Sugar and *HD2* expression:

New insights into the *HD2* plant-specific class of histone deacetylases

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ABSTRACT

The HEADING DATE2 (*HD2*) family of enzymes was identified as a unique class of histone deacetylases, originally identified in maize, that share no sequence homology to previously-identified histone deacetylases. Homologues have now been identified in *Zea mays*, *Solanum chacoense* and *Arabidopsis thaliana*. *A. thaliana* contains four *HD2* genes named *HD2A, HD2B, HD2C* and *HD2D.* Previous research hypothesized that *HD2A* may have evolved a specific role in transcriptional modulation during embryogenesis since suppression-of-function (antisense *HD2A)* lines showed an aborted-seed phenotype. The results of this analysis indicate that the former hypothesis can no longer be supported. An analysis of available microarray data identified the induction of *HD2* transcription in response to the herbicides isoxaben, imidazolinone and within the starch-free *pgm* mutant. The modulation of glucose metabolism is common to the herbicides and mutant-expression profiles. Induction of *HD2* expression by glucose, sucrose and fructose was validated by reverse transcriptase polymerase chain reaction (RT-PCR) for *HD2A, HD2B, HD2C* and *HD2D.* A sugar-based hierarchal model of *HD2*-mediated transcriptional repression is discussed where a subset of metabolic genes is repressed upon periods of high glucose. Proposed models relating to cellular memory mechanisms and deacetylation of non-histone proteins are also discussed.

INTRODUCTION

The goal of this investigation is to understand the function of *HD2* proteins in plant development. *HD2* proteins are a plant-specific class of histone deacetylases. This class of proteins is conserved among plants and was previously hypothesized to play a repressive role in chromatin-remodeling during embryogenesis (Wu et al., 2000b). Chromatin-remodeling is a process which alters the position and contact of DNA with histone proteins resulting in transcription or repression of gene targets. The first chapter outlines the current literature on chromatin-remodeling processes. Whenever possible, plant research is used to outline chromatin-remodeling processes but less is known in this field than in other model organisms such as *Saccharomyces cerevisiae*. Specific attention is given to the process of acetylation and deacetylation with a review of the role this modification can play during plant development.

The second chapter focuses on experimental work to understand the developmental function of the *HD2* histone deacetylases in the model organism *A. thaliana*. Suppression-of-function and loss-of-function mutant lines were analyzed for phenotypic abnormalities. Single and double mutant lines displayed no stable phenotype. To understand the developmental function a survey of publicly accessible microarray experiments was undertaken resulting in a model of expression linking the rapid induction of *HD2* transcripts in response to glucose, sucrose and fructose. The implications of this finding are discussed.

The appendix is an investigation into the process of somatic embryogenesis by the APETLALA2 (AP2) transcription factor BABYBOOM (BBM) cloned from *Glycine max*. This study was undertaken for two purposes.

The first purpose was to compare the profile of transgenic *A. thaliana* lines expressing the *G. max* orthologue to transgenic lines expressing the *B. napus BBM* gene. The second purpose was to use the ability of the BBM transcription factors to promote somatic tissue to undergo embryo development, to model the expression of the *HD2* genes, previously hypothesized to have an embryogenic function. This was undertaken before the discovery of the induction of the *HD2* genes by soluble carbohydrates. It is now clear that *HD2* expression is more clearly linked to the processes of cellular proliferation which *BBM* promotes and not specifically to embryogenesis. To achieve the first goal of the study, the global expression profile of transgenic lines, expressing the *G. max* and *B. napus BBM* genes, driven by the 35S cauliflower mosaic virus promoter were analyzed during somatic embryo induction and after embryo development. The microarray analysis failed to identify the expression cascade which results in the formation of an embryo from somatic tissue (eg.*CYTOKININ INDUCTION1 CKI1* (Kakimoto, 1996), *LEAFY COTYLEDON1 (LEC1)*(Lotan et al., 1998), *LEAFY COTYLEDON2 (LEC2)* (Stone et al., 2001), *ENHANCER OF SHOOT REGENERATION1 (ESR1)*(Banno et al., 2001), *SOMATIC EMBRYOGENESIS RECEPTOR KINASE1 (SERK1)*(Hecht et al., 2001),*WUSHCEL (WUS)* and identified no consistent pattern of *HD2* expression but did identify the expression of seed specific markers confirming the molecular phenotype of the newly characterized *G. max* orthologue eg. (seed specific proteases, *2S ALBUMIN, LATE EMBRYOGENESIS ABUNDANCE 76*). The datasets were compared to published datasets of genes which were highly expressed during normal seed

formation. Similarities in gene expression between somatic and zygotic embryogenesis were identified.

CHAPTER 1

CHROMATIN-REMODELING IN PLANTS

1.1 INTRODUCTION

Within the nucleus of the cell, genetic material is compacted into what is referred to as chromatin. The degree of compaction can directly influence the accessibility of DNA to DNA-binding proteins which may seek to alter the transcriptional rate of a given loci. The conversion of loci from an accessible to inaccessible conformation is reversible and is termed chromatin-remodeling. This process may occur by an ATP-dependent or ATP-independent mechanism. The two processes may also be combined. Within a multicellular organism, any given cell may express only a subset of the genes encoded within the entire genome. The set of mRNAs expressed at a given time from a specific cell type or population is referred to as the transcriptome. The set of genes transcribed into mRNA is altered during development and reflects the environmental stressors a cell is experiencing. Most of the known mechanisms underlying chromatin-remodeling were discovered in *S. cerevisiae* and are now beginning to be understood in more complex model organisms. The next section of this review details the structure of chromatin before remodeling research is discussed.

1.2 HISTONE PROTEINS AND CHROMATIN STRUCTURE

Histone proteins are functionally involved in the compaction of genetic material. DNA is wrapped around a histone octamer which is referred to as a nucleosome. A nucleosome contains two copies of the conserved core histone

proteins H2A, H2B, H3 and H4 and has ~146 base pairs of DNA wrapped around the core (Bradbury, 1975; Luger et al., 1997). Compaction of the DNA to fit within the nucleus occurs through the formation of chromatin structures. A nucleosome, each comprised of a histone octamer are separated by 10-80bp of DNA. This is referred to as the 'beads on a string' conceptual model. This compacts the genetic material 5 to 10 fold. Increased compaction is achieved by the 'beads on a string' folding onto itself. This achieves another 5 to 10 fold increase in compaction and is stabilized by the H1 linker histone. Higher levels of chromatin structure are hypothesized to be present but remain poorly characterized (Lam et al., 2004).

The core histone proteins H2A, H2B, H3 and H4 contain two domains: the globular domain that interacts with other histone proteins within the nucleosome and the highly-charged histone tails which protrude from the nucleosome (Luger et al., 1997). The conservation of amino acid sequences between distantly related organisms is most evident in H3 and H4 but is also noted in H2A and H2B (Cheah and Osborne, 1977). In addition to these conserved core histones, there are organism-specific histones in every organism. These organism-specific histones are hypothesized to have a specialized function within the organism. For example, the genome of *A. thaliana* contains HT12, a variant of H3, thought to be an orthologue of CENP-A, a centromere-specific histone (Talbert et al., 2002). Excluding the variants of histone proteins, homology in the core histone proteins indicates that modifications made to amino acids to alter chromatin structure should also be conserved resulting in the hypothesis of a 'histone code'.

1.3 ATP-DEPENDENT CHROMATIN-REMODELING OVERVIEW

Chromatin-remodeling involves the stable alteration of chromatin structure. This can result in the alteration of gene expression. Chromatin-remodeling complexes (CRCs) have been linked to the activation and the repression of genes. Three mechanisms have been proposed in the literature. Nucleosome displacement involves the displacement of histones from the DNA (Owen-Hughes and Workman, 1996), while nucleosome sliding involves the lateral movement of histones along the DNA template (Fazzio and Tsukiyama, 2003). Finally, covalent modification of histone tails by histone-modifying enzymes is thought to modulate chromatin conformations (Bradbury, 1976). A proposed mechanism for remodeling first involves the binding of the remodeling complex to the chromatin directly or through a transcription factor. An example occurs during cold or dehydration stress, as *C-REPEAT/DRE BINDING FACTOR 1* (CBF1) recruits CRCs to inducible promoters (Stockinger et al., 2001). Site specificity may be accomplished through targeting by DNA sequence recognition or recognition of specific chromatin structures. The hydrolysis of ATP provides the energy to displace or slide histone octamers. Three groups of ATP-dependent chromatin-remodeling complexes have been identified and characterized into *MATING TYPE SWITCHING (SWI2)/SUCROSE NON-FERMENTING2 (*SNF2*), IMITATION OF SWITCH (*ISWI*)* and CHD3/Mi-2 families (Vignali et al., 2000).

The *SWI/SNF* complex, the first member of this group to be identified, was originally isolated from yeast and was found to contain 11 subunits including an ATPase. *SWI/SNF* complexes are known to alter the contact points between DNA and histones, which is thought to enhance access of DNA binding proteins. DNA binding proteins, more specifically transcription factors, may promote or suppress the transcriptional rate of a locus. *SWI/SNF2*-like proteins studied in *A. thaliana* have most recently been shown to be involved in DNA repair mechanisms (Shaked et al., 2006) and in the transition to floral development (Wagner and Meyerowitz, 2002). The *ISWI* family of complexes which include *ATP-UTILIZING CHROMATIN ASSEMBLY AND REMODELING FACTOR* (ACF) (Ito et al., 1997), *NUCLESOME REMODELING FACTOR* (NURF) (Tsukiyama and Wu., 1995) and *CHROMATIN ASSEMBLY COMPLEX* (CHRAC) (Varga-Weisz et al., 1997) were originally identified in *Drosophila melangomaster*, and contain the *ISWI* ATPase as a subunit. To date homologues of the *ISWI* protein have been identified in all eukaryotes studied thus far including yeast, plants, nematodes, flies, mice and humans. *ISWI* complexes have been implicated in nucleosome sliding but have not been demonstrated to displace histone octamers from the DNA strand.

The final group of complexes has recently been purified from human tissue culture and *X. laevis* (Wade et al., 1998; Zhang et al., 1998; Xue et al., 1998; Tong et al., 1998). The *Mi-2* proteins contain an *SNF2* ATPase as well as two *PhD* zinc-finger motifs and two chromodomains. These complexes, unlike *SWI/SNF* and *ISWI*, have been found to contain histone deacetylase activity.

This is an interesting finding as it incorporates the two mechanisms, ATP-dependent and covalent modification of histones, into a CRC.

The previously-mentioned examples detail chromatin-remodeling in an ATP-dependent mechanism. ATP-Independent remodeling occurs by the covalent modification of histone tails. The two processes are intricately linked *in vivo* as one mechanism (covalent modification) may be required to recruit ATP-dependent CRCs to a loci or the cell may combine the two processes into a single protein complex (eg. *Mi-2* complex which contains ATPase domains in addition to histone deacetylase activity (Wade et al., 1998; Zhang et al., 1998; Xue et al., 1998; Tong et al., 1998)). For the ease of discussions, the processes will be discussed independently.

1.4 ATP-INDEPENDENT CHROMATIN-REMODELING

The N-terminal histone tails which protrude from the nucleosome are the primary focus of ATP-independent chromatin-remodeling study as it is believed these play a critical role in the conformational remodeling of a locus. Covalent modifications can occur within the globular domain but the tail is thought to play a crucial role in mediating the strength of DNA-protein interaction by lessening the charge on the tail, preventing access of transcriptional machinery to the gene by steric hindrance, or acting as a possible signal for the recruitment of chromatin-remodeling factors (Wagner, 2003).

Both the position and the nature of the modification have been found to be crucial to the outcome of repression or activation. For example, investigations

using mammalian tissue culture indicate that methylation at lysine residues within the histone tail is correlated with both repression and activation of transcription (Boggs et al., 2002). No single modification is responsible for the alteration of chromatin architecture. The sum of many covalent modifications may result in the up- or down-regulation of a gene. In all species investigated, methylation of lysine 9 of histone H3 leads to the phosphorylation of serine 10 influencing the acetylation at lysine 14 (Cheung et al., 2000). This supports a 'histone code' hypothesis of a cascade of modifications resulting in an alteration of chromatin structure and gene expression. The number of modifications at a specific site (mono, di, tri) is dependent upon the concentration and activity of histone-modifying enzymes within the nucleus of a cell. Some modifications like the addition of an acetyl group have a half-life of minutes making the modification highly reversible while methylation is thought to be a more stable modification suitable for long-term alteration in chromatin architecture.

Methylation, acetylation, phosphorylation and ubiquitination are all thought to influence the levels of gene expression of a target gene (Lusser, 2002). The enzymes responsible for the addition of these covalent groups modify serine, arginine and lysine residues of the histone protein. Known modifications are listed in Table 1. Most are of unknown function.

Table 1. Covalent modifications of amino acids residues of plant histones with assigned function

Histone	amino acid residue	modification	activation/repression	reference
H2A	Ser1	Phosphorylation	-	
	Lys5	Acetylation	-	
	Lys9	Acetylation	-	
	Lys119	Ubiquitination	-	
H2B	Lys5	Acetylation	-	
	Lys12	Acetylation	-	
	Ser14	Phosphorylation	-	
	Lys15	Acetylation	-	
	Lys20	Acetylation	-	
	Lys120	Ubiquitination	-	
H3	Arg2	Methylation	-	
	Lys4	Methylation	Activation	Noma et al., 2001, Boggs et al., 2001
	Lys9	Methylation	Repression	Noma et al., 2001, Boggs et al., 2001
	Lys9	Acetylation	-	
	Ser10	Phosphorylation	-	
	Lys14	Methylation	-	
	Lys14	Acetylation	-	
	Arg17	Methylation	-	
	Lys18	Methylation	-	
	Lys18	Acetylation	-	
	Lys23	Methylation	-	
	Lys23	Acetylation	-	
	Arg26	Methylation	-	
	Lys27	Methylation	-	
	Ser28	Phosphorylation	-	
	Lys36	Methylation	-	
	Lys79	Methylation	-	
H4	Arg3	Methylation	-	
	Lys5	Acetylation	-	
	Lys8	Acetylation	-	
	Lys12	Acetylation	-	
	Lys16	Acetylation	-	
	Lys20	Methylation	-	
	Lys20	Acetylation	-	

The addition of covalent modifications to histone proteins and the subsequent alteration in gene expression provides a method for regulating the transient expression of genes, required for tissue differentiation during growth and development. The ability to reverse the modification by simple dilution through replication of the genetic material during cell division or active removal by enzymatic means (deacetylases, demethylases etc.) allows the cell to monitor and change the expression of gene targets rapidly. This may be crucial in developmental pathways where step-wise, sequential expression of transcription factors is employed for tissue differentiation during growth and development. The role of methylation in chromatin-remodeling is well-studied. There is little evidence in plants which has closely linked the processes of acetylation/deacetylation and methylation/demethylation despite these modifications occurring at the same amino-acid residue of histones. The HISTONE DEAETYLASE6 provides a link between the two processes. Analysis of the *hda6* mutant has shown that deacetylation may enhance the processes of histone methylation and DNA methylation at non-endogenous genes (Murfett et al.,2001) but this may be different to the modification process which occurs at an endogenous genetic locus. In addition, no complex has yet been purified which combines both histone methyltransferase and histone deacetylase activity.

1.5 H1 LINKER HISTONE AND CHROMATIN-REMODELING MECHANISMS

Recently, much research has been completed to investigate the covalent modification of H1 linker histone as well. The H1 histone is comprised of two tails and a central globular domain. The globular domain is thought to interact directly with the DNA strands (Travers, 1999). *A. thaliana* contains three H1 variants within the genome. Suppression of all three genes led to heritable developmental defects at all stages of plant development (Wierzbicki and Jerzmanowksi, 2005). These mutants displayed a disruption in DNA methylation at specific sites connecting the processes of DNA methylation and chromatin structure formation. Within the yeast system, the presence of H1 partially inhibited ATP-dependent chromatin-remodeling *in vivo* indicating that the structures formed by the incorporation of H1 linker histones must be included in any model of chromatin-remodeling (Jerzmanowski, 2007). Investigations of the mammary mouse tissue virus (MMTV) promoter have shown a decrease in H1 histones at the promoter in response to the activation of expression (Bresnick et al., 1992). This indicates structural alteration of the chromatin during the activation of expression. Finally, prolonged exposure to the hormone induction of MMTV leads to the dephosphorylation of H1 and the concomitant decrease in gene expression from the MMTV promoter (Lee and Archer, 1998). Within *A. thaliana*, H1 related chromatin-remodeling is linked to drought stress (Wei and O'Connell, 1996). A drought-inducible H1 has been shown to accumulate within the chromatin of plants in response to water-deficit stress (Scippa et al., 2000). This indicates a

unique and interesting model to study where chromatin structure may be modified at specific genetic loci in response to an environmental stressor. These mechanisms of chromatin-remodeling are not well-characterized. For this reason, the remainder of the review will focus on the acetylation and deacetylation of histones and how these enzymes affect plant development.

1.6 STRUCTURE OF HISTONE ACETYLTRANSFERASES (HATS) AND HISTONE DEACETYLASES (HDACS)

The structures of HATs are highly conserved among yeast, plants and animals. This has led to the identification of 12 HAT genes within *A. thaliana* based upon sequence similarity to previously identified HAT genes in other eukaryotic systems (URL; http://www.chromdb.org). The *A. thaliana* genome contains 5 members of the GCN5-RELATED N-TERMINAL ACETYLTRANSFERASES (GNAT)/ **M**OZ, **Y**BF2/SAS3, **S**AS2, **T**IP60) (MYST), 5 members of the CREB-BINDING PROTEIN (CBP), and 2 members of the TATA BINDING PROTEIN-ASSOCIATED FACTORS ($TAF_{II}250$) families. The following table (Table 2) contains the identified HATs within the *A. thaliana* genome (URL http://www.chromdb.org).

Table 2. Histone acetyl transferases of *A. thaliana* identified by sequence similarity to previously identified HAT enzymes in other model organisms.

HAT Family	Subfamily	Gene Name	AGI Code[1]
GNAT-MYST	GCN5	AtHAG1(AtGCN5)	At3g54610
	ELP3	AtHAG3	At5g56740
	HAT1	AtHAG2	At5g50320
	MYST	AtHAM1	At5g64610
		AtHAM2	At5g09740
CBP		AtHAC1	At1g79000
		AtHAC2	At1g67220
		AtHAC4	At1g55970
		AtHAC5	At3g12980
		AtHAC12	At1g16710
TAFII250		AtHAF1	At1g32750
		AtHAF2	At3g19040

[1] AGI Gene codes were designated by the Arabidopsis Information Resource (TAIR) when the *A. thaliana* genome was annotated. An AGI gene code is a unique identifier for each gene in the genome.

Sequence homology exists within members of each HAT family but little similarity exists between differing families. Members of the GNAT family are identifiable by the conserved HAT domain containing 4 conserved motifs named A-D. The GNAT family contains an N-terminal domain and a C-terminal catalytic HAT domain divided by a hydrophobic cleft within the protein. The histone binds within the A and B motifs of the HAT domain. The enzyme catalyzes the transfer of an acetyl moiety from acetyl-CoA to the ε-amino group of a lysine residue. These proteins also contain a bromodomain, originally identified in *D. melangomaster*, and hypothesized to mediate protein to protein interaction.

The CBP family of HATs share some sequence homology to the A, B, and D motifs of the *GNAT* family. Plant CBPs differ from animal CBP by the absence of a bromodomain and KIX domain as well as the presence of one zinc finger instead of two. The $TAF_{II}250$ HATs within *A. thaliana* are known to contain a HAT catalytic domain, c-terminal bromodomain and a zinc-finger motif.

Sequence homology has also led to the identification of fourteen HDACs within the *A. thaliana* genome (URL, http://www.chromdb.org). Twelve of the HDAC genes belong to the REDUCED POTASSIUM DEFICIENCY3 (RPD3)/HISTONE DEACETYLASE A1 (HDA1) family originally identified in a yeast auxotrophic mutant screen (Rundlett et al., 1996) but only ten contain a complete HDAC domain. Two belong to the SILENT INFORMATION REGULATOR2 (SIR2) also originally identified in yeast as transcriptional silencer of silent mating loci, telomeres, and ribosomal DNA (Fox et al., 1997; Bryk et al., 1997; Smith and Boeke, 1997; Gottschling et al., 2000). The remaining 4 belong

to the *HD2* family originally identified in maize and are unique to the plant kingdom. The *HD2* family of enzymes was originally identified based upon enzymatic function from maize embryo protein extracts (Lusser et al., 1997). Table 3 contains known HDAC genes within *A. thaliana* based upon sequence similarity to other model organisms.

Table 3. HDACs of *A. thaliana* identified by sequence similarity to HDAC enzymes in other model organisms

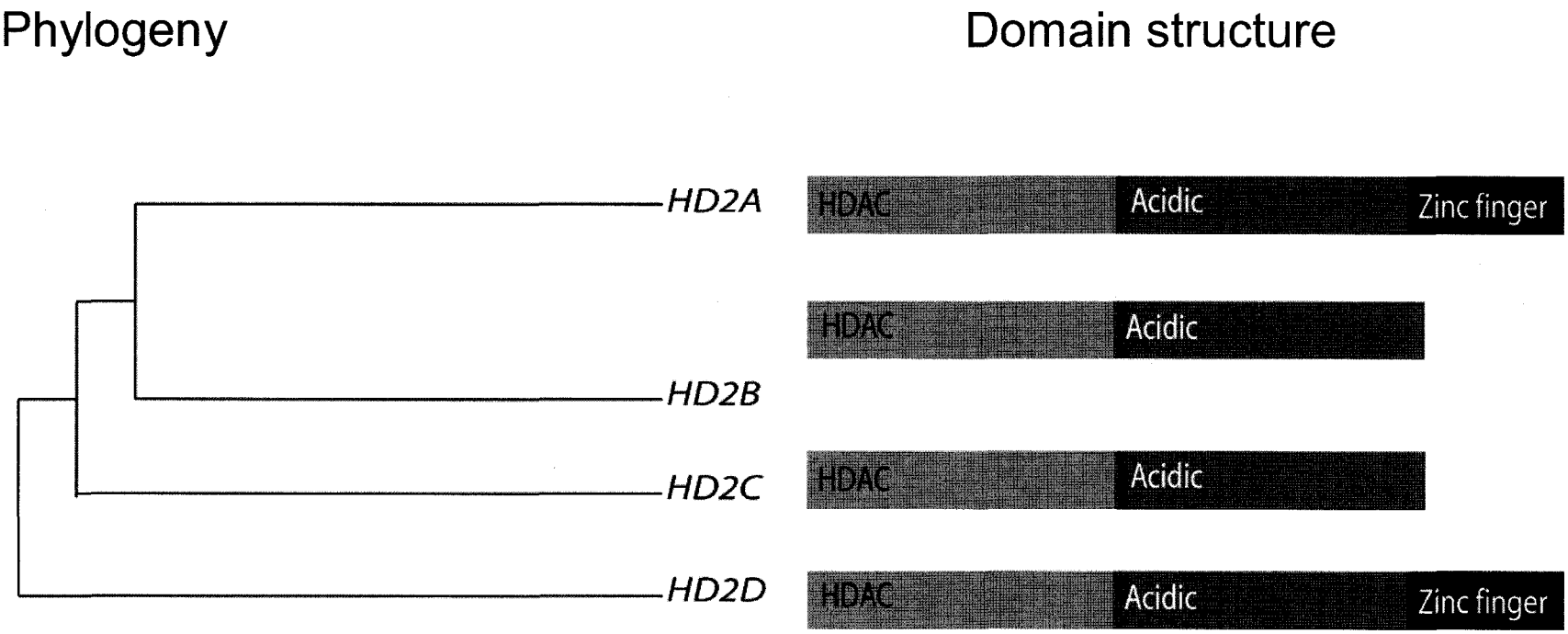
HDAC Family	Gene Name	AGI code[2]	Synonyms
HDA1/RPD3	*HDA2*	At5g26040	
	HDA5	At5g61060	
	HDA6	At5g63110	*AtRPD3B*
	HDA7	At5g35600	
	HDA8	At1g08460	
	HDA9	At3g44680	
	HDA10	At3g44660	
	HDA14	At4g33470	
	HDA15	At3g18520	
	HDA17	At3g44490	
	HDA18	At5g61070	
	HDA19	At4g38130	*HDA1,HD1, OOK, AtRPD3A*
SIR2	*HDA12*	At5g55760	
	HDA16	At5g09230	
HD2	*HD2A*	At3g44750	*HDA3, AtHD2A1*
	HD2B	At5g22650	*HDA4, AtHD2B*
	HD2C	At5g03740	*HDA11, AtHD2C*
	HD2D	At2g27840	*HDA13, AtHD2D*

[2] AGI Gene codes were designated by the Arabidopsis Information Resource (TAIR) when the *A. thaliana* genome was annotated. An AGI gene code is a unique identifier for each gene in the genome.

