ROLE OF ADA2b AND GCN5 IN *COR* GENE EXPRESSION DURING COLD ACCLIMATION IN *ARABIDOPSIS*

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ABSTRACT

ROLE OF ADA2b AND GCN5 IN *COR* GENE EXPRESSION DURING COLD ACCLIMATION IN *ARABIDOPSIS*

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Covalent modifications of histones play important roles in the regulation of transcription. Acetylation of lysine residues on the amino-terminal tails of histones is associated with transcriptionally active genes and is catalyzed by histone acetyltransferases (HAT). GCN5 (a HAT) and ADA2 are components of coactivator complexes such as SAGA in yeast. The *Arabidopsis* genome encodes one homologue of GCN5 and two homologues of ADA2 (ADA2a and ADA2b).

Arabidopsis ADA2 and GCN5 physically interact with the transcriptional activator CBF1 *in vitro* which activates the expression of cold-regulated (*COR*) genes during cold acclimation. Cold acclimation is the process by which plants increase freezing tolerance upon exposure to low non-freezing temperatures. CBF1 binds to the cold/dehydration responsive element (CRT/DRE) present in *COR* gene promoters. *ada2b* and *gcn5* mutants show a delay in activation and a reduction in expression of *COR* genes during cold acclimation. Chromatin immunoprecipitation (ChIP) assays show that the acetylation of histone H3 at the *COR* promoters increases upon cold acclimation. Thus we hypothesized that CBF recruits GCN5 and ADA2b to the *COR* gene promoters to acetylate histones and thus help activate *COR* genes. ChIP assays on plants overexpressing CBF1 show increased acetylation of histone H3 even without cold stress

and the acetylation levels increase further upon cold acclimation. Thus, CBF is sufficient to bring about an increase in acetylation of histone H3 at the *COR* gene promoters.

The acetylation levels of histone H3 in *ada2b* and *gcn5* mutants are similar to wild type plants upon cold acclimation and thus ADA2b and GCN5 are not required for histone H3 acetylation at the *COR* gene promoters. The *Arabidopsis* genome encodes 12 potential HATs and the HAC1, HAC5, HAC12 and TAF1 HATs were tested for their ability to regulate *COR* gene expression. None of these HATs were required for the acetylation of histone H3 at the *COR* gene promoters as determined by ChIP assays. Thus we conclude that the acetylation of histone H3 at the *COR* gene promoters is not solely dependent on any of the HATs tested.

This thesis is dedicated to the three most important people in my life Aai, Baba and Amol.

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LIST OF ABBREVIATIONS

ChIP chromatin immunoprecipitation

CRT/DREs cold/dehydration responsive elements

HAT histone acetyltransferase

HDAC histone deacetylase

IP immunoprecipitation

PIC pre-initiation complex

TSA trichostatin A

Chapter I

Histone acetyltransferases in plants

1.1 Introduction

Assembly of the general transcription factors to form a pre-initiation complex (PIC) is a requirement for genes transcribed by RNA Polymerase II [1]. The eukaryotic genome is packed into a tight chromatin structure and hence the ground state of transcription in eukaryotes is restrictive [2]. Various factors are required to overcome this restriction, facilitate formation of the PIC and activate gene transcription. Transcriptional activators can facilitate PIC assembly by recruiting several coactivators that help counteract the nucleosome barrier and activate transcription [3]. Transcriptional coactivator complexes can be divided into two broad categories. The first category includes coactivators that interact with or are a part of the RNA polymerase II transcription machinery. The second type of coactivators are chromatin modifying complexes that modify chromatin to overcome the nucleosomal barrier to transcription [4].

Chromatin is made of DNA wrapped around nucleosomes. Each nucleosome particle consists of a histone octamer comprising two copies each of histones H2A, H2B, H3 and H4 [5, 6]. Adjacent nucleosomes are bound by the linker histone H1 and the binding of the linker histones leads to further compaction of the chromatin into a ~30 nm fiber. Two models have been proposed for the structure of the ~30 nm chromatin structure. The first is the one-start helix or the solenoid model in which consecutive nucleosomes are placed adjacent to each other bound by linker DNA to form a left-

handed single start helix. The second model proposes that nucleosomes are arranged in a zigzag manner such that alternate nucleosomes are stacked on top of each other. The adjacent nucleosomes are connected by linker DNA [7-9]. This compact structure of chromatin needs to be opened in order for processes like DNA replication and transcription to proceed.

Chromatin modifying coactivator complexes can be divided into two categories: ATP-dependent chromatin modifying complexes (like SWI/SNF and ISWI) and complexes that covalently modify histones (such as SAGA and ADA). ATP-dependent chromatin modifying complexes use energy from ATP hydrolysis to destabilize the chromatin structure by displacement or removal of histones [10, 11]. Coactivator complexes that can covalently modify histones can bring about various modifications of histones such as acetylation, methylation, ubiquitination, and phosphorylation. Some modifications of histones act as transcriptional repressors while others serve to activate the process of transcription. The 'histone code' hypothesis proposes that covalent modification of the histone tails direct the recruitment of chromatin modifying complexes that direct other covalent modifications and help the activation of transcription [12, 13]. Recent work has also shown that some of the histone covalent modifications in plants differ considerably from other eukaryotes [14, 15].

Histone acetylation is usually associated with the activation of genes. The enzymes responsible for histone acetylation are known as histone acetyltransferases (HATs) and are often a part of large coactivator complexes. HATs acetylate lysine residues in the amino terminal tails of histones. Acetylation of lysine residues neutralizes the charge on the lysine residue and is thought to weaken the histone-DNA interaction.

Acetylation of lysine residues can also alter internucleosomal histone-histone contacts and affect interactions between histones and regulatory proteins [16]. The acetylation of lysine residues on the N-terminal tails of histone has been correlated with transcriptional activation [17, 18]. Some transcription factors also show an increased affinity towards acetylated histones [19, 20].

HATs can be divided into two types; nuclear HATs or HAT A and cytoplasmic or type B HATs [21]. Type A HATs acetylate nucleosomal histones during transcription whereas Type B HATs function in the acetylation of newly synthesized histones [22, 23]. Type B HATs acetylate lysine 5 and 12 on histone H4 before the histones are assembled into newly replicated chromatin [24, 25]. Nuclear HATs are associated with transcription and this review will specifically focus on nuclear HATs and their function in plants.

In addition to acetylation of histones, nuclear HATs are also known to acetylate other non-histone proteins and modulate their activity. p300/CBP acetylates the transcription factor p53 and acetylated p53 has a higher binding affinity to its target promoters [26, 27]. PCAF can acetylate the transcription factor E2F1 that acts as an activator of gene expression during the S-phase of the cell cycle [28]. In addition to acetylating transcription factors HATs can also acetylate members of the general transcription machinery TFIIEβ, TFIIF [29]. Due to this function of HATs it has been suggested that HATs can play a role in signal transduction [30]. HATs have been extensively studied in eukaryotes like yeast, flies, mice and humans [16, 17, 31-33]. Nuclear HATs can be further divided into four families, GNAT-MYST, p300/CBP, TAFII250 and the nuclear receptor coactivator family. The nuclear receptor family is not present in plants [34]. The HAT families in plants are further discussed in section 2.

Acetylation of histones is reversible and the enzymes that can deacetylate histones are known as histone deacetylases (HDAC). *Arabidopsis* has three HDAC families with a total of 18 members [34]. Members belonging to RPD3/HDA1 family are present in all eukaryotes. Members of the Silent Information Regulator 2 (SIR2) family share no structural similarity with other HDACs and use NAD as a cofactor. The HD2 family of HDACs is unique to plants. Histone deacetylases are known to deacetylate histones and repress gene expression. Disruption of the HDAC HD1 in *Arabidopsis* causes a change in expression of 7% of the total genes. The changes in acetylation are found mostly at the promoter sites which causes repression of specific genes [35].

This review will focus on the type A HATs in plants and their function during the various cellular, biochemical and physiological aspects of plant growth and development. Plant HATs have some features that are distinctively different from the animal HATs and this may contribute to some plant specific functions of these HATs. In this review we will also bring out these differences and predict how these differences might contribute to plants specific functions in addition to transcription activation.

1.2 Histone acetylation in plants

Plants are subject to a variety of environmental and biological insults due to their sessile nature. Changes in histone acetylation of target genes in response to abiotic and biotic stress, biosynthesis, plant growth and development occur in plants [36, 37]. The HATs that are responsible for the increase in acetylation at these target genes have not been identified. Investigating the mode of action of HATs in controlling gene expression by histone or factor acetylation in response to various stimuli is the next step.

1.2.1 Histone acetylation and plant pathogens

Hyperacetylation of histones has been observed in maize in response to various biotic and abiotic stresses [38, 39]. For example, histones H3 and H4 are hyperacetylated in response to the host selective HC toxin produced by the fungal pathogen *Cochliobolus carbonum*. This is thought to occur via the inhibition of histone deacetylases in maize. The pathogen has no effect on the acetylation status of histones H2A and H2B. The hyperacetylation of histones H3 and H4 due to the inhibition of histone deacetylase might interfere with the expression of the maize defense genes and thus give an edge to the pathogen [38]. Plant hormones ethylene, jasmonic acid (JA) and salicylic acid (SA) are involved in defense responses upon infection by plant pathogens. JA was shown to induce the expression of the deacetylases *HAD6* and *HAD19* [40, 41]. Overexpression of *HAD19* was responsible for the induction of the JA dependent plant defense genes [41]. HDA19 most likely induces the expression of the defense response genes by inhibiting a repressor of these genes. HDA6 was found to physically interact with the F-box protein COI1 *in vivo*. It is possible that the F-box protein COI1 can target HDA6 for ubiquitination and control the expression of the defense genes induced by JA. Whether COI1 targets HDA6 for ubiquitination has not been determined [40]. It will be interesting to find out whether the regulation of defense response genes by the HDACs occurs directly or indirectly. Gene expression of JA induced defense genes seems to be regulated by histone acetylation and deacetylation in plants.

1.2.2 Histone acetylation and C_4 photosynthesis

Maize is a C_4 plant where the fixation of CO_2 occurs in two different types of photosynthetic plant cells, mesophyll and bundle sheath cells. C_4 specific

phosphoenolpyruvate carboxylase (C_4-PEPC) is involved in the fixation of CO_2 in mesophyll cells. The expression of C_4-PEPC is differentially regulated in the mesophyll and the bundle sheath cells in the presence of the same external light stimulus. The expression of C_4-PEPC is low in etiolated plants but is highly induced in green plants. Histone H4 hyperacetylation occurs at the C_4-PEPC promoters in green plants although the hyperacetylation of histone H4 was not sufficient for the transcription of the C_4-PEPC gene [34]. Light signal was shown to be necessary and sufficient for the hyperaceylation of histone H4 at the C_4-PEPC promoter. Thus, histone acetylation may be involved in the signal transduction pathway that leads to the induction of genes involved in C_4 photosynthesis in maize.

1.2.3 Histone acetylation in *Arabidopsis* during flowering

The flowering in *Arabidopis* is controlled by four important pathways; autonomous, vernalization, photoperiod and gibberellins [42]. The autonomous and vernalization pathways induce flowering by repressing *FLC* which acts as a repressor of flowering. The autonomous pathway consists of six genes, one of which (*FLD)* is homologous to a human protein that is a part of the histone deacetylase 1, 2 co-repressor complex [43, 44]. The *fld* mutant flowers later than the wild type plants. As the FLD homologue in humans is a part of a co-repressor complex, it was thought that FLD might act by controlling the acetylation of histones at the *FLC* promoters. Indeed, the *FLC* promoters showed hyperacetylated histone H4 in the *fld* mutants as compared to the wild type plants. Thus, FLD might be a part of a histone deacetylase co-repressor complex in *Arabidopsis* and might repress *FLC* expression by deacetylating histones.

1.2.4 Histone acetylation and root patterning

Root patterning in *Arabidopsis* occurs by the differentiation of epidermal root cells into hair and non-hair cells. The cellular differentiation depends on the correct spatial expression of six key genes. In order to understand the role of histone acetylation during root patterning the root cells were treated with trichostatin A (TSA), an inhibitor of histone deacetylases [45]. Treatment of root cells with TSA disrupts the cellular patterning and increases expression levels of the key genes by inhibiting HDACs. Investigation of the promoters of these genes showed that the levels of acetylated histones H3 and H4 were higher than in the plants without the TSA treatment [46]. Thus the balance between HDACs and HATs controls the spatial expression of the key genes during root patterning. HDA18 is the main HDAC involved but the identity of the HAT or HATs involved is yet to be determined.

1.2.5 Histone acetylation and seed germination

Seed germination in *Arabidopsis* marks the end of the embryonic stage and the beginning of the vegetative phase. This transition is manifested by a decrease in expression of genes involved in embryonic development. In order to identify whether acetylation of histones plays a key role in this switch, the seeds were treated with TSA, resulting in the upregulation of 45 genes and down regulation of 27 genes during the process of seed germination. Eight of these genes (four of which were late embryonic genes) were highly expressed in dry seeds but imbibition of seeds caused a rapid repression of these genes that continued till three days after imbibition [47]. This lead to deacetylation of histone H4 at the promoters and coding regions of these genes one day after imbibition but acetylation was restored to basal levels after three days. Treatment

with TSA did not affect the onset of repression and there was no difference in gene expression observed one day after imbibition in TSA treated and untreated samples. The expression of these genes was higher after three and six days after imbibition in the presence of TSA than in untreated seeds suggesting that the rate of repression may be slower in these plants but the onset of repression is not. However, TSA-treated plants did not show a deacetylation of histone H4 at the promoters or coding regions of these eight genes one day after imbibition as seen in untreated plants. Thus repression of these genes after imbibition occurs in the TSA-treated seeds even though higher histone H4 acetylation levels are maintained at the target genes. The deacetylation occurring one day after imbibition at the target genes might play a critical role in the transition from embryonic stage to vegetative stage.

The phaseolin gene from bean is repressed in vegetative tissues but is activated during embryogenesis. The activation of the phaseolin gene from its repressed state occurs in two stages potentiation and activation [35]. Distinct histone modifications occur during the two stages. The repressed state is marked by the presence of a nucleosome over the TATA box and methylation of lysine 20 of histone H4. The potentiation stage shows a decrease in the occupancy of histones H3 and H4 at the promoters as well as a significant increase in the acetylation of lysine 9 of histone H3 and lysine 12 of histone H4. The activation stage is marked by an increase in the acetylation of lysine 14 of histone H3 and lysine 5 of histone H4 along with an increase in the trimethylation of lysine 4 of histone H3. Thus, the *phaseolin* promoter serves as a good model to study the relationship between histone acetylation within and between histones as well as with

other covalent modifications. Thus plants seem to have their own 'histone code' that specifies different stages of gene activation.

1.3 Histone acetyltrasferases in plants

Most of the work on histone acetyltransferases in plants has been from *Arabidopsis* and maize. In plants, the first correlation between increased histone acetylation and gene activation was seen in the pea plastocyanin gene (*PetE*). The promoter region of the *PetE* showing increased histone H3 and H4 acetylation was also more accessible to DNaseI and micrococcal nuclease [48, 49]. Since then there have been numerous reports of HATs and their role in gene expression in plants.

The *Arabidopsis* genome contains 12 HAT enzyme coding genes. These genes can be further classified into three families [34]. The GNAT-MYST superfamily consists of the GNAT and the MYST families. The GNAT (GCN5 like histone acetyltransferase) family in *Arabidopsis* has three members, designated HAG1 (GCN5), HAG2 and HAG3. GCN5 is the most extensively characterized member of this family [37, 50, 51]. The MYST family contains two members, HAG4 and HAG5. None of the members of the MYST family have been studied in *Arabidopsis* as yet. The p300/CBP family contains five members HAC1, HAC2, HAC4, HAC5 and HAC12. Some members of this family play a role in regulating gene expression of the vernalization pathway of flowering [52, 53]. The TAFII250 family has two members, HAF1 and HAF2 (TAF1). The TAF1 protein in *Arabidopsis* has been shown to govern the expression of light-regulated genes by controlling the acetylation of histones [54, 55].

Histone acetyltransferases are parts of large multisubunit complexes in yeast, flies, mice and humans [56-58]. These coactivator complexes are recruited by various

transcription factors to the promoters of target genes. The HATs act as the catalytic subunits of these complexes acetylating histones at the promoters and activating transcription. Various components of such HAT complexes have been genomically identified in plants but the complexes have not yet been characterized biochemically [50, 59].

Histone acetylation is involved in the regulation of flowering, seed development, embryogenesis and cellular patterning in root cells. The enzymes involved in these pathways have not been characterized. HATs in animal systems are usually parts of large coactivator complexes that are specifically recruited by activators. Coactivator complexes containing HATs have not been identified in plant systems. Some plant HATs differ considerably in their structure from the animal HATs and hence it is very likely that the structure and composition of HAT coactivator complexes in plants would be very different.

1.3.1 GCN5

One homologue of GCN5 has been identified in maize. In order to understand the role of GCN5 in maize gene expression was tested in plants treated with TSA and in plants expressing the *ZmGCN5* antisense transcript. Treatment of maize with TSA caused hyperacetylation of histones, increase in the transcription of histone mRNA and a reduction in the expression of *GCN5*. The expression of antisense *GCN5* transcript caused hypoacetylation of histones, an increase in the transcript levels of histones and a reduction in the expression of the HDAC *RPD3* in maize [60]. Thus, the loss of a HAT or HDAC in maize causes a shift in the balance of acetylated and unmodified histones. The level of histone transcripts increased in both the treatments but did not result in an

increase in the protein levels of histones. The shift in the balance between acetylated and total histones during TSA treatment is probably compensated by the degradation of modified histones and replacement with *de novo* synthesized histones. In plants expressing the antisense GCN5 transcript the balance is probably restored by the replacement of unmodified histones with *de novo* acetylation of the newly synthesized histones. The balance between modified and total histones in plants is maintained by regulating the expression levels of HATs, HDACs and histones.

ZmGCN5 interacts with the coactivator ZmADA2 *in vivo* as has been observed in other eukaryotes [51, 61, 62]. ZmADA2 in turn interacts with the acidic activator ZmO2 *in vivo* as seen by Forster resonance energy transfer assay. The b-ZIP transcription factor ZmO2 controls the expression of genes involved in the regulation of seed storage proteins [63-65]. It was hypothesized that the activator ZmO2 recruits a GCN5-ADA containing complex in order to activate target gene expression. In order to test this hypothesis expression of the *GUS* reporter gene fused to the promoter of the ZmO_2 target gene *b32* was tested in the presence of GCN5 and ADA2. Results showed that the expression of the reporter gene by ZmO2 was repressed in the presence of ZmGCN5 and ZmADA2. This inhibition was relieved by the expression of a mutant form of ZmGCN5 that is unable to interact with ZmADA2. Thus ZmO2 and ZmGCN5 might compete for the binding of ZmADA2 which in turn causes repression of the reporter gene in the presence of these two coactivators. Thus, GCN5 seems to repress gene expression whereas ADA2 activates it. GCN5 and ADA2 might have overlapping functions but may also work independently of each other in plants [37]. GCN5 and ADA2 are parts of the same coactivator complexes in animals but that may not be the case in plants. Determining the structure of

such coactivator complex/es will shed more light on the functions of these two coactivaor proteins in plants.

Arabidopsis contains one homologue of the enzyme GCN5. The structure of GCN5 can be divided into four domains. The N-terminal domain of *Arabidopsis* as well as maize GCN5 is longer than its yeast counterpart but shorter than those found in flies and humans [50]. The function of the N-terminal domain in *Arabidopsis* is unknown but it is thought to contain the nuclear localization signal (NLS). The HAT domain of GCN5 is conserved in the plant and animal systems. The third domain is the ADA2 interaction domain which facilitates the interaction of GCN5 with the ADA2 proteins in the formation of the large coactivator complexes described in the animal systems. *Arabidopsis* GCN5 can physically interact with the two ADA2 homologues ADA2a and ADA2b *in vitro*. The full length, the N-terminal and middle domains of the ADA2 proteins can bind to the full length GCN5 protein. The middle regions of the ADA2 proteins are sufficient for binding the HAT domain of GCN5. In addition to this interaction GCN5 can also acetylate the two ADA2 proteins in *Arabidopsis in vitro* and may control their biological function [51]. This is the first report of GCN5 acetylating the ADA2 proteins and acting as a factor acetyltransferase (FAT) in *Arabidopsis*. The C-terminal domain of GCN5 is the bromodomain which is known to recognize acetylated histone proteins in animal systems [66, 67] .

The GCN5 enzyme in *Arabidopsis* was first shown to be required for the activation of a reporter gene driven by the activator protein CBF in yeast [50]. The CBF family of transcription factors is involved in controlling the cold-responsive (*COR*) gene expression during the cold acclimation process in *Arabidopsis* [68]. GCN5 can physically

interact with the DNA binding domain of CBF *in vitro.* T-DNA insertion mutation in *GCN5* shows a delay in the activation and overall expression of *COR* genes activated by CBF during cold acclimation [37]. CBF might recruit a GCN5-containing complex to the *COR* gene promoters in order to activate *COR* gene expression during cold acclimation. It is thought that GCN5 may control the expression of *COR* genes by acetylation of histones at the *COR* gene promoters during cold acclimation.

GCN5 has also been shown to acetylate histones at the light-regulated genes *CAB2* and *RBCS-1A* [54]. These genes are activated by the transcription factor HY5 that is involved in the light-signaling network [69-72]. The expression of the light-regulated genes is repressed in two HAT mutants *taf1* and *gcn5*. This effect is further enhanced in the *taf1gcn5* double mutant. The expression of *CAB2* and *RBCS-1A* is controlled by both GCN5 and TAF1 but the *IAA3* gene is regulated only by GCN5 but not by TAF1. Thus the genes in response to the light stimulus are controlled by the concerted action of the two HAT enzymes but they also work independently at some genes. Differences are also observed in their ability to acetylate histones H3 and H4. The acetylation of histone H3 at lysines 9 and 27 and of histone H4 is controlled by a combination of GCN5 and TAF1 [54, 55]. GCN5 is solely responsible for the acetylation of histone H3 at lysine 14. Thus, these two HATs seem partially redundant for the expression of light-responsive genes in *Arabidopsis.* Control of gene expression by combination of HATs could be the norm in *Arabidopsis* rather than an exception.

GCN5 is required for root and shoot development in *Arabidopsis*. The TOPLESS protein in *Arabidopsis* is responsible for the normal development of an embryo into shoot and root [73, 74]. The *tpl-1* mutant shows a double root phenotype that develops as a

result of the conversion of the apical shoot pole into a root pole. Mutation in the HAT GCN5 was identified as a suppressor of the double root phenotype of *tpl-1* [73]. GCN5 is necessary for the formation of the root in place of the apical shoot in the *tpl*-1 mutant but is not required for the formation of the basal root. Thus the development of the shoot and roots in *Arabidopsis* depends on the interaction of HATs with the activator-repressor proteins involved in the development of embryos. Whether GCN5 controls the formation of the apical root by changes in the histone acetylation of these promoters is yet to be determined.

Recently a GCN5-interacting protein, AtEML, was identified in *Arabidopsis* that is upregulated in response to abiotic stresses like cold and salt [75]. GCN5 is required for the activation of the reporter gene *lacZ* by AtEML in yeast. The histones at the promoter of this reporter gene are hyperacetylated during the activation of the reporter gene. The expression of *AtEML* in *Arabidopsis* is higher in the *gcn5-1* loss-of-function mutant. Thus GCN5 seems to act as a repressor of AtEML. GCN5 was shown to enhance the AtEML driven transactivation of a reporter gene in yeast. The direct targets of AtEML in *Arabidopsis* have not been identified as yet but it is hypothesized that GCN5 might regulate the target genes by regulating the activity of the AtEML protein itself [75].

GCN5 has also been shown to be involved in the expression of genes forming the floral meristem [76]. A T-DNA insertion mutation in the bromodomain of *GCN5* gene in *Arabidopsis* produced plants with homeotic mutations in flowers as well as the production of terminal flowers. The mutant plants had a higher expression and an expansion of the expression zones of two of the key regulatory genes *WUS* and *AGA* [76]. WUS is a direct activator of the *AGA* gene. An increase in the expression of *WUS* most

likely causes the increased expression of the *AGA* gene [77-79]. The overall acetylation levels of histone H3 were lower in the *gcn5* mutant as compared to wild type plants. Whether the regulation of *WUS* and *AGA* by GCN5 is direct or indirect remain to be investigated.

Microarray analysis indicated that a *gcn5* mutant in *Arabidopsis* affected the expression of 5% of the 8,200 genes involved in the study. Most of the affected genes were upregulated in the *gcn5* mutant suggesting that GCN5 acts to repress most of its target genes. Whether the repression of these genes by GCN5 is direct or indirect is not known. In contrast, GCN5 in animal systems is mostly involved in the activation of genes and only a few examples of gene repression have been reported [80].

The preceding paragraphs indicate that GCN5 controls the expression of genes in response to external stimuli particularly light and temperature. GCN5 seems to be involved in gene repression in most of the cases rather than gene activation. Gene repression by GCN5 seems to be more common in plants than in animal systems. GCN5 can also act as a factor acetyltransferase in addition to histone acetyltrasferases in *Arabidopsis*. GCN5 can acetylate coactivator proteins ADA2a andADA2b in *Arabidopsis*. ADA2a and ADA2b are found in separate complexes with GCN5 in *Drosophila* which serve distinct functions [81]. Similarly GCN5 in *Arabidopsis* might be working with the two ADA2 proteins in different complexes to control the expression of distinct genes under a variety of external or internal stimuli. Identifying GCN5 containing coactivator complexes in plants will help us understand the function of GCN5 and the proteins required for the biological function of GCN5.

1.3.2 p300/CBP

Plants are exposed to various abiotic stresses like high salinity, drought, cold, and UV radiation. The exposure to UV radiation causes DNA damage in plants. The transcription factor HY5 regulates the UV response in *Arabidopsis* [82]. Maize ecotypes from high altitudes show higher adaptation to UV tolerance than ecotypes from the temperate regions. The ecotypes with higher adaptation express chromatin remodeling factors to higher levels. Lines that are more tolerant to UV show an enrichment of hyperacetylated of histones H3 and H4 after irradiation at the promoter and transcribed regions of two UV-B upregulated genes [39]. This increase in acetylation is correlated with an increase in transcript levels of the UV-B upregulated genes as well as loosening of the chromatin structure as assessed by DNaseI and microccocal accessibility assay. Thus, covalent histone modifications and chromatin remodeling seem to be working in concert to regulate the induction of genes involved in UV-B tolerance. Chromatin immunoprecipitation assays showed that CBP was associated with the promoters of these genes. CBP is known to acetylate histones as well as non-histone proteins in animals and plants [21, 52]. The CBP HAT associates with the promoter region in the UV-B tolerant lines and is thought to have a direct role in the regulation of acetylation of histones in the UV-B tolerant lines.

p300/CBP HATs have been identified in higher eukaryotes like flies, mice and humans. No members of the p300/CBP members have been identified in the yeast *Saccharomyces cerevisiae* [21]. The five members of the p300/CBP family identified in *Arabidopsis* known as HAC1, HAC2, HAC4, HAC5 and HAC12 [83]. The primary structures of the members of the HAC family have some regions of sequence similarity but there are also considerable differences. The members show sequence similarities

between the cysteine/histidine domains 2 and 3 and the acetyltransferase domain. Surprisingly all the members of the HAC family lack the bromodomain and the CREB transcription factor binding domain which are hallmarks of p300/CBP family members in animals [16]. Thus the function of the plant p300/CBP proteins may be different than in animals.

T-DNA insertion mutations in all the five HAC members have been studied. The *hac1-1* mutant flowered later than wild type plants [52, 53]. Gene expression analysis showed that the expression of *FLC*, the repressor of flowering, was increased in this mutant. Double mutants *hac1hac5* and *hac1hac12* showed a further delay in flowering as compared to the *hac1* single mutant. Thus, the HAC1, HAC5 and HAC12 HATs seem to be at least partially redundant in the control of flowering. The acetylation levels of histones H3 and H4 at the *FLC* promoter in the *hac* mutants was no different than that in wild type Col plants as tested by chromatin immunoprecipitation assays. The regulation of *FLC* by the HACs is probably not through the direct acetylation of the *FLC* promoters. Thus, the activation of *FLC* by HACs might occur via an indirect method by the repression of a protein that acts as a repressor of *FLC*. It is possible that the HAC enzymes regulate the function of the direct activator or repressor of *FLC* by modulating the acetylation of those proteins which in turn control *FLC* expression. The HAC family of acetyltrasferases is involved in the repression of *FLC* rather than its activation. This is one more example of HATs acting to repress genes rather than activate genes in plants.

The biggest difference between p300/CBP enzymes in animal and plants is the absence of the bromodomain in plants. Bromodomains are known to bind acetylated histones in animals and help target bromodomain containing proteins to the target

promoters/genes. Members of the other HAT families in *Arabidopsis* have a bromodomain and thus the absence of bromodomain in the HAC family members might provide them with specific functions [52, 83].

It is possible that the HAC proteins might be the HATs that are responsible for the first round of acetylation of histones during gene expression. HAC members could be the first HATs recruited to promoters of genes that have hypoacetylated histones. The acetylation of these histones by the HAC proteins might then recruit other HATs that contain the bromodomain able to recognize acetylated histones. These HATs in turn can bring about the full extent of acetylation required for gene activation. Thus the HAC members might 'poise' the genes for activation by establishing a 'histone code'. Crosstalk between these and other histone modifying enzymes will result in the full activation of the target genes.

HAC proteins might be part of coactivator complexes than contain bromodomain-containing proteins. These bromodomain containing proteins might direct the recruitment of the HAC members to the appropriate target genes. HAC members in turn acetylate histones at the target gene promoters and thus activate gene expression.

