the toxic effects of this cation. Also, plants accumulate a variety of organic osmoprotectant solutes through a biochemical mechanism which improves their ability to withstand stresses. Of these solutes, betaines (fully N-methylated amino acids) appear to play a major role in conferring resistance to drought, salinity and temperature stresses. Additionally, when stomata remain open, Co_2 is fixed and the plants photosynthetic accumulation increases. Therefore, the paradoxical finding of open stomata in the transgenic plants during drought stress indicates that the *35S:AtHD2C* plants are using alternate biochemical pathways other than shutting down stomata to achieve stress tolerance. HD2C might upregulate pathways leading to the accumulation of osmoprotectants such as glycine betaine or may be enhancing photosynthetic efficiencies by increasing Co_2 fixation. These theories have to be investigated to identify the molecular reason behind the open stomata supporting stress tolerance phenotype. As we established a negative regulatory role for AtHD2C in the germination

stage, it was expected that the ABA insensitive plants would succumb to environmental stress. This paradoxical finding was further investigated at the gene expression level. RNA collected from mature rosette leaves of the overexpression lines and several ABA signaling pathway genes were examined. It was observed that *RD29B* that was repressed in the seedling stage, was up-regulated in the vegetative phase that could explain the survival of the transgenic plants for longer periods of time. Additionally, the *AREB* gene that was down-regulated during the germination phase was also up-regulated in the vegetative phase supporting the up-regulation of the RD29B gene. *ABI1* that is a negative regulator of ABA signaling was unaffected in the transgenic lines indicating that HD2 protein regulation of the signaling cascade was independent of ABI1 targeting. On the other hand, ABI2 was distinctly down-regulated. ABI1 and ABI2 act either at distinct steps or in parallel pathways (Pei et al., 1997). Yeast two-hybrid studies have shown an interaction between ABI2 and SOS2 (Xiong and Zhu, 2001). SOS2 is a serine/threonine protein kinase identified on the basis of its role in salt-stress signaling (Liu et al., 2000). Both the ABI4 and ABI5 gene products contain ser/thr-rich domains that could be sites of phosphorylation (Finkelstein et al., 1998; Finkelstein and Lynch., 2000) and recent studies have demonstrated that ABI5 protein is stabilized by ABA induced phosphorylation (Lopez-Molina et al., 2001). It is quite possible that the PP2C proteins might dephosphorylate ABI4/5 and thereby inhibit their accumulation and ABA signal. The down-regulation of *ABI2* and ABI2 controlled target ADH1 (de Bruxelles et al., 1996) indicated that the HD2 proteins may play a positive regulatory role during this developmental stage.

To further support this idea, the K^+ inward rectifying channels KAT1 and KAT2 genes were down-regulated in the transgenic lines. Stomatal closing is another essential

aspect of coping with water deficit in addition to accumulation of LEA proteins that help overcome desiccation (Liu et al., 2000). Stomatal closing is induced by ABA by inhibiting K^+ inward rectifying channels so that the guard cells can be de-polarized (Gaymard et al., 1998; Allan et al., 1994). Therefore, down-regulation of these ABA-regulated K^+ inward rectifying channels in the *AtHD2C* overexpression lines indicates that AtHD2C may be involved in helping mediate the ABA inhibition of these channels. SKOR is a K^+ outward rectifier that is repressed by ABA to maintain K^+ content in the apoplast during loss of K^+ from the individual cells (Lancombe et al., 2000). The repression of this gene in the *AtHD2C* overexpression lines indicates that AtHD2C helps maintain apoplastic osmotic potential by enhancing ABA mediated repression of K^+ outward rectifiers in this location. All the results obtained from this part of the study underscore the relevance of a new type of regulator that can exercise opposite effects in different developmental stages.