The RPD3/HDA1 family contains an N-terminal catalytic domain and a short variable C-terminal domain. The SIR2 histone deacetylases share no sequence homology to other histone deacetylases and require NAD+ as cofactor for enzymatic function. The structures of the *HD2* genes within *A. thaliana* have been well studied and will be discussed extensively in the later part of this review.

The domains of the *HD2* proteins have been investigated to understand the chromatin-modifying mechanism and function. Initial work characterized an extended acidic domain consisting of amino acids 149 through 196 from the cDNA sequence within maize (Lusser et al., 1997). Using the sequence of maize *HD2*, two genes, *HD2A* (found on chromosome III) and *HD2B* (found on chromosome V) were identified from the EST database within *A. thaliana,* sharing 44 and 46% amino acid similarity respectively (Wu et al., 2000). Each gene is present within the genome as a single copy. Two additional genes named *HD2C* (found on chromosome V) and *HD2D* (found on chromosome II) were identified through PCR amplification of a cDNA library using conserved primer sequences (Dangl et al., 2001). These genes share sequence homology to previously identified *HD2* genes, encoding a predicted length of 295 and 204 amino acids respectively, each containing eight exons (Figure 1).

Figure 1. Domain structure and phylogeny of *HD2A, HD2B, HD2C, HD2D* Amino acid sequences were aligned using DNAsis Max (Hitachi Corporation) using CLUSTALW. The phylogenetic tree generated from the sequence alignment shows that *HD2A, HD2B* and *HD2C* are the most similar in sequence. *HD2D* is the most divergent of *HD2* proteins within *A. thaliana.* Gene structure is shown. The light grey boxes indicate the presence of an *HD2* HDAC domain at the N-terminus of all four genes followed by an acidic region (medium grey). The zinc-finger motif is shown in black and occurs only in *HD2A* and *HD2C.*



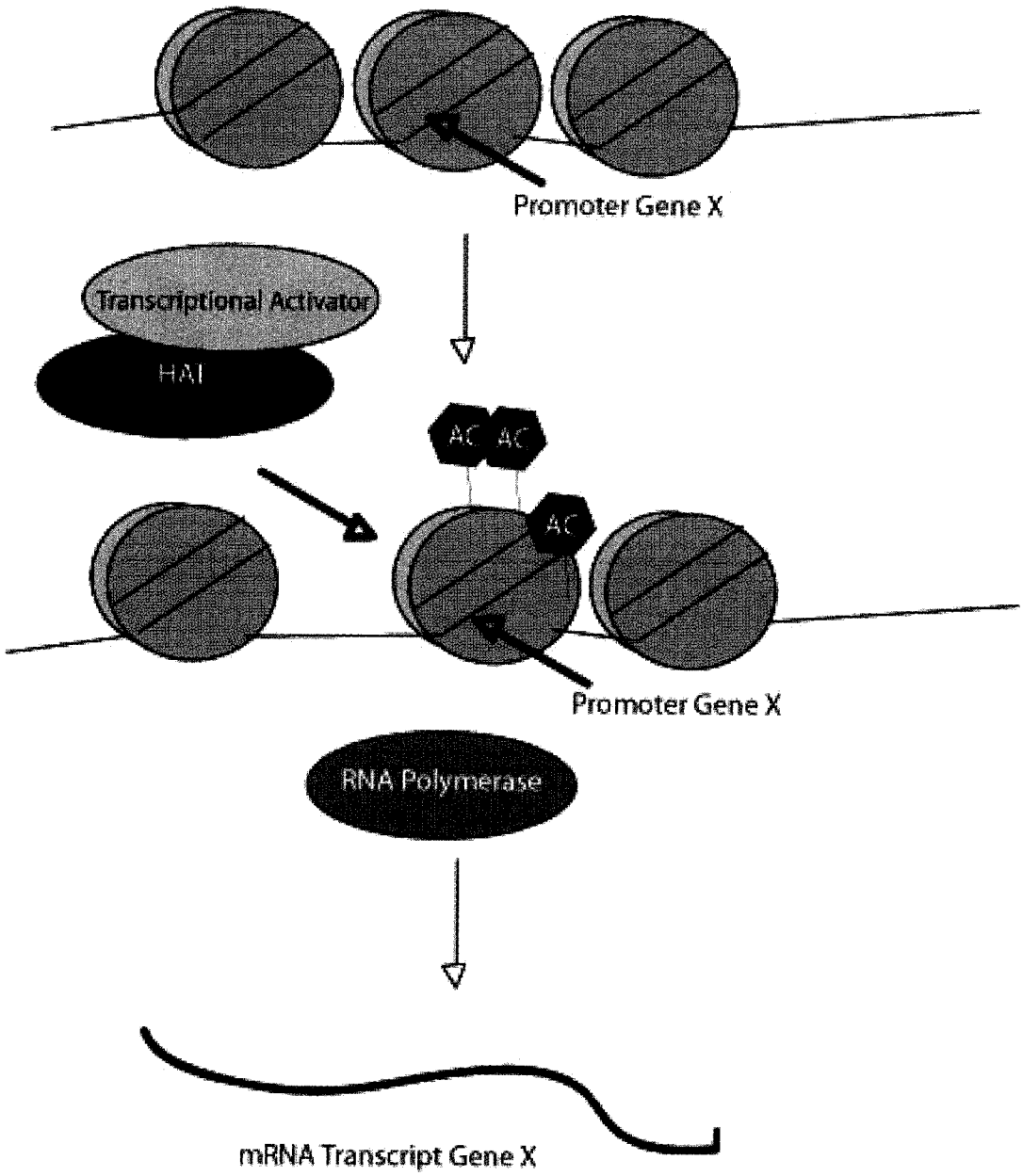
Soon after the identification of the *HD2* genes within both maize and *A. thaliana*, an alignment of the sequence revealed a significant similarity to peptidyl-prolyl cis-trans isomerases in insects, with a conserved aspartic acid, and histidine residues postulated to be involved in lysine deacetylation (Aravind et al., 1998). A region containing acidic amino acid residues was also detected and thought to mediate nucleolar localization and interactions with basic residues within N-terminal tails of histones. Nucleolar localization was first shown in maize (Lusser et al., 1997) and later in *A. thaliana* (Zhou et al., 2004). Further work identified three conserved domains: the N-terminal deacetylase domain, an acidic region found in all four At*HD2* genes, and a zinc finger motif found in *HD2A* and *HD2C* (Wu et al., 2000; Dangl et al., 2001). The predicted protein structure can be seen in Figure 1.

The zinc-finger domain may mediate protein-protein interactions and may therefore be involved in protein complex formation. The presence of a single zinc-finger motif is not sufficient to act as a DNA sequence-specific recognition factor. It has previously been shown that *HD2A, HD2B*, and *HD2C* are more closely related to one another than *HD2D*. This is shown in the phylogenetic tree in Figure 1. In addition, the expression domains of *HD2A-C* are similar whereas *HD2D* shows a lower expression level and more unique expression pattern than the other family members (Zhou et al., 2004). Therefore it has been speculated that *HD2D* may have evolved a distinct function within *A. thaliana*.

1.7 FUNCTION OF ACETYLATION AND DEACETYLATION IN CHROMATIN-REMODELING

The addition of acetyl groups to specific lysine residues has been correlated with transcriptionally-active regions of DNA (Turner, 1993; Utley et al., 1998). The acetyl group is transferred to the ε-amino group of lysine residues in the histone tail. Although the exact mechanism of how this covalent modification modulates gene expression is unclear, the addition of acetyl groups are proposed to neutralize the positively charged histone tail thereby lessening the strength of interaction between DNA and protein (Wagner, 2003). This would allow access of transcriptional factors, resulting in the expression of a target gene. Figure 2 shows a conceptual model of how acetylation of a genetic region results in the modulation of expression of the mRNA transcript.

Figure 2. Conceptual model of acetyl moiety on gene expression. Acetylation is correlated with actively transcribed regions of genome. In this model, a transcriptional activator (positive regulator of transcription) targets a histone acetyltransferase to a gene X. Acetyl groups are transferred to the histone tail which protrude from the nucleosome. This alters the accessibility of regulatory sites allowing RNA polymerase to bind to the DNA template. Gene X is then transcribed into mRNA. The process can be readily reversed by histone deacetylases.



Transferring the acetyl moiety from acetyl-coA to a lysine residue on a histone tail might also act as a signal resulting in the recruitment of ATP-dependent CRCs to the locus. The CRCs would act on the chromatin, resulting in transcript expression. A final proposed mechanism is that acetyl groups themselves may loosen the DNA-protein interaction by steric hindrance. Any of these mechanisms might explain the correlation of enriched acetylated lysine residues of histones within actively expressed regions of a genome (Wagner, 2003).

Deacetylation enzymes are antagonistic to histone acetyltransferases. This functional class of enzymes removes acetyl groups from lysine residues resulting in a modulation of gene expression. These enzymes may function to down-regulate a target gene by increasing the strength of DNA-histone interaction, by removing the signal to recruit CRCs, or by lessening steric hindrance.

Histone acetyltransferases and histone deacetylases act in concert to coordinate the expression of genes within a genome. The concentration and specificity of both types of enzymes at specific loci can modulate chromatin structure. This in turn can alter gene expression. Both HATs and HDACs lack specificity and require additional factors to target them to a specific locus. HATs have been found to exist in highly heterogeneous complexes which have specific targets resulting from associated factors (Lusser et al., 2001). The regulation of gene expression is dependent upon the assembly and targeting of multi-subunit complexes adding additional layers of control mechanisms for the cell. Histone deacetylases have also been found to exist in complexes. *AtRPD3* type

deacetylase, found in both *A. thaliana* and maize, is found to associate with a retinoblastoma-related (ZmRBR1) homologue to silence a specific locus within maize (Rossi et al., 2003). The ZmRPD3/HD1 histone deacetylase found in maize was co-fractionated with the RETINOBLASTOMA RELATED PROTEIN 46-48 (RBAP46-48) protein thought to target enzymes to histones (Lusser et al., 1999). The association of histone acetyltransferases and deacetylases with targeting factors reinforces a conceptual model of highly-coordinated, specific histone modification to control the entire spectrum of genes required for developmental processes.

Research in yeast, amphibian and human tissue culture has recently linked covalent modification of histones, specifically deacetylation, with an ATP-dependent chromatin-remodeling mechanism. Within a *X. laevis* the Mi2-NuRD complex has been identified as a unique complex that combines histone deacetylase activity with a SNF-related ATPase (Wade et al., 1998; Zhang et al., 1998; Xue et al., 1998; Tong et al., 1998). The identification of the Mi2-NuRD complex represents a shift in the chromatin-remodeling paradigm, linking the two processes directly within a single complex to modulate gene expression. Table 4 contains all identified components within each isolated complex. Subunits identified in the human NuRD complex include *HDAC1* and *HDAC2*, both with homologues in plants, *Mi2α, MTA1* (*METASTASIS ASSOCIATED PROTEIN1*) and *MTA2, MBD3A-MBD3B*, and *RbAp48* (Zhang et al., 1998). Due to the isolation of these NuRD complexes from many organisms and tissue types,

employing differing methods for purification, it is difficult to confirm the exact subunit makeup.

Table 4. Identified components of NuRD complexes purified from human and *X. laevis* tissue culture and identified by mass spectrometry

Organism	Components of NuRD	Histone Deacetylase Component
Human NuRD	Mi-2β Mi-2α MTA1 RbAp48	HDAC1/HDAC2
Human NuRD	Mi-2β MTA1 RbAp48/p46	HDAC1/HDAC2
Human NuRD	Mi-2β MTA2 MBD3a MBD3b RbAp48/p46	HDAC1/HDAC2
X. laevis Mi-2	Mi-2 MTA1-like P66 PrZp48/p46 MBD3 MDB3LF	RPD3

MBD2 was recently been co-purified with the human NuRD complex (Feng and Zhang, 2001). The indication of methylated-DNA binding affinity within mammalian histone deacetylase species leads to speculation of a role within a cascade of modifications to down-regulate transcription. Within plants, *PICKLE (PKL)* a chromatin-remodeling factor and homologue of Mi-2 indicates a possible link for dual-mechanism remodeling complexes (Ogas et al., 1999). In the *pkl* mutant, plants express embryonic traits post germination indicating that this chromatin-remodeling factor is required for the repression of the embryonic developmental program. In other words, *PICKLE* serves as a clear link between the processes of chromatin-remodeling and development.

1.8 ROLE OF ACETYLATION AND DEACETYLATION IN DEVELOPMENT

The role of acetylation in chromatin-remodeling is uniquely suited to quick, transient changes in gene expression. Acetyl groups can be removed enzymatically or decay rapidly. Therefore, to maintain a locus in a euchromatic or transcriptionally-active state for any prolonged length of time requires dedicated monitoring of the genetic region. The short half-life ensures that modified sites will decay unless the cell actively maintains the covalent modification. The histone deacetylases may function to provide even finer control over the transcriptional activity of a locus. The removal of acetyl groups may quickly silence transcription when fine control of expression is required. Theoretically, acetylation and deacteylation may be well suited to the process of development. The fine control over expression due to well-characterized enzymes responsible

for the addition and removal of groups, specific factors employed for targeting, and the possibility of short, transient changes in gene expression provides the necessary mechanism for sequential gene expression with temporal and spatial specificity. Acetylation and deacetylation may play a critical role in the coordinated expression of transcription factors and all of the subsequent gene targets employed in plant tissue differentiation and development. The disruption of histone deacetylation by chemical inhibitors like trichostatin A (TSA), and the silencing of genes *HISTONE DEACETYLASE19 (HDA19, AtRPD3A)* and *HD2A* indicate that the processes of acetylation and deacetylation are critical to normal plant development (Xu et al., 2005; Wu et al.,2000a; Tian and Chen, 2001; Wu et al., 2000b).

TSA is a potent specific inhibitor of histone deacetylases (Yoshida et al., 1990). Originally isolated from *Streptomyces platensis* as a fungistatic antibiotic (Tsuji et al., 1976) it has been found to cause hyperacetylation of histones at nanomolar concentrations and inactivity of the S-enantiomer indicates high stereospecificity and inhibition of a specific target (Yoshida et al., 1990). This target was identified as HDAC enzymes. Xu et al. (2005) noted changes in cellular patterning of roots upon exposure to TSA. This paper noted hyperacetylation of core histones within one hour of exposure. TSA increased the number of root hairs in a concentration-dependent fashion. The presence of TSA promoted the conversion of epidermal cells to a hair-cell fate. The fate of a cell is usually determined by the spatial relationship to cortical cells. The conclusion of this paper was that the processes of acetylation and deacetylation are critical to

the coordination of gene expression in a multicellular organism. Disruption of these enzymes leads to a disruption of the transcription factor responsible for normal cell differentiation. Within the root, three of the transcription factors responsible for coordination of root cellular fate; *CAPRICE (CPC)* (Wada et al., 1997), *GLABRA2 (GL2)* (Di Cristina et al., 1996) and *WEREWOLF (WER) (Li et al., 1997),* showed altered expression upon TSA treatment (Xu et al., 2005) resulting in an altered developmental pattern in the developing root.

AtRPD3A (AtHD1,HDA19) has also been implicated in development. The gene was cloned and introduced in antisense orientation driven by the strong – *394tCUP* promoter. Expression of the antisense transcript resulted in a decrease in endogenous levels of *AtRPD3A*. This led to delayed flowering time (Wu et al, 2000a). The role of *AtRPD3A (AtHD1, HDA19)* was investigated in *A. thaliana* ecotype Columbia using antisense technology by Tian and Chen, 2001. The gene, in antisense orientation and driven by a constitutive promoter (35S cauliflower mosaic virus), was introduced into a plant line in order to investigate the role this deacetylase may play in growth and development.

The choice of this specific deacetylase was guided by research carried out in yeast, *D. melanogaster* and mouse model systems. Within yeast, the homologue is thought to be a global transcription regulator. Within mouse tissue culture, HISTONE DEACETYLASE1 (HD1*)* has been implicated in cell cycle progression with a severe delay in growth rates when the cultures overexpress the HD1. This global function of histone deacetylases found in yeast may be also found in *A. thaliana* functioning as a housekeeping deacetylase over a wide-

range of gene targets required for development. A number of phenotypic abnormalities were noted in a population of transformed plants including early senescence, reactivation of silenced genes in a non-specific manner, loss of apical dominance, heterochronic shift to juvenility, homeotic transformation of tissue, defective flowers, and sterility, indicating that this deacetylase gene, and more generally the process of deacetylation, may play a role in many developmental processes (Tian and Chen, 2001). The variety of phenotypes seen can be explained by a set of developmental genes that this deacetylase may repress during growth and development; however, many of these specific gene targets remain uncharacterized. Variation in phenotypes can also be explained by varying levels of abolition of *AtHD1* function by antisense expression. From the results of Wu et al. (2000b) and Tian and Chen (2001) it seems that deacetylation plays a role in embryogenesis as a significant number of plants had malformed primary leaves which form during early development.

The *HD2* family of histone deacetylases has also been implicated in embryogenesis and plant development. Initial work investigated the tissue expression patterns of *HD2* genes. Reverse transcriptase PCR (RT-PCR) was used to investigate expression within selected tissue during plant development. *HD2A, HD2B,* and *HD2C* were detected using RT-PCR in leaves, roots, stems, young plantlets, within a flower, and siliques with the highest levels being detected within stems, flowers and young siliques followed by seedlings (Zhou et al, 2004). *In situ* hybridization was also performed, detecting strong expression levels of mRNA in ovules, embryos, shoot apical meristems, and primary leaves.

The expression patterns of *HD2A, HD2B,* and *HD2C* are consistent with a repressive role in embryogenesis and plant development proposed through phenotypic analysis by antisense suppression lines (Wu et al., 2000b). These genes, in association with targeting factors, may function to repress specific gene targets to facilitate the coordinated expression of developmental genes.

The similarity in expression profiles indicates that genes of this family may function in a redundant manner. For example, *HD2B* may be able to compensate in some functional capacity if *HD2A* was lost through loss-of-function mutation or mRNA suppression. The actual level of translation of mRNA must also be considered. The mRNA expression of these genes can be deceiving, as the amount of mRNA accumulation does not necessarily approximate the levels of translation. Post-translational modifications of *HD2A, HD2B, HD2C,* and *HD2D* remain largely uncharacterized and may also play a critical role in determining the activity of *HD2* deacetylases *in vivo*. *HD2D* was detected in stems, flowers and young siliques, indicating a possible different development role for this gene. *HD2D* is the least characterized of the *HD2* family in *A. thaliana*.

A paper published in 2003 (Calikowski et al., 2003) characterized the nuclear matrix of *A. thaliana* cells. A protein bearing sequence similarity to *HD2A* was isolated from the nucleoli and identified by mass spectrometry. The subcellular localization of this protein was confirmed by Zhou et al. (2004) through microscopic analysis of GFP-*HD2A* reporter complex. Involvement of *HD2A* in seed development is suggested based on the observation that nine genes involved in seed development and maturation showed altered expression

in the *GFP-HD2A* overexpression lines. Suppression of *HD2A* resulted in aborted seed development with no other phenotypic developmental abnormalities indicating a distinct role in embryogenesis (Wu et al., 2000b). Within the Zhou et al. (2004) investigation on the *HD2* family, *in situ* hybridizations were carried out on embryonic tissue generated through the expression of the *B. napus* transcription factor *BABYBOOM (BBM)*. Overexpression lines show ectopic embryo development during vegetative growth and therefore this transcription factor is capable of promoting the process of somatic embryogenesis (Boutilier et al., 2002). Somatic embryogenesis has been historically used as a model for zygotic embryogenesis which can be difficult to study within *A. thaliana* due to its small embryo size. Somatic embryogenesis enabled tissue to be sectioned and analyzed for the expression of *HD2* mRNA transcripts within a developing embryo.*HD2A* and *HD2B* expression was highly induced within the developing embryo but was expressed at a lower concentration in the surrounding cotyledon tissue. This in combination with the antisense and overexpression data indicates that *HD2A* and *HD2B* play a critical role in the developing embryo.

CHAPTER 2

INVESTIGATIONS INTO THE DEVELOPMENTAL SIGNIFICANCE OF *HD2* PROTEINS IN RESPONSE TO SUGAR SIGNAL

2.1 ABSTRACT

The *HD2* family of enzymes was discovered to be a unique class of histone deacetylases. They were originally identified in maize and share no sequence homology to previously identified histone deacetylases. Homologues have now been characterized in *Zea mays*, *Solanum chacoense* and *A. thaliana*. *A. thaliana* contains four *HD2* genes named *HD2A, HD2B, HD2C* and *HD2D.* Previous research hypothesized that *HD2A* may have evolved a specific role in transcriptional modulation during embryogenesis since suppression-of-function (antisense *HD2A)* lines showed an aborted seed phenotype. Inconsistent with a specialized function during embryogenesis, *HD2A-D* have wide-expression domains and can be detected in all tissues analyzed. Higher levels of transcription were noted in rapidly dividing tissue (SAM, RAM, young flowers and imbibed seeds).

An analysis of available microarray data identified the induction of *HD2* transcription in response to the herbicides isoxaben and imidazolinone. From this analysis, it was identified that *HD2* expression was directly linked to the concentration of soluble carbohydrates. Analysis of the *pgm* mutant verified that *HD2* expression followed the rise and fall of glucose during a 12 hour light/12 hour dark cycle. The correlation with glucose level was validated by RT-PCR for

HD2A, HD2B, HD2C and *HD2D* after four hours of exposure to glucose, sucrose, fructose. Mannitol, ABA, and non-metabolized sugars had no effect on the gene expression levels of *HD2* genes. A transcriptional model of *HD2* function is proposed in which a subset of genes is repressed during periods of high glucose since *HD2* expression levels are rapidly induced upon modulation of the sugar environment. An alternative model is identified that hypothesizes that *HD2* proteins function to replicate the acetylation state of the parent cell in rapidly dividing tissue and serve as a mechanism to maintain the expression of a developmental program.

2.2 INTRODUCTION

2.2.1 Overview

The *A. thaliana* genome contains eighteen known genes encoding sixteen functional histone deacetylases (HDACs) (URL http://www.chromdb.org). The majority of these genes (fourteen) have conserved functional HDAC domains originally identified in *S. cerevisiae* (Dangl et al., 2001). Twelve of these genes are classified as RPD3/HDA1-like genes, and two belong to the NAD+-dependent SIR2 class of enzymes. The four remaining genes encode members of *HD2* family, named *HD2A*, *HD2B*, *HD2C*, and *HD2D,* which is unique to the plant kingdom. The role of acetylation and deacetylation during plant growth and development can be found in the previous chapter.