It is also likely that the HAC proteins in plants act primarily as FATs instead of HATs. In this case the recruitment of the HACs to the target proteins will not require the presence of a bromodomain. p300/CBP HATs in animals are known to acetylate a vast array of non-histone proteins and regulate the activity of those proteins. The HAC members in plants might serve the same purpose.

Arabidopsis is the only eukaryote with five members in the HAC family. In order to further our understanding of the biological role of the HAC members it is also

important to know whether the five members act independently to control gene expression or are functionally redundant.

1.3.3 TAF1

The TAF1 family in *Arabidopsis* has two members, HAF1 and HAF2/TAF1. In contrast, animals only have a single member in the TAF1 family [55]. T-DNA insertion mutants in the two genes *HAF1* and *HAF2* were analyzed in order to gain insight into the function of these genes in *Arabidopsis* growth and development. T-DNA insertion in the *HAF1* gene did not result in any aberrant phenotype. The *haf2* mutants were paler than wild type plants due to a 50% reduction in their chlorophyll content as compared to wild type plants. The HAF2/TAF1 protein in *Arabidopsis* contains an ubiquitin, a HAT and a bromodomain. Analysis of the *taf1* mutant showed that TAF1 is involved in controlling the expression of light regulated genes such as *RBCS1* and *CAB2*. Analysis of the acetylation of histones H3 and H4 showed that these histones were hypoacetylated at the *RBCS1* and *CAB2* promoters in the *taf1* mutants as compared to wild type [54]. The acetylation of histones H3 and H4 was dependent on the activity of GCN5 in addition to TAF1. TAF1 was solely responsible for the acetylation of lysine 9 of histone H3 at the target gene promoters. Thus once again we observe an effect on gene expression which depends upon the concerted effort of more than a single HAT protein.

Microarray analysis showed that the expression of about 3% of *Arabidopsis* genes were affected in the *taf1* mutant and almost an equal number of genes were up or down regulated. TAF1 could be regulating gene expression in *Arabidopsis* by acetylating histones at the promoters of target genes. This in turn might govern the recruitment of the TFIID complex to the gene promoters which then leads to activation of transcription.

Loss of TAF1 results in the repression of light responsive genes and this is associated with deacetylation of histone H3 and H4. The repression of these genes is exacerbated by the absence of another HAT GCN5. Members of GCN5 containing coactivator complexes are known to interact with members of the TFIID complex in animal systems [84, 85]. It will be interesting to find out whether such an interaction takes place in plants. HY5 controls the expression of the light-regulated genes but whether it recruits the GCN5 and TAF containing complexes remains to be investigated.

The TAF1 family in *Arabidopsis* contains two members designated HAF1 and TAF1. Whether both the proteins are part of the same TFIID complex in *Arabidopsis* or whether they are components of two different complexes is not known. The presence of two TAF1 members is a unique feature of the plant TAF1 HAT family [55, 76]. The function of the HAF1 enzyme in *Arabidopsis* is unknown and the *haf1* mutant does not show any visible phenotype. It is possible that the functions of the HAF1 and TAF1 enzymes are partially redundant. To unravel the biological functions of these two proteins is an important next step. It will be interesting to find out whether the *haf1taf1* double mutant has an effect on the normal growth and development of *Arabidopsis*. Answering these questions would help elucidate the biological functions of these enzymes in plants.

1.4 Novel histone acetyltrasferases in *Arabidopsis*

The X-ray crystal structure and NMR solution structure of a histone acetyltransferase belonging to the GNAT superfamily in *Arabidopsis* was recently described by Tyler et al [86]. The At1g7750 gene product has an acetyltransferase domain that is considerably smaller than the other members of the GNAT family. Thus, this may be a 'minimal' acetyltransferase that requires the help of a partner protein for its

AT function. The rate of acetylation of histones by this enzyme is lower than for other members and hence histones may not be the physiological substrates. The function of this protein in *Arabidopsis* is unknown.

A nuclear shuttle protein interactor from *Arabidopsis* (AtNSI) has been shown to have histone acetyltransferase activity [87]. This protein interacts with the movement protein nuclear shuttle protein (NSP) that is required for the movement of virus particles from the nucleus to the cytoplasm and neighboring cells. This protein, encoded by certain plant viruses, is required for the establishment of viral infection in plants. NSP interacts with the viral coat protein (CP), sequesters the replicated genome and shuttles it to the cytoplasm. Although AtNSI interacts with NSP and not with CP, it is able to acetylate CP and is thought to function in modulating the interaction between NSP and CP. The AtNSI acetyltransferase domain belongs to the GNAT family but is only distantly related to the well known members like the GCN5 from yeast, flies and *Arabidopsis*. The residues essential for the acetytransferase activity were conserved in the AtNSI. The AtNSI was able to acetylate histones H3, H2A and very weakly histone H4 *in vitro*. The AtNSI lacks a bromodomain and does not function as a transcriptional coactivator as observed by *in vitro* assays.

Thus, plants encode for a large number of acetyltransferases but only 12 of those are, to date, recognized as legitimate histone acetyltransferases or as likely homologs of known HATs. These unclassified proteins with acetyltransferase activity might exclusively act as factor acetyltransferases and function in specialized pathways in plants. Determining the proteins they acetylate and the pathways in which they function will help further our understanding of their biological function in plants.

1.5 Hypothesis and experimental design

The main aim of this doctoral thesis work is to investigate the role of the coactivator proteins ADA2b and GCN5 in cold-regulated (*COR*) gene expression during the cold acclimation process in *Arabidopsis*. Members of the CBF family of activator proteins (CBF1, CBF2 and CBF3) bind to cis-elements in the promoters of the *COR* genes and activate their expression during the cold acclimation process. ADA2b and GCN5 can physically interact with the DNA binding domain of CBF *in vitro*. The expression of *COR* genes during cold acclimation was delayed and reduced in the *ada2b* and *gcn5* mutants.

It was hypothesized that CBF recruits ADA2b and/or GCN5 containing complex to the *COR* gene promoters during cold acclimation to activate the expression of *COR* genes. GCN5 then acetylates histones at the *COR* gene promoters and activates the expression of *COR* genes. Chromatin immunoprecipitation assays were performed using antibodies that specifically recognize acetylated histone H3 (at lysine 9 and/or 14) in order to determine the levels of acetylated histone H3 at the *COR* gene promoters before and after cold acclimation. Hyperacetylation of histone H3 at *COR* gene promoters was observed in wild type plants upon cold acclimation and CBF was sufficient to drive this acetylation. Hyperacetylation of histone H3 at *COR* gene promoters was also observed in the *ada2b* and *gcn5* mutants. Therefore, GCN5 is not the HAT responsible for acetylation of histone H3 at the *COR* gene promoters during cold acclimation. Surprisingly, the occupancy of histone H3 at the *COR* gene promoters in the *ada2b* and *gcn5* mutants did not decrease during cold acclimation as observed in wild type plants. ADA2b and GCN5 are required for the removal of nucleosomes from the *COR* gene promoters but are

dispensable for the histone H3 acetylation. Thus an ADA2b-GCN5containing complex might be required for the recruitment of a chromatin remodeling complex which clears nucleosomes off the *COR* gene promoters.

The *Arabidopsis* genome encodes 11 HAT enzymes in addition to GCN5 and any of these HATs may be responsible for acetylation of histone H3 at the *COR* gene promoters. A few candidate HATs from the HAC and TAF1 families were tested for their ability to regulate *COR* gene expression by acetylating histone H3 at the *COR* gene promoters. Results showed that all the HAT enzymes tested were dispensable for the acetylation of histone H3 at the *COR* gene promoters. Thus, acetylation of histone H3 at the *COR* gene promoters is not dependent on any of the individual HATs tested.

In order to test whether the CBF driven expression of *COR* genes requires ADA2b and/or GCN5 two approaches were utilized. The expression of *COR* genes was compared in Wt, *ada2b* and *gcn5* plants overexpressing CBF. In the second approach the expression of the reporter gene *GUS* fused to four tandem repeats of CRT was compared in Wt, *ada2b* and *gcn5* plants after cold acclimation. Results showed that the CBF-dependent expression of *COR* genes via CRTs does not require ADA2b and GCN5.

We can conclude from the results that GCN5 is not the HAT responsible for the acetylation of histone H3 at the *COR* gene promoters. GCN5 and ADA2b are required for the removal of nucleosomes from the *COR* gene promoters during cold acclimation. GCN5 and ADA2b are not required for the CBF-dependent expression of *COR* genes and probably regulate *COR* gene expression indirectly.

Chapter II

Increase in acetylation of histone H3 corresponds to activation of *COR* genes during cold acclimation.

2.1 Introduction:

The presence of chromatin in eukaryotes serves as a barrier to transcription [88]. Eukaryotes have devised ways to facilitate gene expression even in the presence of chromatin. Transcriptional activators that bind to specific cis-elements present in the promoters of genes specifically recruit the general transcription machinery and thus facilitate transcription of target genes [89, 90]. Activators also recruit proteins known as chromatin modifying proteins that can aid the process of transcription. There are two types of chromatin modifying factors. The ATP-dependent coactivator proteins include SWI/SNF proteins that are able to slide or remove nucleosomes and thus allow transcription to proceed. The second type of chromatin remodeling proteins are enzymes that covalently modify histones by acetylation, methylation, ubiquitination or phosphorylation that are either associated with transcriptional activation or repression or both .

Cold acclimation is the process by which plants can increase their freezing tolerance when exposed to low non-freezing temperatures [91]. The CBF family of transcription factors acts as 'master regulators' during the process of cold acclimation. The transcription of the CBF genes (CBF1, 2 and 3) is induced within 15 minutes of exposure to cold temperatures [92-94]. The CBF proteins bind specific cis-elements known as the cold/dehydration responsive elements (CRT/DREs) in the promoters of

their target genes [95-97]. The CBF proteins activate their target genes, including the cold-regulated (*COR*) genes, at about 2-4 hours after exposure to cold temperature [98]. Overexpression of CBF results in constitutive expression of the *COR* genes and these plants are also constitutively freezing tolerant [93, 94]. CBF1, a transcriptional activator, can physically interact with at least two co-activator proteins, ADA2b and GCN5 (a histone acetyltransferase) [37, 50, 51]. ADA2 and GCN5 are components of large multi-subunit co-activator complexes in eukaryotic organisms like yeast, flies and humans but the function of these enzymes in *Arabidopsis* is not completely known as yet [61, 99-103]. ADA2 is able to enhance the ability of GCN5 to acetylate nucleosomal histones [104, 105]. *Arabidopsis* GCN5 is able to acetylate histone H3 *in vitro* specifically at lysine 9 and 14 [106]. Homozygous T-DNA insertion mutants of GCN5 (*gcn5-1*) and ADA2b (*ada2b-1*) show pleiotropic phenotypes with defects in growth and development. *gcn5-1* and *ada2b-1* mutant plants show delayed activation and reduced expression of *COR* genes during cold acclimation. The freezing tolerance of *ada2b-1* and *gcn5-1* plants is similar to wild type plants but their ability to activate *COR* gene expression is compromised. Thus we hypothesized that upon cold acclimation CBF recruits to the *COR* gene promoters a coactivator complex containing ADA2b and GCN5. This co-activator complex in turn acetylates histone H3 at the promoters of *COR* genes and thus facilitates the activation of *COR* gene upon cold acclimation.

This chapter will answer three important questions related to the regulation of *COR* gene expression during cold acclimation. The first question is whether acetylation of histone H3 increases at the *COR* gene promoters during cold acclimation. The second question that will be addressed is if CBF is necessary and/or sufficient to drive histone

H3 acetylation at the *COR* gene promoters. The last question that will be addressed is whether GCN5 and/or ADA2b are necessary to bring about acetylation of histone H3 at the *COR* gene promoters.

To answer these questions chromatin immunoprecipitation assays were carried out using antibodies against acetylated histone H3 (acetylated lysine 9, lysine 14) and an antibody that recognizes the C-terminal region of histone H3 irrespective of the modifications on the N-terminal tail.

2.2 Materials and methods:

2.2.1 Plants used and growth conditions

Wild type plants of the Wassilewskija-2 (Ws) ecotype were used in this study. *ada2b-1* and *gcn5-1* plants obtained from the Wisconsin T-DNA mutant collection and characterized in the Triezenberg lab were used [37] . Seeds for the CBF1 over-expressing plants and plants expressing a C-terminal truncation mutant, CBF2ΔC, in the Ws background were obtained from the Thomashow lab (Gilmour, unpublished data). Seeds were sterilized using a 40 % bleach solution containing 1 % Triton-X 100 for 10 minutes at room temperature. The seeds were washed 3 times with sterilized MilliQ water for 10 minutes each. The seeds were stratified at 4°C for 4 days before plating out. Plants were grown on Gamborg's basal medium with sucrose and 1 % phyta agar [107, 108]. Plants were grown in a growth chamber at a constant temperature of 22°C and continuous lights at 120 $\mu mol/m^2sec$.

2.2.2 Cold acclimation

Cold treatment of plants was carried out in chambers maintained at a temperature of 4°C and continuous lights at 120 μmol/m^2sec. Plants were treated for either 4 h or 24 h.

2.2.3 Tissue harvesting and storage

Whole seedlings (0.65 g tissue per IP) were harvested from plates using tweezers and transferred to a flask containing a solution of 1 % formaldehyde. After cross linking plants were stored in 50 ml tubes, flash frozen in liquid nitrogen and stored at -80°C until further use.

2.2.4 Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assays were adapted from previously published methods [109, 110]

15-day old seedlings (before bolting) grown on plates were harvested in 1 % formaldehyde and placed under vacuum for 25 minutes. Glycine was added to final concentration of 0.125 M to quench the reaction and placed under vacuum for 5 minutes. The tissue was rinsed with water twice, frozen in liquid nitrogen and stored at –80°C.

The tissue was ground in liquid nitrogen (to keep it cold) using mortar and pestle. The tissue was resuspended in Solution I (0.4 M sucrose, 10 mM Tris pH 8 and 10 mM $MgCl_2$) and ground again. The tissue was then filtered through four layers of cheesecloth and two layers of Miracloth into Oakridge tubes and spun in an SS34 rotor at 10,000 rpm for 10 minutes. The supernatant was discarded; the pellet was resuspended in Solution II (0.4 M sucrose, 10 mM Tris pH 8, 10 mM $MgCl_2$ and 1% Triton X-100) and spun at 10,000 rpm for 10 minutes. The supernatant was discarded; the pellet was homogenized (hand dounced using a homogenizer) in solution III (1.7 M sucrose, 10 mM Tris pH 8, 2

mM $MgCl_2$ and 0.15% Triton X-100) and the homogenate was layered over equal volume of Solution III. The tube was centrifuged at 15,000 rpm for 30 minutes after which the homogenate was poured off. A protease inhibitor solution containing 5 mM β-mercaptoethanol, 0.1 mM phenylmethanesulphonylfluoride and EDTA-free complete protease inhibitor tablets from Roche was added to solutions I, II and III just before use.

The pellet was resuspended in sonication buffer (1% sodium dodecyl sulfate, 10 mM EDTA and 50 mM Tris pH 8.1) using, 150 µl buffer per 0.65 g of original tissue, and kept on ice 5-15 minutes. A protease inhibitor solution containing 0.5 mM phenylmethanesulphonylfluoride, 100 µg/ml leupeptin and 100 µg/ml aprotinin was added to the sonication buffer just before use. The solution was sonicated 5 times for 20 sec (with a Microtip sonicator, on continuous cycle, output control 3), rested on ice for 5 min between pulses and spun in microfuge at full speed for 10 min at 4°C. 100 µl aliquot of the supernatant was taken as size sample (to run on gel to assess shear size). VP16 viral plasmid DNA (to assess non-specific binding of antibody) was added to the rest to a final concentration of 2.6 pg/µl. An aliquot (30 µl) of this input DNA was taken and the size and the input aliquots were stored at -20°C.

Protein G beads (50% slurry in Triton-X lysis buffer with 10 µg/ml salmon sperm DNA) were added using 60 ul protein G / 1 ml extract and incubated at 4°C for 2 hours on rotator. Sample was centrifuged at 5000 rpm for 5 min; and 900 µl Triton-X lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM sodium chloride, 1% Triton X-100 and 1% bovine serum albumin) was added per 150 µl supernatant for each sample. Antibodies were added to appropriate samples and incubated for 8 hours at 4°C on rotator. 40 µl of protein G beads was added and samples incubated overnight at 4°C on rotator.

Samples were centrifuged at 5000 rpm for 5 min at 4°C and beads were then washed with buffers (Triton wash buffer - 50 mM Tris-HCl pH 7.4, 150 mM sodium chloride and 1% Triton X-100, Lysis buffer 500 – 0.1% sodium deoxycholate, 1 mM EDTA, 50 mM HEPES pH 7.5, 500 mM sodium chloride, LiCl/detergent – 0.5% sodium deoxycholate, 1 mM EDTA, 250 mM LiCl, 0.5% IGEPAL CA-630 and 10 mM Tris pH 8.0, TE pH 8.0) for 10 minutes on rotator at RT followed by 2 minute spin at 5000 rpm. 100 µl elution buffer (10 mM EDTA, 1% SDS and 50 mM Tris pH 8) was added to beads and incubated at 65°C for 10 minutes (inverting several times during incubation). The samples were centrifuged 2 min at 5000 rpm and the supernatant was transferred to fresh tubes. 150 µl TE/0.67 % SDS was added to beads, inverted and centrifuged at top speed for 1 min and then combined with first elution. The final volume was 250 µl for each IP, input (30 µl input + 220 µl water) and size (100 µl size sample + 150 µl water) samples. To each tube 10 µl of 5M NaCl and 1 µl RNaseA was added and incubated at 65°C overnight.

DNA was precipitated with 2 volumes of ethanol (incubated at –20°C for 15 minutes, centrifuged at 4°C in microfuge at full speed for 15 minutes) and the DNA pellet was resuspended in 100 µl TE pH 7.5, 25 µl proteinase K buffer (1.25% SDS, 50 mM Trsi-HCl pH 8) and 1 µl proteinase K. Samples were incubated at 42°C for 2 hours and the DNA was extracted with 25:24:1 (v/v) phenol: chloroform: isoamyl alcohol (equal volume, centrifuged in microfuge at full speed for 3 minutes). The upper phase was transferred to a fresh tube and DNA was precipitated with 1/10 volume NaOAc pH 5.2 and 2 volumes 100% ethanol (incubated at –20°C for 15 minutes, spun at 4°C in microfuge at full speed for 30 minutes). The pellet was washed with 250 µl 75% ethanol

once and resuspended in 80 µl nuclease free water (except size sample in 10 µl to run on gel). 3 µl of DNA samples were used per PCR reaction of 30 µl.

2.2.5 Antibodies

Commercially available antibodies were used for chromatin immunoprecipitation assays. The anti-acetyl-H3 antibody (rabbit polyclonal IgG, #06-599) was purchased from Upstate Biotechnology and the anti-histone H3 antibody (rabbit polyclonal IgG, ab1791) was purchased from Abcam Inc. 2 µl of the anti-acetyl-H3 antibody and 1 µl of the anti-histone H3 antibody was used per 0.65 g of plant tissue.

2.2.6 PCR conditions and data analysis

Real-time PCR was carried out using 10X buffer containing SYBR Green, 0.25 µM of each primer, 3 mM $MgCl_2$, 12.4 mM dNTPs and 0.15 µl Ampli Taq Gold DNA polymerase enzyme (all reagents were obtained from ABI) on the purified DNA samples. Input samples were diluted and used as a standard curve. The amount of DNA pulled down by the acetylated H3, the C-terminal H3 antibodies and the no-antibody control were calculated using the input standard curve. PCRs were carried out using primers that span the CRT region of the *COR* gene promoters (*COR6.6, COR15a, COR47, COR78* and *GolS*). The *ACT2/7* promoter region was used as a control. The % input values obtained for the no-antibody control were subtracted from the values obtained for the anti-acetylated histone H3 IP and the anti-histone H3 IP. The % input values thus obtained for the *COR* gene promoters were normalized to the values obtained for the *ACT2/7* promoter. The values were expressed as a ratio of normalized acetylated histone H3 to normalized total histone H3 or as level of normalized total histone H3 at the *COR* gene promoters. Values for the *ACT2/7* promoter were not normalized and were

expressed as a ratio of acetylated histone H3 to total histone H3 or as total histone H3. Data were analyzed using single-factor ANOVA using Microsoft Excel 2007 software. To determine whether the difference between two values is significant, the least significant difference (LSD) method at $p \leq 0.05$ was used.

2.3 Results:

2.3.1 Acetylation of histone H3 at the *COR* gene promoters increases during cold acclimation.

Activation of genes is sometimes also associated with the removal of histones in addition to histone modifications and thus when analyzing the data it is also important to monitor the changes in the level of total histone H3 during *COR* gene expression upon cold acclimation. To test if the acetylation levels of histone H3 at the *COR* gene promoter change upon cold acclimation, chromatin immunoprecipitation (ChIP) assays were carried out in Ws wild type plants. Plants were cold treated at 4°C for 4 or 24 hours. Plants grown at 22°C were used as controls. ChIP assays were carried out using two different antibodies. One antibody recognizes histone H3 acetylated at lysines 9, 14 or both. The second antibody recognizes the C-terminal region of histone H3 irrespective of the modifications on the N-terminal tail. The changes in the acetylation of histone H3 at the *COR* gene promoters are represented as a ratio of acetylated histone H3 to total histone H3 to account for changes occurring in the total level of histone H3 during *COR* gene expression.

Results (Figure 1) show that the acetylation of histone H3 at the *COR* gene promoters increased at least two-fold upon cold acclimation in wild type Ws plants as

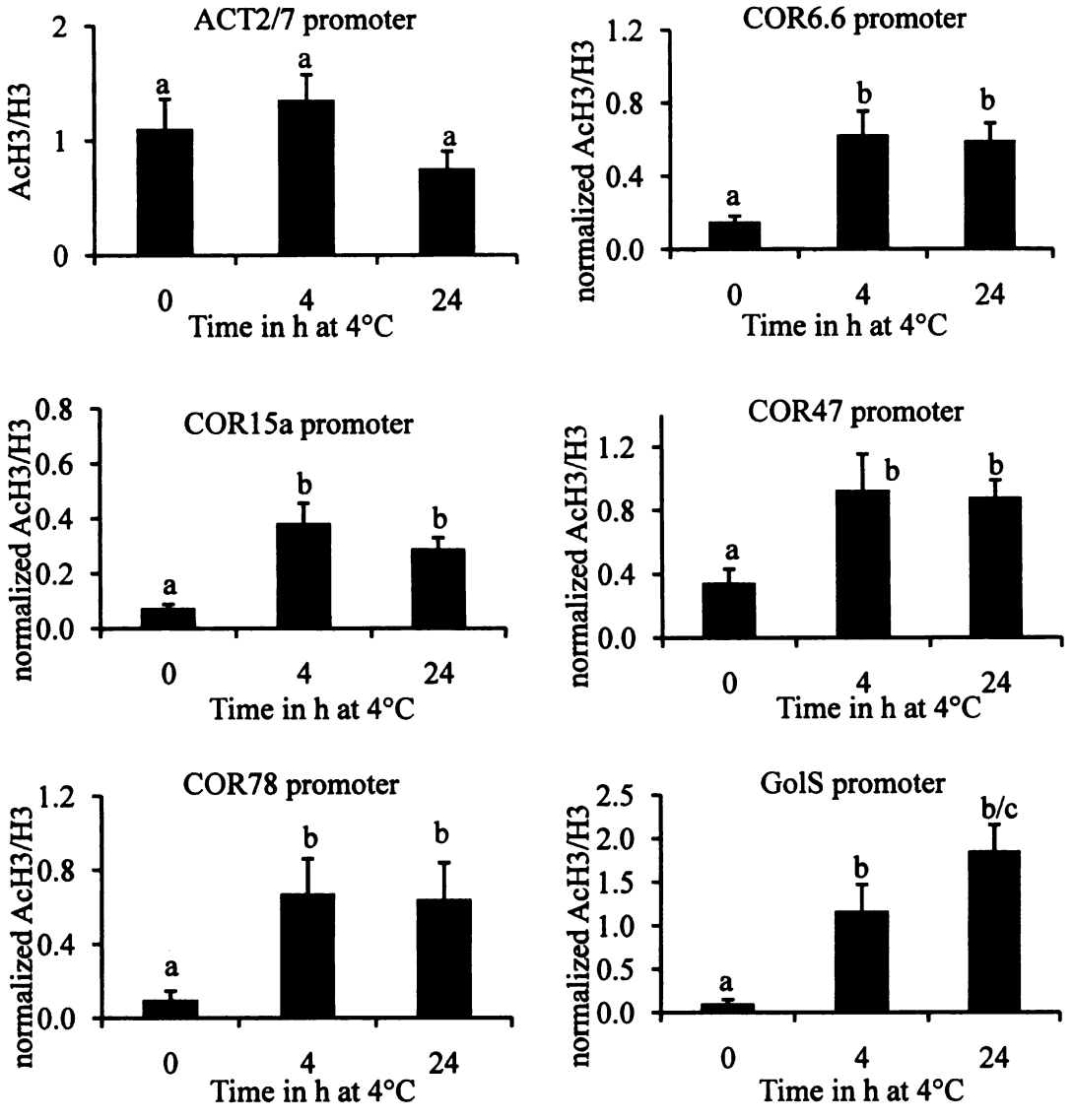


Figure 1: Ratio of acetylated histone H3 to total histone H3 at *COR* gene promoters increases upon cold acclimation. ChIP assays were performed on wild-type plants cold acclimated at 4°C for 0, 4 and 24 hours using antibodies specific for acetylated histone H3 (K9, K14) and the C-terminal of histone H3. Samples were analyzed using real-time PCR to quantify promoter DNA enriched for acetylated histone H3 and total histone H3 at various *COR* gene promoters. Ct values were normalized to values obtained for *ACT2/7* promoter. Data were represented as ratio of acetylated histone H3 to total histone H3 at various *COR* gene promoters. Values at the *ACT2/7* promoter were not normalized and were represented as ratio of acetylated histone H3 to total histone H3. Error bars represent SE where N (number of experiments) = 5. Data were analyzed using single factor ANOVA. Values of bar graphs denoted by the same letter are not significantly different as determined by LSD at $p \leq 0.05$.

compared to non-acclimated plants. The increase in acetylation occurred within 4 hours of cold treatment at all the *COR* gene promoters. Histone H3 remained hyperacetylated even after 24 hours of cold acclimation. The acetylation levels of histone H3 at the *ACT2* promoter did not change significantly upon cold acclimation.

H3 histones at the *COR* gene promoters are hypoacetylated in non-acclimated plants when then *COR* are inactive. Upon cold acclimation H3 histones are hyperactetylated and this increase in acetylation at the *COR* gene promoters corresponds to the activation of *COR* genes. We can conclude from the results that the acetylation of histone H3 at all *COR* gene promoters increase during cold acclimation.

2.3.2 The level of total histone H3 at some *COR* gene promoters decreases upon cold acclimation.

Activation of gene expression involves chromatin remodeling in addition to covalent histone modifications. Chromatin remodeling enzymes are known to slide or remove nucleosomes from genes in order to facilitate transcription activation. To test if nucleosome removal occurs at the *COR* promoters, the changes in level of histone H3 were monitored during cold acclimation. Chromatin immunoprecipitation (ChIP) assays were carried out in Ws wild type plants that were cold treated at 4°C for 4 or 24 hours and plants grown at 22°C were used as controls. ChIP assays were carried out using an antibody that recognizes the C-terminal of histone H3 irrespective of the modifications on the N-terminal tail.

Results (Figure 2) show that the occupancy of histone H3 at *COR6.6, COR78* and *GolS* promoters decreased upon cold acclimation. The occupancy of histone H3 at these

COR gene promoters decreased significantly after 24 hours of cold treatment. The level of histone H3 at the *ACT2, COR15a* and *COR47* promoters remained unchanged after cold acclimation.

The activation of a subset of *COR* genes involved removal of nucleosomes as seen by the decrease in the level of total histone H3 during cold acclimation. This removal of nucleosomes was only evident after 24 hours of cold acclimation (Figure 2), whereas the acetylation of histone H3 was evident as early as 4 hours after cold acclimation. We conclude that histone H3 acetylation at the *COR* gene promoters precedes removal or loss of nucleosomes at a subset of *COR* gene promoters. However, removal is not a certain consequence of acetylation, since acetylation occurs at promoters where no histone loss was evident. Moreover, these results suggest that even though the activation profile of these *COR* genes was similar, subtle differences may exist in the manner in which the individual *COR* genes are regulated.

2.3.3 Levels of acetylated histone H3 and total histone H3 are restored to non-acclimated levels within 24 hours of de-acclimation.

