Characterizing the role of the transcriptional adaptor ADA2: an integrating node in the cold response mechanism of Brachypodium distachyon

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Abstract

Freezing stress limits crop productivity and generates substantial economic losses every year. While most temperate cereal crops exhibit some degree of cold tolerance, they require exposure to low, non-freezing temperatures in order to acclimate. This involves the induced expression of cold-regulated (COR) genes. COR gene promoters contain sequences that are recognized by C-repeat Binding Factor 1 (CBF1), a transcription factor that may mediate gene expression via the SAGA (SPT-ADA2-GCN5-acetyltransferase) complex in response to cold stress. The goal of this study was to characterize the function of the adaptor protein ADA2, a member of the SAGA complex that may link CBF1 to chromatin remodeling proteins. Expression analysis confirmed that *Brachypodium* CBF1 and ADA2 were expressed synchronously in response to cold treatment. Bimolecular fluorescence complementation (BiFC) analysis demonstrated that ADA2 and CBF1 interact directly *in planta*. These results further support the hypothesis that the SAGA complex exists in *Brachypodium* and that it may play an important role in mediating the cold response mechanism.
Résumé

Pour les pays nordiques, les épisodes de gel précoces et tardifs limitent considérablement le rendement des cultures et génèrent de lourdes pertes économiques.Bien que la plupart des plantes céréalières cultivées au Canada possèdent d'emblée un certain niveau de tolérance au gel, elles nécessitent toutes une période d'exposition à de basses températures (de 2 à 10°C) afin de maximiser leurs niveaux de tolérance. Ce processus d'acclimatation repose sur l'expression induite des gènes COR (Cold-Regulated Genes) contrôlés en grande partie par le régulateur CBF1 (C-repeat Binding Factor 1). Récemment, il a été proposé que CBF1 pourrait interagir avec le complexe chromatinien SAGA dans le but d'accomplir sa fonction. Cette interaction serait média-Re par une sous-unité du complexe SAGA, la protéine adaptatrice ADA2. Cette dernière représenterait donc le lien moléculaire unissant les mécanismes de régulation génique traditionnels et chromatiniens impliqués dans le développement de la tolérance au gel des plantes. Le but de cette étude était de caractériser l'interaction physique entre CBF1 et ADA2 dans un contexte in planta. Des analyses d'expression en temps réel ont démontré que BradiCBF1 et BradiADA2 sont exprimés de façon similaire en réponse aux basses températures. De plus, des analyses d'interactions utilisant la technique de complémentation bimoléculaire de fluorescence (BiFC) ont démontré pour la première fois une interaction in planta entre les protéines BradiCBF1 and BradiADA2. Les résultats présentés ici suggèrent fortement qu'un complexe apparenté au complexe SAGA existe chez Brachypodium distachyon et que ce dernier pourrait jouer un rôle important lors du développement de la tolérance au gel chez les plantes céréalières.
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I would like to thank my other current, former and future lab members: my friends Lisa Rosenberger and Rachel Dionne who contributed data to my project and random entertainment, and Boris Mayer for helping me create the transgenic calli and being the only person who knows how the lab microscope works.

Lastly, I would like to thank my mom, my sister and my guinea pigs.
List of abbreviations

2, 4-D 2, 4-dichlorophenoxy acetic acid
ABA abscisic acid
ADA transcriptional adaptor
AP2 APETALA 2
AtADA2a Arabidopsis thaliana transcriptional adaptor 2a
AtADA2b Arabidopsis thaliana transcriptional adaptor 2b
AtCBF1 Arabidopsis thaliana C-repeat binding factor 1
ATP adenosine triphosphate
bHLH basic helix-loop-helix
BradiADA2 Brachypodium distachyon transcriptional adaptor 2
BradiCBF1 Brachypodium distachyon C-repeat binding factor 1
BLAST basic local alignment search tool
bp base pairs
CaMV cauliflower mosaic virus
CBF C-repeat binding factor
cDNA complementary DNA
CEC compact embryogenic calli
COR cold response
CRT/DRE C-repeat/dehydration response element
DAPI 4’, 6-diamidino-2-phenylindole
DNA deoxyribonucleic acid
DNase deoxyribonuclease
DNMT DNA methyltransferase
EDTA ethylene diamine tetraacetic acid
EYFP enhanced yellow fluorescent protein
FAO Food and Agriculture Organization
FPLC fast protein liquid chromatography
GCN5 general control non-repressible 5
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>ICE</td>
<td>inducer of CBF expression</td>
</tr>
<tr>
<td>JA</td>
<td>jasmonic acid</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MS</td>
<td>Musharige and Skoog</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>Nos</td>
<td>nopaline synthase</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PTGS</td>
<td>post-transcriptional gene silencing</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNAP</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SA</td>
<td>salicylic acid</td>
</tr>
<tr>
<td>SAGA</td>
<td>SPT-ADA2-GCN5-acetyltransferase</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>switch/sucrose non-fermentable</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>YC</td>
<td>EYFP C-terminus</td>
</tr>
<tr>
<td>YN</td>
<td>EYFP N-terminus</td>
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Chapter 1: Introduction

1.1 Introduction

Failure to survive in response to environmental stress limits crop productivity and generates substantial economic losses every year. Freezing stress is thus a very real issue for crops in many parts of Canada, with its short growing season, unpredictable periods of frost (in May or even June), and late cold spells. Quebec has three major vegetative zones: the tundra, the taiga (land north of 50° N latitude) and the temperate zone (south of 50°N latitude). Much of Quebec's agricultural crops are grown in the temperate zone, which covers 25% of Quebec's land. Cereal crops do not thrive north of 50°N latitude, which comprises the remaining 75% of the area of Quebec. In fact, there are 39 million hectares of arable land located above 55° N latitude that could be cultivated, but currently are not due to the suboptimal temperatures encountered there (Mills, 1994), as well as the presence of the Canadian Shield. The land available for crops is scarce due to the fact that natural genetic heritability available for developing crop freezing tolerance is low.

Most temperate cereal crops such as rye, wheat, and barley, are cold tolerant. However, they still require some exposure to low but non-freezing temperatures in order to acclimate themselves to eventual freezing temperatures. A sudden episode of frost, without the benefit of a period of cold acclimation, can result in extensive damage. These characteristics are highly unfortunate when one considers the amount of potential crop-growing land available in this country that cannot be exploited. Naturally, there have been efforts to produce cold hardy crops through traditional breeding methods, although these have more or less failed to enhance the traits in question (Thomashow et al., 1990). In order to take advantage of the available land more direct methods of plant modification must be undertaken to develop cold-tolerance capabilities in crop plants, and by characterizing the molecular mechanisms of cold-response in crop plants we hope to gain knowledge into how cold-tolerance occurs and how this can be used to our advantage.

This study will therefore focus on characterizing the cold response mechanism of cereal crop plants, particularly the molecular mechanism that links the cold response to
chromatin changes in the cell. The focus will be placed on CBF1, a transcription factor that binds to the promoters of a wide array of cold response genes. Studies suggest that CBF1 activates the expression of cold response genes by recruiting chromatin modification complexes to the DNA. This recruitment, as previous in vitro assays for yeast and Arabidopsis have shown, is indirect and may involve the transcriptional adaptor protein ADA2.

1.2 Hypotheses

We hypothesize that ADA2 represents an integration node between transcriptional machinery and chromatin modifiers. In order to structure this project the following sub-hypotheses have been developed:

1. A SAGA-like complex exists in Brachypodium distachyon

2. Temporal and spatial expression of BradiCBF1 and BradiADA2 in response to cold temperatures coincides within Brachypodium

   • Expression of these genes is induced by cold temperatures

   • BradiCBF1 and BradiADA2 are expressed synchronously in response to cold temperatures.

   • BradiCBF1 and BradiADA2 are expressed in the same tissues in response to cold temperatures

3. BradiCBF1 and BradiADA2 interact directly in planta
1.3 Objectives

The overall goal for this research project was to characterize the physical interaction between BradiCBF1 and BradiADA2 *in planta*. To accomplish this goal a set of objectives was put forward:

**Objective 1:** to determine whether orthologs of CBF1 and ADA2 exist in *Brachypodium distachyon*

**Objective 2:** to clone these genes for various downstream applications

**Objective 3:** to determine whether the expression levels of BradiCBF1 and BradiADA2 follow a similar trend in time and space when induced by cold temperatures

**Objective 4:** to show that this interaction occurs in an *in planta* setting for *Brachypodium* CBF1 and ADA2 using bimolecular fluorescence complementation (BiFC) assay

**Objective 5:** a BradiADA2 overexpression line of *Brachypodium* Bd21 will be developed
Chapter 2: Literature review

2.1 The effects of abiotic stress on plants

In the natural environment, plants are constantly being exposed to abiotic conditions that limit the productivity of crops, such as drought, salinity, oxidative stress, light and temperature extremes, all of which they must adapt to in order to survive (Charron et al., 2002a, 2002b, 2008). These stresses generally lead to a plethora of cellular irregularities that upset the cellular homeostasis. These include changes in the osmotic and energy balances, loss of integrity of cellular membranes and organelles, as well as damage to genetic material.

The uncontrolled production and/or accumulation of reactive oxygen species (ROS) such as hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (-OH) are believed to be one of the major contributors to these cellular irregularities associated with abiotic stresses. Extensive literature is available on the subject and has demonstrated that ROS buildup is linked to various types of molecular damage in the cell, such as lipid peroxidation, protein fragmentation and DNA lesions (Bowler et al., 1992).

Dehydration is also an underlying cause of cellular damage. It can lead to decreased stomatal conductance, whereby plants cannot take up carbon dioxide as a result and therefore cannot perform photosynthesis (Franks and Farquhar, 2001). Environmental stresses such as drought and temperature extremes both cause dehydration stress. Thus there is considerable overlap between the cellular responses pathways triggered by cold and dehydration in plants. Furthermore, cold and dehydration response transcription factors recognize many of the same sequence elements in the promoters of genes that protect against dehydration and freezing stress (Liu et al., 1998).

Freezing stress injury to cereal crop plants

Freezing stress in particular is a serious threat to plants, as its effects are often severe and irreversible when the plant is returned to normal temperature conditions. The
plasma membrane of the plant cell is the primary site for freezing injury, as it is highly sensitive damage. If plants are exposed to temperatures between -3°C and -5°C and subsequently return to normal temperatures, lysis of the cells will occur (Steponkus, 1984).

Cell lysis can have many causes: lamellar-to-hexagonal II phase transition damage, in which the lipid bilayers of the protoplast and chloroplast membranes merge and compromise the semi-permeability of the cell membrane (Steponkus, 1984); expansion-induced lysis, which is caused by osmotic contractions of the cell membrane during repeated freeze-thaw cycles; and fracture-jump lesions, which occur during plant freezing (Webb, 1994).

Thus it is important to the plant to minimize these types of damage as much as possible. Many species of plants have evolved mechanisms to protect the cell membranes from freeze-induced damage. The process of cold acclimation is able to prevent expansion-induced lysis and the formation of hexagonal-II phase lipids to varying degrees in plants. Cold acclimation is triggered by particular changes in the lipid membrane that occur as a result of cold stress, as well as the buildup of sugars and cold-induced protein denaturation.