2.2.2 Glucose metabolism and cell cycle progression

Glucose is a critical molecule during all aspects of growth and development of plants. Due to the importance of glucose at the cellular and developmental levels, it is one of the most-studied and well-characterized fields of study in plants. Plant metabolism is considered to be more fluid than in animals. The sessile nature of plants requires that sugar be able to be stored and mobilized to survive periods of environmental stressors. Within the cell, two reservoirs of sugar are maintained within the cytosol, the hexose and the triose pools (Buchanan et al., 2000). Sucrose is the main transport form of sugar in the cell. It is cleaved by cell wall invertases upon entry into the cell (Schwebel-Dugue et al., 1994). The glucose and fructose molecules are phosphorylated

components of the hexose sugar pool. From this pool, molecules can be combined to form structural sugars like cellulose, hemicellulose and become incorporated into the cell wall or can be incorporated into starch within the plastid. An alternative path for these sugars is catabolic which results in three carbon molecules within the triose sugar pools. The components of the triose sugar pool can then be oxidized in the glycolytic pathway or can feed into biosynthetic pathways to form amino acids, nucleic acids or lipids (Buchanan et al, 2000). The degradation of glucose or fructose to three carbon molecules is reversible. In fact at any point in the pathway, sucrose can be reformed allowing the plant to respond and modulate the metabolism in response to environmental cues. For example, during photosynthetic periods glucose formation exceeds the metabolic demands of the cell resulting in starch formation (Caspar et al., 1985). When the sugar pool is low during periods of dark, starch is degraded within the plastid and exported to the cytosol. The enzymes involved in sugar metabolism are regulated by a complex regulatory network with numerous negative feedback loops. The hexokinases are thought to detect and regulate the size of the hexose sugar pool by feedback inhibition by phosophorylated sugar molecules (Jang and Sheen, 1994). Beyond the scope of a single cell, sugar metabolism is also highly regulated within the plant. Glucose metabolism is often described in terms of source and sink. Source organs form glucose through high levels of photosynthetic activity and export it to other organs of the plant by loading sucrose into the phloem for transport. Source organs have higher expression levels of genes relating to photosynthetic activity, sucrose synthase genes, and

specific transporter molecules which export the sugar out of the cell. Sink organs have metabolic demands which exceed the capacity of the tissue to meet cellular demands. This occurs during rapid growth and development. Within the plant this is often thought of as a developmental trigger. For example, during seed formation expression of invertases is higher during embryo formation resulting in a high hexose to sucrose ratio (Borisjuk et al., 2004). This has been shown to promote cell growth and division in *Vicia faba* (Borisjuk et al., 1998). At later stages of embryo development after, the expression of invertase enzymes decreases resulting in a reduction in the hexose to sucrose ratio. Experimental evidence indicates that the introduction of exogenous sucrose during embryo formation favours cellular elongation and the storage of sugars. If sucrose is applied prematurely, the embryo does not complete the cellular divisions compared to a normal embryo resulting in premature maturation (Borisjuk et al., 1998).

The relation of glucose as a promoter of mitotic activity has been well established in a variety of plant tissues and organs. The cell cycle, common to all eukaryotes occurs in a tightly regulated fashion. Cells proceed through a DNA synthesis phase (S) and a mitotic phase when the cell divides into two daughter cells. These phases are separated by the G1 (after M, precedes S) and G2 (after S, precedes M) gap phases. Progression through the cycle is regulated by the expression of *CYCLIN DEPENDENT KINASES* (*CDK*s) which activate *CYCLINs* by phosphorylation. Plants contain 32 *CYCLINs* which promote the cellular processes required at each stage of the cell cycle (Inze, 2005). The

CYCLIN/CDK complexes can be inactivated by Kip-related proteins (*KPR*s) which halt cell cycle progression. Glucose has been shown to modulate the rate of cell cycle progression in tobacco cell tissue culture (Hartig, 2005). When the cells have accumulated sufficient starch, they are able to proceed through the cycle without the application of exogenous sugars. The rate of cell cycle progression correlates to endogenous sugar levels and not to glucose uptake of the cell indicating that hexokinase signaling may be involved in the detection of endogenous sugar levels (Sheen et al., 1999). The expression of cyclin proteins *CYCD2, CYCD1, CYCD3, CYCD2 AND CYCA3, CYCD3* correlate to the levels of endogenous sugars and the length of each G1 and G2 phases could be lengthened or shortened by application or removal of sugar (Hartig, 2005). This indicates that sugars plays a key role in coordinating the genes regulated to cell cycle progression. This impacts the development of all tissue and organs within the plant.

2.2.3 Effect of glucose and plant hormones on plant development

Mutant screens have recovered mutants in many of the genes involved in the glucose response pathway (Zhou et al., 1998; Laby et al., 2000; Gibson et al., 2001). These mutants show an altered glucose response, but also often show altered responses to phytohormones. Screens for phytohormone response mutants (eg. abscissic acid) have been mapped to loci identified in mutant glucose-response screens indicating that glucose plays a role in how plants respond to hormones. This is reviewed in Gibson (2004). Glucose is also

hypothesized to serve as a signaling molecule during specific stages of development. For example, by increasing the strength of the glucose sink in the apical meristem through the tissue-specific overexpression of a cell wall invertase (cleaves sucrose to glucose and fructose), transgenic lines flowered earlier (shorter vegetative phase), showed a 28% increase in seed yield, and had increased axillary inflorescences indicating that the source/sink relationship plays a key role in regulating the reproductive phase of plant growth (Heyer et al., 2004). In addition, the presence of glucose, sucrose or fructose caused the formation of adventitious roots in dark grown plants, a phenotype not seen under long day conditions (16h light/8h dark cycle) (Takahashi et al., 2003). The induction of adventitious roots occurred at sites where the hypocotyl remained in contact with the media indicating a local absorption of the carbohydrate and not sucrose translocation through the primary root. These are selected examples to illustrate the role of glucose in plant development. The role of glucose as a signaling molecule is reviewed in Gibson (2005).

2.2.4 Chromatin-remodeling and hierarchical control by glucose

Underlying the global changes in mRNA abundance that occurs during transcriptional modulation is the concept of chromatin-remodeling. If the set of genes expressed under normal conditions is not sufficient to maintain cellular function or integrity, the transcriptome may undergo changes to facilitate the survival of the organism. The histone code (Cheung et al., 2000) hypothesizes that the chemical modifications present on the N-terminus of histone proteins

within a genetic region may dictate the accessibility of a genetic locus thereby directly influencing the abundance of the mRNA transcript. Although the hierarchical mechanisms that govern transcriptional control are only beginning to be understood in model organisms like *S. cerevisiae*, they remain poorly characterized in plants. Considering the sphere of influence glucose has over all aspects of plant growth and development, it would not be surprising to find a strong link between glucose-based epigenetic and transcriptional control mechanisms. This field of study is not well-characterized at present, but a few microarray studies are beginning to make a clear link.

Recent papers published by Nicolaï et al. (2006) and Blasing et al. (2005) have linked glucose to chromatin-remodeling mechanisms. Nioclaï et al. (2006) examined the effect of sucrose starvation on the transcriptome of *A. thaliana* cell suspension cultures. This study noted the repression of the histone deacetylase *AtHD1* (At4g38130) during the period of sucrose starvation and the concomitant increase in H4 acetylation measured by the immunoprecipitation of chromatin with an H4-acetylation binding antibody. This links the modification state of histone proteins as a function of the metabolic state of the cell.

Analysis of the *phosphoglucomutase (pgm)* mutant, a mutant identified to be deficient in starch production (Corbesier et al., 1998) showed two histone deacetylases that show greater than two-fold increase in transcript abundance during a dark/light cycle (Blasing et al., 2005). Several other genes involved in DNA, RNA and protein synthesis were also highly induced during the period of high glucose (light) and repressed during periods of low glucose (dark) conditions

(Blasing et al., 2005). The information available linking the processes of glucose and chromatin-remodeling is limited at this time.

To understand the functional significance of this family, suppression-of-function and loss-of-function mutant lines were analyzed for developmental abnormalities. This study will make use of publicly-available microarray data to screen for chemicals that disrupt the expression (induction or repression) of the *HD2* family. Microarrays which were intended for use in characterizing and deciphering the glucose response have been analyzed (Villadsen and Smith, 2004; Blasing et al., 2005; Li et al., 2006). This analysis showed a definitive link between soluble carbohydrates (glucose, sucrose) and *HD2* gene expression indicating that deacetylation of histones by *HD2* proteins functions in carbohydrate response. Reverse transcription PCR (RT-PCR) was used to validate microarray findings. A variety of metabolized and non-metabolized sugars were analyzed to decipher which sugar-sensing pathways activate *HD2* expression. This might give insight into potential gene targets of transcriptional repression.

2.3 METHODS

2.3.1 Mutants and transgenic plants

Publicly available collections of mutant plants were searched and all available T-DNA (loss-of-function) and RNAi (suppression-of-function) lines were obtained. Seeds of Columbia-0, T-DNA and RNAi seeds were surface-sterilized with a solution of 70% ethanol for 1 min and then soaked in sterilization solution (25% CLOROX® v/v (The Clorox Company, CA, USA) 0.01% Triton-X (v/v) (Fischer Scientific, NH, USA)) for 30 minutes. Seeds were then rinsed three times with sterile water. Seeds were then stratified in 0.1%(w/v) agarose solution in the dark for 48 hours at 4° Celsius before plating on ½ Murashige and Skoog Basal Salt Mixture (MS)(Sigma-Aldrich, UK) (w/v), 3% sucrose(w/v), 0.8% Phytagar (Sigma-Aldrich, UK) (w/v), pH 5.7 plates. Plants were grown in continuous light at 22° Celsius. Suppression-of-function (RNAi) lines and possible loss-of-function T-DNA lines were screened on selective media. Plants were then transferred to soil and grown in 16 hour light/8 hour dark cycle. Mature seed was then harvested.

2.3.2 Genotyping of loss-of-function T-DNA lines

A. thaliana ecotype Columbia (Col) was used as the wild type control for this study. Two *HD2C* alleles (SALK_129799 and SAIL1259_E_06) in the Columbia-0 background were obtained from Arabidopsis Biological Resource Centre (ABRC, Columbus, OH, USA). DNA extraction was carried out using Extract-N-Amp™ Plant PCR Kit (Sigma-Aldrich, UK). Plants homozygous for a T-

DNA insertion in the *HD2C* allele were identified using PCR with primers which flank the T-DNA insertion: SALK_129799 forward primer 5'-GCTGGTTTCAAAGGTAAATGGG-3', reverse primer 5'-CAAGCACATAAAAGAGACTGTCATG-3', SAIL1259_E_06 forward primer 5'-ATCAACGCAGCTTGGGTACTCAATG-3', reverse primer 5'-GGGCCTCTCGTGTTTAGATTTAG-3'. The T-DNA insertion was confirmed using the reverse primer for the *HD2C* allele and a primer designed from the left border (LB) of the T-DNA: SALKLba1 5'-TGGTTCACGTAGTGGGCCATCG-3' (used for SALK_129799), SAILLb1 5'-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3' (used for SAIL1259_E_06). SALK_129799 and SAIL1259_E_06 were renamed *HD2C-1* and *HD2C-2* respectively. An *HD2A* allele was identified in the GABI-Kat collection of T-DNA mutagenised seeds. As outlined above, three primers were used for the confirmation of this allele: 5'-CCACGTTTCTCAGGTTTAGTATC-3', 5'-TCTGTCTAGTTTAAGTACCAGCAGG-3', and LB- 5'-CCCATTTGGACGTGAATGTAGACAC-3'.

2.3.3 Loss-of-function mutant confirmation by RT-PCR

Total RNA was harvested from the lines *HD2A*, *HD2C-1*, and *HD2C-2* after growth for 7 days on solid media as previously described. Tissue was homogenized with a Eurostar power-b tissue homogenizer (Eurostar IKA-Labotechnik, Staufen, Germany) and RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. RNA

quality and quantity was assessed by spectrophotometric measurement at 280 and 260nm (Biomate3, Thermo Electron Corporation, Waltham, MA, USA). Two micrograms of total RNA from each line was run on 1% agarose, TAE gel to ensure accuracy of RNA quantification. Total RNA (2µg), in the presence of a poly-T_{20} primer, was then transcribed into cDNA by SuperScript III first strand cDNA synthesis system (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. The presence of a truncated *HD2A* mRNA transcript was assessed in the *HD2A* mutant line by using two primers upstream of the mapped insertion. The primer sequences used for cDNA amplification during PCR were forward 5'- ATGGAGTTCTGGGGAATTGAAG-3' and reverse 5'-CAAACTCTCCCTTCTTGTTTTT-3'. The same forward primer previously described and a primer downstream of the insertion site, reverse 5'-ACATGAGACTTGACTGGCCGAC-3' were used during PCR amplification to detect a full-length transcript. The primers were designed to anneal to exon junctions to eliminate amplification of genomic DNA contamination. The annealing temperature for each reaction was 54° Celsius with 30 cycles of amplification.

An identical strategy was used for *HD2C-1* mutant line using the forward primer 5'-ATGGAGTTCTGGGGTGTTGAAG-3' and the reverse primers 5'-GAAACCAGCAGCCTCAAGTTGATC-3' and 5'-TCAAGCAGCTGCACTGTGTTTG-3'. The location of T-DNA insertion in *HD2C-2* is upstream of the transcriptional start site so only one primer pair was used for this line.

2.3.4 Analysis for transcriptional compensation

HD2B and HD2D genes were analyzed for potential transcriptional up-regulation as a compensatory mechanism for gene loss in mutant lines *HD2A*, *HD2C-1* and *HD2C-2*. RNA was extracted as previously described. The primers employed for the amplification of *HD2B* were forward 5'-ATGGAGTTCTGGGGAGTTGC -3' and reverse 5'-TGTTTGCCTGAGTTGAATGG-3'. The amplification of *HD2D* mRNA transcript was carried out with forward primer 5'-ATGGAGTTTTGGGGTATCG-3' and reverse primer 5'-TCCACCTTTAGCTATCTCCAAATG-3'. As an internal control, β-TUBULIN (*TUB8*) was run to ensure accuracy and efficiency of the reaction using the gene specific primers, forward 5'-GGGCTAAAGGACACTACACTGAAGG-3' and reverse 5'-CCTCCTGCACTTCCACTTCGTCTTC-3'.

2.3.5 Induction of *HD2* gene expression by soluble carbohydrates

A. thaliana ecotype Columbia (Col) seeds were sterilized and stratified as previously described. Seeds were sown on ½ MS, 0.8% (w/v) Phytagar, pH 5.7 glucose-free media on dialysis membrane backing (BIO-RAD, California USA). Seeds were germinated in continuous light for 24 hours. Plants were then incubated in the dark for 4 days. RNA was harvested from one plate at the end of the period and served as a no-treatment control. The remaining plates were incubated with 15 ml H_2O, or 3% (w/v) glucose solution for 4 hours in the absence of light. RNA was then harvested as previously described. Reverse

Transcription-PCR (RT-PCR) was then carried out on the members of the *HD2* gene family as previously described. This process was repeated for fructose, sucrose, glucose-6-phosphate, fructose-6-phosphate, mannitol, and O-methylglucose.

2.3.6 Analysis of expression profiles in response to modulation of glucose metabolism

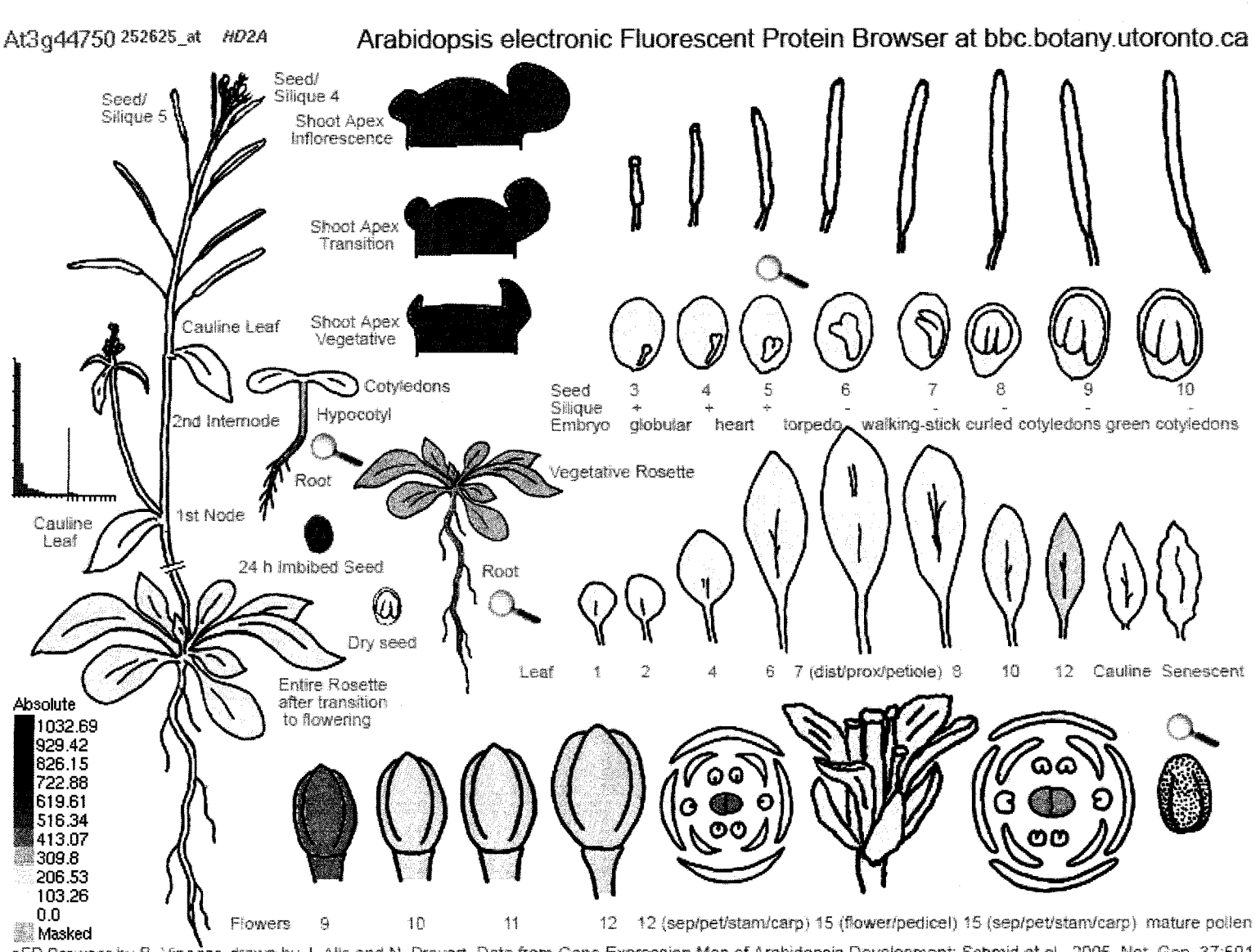
Pre-existing microarray data was also used for discovery. Data for these microarrays was downloaded from Nottingham Arabidopsis Stock Centre Arrays (NASCarrays) website (http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl) (Blasing et al., 2005; Li et al., 2006; Manfield et al., 2004; Villadsen and Smith, 2004). Each dataset was normalized by Robust Multi-array Analysis (Irizarry et al., 2003) using R (http://www.r-project.org)- Bioconductor (http://www.bioconductor.org) (Gentleman et al., 2004) software with the affylmGUI package (Wettenhall et al., 2006). affylmGUI was used to generate tables of differentially expressed genes. For each microarray, Microsoft® Access (Microsoft ® Corporation, USA) was used to create a searchable database to query known chromatin and glucose related genes. Known chromatin-related genes were downloaded from Plant Chromatin Database (http://www.chromdb.org).

2.4 RESULTS

2.4.1 Mutant characterization

Suppression-of-function and loss-of-function mutant lines were obtained from Arabidopsis Biological Resource Centre (ABRC) and Nottingham Arabidopsis Stock Centre (NASC). Thirteen suppression-of-function (RNAi lines) created by the Chromatin Functional Genomics Consortium, were obtained and screened. From previous research (Wu et al., 2000) an aborted seed phenotype was anticipated as a possible phenotype. Due to the wide expression domain (Zhou et al., 2004), the possibility of a phenotype at a different stage of development was monitored. The AtGENEXPRESS eFP (URL http://bbc.botany.utoronto.ca/) browser was used to visualize the expression of the *HD2* genes in the entire plant. The high level of expression in rapidly dividing tissue was noted for all *HD2* genes (Figure 3). The Arabidopsis Root Expression Database (AREX) was used to visualize *HD2* expression in the root. *HD2A, HD2B, HD2C* and *HD2D* were found to have identical expression patterns in the developing root (Figure 4). Expression was highest in rapidly dividing tissue located near the root apical meristem. Expression levels decreased with the age of the tissue.

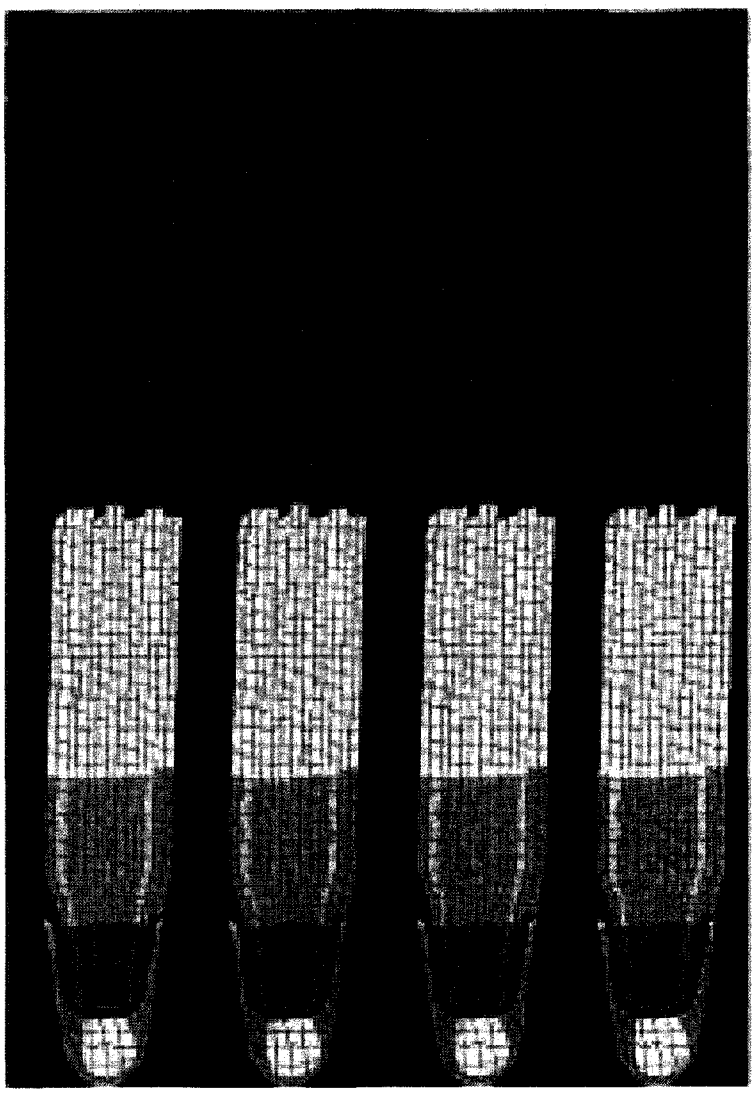
Figure 3. Expression data for *HD2A* generated by eFP browser. Figure 3 was generated using AtGenExpress Tissue Data Set (Schmid et al., 2005) electronic Fluorescent Protein browser (http://bbc.botany.utoronto.ca) generated using average expression values. The figure shows expression levels in specific tissue throughout plant development. The right side of the figure shows a developmental series for siliques, embryo, leaf and floral development. The left side shows the expression in the SAM, root, imbibed seed and within the first node, 2nd internode and late stage siliques. High levels of expression are indicated in red, intermediate levels in orange, and low levels of expression in yellow. High levels of expression are noted in the vegetative shoot apex, inflorescence shoot apex, imbibed seed (24 hour). High levels of expression were found in identical tissues for the remaining *HD2* (*HD2B, HD2C* and *HD2D*) genes (data not shown).



eFP Browser by B. Vinegar, drawn by J. Alis and N. Provart. Data from Gene Expression Map of Arabidopsis Development: Schmid et al., 2005, Nat. Gen. 37:501, and the Nambara lab for the imbibed and dry seed stages. Data are normalized by the GCOS method, TGT value of 100. Most tissues were sampled in triplicate.

Figure 4. *HD2* Expression in developing root (AREX DB). The figure is a graphical map of expression values obtained in a growing *A. thaliana* root. The data is average expression values obtained within specific regions of the root from the Arabidopsis Root Expression database (http://www.arexdb.org). All four *HD2* genes are shown. High levels of expression are shown in red, intermediate in pink and low levels in white. An identical pattern of expression is noted in all four genes. High levels of expression are concentrated in the root apical meristem of the developing root. Expression levels decrease as the age of tissue increases.