Activation of *COR* gene expression is associated with both an increase in histone H3 acetylation as well as decrease in histone H3. De-acclimation of plants leads to a decrease in *COR* gene expression back to non-acclimated levels within 24 hours [111]. To test whether deacclimation also affects the levels of acetylated histone H3, ChIP assays were carried out in non-acclimated, cold acclimated and de-acclimated Ws wild type plants. For de-acclimation, the plants were first cold acclimated at 4°C for 24 hours and then placed at 22°C for 24 hours. ChIP assays were carried out using an antibody that

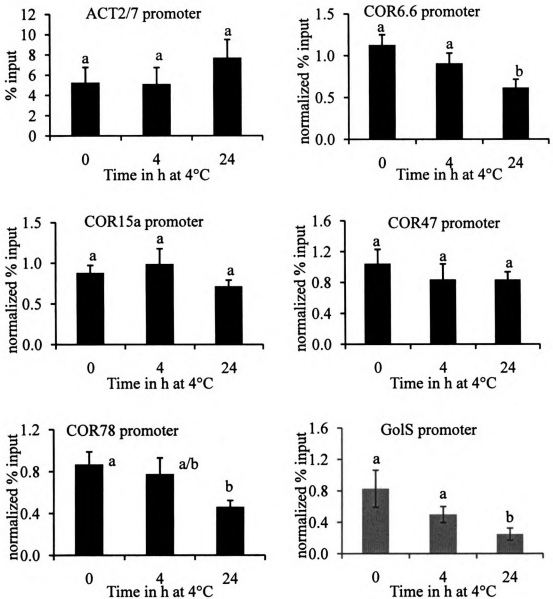


Figure 2: Level of total histone H3 at *COR* gene promoters decreases upon cold acclimation. ChIP assays were performed on wild-type plants cold acclimated at 4°C for 0, 4 and 24 hours using an antibody specific for the C-terminal of histone H3. Samples were analyzed using real-time PCR to quantify promoter DNA enriched for total histone H3 at various *COR* gene promoters. Ct values were normalized to values obtained for *ACT2/7* promoter. Data were represented as normalized % input levels of total histone H3 at various *COR* gene promoters. Ct values for the *ACT2/7* promoter were not normalized and were represented as % input. Error bars represent SE where N (number of experiments) = 5. Data were analyzed using single factor ANOVA. Values of bar graphs denoted by the same letter are not significantly different as determined by LSD at $p \leq 0.05$.

recognizes acetylated histone H3 and an antibody that recognizes the C-terminal region of histone H3 irrespective of the modifications on the N-terminal tail.

The results in figure 3 show that H3 histones at the *COR* gene promoters were hypoacetylated in non-acclimated plants. Within four hours of cold acclimation H3 histones at the *COR* promoters were hyperacetylated and high levels of acetylated histone were maintained even after 24 hours of cold acclimation. There was at least a two-fold increase in acetylation of histone H3 at the *COR* gene promoters in the acclimated plants as compared to the non-acclimated plants. Upon de-acclimation the H3 histones were deacetylated and acetylation levels were restored to non-acclimated levels after 24 h.

Thus, activation of *COR* genes in wild type plants is associated with an increase in acetylation of histone H3 whereas the repression of *COR* genes during de-acclimation is linked to a decrease in the acetylation of histone H3 at the *COR* gene promoters.

The histone H3 occupancy at *COR* gene promoters during de-acclimation was also tested. Results (Figure 4) show that the occupancy of histone H3 decreased significantly at the *COR6.6, COR78* and *GolS* promoters upon 24 hours of cold acclimation. When plants were de-acclimated the levels of histone H3 were restored to non-acclimated levels at these promoters. The levels of histone H3 occupancy at the *ACT2/7, COR15a* and *COR47* did not decrease upon cold acclimation or de-acclimation as compared to non-acclimated plants. Thus, activation of some *COR* genes in wild type plants is associated with a decrease in histone H3 occupancy whereas the repression of *COR* genes during de-acclimation leads to the restoration of histone H3 at the target *COR* gene promoters.

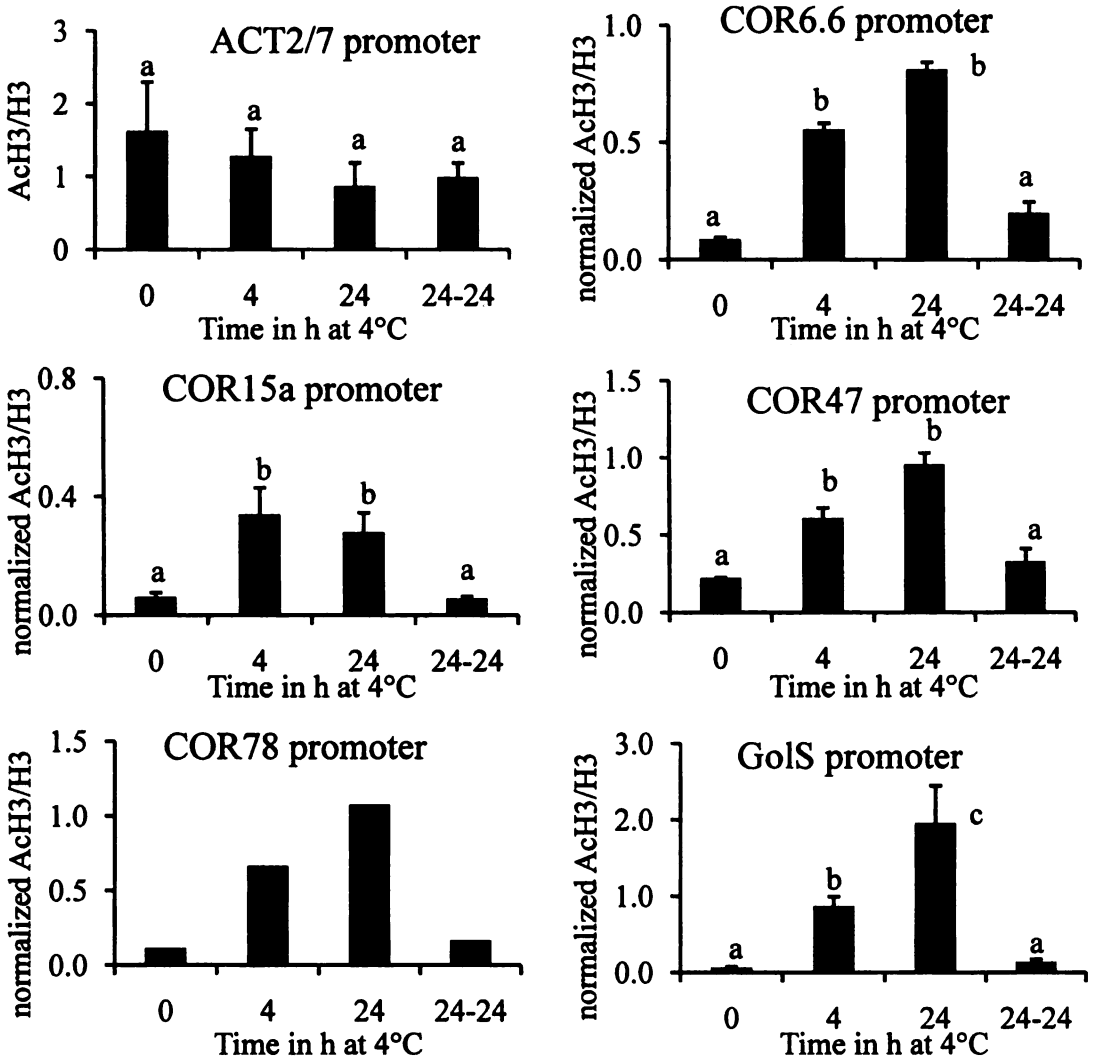


Figure 3: Ratio of acetylated histone H3 to total histone H3 at *COR* gene promoters increases upon cold acclimation and is restored upon de-acclimation. ChIP assays were performed on wild-type plants cold acclimated at 4°C for 0, 4 and 24 hours using antibodies specific for acetylated histone H3 (K9, K14) and the C-terminal of histone H3. One set of plants was acclimated at 4°C for 24 hours and then de-acclimated by placing them at 22°C for 24 hours. Samples were analyzed using real-time PCR to quantify promoter DNA enriched for acetylated histone H3 and total histone H3 at various *COR* gene promoters. Ct values for the *COR* promoters were normalized to the Ct values of *ACT2/7* promoter. Data were represented as ratio of acetylated histone H3 to total histone H3 at various *COR* gene promoters. Ct values obtained for the *ACT2/7* promoter were not normalized and were represented as a ration of acetylated histone H3 to total histone H3. Error bars represent SE where N (number of experiments) = 2, except *COR78* where N = 1. Data were analyzed using single factor ANOVA. Values of bar graphs denoted by the same letter are not significantly different as determined by LSD at $p \leq 0.05$.

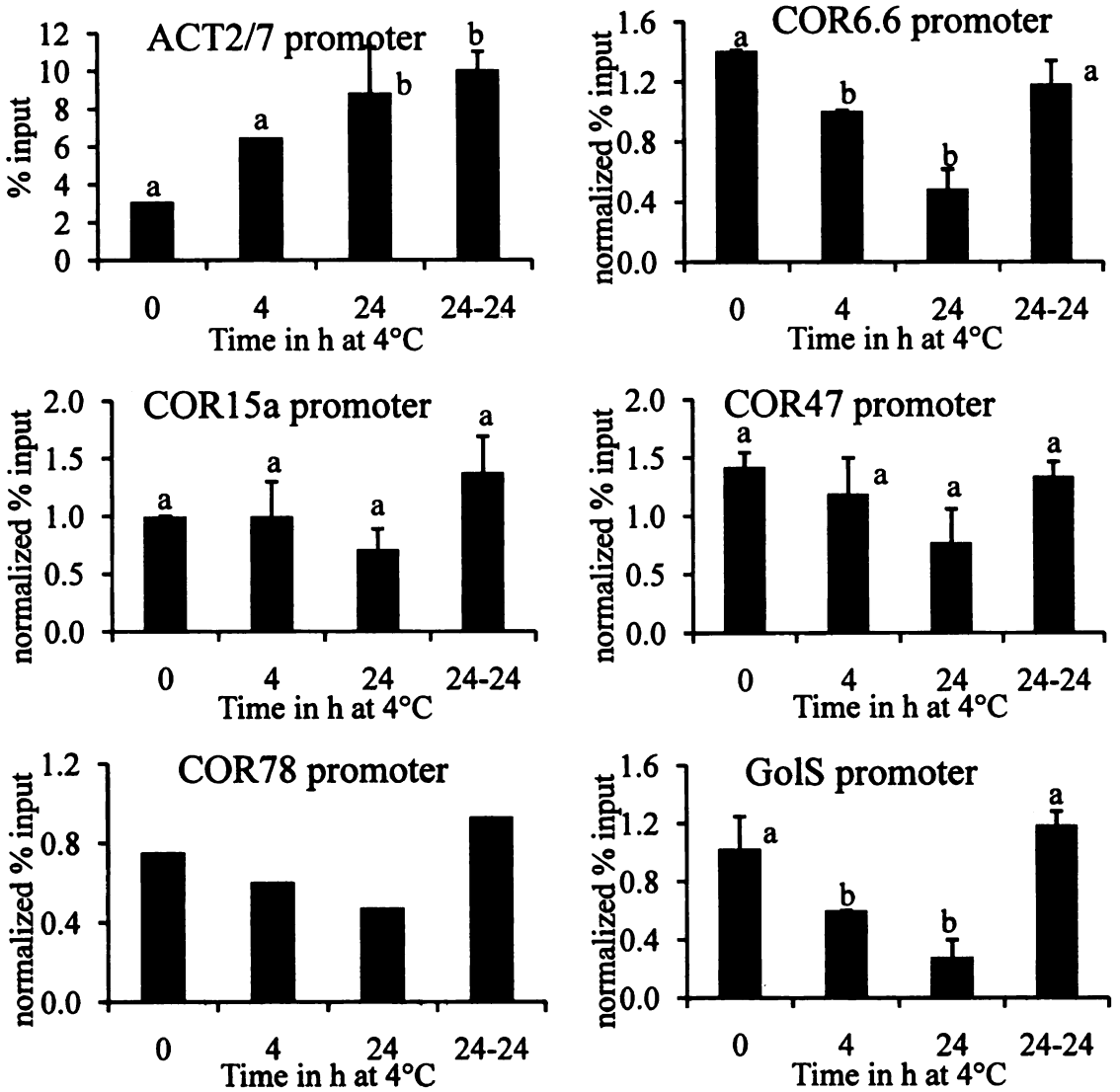


Figure 4: Levels of total histone H3 at some *COR* gene promoters decreases upon cold acclimation and is restored upon de-acclimation. ChIP assays were performed on wild-type plants cold acclimated at 4°C for 0, 4 and 24 hours using antibodies specific for the C-terminal of histone H3. One set of plants was acclimated at 4°C for 24 hours and then de-acclimated by placing them at 22°C for 24 hours. Samples were analyzed using real-time PCR to quantify promoter DNA enriched for acetylated histone H3 and total histone H3 at various *COR* gene promoters. Ct values were normalized to values obtained for *ACT2/7* promoter. Data represented as levels of total histone H3 at various *COR* gene promoters. The Ct values for the *ACT2/7* promoter were not normalized and represented as % input values for total histone H3. Error bars represent SE where N (number of experiments) = 2, except for the *COR78* promoter where N=1. Data were analyzed using single factor ANOVA. Values of bar graphs denoted by the same letter are not significantly different as determined by LSD at $p \leq 0.05$.

One unexplained discrepancy was observed in the experiments using de-acclimated plants. The level of total histone H3 at the *ACT2/7* promoter was significantly different in the acclimated (24 h sample) and de-acclimated samples, compared to the 4 h acclimated sample and non-acclimated control. This effect was not observed in the comparable experiment shown in Figure 2. Compared to the previous experiment (figure 2) the value of total histone H3 in the non-acclimated control was twofold lower. The values of total histone H3 in the non-acclimated and the 4 h acclimated samples in the two independent experiments (figure 4) were identical and the samples showed no variation. This could account for the difference between these samples and the 24 h acclimated and de-acclimated samples.

We conclude that the activation of *COR* genes involves both an increase in the acetylation of histone H3 as well as a removal of nucleosomes (as observed by the removal of histone H3) during cold acclimation. Both these marks of activation are transient and are restored to basal levels once the expression of *COR* genes decreases (as observed by the de-acclimation experiment).

2.3.4 CBF is sufficient for the acetylation and removal of histone H3 at the *COR* gene promoters.

Overexpression of CBF in wild type plant leads to constitutive expression of *COR* genes. These plants are also constitutively freezing tolerant [94]. Results in section 2.3.1 show that the expression of *COR* genes in wild type plants upon cold acclimation is associated with hyperacetylation of histone H3. Results in section 2.3.2 show that expression of some *COR* genes is also associated with a loss of histone H3 occupancy. It

is possible that these marks of gene activation occur at the *COR* gene promoters in the CBF overexpressing plants even without cold acclimation. To test whether CBF is sufficient to drive the acetylation and/or removal of histone H3 at the *COR* gene promoters, ChIP assays were carried out using plants that overexpress CBF1. Plants overexpressing CBF1 were cold acclimated at 4°C for 4 or 24 hours. Plants grown at 22°C were used as controls. ChIP assays were performed using the anti-acetylated histone H3 antibody and the anti-histone H3 antibody.

The levels of acetylated histone H3 and total histone H3 for the plants overexpressing CBF1 were compared to the results obtained for Ws wild type plants (Figures 1 and 2). Results shown in Figure 5 indicate that the acetylation levels of histone H3 at *COR* gene promoters in plants overexpressing CBF1 under non-acclimated conditions were significantly higher than in the wild type plants. The acetylation levels of histone H3 in the non-acclimated CBF1 overexpressing plants approached the acetylation levels observed for the cold acclimated wild type plants. When the CBF1 overexpressing plants were cold acclimated, the acetylation levels of histone H3 increased further.

We conclude that CBF1 is sufficient to drive the acetylation of histone H3 at the *COR* gene promoters even without cold acclimation. Although overexpression of CBF is sufficient to drive acetylation of histone H3 at the *COR* gene promoters, it is not sufficient for the further increase in acetylation of histone H3 upon cold acclimation. This increase could be due to the activation of endogenous CBFs or some other transcription factors upon cold acclimation.

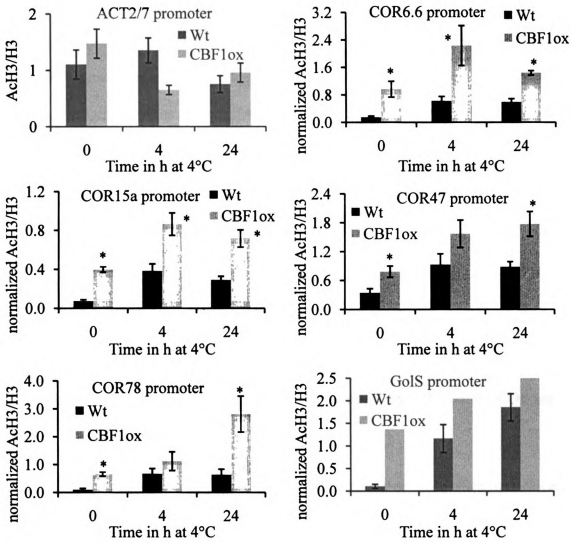


Figure 5: Ratio of acetylated histone H3 to total histone H3 at *COR* gene promoters increases upon cold acclimation. ChIP assays were performed on wild-type and CBF1 over-expressing plants cold acclimated at 4°C for 0, 4 and 24 hours using antibodies specific for acetylated histone H3 (K9, K14) and the C-terminal of histone H3. Samples were analyzed using real-time PCR to quantify promoter DNA enriched for acetylated histone H3 and total histone H3. Ct values obtained were normalized to values obtained for *ACT2/7* promoter. Data were represented as ratio of acetylated histone H3 to total histone H3 at various *COR* gene promoters. The Ct values for the *ACT2/7* promoter were not normalized and represented as ratio of acetylated histone H3 to total histone H3. Results for the wild type plants were taken from figure 1. Error bars represent SE where N (number of experiments) = 3, except N=1 for *GolS*. Comparison between wild type and CBF1ox plants was carried out using the t-test. * represents values of CBF1ox plants significantly different than wild type plants at $p \leq 0.05$ at that particular time point.

The levels of histone H3 occupancy at *COR* genes were also examined. The results shown in figure 6 reveal that the occupancy of histone H3 at the *COR6.6, COR15a* and *COR78* promoters in CBF1 overexpressing plants under non-acclimated conditions were significantly lower as compared to the level of histone H3 in Ws wild type plants. These levels were not further depleted upon cold-acclimation. The levels of histone H3 at these promoters were similar to the levels of histone H3 after 24 h of cold acclimation in wild type plants.

Thus, CBF1 is sufficient to decrease the occupancy of histone H3 at the *COR6.6, COR78* and *GolS* promoters without cold acclimation.

2.3.5 The activation domain of CBF is not necessary to drive acetylation of histone H3 upon cold acclimation but is required for the removal of histone H3 from the *COR* gene promoters.

The previous section indicated that CBF overexpression was sufficient to drive histone H3 acetylation and depletion of nucleosomes at the *COR* gene promoters. To find out whether the activation domain of CBF is necessary to drive the acetylation of histone H3 at the *COR* gene promoters, a mutant form of CBF was used. This mutant form of CBF (CBF2ΔC) contains an intact DNA-binding domain but lacks the C-terminal activation domain because of a nonsense mutation in the *CBF2* gene. has a stop codon just before the activation domain and thus lacks the C-terminal activation domain. The phenotype of plants overexpressing CBF2ΔC is similar to wild type plants. Plants overexpressing CBF2ΔC are neither constitutively freezing tolerant nor do they show constitutive expression of *COR* genes. The freezing tolerance of plants overexpressing

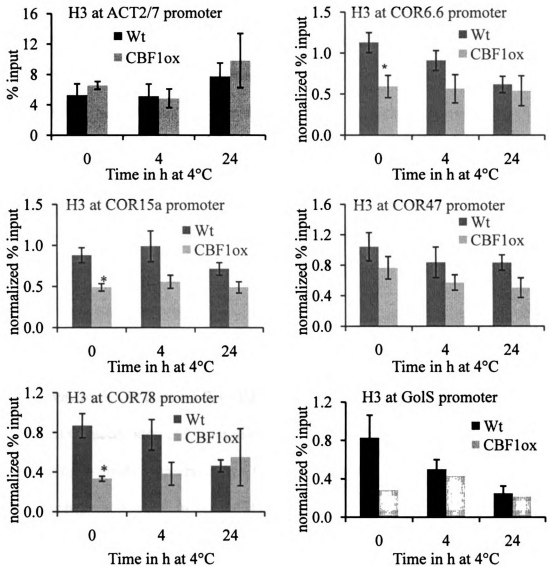


Figure 6: Level of total histone H3 at *COR* gene promoters decreases upon cold acclimation. ChIP assays were performed on wild-type and CBF1 overexpressing plants cold acclimated at 4°C for 0, 4 and 24 hours using antibodies specific for acetylated histone H3 (K9, K14) and the C-terminal of histone H3. Samples were analyzed using real-time PCR to quantify promoter DNA enriched for acetylated histone H3 and total histone H3. Ct values obtained were normalized to values obtained for *ACT2/7* promoter. Data were represented as normalized levels of total histone H3 at various *COR* gene promoters. Ct values for the *ACT2/7* promoter were not normalized and were represented as % input. The wild type results were taken from figure 2. Error bars represent SE where N (number of experiments) = 3 except *GolS* where N = 1. Comparison between wild type and CBF1ox plants was carried out using the t-test. * represents values of CBF1ox plants significantly different than wild type plants at $p \leq 0.05$ at that particular time point.

CBF2ΔC is similar to wild type plants but the expression of *COR* genes is compromised upon cold acclimation (Gilmour, unpublished data and Doherty, unpublished data). CBF2ΔC is thought to act as a dominant negative where it is able to occupy most of the CBF binding sites and thus cause a reduction of *COR* gene expression in plants overexpressing CBF2ΔC.

To determine whether the activation domain of CBF is necessary to drive acetylation at the *COR* gene promoters, ChIP assays were carried out on plants overexpressing CBF2ΔC and cold treated at 4°C for 4 and 24 hours. Non-acclimated plants were used as controls.

Results obtained for the CBF2ΔC overexpressing plants were compared with results previously obtained for Ws wild type plants (Figures 1 and 2). Results (Figure 7) show that the acetylation of histone H3 at the *COR* gene promoters increased upon cold acclimation in plants overexpressing CBF2ΔC. The non-acclimated CBF2ΔC plants did not show hyperacetylation of histone H3 as observed in non-acclimated CBF1ox plants. Figure 8 shows that the level of histone H3 in plants overexpressing CBF2ΔC did not decrease upon cold acclimation as observed in wild type plants. The level of histone H3 before and after cold acclimation was similar in the CBF2ΔC overexpressing plants. Thus, the activation domain of CBF is not necessary for the acetylation of histone H3 at the *COR* gene promoters during cold acclimation. However, in the absence of the activation domain non-acclimated plants do not show hyperacetylation of histone H3 as observed in the plants overexpressing CBF1ox. Thus even though the activation domain is not necessary for the cold induced increase in acetylation histone H3 it seems to be required for the acetylation of histone H3 in some way. The activation domain of CBF is

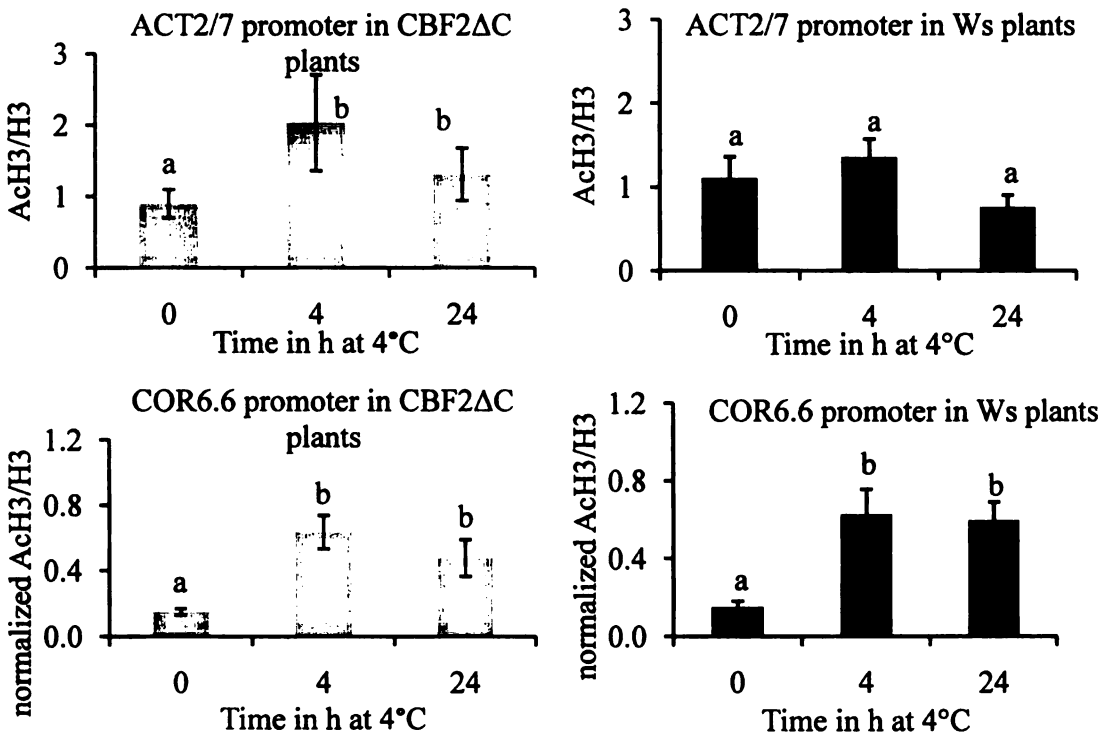
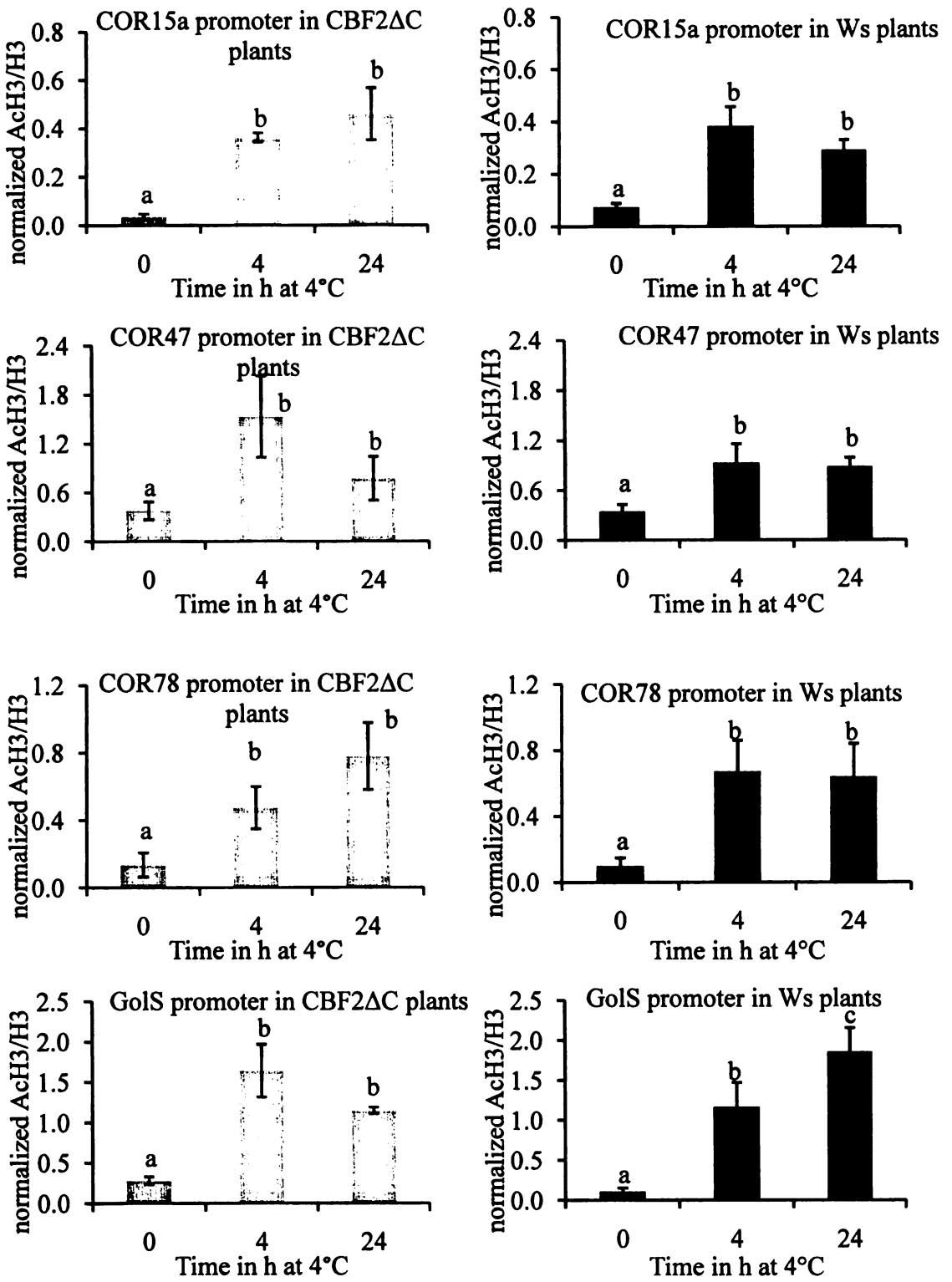


Figure 7: Level of acetylated histone H3 at *COR* gene promoters increases upon cold acclimation. ChIP assays were performed on CBF2ΔC plants cold acclimated at 4°C for 0, 4 and 24 hours using antibodies specific for acetylated histone H3 and C-terminal of histone H3. Samples were analyzed using real-time PCR to quantify promoter DNA enriched for acetylated and total histone H3. Ct values were normalized to values obtained for *ACT2/7* promoter. Data were represented as ratio of normalized levels of acetylated histone H3 to normalized levels of total histone H3 at various *COR* gene promoters. Ct values for *ACT2/7* were not normalized and represented as ratio of acetylated histone H3 to total histone H3. Data for wild type plants were taken from figure 1. Error bars represent SE where N (number of experiments) = 3. Data were analyzed using single factor ANOVA. The trend of change of histone H3 acetylation in plants overexpressing CBF2ΔC was compared with the trend observed in Ws wild type plants. Values of bar graphs denoted by the same letter are not significantly different as determined by LSD at $p \leq 0.05$.