(iv) AtHD2B and AtHD2C do not interact with AtHDA1

Based on the structural analysis of the HD2C protein sequence, we expected it to associate with a protein complex as HD2 proteins were first isolated in a large complex (Lusser *et al.*, 1997). The reason for our expectation was the presence of a Zn finger motif at the C-terminus as the Zn finger is instrumental in direct DNA contact and protein interactions (Dangl *et al.*, 2001). Additionally, Zn-finger proteins have been identified to be involved in stress responses by directly contacting core ACGT sequences (Sakamoto *et al.*, 2004). This led us to speculate if HD2C might have ability to directly contact target genes. However, the ideal way to examine the complex members would have been a library screen. We started the process by conducting a two-hybrid screen. We chose to test interaction between AtHD2C and AtHDA1 (Class I HDAC) because there are reports of HDACs being recruited to target

sites as HDAC complexes consisting of different class members. Therefore, we wanted to investigate if HD2 proteins also participate in this HDAC complex targeting. Neither AtHD2B nor AtHD2C interact with AtHDA1 proteins (Figure 28). However, we don't rule out the possibility that there might be interaction between these proteins, owing to the non-reliable heterologus yeast system to examine plant protein interactions.

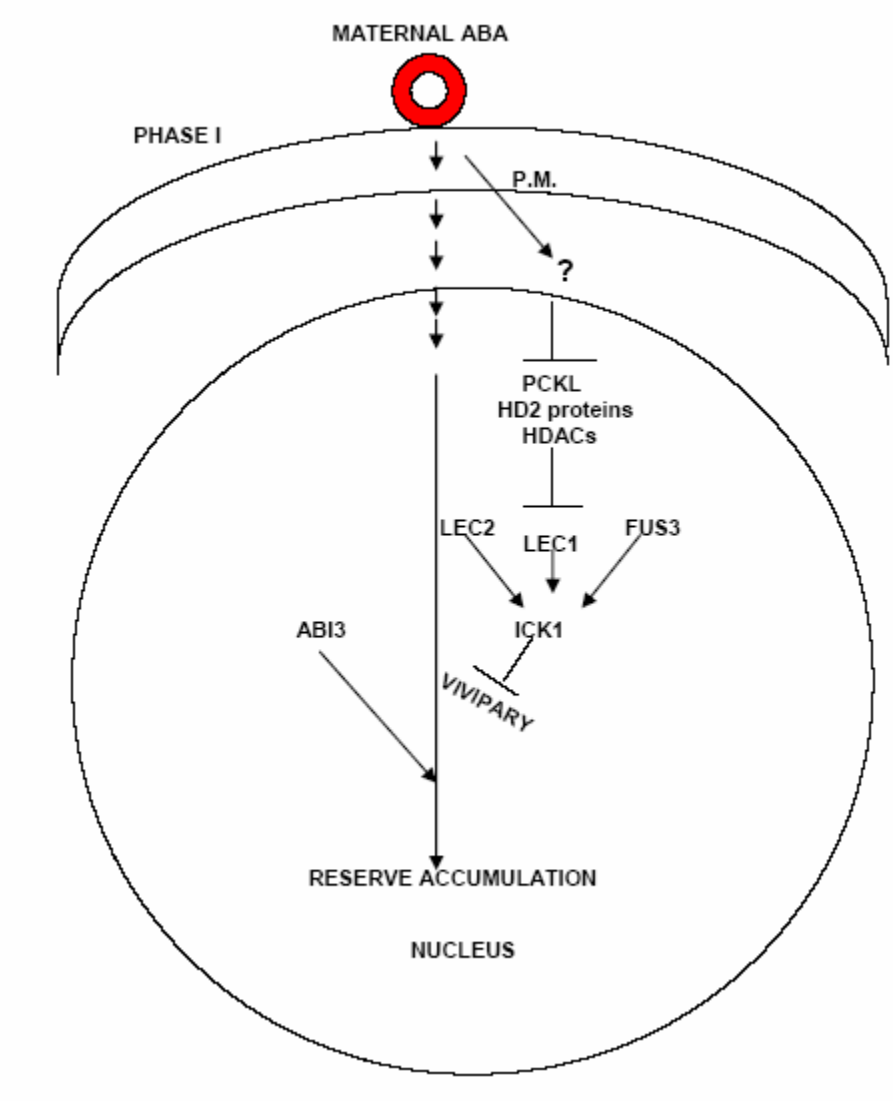
SPECULATION

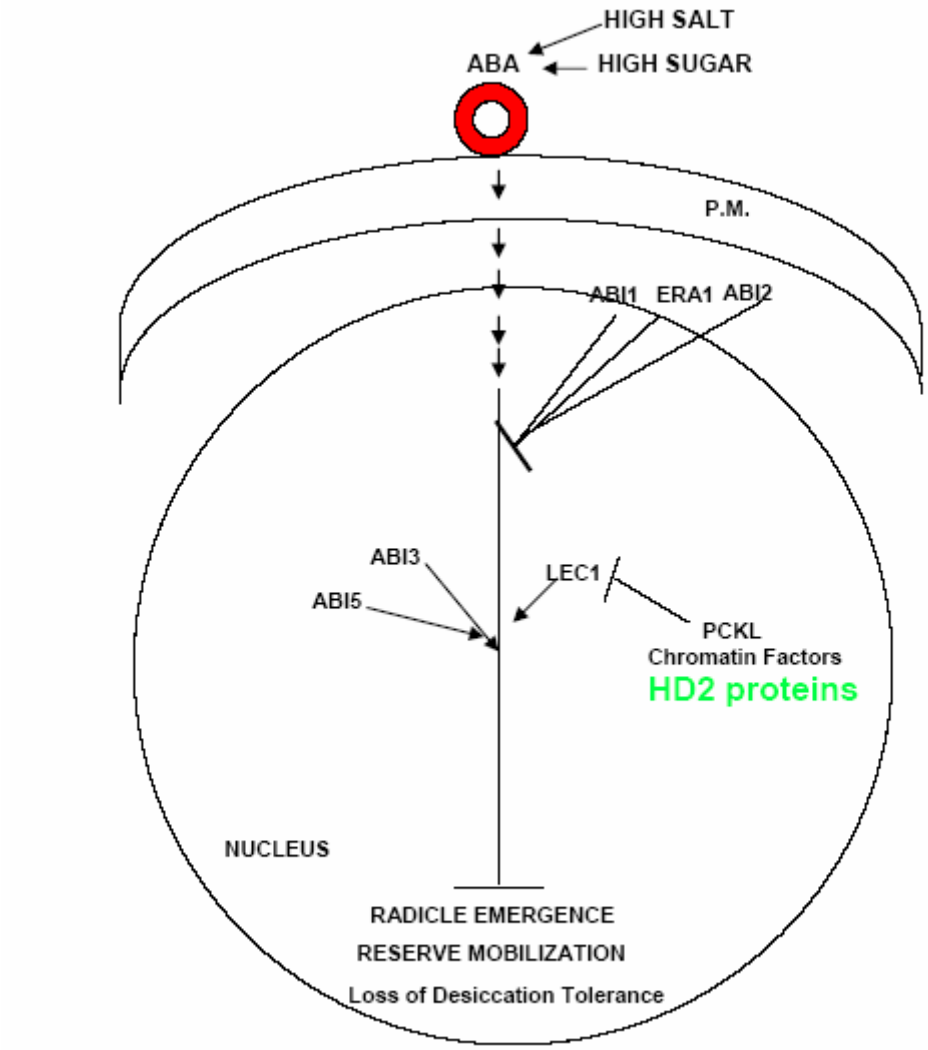
(i) Model during seed maturation