HD2A HD2B HD2C HD2D



None of the lines that suppress a single *HD2* gene showed an observable phenotype under normal growth conditions. Five lines identified to have T-DNA insertions within the *HD2C* locus, two within the *HD2A* locus, and two within the *HD2D* locus were screened for homozygous plants. All of the mutants analyzed to date are listed in Table 5.

Table 5: *HD2* mutant in *A. thaliana* analyzed for phenotypic abnormality

Gene	Stock #[3]	Ecotype	Selectable Marker[4]	Type[5]	Mutant Name[6]
HD2A	GK355H03	Columbia-0	Sulfadiazine	T-DNA	*hd2a*
	N371554	Columbia-0	Sulfadiazine	T-DNA	-
	CS3969	Wassilewskija	hygromycin	DsRNAi	-
	CS3970	Wassilewskija	hygromycin	DsRNAi	-
HD2B	CS30871	Wassilewskija	hygromycin	DsRNAi	-
	CS30872	Wassilewskija	hygromycin	DsRNAi	-
	CS30873	Wassilewskija	hygromycin	DsRNAi	-
	CS30874	Wassilewskija	hygromycin	DsRNAi	-
	CS30875	Wassilewskija	hygromycin	DsRNAi	-
HD2C	CS24027	Wassilewskija	hygromycin	DsRNAi	-
	CS24028	Wassilewskija	hygromycin	DsRNAi	-
	CS24029	Wassilewskija	hygromycin	DsRNAi	-
	SAIL_1259_E06	Columbia-0	BASTA	T-DNA	*hd2c-2*
	SALK_039784	Columbia-0	kanamycin	T-DNA	-
	SALK_129799	Columbia-0	kanamycin	T-DNA	*hd2c-1*
	SALK_136925	Columbia-0	kanamycin	T-DNA	-
HD2D	CS30957	Wassilewskija	hygromycin	DsRNAi	-
	CS30958	Wassilewskija	hygromycin	DsRNAi	-
	CS30965	Wassilewskija	hygromycin	DsRNAi	-
	N371578	Columbia-0	Sulfadiazine	T-DNA	-
	N369104	Columbia-0	Sulfadiazine	T-DNA	-

[3] Indicates the stock number of the mutant line from either The Arabidopsis Information Resource (TAIR) or Nottingham Arabidopsis Stock Centre (NASC)

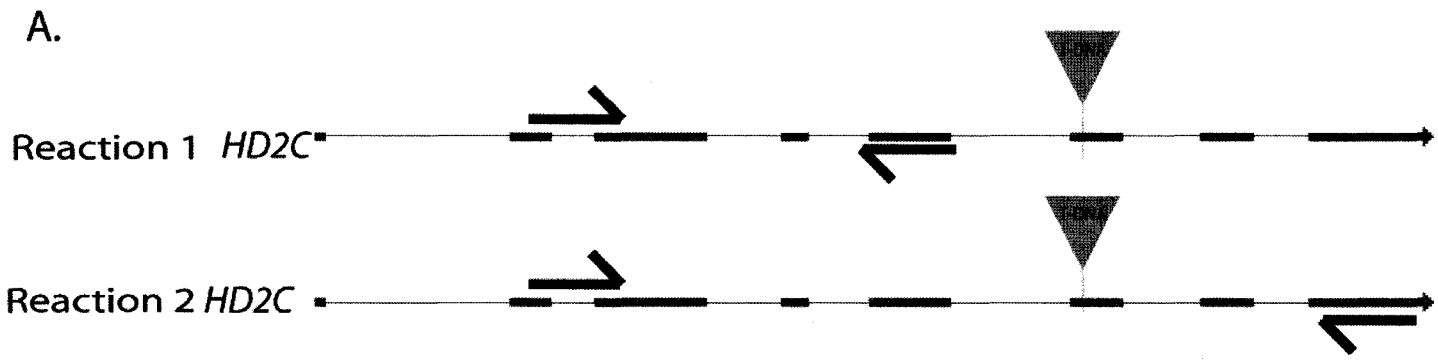
[4] Indicates the transgene carried within the mutant line in order to select for the genetic material inserted into the genome

[5] Indicates the type of mutant used in analysis. Mutants were obtained through TAIR and NASC. T-DNA mutants were generated by *Agrobacterium sp.* infiltration with a strain carrying the Ti plasmid. Portions of the Ti plasmid are transferred to the plant host cell. This DNA integrates into the genome and may disrupt the expression of gene in the immediate vicinity of the insertion. DsRNAi mutants were obtained from TAIR. These mutants were transformed with a vector containing a short fragment of the target gene. This may trigger the suppression of the endogenous gene by the RNAi mechanism.

[6] mutant names were assigned after characterization of that mutant line

These plants were screened for the absence of a full-length or shortened mRNA transcript. Three mutant lines: SALK_129799, SAIL_1259_e06 and GK355H03 were found to be loss-of-function alleles and were renamed *HD2C-1, HD2C-2* and *HD2A* respectively (Figure 5, Figure 6, Figure 7). No shortened or full length mRNA transcript of *HD2A* or *HD2C* was identified after 30 cycles of PCR. β-tubulin served as a positive control to ensure the quality of RNA for all samples. No 'loss-of function' alleles were obtained for the *HD2D* gene.

Figure 5. *hd2c-1* mutant RT-PCR analysis. The location of the T-DNA insert was mapped using SIGnAL "T-DNA Express" Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress). RNA was harvested from 7-day old Columbia-0, heterozygous (*HD2C-1 hd2c-1*) and homozygous (*hd2c-1 hd2c-1*) plants grown in continuous light on 1/2MS salts, 0.8% phytagar, 3% sucrose medium. 2 µg of total RNA was converted to cDNA which served as a template for 30 cycles of PCR. Panel A contains a schematic of the two PCR reactions (1 and 2) carried out to characterize mutant. Green arrows represent exons of the gene. Reaction 1 was designed using primers which span exon-junctions upstream of the T-DNA insert. Reaction 2 contained primers which span exon junctions flanking the site of T-DNA insertion. Panel B contains the PCR reactions carried out on cDNA generated from Columbia 0, heterozygous and homozygous plant lines. No shortened mRNA transcript was detected in the homozygous plants but was detected in heterozygous and Columbia-0 lines. No full length transcript was detected in the homozygous line but was detected in heterozygous and Columbia-0 lines. *β-TUBULIN* served as apositive control and was amplified in all samples.



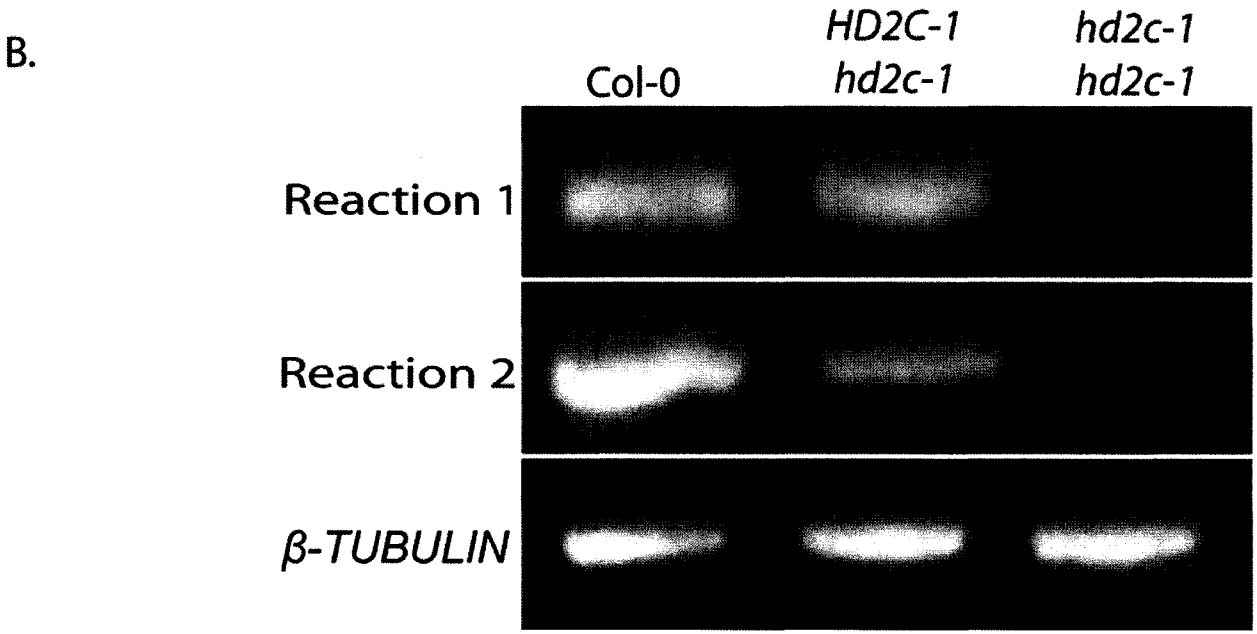
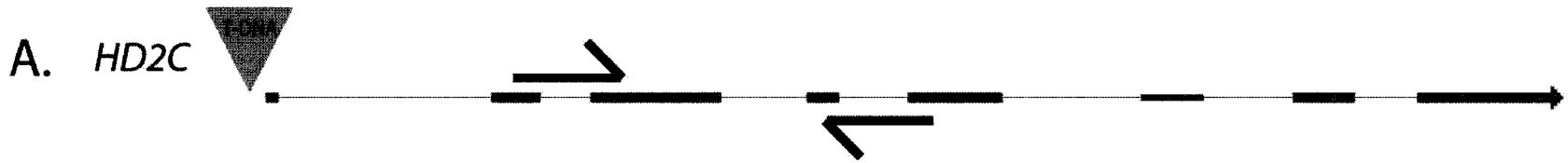


Figure 6. *hd2c-2* mutant RT-PCR analysis. The location of the T-DNA insert was mapped using SIGnAL "T-DNA Express" Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress). RNA was harvested from 7-day old Columbia-0, and homozygous (*hd2c-2 hd2c-2*) plants grown in continuous light on 1/2MS salts, 0.8% phytagar, 3% sucrose medium. 2 µg of total RNA was converted to cDNA which served as a template for 30 cycles of PCR. Panel A contains a schematic of the reaction designed to characterize the mutant. Green lines represent exons of the gene. Panel B contains the results of the PCR amplification of cDNA from Columbia-0 and homozygous plant lines. No shortened mRNA transcript was detected in the homozygous plants but was detected in Columbia-0. *β-TUBULIN* served as a positive control and was successfully amplified in both samples.



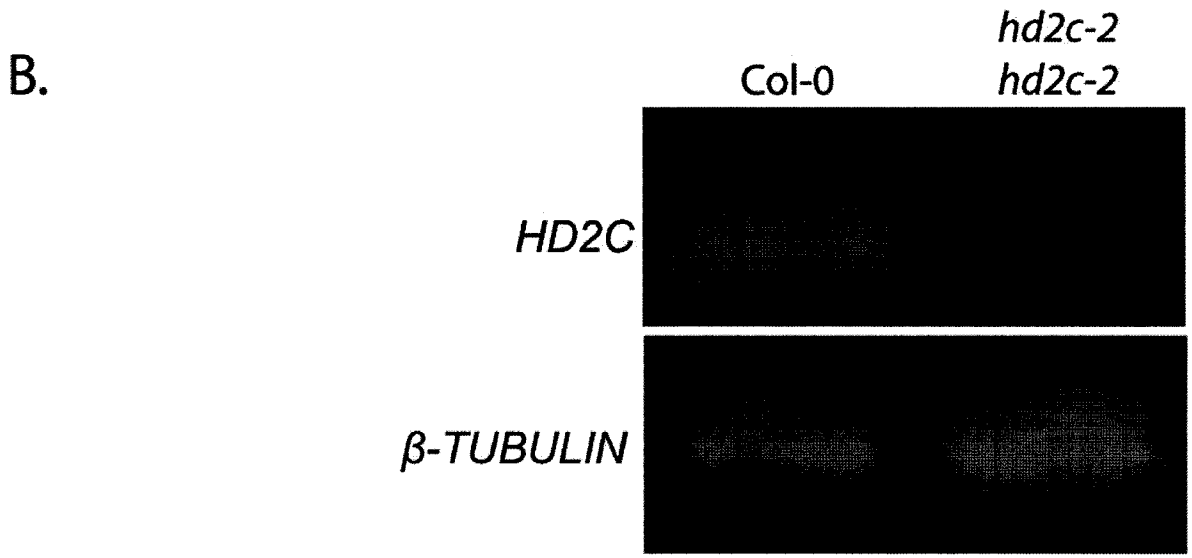
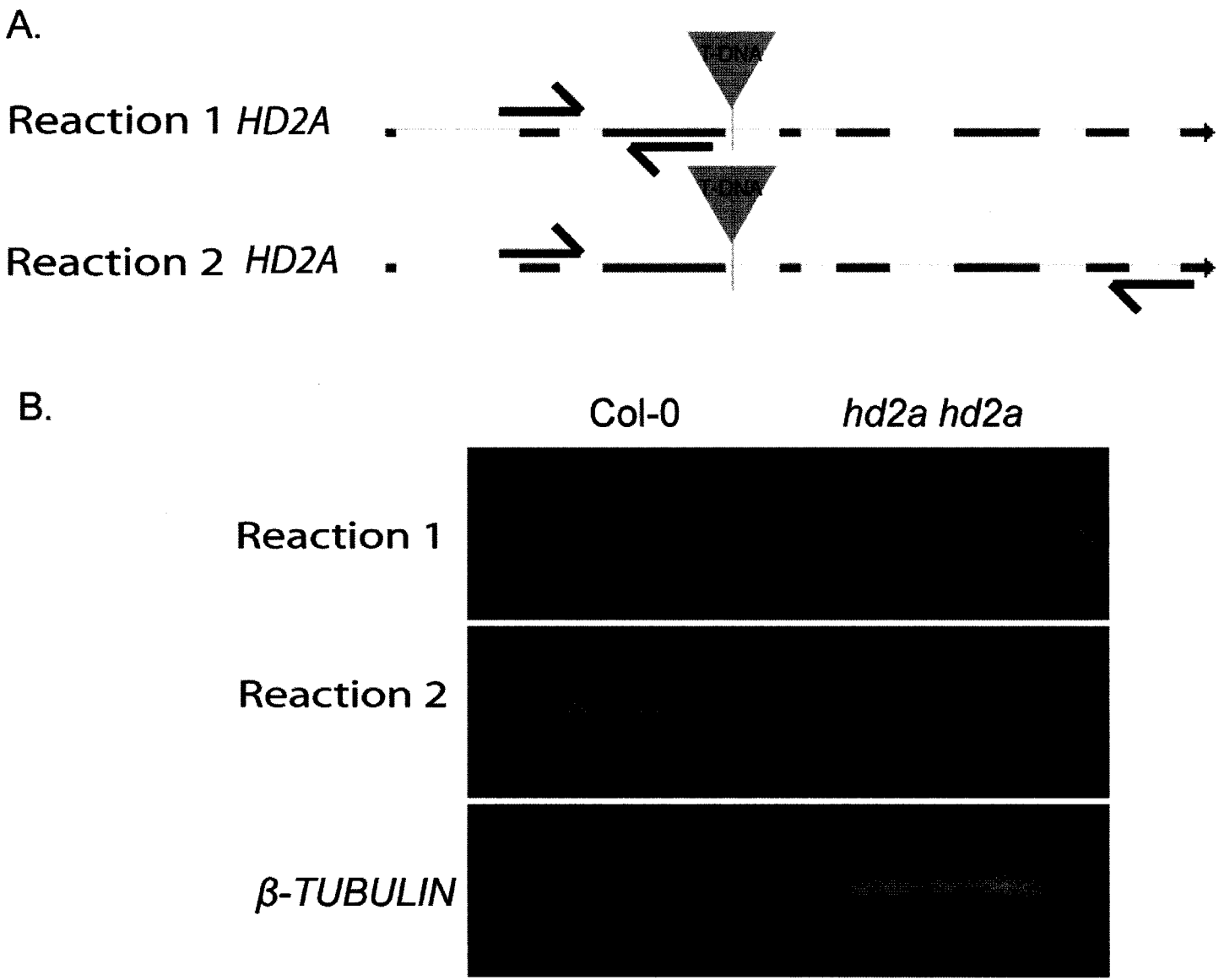
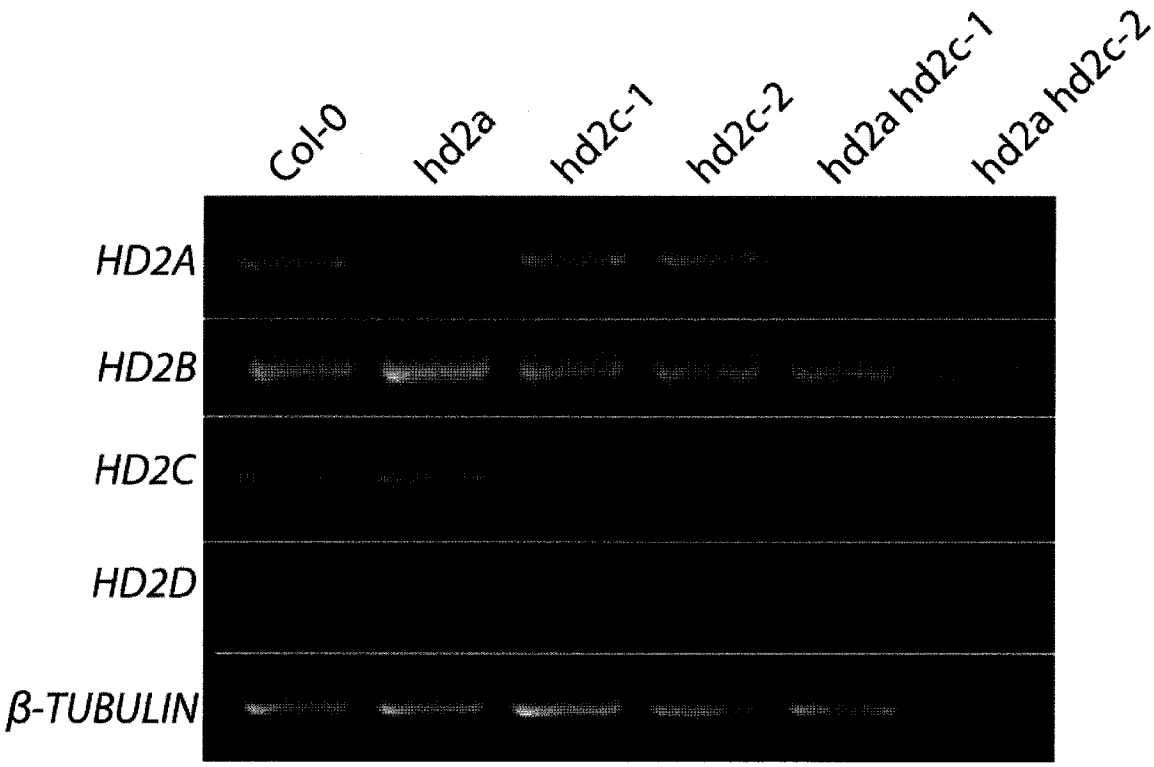


Figure 7. *hd2a* mutant RT-PCR analysis. The location of the T-DNA insert was mapped using SIGnAL "T-DNA Express" Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress). RNA was harvested from 7-day old Columbia-0, homozygous (*hd2a hd2a*) plants grown in continuous light on 1/2MS salts, 0.8% phytagar, 3% sucrose medium. 2 µg of total RNA was converted to cDNA which served as a template for 30 cycles of PCR. Panel A contains a schematic of the PCR reactions carried out to characterize the mutant. Green lines indicate the exons of the gene. Reaction 1 was done used primers which spanned exon junctions upstream of the T-DNA insert. Reaction 2 was designed with primers which span exon junctions flanking the site of T-DNA insertion. Panel B contains the PCR reactions carried out on cDNA generated from Columbia 0, and homozygous plant lines. No shortened mRNA transcript was detected in the homozygous plants but was detected in Columbia-0 tissue. No full length transcript was detected in the homozygous line but was detected in Columbia-0 tissue. *β-TUBULIN* served as a positive control and was amplified in all samples.



These *HD2A*, *HD2C*-1 and *HD2C*-2 alleles were crossed to yield double mutants. These lines showed an unstable aborted seed phenotype in the first homozygous generation which lessened in subsequent generations. The unstable aborted seed phenotype was similar to phenotype of *HD2A* suppression-of-function antisense lines (Wu et al., 2000). RT- PCR was done on single and double mutants to ascertain whether there was compensation at the transcriptional level when one or two of the genes were disrupted by T-DNA insertion. The results of this (Figure 8) indicate that the transcriptional rate of *HD2* genes was stable when either *HD2A, HD2C* or both are disrupted by T-DNA insertion.

Figure 8. RT-PCR of Characterized *HD2* mutant lines. RT-PCR analysis was carried out to detect upregulation of other *HD2* genes in the absence of one or two functional *HD2* genes. Plant lines were grown for seven days on ½ MS, 0.8% phytagar, 3% (w/v) sucrose in continuous light. The following plant lines were grown and RNA extracted: lane A- Columbia-0, lane B- *hd2a hd2a*, lane C- *hd2c-1 hd2c-1*, lane D- *hd2c-2 hd2c-2*, lane E- *hd2a hd2a hd2c-1 hd2c-1*, F- *hd2a hd2a hd2c-2 hd2c-2*. 2µg of total RNA was converted to cDNA which served as a template for 30 cycles of PCR. In the absence of *HD2A, HD2C* or both *HD2A* and *HD2C* no compensatory transcriptional upregulation occurs in the remaining functional *HD2* genes. This was consistently noted for all samples. *β-TUBULIN* served as a positive control and was successfully amplified in all of the samples.

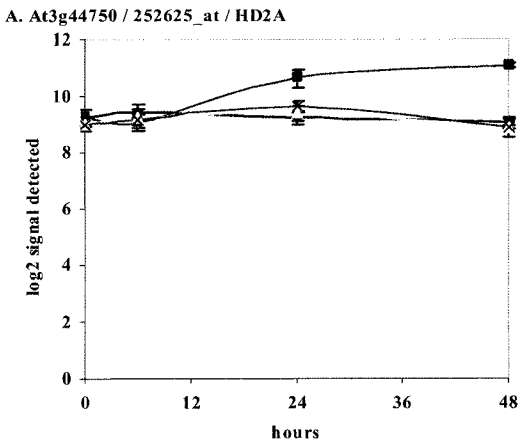


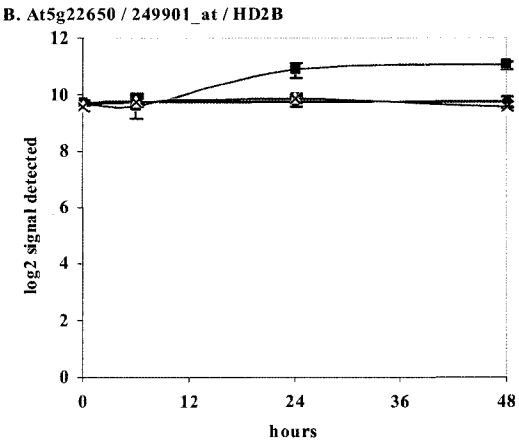
In parallel to the analysis of publicly available *HD2* mutants, suppression-of-function (RNAi) lines were created to simultaneously target *HD2B*, *HD2C* and *HD2D* in the *HD2A* mutant background. These lines were constructed using the pHANNIBAL vector (CSIRO, http://www.pi.csiro.au). The lines obtained showed no observable phenotype. No analysis was done to determine if suppression of *HD2* gene family was occurring.