Like other types of abiotic stress, cold induces the expression of genes that protect the plant and protect it from freezing damage, including temperature-induced lipocalins in Arabidopsis thaliana and wheat (Triticum aestivum) (Charron et al., 2002a) and CBFs in Arabidopsis and wheat (Stockinger et al., 1997; Jaglo-Ottosen et al., 1998; Badawi et al., 2007).

**Cold acclimation and freezing tolerance**

Cold acclimation is the development of freezing tolerance that is obtained during plant exposure to low, non-freezing temperatures and shortened days. During the process of cold acclimation, plants increase their freezing tolerance ability. Acclimated plants are able to withstand freezing temperatures markedly better than their non-acclimated counterparts (Jaglo-Ottosen et al., 1998). Two common types of cereal crop, wheat (Danyluk et al., 1991) and rye, are able to tolerate temperatures as low as -20°C and -30°C respectively, provided they are properly acclimated prior to exposure to freezing
temperatures (Thomashow et al., 1990, 1993). In the absence of acclimation freezing temperatures as high as -5°C will be sufficient to kill the plants. Cold acclimation has also been observed in Arabidopsis, albeit to a limited degree (Thomashow et al., 1990).

In 1970 it was first suggested that cold acclimation signals could alter gene expression in plants (Weiser et al., 1970). In 1985, Guy et al. determined that changes in the expression of protective proteins occurred during cold acclimation. By now, it is well established that some plants can acclimate to low, non-freezing temperatures prior to frost exposure. These plants are able to tolerate freezing temperatures by limiting injury at the molecular level (Thomashow, 1999).

**Cold-regulated (COR) genes**

The cold acclimation process revolves around the cold-induced expression of cold-regulated (COR) genes, whose functions are crucial for protection against freezing stress (Thomashow, 1999). The first COR genes were isolated as cDNA from Arabidopsis (Hajela et al., 1990). Recent findings obtained with large scale genomic approaches have shown that the expression of roughly 10% of the plant genome is modulated by low temperature. These large-scale projects have lead to the creation of three general categories of COR genes: genes that encode structural proteins; genes that encode components of osmoprotectant biosynthesis pathways; and genes that encode components of signal transduction pathways, such as transcription factors. Overall, COR genes largely code for proteins that play a protective role in freezing tolerance (Thomashow et al., 1990, 1993). As many as 2500 to 3000 genes may be regulated by cold (Seki et al., 2001), although the functions of roughly 60 to 70 COR have been extensively studied so far.

Interestingly, the promoters of most COR genes were found to contain sequences (5’-[AC]-CCGAC-3’) that became known as C-repeat/dehydration-response elements (CRT/DREs), cis-acting DNA regulatory sequences. These elements are recognized by a family of transcription factors designated the C-repeat binding factors (CBFs) (Stockinger et al., 1997).
The CBF (C-repeat binding factor) family of transcription factors

The CBF proteins are characterized by the presence of a DNA-binding AP2 domain that is flanked by CBF “signature” motifs. These motifs determine the specificity of CRT/DRE sequence binding by the appropriate CBFs (Jaglo et al., 2001). The CBF family of genes has numerous members found in a wide variety of plants, and is particularly elaborate within the Poaceae family. The Poaceae branched off into the subfamilies of Oryzaceae, Panicoideae and Pooidae 55-70 million years ago (Kellogg, 2001). The Triticaceae and Poeae tribes of the Pooidae subfamily emerged some 35 million years ago. Within the Triticaceae tribe barley and rye diverged from wheat 11 and 7 million years ago respectively (Huang et al., 2002). Out of the ten classified groups of CBF genes, six are found specifically in Pooidae plants, and five of the groups specific to Pooidae are expressed in response to cold (Badawi et al., 2007).

Additional characterization of the CBF family of genes has been done in Arabidopsis. Prior to cold-induced expression being observed in wheat, three CBF transcripts designated CBF1, CBF2 and CBF3 (Jaglo-Otto ten et al., 1998) were shown to accumulate within 15 min of the plant being exposed to low temperatures. In wheat the expression of the CBF2, CBF3 and CBF4 gene families were observed to peak 4h following exposure to 4°C temperatures (Badawi et al., 2007).

The CBF family of genes is also involved in response mechanisms to other types of stress. CBF2 overexpression in Arabidopsis has been shown to suppress ethylene-induced leaf senescence and chlorophyll degradation, as well as leaf senescence induced by abscisic acid, salicylic, and jasmonic acid. ABA biosynthesis was actually induced by CBF2, and in parallel expression of SA and JA pathway genes were repressed. The lifespan of the plant is extended by two weeks due to the effect of CBF2 overexpression. Transcriptome analysis revealed that CBF2 overexpression upregulated 286 different genes in mature leaf tissue, including 30 stress-related genes, 24 transcription factors, and 20 protein metabolism genes. Also characteristically of CBFs, CBF2 overexpression increased the freezing tolerance ability of the plants (Sharabi-Schwager et al., 2010a, 2010b).

Overexpression of CBF3 in Arabidopsis (Maruyama et al., 2004), tobacco (Nicotiana tabacum) and wheat increased tolerance to salinity, drought and freezing. CBF3-
overexpressing maize lines tolerant to various types of abiotic stress were developed in 2003 (Sairam et al., 2003).

Drought stress and cold stress are similar, as both result in dehydration and cause several of the same types of damage (Pearce, 1999). Both types of stress evidently trigger the same pathways in plants, as mild drought stress can acclimate plants to cold (Clavitier and Siminovitch, 1982; Siminovitch and Cloutier, 1983; Guy et al., 1992), demonstrating the amount of overlap between the two pathways.

Overexpression of CBF1, CBF2 or CBF3 in Arabidopsis increases the plant’s cold and drought tolerance (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000). CBF4, on the other hand, is induced by drought stress, but not by cold. Arabidopsis CBF4 overexpression show slowed growth, and induced COR gene expression (Haake et al., 2002).

CBF1, the cold response element (CRE) binding factor 1

Of all of the members of the CBF family of genes, CBF1 may be the most crucial to the activation of the cold acclimation process in cereal crop plants. CBF1 is a transcription factor responsible for regulating transcription of cold-response genes and induced by ICE (inducer of CBF expression) in response to low temperatures (Stockinger et al., 1997; Jaglo-Ottosen et al., 1998; Chinnusamy et al., 2003). It represents one of the first steps in the cold response pathway and was first isolated in 1997. Sequence analysis revealed a protein with a molecular weight of 24 kilo Daltons, with a putative acidic activation domain, a DNA-binding domain and a nuclear localization signal, both of which are characteristic of transcription factors. CBF1 binds to CRT/DRE repeat sequences via its AP2 domain (Stockinger et al., 1997) when induced by cold treatment (Gilmour et al., 1998).

Wild type Arabidopsis showed increased resistance to freezing temperatures if they were exposed to low non-freezing temperatures prior to freezing exposure. Arabidopsis transgenic lines overexpressing CBF1 induced COR gene expression and increases freezing tolerance of non-acclimated plants. The freezing tolerance ability of the CBF1-overexpressing plants without cold acclimation was comparable to the freezing tolerance
ability of acclimated wild type *Arabidopsis* plants (Jaglo-Ottosen et al., 1998). However, plants overexpressing AtCBF1 display a dwarf phenotype, due to the increased expression of DELLAs, which repress plant growth (Achard et al., 2008). This undesirable side effect has hindered the development of transgenic plants overexpressing CBF1.

CBF1 can be considered a master regulator of cold response, as it activates a massive downstream regulon of genes involved in cold acclimation (Thomashow et al., 2001). The next logical step is to determine through what mechanisms CBF1 transcription factors activated transcription of COR genes other than the simple direct binding to the promoter of COR genes. One potential mechanism by which CBF1 acted was through chromatin modification. CBF1 has not been shown to bind any chromatin modification proteins directly, so the focus was placed on identifying an adaptor that would link the transcription factor with a chromatin-modifying complex.

### 2.2 Regulation of gene transcription

Transcription is the process of producing an RNA transcript from the DNA strand. The transcript is produced by RNA polymerase from the template strand of the open reading frame of the gene. RNA polymerases are recruited to the promoters of genes by a wide array of different transcription factors. These proteins bind to various sequence elements in the promoter, as well as upstream of the promoter and/or downstream of the promoter.

There are several types of RNA molecules in plants, each transcribed by a different type of RNA polymerase (RNAP): mRNA (messenger RNA) transcribed by RNAP II, pri-miRNA (primary micro RNA) transcribed by RNAP II, pri-siRNA (primary small interfering RNA) transcribed by RNAP IV, siRNAs involved with heterochromatin formation transcribed by RNAP V, tRNA (transfer RNA) and rRNA (ribosomal RNA), both transcribed by RNAP III and I, respectively (Archambault et al., 1993).

There are two types of transcription complexes that can form around these RNA polymerases, depending on the gene to be transcribed; basal transcription of constitutively
expressed housekeeping genes requires the formation of the pre-initiation transcription complex. This complex is characterized by the presence of TBPs (TATA-binding proteins), which binds to the gene promoters. The entire complex contains many general transcription factors and RNA polymerases. As opposed to basal transcription, differential transcription occurs when genes are activated in a particular tissue and/or in response to an external signal. Transcription factors can bind to particular sequence elements in the promoters and/or intergenic regions in response to different signals. These factors then recruit co-factors, including adaptor proteins, polymerases and chromatin-modifying machinery that can enact gene expression (Triezenberg, 1995; Lee and Young, 2000).

**Transcription factors**

Transcription factors are nuclear proteins that bind directly to the DNA strand and enact transcription. These proteins, when binding directly to the DNA, may recruit RNA polymerases, and/or recruit additional transcription factors that recruit polymerases, thereby indirectly enacting transcription. Conversely, transcription factors may block the binding of polymerases to the DNA and repress expression of the gene. Transcription factors often dimerize in response to signals in the cell, which exposes a DNA-binding site. These proteins bind to promoter elements on DNA strands, and recruit co-activators or co-repressors and RNA polymerases to form transcription complexes. Transcription factors are small proteins that usually have at least two specialized domains: one, a DNA binding domain, and the second, an activation domains through which it is able to recruit co-factors and RNA polymerases. DNA-binding domains are composed particular motifs (bHLH, basic leucine zipper (bZIP), zinc fingers and helix turn helix motifs) that interact with other proteins and/or with DNA (Triezenberg et al., 1995).

There are four categories of eukaryotic activators, based on the type of activation domain they contain: acidic, glutamine-rich, proline-rich and serine/threonine rich (Triezenberg et al., 1995). Specificity in binding is determined by flanking residues, which can vary in number, from many to one. Yeast GCN4P contains seven different motifs (Drysdales et al., 1995), for example, while maize (Zea maize) anthocyanin biosynthetic
pathway transcriptional regulator C1 has a single leucine residue that determines binding specificity and capability (Sainz et al., 1997). CBF1s contain an N-terminal DNA- and protein- binding AP2 (APETELA2) domain, as well as a variably sized C-terminal acidic activation domain (Mao et al., 2006).