Seed maturation consists of two phases; early and late. In the early phase, ABA- inducible LEC/FUS3 repress cell-division via recruiting mediators such as ICK1 to the CDPKs (Finklestein and Rock., 2002). From our results, we will tentatively place AtHD2C (and other HD2 proteins) upstream of *LEC1* based on the down-regulation of this gene in our investigation. Therefore, at this upstream position if HD2 proteins can inhibit cell-cycle arrest by indirectly inhibiting *ICK1* expression, then they can inhibit the protein accumulation in the second phase or affect AREB (ABI5), RD29B (LEA) expression. This is sequence of events might be executed in the post-germinative growth phase when ABA signaling is not needed. However, in the embryo-maturation phase, although HD2 proteins are present at particular positions, they do not execute their function as they are silenced by ABA peaks in these stages. This idea stems from the observation that the HD2 genes were down-regulated by ABA application. What ABA-signaled factors silence the HD2 genes is not known (Figure 29). It could it be the cell-cycle regulation factors, as there is a cell-cycle regulation motif in the promoter of *AtHD2C*..

(ii) Model during post-germination growth

Essentially, the paradigm here is a continuation of the model presented above. As we established HD2 proteins upstream to *LEC1* in the seed maturation phase, the repressor function is not executed due to down-regulation of HD2 genes expression by unknown ABA signaled factors. But in the post-germination phase, as the ABA