Figure 7 continued



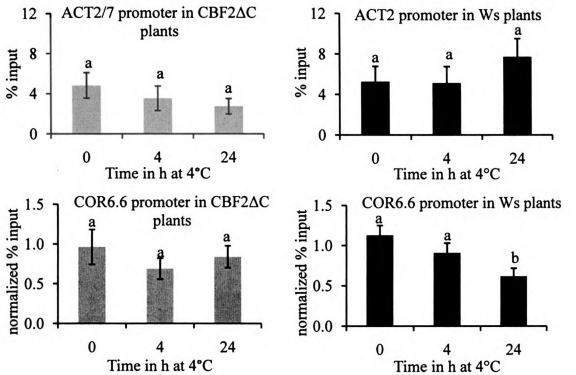
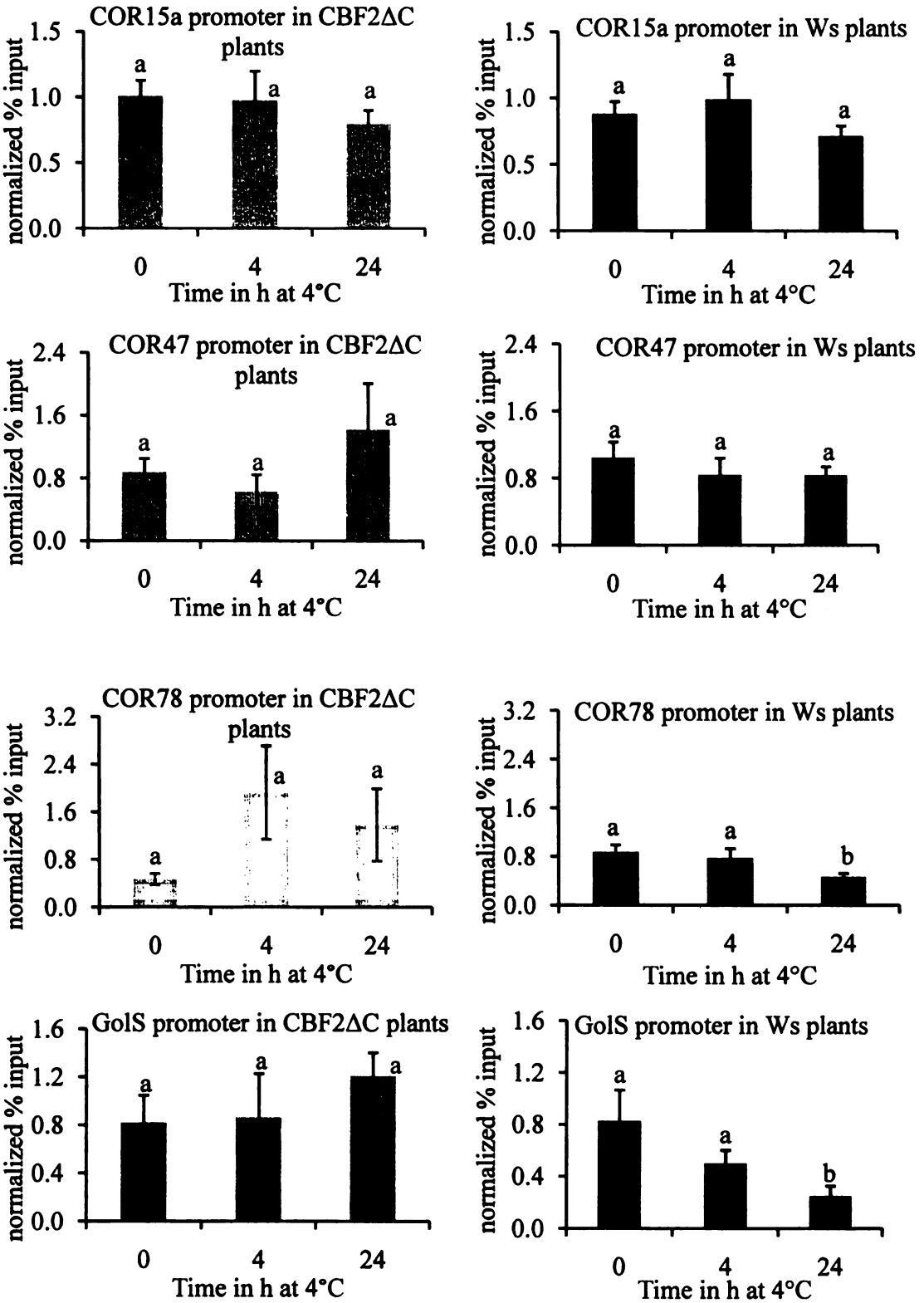


Figure 8: Level of total histone H3 at *COR* gene promoters remains unchanged upon cold acclimation in CBF2ΔC plants. ChIP assays were performed on CBF2ΔC plants cold acclimated at 4°C for 0, 4 and 24 hours using an antibody specific for the C-terminal of histone H3. Samples were analyzed using real-time PCR to quantify promoter DNA enriched for total histone H3. Ct values were normalized to values obtained for *ACT2/7* promoter. Data represented as normalized levels of total histone H3 at various *COR* gene promoters. Ct values or the *ACT2/7* promoter were not normalized and represented as % input. Error bars represent SE where N (number of experiments) = 3. Data for wild type plants were obtained from figure 2. Data were analyzed using single factor ANOVA. The trend of change of histone H3 acetylation in plants overexpressing CBF2ΔC was compared with the trend observed in Ws wild type plants. Values of bar graphs denoted by the same letter are not significantly different as determined by LSD at $p \leq 0.05$.

Figure 8 continued



also required for the loss of histone H3 occupancy at the *COR6.6, COR78* and *GolS* promoters during cold acclimation.

These experiments reveal three interesting aspects of CBF activity:

1. The histone H3 acetylation profiles of plants over-expressing CBF2ΔC were similar to wild type plants. These plants did not show constitutively high levels of acetylated histone H3 like plants over-expressing CBF. Thus, the activation domain of CBF is necessary to drive acetylation of histone H3 without cold acclimation.
2. The *COR* gene expression levels in the CBF2ΔC over-expressing plants upon cold acclimation were lower as compared to wild type plants, but the acetylation of histone H3 did not seem to be affected in these plants and was similar to wild type plants. Thus, the DNA-binding domain of CBF is sufficient to recruit chromatin modifying factors necessary to carry out acetylation of histone H3 at the *COR* gene promoters but is not able to activate transcription. It is also possible that other cold induced transcription factors bring about the increase in histone H3 acetylation at *COR* gene promoters but CBF2ΔC blocks the trans activation of *COR* genes during cold acclimation.
3. The levels of total histone H3 did not decrease at the *COR6.6, COR78* and *GolS* promoters in the CBF2ΔC plants upon cold acclimation. Thus the activation domain of CBF is necessary to recruit chromatin remodelers that can clear histone H3 from the *COR* gene promoters and the DNA-binding domain lacks this ability.

Thus, overall we can conclude that the activation domain of CBF is necessary to drive acetylation of histone H3 and the removal of histone H3 at the *COR* gene promoters in

the absence of cold acclimation. The activation domain of CBF is not necessary to drive acetylation of histone H3 at the *COR* gene promoters upon cold acclimation as overexpression of the DNA-binding domain is sufficient to increase acetylation of histone H3 upon cold acclimation. The activation domain of CBF seems necessary for the removal of histone H3 at the *COR* gene promoters during cold acclimation as overexpressing the DNA-binding domain by itself is unable to bring about this change.

2.3.6 GCN5 is not required for the acetylation of histone H3 at the *COR* gene promoters but is required for the removal of histone H3 from the *COR* gene promoters during cold acclimation.

Arabidopsis GCN5, a histone H3 specific HAT, can physically interact with the activator CBF *in vitro* [106]. CBFs bind to the CRT/DREs cis-elements in the promoters of *COR* genes and activate *COR* gene expression upon cold acclimation [68, 98]. *gcn5-1* mutants show a delay in the activation and a reduction in the overall expression of *COR* genes during cold acclimation [37]. Thus we hypothesized that, upon cold acclimation, CBF recruits a GCN5 containing complex to the *COR* gene promoters. This complex in turn acetylates histone H3 at the *COR* gene promoters and facilitates the activation of *COR* genes. According to this model, the acetylation of histone H3 at the *COR* gene promoters in *gcn5-1* plants would be hampered, resulting in a delay in the activation and reduction in the overall expression *COR* genes. To test this hypothesis, ChIP assays were carried out in *gcn5-1* plants that were cold acclimated at 4°C for 4 or 24 hours. Plants grown at 22°C were used as controls.

Results obtained for the *gcn5-1* plants were compared with results previously obtained for Ws wild type plants (Figures 1 and 2). Figure 9 shows that the acetylation of

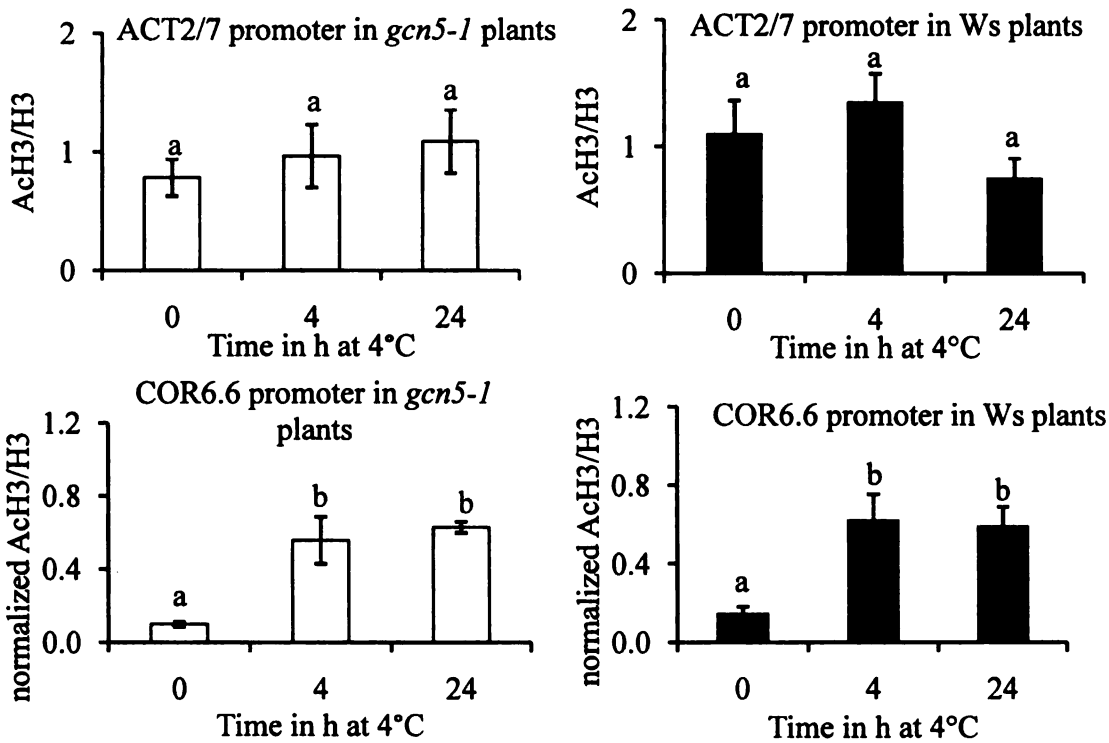
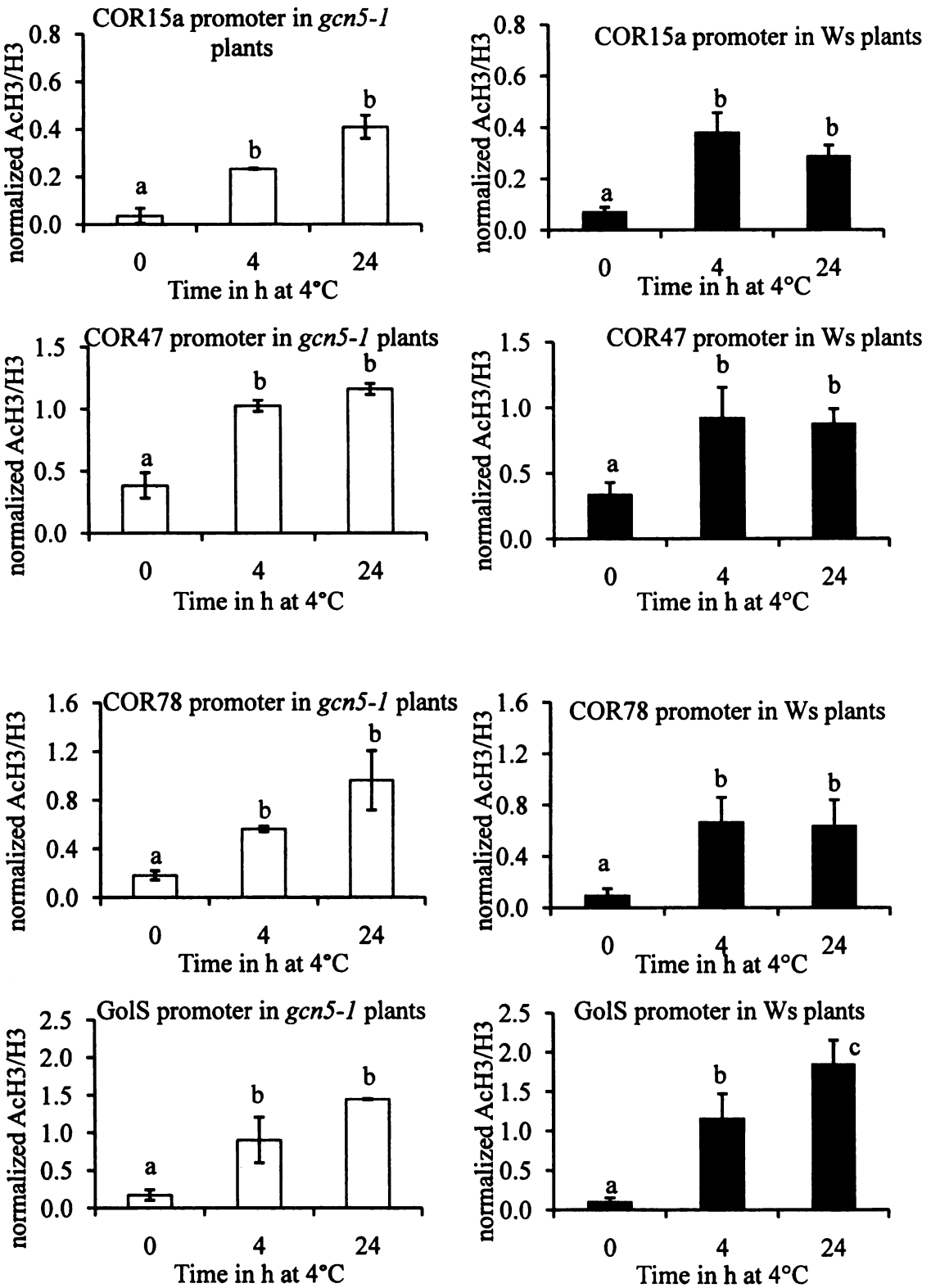


Figure 9: Ratio of acetylated histone H3 to total histone H3 at *COR* gene promoters increases upon cold acclimation. ChIP assays were performed on wild-type and *gcn5-1* plants cold acclimated at 4°C for 0, 4 and 24 hours using antibodies specific for acetylated histone H3 (K9, K14) and the C-terminal of histone H3. Samples were analyzed using real-time PCR to quantify promoter DNA enriched for acetylated histone H3 and total histone H3. Ct values obtained were normalized to values obtained for *ACT2/7* promoter. Data were represented as ratio of acetylated histone H3 to total histone H3 at various *COR* gene promoters. Ct values for the *ACT2/7* promoter were not normalized and represented as ratio of acetylated histone H3 to total histone H3. Data for wild type plants were obtained from figure 1. Error bars represent SE where N (number of experiments) = 3. The trend of change of histone H3 levels upon cold acclimation over time in wild and *gcn5-1*plants was analyzed using single-factor ANOVA. Values of bar graphs denoted by the same letter are not significantly different as determined by LSD at $p \leq 0.05$.

Figure 9 continued



histone H3 at the *COR* gene promoters increased upon cold acclimation in *gcn5-1* plants to a degree similar to that observed for Ws wild type plants. Low levels of acetylated histone H3 were observed at all the *COR* gene promoters in non-acclimated control plants in the *gcn5-1* mutants. The acetylation levels of histone H3 at the *COR* gene promoters increased at least two-fold after 4 h of cold acclimation and high levels of acetylated histones were maintained after 24 h. Interestingly, results in figure 10 show that the decrease in the occupancy of histone H3 at the *COR6.6, COR78* and *GolS* promoters in *gcn5-1* plants was not significant upon cold acclimation as observed in wild type plants. The histone occupancy at the *COR78* and *GolS* promoters did not decrease after 24 h of cold acclimation as observed in wild type plants. Surprisingly, loss of histone H3 was observed at the *COR15a* promoter after 24 h of cold acclimation in the *gcn5-1* plants. Such a decrease in histone H3 occupancy was not observed in wild type plants. The level of histone H3 at the *COR15a* promoter in non-acclimated *gcn5-1* plants was higher than in non-acclimated wild type plants. The levels of histone H3 in the *gcn5-1* and wild type plants were similar after cold acclimation. Thus the decrease in the occupancy of histone H3 at the *COR15a* promoter in *gcn5-1* plants could be because of the higher level of histone H3 in non-acclimated plants.

We can conclude from the results that GCN5 is not required for acetylation of histone H3 at the *COR* gene promoters during cold acclimation. However, GCN5 seems to be required for the loss of histone H3 occupancy at the *COR6.6, COR78* and *GolS* promoters during cold acclimation. Nevertheless, the expression levels of *COR* genes are delayed and lowered in these mutants. Thus GCN5 might regulate the expression of *COR* genes independent of its HAT activity.

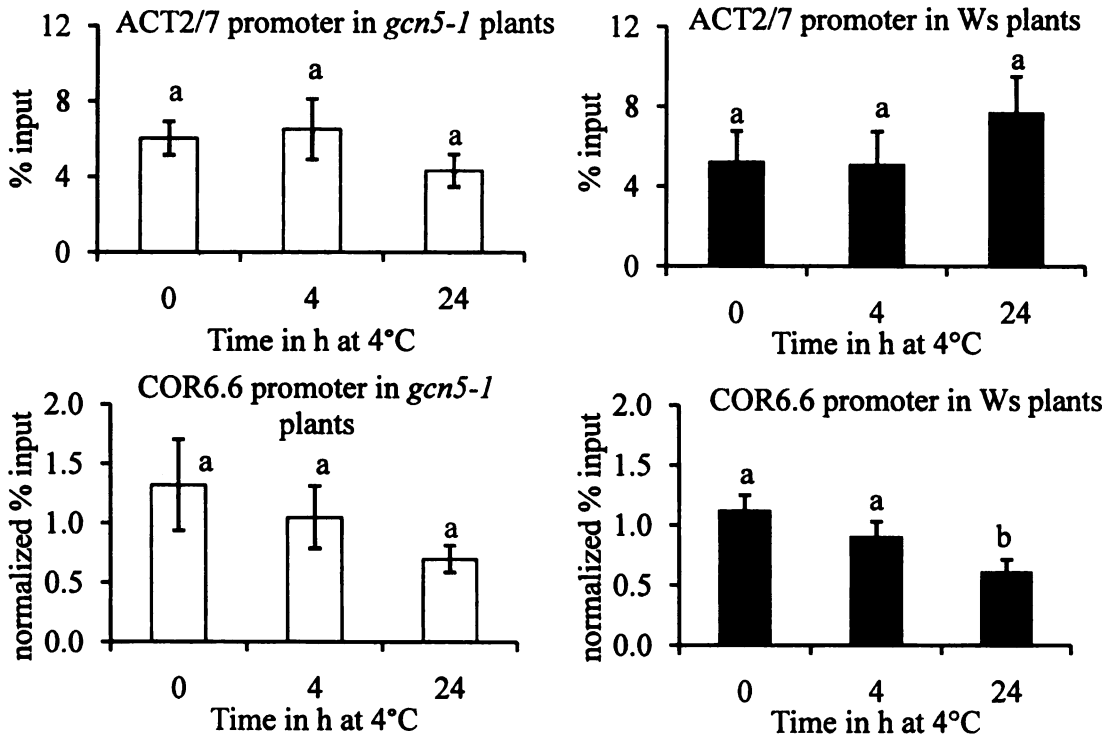
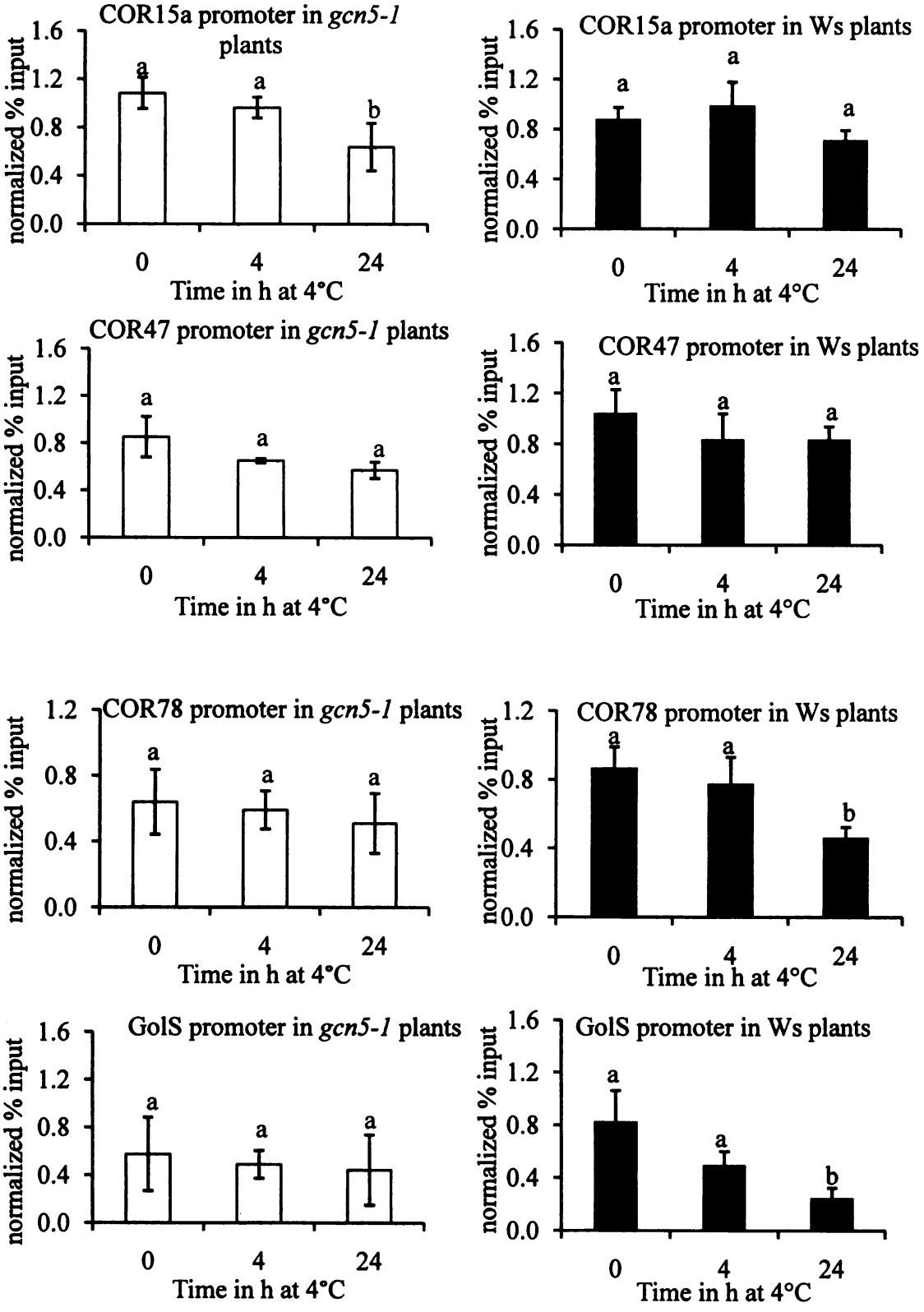


Figure 10: Level of total histone H3 at *COR* gene promoters decreases upon cold acclimation in wild type plants but not in *gcn5-1* plants. ChIP assays were performed on wild-type and *gcn5-1* plants cold acclimated at 4°C for 0, 4 and 24 hours using an antibody specific for the C-terminal of histone H3. Samples were analyzed using real-time PCR to quantify promoter DNA enriched for total histone H3. Ct values obtained were normalized to values obtained for *ACTIN2* promoter. Data were represented as normalized levels of total histone H3 at various *COR* gene promoters. Ct values for the *ACT2/7* promoter were not normalized and represented as % input. Data for wild type plants were taken from figure 2. Error bars represent SE where N (number of experiments) = 3. The trend of change of histone H3 levels upon cold acclimation over time in wild and *gcn5-1* plants was analyzed using single-factor ANOVA. Values of bar graphs denoted by the same letter are not significantly different as determined by LSD at $p \leq 0.05$.

Figure 10 continued



2.3.7 ADA2b is not necessary for the acetylation of histone H3 at the *COR* gene promoters but is required for the removal of histone H3 from the *COR* gene promoters during cold acclimation.

Arabidopsis GCN5 is a histone H3 specific histone acetyltransferase and its ability to acetylate nucleosomal histones is enhanced by the presence of ADA2b [51, 106]. ADA2b can physically interact with GCN5 and CBF *in vitro*. *ada2b-1* plants show delayed activation and an overall reduction in the expression of the *COR* genes activated by CBF. Thus we hypothesized that upon cold acclimation CBF recruits a GCN5-ADA2b containing complex at the *COR* gene promoters. This complex in turn acetylates histone H3 at the *COR* gene promoters and facilitates the activation of *COR* genes. According to this model, acetylation of histone H3 at the *COR* gene promoters in the *ada2b-1* plants would be hampered resulting in a delay in the activation and reduction in the overall expression *COR* genes. In order to test this hypothesis, ChIP assays were carried out in *ada2b-1* plants cold acclimated at 4°C for 4 or 24 hours. Non-acclimated plants were used as controls.