2.3 Chromatin-mediated transcriptional activation

Transcription factors represent only one mechanism through which the cell can regulate the expression of genes. Gene expression can also be controlled through the modification of the DNA structure itself, through siRNA/miRNA-mediated silencing mechanisms, as well as through modifying the interaction of the DNA strand with proteins that are used to package the DNA.

DNA methylation

DNA methylation serves to both repress gene expression and develop heterochromatin in non-transcribed regions such as telomeres and centromeres. DNA methyltransferases modify cytosine residues by adding a methyl group to the 5’ position on the cytosine ring. In plants cytosine nucleotides can be methylated at three different sites: CpG, CpHpG, and CpHpH sites (where H can be cytosine, adenosine or thymine). In animals cytosines are typically methylated in the context of CpG islands. CpG islands and similar motifs are generally found in gene promoters. Two broad categories of methylation occur in the cell: maintenance methylation, where methyl groups are added during DNA replication in order to maintain epigenetic modifications on the DNA strand; and de novo methylation, which can allow the plant to adjust gene expression according to environmental cues (Cao and Jacobsen, 2002).
**RNA silencing**

RNA silencing is a highly conserved epigenetic mechanism of gene regulation that exists in both plants and animals. In plants the process is known as PTGS; its animal counterpart is known as RNAi (Voinnet et al., 2003). PTGS often targets viruses by processing siRNAs from viral transcripts (Klahre et al., 2002). SiRNA and miRNA are both produced from double stranded RNA processed into single stranded RNA, following similar, overlapping pathways (Dunoyer et al., 2004). The primary difference between siRNAs and miRNAs is that miRNAs are encoded in the plant’s genome, while siRNAs are usually processed from exogenous transcripts. Less frequently, siRNAs are processed from endogenous double-stranded RNA transcripts produced by inverted repeat transcription or RNA-dependent RNA polymerases (Voinnet et al., 2003). The miRNA pathway exists in plants as an endogenous mechanism of gene regulation at the epigenetic level. There is some evidence that plants also inhibit translation via miRNAs (Chen, 2003), although this process is more common in animals. SiRNA is also involved with the formation of heterochromatin in plant genomes (Wierzbicki et al., 2009). Double-stranded RNA is produced from transposon transcripts via DNA-dependent RNA polymerase IV in silent chromatin (heterochromatin) in order for them to be silenced via an siRNA-mediated pathway. Transposon loci without inverted repeats (to make hairpin loops) depend on DNA methylation to recruit the appropriate siRNA-producing machinery (Chan, 2008).

Viruses have evolved mechanisms to inhibit plant post-transcriptional gene silencing (PTGS). The tomato bushy stunt virus encodes the p19 suppressor. Once the plant is infected with the virus, the p19 protein presumably binds siRNA, sequestering them in order to prevent cleavage of siRNA targets (Voinnet et al., 2003). At this point, however, p19 binding of siRNA has only been demonstrated in vitro (Dunoyer et al., 2004).

The p19 suppressor also targets the miRNA pathway. It binds poorly to 21-nt single-stranded miRNA but binds strongly to miRNA duplexes in vitro (Dunoyer et al., 2004), suggesting that p19 blocks the miRNA-processing pathway upstream of Dicer cleavage.
**Chromatin modification**

A DNA strand in a eukaryotic cell can be as long as several metres, and thus needs to be stored in an organized fashion in order to fit into a nucleus that is approximately only 10 μM in diameter, and still be made accessible to transcription machinery (Van Holde, 1989). Chromatin falls into two categories: euchromatin, which is loosely packed (composed of nucleosome ‘beads on a string’) and actively transcribed (Worcel, 1977), and heterochromatin, which is densely packed and thus not accessible to transcription machinery. Heterochromatin can be permanent, such as the DNA forming chromosomal centromeres, or facultative, in that it can be covered into a more loosely packed state and transcribed. Heterochromatin also represents a higher level of DNA packaging. It is composed of a 30 nm fibre that is formed from further packaging of the ‘beads on a string’ structure (Lowary, 1989). During cell metaphase DNA packs into a higher order structure to form the chromosome (Ratner and Hamkalo, 1978).

The expression of genes can also be controlled through the modification of the DNA structure and the interaction between the DNA strand and the proteins that are used to package the DNA. In order to control the expression of genes, the chromatin structure can be modified to make it more or less available to transcription machinery. Specialized chromatin-modifying enzymes are responsible this dynamic behaviour. These enzymes, however, are generally recruited to the DNA in response to a particular signal. This recruitment can occur through the interaction of a particular transcription factor or adaptor protein that acts as an intermediate between the chromatin-modifying enzyme and the transcription factor, which may allow the transcription factor to maximize the variety of modifying enzymes it can recruit to the DNA, and thusly, the variety of functions it can perform.

**The nucleosome**

In order to effectively store an organism’s entire genome into the nucleus of the cell, it must be packaged in a highly ordered way. This is achieved through the wrapping of the
DNA strand around a protein structure called a nucleosome. Nucleosomes are composed of 4 pairs of histone subunits, forming a protein octamer. All histone proteins contain the “histone fold” – a structural protein motif composed of three alpha-helices, separated by two loops. The histone octamer is composed of a H3-H4 tetramer flanked by two H2A/H2B dimers. Nucleosomal DNA has 14 binding regions around the histone octamer (H-bonds and salt links) (Luger et al., 1998), for a total of 120 direct interactions (Davey and Richmond, 2002). The N-terminal regions of the histone proteins form long, protruding tails and present sites for histone modification. Histone H2B and H3 tails protrude through minor grooves of DNA strand, every 20 base pairs. The octamer is made up of histones H2A, H2B, H3 and H4. Outside of the core are histones H1 and H5, which secure the DNA loop around the histone octamer. As the histones subunits are positively charged it encourages the binding of the phosphate backbone of the DNA strand to it. The DNA wraps around the histone core 1.65 times left-handedly and forms a length of 147 base pairs of DNA. The nucleosomes are separated from one another by 50 base pairs of DNA, which form the “linker” regions of the strand. In addition to storing the DNA molecule the nucleosome also serves to control gene accessibility (Ramakrishnan, 1994).

**ATP-dependent nucleosome remodeling**

ATP-dependent nucleosome remodeling enzymes use the energy produced by ATP hydrolysis to disrupt the contacts between histones and nucleosomal DNA (Goldman et al., 2010). For example, the yeast RISC complex uses ATP hydrolysis to disrupt the structure of the nucleosome, and to recruit transcriptional activators to the DNA in the nucleosomes (Cairns, 1996). The human and yeast SWI/SNF has been shown to remodel the chromatin structure both *in vivo* and *in vitro*. This remodeling then recruits transcriptional activators and basal transcription machinery binding to nucleosomal DNA (Sterner et al., 1999).

Another important factor in chromatin-related gene regulation are variant histones. The chromatin remodeler SWR1 (an ATP-dependent histone-variant exchanging complex) replaces histone H2A with H2AZ in the histone octamer, which increases remodeling activity and subsequently activates gene expression (Goldman et al., 2010).
**Nucleosome sliding**

Nucleosome sliding is the transportation of a histone octamer to a location on the DNA strand downstream. Nucleosome sliding is one aspect of nucleosome remodeling activities. ATPase ISWI is an example of a remodeling complex that catalyzes nucleosome sliding (Längst and Becker, 2001). ISWI belongs to SWI2/SNF2 family of chromatin remodelers (Eisen et al., 1995), which are characterized by C-terminal SANT-like modules. Other remodeling complexes can also disrupt interactions between the nucleosome and the DNA without disrupting the octamer, or can remove the octamer altogether, which is then moved to an entirely new segment of DNA in *trans* (Lorch et al., 1999; Phelan et al., 2000).

In addition to covalent modifications, histone tail modifications may also recruit chromatin-remodeling complexes that can relocate nucleosomes (Yang et al., 2007). Recent studies have shown that nucleosome location may depend on the actual sequence of the DNA strand, where the sequence affects the structure of the strand (Segal et al., 2006). The tail domains of histone proteins may in fact play a role in the recognition of the DNA sequence, as removal of tail domains from histones lead to redistribution of nucleosomes along the DNA strand (Yang et al., 2007).

**Histone modification**

There are many types of covalent histone modifications: acetylation, methylation, demethylation, phosphorylation, ubiquitination, SUMOylation, ADP-ribosylation, deimination and proline isomerization. Histones can be modified on multiple sites with multiple types of modifications. These modifications can either activate or repress transcription, depending on both the type and location of the modifications. Different combinations often have different effects on transcription (the “histone code”) (Berger, 2002; Jenuwein and Allis, 2001).

The chromatin structure can be modified via the tails of the histone proteins forming the nucleosome. Residues on the histone tails are covalently modified in a bipartite manner, leading either to a loosening or a tightening of the DNA strand around the histone octamer.
Modifications such as the addition of a negatively charged acetyl group to the lysine residues of histone tails repels the negatively charged DNA backbone from the nucleosome, creating a more open structure that allows transcription factors and polymerases to bind to the DNA strand. Conversely, other modifications such as methylation fail to neutralize the positive charge of the histones, leading to a more packed chromatin structure that prevents activation complexes from binding. In order to control this process, specialized proteins that add or remove residues from the histone tails are available downstream of signaling pathways in order to activate or repress gene expression when necessary. More rarely, arginine residues on histone tails may be modified. The histone tail arginine methyltransferase PRMT6 methylates H3R2. These are typically repressive modifications that serve to prevent the binding of H3 activation proteins to acetylated lysine residues (Iberg et al., 2008).

**Histone acetylation**

Histone acetylation has long been associated with gene expression (Turner, 1991; Turner and O’Neill, 1995). *In vivo* assays have shown that the histones in transcriptionally active nucleosomes were hyperacetylated, while heterochromatic regions lacked the same degree of acetylation. Acetylated histones were shown to be bound by various transcriptional adaptors *in vitro* (Sterner et al., 1999). Acetylated lysine resides on the N-terminal tails of histone proteins in nucleosomes, changes affinity of DNA for nucleosomes and changes the structure of the chromatin (Grant et al., 1997).

There are two classes of histone acetyltransferases: the first group, known as type A HATs, are localized to the nucleus and acetylate histones in nucleosomes. Type A HATs activate transcription of the genes (Brownell and Allis, 1996). Examples of type A HATs include GCN5, which is found in humans, yeast, plants and drosophila (Ciurciu et al., 2006); and TAFs (TBP-associated factors), which are found in humans, yeast, and drosophila. The importance of type A HATs has been demonstrated through *in vitro* assays; for example, GCN5 mutations in yeast lead to slow growth and reduction in transcriptional activation. Acetylated histone tails, in addition to repelling the DNA backbone, interact with ATP-
dependent remodeling complexes (Clapier et al., 2002). Yeast SWI/SNF complexes, for example, bind directly to acetylated histone tails (Hassan et al., 2001).

The second group of histone acetyltransferases is the type B HATs: these enzymes are cytoplasmic and acetylate free histones, leading to their assembly in chromatin (Kleff et al., 1995).