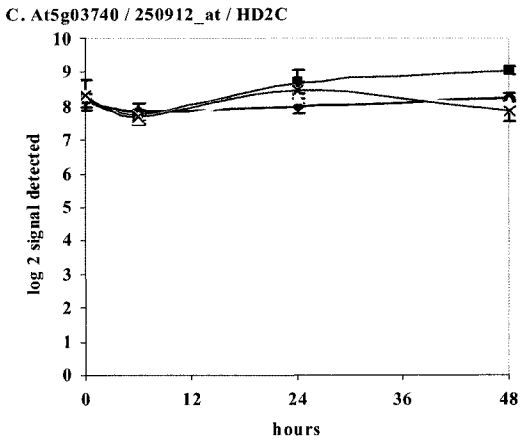
2.4.2 Induction by imidazolinone

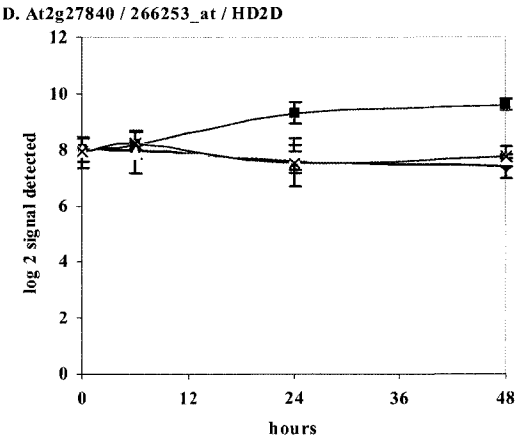
It is current practice to make microarray data available to the public after a group has published a dataset. This enables other researchers to use datasets downloaded from online repositories for quick hypothesis testing. Publicly accessible data was used in this analysis for the exploration of *HD2* function. A microarray analysis was carried out to measure the stability of the transcriptome when stressed by imidazolinone. Imidazolinone inhibits branch-chain amino acid synthesis (Ray, 1984; Shaner et al., 1984). This analysis, completed by Yuzuki Manabe, compared resistant and non-resistant plant lines at 0, 6, 24 and 48 hours exposure. *HD2* expression was induced at 24 hours after exposure of non-resistant (Columbia-0) plants to imidazolinone (100μM) and this level of expression was sustained for *HD2A, HD2B, HD2C, and HD2D* at subsequent time points. Three replicates were analyzed at each time point. Error bars indicate one standard deviation above and below the calculated mean expression value (Figure 9, Yuzuki Manabe, unpublished data).

Figure 9. Graphical representation of *HD2* expression in response to imidazolinone (100μM) exposure (unpublished data, Yuzuki Manabe). Columbia-0 and an imidazolinone-resistant line were exposed to both mock treatment (H_2O) and imidazolinone treatment for a period of 48 hours. Plant shoots were sampled at 0, 6, 24 and 48 hours of exposure to H_2O or imidazolinone. Error bars indicate one standard deviation above and below the mean expression value of three replicates.The pink line represents signal data obtained for the Columbia-0 plants treated with imidazolinone. The remaining lines represent resistant plants treated with imidazolinone (turquoise), mock treated Columbia-0 (Blue) and mock-treated imidazolinone-resistant plants (yellow). An increase in expression (pink line) was noted for all *HD2* genes beginning after 12 hours of exposure and remaining elevated for the remainder of the timepoints. *HD2A* can be found in Panel A, *HD2B* in Panel B, *HD2C* in Panel C and *HD2D* in Panel D. This upregulation in *HD2* transcription was not noted in mock treated samples or in the imidazolinone-resistant imidazolinone treated samples.









The change of expression observed was not always statistically significant (depending upon the cut-off used for the analysis) but the uniform response of the *HD2 (HD2A, HD2B, HD2C* and *HD2D*) family indicated the possibility of a link between herbicide exposure and *HD2* expression. The expression levels of the 14 remaining HDACs within the *A. thaliana* genome were not altered (data not shown). In this analysis, treatment of imidazolinone-resistant plant lines, and a mock treatment (exposure to H_2O) failed to induce *HD2* expression indicating that the response was specific. The induction of *HD2* expression was not validated by RT-PCR experiments.

2.4.3 Modulation of *HD2* expression by biotic and abiotic stressors

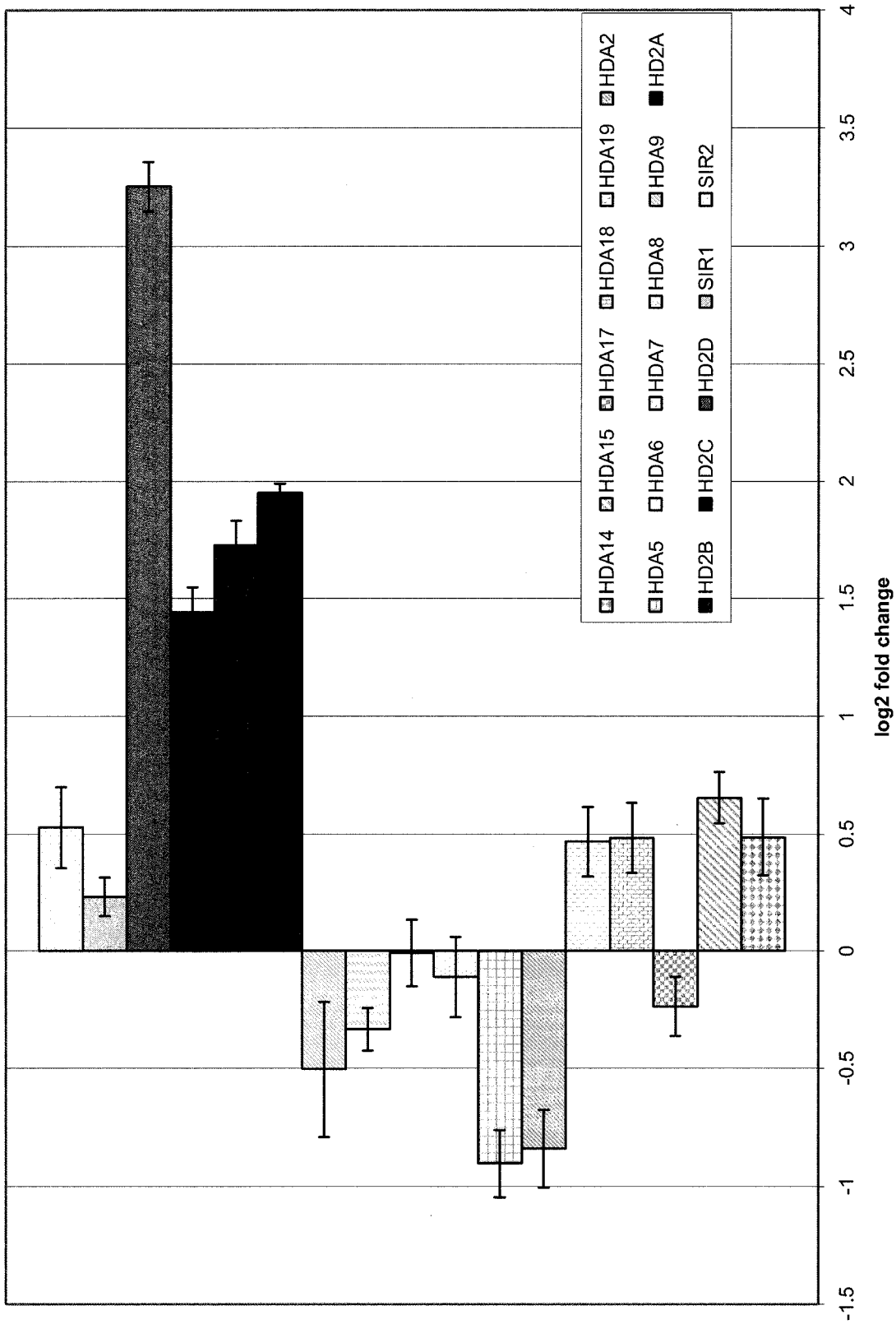
The effect of other herbicides, and chemical stressors on A. *thaliana* plants were investigated to determine if this was a function of a generalized stress response through AtGENEXPRESS tissue series, (Schmid et al., 2005), NASCArrarys (Craigon et al., 2004) GENEVESTIGATOR (Zimmermann et al., 2004).

No modulation of *HD2* expression was identified in the biotic and abiotic stress series (Schmid et al. 2005) indicating that the response to imidazolinone was specific and not part of a generalized stress response. During this analysis, it was noted that most herbicides had no effect on *HD2* expression with the exception of isoxaben.

2.4.4 Induction of *HD2* expression by isoxaben

HD2 expression was highly induced upon treatment of isoxaben (Manfield et al., 2004). Three replicates were analyzed for habituated and non-habituated cells. Error bars indicate one standard deviation above and below the calculated mean expression value (Figure 10).

Figure 10. Graphical representation of HDAC response to herbicide isoxaben (Manfield et al., 2004). Plant tissue was grown in liquid suspension cultures. Plants were habituated to isoxaben exposure and compared to non-habituated cells. This graph shows the measured expression levels in habituated cells using the non-habituated as a control for all of the HDAC genes within Arabidopsis. Error bars indicate one standard deviation above and below the mean expression value for three replicates. *HD2* expression is elevated in the isoxaben habituated population of cells. All of the *HD2* genes show a greater than two-fold induction in expression in treated cells. *HD2D* shows the largest change with a greater than 8 fold induction of mRNA expression. *HD2C* shows the smallest increase with a 2.6 fold induction of expression. None of the remaining HDACs showed a significant change in expression.



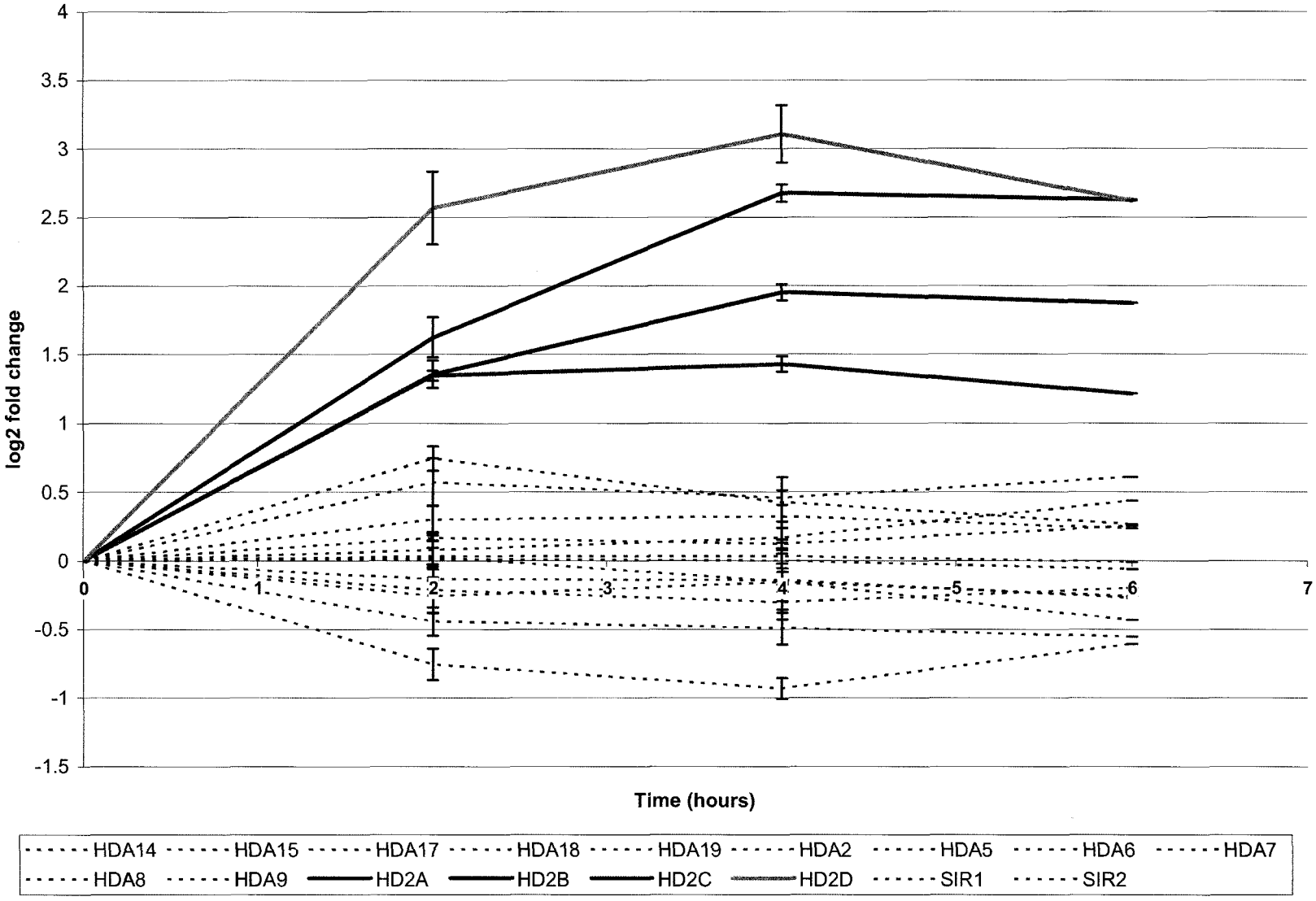
This plant herbicide specifically blocks cellulose synthesis in higher plants (Scheible et al., 2001). In this analysis, *HD2* expression was highly induced and like imidazolinone, this response was not mimicked by the remaining known HDACs.

2.4.5 Specificity of *HD2* induction by glucose

HD2 expression is up-regulated during imidazolinone and isoxaben treatment. Glucose is the precursor of both cellulose (structural sugar) and *de novo* amino acid synthesis in *A. thaliana* (Buchanan et al., 2000). A disruption of either pathway may therefore lead to an increase in intracellular glucose concentrations. The hypothesis that glucose may induce expression of *HD2* genes during normal plant growth and development was tested using additional microarray datasets.

An analysis of gene expression and the interrelated transcriptional network of glucose and ABA were carried out by Li et al. (2006). *HD2* expression was highly induced upon 3% (w/v) glucose reintroduction. Three replicates were analyzed for the 0, 2 and 4 hour time points. The original experimental design included one replicate at the 6 hour time point. Error bars indicate one standard deviation above and below the calculated mean expression value for the 2 and 4 hour time points (Figure 11).

Figure 11. HDAC response to 3% glucose (w/v) (Li et al., 2006). Plant tissue was grown in liquid MS media. Whole plants were grown for seven days in the presence of 0.5% (w/v) glucose in constant light. Glucose was removed for 24 hours and then reintroduced (3%w/v). Columbia-0 whole plant tissue was harvested at 0, 2, 4, and 6 hours of exposure to glucose. Error bars indicate one standard deviation above and below the mean expression value determined from three replicates at the 0, 2 and 4 hour timepoints. No error bars are displayed at the 6 hour time point as the measured expression value was determined from a single replicate. *HD2* expression is transcriptionally up-regulated in response to glucose. *HD2A* and *HD2D* show the largest induction with greater than 5.6 fold increase in expression at the 4 hour time point. Both *HD2B* and *HD2C* show significant induction of greater than 2.7 fold increase. Expression remains elevated at 6 hours exposure. None of the remaining HDACs show a significant alteration in expression level during the time points examined.



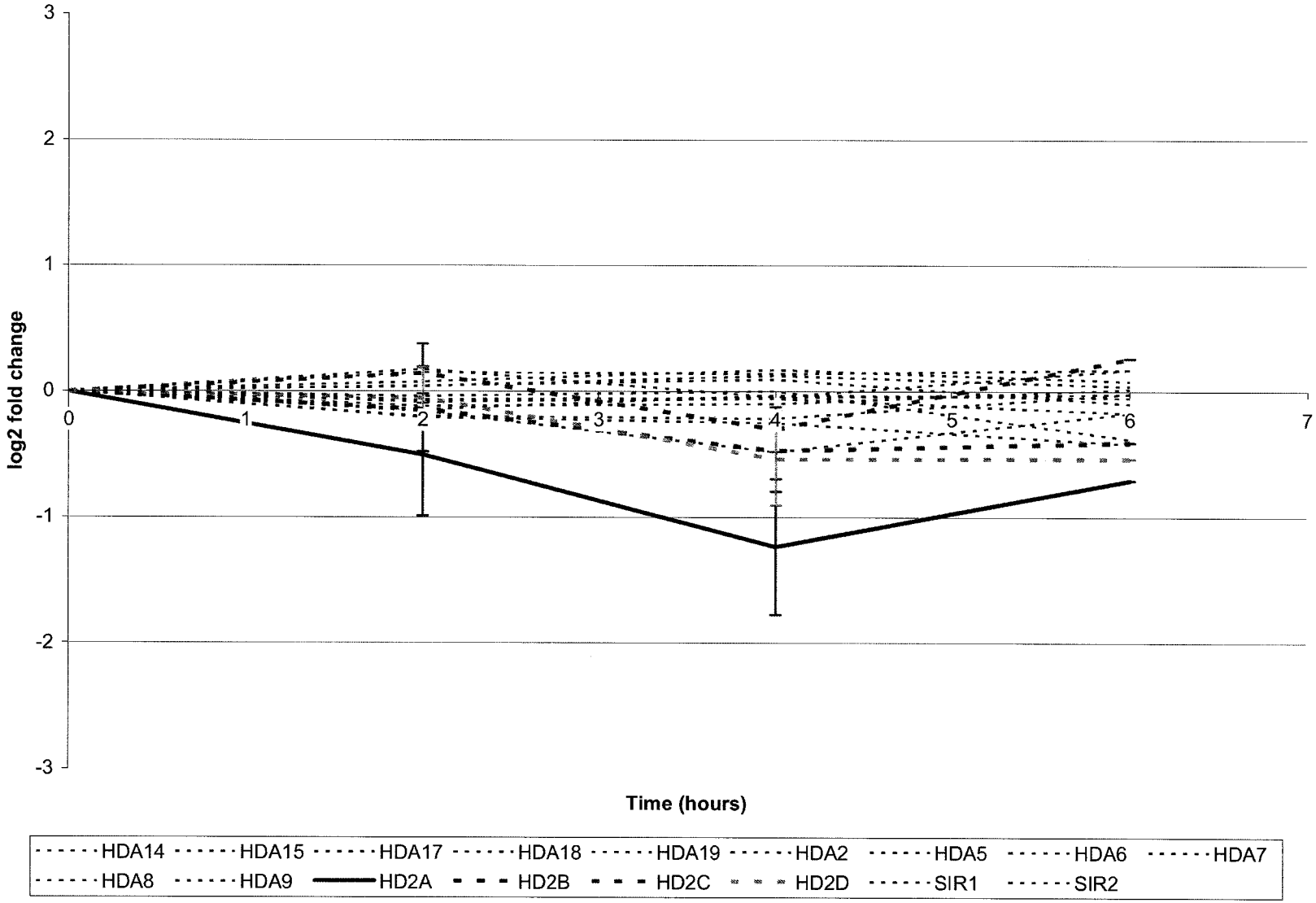
HD2D and *HD2A* showed the highest levels of induction, a trend previously seen in the *HD2* response to isoxaben (Figures 10 and 11). Expression levels peaked at 4 hours but remained high at the 6 hour time point for *HD2A, HD2B, HD2C* and *HD2D*. This response was unique to the *HD2* class of HDACs and was not part of a generalized HDAC response as the HDA1/RPD3 or SIR2 showed no significant alteration of expression, using a two-fold change of expression as cut-off.

A list of chromatin related genes was downloaded from ChromDB:The Plant Chromatin Database (http://www.chromdb.org) to detect simultaneous transcriptional modulation of chromatin-related genes within this dataset. The goal of this was to develop a mechanistic model of *HD2* modulation of target genes and identify possible interacting partners. Other histone-modifying enzymes (demethylases, histone acetyltransferases) did not show a significant change during the reintroduction of glucose (data not shown). Since the acetylation state of the chromatin is dependent on the activity of histone acetyltransferases and histone deacetylases, overall chromatin acetylation marks should decrease during the reintroduction of glucose due to the induction of the *HD2* class and the absence of induction of HATs.

The *HD2* induction of expression was not due to osmotic stress as mannitol (3% w/v) had little effect on HDAC expression. *HD2A* showed two-fold repression at the 4 hour time point but this was the only *HD2* gene to be modulated during the time points monitored. Three replicates were analyzed for the 0, 2 and 4 hour time points. The original experimental design included one

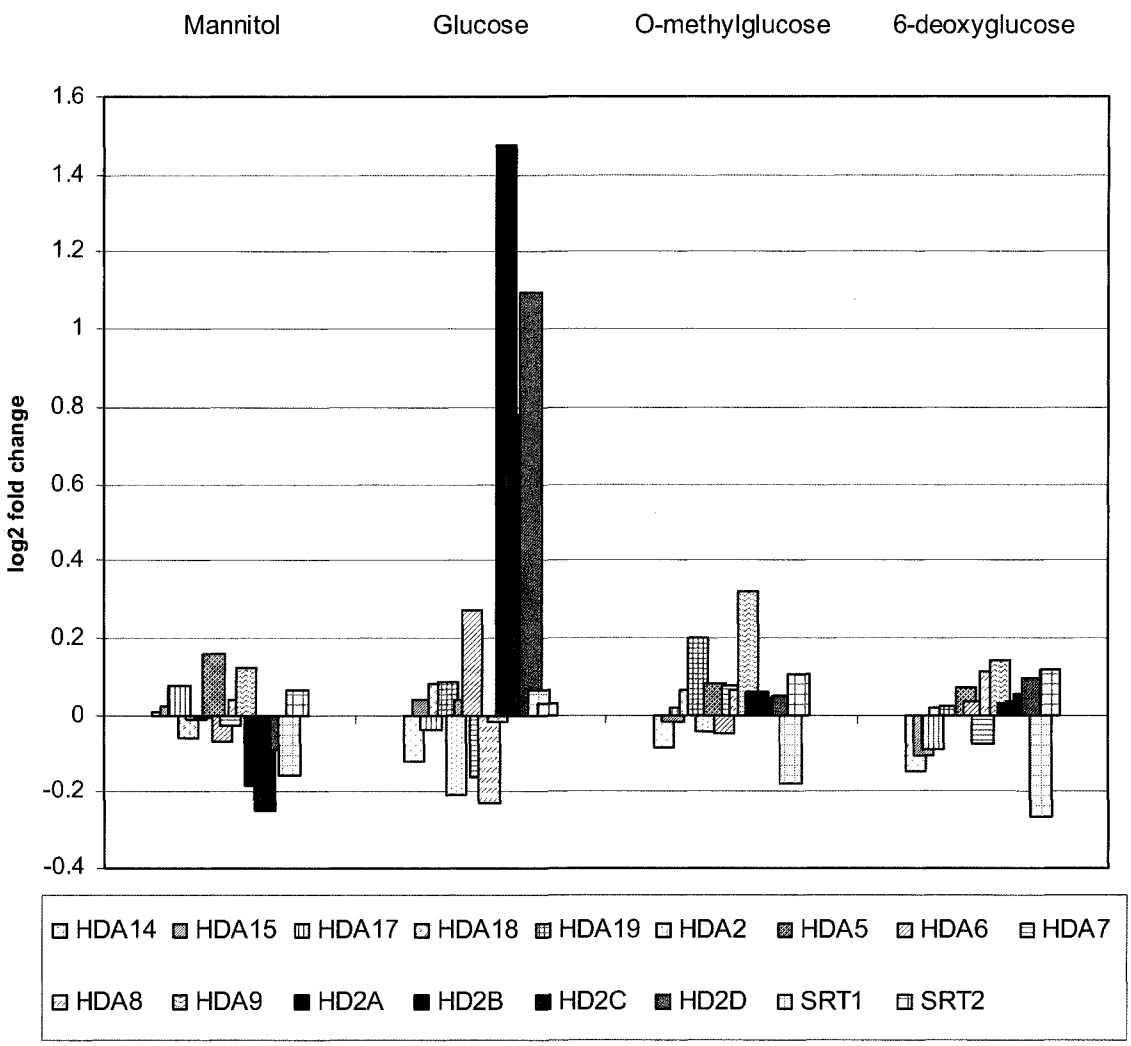
replicate at the 6 hour time point. Error bars indicate one standard deviation above and below the calculated mean expression value for the 2 and 4 hour time points (Figure 12).

Figure 12. HDAC response to mannitol (3% w/v) (Li et al., 2006). Plant tissue was grown in liquid MS media. Plants were grown for seven days in the presence of 0.5% (w/v) glucose in constant light. Glucose was removed for 24 hours prior to the introduction of mannitol(3%w/v). Columbia-0, whole plant tissue was harvested at 0, 2, 4, and 6 hours of exposure to mannitol. Error bars indicate one standard deviation above and below the mean expression value determined from three replicates at the 0, 2 and 4 hour timepoints. No error bars are displayed at the 6 hour timepoint as the measured expression value was determined from a single replicate Only *HD2A* showed a significant alteration in expression during the time points measure. *HD2A* showed a decrease in expression the the 4 hour time point. None of the remaining HDACs showed a significant alteration in expression.