Results obtained for the *ada2b-1* plants were compared with results previously obtained for Ws wild type plants (Figures 1 and 2). Results (Figure 11) show that the acetylation of histone H3 at all the *COR* gene promoters except the *COR47* promoter increased upon cold acclimation in *ada2b-1* plants. The increase in acetylation of histone H3 at the *COR47* did not increase significantly at $p \leq 0.05$ as determined by ANOVA. The large variation observed in the 24 h cold acclimated sample was probably the reason

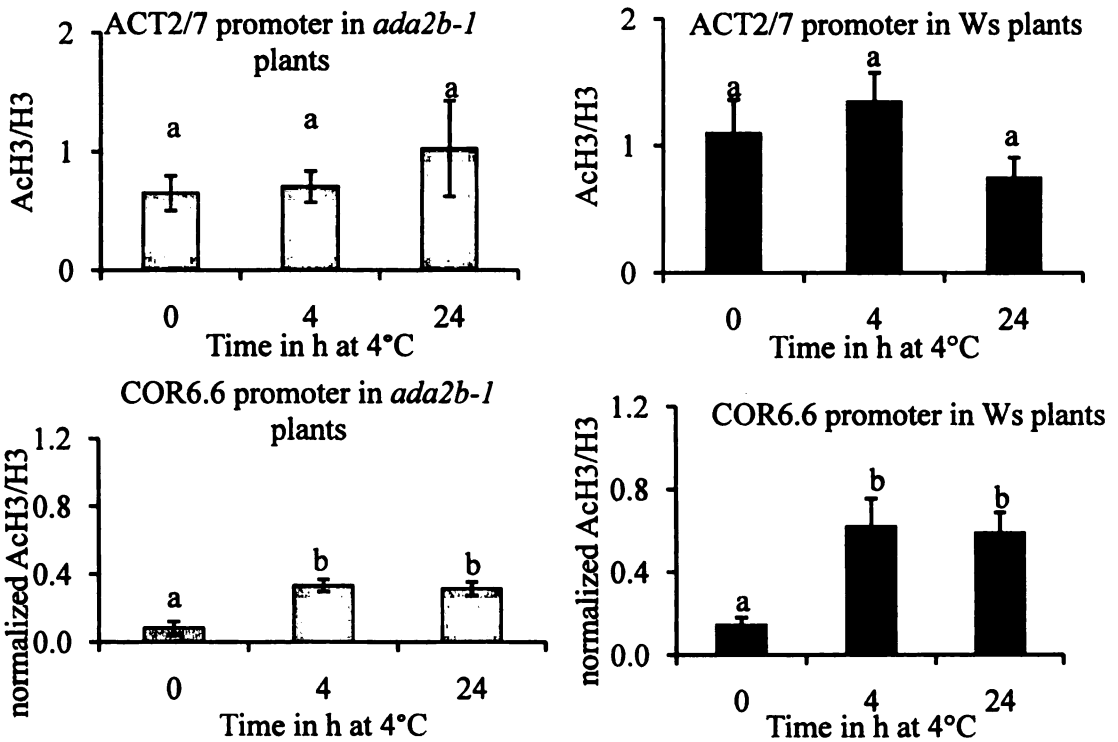
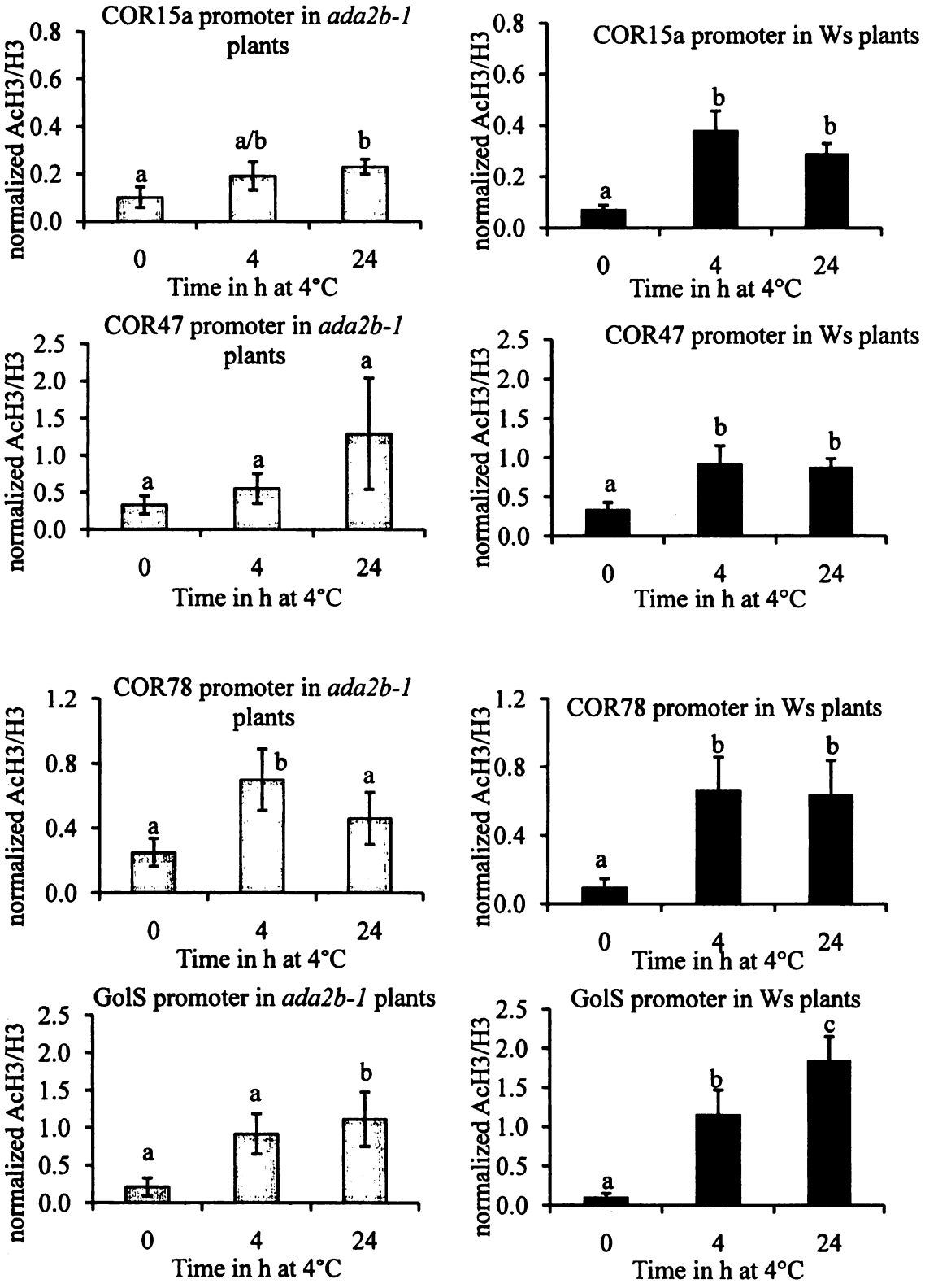


Figure 11: Ratio of acetylated histone H3 to total histone H3 at *COR* gene promoters increases upon cold acclimation. ChIP assays were performed on wild-type and *ada2b-1* plants cold acclimated at 4°C for 0, 4 and 24 hours using antibodies specific for acetylated histone H3 (K9, K14) and the C-terminal of histone H3. Samples were analyzed using real-time PCR to quantify promoter DNA enriched for acetylated histone H3 and total histone H3. Ct values obtained were normalized to values obtained for *ACT2/7* promoter. Data were represented as ratio of acetylated histone H3 to total histone H3 at various *COR* gene promoters. Ct values for *ACT2/7* promoter were not normalized and were represented as ratio of acetylated histone H3 to total histone H3. Data for wild type plants were taken from figure 1. Error bars represent SE where N (number of experiments) = 3. The trend of change of histone H3 levels upon cold acclimation over time in wild and *ada2b-1*plants was analyzed using single-factor ANOVA. Values of bar graphs denoted by the same letter are not significantly different as determined by LSD at $p \leq 0.05$.

Figure 11 continued



for this.

We can conclude from the results that ADA2b is not required for the acetylation of histone H3 at the *COR* gene promoters during cold acclimation.

Results in Figure 12 show that the occupancy of histone H3 in *ada2b-1* plants did not decrease at the *COR6.6, COR78* and *GolS* promoters even after 24 h of cold acclimation as observed in wild type plants. We conclude that ADA2b is required for the decrease in histone H3 occupancy at the *COR6.6, COR78* and *GolS* promoters during cold acclimation.

2.4 Discussion:

Any valid model for mechanisms of inducing eukaryotic gene regulation in response to external signals must account for the barrier imposed by chromatin. The standard mechanism as developed largely in yeast experimental models involves both histone-modifying enzymes (especially HATs) and remodeling enzymes. This chapter reports evidence that several features of this standard model apply to the activation of *COR* genes upon cold acclimation. My results reveal that histone H3 at the *COR* gene promoters is hypoacetylated in non-acclimated plants but the acetylation of histone H3 at the *COR* gene promoters increases upon cold acclimation in wild type plants. The occupancy of histone H3 decreases at some *COR* gene promoters (*COR6.6, COR78* and *GolS*) after 24 h of cold acclimation. Acetylation of histone H3 seems to precede removal of histone H3 at these *COR* gene promoters. The increase in acetylation is a dynamic change occurring within 4 hours of cold stress and stable levels of acetylation are

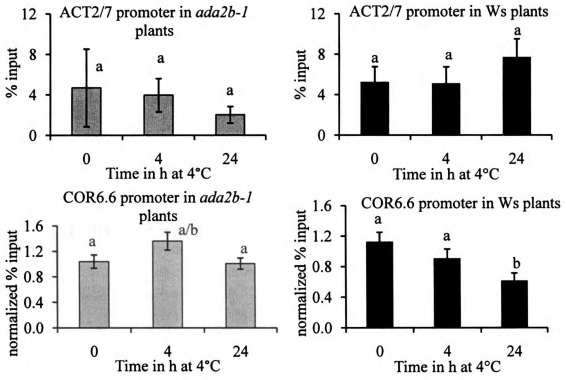
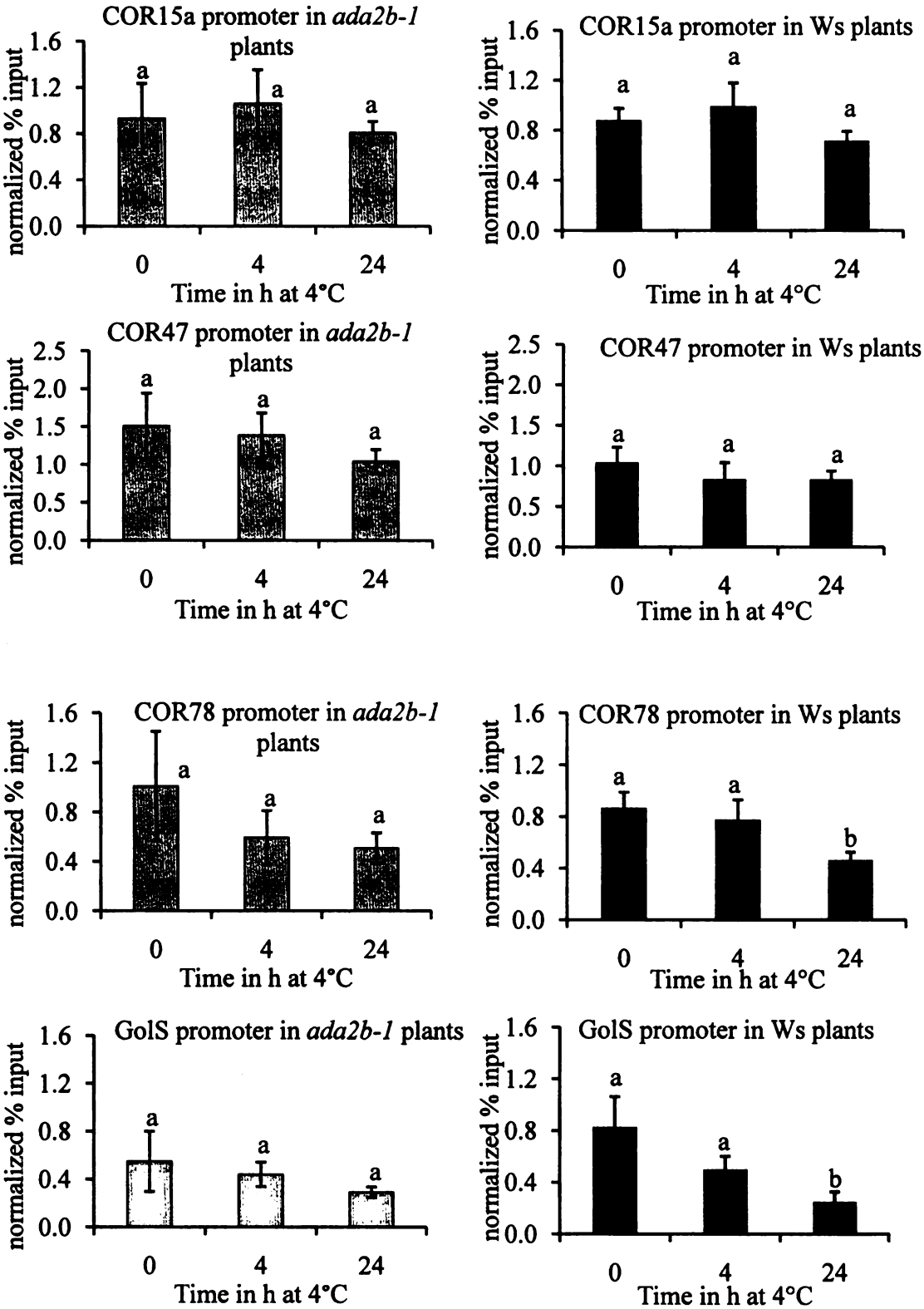


Figure 12: Level of total histone H3 at *COR* gene promoters decreases upon cold acclimation in wild type plants but not in *ada2b-1* plants. ChIP assays were performed on wild-type and *ada2b-1* plants cold acclimated at 4°C for 0, 4 and 24 hours using an antibody specific for the C-terminal of histone H3. Samples were analyzed using real-time PCR to quantify promoter DNA enriched for total histone H3. Ct values obtained were normalized to values obtained for *ACT2/7* promoter. Data were represented as normalized levels of total histone H3 at various *COR* gene promoters. Ct values for *ACT2/7* were not normalized and were represented as % input. Data for wild type plants was obtained from figure 2. Error bars represent SE where N (number of experiments) = 3. The trend of change of histone H3 levels upon cold acclimation over time in wild and *ada2b-1* plants was analyzed using single-factor ANOVA. Values of bar graphs denoted by the same letter are not significantly different as determined by LSD at $p \leq 0.05$.

Figure 12 continued



maintained even after 24 hours. Upon de-acclimation the acetylation level of histone H3 decreases to non-acclimated levels in 24 hours and the levels of total histone H3 are restored to levels similar to non-acclimated plants. Acetylation of histones and depletion of nucleosomes seem to be hallmarks of the *COR* gene activation process in *Arabidopsis* as in other eukaryotes [110, 112, 113].

The *COR* genes are a set of cold-regulated genes whose transcription is stimulated by CBF. Other transcription factors also bind to *COR* genes and so it was important to dissect whether CBF itself was responsible for the chromatin modifications at the *COR* gene promoters. Results show that overexpression of CBF1 is sufficient to drive the acetylation and depletion of histone H3 from the *COR* gene promoters in the absence of cold acclimation. The level of acetylated histone H3 further increased when the plants were cold-acclimated, which can probably be attributed to the function of the endogenous CBF proteins or to the activity of other transcription factors induced during cold acclimation. To determine whether the activation domain of CBF is necessary to drive the acetylation of histone H3 at the *COR* gene promoters, ChIP assays were carried out in plants overexpressing a mutant form of CBF, lacking the C-terminal activation domain (CBF2ΔC). Plants overexpressing CBF2ΔC showed lower *COR* gene expression as compared to wild type plants. ChIP assays revealed that the activation domain of CBF is not necessary to drive acetylation of histone H3 upon cold acclimation but is required for the removal of histone *H3* from some *COR* gene promoters. However, overexpression of CBF2ΔC did not lead to increased acetylation of histone H3 in non-acclimated plants as observed in plants overexpressing CBF. Thus, the activation domain might play a role in driving acetylation of histone H3 in the absence of cold signal. The activation and DNA-

binding domains of CBF seem to control different aspects of chromatin remodeling during cold acclimation. The DNA-binding domain of CBF might recruit chromatin modifying proteins that can acetylate histone H3 whereas the activation domain might recruit chromatin remodeling enzymes. The GCN5 and ADA2 proteins in *Arabidopsis* physically interact with the DNA binding domain of CBF *in vitro* which is unique given that most activator proteins bind coactivators via their activation domain. Although this is an unusual finding in *Arabidopsis* examples of coactivator proteins binding to DNA-binding domains have been observed in eukaryotes [114]. Thus CBF might be able to recruit ADA2b-GCN5 containing chromatin modifying complexes to the *COR* gene promoters via interaction with its DNA binding domain.

Activation domains of transcription factors are known to bind members of coactivator complexes. Mutation of an activation domain can lead to loss of binding of these coactivator complexes and also reduce their activation potential [89, 115]. The CBF2ΔC form of CBF retains the ability to acetylate histone H3 after cold acclimation but cannot activate *COR* gene expression to wild type levels. However, the CBF2ΔC form of CBF is unable to deplete nucleosomes from the *COR* gene promoters. The activation domain might recruit chromatin remodeling factors that have the ability to remove nucleosomes off promoters and regulate *COR* gene expression. Deletion of the activation domain of CBF seems to affect its activation potential but not its ability to bring about histone modifications. Thus, acetylation of histone H3 seems to precede gene activation and acetylation at lysines 9/14 of histone H3 is not sufficient for transcription.

GCN5, a histone acetyltransferase, and its likely interaction partner protein, ADA2b, were prominent candidates for the role of carrying out histone H3 acetylation at

the *COR* gene promoters. The two proteins can interact in vitro not only with each other but also with CBF *in vitro*. Homozygous mutants of *GCN5* and *ADA2b* show a delay in activation and two-fold lower expression of *COR* genes upon cold acclimation. However, ChIP assay results show that the increase in acetylation of histone H3 in the *gcn5-1* mutant is similar to the increase in wild type plants upon cold acclimation. Thus GCN5 is not required for the acetylation of histone H3 (lysines 9/14) at the *COR* gene promoters. Nonetheless, GCN5 is required for the removal of histone H3 from the *COR* gene promoters, as the occupancy of histone H3 did not decrease in the *gcn5-1* mutants upon cold acclimation. Therefore, a GCN5-containing complex might be directly required for the histone H3 removal from the *COR* gene promoters. Alternatively, the function of such a complex might be essential for the recruitment and removal of histone H3 by an ATP-dependent chromatin remodeling complex. In addition to functioning as a histone acetyltransferase, GCN5 can also act as an adaptor protein facilitating the binding of the general transcription factors and the activator at the promoter of target genes. Thus deleting GCN5 might delay the recruitment of the general transcription factors at the *COR* gene promoters and in turn cause a delay in the activation of transcription.

The acetylation of histone H3 at the *COR* gene promoters increases in the *ada2b-1* mutants upon cold acclimation. Thus, ADA2b is not required to bring about acetylation of histone H3 at the *COR* gene promoters upon cold acclimation. ADA2b is required for the loss of histone H3 occupancy at the *COR6.6, COR78* and *GolS* promoters. The human ADA2β protein can interact *in vitro* with the chromatin remodeling enzyme BRG1 and activate gene expression [61]. The *Arabidopsis* ADA2b might be able to interact with chromatin remodeling complexes and regulate *COR* gene expression.

ADA2b and GCN5 are known to act as adaptors facilitating the interaction of activators with the general transcription factors and thus activating transcription [84]. GCN5 and ADA2b are parts of large multi-subunit complexes that act to recruit TBP to promoters and thus facilitate transcriptional activation [116]. Absence of either GCN5 or ADA2b might disrupt such a complex or de-stabilize the complex which in turn causes delay in the activation of target genes and lower transcription. In addition to the HAT domain, GCN5 also contains a bromodomain that is known to bind acetylated histones as well as other transcription factors [66]. The bromodomain of GCN5 may be required for the recruitment of the chromatin remodeling enzymes that remove nucleosomes from the *COR* gene promoters.

Recent studies show that the expression of *COR* genes is not solely CBF-dependent but is a result of the interplay of various transcription factors (Doherty, unpublished data). Thus, GCN5-ADA2b might be regulating the expression of *COR* genes in a CBF-independent manner. It will be interesting to find out whether GCN5 and /or ADA2b interact with transcription factors that regulate *COR* gene expression in a CBF-independent manner.

Chapter III

The activation of *COR* genes by CBF occurs in an ADA2b and GCN5 independent manner.

3.1 Introduction:

Cold acclimation is the process by which exposure to low non-freezing temperatures increases the ability of some plants to survive freezing stress [117]. The CBF family of transcription activators acts as 'master regulators' during the cold acclimation pathway in *Arabidopsis* [118]. In wild type plants, the expression of the CBF family of activators is induced within 15 minutes of cold stress, which in turn leads to the transcriptional activation of the cold-regulated (*COR*) genes [68]. Overexpression of the CBF proteins is sufficient to drive *COR* gene expression and causes the plants to be constitutively freezing tolerant [93, 94]. CBF can bind to specific cis-elements in the promoters of these genes known as the cold/dehydration responsive elements (CRT/DREs) and activate their expression [98]. Studies in yeast have shown that the *Arabidopsis* CBF1, CBF2 and CBF3 proteins activate the expression of a reporter gene fused to the CRT promoter elements. One-hybrid study conducted in yeast showed that the activation of the reporter gene fused to the CRT by CBF depends upon the presence of two co-activator proteins ADA2 and GCN5 [50].

Arabidopsis encodes two homologues of ADA2 (ADA2a and ADA2b) and one homologue of GCN5. Homozygous null mutants *ada2b-1* and *gcn5-1* show pleiotropic phenotypes whereas the *ada2a-1* mutant is phenotypically similar to wild type plants [37]. The *ada2b-1* and *gcn5-1* mutants show a delay in activation and a two-fold

reduction in overall expression of the CBF regulated cold regulated (*COR*) genes [37]. The ability of *gcn5-1* plants to withstand freezing temperatures is similar to wild type plants whereas *ada2b-1* plants are constitutively freezing tolerant. This constitutive freezing tolerance of the *ada2b*-1 plants is thought to occur in a CBF-independent manner because the expression of CBF or its target genes is not elevated in these mutants. ADA2b and GCN5 can physically interact with each other as well as with CBF [50, 51]. *Arabidopsis* GCN5 can acetylate histone H3 *in vitro* [51].

Results from the previous chapter indicate that the acetylation of histone H3 increases at the *COR* gene promoters upon cold acclimation. GCN5 is not required for the acetylation of histone H3 at the *COR* gene promoters but is required for the depletion of histone H3 from some *COR* gene promoters. ADA2b is required for driving the increase in acetylation at some *COR* gene promoters. ADA2b and GCN5 can physically interact with CBF *in vitro*. Whether they interact with CBF *in vivo* and regulate the expression of *COR* genes via CBF is not known. It is possible that ADA2b and GCN5 control *COR* gene expression independently of CBF.

To determine whether the CBF-dependent activation of *COR* genes requires ADA2b and/or GCN5, two approaches were used. In the first approach, a promoter-reporter plasmid was constructed which contained four tandem repeats of the CRT fused to the reporter gene *GUS*. The expression of the reporter gene in wild type plants was compared with the expression in the *ada2b-1* and *gcn5-1* plants upon cold acclimation. The rationale behind this approach was that CBF regulates the expression of the *COR* genes via the CRTs. The promoters of the *COR* genes contain various other cis-elements that could be potentially be regulated by non-CBF transcription factors [119]. Thus

fusing a reporter gene specifically to the CRTs would render that promoter-reporter gene solely under the regulation by CBFs. Any difference between the expression of the reporter gene between wild type and mutant plants would be due to a regulation of the reporter gene via a CBF dependent pathway. Thus by using a CRT driven promoter we are eliminating the possibility that the reporter gene could be regulated by non-CBF transcription factors. In the second approach, the CBF2 gene was overexpressed in the *ada2b-1* and *gcn5-1* background. The expression of the *COR* genes in these plants was compared with plants that overexpressed CBF2 in the wild type background without cold acclimation. Overexpression of CBF leads to constitutive expression of *COR* genes without the requirement of cold stress. Thus any difference in *COR* gene expression in between wild type and mutant plants overexpressing CBF must likely occur in a CBF dependent manner.

3.2 Materials and methods:

3.2.1 Construction of *ADA2b-1+/-* and *GCN5-1+/-* lines containing the 4X-CRT-GUS promoter-reporter gene.

To make these transgenic lines, a promoter-reporter plasmid was constructed by fusing four tandem repeats of the CRT and a 35S minimal promoter to the *GUS* gene. The 35S minimal promoter fused to the *GUS* reporter gene taken from pBI101.1 plus (Van Buskirk, unpublished data) was inserted into the pCAMBIA3300 vector (Cambia, Canberra, Australia) that encodes kanamycin resistance (for selection in bacteria) and glufosinate (Basta) resistance for selection in plants. A double-stranded oligonucleotide bearing four copies of CRT was then inserted between the *XbaI* and *BamHI* sites of the pCAMBIA3300 plasmid. The plasmid containing the 4X-CRT-35Sminimal-GUS

sequence in the pCAMBIA3300 vector is now referred to as 4X-CRT-GUS. This 4X-CRT-GUS clone was then transformed into electrocompetent *Agrobacterium tumifaciens* GV3101 (pMP90) cells. The *Agrobacterium* cells transformed with the plasmid were then used to transform *ADA2b-1* +/- and *GCN5-1* +/- plants. The seeds from the transformed plants were repeatedly selected for Basta resistance through three generations (T3) to homozygosity. Two lines containing 4X-CRT-GUS in the *ADA2b-1* +/- background (lines # 1and 14) and six lines in the *GCN5-1* +/- background (lines # 2, 4, 5, 6, 9 and 10) were selected for gene expression analysis.

3.2.2 Creating wild type, *ada2b-1* and *gcn5-1* plants overexpressing CBF2

ADA2b-1 +/- and *GCN5-1* +/- plants were transformed with *Agrobacterium* cells transformed with a pCAMBIA3300 plasmid containing the *CBF2* gene driven by the 35S promoter (Sarah Gilmour, unpublished data). *ADA2b-1* +/- and *GCN5-1* +/- plants containing the transgenic *CBF2* were selected on Basta plates and confirmed by genotyping. Six lines containing the transgenic *CBF2* in *ADA2b-1* +/- background (lines 1, 5, 16, 22, 41 and 107) and three lines containing the transgenic *CBF2* in *GCN5-1* +/- background (lines 1, 3 and 1S2) were selected for gene expression analysis.

3.2.3 RNA isolation and gene expression analysis

Six to eight 15 day old whole seedlings were harvested in eppendorf tubes and flash frozen in liquid nitrogen. The tissue was stored at -80°C until further use. For RNA extraction the frozen tissue was ground in liquid nitrogen using a pestle. RNA extraction was carried out using 0.5 ml of cold (4°C) Plant RNA Reagent from Invitrogen per 0.1 gram tissue. The mix was briefly vortexed and then the tube was incubated by laying it horizontally at room temperature for 5 minutes. The solution was clarified by

centrifuging for 2 minutes at 12,000 x g in a microcentrifuge at room temperature. The supernatant was transferred to an RNase-free tube and then 0.1 ml of 5 M NaCl was added to the clarified extract. The solution was mixed and then 0.3 ml of chloroform added and the tube was inverted to mix the solution. The sample was centrifuged at 4°C for 10 minutes at 12,000 x g to separate the phases. The top aqueous phase was transferred to an RNase-free tube and an equal volume of isopropyl alcohol was added to it. The solution was mixed and incubated at room temperature for 10 minutes. The sample was centrifuged at 4°C for 10 minutes at 12,000 x g. The supernatant was discarded and the pellet was washed with 1 ml of 75% ethanol. The pellet was dried by removing all the residual ethanol and then dissolved in 50 µl RNase-free water to dissolve the RNA. The RNA was stored at -20°C.

DNase treatment was carried out by adding 5 µl 10X buffer and 1 µl DNase (10U) to 50 µl of RNA solution. The mix was incubated for 2 hours at 37°C. After the incubation, 50 µl water was added to the mix to increase volume. Samples were extracted using 1 volume (100 µl) phenol, chloroform, isoamyl alcohol (25:24:1 v/v). The mix was vortexed and then centrifuged at top speed in a microcentrifuge for 5 minutes. The upper aqueous phase was transferred to a new tube and RNA was precipitated by adding ½ volume 7.5 M ammonium acetate (50 µl) and 2 volumes 100% ethanol (200 µl). The resulting RNA pellet was washed with 500 µl of 75% ethanol. The purified RNA pellet was resuspended in 50 µl nuclease-free water.

Levels of specific mRNAs were assayed using reverse transcription and real-time PCR. 1 µg of total RNA was used to make cDNA using the Reverse Transcriptase kit from Promega. The final reaction volume was 20 µl. After the reaction the samples were

diluted 10 times in nuclease-free water. Real-time PCR was carried out using 10X buffer containing SYBR Green, 0.25 μM of each primer, 3 mM $MgCl_2$, 12.4 mM dNTPs and 0.15 μl Ampli Taq Gold DNA polymerase enzyme (all reagents were obtained from ABI) on the cDNA samples. 3 μl of cDNA sample was used in a 30 μl reaction. PCRs were carried out by using primers that detect the open reading frames of the *COR* genes (*COR6.6, COR15a, COR47, COR78* and *GolS*) and the *ACT3* open reading frame as a control. The Ct values thus obtained for the *COR* genes were normalized to the values obtained for the *ACT3* promoter. The values were expressed as *ACT3*- normalized expression levels of the target genes. The Ct values for the *ACT3* gene were not normalized and were represented as average Ct. t-tests were conducted to compare the expression of the target genes between wild type and mutants plants to determine whether the difference was significant at $p \leq 0.05$.

3.3 Results:

3.3.1 The 4X-CRT driven expression of the reporter gene *GUS* by CBF is neither dependent on ADA2b nor GCN5

In order to determine whether the CBF dependent expression of *COR* genes via CRTs requires ADA2b and GCN5, expression of the reporter gene *GUS* fused to 4X-CRT was compared in wild type, *ada2b-1* and *gcn5-1* plants. *ADA2b-1+/-* and *GCN5-1+/-* plants were transformed with *Agrobacterium* cells containing the 4X-CRT-GUS plasmid by the floral dip method. T3 lines homozygous for the 4X-CRT-GUS reporter gene were selected and screened for the cold-induced *GUS* expression. Lines showing at least a two-fold induction of *GUS* after cold acclimation at 4°C for 24 hours as compared

to non-acclimated plants were selected. Seeds from six lines containing the reporter gene in the *GCN5-1*+/- background and two lines containing the reporter gene in the *ADA2b-1*+/- background were sown on Gamborg media plates containing Basta in order to select for the reporter gene. Seeds from the *ADA2b-1*+/- plants generated Wt, *ADA2b-1*+/- and *ada2b-1* -/- plants. Seeds from the *GCN5-1*+/- plants generated Wt, *GCN5-1*+/- and *gcn5-1* -/- plants. The plants heterozygous for either ADA2b or GCN5 are phenotypically wild type whereas mutant plants have a dwarf phenotype. Plants heterozygous for *ADA2b* or *GCN5* have no known effect on *COR* gene expression during cold acclimation and hence this was not thought to have any effect on the expression of the reporter gene *GUS*. Wild type or mutant plants were collected before or after cold treatment at 4°C for 24 hours. Gene expression analysis was performed to compare the expression of the *GUS* reporter gene in the Wt and the mutant (*gcn5* or *ada2b*) background.

Figure 13 compares the expression of the reporter gene *GUS* between Wt and *ada2b-1* plants from two independent lines. Results are represented as expression levels of *GUS* relative to *ACT3* from two independent biological experiments for each line. The expression level of *GUS* between the two experiments showed a lot of variance in the wild type plants. In the first experiment the expression of *GUS* in wild type plants was higher as compared to *ada2b-1* plants but in the second experiment the expression levels were similar. To determine whether the difference observed in the *GUS* expression was significantly different between the wild type and *ada2b-1* plants, the data were analyzed using a paired t-test, which showed that the expression of the reporter gene *GUS* after cold acclimation was not significantly different in wild type and *ada2b-1* plants at $p \leq 0.05$.