**Cold-induced histone modification**

Histone modification is often part of the cell’s response to stress. Cold stress, for example, has been shown to lead to chromatin modification in genes involved in the cold response mechanism. In Arabidopsis, AtCBF1 overexpression leads to constitutive H3 acetylation and decreased nucleosome occupancy in the promoters of COR genes, suggesting that CBF1 expression is crucial in activating COR gene expression. This acetylation decreased when the plants were de-acclimated (Pavangadkar et al., 2010).

This was also observed by an earlier study on Arabidopsis methylation markers. Conserved H3K27me3 markers are present in inactive genes in Arabidopsis, but were lost in the promoters of in two COR genes (COR15A and ATGOLS3) when the plant was cold-treated. While transcription of these genes returned to normal 24h following the return of the plant to normal temperatures, the methylation markers were not restored (Kwon et al., 2009). Both of these studies appear to suggest that the histone modification process alone, although important, is not sufficient for activation and repression of gene expression.

**2.4 Transcription factors and chromatin dynamics**

One candidate for the integration of transcription and chromatin modification is the SAGA model. SAGA (SPT/ADA2/GCN5-acetyltransferase) is a chromatin modification complex that functions by being recruited to the promoter by transcription factors. This complex originally isolated from Saccharomyces cerevisiae is comprised of four major
components: a HAT protein that acetylates lysine residues (GCN5), a transcription factor that binds to sequence elements in response to extracellular stress (CBF1), proteins that imparts nucleosome remodeling activity (SPT) and an adaptor protein that binds both the transcription factor and the HAT protein (ADA2) (Grant et al., 1997).

**The SAGA complex in *Saccharomyces cerevisiae***

The first discovery in relation to the SAGA complex took place when the transcriptional adaptor ADA2 was isolated (Berger et al., 1992). Since then, the function of ADA2 as a transcriptional co-activator in yeast has been well established (Grant et al., 1997; Mao et al., 2006; Gordon and Hark, 2007). Yeast ADA2 was then shown to interact with yeast GCN5 *in vitro* assays (Marcus et al., 1994) GCN5 mutants also exhibited slowed growth, temperature sensitivity and reduced gene activation via the VP16 (herpes simplex virus protein) and GCN4 activation domains, suggesting that GCN5 was crucial for gene expression (Marcus et al., 1994). In 2009 Jacobson et al. supported this discovery by revealing that ADA2 increases the histone acetyltransferase activity of GCN5. This association suggested that ADA2 a crucial component of chromatin modification mechanisms in the yeast cell.

Yeast two-hybrid and co-immunoprecipitation studies revealed that ADA2 formed a complex with GCN5. This complex acetylated free histone H3 preferentially and acetylated free H2B weakly. It also acetylated nucleosomal histones *in vitro* (Grant et al., 1997; Balasubramanian et al., 2001). The presence of ADA2 was crucial for GCN5 activity (Grant et al., 1997). The ADA2 SANT domain may regulate GCN5 by altering the structure of the GCN5 histone-tail binding pocket. Deletion of the SANT domain reduced the H3 acetylation ability of an ADA2-GCN5 complex in yeast significantly (Balasubramanian et al., 2001). The acetylation of histones in nucleosomes also required the presence of the GCN5 bromodomain. Without this domain GCN5 is still able to acetylate free histones, but cannot acetylate nucleosomal histones (Sterner et al., 1999).

An additional crucial component in the SAGA complex is SPT, a relatively less-characterized protein. These are transcriptional regulators that fall into two functional
groups: one encodes genes for histones and chromatin regulators; the second encodes related genes that form part of the SAGA complex. The proteins SPT3, SPT7, SPT20/ADA5 and ADA2 were found to co-purify with GCN5 (Grant et al., 1997), suggesting that ADA2 and GCN5 functioned as part of a large activation complex. SPT3 interacts with TBP/SPT15 in the SAGA complex in yeast, suggesting that the SPT group of proteins assists in linking SAGA complex HAT activity with transcription (Sterner et al., 1999).

The SAGA complex in *Arabidopsis thaliana*

In 2001 Stockinger et al. reported the existence of two ADA2 homologs in *Arabidopsis*, AtADA2a and an N-terminally truncated form, AtADA2b. A homolog of GCN5 was also found in *Arabidopsis*. GST-AtCBF1 pull-down assays demonstrated that AtCBF1 interacted with both AtADA2 isoforms, as well as GCN5. GST-AtGCN5 pull-down assays showed that AtGCN5 interacted with both isoforms of AtADA2. AtADA2a also enhanced the HAT activity of AtGCN5 five fold *in vitro* (Mao et al., 2006). When expressed in yeast, AtCBF1 was able to harness transcription machinery and activate the expression of reporter genes with promoters containing minimal C-repeats (Stockinger et al., 2001), suggesting that a similar complex was involved in the expression of COR genes.

ADA2 transcriptional adaptors may thus serve as the contact points through which CBF1 recruits HAT complexes to specific promoters in response to freezing stress. These data suggested that a complex very similar to the SAGA complex found in yeast existed in *Arabidopsis*. The fact that CBF1 was possibly one of the main factors recruiting the SAGA complex to the DNA suggested that this complex was involved in cold acclimation.

2.5 *Brachypodium distachyon*, a model for cereal crop research

The usefulness of *Arabidopsis* as a model plant system is well established. However, despite its rapid life cycle, small stature, ease of cultivation and transformation, as well as
having a sequenced genome, it is not a perfect system for studying cold stress in cereal crop plants, as it a dicot and thus not closely related to many cereals. The functions of genes characterized in Arabidopsis may then not be directly translatable to their monocot counterparts. Rice (Oryza sativa) is currently used as a model system for crop grasses, although it can be a challenging and expensive plant to grow. Despite this, it is still used as a model organism, due to the fact that its entire genome has been sequenced and entered into databases. Yet while rice is becoming a more popular model for cereal crop study, it is only very distantly related to modern crops, having diverged from a common ancestor some 50 million years ago (Zhang et al., 2005). A more appropriate model system, one more closely related to a wider variety of crop grasses and simpler to cultivate, is thus becoming a necessity in crop research. One such plant being put forth is Brachypodium distachyon, commonly known as purple false brome.

Brachypodium is a small wild grass, some accessions growing to be only 20 centimetres in height. It is a monocot and a member of the grass family Poaceae. Cultivating this plant proves to be relatively simple, requiring little maintenance and infrequent watering. Under optimal conditions it completes its entire life cycle in less than two months, comparable to Arabidopsis. Brachypodium is also self-pollinating and produces a respectable number of seeds (Draper et al., 2001). Efficient protocols for transformation have been developed in recent years (Alves et al., 2009; Christiansen et al., 2005; Vain et al., 2008), making it a potentially exceptional model for cereal crop research and modification. The Brachypodium distachyon inbred accession Bd21 was developed in response to the need for a model crop plant that could be transformed efficiently and lead to the development of transgenic plant lines for mutagenic analysis (Vain et al., 2008).

Brachypodium is particularly desirable for genetic manipulation as it has a fully sequenced, relatively small genome, approximately 2% the size of the wheat genome. Nearly as small as that of Arabidopsis, and much smaller than that of rice, it is composed of five easily distinguishable chromosomes. It is also ideal for crop plant research due to its evolutionary history; it diverged from its ancient Pooideaen ancestors prior to the emergence of what are known as the core pooids: Triticeae, Bromeae, Poeae, and Aveneae, all modern species of crop grass (Draper et al., 2001; Rathore and Shekhawat, 2009). Brachypodium thus shares a common ancestry with a wide range of modern crops.
2.6 Importance of cereal crops

Modern cereal crops, such as wheat, rye, sorghum and barley, have for many thousands of years been the main source of food for both humans and livestock. Wheat is the oldest, followed by rice and maize. Cereals (of the family Gramineae) provide important nutrients such as carbohydrates, vitamin B complexes, trace minerals, iron and fibre. Nearly fifty percent of the protein and energy required for our diet is derived from cereal crop harvests, highlighting their overall importance to our existence throughout history.

In recent times, however, cereal crop production has been declining, while consumption has increased steadily since the 1970s. At this point in time it appears that the direct consumption of crops (that is, cereal grains consumed by humans and not indirectly by feeding to livestock) will increase at a rate slightly above the overall population, making it imperative that the production of cereal crops improves and increases to match the growing need. World cereal production in 2012 has dropped 2.6% from 2011, continuing a trend that has formed over the past several years. This drop is due primarily to excessive drought periods resulting in greatly reduced crop yields, mainly in Europe but in many other parts of the world as well (FAO).

2.7 The bimolecular fluorescence complementation (BiFC) assay

Protein-protein interactions can be identified through the use of in vitro assays. Yeast two-hybrid assays, co-immunoprecipitation and pull-down assays have the advantages of being straightforward and efficient. The major drawback of the in vitro assay is that the results cannot necessarily be extrapolated to a biological situation. Ideally, a protein-protein interaction should be demonstrated in vivo to reconstruct the natural setting of the interaction. Fluorescence resonance energy transfer (FRET) (Gadella et al., 1999), transgenic organisms, and bimolecular fluorescence complementation (BiFC) (Brachi-Drori et al., 2004) are popular examples of in vivo assays.

BiFC is now frequently being utilized in the field of plant science to observe
interactions between two proteins of interest in planta. It was first developed in mammalian tissue culture cells (Hu et al., 2002) but has since been adapted to intact plant tissue. The assay involves putative interacting partners, each fused to the N-terminal or C-terminal half of enhanced yellow fluorescence protein (EYFP), in both orientations, that interact and bring the two halves of EYFP together, forming a stable, fluorescent complex (Brachi-Drori et al., 2004, 2007). The two halves of EYFP do not interact spontaneously, which has been shown through the use of free non-fused EYFP halves (Brachi-Drori et al., 2004). The two interactors tethered to the EYFP halves must interact in order for EYFP to be reconstituted (Brachi-Drori et al., 2007). Because reconstituted EYFP is highly stable, this system can be used to analyze weak and/or transient interactions (Hu et al., 2002), although admittedly this stability precludes the possibility of analyzing protein dissociation dynamics. This system also allows one to determine the localization of the protein complex. This is demonstrated by the dimerization of Arabidopsis bZIP63, a member of the subfamily C of Arabidopsis bZIP factors. The bZIP dimer interaction was confirmed by the appearance of fluorescent nuclei (Figure 2.1a). When the binding site of one interactor was disrupted (bZIP63pp), the interaction was disrupted and no fluorescent nuclei were observed (Figure 2.1b) (Walter et al., 2004).

Once the proteins have been expressed the formation of a complex, if the pair does in fact interact, generally occurs with a $t_{1/2}$ of 1 second. EYFP reconstitutes in a similar amount of time once the complex has formed. The development of a fluorescent EYFP protein takes an additional 50 min. In mammalian cells, formation of the stable GFP complex occurs in approximately 1 min without fused protein partners (Hu et al., 2002), although fluorescence production takes approximately 50 min to occur. This may present a disadvantage to the system; however, it is not known if the kinetics of fluorophore complex formation will be the same for this assay when performed in plant tissue. Expression levels of the fusion proteins should also be kept low to avoid appearance of non-specific interactions due to high levels of EYFP fragments that interact on their own and bring the proteins of interest together (Brachi-Drori et al., 2004).