A dataset published by Villadsen and Smith et al., (2004) analyzed the effect of non-metabolized sugars on the transcriptome. The non-metabolized sugars 6-deoxyglucose (DOG) and O-methylglucose (OMG) did not significantly modulate the expression of the *HD2* or other HDAC genes. Error bars are not displayed as a single replicate was analyzed for each treatment (Figure 13).

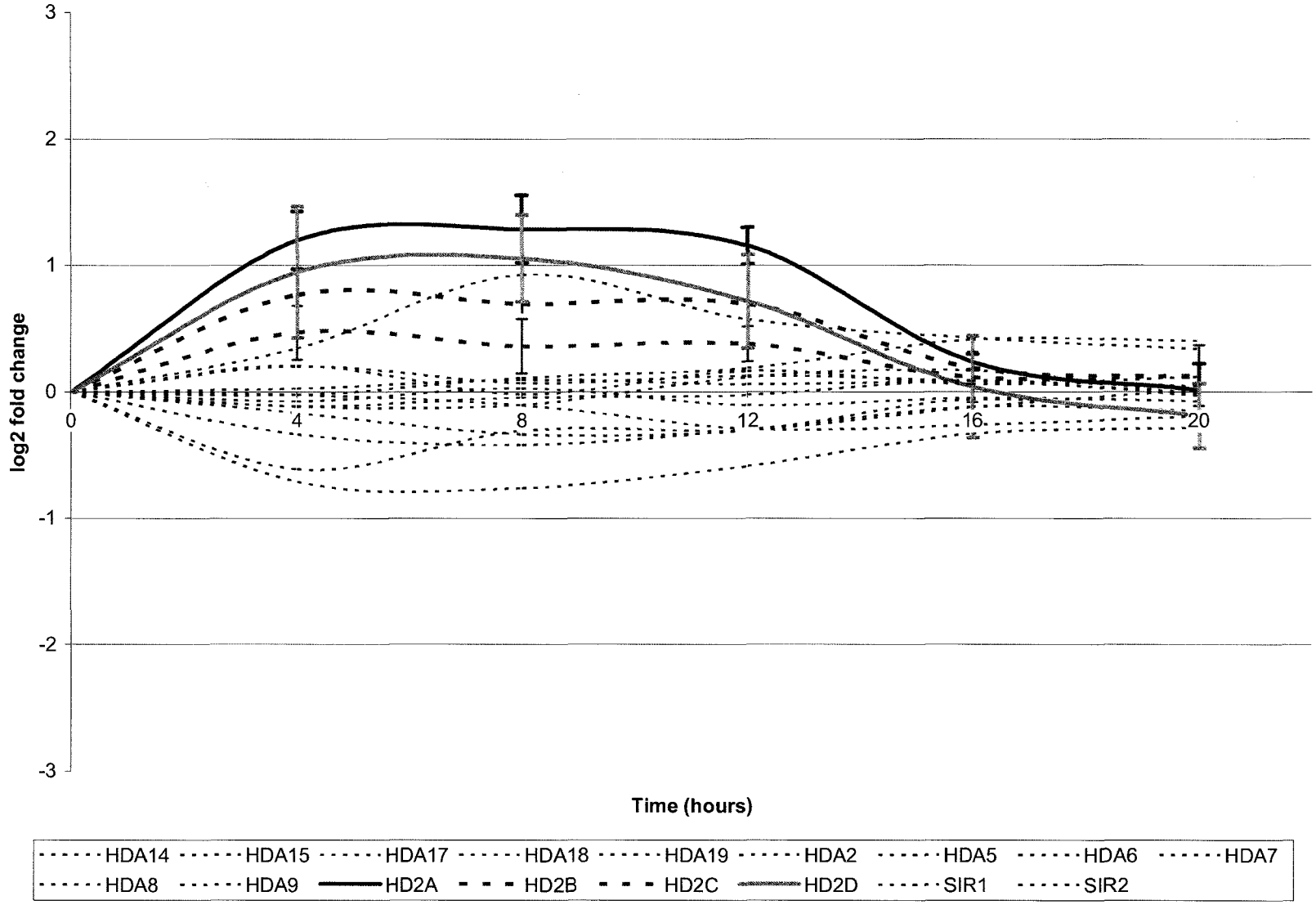
Figure 13. HDAC response to glucose analogues (Villadsen and Smith, 2004). Plants were exposed to 30mM mannitol, glucose, O-methylglucose and 6-deoxyglucose for 8 hours. RNA was extracted for mRNA transcript profiling from whole plants. No error bars are displayed as the measured expression value for each gene was determined from a single replicate for each treatment. Only *HD2A* and *HD2D* show greater than two-fold induction of expression at the time point measured. None of the remaining HDACs show a significant alteration of expression by an osmotic stressor (mannitol) or non-metabolized sugars,



There was a lower level of induction for the *HD2A*, *HD2B*, *HD2C* and *HD2D* in this experiment as compared to the Li et al. (2006) dataset (Figure 11). The glucose concentration for this experiment was lower 30mM (~1% w/v) compared to the Li et al, (2006) protocol. This indicates that induction of *HD2* expression may be proportional to the intracellular glucose concentration of the cell.

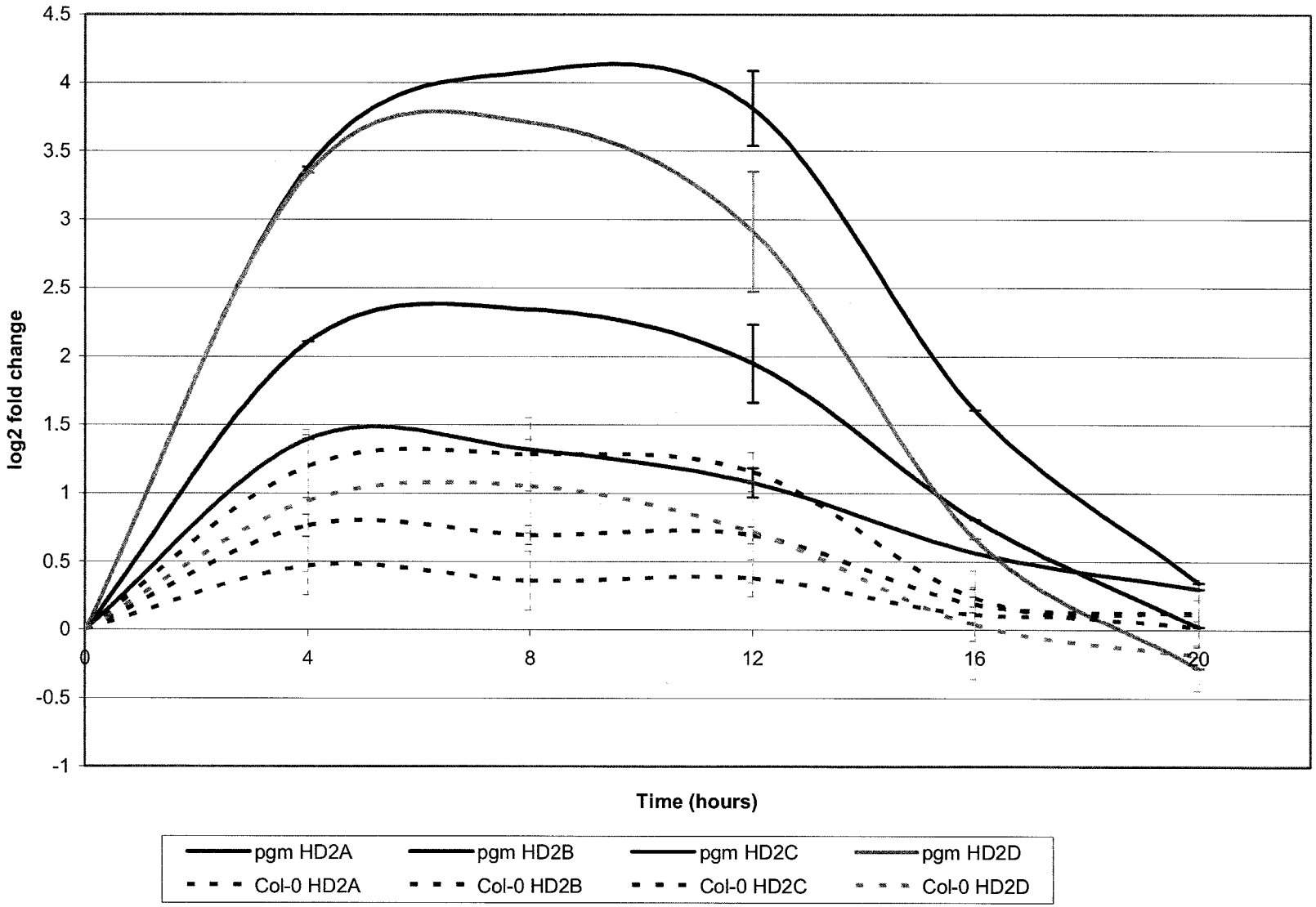
The final microarray analysis that was analyzed for modulation of HDAC expression was Blasing et al. (2005) which compared the diurnal expression of the genome within Columbia-0 to the *pgm* mutant to understand the role glucose plays in driving the expression of diurnally expressed genes. In the Columbia-0 population *HD2A* and *HD2D* show greater than two-fold induction of expression within 4 hours of exposure to light. This level of expression remains relatively constant until the 12 hour dark period when it rapidly decreases in abundance. This indicates that *HD2* expression is linked directly to the rise and fall of intracellular sugars during period of photosynthesis and dark (Figure 14) Error bars indicate one standard deviation above and below the calculate mean expression value for each gene. Three replicates were analyzed at each time point.

Figure 14. Graphical representation of diurnal cycle of expression in Columbia-0 rosette leaves (12 hour light/12 hour dark cycle)(Blasing et al., 2005) Plants were grown on soil. Rosette leaves were harvested at 0, 4, ,8, 12, 16 and 20 hours. Error bars indicate one standard deviation above and below the mean expression value determined from three replicates. Only *HD2A* and *HD2D* show a greater than two-fold fluctuation change during the time period and are solid lines. The remaining HDACs (indicated by dashed lines) do not change significantly during the timepoints measured.



As a comparison to the Columbia-0 populations, researchers completed the same experiment with a *pgm* mutant. Due to the disruption of starch formation in this mutant, glucose levels rise and fall dramatically during photosynthetic and non-photosynthetic periods respectively. In the *pgm* mutant, all four *HD2* genes exceed a two-fold change in expression within 4 hours of light exposure (Figure 15) Error bars indicate one standard deviation above and below the calculate mean expression value from two replicates, for each gene at the 12 hour time point. A single replicate was analyzed at all other time points.

Figure 15. Graphical representation of diurnal cycle of expression in *pgm* rosette leaves (12 hour light/12 hour dark cycle) in comparison two Columbia-0 plants. Plants were grown on soil. Rosette leaves were harvested at 0, 4, 8, 12, 16 and 20 hours. Error bars for the Columbia-0 dataset indicate one standard deviation above and below the mean expression value for each gene. Error bars are displayed at the 12 hour timepoint of the *pgm* mutant as this timepoint had two replicates. All other expression values were determined from a single replicate. *HD2* induction is marked in this mutant. *HD2A, HD2D* show a greater than 13 fold increase after being exposed to light for 8 hours. *HD2B* and *HD2C* both exceed a fold induction of expression during the exposure to light. *HD2* expression rapidly declines within 4 hours of the onset of dark indicating a strong diurnal expression pattern for *HD2* family of genes. The expression profile in Columbia-0 is shown in dashed lines while expression values calculate from the *pgm* mutant are shown as solid lines.



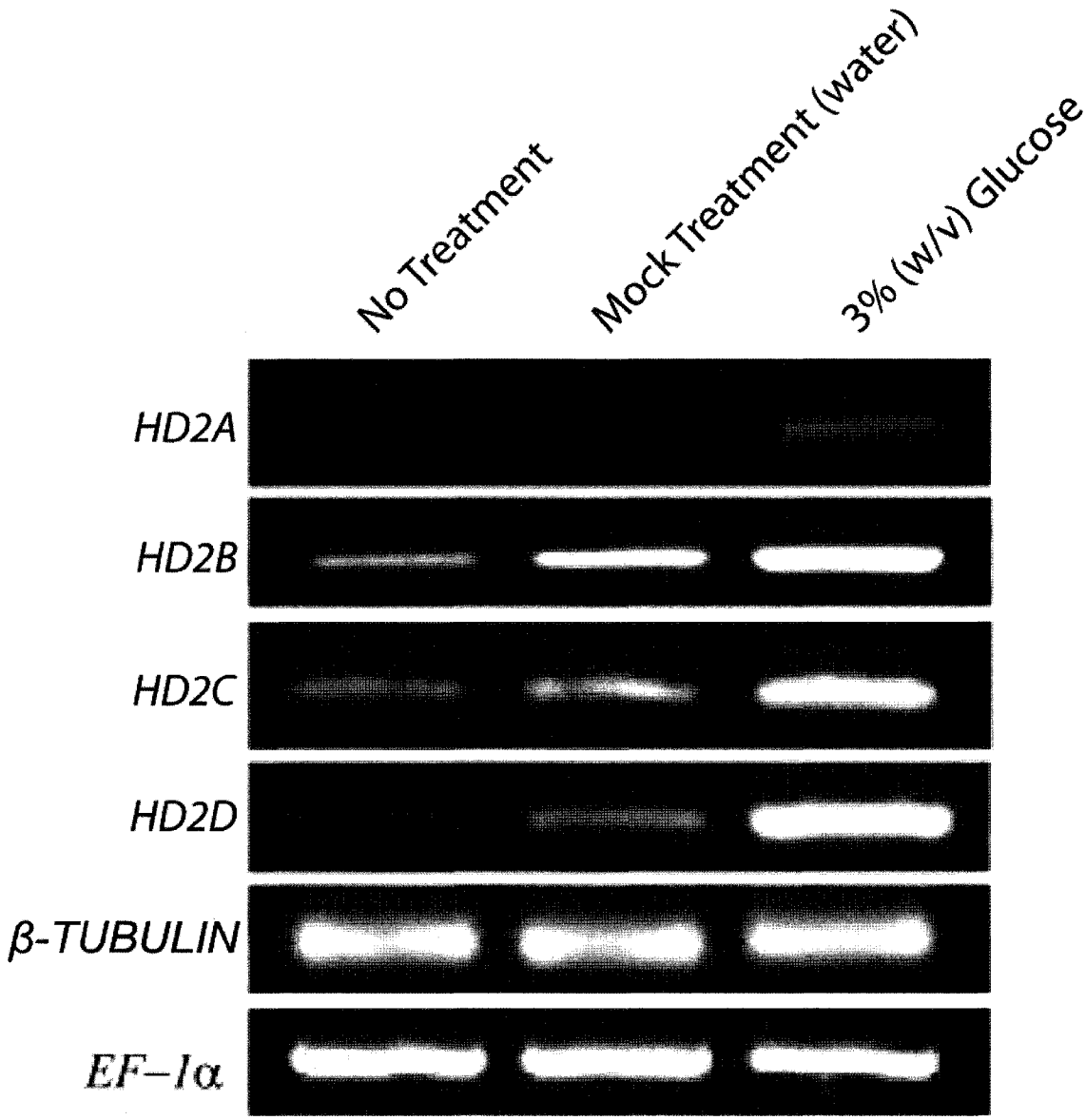
Similar to previous findings, *HD2A* and *HD2D* show the largest changes in expression compared to *HD2B* and *HD2C,* with a greater than 16 and 11 fold induction of expression at the 8 hour time point respectively. In the absence of light, glucose levels begin to decrease. At this time, *HD2* expression decreases simultaneously. The half-life of the mRNA transcript *HD2A, HD2B, HD2C and HD2D* is estimated to be less than 4 hours as the transcript abundance diminishes rapidly within 4 hours of dark.

2.4.6 Validation of glucose induction of *HD2* genes

An analysis was carried out to validate the microarray data previously discussed. This was done in Columbia-0 and not in the characterized *HD2* mutants. Since previous findings indicate that *HD2* is expressed in all cells containing glucose tissue, a method was devised to starve tissues of sugar before glucose reintroduction. The method used in the microarray study Li et al. (2006) was not suitable because it was a liquid culture setup. Plants were germinated in the absence of glucose and presence of light for one day before being covered. Plants were then grown on sugar-free media in the absence of light for 4 additional days. After 4 days, a 3% glucose solution was added to the plate and total RNA was harvested after 4 hours. The control genes *β-TUBULIN* and *ELONGATION FACTOR-1α* were amplified from cDNA from all samples to ensure the quality of RNA harvested and the efficiency of the PCR reaction. After 4 hours of exposure to 3% w/v glucose solution in the absence of light, *HD2A*, *HD2B*, *HD2C* and *HD2D* expression was highly induced indicating the validity of

the microarray data and linking *HD2* expression to intracellular glucose levels (Figure 16).

Figure 16. RT-PCR analysis of induction of *HD2* expression by 3% (w/v) glucose solution (4 hour exposure). Plants were germinated in the light on ½ MS, 0.8% phytagar for 24 hours after 48 hours of stratification (4° Celsius). Plants were then removed from the light for four days. After 96 hours, solutions of H_2O and 3% glucose were added to the plate. RNA was extracted after 4 hours of exposure. Lane A, RNA sample which received no treatment, Lane B the sample exposed to H_2O, and Lane C the RNA sample from plants exposed to 3% (w/v) glucose. The highest level of *HD2* induction occurred when the plants were exposed to 3% glucose. A consistent induction of expression was noted for *HD2A, HD2B, HD2C* and *HD2D*. β-*TUBULIN* and *EF-1α* were successfully amplified in all of the samples and served as positive controls.

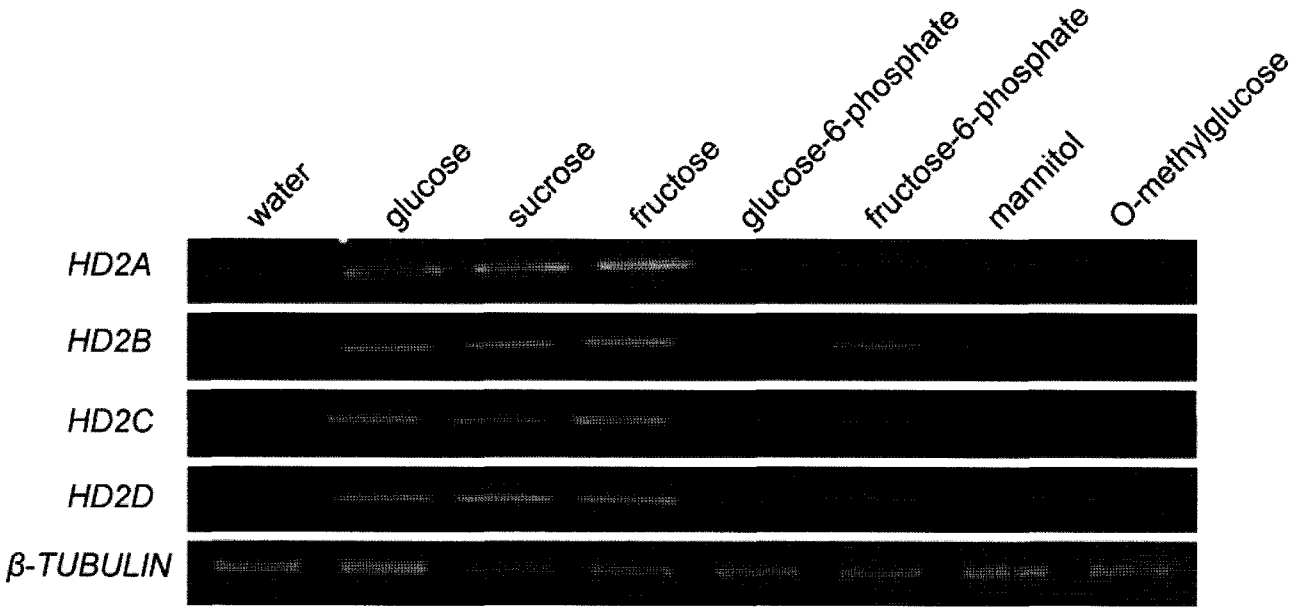


The result of this analysis was significant and led to the investigation of the induction of expression by a variety of soluble sugars.

2.4.7 Modulation of *HD2* expression by soluble sugars

A variety of soluble sugars play critical roles during plant development. Sugar is stored in the form of starch, transported in the form of sucrose which is cleaved to glucose and fructose by cell wall invertase enzymes during normal plant growth and development. The possibility that *HD2* expression may be linked to other functionally significant soluble carbohydrates was analyzed. Mannitol and the non-metabolized sugar O-methylglucose were included as experimental controls. *β-TUBULIN* served as the positive control. *HD2A, HD2B, HD2C,* and *HD2D* were found to have similar profiles in response to sugar exposure (Figure 17).

Figure 17. RT-PCR analysis of soluble sugars (4 hour exposure in the absence of light). Plants were germinated in the light on ½ MS, 0.8% phytagar for 24 hours after 48 hours of stratification (4° Celsius). Plants were then removed from the light for four days. After 96 hours, solutions of H_2O and 3% (w/v) soluble sugar solutions were added to the plate. RNA was extracted after 4 hours of exposure. 2µg of total RNA was converted to cDNA which served as a template for 25 cycles of PCR amplification. The controls for this experiment include H_2O and mannitol. The sugar solutions Columbia-0 plants were exposed to include glucose, sucrose, fructose, glucose-6-phosphate, fructose-6-phophate and O-methylglucose. All solutions were 3% (w/v). The four *HD2* genes (*HD2A, HD2B, HD2C* and *HD2D*) were highly induced by glucose, sucrose and fructose. These genes were moderately induced by glucose-6-phosphate. Mannitol and O-methylglucose did not seem to modulate *HD2* expression. A lower level of induction was seen by fructose-6-phosphate for all four *HD2* genes as compared to glucose, sucrose and fructose. β-*TUBULIN* served as a positive control and was successfully amplified in all of the PCR reactions.



HD2 expression was highly induced after exposure to glucose, sucrose, fructose, and fructose-6-phosphate. Moderate to low levels of induction were noted for H_2O and glucose-6-phosphate. Mannitol and O-methylglucose did not seem to modulate *HD2* expression. Microarray data from Li et al. (2006) (Figure 12) showed a repression of *HD2A* in response to 3% (w/v) mannitol. The amplification of *HD2A* occurred at similar levels to the mock treatment (H_2O) indicating that repression of this gene was not occurring. The results of this analysis strongly indicate that *HD2* expression is a component of the sugar response and may play an integral role in controlling the transcriptome in response to metabolic needs.

2.5 DISCUSSION

2.5.1 Mutant analysis

No stable phenotype was found within single or double loss of function *hd2* alleles. This does not necessarily indicate that these plants would not display a stable phenotype if the right environmental condition or chemical stressor was applied. The first generation of the *hd2a hd2c* loss of function lines displayed an aborted seed which affected 8-50% of the seeds formed. This phenotype lessened in the next generation and was therefore deemed unstable. The difference in the phenotype may be attributable to a difference in the environmental conditions experienced by the populations during growth and development as the two generations were grown in different growth chambers due to constraints on growth cabinets during the study period. Since single loss-of-function or suppression-of-function *hd2* alleles did not display a phenotype, it is likely that the *HD2* family of enzymes is functionally redundant. Therefore, any phenotype viewed might be more evident within combinatorial *hd2* mutants.

HD2 expression is highest in tissues which are mitotically active due to the induction of expression by intracellular sugars (Borisjuk et al., 1998). Therefore, any *HD2* developmental phenotype is likely to originate within mitotically active tissue. Within the plant a phenotype might be expected in the SAM, RAM, floral organogenesis, embryo formation or germination.

It is also likely that any environmental condition or stressor which modulates the intracellular glucose would result in an altered developmental response in hd2 mutant lines. This analysis links *HD2* expression as a

component of the glucose response. The application of glucose and other forms of sugars within the plant have been shown to modulate the timing of developmental events such as flowering (Corbesier et al., 1998) and senescence (Paul and Pellny, 2003). Since glucose levels within the plant are related directly to photosynthetic activity, modulation of the day length, temperature or carbon dioxide levels may result in altered plant development within *hd2* mutant lines, however these conditions have not been tested.