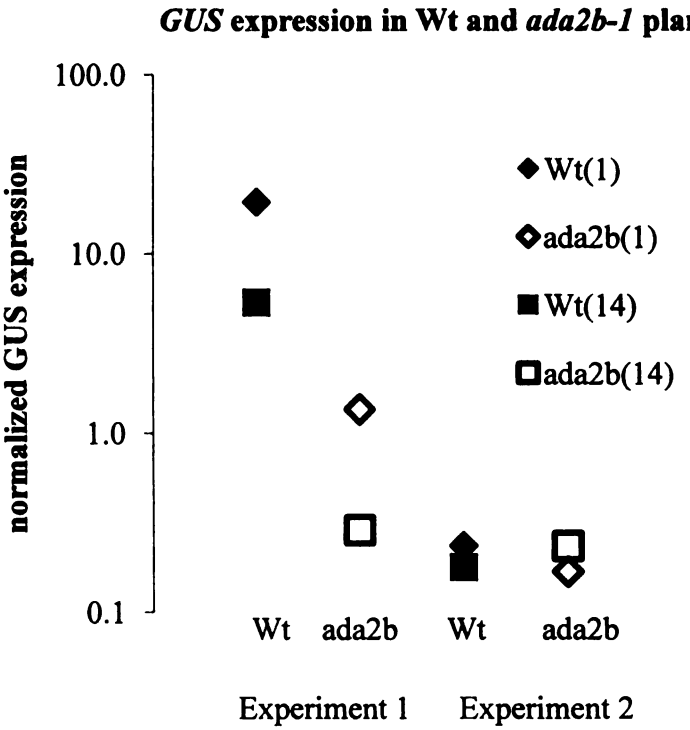


Figure 13: The expression of the reporter gene *GUS* fused to 4X-CRT is similar in wild type and *ada2b-1* plants. The expression of the reporter gene *GUS* fused to the promoter element 4X-CRT was compared in wild type and *ada2b-1* plants in two independent lines (lines 1 and 14). Gene expression analysis was performed in Ws wild type and *ada2b-1* plants after cold treatment at 4°C for 0 and 24 hours. Real-time RT PCR was performed using primers for the *ACT3* and *GUS* genes. The Ct values for the GUS gene were normalized to the Ct values for the *ACT3* gene. Results were represented as normalized expression levels of *GUS* after cold treatment at 4°C for 24 hours using the DCt method. The results shown are from two independent experiments.

The expression of the *GUS* reporter gene in wild type and *ada2b-1* plants measured in the two independent experiments show different results. In the first experiment the expression of *GUS* is lower in the *ada2b-1* plants as compared to wild type plants whereas the expression of *GUS* in the second experiment is similar in *ada2b-1* and wild type plants. Even though the paired t-test shows that the expression of *GUS* is similar in the wild type and *ada2b-1* plants at $p \leq 0.05$ we have to state that the experiment is inconclusive. We cannot conclude whether the CBF regulation of *COR* genes via CRTs requires ADA2b.

The expression of the 4X-CRT-GUS reporter gene was also compared between Wt and *gcn5-1* plants from six independent lines. Figure 14 shows the expression levels of *GUS* normalized to *ACT3* from two independent biological experiments. The expression level of *GUS* was similar in the wild type and *gcn5*-1 plants within an experiment, but the expression levels between the experiments showed a lot of variation. Expression of the *GUS* reporter gene in four out of the six independent lines were slightly higher in wild type plants as compared to *gcn5-1* mutants in the first experiment. In the second experiment expression of the *GUS* reporter gene was slightly higher in the *gcn5-1* mutants as compared to wild type plants. To determine whether the differences observed were significant, the data were analyzed using a paired t-test. Analysis of the data by paired t-test show that the difference between expression of the reporter gene *GUS* after cold acclimation is not significantly different in wild type and *gcn5-1* plants at $p \leq 0.05$.

Since the CRT-driven expression of the reporter gene *GUS* during cold acclimation was similar in wild type and *gcn5-1* plants, we conclude that the CBF driven expression of the reporter gene *GUS* fused to the 4X-CRT does not require GCN5. Thus,

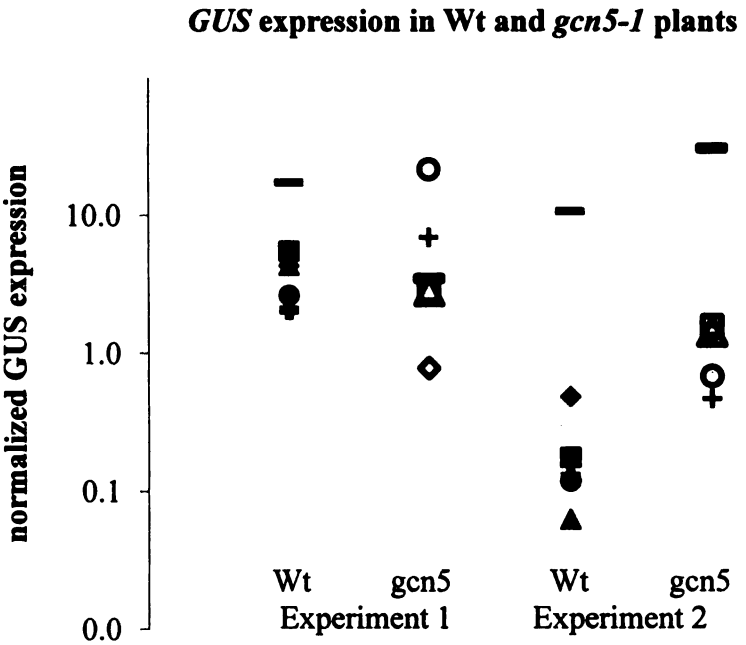


Figure 14: The expression of the reporter gene *GUS* fused to 4X-CRT is similar in wild type and *gcn5-1* plants. The expression of the reporter gene *GUS* fused to the promoter element 4X-CRT was compared in wild type and *gcn5-1* plants in six independent lines. Gene expression analysis was performed in Ws wild type and *gcn5-1* plants after cold treatment at 4°C for 0 and 24 hours. Real-time RT PCR was performed using primers for the *ACT3* and *GUS* genes. The Ct values for the *GUS* gene were normalized to the Ct values for the *ACT3* gene. Results were represented as normalized expression levels of *GUS* after cold treatment at 4°C for 24 hours using the DCt method. The results shown are from two independent experiments.

the CBF-dependent activation of *COR* gene expression via CRT elements does not require GCN5.

3.3.2 ADA2b and GCN5 are not required for the CBF-dependent expression of the *COR* genes.

Overexpression of CBF is sufficient to drive the expression of *COR* genes in non-cold-acclimated plants. Thus, the expression of *COR* genes in these plants is solely CBF-dependent. Thus any difference in *COR* gene expression in wild type, *ada2b-1* and *gcn5-1* plants overexpressing CBF would be through the CBF-dependent pathway of *COR* gene regulation. To test whether the CBF-dependent expression of *COR* genes requires ADA2b and/or GCN5, the expression of the *CBF2* and *GolS* gene was compared in wild type, *ada2b-1* and *gcn5-1* plants overexpressing CBF2. Seeds from *ADA2b-1* +/- and *GCN5-1* +/- plants overexpressing CBF2 were sown on Gamborg plates containing Basta to select for plants containing the *CBF2* transgene. Six independent lines overexpressing *CBF2* in the *ADA2b-1* +/- background and three independent lines overexpressing *CBF2* in the *GCN5-1* +/- background were chosen. Wt-like and mutant plants were identified phenotypically. Tissue from each line was harvested and used for gene expression analysis. The expression of *CBF2* and the *COR* gene *GolS* in the wild type and mutant backgrounds was assayed using real-time RT-PCR. Results in figures 15 and 16 are represented as the normalized expression levels of *CBF2* and *GolS* obtained in two biological replicate experiments.

The overexpression levels of *CBF2* varied widely in the wildtype plants derived from transformation of heterozygous ada2b plants (Fig. 15). However, the loss of

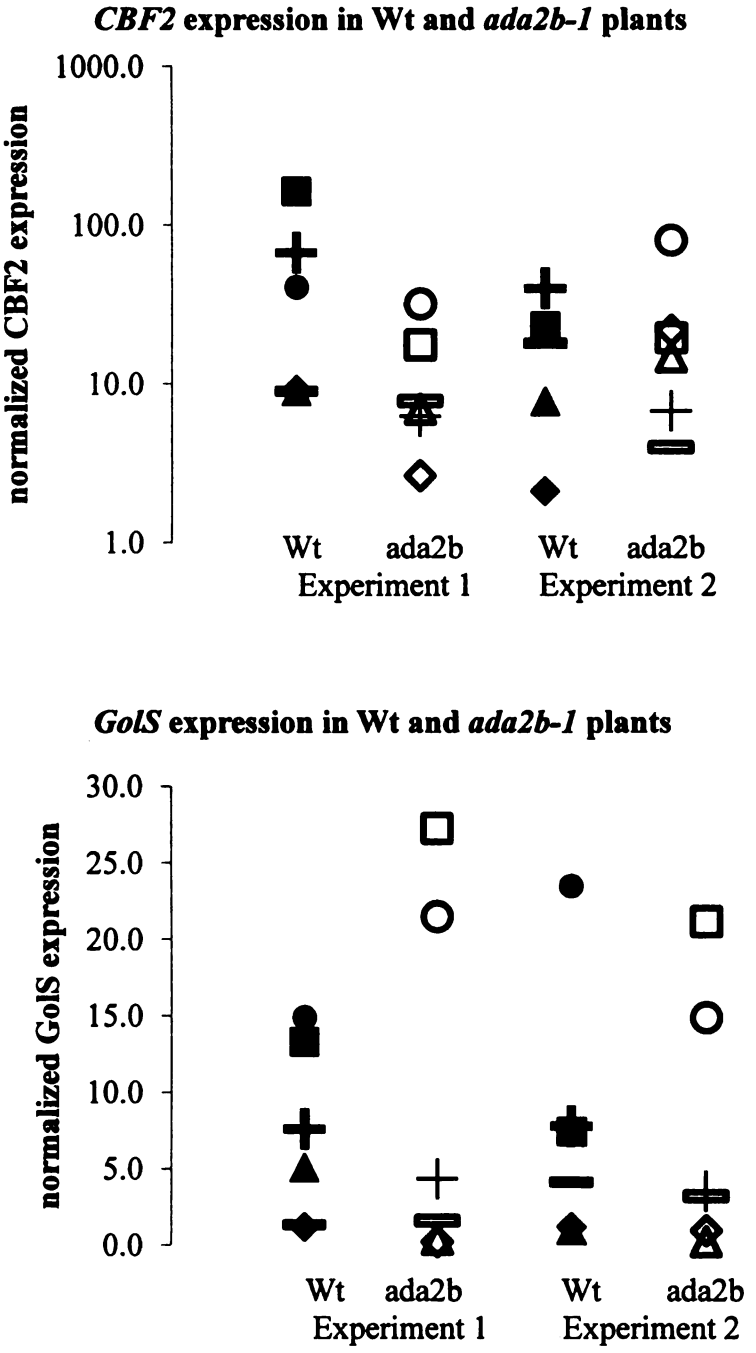


Figure 15: The expression of *CBF2* and *GolS* is similar in wild type and *ada2b-1* plants overexpressing *CBF2*. The expression of *CBF2* and *GolS* was compared in wild type and *ada2b-1* plants in six independent lines. Gene expression analysis was performed on non cold acclimated Ws wild type and *ada2b-1* plants. Real-time RT PCR was performed using primers for the *ACT3, CBF2* and *GolS* genes. The Ct values for the *CBF2* and *GolS* genes were normalized to the Ct values for the *ACT3* gene. Results were represented as normalized expression levels of *CBF2* and *GolS* using the DCt method. The results shown are from two biological replicate experiments.

ADA2b did not have a significant effect on the overexpression levels of *CBF2* as seen by t-test at $p \leq 0.05$. Also, the variation between the two biological replicate experiments was not large. The expression of *GolS* in the six independent wild type lines showed a large variation between lines. This was most likely due to the variation in CBF2 levels between different lines. Again the variation between the two biological experiments was very small. The expression of *GolS* in the *ada2b-1* plants showed a lot of variation between the six independent lines. Results from the t-test showed that the loss of ADA2b did not significantly affect the expression levels of *GolS* at $p \leq 0.05$.

Figure 16 shows that the overexpression levels of *CBF2* varied widely in the three independent Ws wild type lines. The expression of *CBF2* in the *gcn5-1* mutants was higher as compared to wild type plants but this difference was not significant at $p \leq 0.05$ as analyzed by the paired t-test. The expression of *GolS* in the three independent wild type lines also showed a large variation between lines. This was probably due to the variation in CBF2 levels between different lines. The expression of *GolS* in the *gcn5-1* plants showed a lot of variation between the three independent lines. Results from the t-test showed that the loss of GCN5 did not significantly affect the expression levels of *GolS* at $p \leq 0.05$.

From the results we can conclude that the CBF-dependent expression of *COR* genes does not require ADA2b and GCN5.

3.4 Discussion

CBF is a major activator of *COR* gene expression during cold acclimation. Overexpression of CBF is sufficient to drive the expression of *COR* genes in non-cold-acclimated plants. CBF is able to interact with coactivators ADA2b and GCN5 *in vitro*

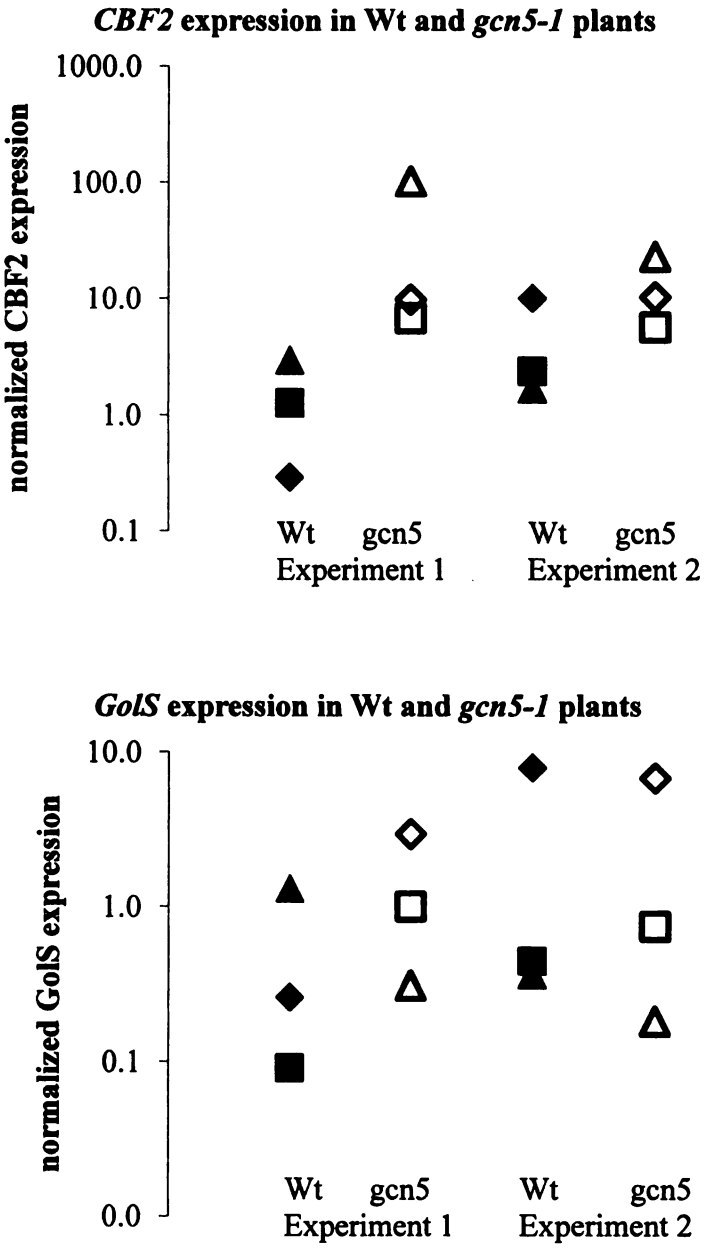


Figure 16: The expression of *CBF2* and *GolS* is similar in wild type and *gcn5-1* plants overexpressing *CBF2*. The expression of *CBF2* and *GolS* was compared in wild type and *gcn5-1* plants in three independent lines. Gene expression analysis was performed on non cold acclimated Ws wild type and *gcn5-1* plants. Real-time RT PCR was performed using primers for the *ACT3, CBF2* and *GolS* genes. The Ct values for the *CBF2* and *GolS* genes were normalized to the Ct values for the *ACT3* gene. Results were represented as normalized expression levels of *CBF2* and *GolS* using the DCt method. The results shown are from two biological replicate experiments.

and these coactivators are known to regulate *COR* gene expression. Hence we proposed that the coactivators ADA2b and GCN5 are involved in the CBF-dependent activation of *COR* genes.

However, the results of the experiments presented here show that ADA2b and GCN5 are not involved in the CBF-dependent expression of *COR* genes. The CBF-driven activation of the 4X-CRT-GUS reporter gene was not affected by the loss of GCN5 during cold acclimation. Also, the loss of ADA2b and GCN5 had no effect on the expression of *COR* genes in CBF overexpressing plants. Hence, the regulation of *COR* gene expression by ADA2b and GCN5 most likely occurs in a CBF-independent manner.

The *COR* gene promoters contain several other cis-elements that are potentially regulated by non-CBF transcription factors [120]. The expression of *COR* genes could be controlled by transcription factors other than CBF either synergistically with CBF or totally independent of CBF. Thus, ADA2b and GCN5 might regulate the expression of the *COR* genes through other cis-elements.

ADA2b and GCN5 might regulate the expression of *COR* genes in a CBF-independent manner through another transcription activator. It is also possible that the regulation of *COR* genes by ADA2b and GCN5 occurs indirectly. Thus, ADA2b and GCN5 might be regulating the expression of a transcription factor which in turn regulates *COR* gene expression.

The expression of *COR* genes in *ada2b-1* and *gcn5-1* mutants is delayed and two-fold reduced as compared to wild type plants but not completely repressed. Thus ADA2b and GCN5 might regulate the *COR* gene expression in a subtle way. Overexpression of *CBF2* by passes the requirement of a cold stimulus for the induction of *COR* genes.

Overexpression of CBF2 may also bypass the requirement for ADA2b and GCN5 for *COR* gene induction and regulation as a large amount of CBF2 might be capable of recruiting the transcription machinery required for *COR* gene activation. Hence we are unable to see any effect of ADA2b and GCN5 in the CBF dependent pathway of *COR* gene expression in plants overexpressing CBF.

We conclude from the results that the regulation of *COR* genes by CBF does not require ADA2b and GCN5. The CBF driven activation of *COR* genes via the CRTs is independent of the function of GCN5. Thus, the regulation of *COR* genes by ADA2b and GCN5 may occur through a non-CBF transcription factor binding to cis-elements other than the CRTs or it may occur indirectly.

Chapter IV

Histone acetyltransferases and their effect on *COR* gene regulation during cold acclimation in *Arabidopsis*.

4.1 Introduction

The results described in previous chapters have revealed that the acetylation of histone H3 at the *COR* gene promoters increases in response to cold acclimation. The histone acetyltransferase GCN5 regulates the expression of *COR* genes but is not required for acetylation of histone H3 at the *COR* gene promoters during cold acclimation. To identify the HAT (or HATs) responsible for acetylation of histone H3 at the *COR* gene promoters during cold acclimation, several candidate genes were investigated in the current chapter.

The *Arabidopsis* genome includes 12 HAT encoding genes belonging to four different families. The GNAT family contains members that have a HAT domain characterized by four motifs (A-D) [16]. The GNAT family in *Arabidopsis* has three members, designated GCN5, ELP3 and HAT1 (or HAG1, HAG2 and HAG3 respectively) [34]. The MYST family members have HAT domains similar to those of the GNAT family but comprising only the A motif. The MYST family in *Arabidopsis* contains two members, designated HAG4 and HAG5. The *Arabidopsis* genome contains five members of the CBP/p300 family, designated HAC1, HAC2, HAC4, HAC5 and HAC12. The CBP family members in *Arabidopsis* differ from their animal counterparts in that they lack a bromodomain, which is known to bind acetylated histones [66]. The *Arabidopsis* genome encodes two members of the TAF1 family, designated HAF1 and HAF2/TAF1. The TAF1 protein is

part of the general transcription factor TFIID [121-123]. Other eukaryotes such as yeast, flies and humans all have one single member in the TAF1 family [106, 124].

GCN5 is the most extensively characterized HAT in *Arabidopsis*. Microarray studies showed that GCN5 regulates the expression of 5% of the *Arabidopsis* genes [37]. GCN5 has been shown to physically interact *in vitro* with the transcriptional activator CBF and also with the coactivator protein ADA2 [51]. GCN5 regulates the activity of cold-regulated (*COR*) genes during cold acclimation in Arabidopsis [37]. In addition, GCN5 regulates the floral meristem activity through the WUS/AG pathway [76]. GCN5 along with another HAT protein, TAF1, also regulates the expression of light-responsive genes CAB2 and RBCS-1A that are bound by the transcriptional activator HY5 [54, 55]. Some members of the CBP family are involved in the regulation of flowering by regulating the flowering repressor FLC [52, 53]. Homozygous mutants of *hac1* show delayed flowering due to an increase in the expression of *FLC*. This phenotype is further enhanced in *hac1hac5* and *hac1hac12* double mutants. Members of the HAC family thus act as repressors of FLC rather than as activators. Chromatin immunoprecipitation assays performed using antibodies against acetylated H3 and H4 show that the acetylation profiles at the *FLC* promoter are not different in the mutants than in wild type plants [52, 53, 83]. Thus the HAC genes regulate *FLC* in a manner that is independent of histone acetylation at the *FLC* promoter. The manner in which they regulate the expression of *FLC* is not known as yet.

To find the HAT (or HATs) responsible for acetylating histone H3 at the *COR* gene promoters during cold acclimation, several members of the HAC and TAF1 families were tested for their role in *COR* gene regulation during cold acclimation.

4.2 Materials and methods:

4.2.1 Plants used and growth conditions:

Seeds for the Ws and Col-0 plants were obtained from the Thomashow lab. T-DNA insertion lines were obtained from the ABRC stock center for the *hac1-1*, *hac5-1* and *hac12-1* homozygous mutants. Seeds for the *hac1hac12* double mutant were obtained from the Noh lab in Korea. All of these T-DNA insertion lines were constructed in the Col-0 background. Seeds for the *taf1-1* homozygous mutants were obtained from the Zhou lab in France. This T-DNA line was in the Ws background. Plant growth conditions were as described in 2.2.1

4.2.2 RNA isolation and gene expression analysis:

RNA isolation and gene expression studies were carried out as described in section 3.2.3. Genes expression analysis of the *CBF2* and *COR* genes in wild type and mutant plants were compared by ANOVA using SAS.

4.2.3 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assays were performed on Col-0, *hac1hac12*, Ws-Wt and *taf1-1* plants as described in sections 2.2.2 through 2.2.6.

4.3 Results:

4.3.1 HAC gene family:

We first asked whether members of the HAC gene family of histone acetyltransferase play a role in regulating the expression of *COR* genes upon cold acclimation.

Three genes from the HAC family, *HAC1*, *HAC5* and *HAC12*, were chosen for this study. The *HAC2* gene was excluded because the encoded protein does not have a functional HAT domain [52, 83]. To determine whether any of the HATs from the HAC gene family play a role in regulating *COR* gene expression during cold acclimation, wild type Col-0 and mutant plants were treated at 4°C for various times and gene expression analysis was carried out. The *hac12*-1 homozygous mutant seeds failed to germinate and hence this mutant was not included in this study. The ability of the *hac1-2, hac5-1* and *hac1hac12* mutants to activate *COR* gene expression upon cold acclimation was compared with wild type Col-0 plants. Plants were cold treated at 4°C for 0, 1, 2, 4, 6, 8, 10, 12 and 24 hours. Gene expression analysis was performed using real-time RT-PCR. The figures represent an average of two biological experiments for the *hac1-2* and *hac5-1* mutants and an average of four biological experiments for the *hac1hac12* double mutant.

Figure 17 shows the expression of *ACT3, CBF2* and *COR* genes in wild type and *hac1-2* plants upon cold acclimation. The *ACT3* expression levels did not change with time upon cold acclimation and were similar in the wild type and *hac1-2* plants. The expression of *CBF2* in *hac1*-2 plants was induced upon cold acclimation, peaked between two and four hours and then decreased in concert with wild type plants. The expression of all *COR* genes was induced upon cold acclimation in wild type and *hac1-2* plants. The expression of *COR47* and *GolS* was similar in *hac1-2* and wild type plants. The expression of *COR6.6, COR15a* and *GolS* genes was lower in *hac1-2* plants than in wild type plants. This difference was not significant at $p \leq 0.05$ as analyzed by ANOVA, likely because the expression of these *COR* genes in wild type plants showed a large variation in the two independent experiments. We can conclude that our evidence

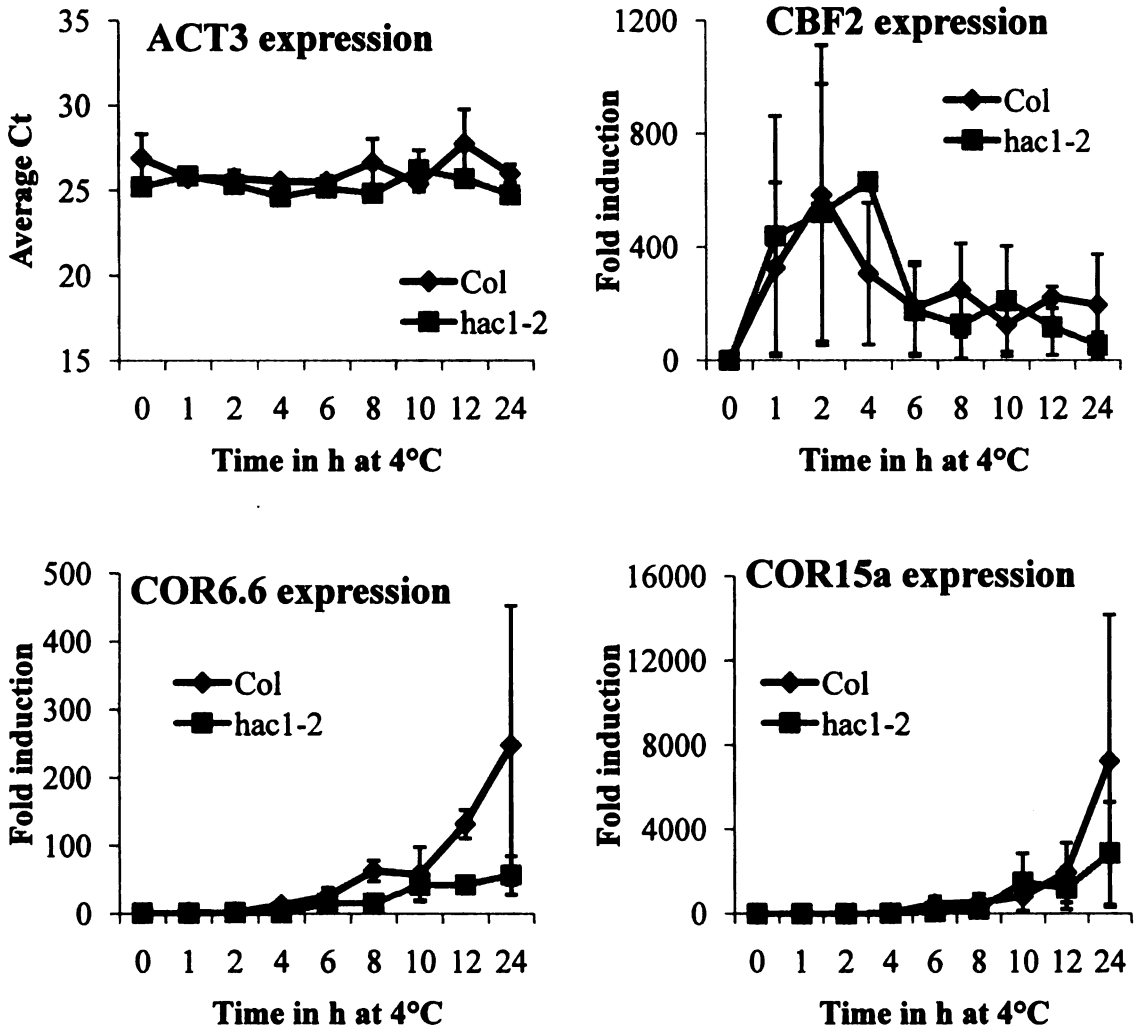
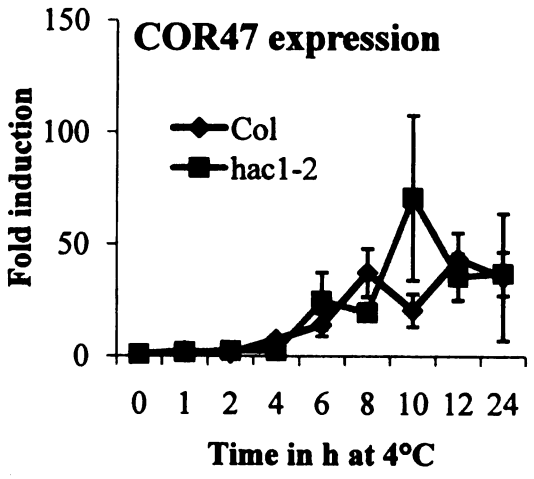
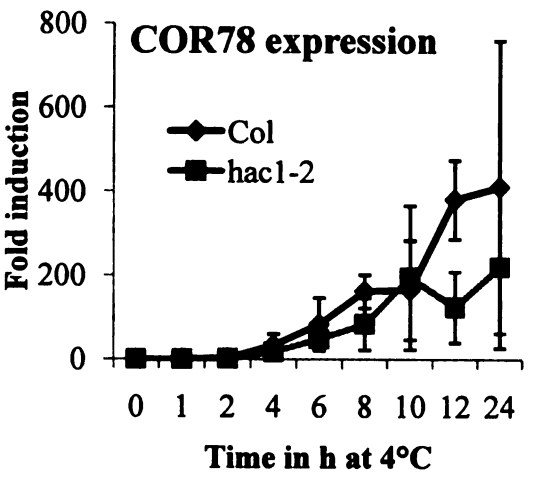
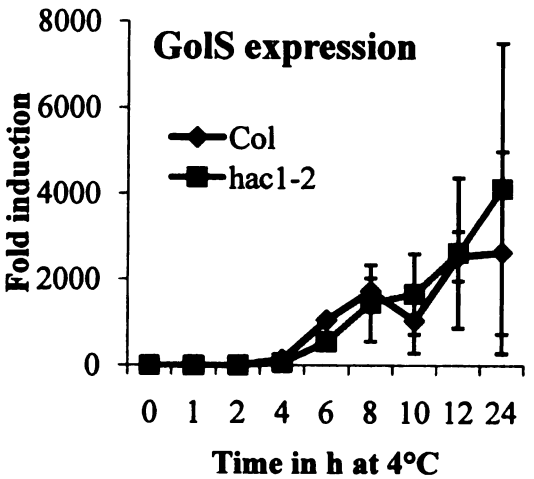


Figure 17: The expression of *CBF2* and *COR* genes increases in wild type Col-0 and *hac1-2* plants upon cold acclimation. Gene expression analysis was performed in Col-0 and *hac1-2* plants after cold treatment at 4°C for 0, 1, 2, 4, 6, 8, 10, 12 and 24 hours. Real-time PCR was performed using primers for the *ACT3, CBF2* and *COR* genes and the Ct values obtained for *COR* genes were normalized to the Ct values obtained for *ACT3*. Ct values for the *ACT3* promoter were not normalized. Fold induction of *CBF2* and *COR* genes after cold treatment were calculated by normalizing the cold treated plants with the untreated sample. Error bars represent standard error where N=2.