This assay is often performed in Nicotiana benthamiana, a dwarf mutant of tobacco that is particularly suited to the assay. Nicotiana can be grown from seed and does not require stratification or vernalization beforehand. The leaves of the plant are large with a
high number of stomata that facilitate agroinfiltration, the infection of plants with *Agrobacterium tumefaciens* strain EHA105 using a needleless syringe. *Agrobacterium tumefaciens* is a gram-negative member of the *Rhizobium* family of bacteria. In nature these bacteria are known for their ability to induce tumours in dicots (crown-gall disease). These bacterium, however, are also exploited by researchers for their ability to pass genes (horizontal gene transfer) to plants via the bacterium Ti (tumour-inducing) plasmid (Hellens *et al.*, 2000). The BiFC assay has been fine-tuned for use in *Nicotiana*, although many technical limitations remain.

Alternatively, the BiFC assay can be performed in onion (*Allium cepa*) epidermal cells (Dong *et al.*, 2007; Diaz *et al.*, 2005; Zhang *et al.*, 2006). This assay has the advantages of not requiring the use of *Agrobacterium*, and performing the assay in a monocot system may also provide stronger evidence for the interaction. Using a particle gun to bombard the plasmid directly into the epidermal cells has the advantages of not requiring *Agrobacterium* and producing transformed cells at a faster rate, but the overall efficiency of this system is lower and it requires specialized equipment.

While the BiFC assay has many advantages over other types of systems that analyze protein interactions, there are many technical limitations that one must be aware of when performing the assay. It is important to attempt the interaction with each of the fluorophore in every possible orientation to the proteins of interest, as this can affect the reconstitution of the fluorophore (Brachi-Drori *et al.*, 2004). The tags are relatively large, so it is quite possible that linking them to proteins can affect the interaction of the pair of interest, either through affecting the folding of the attached protein, or through steric hindrance.

Another major disadvantage of this system is background autofluorescence. Autofluorescence in plants is similar in colour, though considerably weaker in intensity, to the fluorescence produced by reconstituted EYFP in plant cells. It is thus highly important to use a filter set that can distinguish between the two. Much of the autofluorescence observed is due to chlorophyll secondary metabolites (Zhang *et al.*, 2010) produced by cells damaged by the physical pressure placed on epidermal cells during agroinfiltration and the *Agrobacterium* infection itself. Most autofluorescence produced by plants is primarily visible at the same wavelengths of GFP. The use of EYFP generally avoids the interference produced by autofluorescence, as little plant autofluorescence emits light at the same
wavelengths.

It is also important to note that not every in vitro interaction will necessarily be confirmed by the BiFC assay – for example, the interaction between AP3 and PI has been confirmed in vitro, yet the BiFC assay for this interaction was not successful (Brachi-Drori et al., 2007). Considering that there is solid in vitro evidence that these protein pairs do in fact interact (Egea-Cortines et al., 1999), the lack of a signal is likely due to the BiFC assay itself. The EYFP halves linked to the interactors may affect the interactors in such a way that they cannot interact as they normally would. There is also the possibility that these interactions require co-interactors that are not available in order to form a stable complex. In the case of using Nicotiana as the host plant for the assay, the co-interactors may not be present in the epidermal cells, and analyzing two monocot proteins in a dicot host system (such as Nicotiana) co-factors that may be required for the complex to form may not be produced by the host plant. The complex may also require certain stimuli, such as cold or light, in order to form. In general, if no interaction is observed in the BiFC assay, it is important to test the interaction through various in vitro assays to determine if the assay itself is at fault.
2.8 Figures

**Figure 2.1 The BiFC assay.** (a) Reconstitution of split EYFP takes place when the proteins of interest (bZIP63-bZIP63) interact *in planta*. The bZIP63 transcription factors dimerize in the nuclei of *Nicotiana benthamiana* epidermal cells. (b) bZIP63 with a mutated interaction site (bZIP63pp) cannot interact with wild type bZIP63. EYFP is not reconstituted. Figure adapted from Walter *et al.* (2004).
Chapter 3: Materials and methods

3.1 Plant production and transformation

Plant material and growth conditions

Wild type *Brachypodium distachyon* Bd21: seeds were washed for once for 10 min with sterile water and a solution of 10% sodium hypochlorite. Under sterile conditions approximately 50 seeds per 100 mm plate were placed on wet Wattman filter paper. The plates was covered and sealed with Micropore tape (3M). The plates were stratified for 1 week at 4°C in total darkness. After 1 week the seeds were planted in a mixture of soil and Perlite and grown at 22°C under a 16-hour photoperiod with a light intensity of 100 μE m⁻² sec⁻¹. Total RNA was extracted from adult plants using the RNeasy kit (Qiagen). Wild type *Arabidopsis thaliana*: seeds were prepared by sterilizing them with a solution of 10% sodium hypochlorite and rinsing with sterile water three times. The seeds were placed 1 cm apart on 1X MS plates with a pipette tip. The seeds were stratified at 4°C for 3 days in darkness. The plates were incubated in a 22°C growth chamber under a 16-hour photoperiod with a light intensity of 100 μE m⁻² sec⁻¹. Plants were collected at the 6-leaf stage (after approximately 2 weeks of growth) and immediately frozen in liquid nitrogen. Total RNA was extracted from the tissue using the RNeasy kit (Qiagen). *Nicotiana benthamiana*: seeds were planted in a mixture of 70% Pro-mix (Premier) and 30% sand, irrigated from below with standard fertilizer, covered and grown in a chamber set at 25°C under a 16-hour photoperiod with a light intensity of 100 μE m⁻² sec⁻¹. Seedlings were transplanted to individual pots at the 4-leaf stage, kept covered at 25°C, irrigated from below with standard fertilizer until 3 weeks of age. Plants were watered with fertilizer 3h prior to agroinfiltration.
Sequence analysis and primer design

Amino acid sequences from *Arabidopsis thaliana*, wheat (*Triticum monococcum* or *Triticum aestivum*) and rice (*Oryza sativa*) were obtained from The Arabidopsis Information Resource (TAIR) (www.arabidopsis.org) and NCBI (www.ncbi.nlm.nih.gov) to search for similar genes in *Brachypodium distachyon*. Orthologous sequences were mined from the BrachyBLAST database (Brachybase.org). The amino acid sequences were aligned using Clustal Omega Multiple Sequence Alignment software (www.ebi.ac.uk/Tools/msa/clustalo) and homologous residues were highlighted using BoxShade3.21 software. BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi) analysis was also performed on the sequences obtained from *Brachypodium*. Primers were designed using Primer3 software (frodo.wi.mit.edu/primer3/) based on the coding sequences of the putative *Brachypodium* genes. Each set was designed to keep the gene in frame with either downstream or upstream tags and did not contain stop codons (Table 3.1).

Cloning

Total RNA was extracted from wild type *Brachypodium distachyon* Bd21 and *Arabidopsis thaliana* plants using the Qiagen RNeasy kit (Qiagen) and cDNA was produced using the Agilent cDNA AffinityScript kit (Stratagene). This cDNA was used as a template for PCR amplification. AtADA2a was amplified from a pUNI52 vector containing the open reading frame for this locus (Arabidopsis Biological Resource Centre). Each gene was amplified using the following protocols in a Thermal cycler (Bio Rad); BradiADA2: 35 cycles, 2 min initial denaturation at 95°C, 30 sec denaturation at 95°C, 30 sec annealing at 51°C, 2 min extension at 68°C, 10 min final extension; BradiCBF1: 35 cycles, 2 min initial denaturation at 95°C, 30 sec denaturation at 95°C, 30 sec annealing at 53°C, 45 sec extension at 68°C, 10 min final extension; AtADA2a: 35 cycles, 2 min initial denaturation at 95°C, 30 sec denaturation at 95°C, 30 sec annealing at 49°C, 2 min extension at 68°C, 10 min final extension; AtCBF1: 35 cycles, 2 min initial denaturation at 95°C, 30 sec denaturation at 95°C, 30 sec annealing at 45°C, 2 min extension at 68°C, 10 min final extension. BradiCBF1,
BradiADA2 and AtCBF1 were incorporated into the pCR8 vector via topoisomerase-mediated TA-cloning (Gateway®-compatible entry vector; Invitrogen). All reactions were transformed into chemically competent MACH1 DH5α *Escherichia coli* cells. PCR screening identified positive clones. Overnight cultures were grown and prepped using the QIAprep Spin Miniprep kit (Qiagen). Plasmid was digested using EcoRI (New England Biolabs) to confirm the presence of the gene. Further restriction analysis was performed to confirm correct orientation. AtADA2a was cloned into the pGEM T-Easy vector (Promega) and transformed into chemically competent MACH1 DH5α *E. coli*. PCR screening identified positive clones. Overnight cultures were grown and prepped using the QIAprep Spin Miniprep kit (Qiagen). Plasmid was digested with EcoRI (New England Biolabs) for 2h to digest AtADA2a from the cloning vector. This fragment was ligated into EcoRI-digested vector pENTR2B (Gateway®-compatible entry vector; Invitrogen). The ligation reaction was transformed into chemically competent MACH1 DH5α *E. coli*. PCR screening identified positive clones. Overnight cultures of positives were grown and prepped using the QIAprep Spin Miniprep kit (Qiagen). Plasmid was digested with EcoRI (New England Biolabs) for 30 min to confirm presence of the gene (Figures 3.1 and 3.2).

Sixteen separate LR clonase reactions (LR Clonase II plus enzyme kit; Invitrogen) were performed to incorporate each gene into each BiFC assay destination vector. The genes were cloned between the CaMV 35S promoter and the NOS transcriptional terminator in the destination vectors (Table 3.2). All final constructs were sequenced to confirm that the genes were in the correct frame with the tags (Appendix I).

Transformation of BiFC vectors into *Agrobacterium tumefaciens* EHA105

All BiFC vectors were transformed via electroporation into *Agrobacterium tumefaciens* strain EHA105. *Agrobacterium* were plated onto selection plates (25 μg/mL rifampin; 100 μg/mL ampicillin). PCR screening identified positive clones.
The full-length EYFP positive control vector

EYFP cDNA was digested from pSITE-3CA using Ncol/NotI and ligated into Ncol/NotI digested BiFC set vector pSAT4-DEST-nEYFP-C1 (both vectors obtained from Arabidopsis Biological Resource Centre). The ligation was transformed into chemically competent MACH1 DH5α E. coli. PCR screening identified positive clones. Overnight cultures were prepped using the QIAprep Spin Miniprep kit (Qiagen). Plasmid was digested with EcoRI and Ncol/NotI to confirm presence of the gene (New England Biolabs) (Figure 3.3).