Screens for glucose insensitive mutants have shown that ABA and glucose response are interconnected (Zhou et al., 1998; Arenas-Huertero et al., 2000). Because *HD2* expression is induced by glucose, double loss of function alleles may display an ABA-related phenotype. Gain-of-function *HD2C* lines display an ABA-insensitive phenotype while single *hd2c* loss-of-function alleles are ABA-sensitive. Application of TSA during germination led to the induction of the ABA response in imbibed seedlings (Tai et al., 2005) indicating that ABA-related genes are acetylation sensitive. By modulating the level of glucose within the growth medium, double *hd2* loss-of-function mutant lines may become more sensitive to ABA due to the hyperacetylation of chromatin and resulting from the depletion of the *HD2* genes. A TSA-related phenotype might also be visible in double loss of function lines. By modulating the glucose in the medium, these mutants might display hypersensitivity to TSA which causes the conversion of non-hair cells to hair cells in a concentration dependent manner within a developing route (Xu et al., 2005).

On a molecular level these mutants might display selectivity in removing the acetyl moiety from specific histones, although this has not been confirmed *in vivo*. For example, *HD2A* may selectively target H4 acetylation marks where *HD2C* might target H3. This could be detected and analyzed according to the protocol developed by Suttle et al., (2004) which detected variations in acetylation within the meristematic tissue of potato tubers during dormancy break. Due to the inability to determine a specific phenotype during the mutant analysis, an analysis of microarray datasets was undertaken in order to understand *HD2* expression and function within *A. thaliana.*

2.5.2 Microarray analysis to elucidate *HD2* function

Microarray experimentation provides a powerful tool to monitor the transcriptome when a disturbance is created through gene mutation or when an outside stressor is applied to an organism. Both of these strategies were used to gain insight into the function of *HD2* histone deacetylases in plants. Li et al. (2006) monitored the changes in the transcriptome in the six hours after a 3% (w/v) glucose solution was added to sugar-starved plants. The purpose of this experiment was not to identify genes involved in the glucose response but to model the interconnections between glucose and ABA response networks. Extracting the data from individual treatments (glucose, ABA, mannitol) for use in this analysis identified that *HD2* expression was induced by glucose while ABA and mannitol had minimal effects during the analyzed time points.

2.5.3 Transcriptional activation of *HD2* genes by herbicide application

Application of the herbicides imidazolinone and isoxaben also induces *HD2* expression. The pathways that are disrupted upon application include the molecule glucose. Imidazolinone blocks branch-chain amino acid synthesis (Shaner et al., 1984). The precursor of for the *de novo* synthesis of leucine, isoleucine, and valine is glucose. Isoxaben blocks the incorporation of glucose into the structural sugar cellulose (Scheible et al., 2001). This strengthens the evidence for the glucose linkage since two pathways which use glucose as a precursor, when blocked by application of an inhibitor compound, results in the up-regulation of transcription of the four *HD2* genes. Application of non-metabolized sugars did not modulate the expression of *HD2A, HD2B, HD2C*, or *HD2D.* This study which used a 30mM glucose solution compared to the level of induction by 167 mM glucose solution in the Li et al. (2006) analysis indicates that the expression level of all the *HD2* genes may be directly proportional to the concentration of glucose.

2.5.4 Transcriptional activation of *HD2* genes in *pgm* mutant analysis

The *pgm* and Columbia-0 microarray comparison was the last line of evidence to link *HD2* expression to glucose. Since this mutant experiences extreme fluctuations in glucose due to inability to buffer these changes by starch degradation it was hypothesized that the *HD2* genes would mimic the fluctuations in glucose. The data from this experiment strongly supports this hypothesis. Two

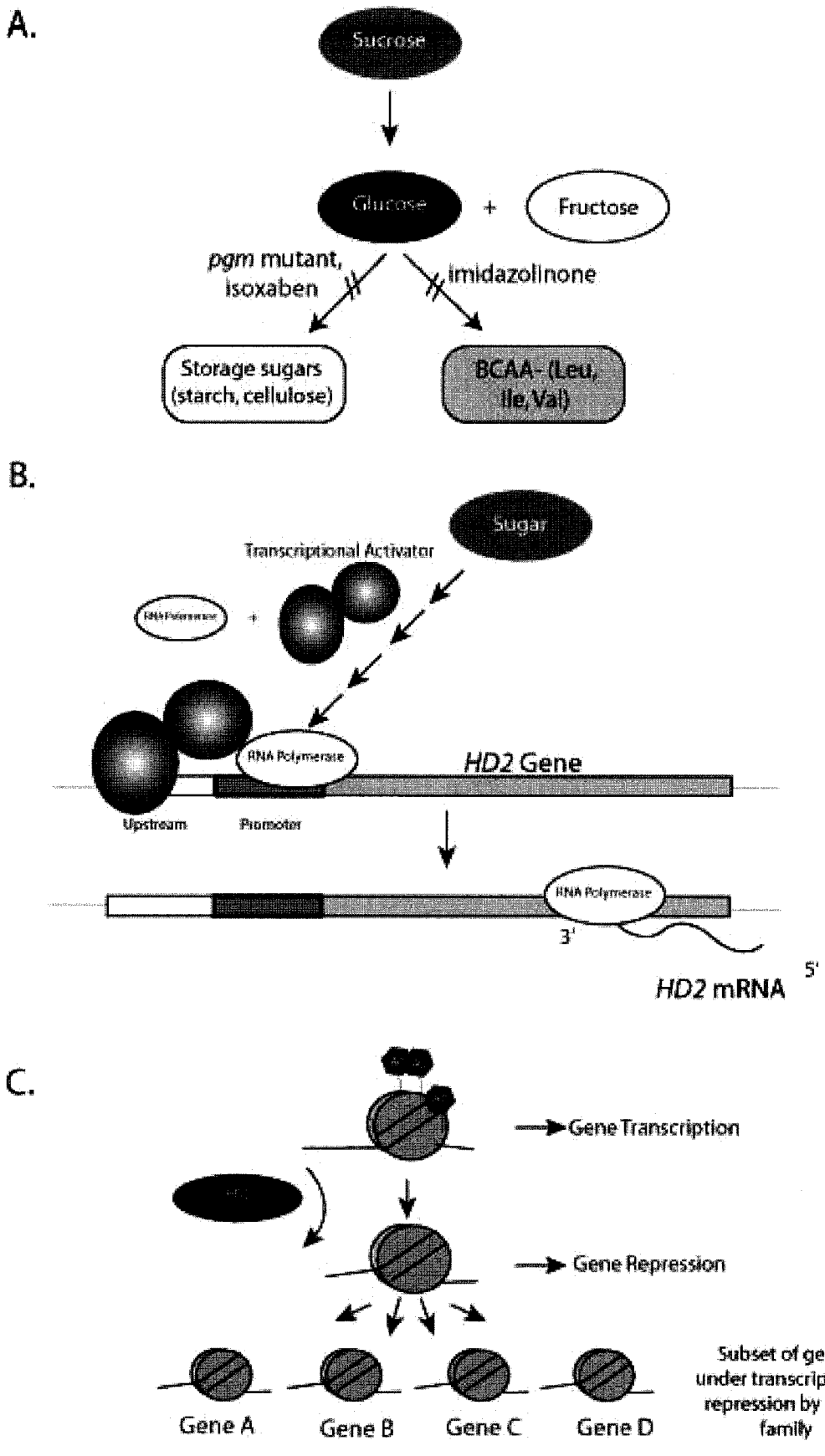
different microarray approaches (chemical stressor and mutant analysis) indicates that this is phenomenon is observable in a variety of datasets which modulate the glucose metabolic pathways. This phenomenon was validated by RT-PCR for all four *HD2* genes. In this experiment it was also noted that application of the mock treatment (water) induced *HD2* expression but to a lesser degree than the glucose. This may be related to the hexose to sucrose ratio. Drought stress has been shown to reduce the hexose sugar pool and increase the amount of sucrose within maize (Andersen et al., 2002). Plants within this experiment might have been under drought stress during growth. The addition of water would therefore increase hexose sugar concentrations through the cleavage of sucrose. Since sucrose cleavage results in glucose and fructose, this could explain why water also induces *HD2* expression.

2.5.5 Development of transcriptional activation model

With the validation of the microarray data by RNA accumulation analysis, the focus of this study shifted to use the available datasets and electronic resources to develop a transcriptional activation model. The DNA sequences upstream of *HD2A, HD2B, HD2C,* and *HD2D* were analyzed using Promomer (http://bbc.botany.utoronto.ca/) in an attempt to identify a DNA binding domain (consensus sequence) which may regulate expression in response to sugar (sugar responsive element–SRE). Promomer is a database of characterized consensus sequences. It is not an effective tool to identify uncharacterized response elements. Therefore, if a previously identified SRE had been

characterized and was functional in *HD2* transcriptional activation, Promomer could detect the enrichment of this sequence upstream of the *HD2* genes. Since Promomer only analyzes the upstream region of the gene enhancer sequences located elsewhere in the genome would not be detected. It is possible that the binding site might be conserved among the *HD2* genes since they all respond in a similar manner to glucose. No consensus sequence was identified in the in the upstream regions of the *HD2* genes indicating that hypothesized SRE element upstream of the *HD2* genes is uncharacterized or may be located in a different region of the genome. The SRE element is included in the new model of *HD2* transcriptional activation in response to glucose (Figure 18).

Figure 18. New proposed model of *HD2* function. Diagram A indicates the flux of metabolic processes through glucose which were disrupted in micoarray analyses which modulated *HD2* expression. Diagram B proposed a hierarchical transcriptional control of *HD2* genes by glucose. The transcriptional activator complex and the steps which result in an up-regulation of an *HD2* genes remain unknown. Diagram C illustrates the chromatin process *HD2* genes promote and indicates that a subset of genes may be under direct *HD2* control in tissues with high levels of intracellular sugars.



The model addressed the major findings of this thesis. Perception of increased intracellular sugars results in the transcriptional activator complex to bind to the SRE upstream of an *HD2* genes. This results in the transcriptional activation of the *HD2* family. The mRNA would then be translated into a protein where it would function to repress gene targets. The targets of *HD2* repression were not identified.

It is now clear that sugar signaling cascade results in *HD2* expression but the model is far from fully developed. The model does not address the phosphorylation cascade which is likely to be involved in *HD2* expression and activation. Phosphorylation was shown to be required for the activity of the maize *HD2* complex purified from germinating embryos (Lusser et al., 1997). The dependence of activity on phosphorylation has not been shown in any dicot species but six to 12 possible phosphorylation sites are found within *HD2A, HD2B, HD2C* and *HD2D* of *A. thaliana* (Pandey et al., 2002). The model also does not address the subcellular localization to the nucleolus of *HD2* genes (Lusser et al., 1997; Zhou et al., 2004).

2.5.6 Modulation of chromatin-related genes in response to glucose reintroduction

An analysis of all the chromatin related genes was undertaken to attempt to build a model of the mechanisms of transcriptional modulation upon glucose reintroduction. Glucose has been hypothesized as a promoter of mitotic activity

during embryo development (Weber et al., 1996). Therefore, during mitotic activity DNA and histone proteins may be synthesized in order to support the rapid cellular division that is occurring in mitotically active tissue. Within the mined data from the Li et al. (2006) dataset, the mRNA transcript of a subset of histone proteins are rapidly induced within 4 hours of exposure to increased intracellular glucose (Table 6). The remaining mRNA transcripts of histones included on the ATH1 array do not show significant alteration.

Table 6. Histone proteins within *A.thaliana* genome which show greater than two-fold induction at peak level of *HD2* induction.

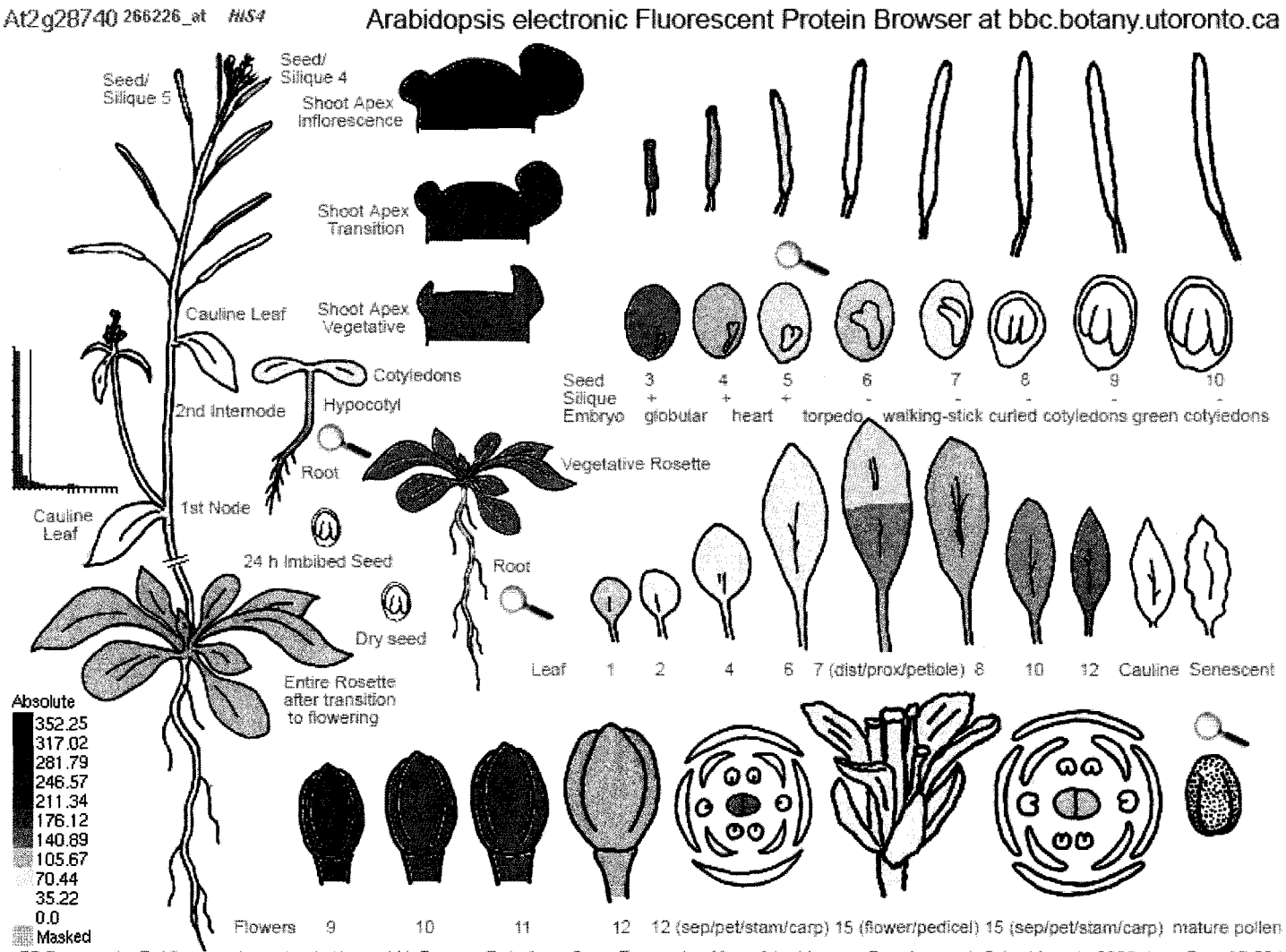
AGI gene code[7]	Histone name	Fold change (4 hour time point)
At1g51060	H2A	3.5
At3g54560	H2A	2.9
At4g27230	H2A	2.2
At1g54690	H2A	5.6
At1g08880	H2A	2.2
At5g59870	H2A	4.2
At1g07790	H2B	4.0
At5g22880	H2B	3.0
At5g65360	H3	3.9
At5g10390	H3	6.4
At1g09200	H3	5.7
At3g27360	H3	4.3
At5g10400	H3	4.1
At2g28740	H4	2.4

[7] AGI Gene codes were designated by the Arabidopsis Information Resource (TAIR) when the *A. thaliana* genome was annotated. An AGI gene code is a unique identifier for each gene in the genome.

A subset of histone proteins were highly induced upon glucose introduction. Consistent with this, NUCLEOSOME ASSEMBLY FACTORS (*NFA1, NFA3, NFA5, NFA6, NFC1*), involved in the deposition of histone octamers in newly synthesized DNA were induced greater than 2 fold after four hours of glucose reintroduction (Zhu et al., 2006).

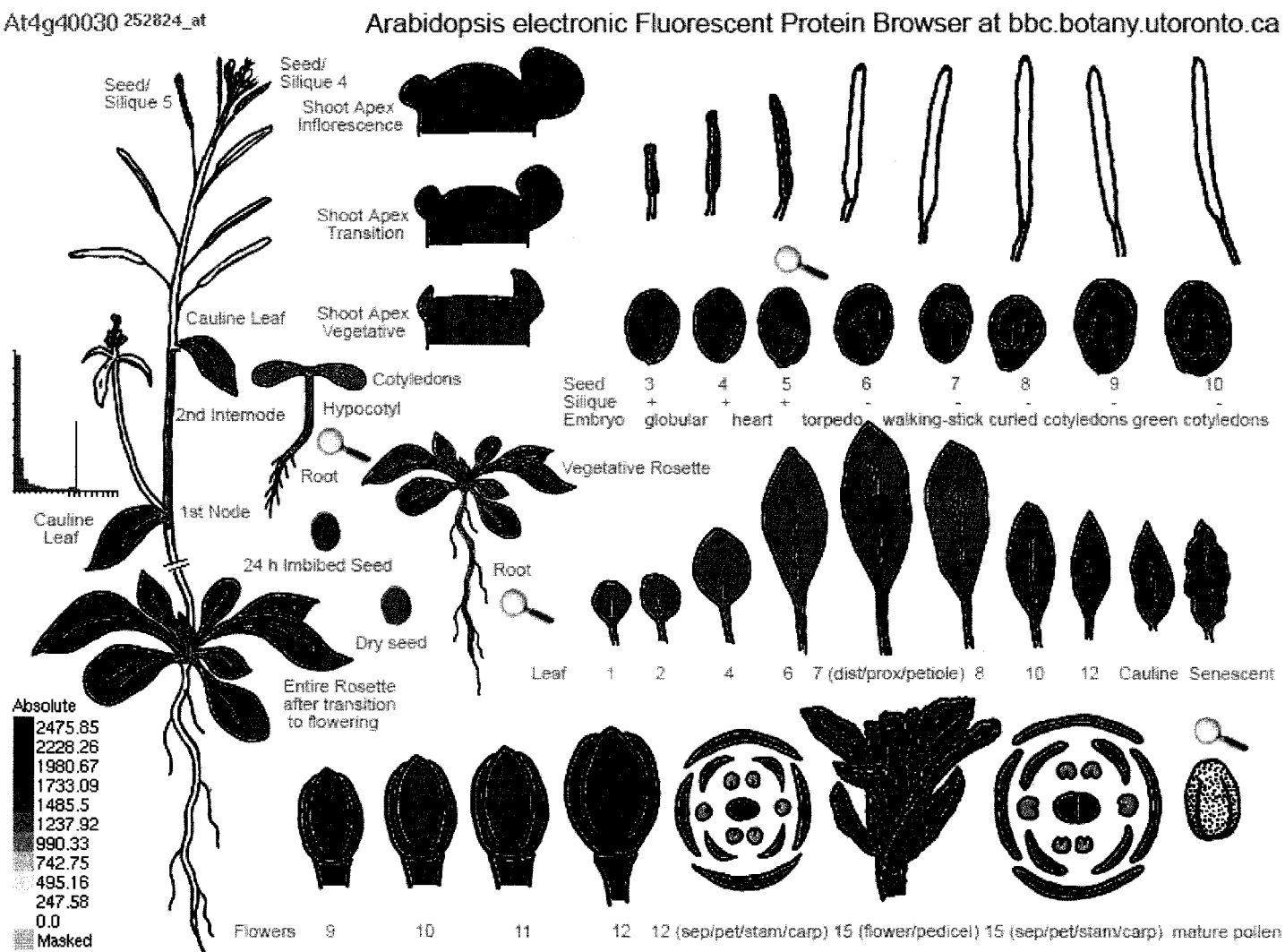
Using the eFP browser (http://bbc.botany.utoronto.ca) histones which showed an induction of expression by glucose, as well as those which remained unchanged were mapped to detect regions of the plant with higher levels of expression of these proteins (Figures 19 and 20).

Figure 19. Expression data for *H4* At2g28740 generated by eFP browser. Figure 19 was generated using AtGenExpress Tissue Data Set (Schmid et al., 2005) electronic Fluorescent Protein browser (http://bbc.botany.utoronto.ca) generated using average expression values. The figure shows expression levels in specific tissue throughout plant development. This gene was selected due to induction of expression in Li et al. (2006). Higher expression is noted in rapidly dividing tissue.



eFP Browser by B. Vinegar, drawn by J. Alls and N. Provart. Data from Gene Expression Map of Arabidopsis Development: Schmid et al., 2005, Nat. Gen. 37:501, and the Nambara lab for the imbibed and dry seed stages. Data are normalized by the GCOS method, TGT value of 100. Most tissues were sampled in triplicate.

Figure 20. Expression data for *H3*, At4g40030 generated by eFP browser. Figure 20 was generated using AtGenExpress Tissue Data Set (Schmid et al., 2005) electronic Fluorescent Protein browser (http://bbc.botany.utoronto.ca) generated using average expression values. The figure shows expression levels in specific tissue throughout plant development. This gene was selected due to an absence of modulation by sugar in Li et al. (2006). Relative even expression is noted throughout the plant.



eFP Browser by B. Vinegar, drawn by J. Alls and N. Provart. Data from Gene Expression Map of Arabidopsis Development: Schmid et al., 2005, Nat. Gen. 37:501, and the Nambara lab for the imbibed and dry seed stages. Data are normalized by the GCOS method, TGT value of 100. Most tissues were sampled in triplicate.

As expected, the mRNA transcripts of histone induced by glucose are more highly expressed in rapidly dividing tissue like the SAM and young floral buds, and mRNA transcripts of histones which show no response to glucose are expressed more evenly expressed throughout the plant. The detection of up-regulation of histone and nucleosome assembly factor genes, at the same time as *HD2* transcriptional activation may indicate that *HD2* expression could be involved in the formation of chromatin structure in rapidly dividing tissue. Chromatin structure is also regulated by other DNA- and histone-modifying enzymes. These were also analyzed.

Newly synthesized DNA is methylated to maintain the methylation state of the parent cell within daughter cells. Of the ten DNA methyltransferases, only *DMT6* (also known as *CMT3*) was induced. *CMT3* is involved in locus specific non-CG methylation (Bartee et al, 2001). There was no induction of *DMT9, DMT10*, both previously implicated in *de novo* CG and non-CG methylation (Cao and Jacobsen, 2002). This raises the likelihood that DNA methylation was not altered globally in response to glucose, but occurred at specific loci relating to *CMT3* function. This is inconsistent with the up-regulation of histone gene expression and nucleosome assembly factors within 6 hours of glucose reintroduction. From this dataset, it appears that non-CG DNA methylation at *CMT3* related gene-function occurs before global CG methylation. It is possible that the reformation of global DNA methylation patterns may occur at a later time point then was analyzed in this dataset.

Within the literature, DNA methylation has been shown to be linked to histone methylation. Histone methylation marks can relate to gene activation and repression. For example, H3K9 is considered a mark of repressive chromatin structure, while methylated H3K4 is considered to be a mark of active or open chromatin (Fuchs et al., 2006). *A. thaliana* contains 32 genes with complete histone methyltransferases (HMTs) domains. Of these 32, only SDG40, is induced greater than two-fold after four hours of glucose exposure. Histone demethylases *HDMA1, HDMA2, HDMA3* and *HDMA4* were not induced. Therefore, the modification of the transcriptome in response to glucose may not be dependent on large changes in methylation of histone octamers. This may also occur at a later time point then was analyzed within this dataset.