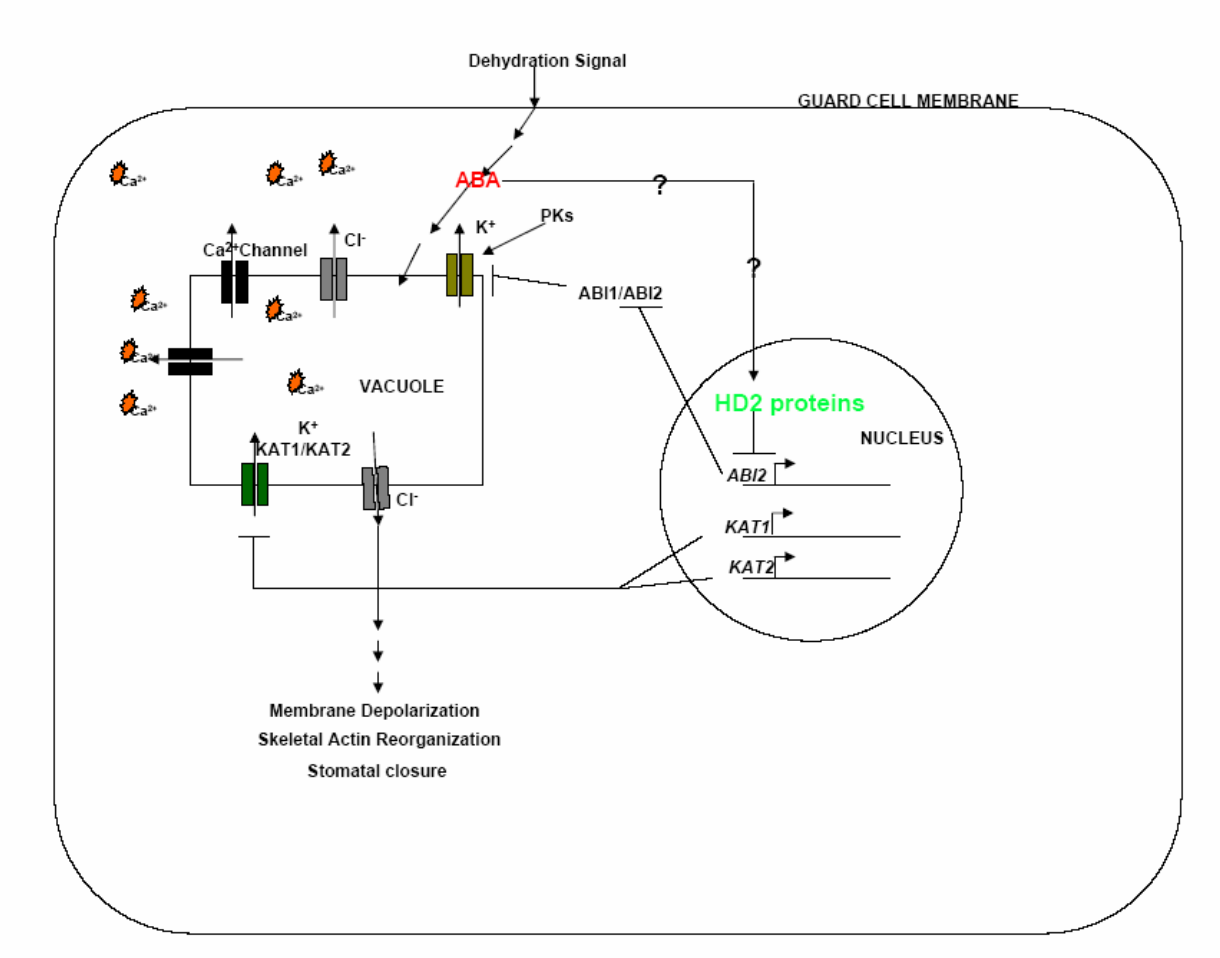

MATERNAL ABA
PHASE I
P.M.
?
PCKL
HD2 proteins
HDACs
LEC2
LEC1
FUS3
ABI3
ICK1
VIVIPARY
RESERVE ACCUMULATION
NUCLEUS


HIGH SALT
ABA
HIGH SUGAR
P.M.
ABI1
ERA1
ABI2
ABI3
ABI5
LEC1
PCKL
Chromatin Factors
HD2 proteins
NUCLEUS
RADICLE EMERGENCE
RESERVE MOBILIZATION
Loss of Desiccation Tolerance

levels go down, the *HD2* expression is de-repressed and thereby, *LEC1* down-regulation mediated repression of the ABA- response pathway is down-regulated to prevent growth inhibition (Figure 30).

(iii) Model during vegetative stress response

In the vegetative phase, a switch in the function of the HD2 proteins was observed. This role reversal led us to speculate that the HD2 proteins can function downstream to some ABA–responsive kinases that can phosphorylate the HD2 proteins and led to their activation. Once the HD2 proteins are activated, they might led to down-regulation of phophoprotein phosphatases (ABI2) that negatively regulate ABA- responsive transcription factors such as ABI3/5 and ser/thr protein kinases. ABI1 and ABI2 take part in a negative feedback regulatory loop that continuously resets the ABA signaling cascade to adjust the response to endogenous ABA levels. Also, protein phosphorylation is