BiFC assay – agroinfiltration of Nicotiana benthamiana

Two millilitre cultures of LB with the appropriate antibiotics (25 μg/mL rifampin; 100 μg/mL ampicillin for BiFC vectors) were inoculated with Agrobacterium tumefaciens EHA105 containing the vectors and grown overnight to an O.D. of ~0.7 at 28°C (200 rpm). The p19 suppressor clone was grown to an O.D. of 1.0 in a 1 mL overnight culture at 28°C (200 rpm). Cells were pelleted (10 min at 5000 rpm) and resuspended in the same volume of infiltration media (LB; 10 mM MgCl₂; 150 μg/mL acetylthiogalactosamine). The cultures were incubated for 3h at room temperature (60 rpm). Prior to infiltration the appropriate Agrobacterium clones were combined and co-infiltrated with the p19 vector in a 4.5:4.5:1 ratio. The Agrobacterium solution was infiltrated into the abaxial side of the leaf of a Nicotiana plant using a 1 mL needleless syringe (Terumo). The plants were covered for 3-5 days at 25°C. To stain the epidermal cell nuclei a solution of 1 mg/mL DAPI + 0.5% Triton X-100 in LB buffer was infiltrated into the Agrobacterium-infiltrated site 2-2.5h prior to observation.
Microscopy

To observe fluorescence a Zeiss V20 Discovery Stereomicroscope (Zeiss) equipped with an X-Cite Series 120Q UV lamp (Lumen Dynamics) was used. Infiltrated Nicotiana leaves were removed from the plant and observed under either the GFP filter (excitation wavelength: 395 nm emission wavelength: 509 nm) or the EYFP-LP filter (excitation wavelength: 514 nm; emission wavelength: 527 nm) filter. DAPI-stained samples were observed under the DAPI filter. Positive control samples were observed 12-24h following agroinfiltration. Interaction assay samples were observed 4-5 days following agroinfiltration. Transformed onion scale samples were observed under the EYFP-LP filter using the Zeiss V20 Discovery stereomicroscope (Zeiss). Nuclei were stained with DAPI or propidium iodide. Samples were observed under the DAPI filter and the RFP/CFP filter respectively. Positive control samples were observed 24h following bombardment. Interaction assay samples were observed 72h following bombardment. All samples were photographed using the Zeiss AxioCam MRc camera (Zeiss). Images were visualized and processed using AxioVision (Release 4.8.2) software (Zeiss).

BradiADA2 overexpression vector

BradiADA2 cDNA was BsrG1-digested from the pCR8-BradiADA2 vector. This fragment was ligated into BsrG1-digested pENTR1A vector (Gateway©-compatible entry vector; Invitrogen). The ligation was transformed into chemically competent MACH1 DH5α E. coli. PCR analysis identified positive clones. Overnight cultures were prepped using the QIAprep Spin Miniprep kit (Qiagen). Plasmid was digested with BsrG1 and EcoRI (New England Biolabs) separately to confirm presence of the gene. The clonase reaction incorporated BradiADA2 into destination vector pMHb7Fm21GW-UBIL (Vlaams Instituut voor Biotechnologie). The clonase reaction was transformed into chemically competent MACH1 DH5α E. coli. PCR screening identified positive clones. Overnight cultures were prepped using the QIAprep Spin Miniprep kit (Qiagen). The pMHb7Fm21GW-UBIL-BradiADA2 vector was transformed via electroporation into Agrobacterium tumefaciens
strain AGL1 and plated on selection plates (100 μg/mL spectinomycin; 50 μg/mL carbenicillin) plates. Colonies were selected at 3 days and 5 mL overnight cultures were grown (28°C, 200 rpm in LB). Cultures were prepped and PCR screening of BradiADA2 and GFP identified positive clones (Figure 3.4). The final construct was sequenced to confirm that BradiADA2 was in the correct frame with the full-length GFP tag (Appendix II).

Expression analysis of BradiCBF1 and BradiADA2

Temporal expression analysis: on accession of Brachypodium distachyon, Bd21 (spring) was selected for analysis due to its characteristic growth habit. Plants were grown as discussed earlier. Cold treatment at 4°C was initiated when the plants were exactly 2 weeks of age. Samples (3 separate plants) were collected at each time point: 0, 0.25, 0.5, 1, 2, 4, 6, 12 and 24h. All samples were frozen immediately in liquid nitrogen. Samples from non-acclimated (22°C) plants were collected at the same time points as controls. Spatial expression analysis: 4h following the initiation of cold treatment Bd21 plants were removed from the soil and dissected into separate crown and leaf tissue samples (15 plants per tissue type). All samples were immediately frozen in liquid nitrogen. Samples from non-acclimated plants were collected at the same time as controls.

RNA was extracted using the RNeasy Extraction kit and treated with DNase I (Qiagen). The Agilent AffinityScript cDNA kit (Stratagene) was used to produce cDNA from all samples. Six microlitres of each RNA sample was used per cDNA reaction. All reactions were run simultaneously in a Thermal Cycler (Bio Rad). To prepare qPCR reactions: cDNA was diluted 1:10 and two microlitres were used per 10 μL qPCR reaction. The primers used for this assay are presented in Table 4.1. Primers were diluted to 300 nM. The iTaq Universal SYBR Green kit was used for the qPCR assay (Bio Rad). Each sample was run in technical triplicate. The Brachypodium distachyon 18S gene (Bradi18S) was used as the normalization control. The analyses were performed in the CFX Connect Real-Time system (Bio Rad). A total of three biological replicates were used for these analyses and similar results were obtained with each replicate. All qPCR amplicons were cloned into the pGEM-T-Easy vector (Promega) and sequenced (data not shown).
Transformation of CEC for development of transgenic Bd21 lines

To develop CEC for transformation: immature seeds were harvested from *Brachypodium distachyon* Bd21 plants following pollination and the lemmae was removed, with palea being left on the seed. The seed was surface sterilized in ethanol for 15 seconds, rinsed in sterile water for 15 seconds and sterilized in 1.3% hypochlorite solution for 15 seconds and washed three times with sterile deionized water. The embryo was then removed from the seed and placed on basic MSB3+CuSO$_4$ solid medium for callus culture for 3 weeks at 25°C in darkness. The pMHb7Fm21GW-UBIL-BradiADA2 vector was transformed via electroporation into the highly virulent *Agrobacterium tumefaciens* strain AGL1 and grown on selection plates (50 μg/mL spectinomycin; 100 μg/mL carbenicillin) plates. Colonies were selected at 3 days and 5 mL overnight cultures were grown in LB (28°C, 200 rpm). Cultures were prepped and PCR analysis of BradiADA2 and GFP in the plasmid prep was performed to screen for positive transformants. One positive clone was selected and grown for 24h at 28°C. Five hundred microlitres of the overnight culture was spread on selection plates (50 μg/mL spectinomycin; 100 μg/mL carbenicillin; 30 μg/mL acetylsyringone). The plates were grown at 28°C for 48h. The plates were scraped and the bacteria were resuspended in 5 mL of sterile liquid 1xMS media (45 μg/mL acetylsyringone; 100 μg/mL spectinomycin). These cultures were grown for 45 min at 28°C, shaking at 200 rpm. *Brachypodium* CEC were saturated in the culture for 5 min. After drying the CEC were transferred onto MSB3 plates (1xMS, 50 μg/mL hygromycin, 300 μg/mL timentin, 0.6 μg/mL CuSO$_4$, vitamin M5) for 2 days. After 2 days the calli were transferred to fresh MSB3 plates (40 μg/mL hygromycin; 225 μg/mL timentin) and grown at 28°C for 3 weeks. Calli expressing GFP were fragmented and grown for another 2 weeks on fresh MSB3 plates. After three weeks of growth on MSB3 plates, calli were transferred to MSR26 regeneration plates (1xMS, Fe-EDTA, 30 g/L sucrose, 0.2 mg/L kinetin, 5 g/L agar, pH 5.8, 50 μg/mL hygromycin, 300 μg/mL timentin).
3.2 Experimental designs

From the studies performed by Stockinger et al. (2001) and Grant et al. (1997) it can be theorized that ADA2 integrates the mechanisms of transcription and chromatin modification. The next step is to confirm that both factors are present in the cell at the same time in response to cold treatment. Lastly, and most importantly, it must be demonstrated that CBF1 and ADA2 interact in an in vivo setting. Due to the fact that we want to apply this research to cereal crops we will be analyzing the functions of CBF1 and ADA2 in Brachypodium Bd21.

If BradiCBF1 and BradiADA2 are indeed part of the same complex it is likely that they would be expressed synchronously when induced by cold. Once the expression peaks of BradiCBF1 and BradiADA2 over a 24-hour period were determined, the aerial tissues of the plants were harvested at roughly the peak of expression for both genes. The expression of BradiCBF1 and BradiADA2 in leaf and crown tissue was compared to expression in non-acclimated tissue to determine where cold acclimation was occurring in the plant.

While the CBF1-ADA2 interaction had been shown to occur in vitro for Arabidopsis CBF1 and ADA2, it had not yet been shown to occur in an in planta setting, nor had it been shown to occur between BradiCBF1 and BradiADA2 either in vitro or in vivo. Both sets of interactors were incorporated into a large set of destination vectors. For each pair of interactors it was necessary to perform 8 different interactions in order to maximize the possibility of observing a positive signal.

In order to ascertain the function of BradiADA2 in the context of a living plant a transgenic Bd21 BradiADA2 overexpression line was developed. The Ti-plasmid for the overexpression line contains full-length BradiADA2 linked to GFP in order to identify successfully transformed CEC. Following the successful production of the transgenic lines, the plants will undergo both phenotypic analysis and freezing tolerance assays in order to determine the effects of the mutation on the cold acclimation ability of the plants.
### 3.3 Tables and figures

#### Table 3.1 Primer set.

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<th>Primers</th>
<th>Sequence (5’-3’)</th>
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Table 3.2 List of BiFC vectors.