Figure 17 continued







does not support the hypothesis that HAC1 regulates the expression of *COR* genes during cold acclimation.

Figure 18 shows the expression of *ACT3, CBF2* and *COR* genes in wild type and *hac5-1* plants upon cold acclimation. Data for the wild type plants were obtained from figure 17. The *ACT3* expression levels did not change with time upon cold acclimation and were similar in the wild type and *hac5-1* plants. The expression of *CBF2* in *hac5-1* plants was induced upon cold acclimation, peaked at two hours and then decreased in concert with wild type plants. The expression of *COR15, COR78* and *GolS* upon cold acclimation was similar in *hac5-1* and wild type plants. *COR6.6* expression was modestly lower in *hac5-1* plants at 10, 12 and 24 hours after cold acclimation as compared to wild type plants. The expression of *COR47* in *hac5-1* plants was lower at 8 and 12 hours after cold acclimation as compared to wild type plants. Both the differences were not significant at $p \leq 0.05$ as analyzed by ANOVA using SAS. Thus, our evidence does not support the hypothesis that HAC5 regulates the expression of *COR* genes during cold acclimation.

The *hac1-1* single mutant was late flowering, likely due to an increase in the expression of FLC, a flowering repressor. This phenotype was enhanced in the *hac1hac12* mutant, suggesting that the HAC1 and HAC12 HATs together regulate the expression of *FLC* [52, 53, 83] . To determine whether the HAC1 and HAC12 HATs act synergistically to control the expression of *CBF2* and/or *COR* genes the *hac1hac12* double mutant was used in this study.

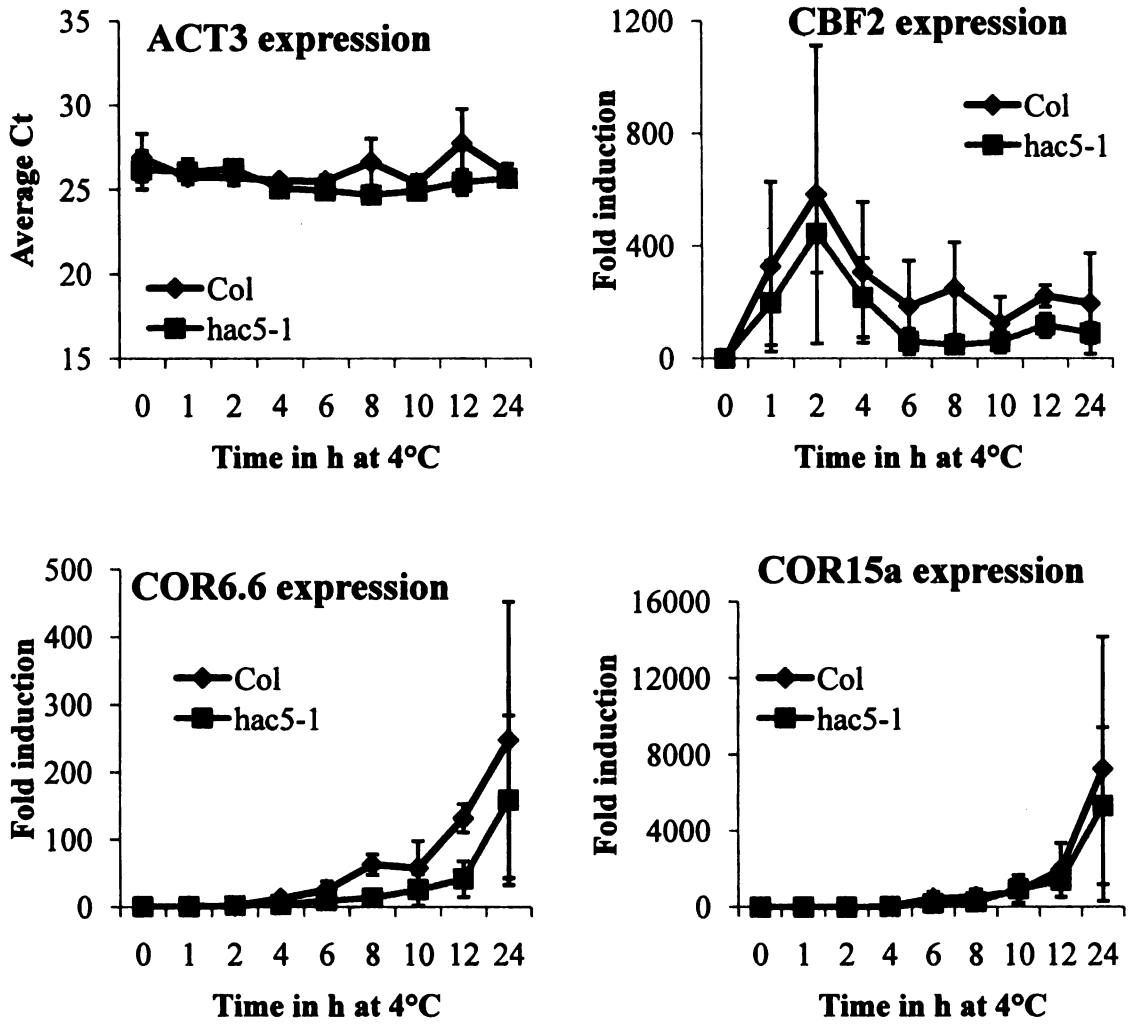
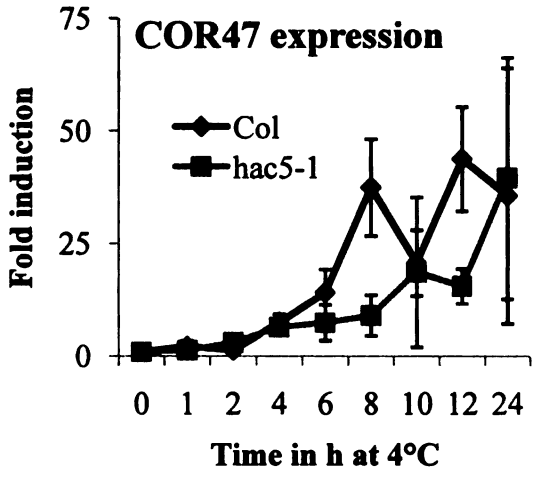
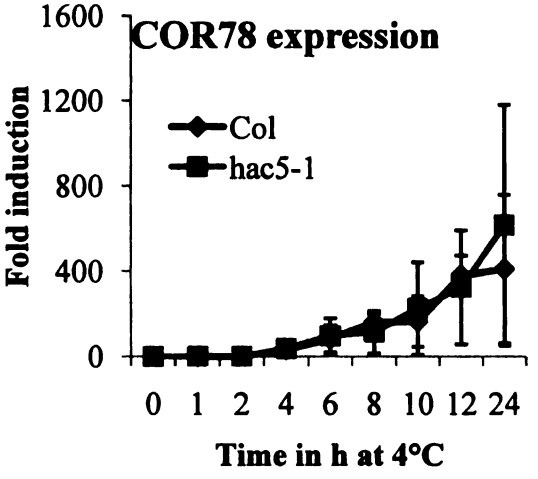


Figure 18: The expression of *CBF2* and *COR* genes increases in wild type Col-0 and *hac5-1* plants upon cold acclimation. Gene expression analysis was performed in Col-0 and *hac5-1* plants after cold treatment at 4°C for 0, 1, 2, 4, 6, 8, 10, 12 and 24 hours. Real-time PCR was performed using primers for the *ACT3, CBF2* and *COR* genes and the Ct values obtained for *COR* genes were normalized to the Ct values obtained for *ACT3*. Ct values for the *ACT3* gene were not normalized. Data for the wild type plants were obtained from figure 17. Fold induction of *CBF2* and *COR* genes after cold treatment were calculated by normalizing the cold treated plants with the untreated sample. Error bars represent standard error where N=2.

Figure 18 continued





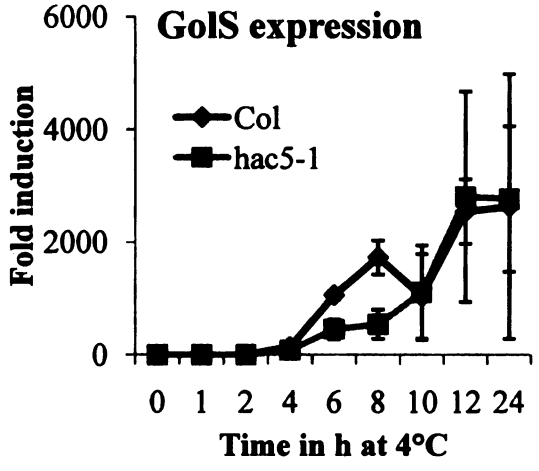


Figure 19 shows the expression of *ACT3, CBF2* and *COR* genes in wild type and *hac1hac12* plants upon cold acclimation. The *ACT3* expression levels did not change with time upon cold acclimation and were similar in the wild type and *hac1hac12* plants. The expression of *CBF2* in *hac1hac12* plants was induced upon cold acclimation, peaked at two hours and then decreased in concert with wild type plants. Although the peak level of *CBF2* expression was lower in the *hac1hac12* mutants as compared to wild type plants this difference was not significant at $p \leq 0.05$. *COR6.6* and *COR15a* expression levels in *hac1hac12* plants were lower than in wild type plants. The expression of these *COR* genes in wild type plants showed a large variation in the two independent experiments, and the difference observed between mutant and wild type plants was not significant at $p \leq 0.05$ as analyzed by ANOVA using SAS. The expression of the *COR47* gene was lower in *hac1hac12* as compared to wild type plants. The expression of COR47 was lower at 8, 10, 12 and 24 hours after cold acclimation. This difference was significant at $p \leq 0.05$ as analyzed by ANOVA using SAS. The evidence presented in these results suggests that HAC1 and HAC12 may be involved in the regulation of *COR47* gene expression during cold acclimation but none of the other *COR* genes or *CBF2*.

The induction of *CBF2* and *COR* genes upon cold acclimation in the *hac1-2* and *hac5-1* mutants was similar to the wild type Col-0 plants. Thus HAC1 and HAC5 genes do not seem to regulate the expression of *COR* genes upon cold acclimation. In the *hac1hac12* double mutant, expression of the *COR47* gene during cold acclimation was significantly lower than in the wild type plants but none of the other *COR* genes are significantly affected. Hence, HAC1 and HAC12 are involved in controlling the expression of *COR47* upon cold acclimation. As the *hac1-2* single does not affect the

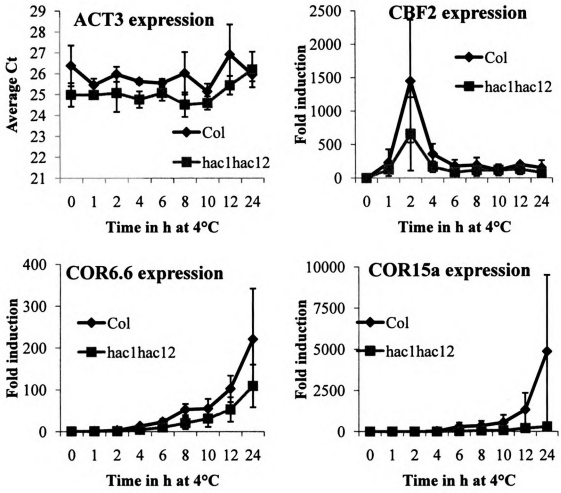
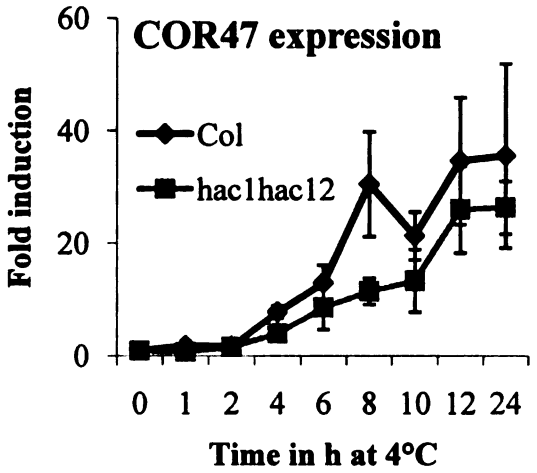
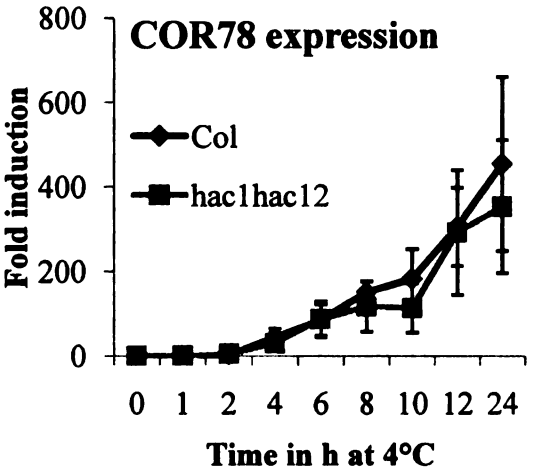
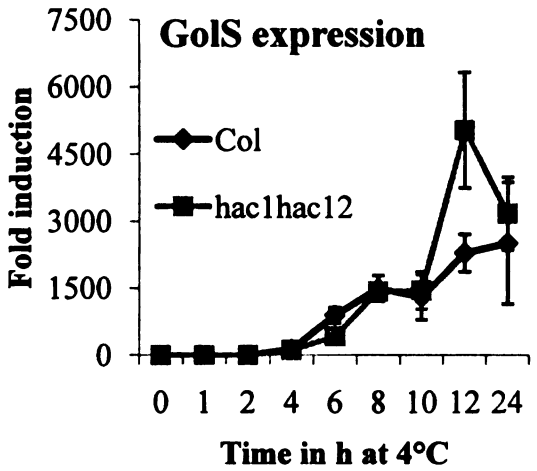


Figure 19: The expression of *CBF2* and *COR* genes increases in wild type Col-0 and *hac1hac12* plants upon cold acclimation. Gene expression analysis was performed in Col-0 and *hac1hac12* plants after cold treatment at 4°C for 0, 1, 2, 4, 6, 8, 10, 12 and 24 hours. Real-time PCR was performed using primers for the *ACT3, CBF2* and *COR* genes and the Ct values obtained for *COR* genes were normalized to the Ct values obtained for *ACT3*. Ct values for the *ACT3* gene were not normalized. Fold induction of *CBF2* and *COR* genes after cold treatment were calculated by normalizing the cold treated plants with the untreated sample. Error bars represent standard error where N=4.

Figure 19 continued







expression of *COR* genes, the effect of the *hac1hac12* double mutant on *COR47* gene expression is most likely due to the loss of the *HAC12* gene.

Since the expression of the *COR47* was affected in the *hac1hac12* mutant we checked whether the acetylation of histone H3 at the *COR47* promoter was also affected in the double mutant. Results in chapter I showed that the acetylation of histone H3 at all the COR gene promoters increased upon cold induction and corresponded with the induction of *COR* genes. HAC1 and HAC12 together may acetylate histone H3 at the *COR47* promoter specifically during cold acclimation and control its gene expression. If HAC1 and HAC12 are responsible for the acetylation of histone H3 at the *COR47* promoter we would expect to see no increase in the acetylation at the *COR47* promoter in the *hac1hac12* mutant upon cold acclimation. The acetylation of histone H3 at the other *COR* gene promoters will be similar to wild type plants and will not be affected in the *hac1hac12* mutant.

ChIP assays were conducted on *Col-0* and *hac1hac12* plants cold treated at 4°C for 4 and 24 hours. Non-acclimated plants were used as controls. ChIP assays were performed using the anti-acetylated histone H3 and the anti-histone H3 antibodies. Figure 20 shows that the acetylation of histone H3 at the *COR47* and the other *COR* gene promoters increased upon cold acclimation in *hac1hac12* mutant plants, in a manner and to a degree similar to that observed for the Col-0 wild type plants. Exceptions were observed at the *COR78* and *GolS* promoters where the acetylation of histone H3 in wild type plants was significantly higher than that observed for the *hac1hac12* mutant. The level of histone H3 at the *ACT2/7* promoter was unaffected in the *hac1hac12* and wild type plants upon cold acclimation. Hyperacetylation of histone H3 and depletion of

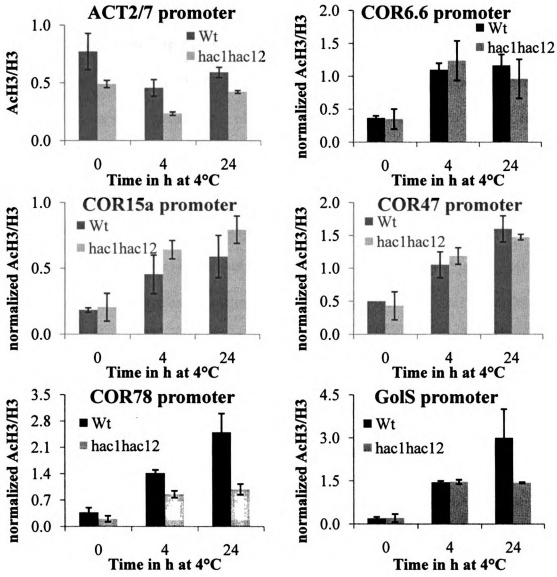


Figure 20: Ratio of acetylated histone H3 to total histone H3 at *COR* gene promoters increases upon cold acclimation. ChIP assays were performed on wild-type Col-0 and *hac1hac12* plants cold acclimated at 4°C for 0, 4 and 24 hours using antibodies specific for acetylated histone H3 (K9, K14) and the C-terminal of histone H3. Samples were analyzed using real-time PCR to detect acetylated histone H3 and total histone H3 at various *COR* gene promoters. Ct values obtained were normalized to values obtained for *ACT2/7* promoter. Data were represented as ratio of acetylated histone H3 to total histone H3. Ct values for the *ACT2/7* promoter were not normalized. Error bars represent SE where N (number of experiments) = 2.

histone H3 occurs at some *COR* gene promoters during cold acclimation. The occupancy of the *COR* gene promoters was tested in *hac1hac12* and wild type plants. Figure 21 shows that the occupancy of histone H3 at the *COR78* and *GolS* promoters decreased during cold acclimation in wild type plants. The histone H3 occupancy at the *COR6.6, COR15a* and *COR47* promoters is unchanged upon cold acclimation in wild type plants. The effect of *hac1hac12* mutant on histone H3 occupancy was also tested. Figure 21 shows that the level of total histone H3 at the *ACT2/7* promoter was lower in the non-acclimated control *hac1hac12* mutants as compared to the wild type plants. This most likely accounts for the increased histone H3 occupancy observed at all the *COR* gene promoters in the non-acclimated *hac1hac12* plants. Data analysis by ANOVA indicated that the level of histone H3 at the *COR47* promoter remained unchanged after cold acclimation in Col-0 and *hac1hac12* plants. Also, the decrease in histone H3 at the *COR78* and *GolS* promoters in the *hac1hac12* mutants was similar to the decrease observed in Col-0 plants.

Given these results, we can conclude that HAC1 and HAC12 histone acetyltransferases together contribute to the regulation of *COR47* gene expression but do so without affecting the acetylation or the occupancy of histone H3 at the *COR47* gene promoter.

4.3.2 TAF1 gene family:

The TAF1 family of histone acetyltransferases in *Arabidopsis* consists of two members, designated HAF1 and HAF2/TAF1 [34, 54, 55]. The TAF1 protein is a part of the general transcription factor complex TFIID [121]. The HAF2/TAF1 protein has been shown to

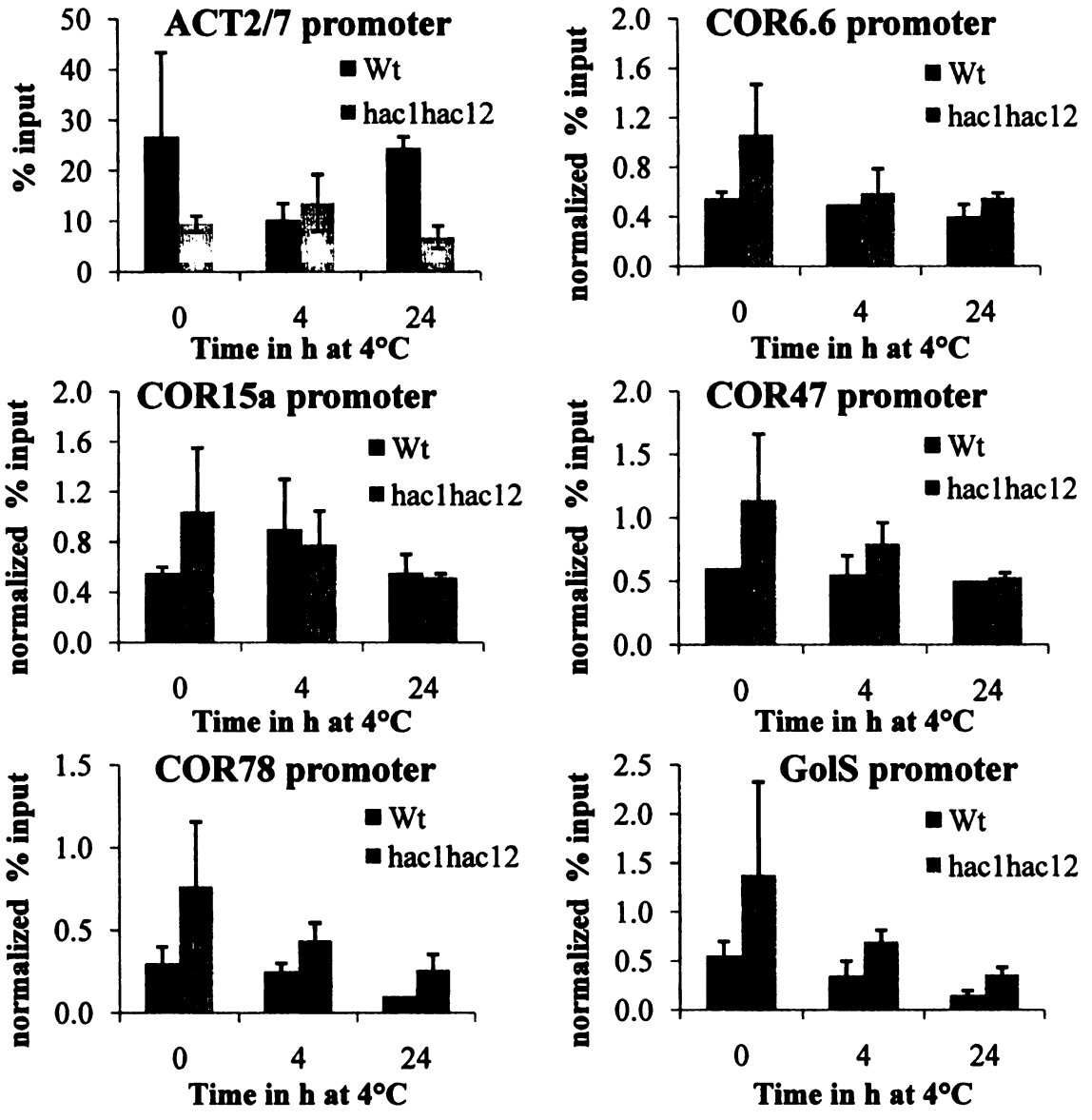


Figure 21: Level of total histone H3 at some *COR* gene promoters decreases upon cold acclimation. ChIP assays were performed on wild-type and *hac1hac12* plants cold acclimated at 4°C for 0, 4 and 24 hours using an antibody specific for the C-terminal of histone H3. Samples were analyzed using real-time PCR to detect total histone H3 at various *COR* gene promoters. Ct values obtained were normalized to values obtained for *ACT2/7* promoter. Data were represented as normalized levels of total histone H3. Ct values for the *ACT2/7* promoter were not normalized and were represented as % input values. Error bars represent SE where N (number of experiments) = 2.

regulate light-responsive genes and homozygous *haf2/taf1-1* mutant plants show decreased acetylation of histones H3 and H4 at the target gene promoters. Microarray studies showed that a homozygous null mutation in *TAF1* affects the expression of 9% of the total genes in *Arabidopsis.* A homozygous null mutant of *HAF1* showed no visible phenotype and hence this gene was not used in this study [54, 55].

We tested whether the histone acetyltransferase HAF2/TAF1 regulates the expression of *COR* genes during cold acclimation. The expression of *CBF2* and *COR* genes was compared in *taf1-1* mutant and wild type Ws plants after treating the plants at 4°C for 0, 1, 2, 4, 6, 8, 10, 12 and 24 hours. RNA was extracted and gene expression was analyzed using real-time RT-PCR, as described previously.

The results (Figure 22) show that the expression of the *ACT3* gene did not change with time and was similar in the wild type Ws and the *taf1-1* plants. The expression of *CBF2* in the *taf1*-1 plants was induced within one hour of cold acclimation, peaked after two hours and then decreased in concert with wild type Ws plants. The expression of all the *COR* genes increased upon cold acclimation in wild type plants. The expression COR6.*6, COR15a* and *GolS* genes in the *taf1-1* mutant plants was significantly lower than in the wild type Ws plants after 8 hours of cold acclimation (at $p \leq 0.05$). A similar trend was observed for the *COR47* and *COR78* genes but this difference was not significant at $p \leq 0.05$ as analyzed by ANOVA using SAS. We conclude that the TAF1 histone acetyltransferase controls the expression of *COR6.6, COR15a* and *GolS* genes during cold acclimation but is not required for the expression of *COR47* and COR78 genes. Thus, TAF1 controls the regulation of a subset of *COR* genes during cold acclimation.