Transcriptional modulation has also been shown to correlate with the acetylation state of chromatin. The induction of HDACs would deactivate chromatin and would act antagonistically to HATs. None of the HATs were induced during the six hours of exposure. Only the *HD2* class of HDACs was highly induced, indicating that chromatin could be deacetylated in an *HD2*-dependent active response to glucose reintroduction. It is speculative to attempt to order the temporal response of chromatin-related genes upon glucose reintroduction because differences may exist depending on the tissue analyzed. Since this analysis used whole plants grown in liquid culture, the results may differ from a dataset generated from apical meristems. If meristematic tissue was the sole RNA source, the sequence of expression may be different. A dataset from meristematic tissue was not available at this time.

2.5.7 Proposed *HD2* function based upon glucose induction

The target proteins which translated *HD2* genes would deacetylate have not been identified. Although studies have shown that *HD2* proteins are capable of the removal of acetyl groups from hyperacetylated histones (Kolle et al., 1999) HATs and HDACs have been shown to add and remove acetyl groups to non-histone proteins in vivo (Loidl, 2004). Therefore it is possible that the targets of *HD2* function could be non-histone proteins within the nucleus of the cell. Previous *HD2A* gain-of-function lines have shown the repression of gene transcripts (Wu et al., 2000b; Zhou et al., 2004). These transcripts were not analyzed due to the lack of phenotype seen in *hd2a hd2c* loss-of-function lines. The possible gene targets of *HD2* will be discussed. *HD2* expression may also be linked to a cellular memory mechanisms relating to the acetylation state of chromatin within the nucleus. Finally, the possibility of non-histone *HD2* targets will be discussed.

Since *HD2* genes are rapidly expressed in response to glucose, it is logical that these genes may modulate the expression of glucose-related genes. Glucose-related genes are modulated in response to glucose introduction but it is not clear if *HD2* expression is directly involved in this modulation. Analysis of the carbohydrate contents of *hd2* loss-of-function mutants might yield differences in glucose metabolism. This proposed analysis might then yield specific gene targets within a metabolic pathway.

Previous work has introduced the possibility that *HD2* gene expression may modulate rRNA expression (Lusser et al., 1997) and genes related to the

ABA response (Zhou et al., 2004; Sridha et al., 2006). An analysis of the rRNA genes included within the Li et al. (2006) dataset indicates that *HD2* expression is not likely to modulate the rRNA genes. A large proportion of the rRNA genes showed a greater than two-fold up-regulation of expression in response to glucose. This is not consistent with previous findings which show *HD2* genes are able to repress the transcription of reporter genes *in vivo*. Some research into nucleolar dominance has indicated that *HD2* genes may function to repress rRNA expression (Lawerence et al., 2004) in *A. suecica*, the allotetraploid plant formed when *A. aeronosa* and *A. thaliana* are crossed. Normally the rRNA genes from *A. aeronosa* are expressed while the rRNA genes from *A. thaliana* are silenced. *hd2a* suppression-of-function lines expressed the normally silenced *A. thaliana* rRNA genes in the tetraploid plant. Nucleolar dominance was not eliminated as only 'trace amounts' of rRNA was detected. It is not clear if this would play a role in the diploid plant.

HD2 genes have also been previously implicated in modulating gene expression in the ABA response (Zhou et al., 2004; Sridha et al., 2006). The ABA treatment carried out in the Li et al, (2006), dataset was used to detect the direct modulation of *HD2* genes in response to ABA application. Extracting the data from individual treatments (glucose, ABA, mannitol) for use in this analysis identified that *HD2* expression was induced by glucose while ABA had no effect during the analyzed time points (data not shown). This finding contradicts a recent paper, Sridha et al. 2006 which indicates that *HD2C* is a modulator of the ABA pathway. Analyzed within Sridha et al. (2006) *hd2c* loss-of-function mutants

displayed no phenotype when grown in the presence of ABA. However, *HD2C* overexpression lines were ABA insensitive compared to Columbia-0 plants. Another recent paper which analyzed the trancriptome profile in response to TSA treatment found that a large number of genes with the highest level of induction were annotated as ABA-responsive (Tai et al., 2005). In the presence of TSA, deacetylation of chromatin is blocked resulting in hyperacetylation of the genome and an alteration in the transcriptome. This paper indicates that the ABA response is strongly modulated by the acetylation state of chromatin. It is likely that the *HD2C* overexpression lines tested resulted in a hypoacetylated chromatin resulting in a reduction in the ABA response and the observed phenotype of ABA-insensitivity. Since ABA is not a direct regulator of *HD2* expression it is unclear whether this modulation would be relevant in a wild type plant.

HD2 expression may also function to create chromatin structure that acts as a cellular memory of gene expression. During cellular division the histone octamers are divided equally between the two daughter cells. Newly synthesized histones which have been acetylated in the cytosol and nucleus by Type B HATs are then imported into the nucleus and deposited into the new chromatin structure (Reyes et al., 2002). Type B HATs have been shown to specifically acetylate lysine 5 and 12 of H4 before incorporation into the nucleosome in maize (Lusser et al., 1999). The acetylation of other newly synthesized histone proteins (H3, H2A, H2B) within a histone octamer are not well characterized. The acetylated new histone proteins could then be deacetylated to correspond to the

region they have been incorporated in. For example, a new histone octamer may be deposited into an inactive region of the genome and would be expected to be more heavily deacetylated than an octamer deposited into an actively transcribed region of the genome. Specific targeting proteins that target specific histone-modifying enzymes to a locus resulting in the replication of chromatin structure to match the parent cell. This could represent a cellular memory mechanism which would allow for the developmental program of the parent to be passed to daughter cells. This might also present a mechanism that would prevent expression of key developmental transcription factors which could trigger a developmental cascade towards a differentiated cell fate.

Since actively dividing regions may contain more heavily deacetylated chromatin, this may repress a developmental set of genes contributing to undifferentiated pool of cells found in the RAM and SAM. This could be crucial to the ability of plants to maintain and coordinate the expression of genes in newly formed organs and tissue. Since none of the other HDACs are shown to be enriched in rapidly dividing tissue and there is no increase in HAT mRNA abundance within this tissue, *HD2* proteins may function to replicate the cellular program of the parental cell through regulation of the acetylation state, or they may repress the developmental genes that trigger differentiation processes.

Studies employing antisense suppression-of-function and TSA have shown results which support the possibility that deacetylation may function as a memory of the developmental program. Tian and Chen (2001) analyzed antisense-*HD1* (*HDA19*) lines. These lines showed numerous developmental

abnormalities including early senescence, ectopic expression of silenced genes, suppression of apical dominance, homeotic transformations, and sterility. Therefore, in many cases the developmental program which would have resulted in normal development was altered when the normal process of deacetylation was disrupted. The application of TSA to the developing root results in the conversion of non-hair cells to hair cells in a concentration-dependent manner by the misexpression of key patterning genes *CARPICE*, *GLABRA2* and *WEREWOLF* (Xu et al., 2005). Therefore, the disruption of the processes of acetylation/deacetylation results in the alteration of the developmental program and a shift in cell fate. Since *HD2* expression is correlated with young tissue, it may function to repress the expression of genes which might trigger a developmental cascade to cellular differentiation.

The possibility that *HD2* genes may function within the nucleolus and through the deacetylation of non-histone proteins is also a possibility. The nucleolus was historically defined as the site of rRNA synthesis. The role of the nucleolus has rapidly expanded beyond this limited scope with the recent molecular analysis of cancer cell-tissue culture models and the recent identification of the human (Scherl et al., 2002, Lam et al., 2005) and plant nucleolar proteome (Pendle et al., 2005). These studies identified over 700 proteins in humans and 213 proteins in plants that are located within the nucleolus. Within mammalian cancer cells, the number of nucleoli is increased (Derenzini et al., 2000). This indicated that the nucleolus might be involved in cellular proliferation. In addition to this, HDAC inhibitors are being heavily

researched as a possible treatment for many types of cancer (Marchion and Munster, 2007). Within *A. thaliana*, fewer proteins were identified but the presence of the exon junction complex which is involved in exon splicing of pre-RNA was detected attributing a novel function to the nucleolus within plants. The analysis also found 23 plant-specific proteins with unknown functions extending the possibility of the addition of more novel functions for the plant nucleolus Since *HD2* studies have repeatedly shown *HD2A-C* localization to the nucleolus, it is possible that *HD2* may deacetylate these proteins and may be involved in a novel but undefined nucleolar function.

A recent paper reconciles the nucleolar localization of the targeting of *HD2* genes with a novel nucleolar function. Analysis of *ASSYMETRIC1* (*as1*) and *ASSYMETRIC2 (as2)* showed that pleiotropic abnormalities were worsened in the presence of the *pkl* mutation. Within mammalian tissue culture (human) and *X. laevis* related proteins have been shown to exist in repressor complexes with HDAC activity. This led researchers to investigate whether acetylation would modulate the as1 as2 phenotype. Application of TSA led to the development of filamentous leaves. Screening of HDAC suppression of function mutants in the as1 as2 background identified that *HD2A* and *HD2B* are involved in the process of leaf development. Application of TSA or suppression of *HD2A* or *HD2B* resulted in the deregulation of the microRNAs (miRNA) thought to control leaf formation. To support this, *HD2* was again confirmed to localize to the nucleolus while *AS1* and *AS2* localized to bodies adjacent to the nucleolus. This is an intriguing finding that *HD2* expression may modulate miRNA expression within

the nucleolus. The finding that *HD2* may be involved in miRNA regulation of patterning genes would reconcile the nucleolar subcellular localization with previous findings of transcriptional regulation of genes outside the nucleolus. This opens the possibility that *HD2* genes may be regulating miRNA expression of other genes as well and leads to further speculation that *HD2* may be functioning in pathways other than gene repression through deacetylation of chromatin.

2.6 FUTURE WORK

From this work a clear link between *HD2* expression and glucose has been established. This work must continue as the genes may be essential to understanding how the plant kingdom recognizes and responds to soluble carbohydrates. Currently, the work is limited by the number of available loss-of-function mutants. As new mutants become available from seed collections, the new lines should be screened in order to obtain loss-of-function mutations in all four of the *HD2* genes if possible. This would greatly increase the power of phenotypic investigation as combinatorial loss-of-function mutants could be created. Mutant TILLING might also be considered (http://tilling.fhcrc.org:9366/) if loss-of-function lines cannot be obtained from public seed collections.

From this investigation, *HD2A* and *HD2D* show the largest change in expression when glucose is introduced, and a double loss-of-function mutant in these genes should be pursued. These mutants, if characterized might also be used in the future to characterize protein interacting partners as *HD2* proteins may function within protein complexes. Since no single loss-of-function mutant or suppression-of-function mutant displayed a phenotype it is likely that multiple *HD2* genes may need to be disrupted to obtain a visible phenotype. Any visible phenotype would likely be displayed in tissue which expresses these genes at a high level (siliques- embryogenesis, SAM, RAM). Research might also be completed to measure the intracellular forms of sugar in the current and all future *HD2* loss-of-function mutant lines. Methods to measure glucose, sucrose and fructose (Geigenberger et al., 1996), as well as starch (Hendriks et al., 2003)

could be used to detect an alteration in sugar metabolism in these mutant lines. This may help to distinguish specific gene targets modulated by *HD2* genes if a molecular phenotype is detected.

Clear phenotypic modulation of an acetylation phenotype would link the expression of the *HD2* genes with the development of a plant. TSA is a potent deacetylase inhibitor and could be used in a glucose screen to monitor the growth of the root when exposed to differing glucose concentrations. Previous studies have shown a dramatic alteration of root development in the presence of TSA (Tian and Chen, 2001). Root development may be more resistant to TSA if a high level of *HD2* expression is present in the root tissue. As the microarray data showed, the remaining histone deacetylases in *A. thaliana* would theoretically remain unaltered by a modulation of the glucose level.

A method has been developed and described in Law and Suttle (2004), which is able to detect global differences in histone acetylation levels. The paper describes a method to isolate chromatin and detect and measured the acetylation levels of all H3, H4, H2A and H2B proteins. This might be used to detect the rapid and possibly reversible deacetylation of histone proteins in response to a change in intracellular sugar concentrations.

APPENDIX

INVESTIGATION OF TRANSCRIPTIONAL PROFILE DURING EXPRESSION OF THE AP2 TRANSCRIPTION FACTOR BBM CLONED FROM *G. MAX*

3.1 ABSTRACT

Previous studies characterized the *B. napus* AP2-type transcription factor BABYBOOM (BBM) as a promoter of cell proliferation and somatic embryogenesis in the absence of exogenous plant hormones. Recent work has characterized the *G. max* orthologue (*GmBBM)* which has shown to initiate somatic embryogenesis resulting in a phenocopy of *BnBBM* when expressed in *A. thaliana*. A microarray analysis was undertaken to compare the expression profiles of *BnBBM* and *GmBBM* during induction and formation of somatic embryos. Cotyledon tissue was harvested after 4 days growth (induction phase) and at 10 days when somatic embryos were present. The expression of seed specific markers: *2S ALBUMIN, 12S CRUCIFERIN*, lipid transfer proteins and *OLEOSINS* was highest in lines showing the strongest penetrance of the somatic embryo phenotype (*BnBBM-1*). *HD2* expression was not found to be induced in a consistent manner within analyzed lines after four or 10 days of growth. The modulation of specific transcriptional regulators that leads to the induction of somatic embryogenesis were not detected. However, induction of seed-specific marker genes: protease inhibitors/lipid transfer proteins, *2S ALBUMIN* storage proteins, and *LATE EMBYROGENESIS ABUNDANCE PROTEIN 76* were found to be differentially expressed in *BnBBM* and *GmBBM* gain-of-function lines

compared to wild type. The differentially expressed genes are consistent with the process of zygotic seed/embryo formation despite the somatic tissue source.

3.2 INTRODUCTION

Biotechnology and plant breeding initiatives require rapid methods to regenerate plant material. Somatic embryogenesis is a process which results in the formation of numerous embryos from somatic tissue. This process can result in the rapid amplification of a parent plant into numerous genetically identical progeny and for this reason is a useful technique to the biotechnology industry. Somatic embryogenesis can be promoted by the application of plant hormones (eg. auxins and cytokinins) or by genetic manipulation. Gain-of-function studies have identified 6 loci: *CYTOKININ INDUCTION1 CKI1* (Kakimoto, 1996), *LEAFY COTYLEDON1 (LEC1)*(Lotan et al., 1998), *LEAFY COTYLEDON2 (LEC2)* (Stone et al., 2001), *ENHANCER OF SHOOT REGENERATION1 (ESR1)*(Banno et al., 2001), *SOMATIC EMBRYOGENESIS RECEPTOR KINASE1 (SERK1)*(Hecht et al., 2001),*WUSCHEL (WUS)*(Zuo et al., 2002), and *BABYBOOM (BBM)*(Boutilier et al., 2002) which promote cell proliferation and the induction of somatic embryogenesis without the application of exogenous hormones.

Soybean is an economically important crop. Molecular work in the model organism *A. thaliana* is leading to the identification and characterization of cellular mechanisms in *G. max.* Recent work identified the first homologues of *APETALA2* (*AP2*) transcription factors in soybean *Gm-DEHYDRATION REPONSIVE ELEMENT BINDING protein a* (*GmDREBa*), *GmDREBb*, and *GmDREBc* (Li et al., 2005). The AP2/ ETHYLENE RESPONSIVE ELEMENT BINDING PROTEIN (EREBP) multigene family are comprised of transcription

factors containing the 60-70 amino acid in length conserved DNA binding domain (Jofuku et al., 1994). The family is subdivided into the AP2 or EREBP subfamilies due to the presence of one (AP2) or two (EREBP) AP2 domains. AP2-type transcription factors are key regulators of plant cell growth and reproduction (Riechmann and Meyerowitz, 1998).

BBM is an AP2-type transcription factor originally isolated from embryonic microspore *B. napus* cell cultures (Boutilier et al., 2002). Gain-of-function analysis in *A. thaliana,* indicated that BnBBM was a promoter of cellular proliferation and morphogenic processes during embryogenesis. Recent work has led to the identification of the *G. max* homologue of *BnBBM* as gain-of-function studies carried out in *A. thaliana* phenocopied *BnBBM* transgenic lines. Recent work has shown similarities in zygotic and somatic embryogenesis with the induction of expression of the plant-specific class of histone deacetylases *HD2* within the developing embryo (Zhou et al., 2004; Lagace et al., 2003). A microarray analysis was undertaken to compare the expression profile of *BnBBM* and *GmBBM* transgenic lines. The goal of this analysis is to confirm the expression of seed specific genes, model the expression of the *HD2* genes during the induction and formation of somatic embryos and compare the similarity in expression profiles of *BnBBM* and *GmBBM* gain-of-function lines.

3.3 METHODS

3.3.1 *BBM* microarray:RNA extraction and bioanalyzer analysis

4-day-old and 10-day-old cotyledons were harvested from three *BnBBM*, and *GmBBM* transgenic lines, and a single Columbia-0 control. Transgenic lines had previously been generated by floral-dip transformation with *Agrobacterium tumefaciens* carrying the GmBBM and BnBBM genes, cloned into the pBINPLUS vector (Boutilier et al., 2002). Transgenic lines were grown in continuous light on ½ MS MEDIA containing 50 mg/L Kanamycin. No visible somatic embryos were visible in the 4-day-old tissue but were present in 10-day-old transgenic tissue. RNA extraction was performed using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocols. After extraction, RNA quality and quantity was assessed using 2100 Bioanalyzer (Agilent, California, USA).

3.3.2 Microarray processing and data analysis

cRNA synthesis, hybridization and scanning were performed at Botany Affymetrix GeneChip Facility, University of Toronto (ON, Canada). The datasets for *BnBBM-1* and *GmBBM-1*, and the Columbia-0 control were normalized by Robust Multi-array Analysis (Irizarry et al., 2003) using R (http://www.r-project.org)- Bioconductor (http://www.bioconductor.org)(Gentleman et al., 2004) software with the affylmGUI package (Wettenhall et al., 2006). affylmGUI was used to generate tables of differentially expressed genes by contrasting the gain-of-function line to the mRNA harvested from 4-day-old and 10-day old Columbia-0 cotyledons.

3.4 RESULTS AND DISCUSSION

BBM is an *AP2* transcription factor isolated from *B. napus (BnBBM),* with identified orthologues in *G. max* and *A. thaliana.* Gain-of-function studies have shown that *BnBBM* is able to promote the occurrence of somatic embryogenesis and the formation of cotyledon-like organs on developing seedlings (Boutilier et al., 2002). Previous work in this lab has characterized the *G. max* orthologue (*GmBBM*) of the originally discovered *B. napus* transcription factor. Similar to *BnBBM,* over-expression lines are able to induce somatic embryogenesis (data not shown).

Although somatic embryogenesis is used as a model for zygotic embryogenesis (Zimmerman et al., 1993), there is little molecular data to confirm the transcriptional regulatory network is similar. *BnBBM* over-expression lines showed the expression of the *2S-ALBUMIN* seed specific marker (Boutilier et al., 2002). This marks the expression of seed storage proteins which occurs in the late stages of embryogenesis and seed formation. A microarray analysis was carried out to compare the expression profiles of the *BnBBM*, and *GmBBM* profiles during the induction of somatic embryos and after the embryos are present. Three lines were analyzed for each orthologue. A range of phenotype penetrance occurred in all lines, with the *BnBBM* (1-3) occurring at rates of 80%, 25% and 14% respectively, and the *GmBBM (1-3)* lines at rates of 28%, 10% and 7%. This may impact the resolving power within the microarray data as it is assumed that the penetrance of phenotype is a direct reflection of the modulation of the transcriptome. Therefore, a high penetrance may have larger alterations to

the transcriptome, specifically within the expression cascade leading to somatic embryo formation. This trend was noted in the data. The *BnBBM-1* line which had the highest penetrance of phenotype (80%) showed the highest level of expression of seed storage proteins and storage lipids. Of the twelve highest differentially expressed genes, three were annotated as 12S seed storage proteins (12S cruciferins: *CRA1*, and *CRB*), three as 2S albumin storage proteins, two oleosin genes, and one lipid transfer protein involved in lipid storage. The detected level of expression was lower in all other transgenic lines analyzed. Induction of seed specific markers was noted in the 4 and 10-day old tissue from *BnBBM-1* and *GmBBM-1* (Table 7).

Table 7. Differentially expressed genes linked to seed formation/embryogenesis expressed in *BnBBM-1 and GmBBM-1* at 4 and 10-day old plants.

Day	AGI[8]	Gene Function	*BnBBM-1* Fold Change	*GmBBM-1* Fold Change
4	At5g64080	seed storage/lipid transfer protein (LTP)	2.7	2.1
	At5g59320	seed storage/lipid transfer protein (LTP)	20.3	2.0
	At1g62500	seed storage/lipid transfer protein (LTP)	3.5	2.1
	At4g27140	2S albumin seed storage protein	58.9	2.7
10	At5g55450	seed storage/lipid transfer protein (LTP)	2.2	3.2
	At3g53980	seed storage/lipid transfer protein (LTP)	22.2	15.9
	At4g33550	seed storage/lipid transfer protein (LTP)	9.5	4.1
	At4g12470	seed storage/lipid transfer protein (LTP)	2.7	3.6
	At3g18280	seed storage/lipid transfer protein (LTP)	3.1	3.3
	At1g62790	seed storage/lipid transfer protein (LTP)	3.1	4.3
	At1g12090	seed storage/lipid transfer protein (LTP)	2.0	2.3
	At4g27140	2S albumin seed storage protein	70.6	4.0
	At3g15670	late embryogenesis abundant (LEA 76)	8.0	4.0

[8] AGI Gene codes were designated by the Arabidopsis Information Resource (TAIR) when the *A. thaliana* genome was annotated. An AGI gene code is a unique identifier for each gene in the genome.

These lines showed a greater than two-fold induction of expression of *2S ALBUMIN SEED STORAGE PROTEIN* (At4g27140) and three lipid transfer proteins at 4-days. A larger number of seed-related genes were induced at 10 days growth when somatic embryos were visible. At this time, the 2S ALBUMIN GENE remained up-regulated compared to expression in wild-type plants. A total of seven seed-specific protease inhibitors/lipid transfer proteins and *LATE EMBRYOGENESIS ABUNDANCE PROTEIN 76* (LEA76) were found to be induced at 10-days growth. This confirms the expression of seed specific markers in each of the *BnBBM* and *GmBBM* overexpression lines consistent with the observed phenotypic data.

This dataset was also used to analyze the similarities between zygotic and somatic embryogenic processes. To address this, the expression data was compared to a subset of the genes which were included on a custom microarray published in Girke et al. 2000. The custom array included ~2600 genes which were selected from a 10 000 cDNA clone library, harvested from developing *A. thaliana* seeds (5 and 13 days after fertilization (White et al., 2000). Genes which showed expression levels 10 times higher in seeds versus vegetative tissue were selected as 'highly preferentially expressed' and were used for comparison in this analysis. Of the 97 genes, two show a greater than two-fold induction in the 4-day old *BnBBM-1 and GmBBM-1* tissue; At1g10760 andAt1g62500, and three genes within 10-day old tissue; At4g36700, At1g74210 and At1g48130. Excluding the *GmBBM-1,* 27 of the 97 genes showed a greater than two fold induction at either of the sampled time points. This analysis identified genes

which were common to both processes. The genes identified are all involved in the metabolic processes required during seed formation. The transcriptional regulators which drive the coordinated expression of genes during embryo formation remain uncharacterized. This is most likely due to small changes in expression levels which are difficult to distinguish using microarray analysis.

Previous research showed the induction of *HD2* expression during somatic embryo formation in *BnBBM* transgenic *A. thaliana* overexpression lines (Zhou et al., 2004) and proposed that *HD2* expression may be required during embryo formation. Within this dataset, no trend emerged regarding the induction or repression of any of the *HD2* genes in either of the sampled populations. The microarray experiment was undertaken before *HD2* expression was clearly linked to intracellular sugars. The induction of *HD2* expression is therefore not directly linked to the expression of *BnBBM* gene but is connected to the process it promotes. The *BnBBM* transcription factor promotes rapid cell proliferation of cells and results in the formation of somatic embryos. The rapid proliferation of cells results in higher intracellular glucose (Borisjuk et al., 1998) which results in the transcriptional up-regulation of the 4 *HD2* genes within that tissue.

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