one of the most important mechanisms in ion transport in guard cells (Liu *et al*, 2000). KAT1 guard cell channel has been shown to be phosphorylated by calcium-dependent and (ABA)-regulated protein kinase activities (Li et al., 1998; Mori et al., 2000). ABI1/2 phosphatases have been implicated in regulating the K^+ opening and closing (Chérel *et al.*, 2002). We propose that the HD2 proteins may have additional level of regulation other than controlling *LEC1* downstream signals. We place HD2 proteins in the early ABA signaling cascade, i.e., on the plasma membrane location. HD2 can repress *ABI2* expression and thereby, prolong the ABA signal relay. Also, the HD2 proteins can regulate K^+ inward rectifying channel gene expression by a mechanism independent of *ABI2*. It cannot be concluded from these results if all the genes affected by HD2 overexpression are directly contacted by the HD2 proteins, but certainly they are in the path of the HD2 regulation loop (Figure 31).


Dehydration Signal
GUARD CELL MEMBRANE
ABA
?
PKs
K+
Ca2+Channel
Cl-
ABI1/ABI2
?
VACUOLE
HD2 proteins
NUCLEUS
K+
KAT1/KAT2
Cl-
ABI2
KAT1
KAT2
Membrane Depolarization
Skeletal Actin Reorganization
Stomatal closure

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