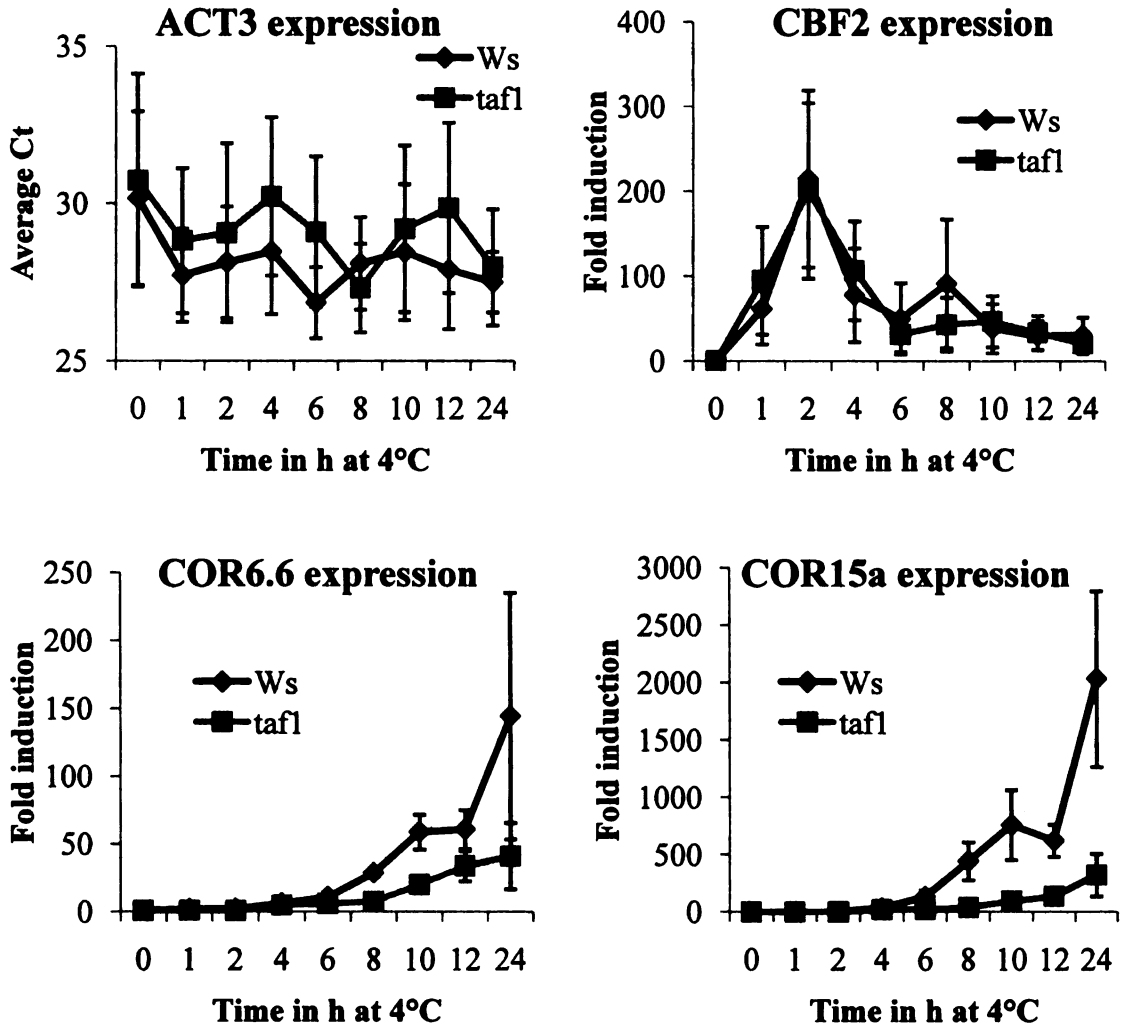
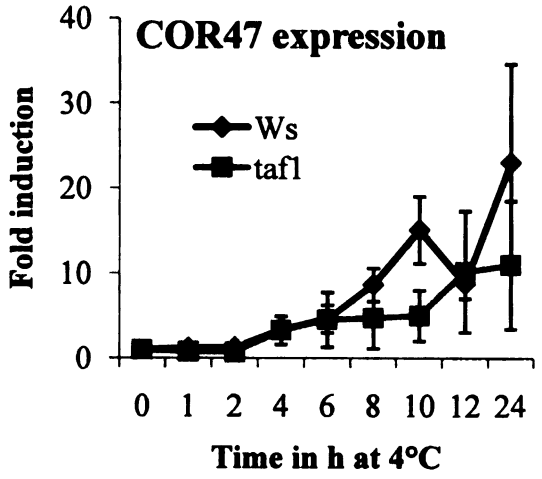
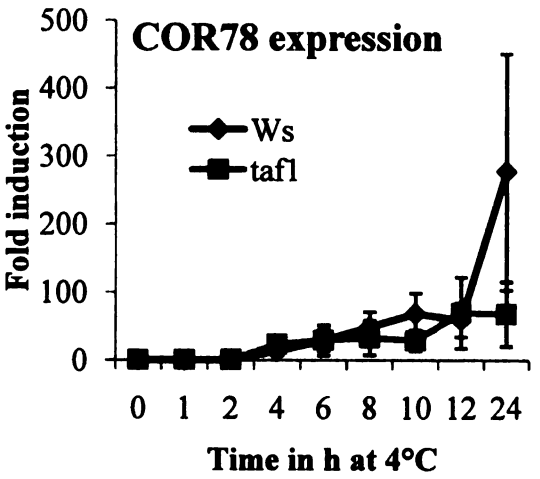
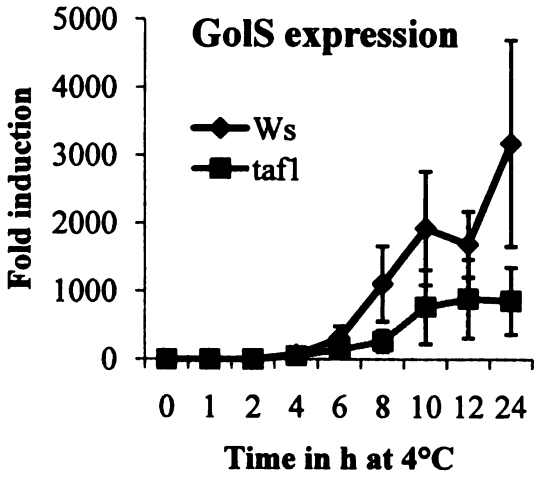


Figure 22: The expression of *CBF2* and *COR* genes increases in wild type Ws and *taf1-1* plants upon cold acclimation. Gene expression analysis was performed in Ws and *taf1-1* plants after cold treatment at 4°C for 0, 1, 2, 4, 6, 8, 10, 12 and 24 hours. Real-time PCR was performed using primers for the *ACT3, CBF2* and *COR* genes. Ct values obtained for *COR* genes were normalized to the Ct values obtained for *ACT3*. Fold induction of *CBF2* and *COR* genes after cold treatment were calculated by normalizing the cold treated plants with the untreated sample. Ct values for *ACT3* were not normalized and were represented as average Ct. Error bars represent standard error where N=4.

Figure 22 continued







Given the result that the disruption of *TAF1* resulted in diminished cold-induced expression of some *COR* genes we tested whether the acetylation or occupancy of histone H3 at these *COR* gene promoters was also affected in the *taf1-1* mutant. Chromatin immunoprecipitation assays were carried out on wild type Ws and *taf1-1* plants cold acclimated at 4°C for 4 and 24 hours using the anti-acetylated histone H3, anti-tetra-acetylated histone H4 and anti-histone H3 antibodies. Non-acclimated plants were used as controls. Real-time PCR was performed by using primers spanning 100-150 bp of the *COR* gene promoters. Data were analyzed using two-factor ANOVA.

The results shown in Figure 23 reveal that the increase in acetylation of histone H3 at the *COR* gene promoters in the *taf1-1* mutants was similar to the increase in wild type plants. The increase in acetylation in the *taf1-1* mutants occurred at all the *COR* gene promoters irrespective of whether the expression of that *COR* gene was affected in the *taf1-1* mutant. The only difference observed was that the increase in acetylation after 24 hours of cold treatment at the *COR78* promoter was higher in wild type plants as compared to the *taf1-1* mutant. However, the expression of *COR78* was not affected in the *taf1-1* mutant upon cold acclimation. We also tested whether histone H3 occupancy at the *COR* gene promoters was affected by the *taf1-1* mutation. Figure 24 shows that the level of total histone H3 at the *ACT2/7* promoter was lower in the 24 hour cold acclimated *taf1-1* mutant as compared to the wild type plants. So any difference between wild type and *taf1-1* plants at the 24 hour time point could be the result of higher levels of histone H3 at the *ACT2/7* promoter in wild type plants. Results indicate that the level of total histone H3 at the *COR78* and *GolS* promoters decreased after 24 h of cold treatment at 4°C in the *taf1-1* mutants in a manner and to a degree similar to wild type plants. A

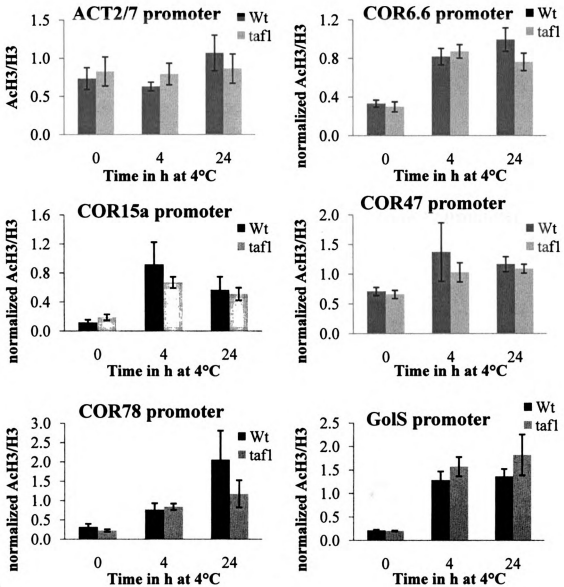


Figure 23: Ratio of acetylated histone H3 to total histone H3 at *COR* gene promoters increases upon cold acclimation. ChIP assays were performed on wild-type Ws and *taf1-1* plants cold acclimated at 4°C for 0, 4 and 24 hours using antibodies specific for acetylated histone H3 (K9, K14) and the C-terminal of histone H3. Samples were analyzed using real-time PCR to detect acetylated histone H3 and total histone H3 at various *COR* gene promoters. Ct values obtained were normalized to values obtained for *ACT2/7* promoter. Data represented as ratio of acetylated histone H3 to total histone H3 at various *COR* gene promoters. Ct values for the *ACT2/7* promoter were not normalized. Error bars represent SE where N (number of experiments) = 3.

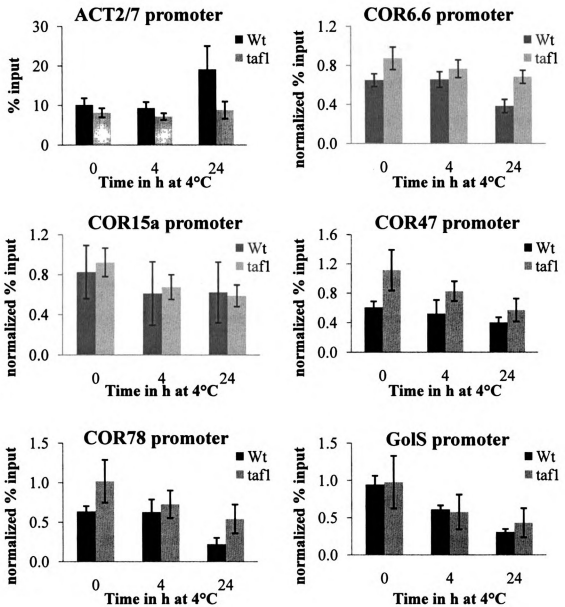


Figure 24: Level of total histone H3 at *COR* gene promoters decreases upon cold acclimation. ChIP assays were performed on wild-type Ws and *taf1-1* plants cold acclimated at 4°C for 0, 4 and 24 hours using an antibody specific for the C-terminal of histone H3. Samples were analyzed using real-time PCR to detect total histone H3 at various *COR* gene promoters. Ct values obtained were normalized to values obtained for *ACT2/7* promoter. Data represented as normalized levels of total histone H3 at various *COR* gene promoters. Ct values for the *ACT2/7* promoter were not normalized and were represented as % input. Error bars represent SE where N (number of experiments) = 3.

decrease in histone H3 occupancy was also observed at the *COR6.6* promoter but the level of histone H3 in wild type plants after 24 hours of cold acclimation was lower as compared to the *taf1-1* mutant. This could be due to the higher level of histone H3 observed at the *ACT2/7* promoter observed in wild type plants. There was no decreased in histone H3 occupancy at the *COR15a* and *COR47* promoters upon cold acclimation in the wild type or mutant plants. Thus, TAF1 is not required for the acetylation or depletion of histone H3 from the *COR* gene promoters upon cold acclimation.

TAF1 histone acetyltransferase might be controlling the expression of *COR* genes by regulating the acetylation of histone H4 at the *COR* gene promoters [31, 125]. Hence, ChIP assays were carried out in wild type Ws and *taf1-1* mutant plants using anti-tetra-acetylated histone H4 antibodies. The results are represented as a ratio of acetylated histone H4 to total histone H3. Histones H3 and H4 exist as dimers in nucleosomes and thus the level of change in total histone H3 upon cold acclimation should reflect the changes occurring with histone H4. We know that the level of histone H3 decreases at some *COR* gene promoters during cold acclimation and hence representing the level of acetylated H4 as a ratio accurately reflects the actual change occurring at the *COR* gene promoters during cold acclimation. Results in figure 25 show that the acetylation of histone H4 at most of the *COR* gene promoters did not increase upon cold acclimation in wild type plants and the level of acetylated histone H4 in the *taf1-1* plants was similar to the levels observed in wild type plants. Acetylation of histone H4 increased at the *GolS* promoter upon cold acclimation but there was no difference in the acetylation levels between wild type and *taf1-1* plants. Acetylation of histone H4 might be involved in the regulation of *GolS* expression but none of the other *COR* genes. We can conclude from

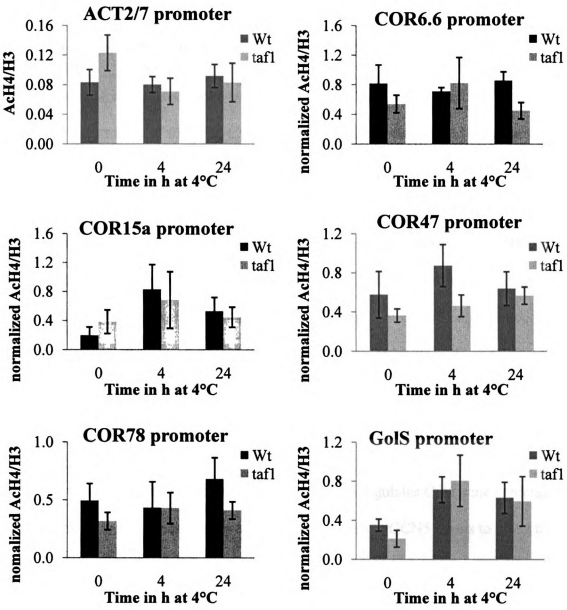


Figure 25: Ratio of acetylated histone H4 to total histone H3 at *COR* gene promoters increases upon cold acclimation. ChIP assays were performed on wild-type Ws and *taf1-1* plants cold acclimated at 4°C for 0, 4 and 24 hours using antibodies specific for tetra-acetylated histone H4 and the C-terminal of histone H3. Samples were analyzed using real-time PCR to detect tetra-acetylated histone H4 and total histone H3 at various *COR* gene promoters. Ct values obtained were normalized to values obtained for *ACT2/7* promoter. Data represented as ratio of acetylated histone H4 to total histone H3 at various *COR* gene promoters. Ct values for the *ACT2/7* promoter were not normalized. Error bars represent SE where N (number of experiments) = 3.

the results that the regulation of *COR* gene expression by TAF1 occurs independently of changes in histone acetylation at the target *COR* gene promoters.

The results presented above indicate that the TAF1 histone acetyltransferase controls the expression of *COR6.6*, *COR15a* and *GolS* genes during cold acclimation but has no effect on the expression of *COR47*, *COR78* or *CBF2* genes. The set of genes affected by TAF1 is different from the set controlled by the HAC1 and HAC12 proteins. Although TAF1 histone acetyltransferase regulates expression of a subset of *COR* genes the level of histone H3 and H4 acetylation and histone H3 depletion during cold acclimation was similar in the wild type and *taf1-1* plants. Thus, TAF1 does not control the expression of *COR* genes by regulating the acetylation of histones H3 and H4 or nucleosome occupancy.

4.4 Discussion

Studies carried out in chapter 2 show that GCN5 regulates *COR* gene expression but this activity is independent of its ability to act as a HAT. GCN5 seems to affect the occupancy of histone H3 and thus nucleosomes at the *COR6.6, COR78* and *GolS* genepromoters. In order to find the HAT responsible for carrying out the acetylation of histone H3 at the *COR* gene promoters, several HAT genes were studied for their possible role in regulation of *COR* gene expression during cold acclimation. Results show that the HAC1 and HAC12 proteins together regulate the expression of the *COR47* gene but none of the other *COR* genes. Chromatin immunoprecipitation assays show the HAC1 and HAC12 are not required for the acetylation of histone H3 at the *COR47*

promoter. The HAC1 and HAC12 genes also do not affect the occupancy of histone H3 at the *COR47* promoter.

The HAC genes may be controlling the regulation of *COR47* by regulating the acetylation of histone H4, by acetylation of a transcription factor or their function might be totally independent of their acetyltransferase activity. The members of the HAC family are broad specificity enzymes and it is possible that they regulate *COR47* gene expression by affecting the acetylation of histone H4 during cold acclimation [106]. Studies were conducted on wild type Wt plants to determine whether the acetylation of histone H4 increases at *COR* gene promoters during cold acclimation. Results show that the acetylation of histone H4 at the *COR* gene promoters (except for the *GolS* promoter) does not increase upon cold acclimation in wild type Ws plants (Fig.25). Hence the regulation of *COR47* by HAC1 and HAC12 is independent of their histone acetyltransferase activity at the *COR47* promoter. CBP and p300 in other eukaryotes are known to acetylate transcription factors and regulate the expression of target genes [26, 27]. Thus the HAC genes might control the expression of *COR47* by acetylating a transcription factor that in turn controls the expression of *COR47*. The ability of GCN5 to regulate *COR47* expression is also independent of its HAT activity. It is possible that in the absence of GCN5, HAC1 and HAC12 might acetylate histone H3 at the *COR47* promoter and thus we fail to see an effect of GCN5 on histone H3 acetylation. Similarly, in the absence of HAC1 and HAC12, GCN5 may suffice for acetylation of histone H3 at the *COR47* promoter. The next step would be to create a *hac1hac12gcn5* triple mutant and to test its ability to activate *COR47* expression and acetylate histone H3 at the *COR47* promoter. If the above hypothesis is true then the acetylation of histone H3 at the

COR47 promoter in the triple mutant should be lower as compared to wild type plants upon cold acclimation.

One more potential candidate studied was the TAF1 protein which is also a member of the transcription factor IID. Data revealed that TAF1 controls the regulation of only a subset of *COR* genes, namely, *COR6.6, COR15a* and *GolS* but does not affect the expression of *COR47, COR78* or *CBF2* during cold acclimation. The regulation of the *COR* genes by TAF1 is also independent of its HAT activity as seen by the chromatin immunoprecipitation assays. TAF proteins can act as adapters that connect the activators with the general transcription machinery and TAF1 could be acting as an adapter at its target *COR* genes and thus regulate their expression. TAF1 and GCN5 together control the regulation of light-responsive genes in *Arabidopsis* by affecting acetylation of histones H3 and H4 at the target gene promoters. This could also be true for the regulation of *COR* genes. The next step would be to test the effect of the *taf1gcn5* double mutant on *COR* gene expression and acetylation of histone H3 at the *COR* gene promoters. If the hypothesis is true then the effect of the double mutant on *COR* gene expression would be greater than any of the single mutant. We should also be able to see a difference in acetylation of histone H3 at the *COR* promoters between wild type and the double mutant.

Thus, it seems no single family of HATs is predominantly responsible for the acetylation of histone H3 at the *COR* gene promoters. Mutating any single HAT protein has an effect on the expression of *COR* genes but not on the acetylation of histone H3 per se. It is possible that in the absence of one HAT the other HATs are sufficient for the acetylation of histone H3 at the *COR* gene promoters. All HATs are known to acetylate

proteins other than histones and thus these HATs might be controlling the activity of a factor upstream of *COR* genes and hence indirectly regulate the expression of the *COR* genes. It is also interesting to note that GCN5 affects all the five *COR* genes used in this study but the other HATs control only a subset of these genes. GCN5 could be the main HAT responsible for acetylation of histone H3 but in its absence other HATs might be sufficient for the acetylation of histone H3 during cold acclimation. Thus, testing the ability of double or triple HAT mutants for their ability to efficiently acetylate histone H3 and activate *COR* gene expression might provide more detailed insight into the regulation of *COR* genes by histone H3 acetylation during cold acclimation.

Chapter V

Discussion

5.1 Histone acetylation and *COR* gene expression:

The results of my study reveal that acetylation of histone H3 increases at all the *COR* gene promoters tested during cold acclimation. The increase in acetylation of histone H3 is evident within 4 hours of cold acclimation. In addition to acetylation of histone H3, a subset of *COR* genes (*COR6.6, COR78* and *GolS*) also show a decrease in nucleosome occupancy upon cold acclimation. This decrease in nucleosome occupancy is evident after 24 hours of cold acclimation. Acetylation of histone H3 seems to precede loss of nucleosome occupancy at some *COR* gene promoters. The results also reveal subtle differences in the manner in which *COR* genes are regulated during cold acclimation. The hallmarks of *COR* gene regulation, histone H3 acetylation and loss of histone H3, are transient, as deacclimation of plants results in decrease in histone H3 acetylation back to non-acclimated levels as well as restoration of histone H3 to non-acclimated levels at the *COR* gene promoters.

5.2 Histone H3 acetylation and CBF:

The CBF family of activators (CBF1, CBF2 and CBF3) act as master regulators of the cold acclimation pathway in *Arabidopsis*. Overexpression of CBF leads to constitutive expression of *COR* genes which in turn leads to constitutive freezing tolerance of the CBF overexpressing plants [93, 94]. In the experiments reported here we found that overexpression of CBF was sufficient for the increase in histone H3 acetylation as well as loss of histone H3 at the *COR* gene promoters in the absence of

cold acclimation. Cold acclimation of plants overexpressing CBF led to a further increase in the acetylation of histone H3 at the *COR* gene promoters. This increase in histone H3 acetylation might be driven by the endogenous CBF proteins or by other transcription factors that are activated during cold acclimation.

The CBF activator is composed of a DNA-binding domain and an activation domain. In order to test whether the activation domain of CBF is sufficient to drive the acetylation of histone H3 as well as removal of histone H3 at the *COR* gene promoters, a mutant form of CBF lacking the activation domain was used. My experiments showed that the mutant form of CBF lacking the activation domain was unable to bring about increase in acetylation of histone H3 and the removal of histone H3 in the absence of cold acclimation. However, acetylation of histone H3 increased upon cold acclimation in plants overexpressing this mutant form of CBF. I conclude that the activation domain is required for driving histone H3 acetylation in the absence of the cold signal but is dispensable in the presence of cold acclimation. Also, the mutant lacking the activation domain of CBF is able to act as a dominant negative with respect to histone removal but not histone H3 acetylation. The increase of histone H3 acetylation observed upon cold acclimation might be due to a non-CBF transcription factor or due to the recruitment of chromatin modifying machinery by the DNA binding domain of CBF. Previous studies showed that the DNA domain of CBF can physically interact with the coactivator proteins ADA2a and ADA2b *in vitro* [51]. Therefore it is possible that chromatin modifying factors that bring about histone H3 acetylation are recruited by the DNA binding domain of CBF upon cold acclimation. In contrast, the recruitment of chromatin remodeling factors bringing about histone H3 removal are probably recruited by the

activation domain of CBF. Thus, the two domains of the CBF proteins seem to play distinct roles with respect to chromatin modifications during *COR* gene regulation.

5.3 Histone acetyltransferases and histone acetylation of *COR* genes:

The coactivator proteins ADA2 can interact *in vitro* with GCN5, a histone acetyltransferase, in *Arabidopsis* [51]. T-DNA insertion mutants *ada2b* and *gcn5* show a delay in activation and two-fold reduction of *COR* gene expression upon cold acclimation, leading to the hypothesis that CBF recruits an ADA2b-GCN5 containing complex at the *COR* gene promoters during cold acclimation [37]. The ADA2b-GCN5 complex might in turn acetylate histones at the *COR* gene promoters and aid the CBF-driven activation of *COR* genes. However, in opposition to this hypothesis, experiments revealed that neither ADA2b nor GCN5 are required for the acetylation of histone H3 at the *COR* gene promoters during cold acclimation. It is possible that GCN5 brings about histone H3 acetylation at the *COR* gene promoters but in the absence of GCN5 other HATs might be capable of bringing about acetylation of histone H3 at the *COR* gene promoters during cold acclimation. However, both ADA2b and GCN5 seem to be necessary for the removal of histone H3 at the *COR* gene promoters. ADA2b is known to interact with the chromatin remodeling enzyme BRG1 in humans and the absence of GCN5 hampers histone removal of some genes in yeast [61, 126, 127] . Thus, ADA2b and GCN5 might be required for the recruitment of a chromatin remodeling enzyme at the *COR* gene promoters to bring about histone removal.

To test the possibility that other HATs may be responsible for acetylation of histone H3 during cold acclimation, members of the HAC and TAF1 gene families of

HATs were tested for their role in *COR* gene expression and acetylation of histone H3 at the *COR* gene promoters. The *hac1hac12* double mutant showed lower expression of *COR47* as compared to wild type plants during cold acclimation. However, the *hac1hac12* mutant had no effect on the histone H3 acetylation nor on histone H3 removal at the *COR47* promoter.

Loss of *taf1* affected the expression of *COR6.6, COR15a* and *GolS* genes during cold acclimation, but the *taf1* mutant did not affect the acetylation or removal of histone H3 at the target *COR* gene promoters. TAF1 has been shown to regulate histone H4 acetylation in *Arabidopsis* and hence the ability of *taf1* mutants to bring about histone H4 acetylation at the *COR* gene promoters was tested [54]. Chromatin immunoprecipitation assay showed that there was no significant increase in the histone H4 acetylation upon cold acclimation at the *COR* gene promoters in wild type and *taf1-1* plants, except for the *GolS* promoter. TAF1 was not required to drive histone H4 acetylation at the *GolS* promoter during cold acclimation. TAF1 is part of the large coactivator complex TFIID and hence loss of TAF1 might interfere with the stability or recruitment of TFIID affecting *COR* gene expression during cold acclimation [122]. Results so far indicate that functional redundancy might exist between HATs controlling the histone H3 acetylation at the *COR* gene promoters. To test this hypothesis the effect of *gcn5taf1, gcn5hac1hac12* and *taf1hac1hac12* mutants on histone H3 acetylation at the *COR* gene promoters needs to be tested.

5.4 ADA2b and GCN5 regulation of *COR* gene expression:

CBFs bind specific cis-elements known as cold/dehydration responsive elements (CRT/DREs) present in the promoters of their target genes and activate their expression [68, 128]. ADA2b and GCN5 regulate the expression of *COR* genes but whether they do so in a CBF-dependent manner is not known [37]. In order to test this possibility two approaches were used. In the first approach, the expression of the reporter gene *GUS* driven by four tandem repeats of CRT fused to a minimal 35S promoter was compared in wild type and *gcn5-1* plants. As the reporter gene *GUS* is driven by CRTs, the induction of *GUS* is solely dependent on CBF. Experiments showed that GCN5 is not required for driving the CBF-dependent expression of *GUS* during cold acclimation. In the second approach the expression of *COR* genes was compared in wild type, *ada2b-1* and *gcn5-1* plants overexpressing CBF. Overexpression of CBF leads to constitutive expression of *COR* genes and any effect on *COR* expression in the mutant plants as compared to wild type plants will be the result of their regulation of the CBF-driven *COR* gene expression. However, experiments revealed that the expression of *COR* genes is not affected in the mutants as compared to wild type plants. Thus, ADA2b and GCN5 do not regulate the CBF-dependent *COR* gene regulation. Recent studies show that the expression of *COR* genes might be regulated by transcription factors other than CBF (Doherty, unpublished data). Thus it is possible that the ADA2b and GCN5 regulate the expression of *COR* genes via some other non-CBF transcription factor(s).

Table 1: Oligonucleotides used in quantitative Real-time PCR analysis of chromatin immunoprecipitation assays. Primers were designed to detect promoters of various cold-regulated genes. Primer locations are in relation to the translation start site +1.

ID#	Gene	Gene sequence	Position
KP1	COR15a(F)	5'-GGCTTGCAAACCCAAATTAAC-3'	-507 to -487
KP2	COR15a(R)	5'-AAGAGGGTCGTTCTCATTTCC-3'	-420 to -400
KP3	COR6.6(F)	5'-AAACGACACGTGATGTCTTGA-3'	-224 to -204
KP4	COR6.6(R)	5'-TGCCACGTGTAATCTGAAACC-3'	-140 to -120
KP5	COR47(F)	5'-GACTTCAAGAAATAAGAGGGGTCA-3'	-266 to -243
KP6	COR47(R)	5'-AGAAGGGTCCAGGCCAAC-3'	-164 to -147
KP7	COR78(F)	5'-ATGGGCCAATAGACATGGAC-3'	-371 to -352
KP8	COR78(R)	5'-GCTTTTTGGAACTCATGTCG-3'	-244 to -225
KP9	ACT2/7(F)	5'-CGTTTCGCTTTCCTTAGTGTTAGCT-3'	-156 to -132
KP10	ACT2/7(R)	5'-AGCGAACGGATCTAGAGACTCACCTTG-3'	-49 to -23
GS1	GolS(F)	5'-TGACGTTAGGACAAAAGAAGAAA-3'	-528 to -506
GS2	GolS(R)	5'-CAAAGCTAATTTGCTGCTCGT-3'	-425 to -405

Table 2: Oligonucleotides used in quantitative Real-time PCR for gene expression analysis. Primers were designed to detect open-reading frames (ORFs) of various cold-regulated genes. Primer locations are in relation to the translation start site +1.

ID#	Gene	Gene sequence	Position
KP13	COR15a(F)	5'-ATGGCTTCTTCTTTCCACAGC-3'	1 - 21
KP14	COR15a(R)	5'-GAAGCTTTCTTTGTGGCCTC-3'	151 – 170
KP17	COR6.6(F)	5'-CTGGCAAAGCTGAGGAGAAG-3'	44 – 63
KP18	COR6.6(R)	5'-ACTGCCGCATCCGATATACT-3'	136 – 155
KP19	COR47(F)	5'-CGGTACCAGTGTCGGAGAGT-3'	497 – 516
KP20	COR47(R)	5'-ACAGCTGGTGAATCCTCTGC-3'	604 – 623
KP21	COR78(F)	5'-GAAAGGAGGAGGAGGAATGG-3'	1908 – 1927
KP22	COR78(R)	5'-AACCAGCCAGATGATTTTGG-3'	2008 – 2027
KP23	ACT3(F)	5'-GGTCGTACTACTGGTATTGTGCT-3'	442 – 464
KP24	ACT3(R)	5'-TGACAATTTCACGCTCAGCT-3'	621 – 640
KP11	GolS(F)	5'-GGAGTGGTTGGTCTGGCTAA-3'	91 – 110
KP12	GolS(R)	5'-TTGGTTATCCGGTGGGTAAA-3'	230 – 249
KP15	CBF2(F)	5'-GGATGCTCATGGTCTTGACATG-3'	431 – 452
KP16	CBF2(R)	5'-TCTTCATCCATATAAAACGCATCTTG-3'	495 - 520

Table 3: Oligonucleotides used in the generation of the 4X-CRT-GUS plasmid.

ID#	Gene sequence
ST 1103	5'-CTAGATACCGACATTACCGACAT TACCGACATTACCGACATG – 3'
ST 1104	5' – GATCCATGTCGGTAATGTCGGTA ATGTCGGTAATGTCGGTAT – 3'

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