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Figure 3.1 The *Arabidopsis thaliana* vector set for BiFC assay. The entire set of BiFC vectors for the AtADA2a and AtCBF1 are shown here. All vectors were digested with EcoRI. AtCBF1 is 682 bp and AtADA2a is 1680 bp. Lane 1: PCR amplification of AtCBF1 from cDNA. Lane 2: PCR amplification of AtADA2a from pUNI51-AtADA2a (obtained from the Arabidopsis Biological Resource Centre). Lanes 3: AtCBF1 was incorporated into the Gateway-compatible entry vector pCR-8. Lane 4: AtADA2a was ligated into the Gateway-compatible entry vector pENTR1A. Lanes 5-8: AtCBF1 was incorporated into the four BiFC destination vectors from the entry vector via LR clonase-mediated recombination. Lanes 9-12: AtADA2a was incorporated into the four BiFC destination vectors from the entry vector via LR clonase-mediated recombination.
**Figure 3.2 The *Brachypodium distachyon* vector set for BiFC assay.** The entire set of BiFC vectors for the BradiCBF1 and BradiADA2 are shown here. All vectors were digested with EcoRI. BradiCBF1 is 700 bp and BradiADA2 is 1742 bp. Lane 1: PCR amplification of BradiCBF1 from cDNA. Lane 2: PCR amplification of BradiADA2 from cDNA. Lanes 3: BradiCBF1 was incorporated into the Gateway-compatible entry vector pCR-8. Lane 4: BradiADA2 was ligated into the Gateway-compatible entry vector pENTR1A. Lanes 5-8: BradiCBF1 was incorporated into the four BiFC destination vectors from the entry vector via LR clonase-mediated recombination. Lanes 9-12: BradiADA2 was incorporated into the four BiFC destination vectors from the entry vector via LR clonase-mediated recombination.
Figure 3.3 EYFP positive control vector for the BiFC assay. A cDNA fragment of enhanced yellow fluorescent protein was ligated into the vector backbone of pSAT4(A)-DEST-nEYFP-N1. The EYFP fragment runs at ~850 bp. Lane 1: undigested pSAT4(A)-DEST-EYFP. Lane 2: pSAT4(A)-DEST-EYFP digested with NotI and EcoRI to remove the EYFP fragment.
Figure 3.4 The BradiADA2 overexpression vector. BradiADA2 is tagged with full-length GFP for screening purposes. Lane 1: pENTR1A-BradiADA2 digested with EcoRI to remove the BradiADA2 fragment. Lane 2: the pMHb7Fm21GW-UBIL-BradiADA2 vector was digested with EcoRV; the vector backbone and BradiADA2 each have a single EcoRV restriction site. Lane 3: PCR amplification of BradiADA2 from the pMHb7Fm21GW-UBIL-BradiADA2 vector. Lane 4: PCR amplification of GFP from the pMHb7Fm21GW-UBIL-BradiADA2 vector.
Chapter 4: Results

4.1 Identification of BradiCBF1 and BradiADA2

Due to the vast amount of information available, the model organism *Arabidopsis thaliana* was used as the primary resource for sequence information. *Arabidopsis* has both CBF1 and ADA2 genes, albeit two highly similar ADA2 genes (AtADA2a (AT3G7740.1) and AtADA2b (AT4G16420.1)). The *Arabidopsis* (AT4G25490.1) CBF1 and *Triticum aestivum* (AAX28967.1) CBF1 amino acid sequences were used as bait. The genomic region Bradi3g51630.1 was selected as it had the highest homology to AtCBF1 (Score (bits): 134; E Value: 5e⁻³²) and TaCBF1 (Score (bits): 194; E Value: 6e⁻⁵⁰). Sequence analysis revealed only one CBF1 gene in *Brachypodium*. The genomic length of this gene is 978 base pairs and the coding sequence is 684 base pairs in length. It does not have introns. The aligned CBF1 sequences are shown in Figure 4.1.

*Arabidopsis* is the only plant species with two copies of the ADA2 gene (Stockinger et al., 2001). AtADA2b is the result of a nearby gene duplication event, and has a truncated 5' region when compared to ADA2a; has an alternate first exon, with 49% homology to AtADA2a (Stockinger et al., 2001). Both AtADA2a and AtADA2b interact with both AtCBF1 and AtGCN5 in vivo. Following a similar strategy as with CBF1, the sequences of *Arabidopsis* and rice ADA2 (Os03g53960.1) genes were used to confirm the identity of the putative *Brachypodium* ADA2 gene. The sequences were blasted against the Brachypodium.org BrachyBLAST database (blastp, 8x release proteins). A region of *Brachypodium* DNA in the genome (Bradi1g08470.1) was obtained that showed the most significant alignment to AtADA2a (Score (bits): 493; E Value: e⁻¹³⁹; for comparison, the second most significant alignment had a score of 76 and an E Value of e⁻¹⁴). Sequence analysis did not reveal the presence of other ADA2 genes in *Brachypodium*. The genomic length of BradiADA2 covers 1236 base pairs with twelve exons and the coding sequence is 1707 base pairs. The aligned ADA2 sequences are show in Figure 4.2.

BLAST analysis revealed the presence of particular interaction domains in the amino acid sequences of the BradiCBF1 and BradiADA2 proteins. Both BradiCBF1 and BradiADA2
have N-terminally located nuclear localization sites. BradiCBF1 features a large, centrally located AP2 domain, which is also present in AtCBF1, TaCBF1 and OsCBF1 (Figure 4.1). The C-terminus of BradiCBF1 shares a single hydrophobic cluster in common with AtCBF1 and OsCBF1. BradiADA2 has two domains within its N-terminus: one is a SANT domain similar to those found in the Myb family of DNA-binding proteins. The other is a cysteine-rich region containing ZZ-type zinc fingers. This region contains six conserved cysteine residues and two conserved histidine residues (Figure 4.2). In the BradiADA2 C-terminus is a DNA-binding SWIRM superfamily domain. These three domains are also found in Arabidopsis ADA2a/2b and rice ADA2.

4.2 Expression analysis of BradiCBF1 and BradiADA2

A key piece of evidence that BradiCBF1 and BradiADA2 interact can be provided through the use of expression analysis, as genes that are expressed synchronously in response to stimuli may function together. In this experiment, it was observed that the relative transcript levels of both BradiCBF1 and BradiADA2 are low and consistent in non-acclimated plants (Figure 4.3a). Following the induction of 4°C cold treatment, BradiCBF1 expression is upregulated and peaks at 2h. BradiADA2 is also upregulated and expression peaks at 6h (Figure 4.3b). The expression of both genes decreases sharply following peak expression, returning to the same level of expression as they were prior to the initiation of cold treatment (0h).

BradiCBF1 and BradiADA2 upregulation is most apparent in the crown tissue of Brachypodium. While the expression of both genes in crown tissue in non-acclimated aerial tissue is negligible (Figure 4.4), BradiCBF1 expression in crown tissue is significantly upregulated following the induction of cold treatment. BradiADA2 expression in crown tissue is also upregulated in response to cold treatment, albeit not to the same degree as BradiCBF1. In non-acclimated leaf tissue the expression of both genes is very low. Both genes are upregulated in leaf tissue response to cold treatment but not to the same degree as they are in crown tissue.
4.3 The bimolecular fluorescence complementation (BiFC) assay

In order to perform this assay it was necessary to create more than 21 different vectors. In addition to entry and intermediary cloning vectors, performing all the interactions necessary for the assay required four different vectors per gene. Each half of EYFP has been cloned in either possible orientation to each gene using Gateway® technology. An expression vector containing a gene for EYFP was also developed as a positive control. Eight separate interactions per interacting pair needed to be tested, as each orientation and partner could potentially affect an interaction, whether through preventing the normal folding arrangement of the protein of interest or steric hindrance. Therefore complementary EYFP halves were fused to either partner, in both orientations, producing a necessary 8-vector set per interacting pair.

Setting up the assay

Before testing the interaction it was necessary to optimize the assay. This was accomplished with the use of two different positive control plasmids, one coding full-length GFP and another coding full-length EYFP (this plasmid was created for this study). The GFP infiltration control was a full-length GFP₆ concatamer with an endoplasmic reticulum-targeting tag (Badawi et al., 2008). This protein did not diffuse into the nucleus of the cell (Figure 4.5a). Untagged proteins diffuse freely throughout the cell but cannot penetrate the large central vacuole of Nicotiana epidermal cells. In these cases the nuclei and cytoplasmic contents are highly fluorescent, as can be seen in the cells expressing untagged, full-length EYFP (Figure 4.5b). For both positive controls roughly 95% of the cells within the infiltration region expressed the fluorescent protein. Fluorescence could be observed as soon as 12h post-infiltration and the signal remained strong as late as ten days post-infiltration. The epidermal cells remained healthy following Agrobacterium infection and the subsequent expression of fluorescent protein. Indeed, damaged and/or necrotic cells lost the fluorescent signal, often rapidly after the integrity of the cell wall was
compromised. This was also observed frequently in samples stained with DAPI + Triton X-100.

Positive control plasmids were co-infiltrated with Agrobacterium containing a plasmid coding for p19, a post-transcriptional gene silencing-suppressing protein from the tomato bushy stunt virus. The plant’s PTGS mechanism typically limits the number of transcripts being produced by expression vectors, thereby drastically reducing the intensity of the fluorescence to a nearly negligible signal (Voinnet et al., 2003) (Figure 4.5c). The p19 suppressor inhibits the PTGS mechanism and leads to a far more intense fluorescent signal being produced by full-length fluorescent proteins (Figure 4.5d) as soon as 12h post-infiltration. The p19 suppressor was thus co-infiltrated with each interacting pair.

In order to confirm that the fluorescent structures were in fact nuclei, it was necessary to label the nuclei with chemical stains that had previously been optimized for this purpose. Propidium iodide and DAPI were selected as both are commonly used to stain nuclei in many different cell types. Propidium iodide stained nuclei at a much faster rate using a considerably lower concentration (100 μg/mL PI + 0.1% Triton). However, chlorophyll also fluoresces, making the stained nuclei often difficult to distinguish (Figure 4.5e). External application of any of the staining chemicals to Nicotiana leaves was not effective at staining epidermal cell nuclei.

The stains were infiltrated into the leaf prior to observation over the previously Agrobacterium-infiltrated area. Infiltrating a live leaf kept the epidermal cells alive longer in order to allow the stains more time to enter the cell. This method was slightly more efficient than simply adding the stain to a leaf removed from the plant, but even 4-5h post-infiltration with DAPI the percentage of cells with stained nuclei was only ~60%. Considering the low percentage of cells that had been observed to show the positive interaction (10-20%), this was not an acceptable level of staining due to the low frequency of overlap.

It was determined that the lack of effectiveness of the stains was due to the fact that the epidermal cell wall provided an extra barrier against the stains. Triton was added to the staining media prior to infiltration to create pores in the cell wall that would allow the stains to pass through more effectively. This method raised the percentage of stained cells within the infiltration radius to >90% (Figure 4.5f). It also reduced the time required to
stain to less than 3h, which became necessary due to the fact that both the high concentration of DAPI and the presence of a detergent in the infiltration media proved to be extremely toxic to *Nicotiana* leaf tissue. Optimizing the concentration of DAPI and Triton allowed the leaves to remain healthy long enough for visualization. Greater than 95% of fluorescent nuclei observed were stained with DAPI. DAPI-stained nuclei did not fluoresce under the EYFP filter on their own (Figure 4.6d, g; Figure 4.7d, g), confirming there was no cross-fluorescence between filter sets.

The AtCBF1-AtADA2a interaction

AtCBF1a and AtADA2a protein have been shown to interact using *in vitro* GST pull-down and yeast two-hybrid assays (Stockinger *et al.*, 2001). However interaction between these two proteins had not been shown *in planta*. Thus AtCBF1 and AtADA2a were used as a positive control for the *in planta* assay. Using the AtCBF1-AtADA2a interaction as a control provided both a positive interaction control and validation for the BiFC system. The infiltration assay was performed ten separate times in order to confirm that any positive results were not random or due to artifacts. After four assays performed with all possible interactions a positive interaction was tentatively identified for AtCBF1 and AtADA2a. Fluorescent nuclei were observed no earlier than 72h post-infiltration (Figure 4.6a), and required uninterrupted exposure to complete darkness in a 25°C growth chamber prior to observation, with constant watering. This result was confirmed through DAPI staining (Figure 4.6b). The overlay of the DAPI image and the fluorescent image is shown in Figure 4.6c.

It was observed that when YN was fused downstream of AtADA2a and YC was fused downstream of AtCBF1, the two proteins interacted and localized to the nucleus of the epidermal cell, producing fluorescent nuclei (Figure 4.6a). This was confirmed by DAPI staining (Figure 4.6b). The overlay of both images is shown in Figure 4.6c. Fluorescent nuclei were not observed when either interactor was replaced with the same interactor with the complementary EYFP fragment fused in the opposite orientation (Figure 4.6d). Replacing one of the interactors with the complementary EYFP fragment in the empty
vector also did not show a positive interaction (Figure 4.6g). The individual interactors on their own or without p19 did not produce fluorescent nuclei, nor did the suppressor on its own or untransformed *Agrobacterium* infiltrated into the leaf produce fluorescent nuclei (data not shown). The negative control interactions were confirmed by DAPI staining (Figure 4.6e, h). The overlays are shown in Figures 4.6f and i.

**The BradiCBF1-BradiADA2 interaction**

Once the *Arabidopsis* interaction had been confirmed it was used as a positive BiFC interaction control for following interaction studies. Because BradiCBF1 and BradiADA2 are highly similar to their *Arabidopsis* counterparts the same fusion orientation was used for the *Brachypodium* interaction. When YN was fused downstream of BradiADA2 and YC was fused downstream of BradiCBF1, a positive interaction was observed (Figure 4.7a). The percentage of positively transformed cells was similar (~10%) to that of the *Arabidopsis* interaction. However, fluorescent nuclei were not observed any earlier than 96 post-infiltration for the *Brachypodium* interaction. This result was confirmed by DAPI staining (Figure 4.7b). The overlay is shown in Figure 4.7c. For this interaction pair fluorescent nuclei were observed consistently over multiple runs of the BiFC assay.

When YC was fused upstream of BradiCBF1, there was no interaction (Figure 4.7d). No interaction was produced by any other combination, or when one of the interacting partners was replaced with an empty vector containing the complementary half of EYFP (Figure 4.7g). These results were confirmed by DAPI staining (Figure 4.7e, h). The overlays are shown in Figure 4.7f and i.

**4.4 Transgenic Bd21 lines overexpressing BradiADA2**

This experiment is currently in progress. The vector expressing GFP-tagged BradiADA2 has been constructed. Compact embryogenic calli (CEC) were successfully
cultured from Bd21 seeds and infected with the *Agrobacterium tumefaciens* AGL1 clone containing the overexpression vector. GFP fluorescing calli were obtained at a rate of >80% following infection. At four weeks post-infection positively transformed calli showed a strong fluorescent signal under the GFP filter (Figure 4.8a). Background fluorescence was observed in untransformed calli (Figure 4.8b) but the signal was considerably weaker than the fluorescent signal produced by positively transformed CEC. At this stage in the experiment positively transformed CEC are being grown on regeneration plates.
### 4.5 Figures

**Figure 4.1** *Brachypodium distachyon* encodes an ortholog of CBF1. Amino acid sequences from *Brachypodium distachyon*, *Triticum aestivum*, *Oryza sativa* and *Arabidopsis thaliana* CBF1 were aligned using Clustal Omega software. Residues that are identical in all four sequences are shown in white text on a black boxshade. Residues with similar properties are shown in black text on a grey boxshade. The location of the nuclear localization signal is indicated by a red line. The conserved AP2 DNA-binding domain is indicated by a black line. The acidic activation domain is indicated by a grey line. C-terminal hydrophobic clusters are indicated by green lines. The conserved hydrophobic residues in each cluster are surrounded by green boxes.
Figure 4.2 *Brachypodium distachyon* encodes an ortholog of ADA2. Amino acid sequences from *Brachypodium distachyon*, *Triticum aestivum* and *Oryza sativa* ADA2, and *Arabidopsis thaliana* ADA2a and ADA2b were aligned using Clustal Omega software. Residues that are identical in all four sequences are shown in white text on a black boxshade. Residues with similar properties are shown in black text on a grey boxshade. The location of the nuclear localization signal is indicated by a red line. The ZZ-rich zinc finger domain is indicated by a black line. The conserved cysteine residues are surrounded by orange boxes and the conserved histidine residues are surrounded by blue boxes. The Myb-like SANT domain is indicated by the double line. The SWIRM domain is indicated by the purple line.
Figure 4.3 Temporal analysis of BradiCBF1 and BradiADA2 expression in Bd21, non-acclimated vs. acclimated. (a) Expression of BradiCBF1 (grey) and BradiADA2 (checkered) over a 24-hour period in non-acclimated *Brachypodium distachyon* Bd21. (b) Expression of BradiCBF1 (grey) and BradiADA2 (checkered) over a 24-hour period in acclimated Bd21. The expression levels of BradiCBF1 and BradiADA2 have been normalized to Bradi18S expression. (a) and (b) are presented using the same scale for best comparison.
Figure 4.4 Tissue-specific gene expression in Bd21, non-acclimated vs. acclimated. Expression levels of BradiCBF1 and BradiADA2 in non-acclimated (NA) cold-and acclimated (CA) aerial tissue. The expression levels of BradiCBF1 and BradiADA2 have been normalized to Bradi18S expression.
Figure 4.5 Positive control expression and nuclei staining in *Nicotiana benthamiana*. 
(a) GFP<sub>6</sub> expression in epidermal cell, 225x magnification, GFP filter. (b) Full-length EYFP in epidermal cell, 225x, YFP-LP filter. (c) Agroinfiltration of *Agrobacterium tumefaciens* EHA105 containing GFP<sub>6</sub> expression plasmid, 65x magnification, GFP filter. (d) Co-infiltration of p19 suppressor with *Agrobacterium tumefaciens* EHA105 containing ER-tagged GFP<sub>6</sub> expression plasmid, 65x, GFP filter. (e) Propidium iodide staining of epidermal cell nuclei, 65x magnification, RFP/CFP filter. (f) DAPI + 0.5% Triton X-100 staining of epidermal cell nuclei, 65x magnification, DAPI filter. Scale bars are 100 μM.
Figure 4.6 The AtCBF1-AtADA2a interaction in *Nicotiana* epidermal cells. (a) A positive interaction was observed when EYFP halves were fused downstream of AtCBF1 and AtADA2a. The fluorescent nuclei were stained with DAPI (b). The images of the positive result are merged in (c). The interaction was disrupted when other combinations were used (d). Replacing one interactor with an empty vector did not produce a positive result (g). Both negative results were confirmed with DAPI (e, h). The images of the negative results are merged in (f) and (i). Each interaction was photographed using the YFP-LP (a, d, g) and DAPI filters (b, e, h), 225x magnification, 3 days post-infiltration.
**Figure 4.7 The BradiCBF1-BradiADA2 interaction in *Nicotiana* epidermal cells.** (a) A positive interaction was observed when EYFP halves were fused downstream of BradiCBF1 and BradiADA2. The fluorescent nuclei were stained with DAPI (b). The images of the positive result are merged in (c). The interaction was disrupted when other combinations were used (d). Replacing one interactor with an empty vector did not produce a positive result (g). Both negative results were confirmed with DAPI (e, h). The images of the negative results are merged in (f) and (i). Each interaction was photographed using the YFP-LP (a, d, g) and DAPI filters (b, e, h), 225x magnification, 4 days post-infiltration.
Figure 4.8 *Brachypodium* Bd21 CEC expressing BradiADA2-GFP. (a) Four-week old positively transformed Bd21 CEC expressing BradiADA2-GFP. (b) Untransformed CEC. The calli were photographed using a GFP filter under 225x magnification.
Chapter 5: General discussion

The importance of the SAGA complex model

The importance of the SAGA complex is summarized by its complexity. By integrating the mechanisms of transcription and chromatin modification, it serves to play a crucial role in an organism's epigenetic response. In addition to acetylation (Grant et al., 1997), the SAGA complex has been found to perform other types of histone modifications, such as methylation, phosphorylation and ubiquitination, all of which have varying and often opposing effects on gene expression (Baker and Grant, 2007). SAGA function also changes depending on the components present in the complex, many of which can vary depending on the type of SAGA complex, and many of which remain to be fully characterized (Daniel et al., 2004; Daniel et al., 2005; Stockinger et al., 2001).

The functions of the SAGA complex are wide-ranging; in plants, this complex serves to modulate the expression of certain genes in response to varying different conditions, such as in the case of a plants response to abiotic stress, as well as mediating its normal development (Hark et al., 2009; Vlachonasios et al., 2003). In other organisms, the SAGA complex has also been found to play a role in transcriptional elongation, mRNA export and nucleotide excision repair (Baker and Grant, 2007). Thus, further characterization of the SAGA complex will further our understanding of transcription and transcriptional control.

Characterization of the SAGA-like complex in Brachypodium distachyon

Sequence analysis and alignment of the putative BradiCBF1 and BradiADA2 genes has shown that we have in fact isolated CBF1 and ADA2 orthologs in Brachypodium distachyon. BLAST analysis identified domains in BradiCBF1 and BradiADA2.

BradiCBF1 has all of the characteristic features of a transcription factor: a nuclear localization signal, a DNA-binding domain (AP2) and an acidic transactivation region (Figure 4.1). The AP2 domain binds to the C-repeats in the promoters of COR genes and
likely recruits RNA polymerases to the DNA via its transactivation domain (Thomashow et al., 1997; Ptashne and Gann, 2002). AtCBF1 interacts with AtADA2a/2b via the AP2 domain, a dual-purpose DNA- and protein-binding domain (Mao et al., 2006). Most importantly, BradiCBF1 is highly similar in sequence and domain identity to AtCBF1, OsCBF1 and TaCBF1 (Figure 4.1).

Another important but less characterized feature of CBF1 is the group of hydrophobic clusters within the transactivation domain. In Arabidopsis, the most C-terminal 98 residues of AtCBF1 are responsible for its transactivation function and are sufficient to target CBF1 to COR gene promoters (Wang et al., 2005). Within this region are several conserved clusters of hydrophobic residues embedded amongst the acidic residues. BradiCBF1 shares two of the C-terminal hydrophobic clusters with Arabidopsis CBF1, CBF2 and CBF3 (Wang et al., 2005), albeit with varying degrees of conservation. Hydrophobic cluster 6 (HC6) (Wang et al., 2005) is located at the very C-terminal end of CBF1 genes and is identical in BradiCBF1 and AtCBF1. HC4 is partially conserved in BradiCBF1. Deletion analysis revealed that when HC6 in particular was removed, AtCBF1 activity increased dramatically (Wang et al., 2005). This particular cluster appears to provide some sort of attenuation of AtCBF1 and BradiADA2 function. These clusters are not well conserved in wheat and rice CBF1. The in vivo biological significance of these clusters is as yet unknown.

BradiADA2 has three distinct domains, as well as a nuclear localization signal, all of which are highly conserved and shared with Arabidopsis ADA2a and 2b and rice ADA2 (Figure 4.2). The most N-terminal domain is a ZZ-type zinc finger protein-binding domain. These are found in proteins that perform a wide variety of functions, particularly those involved in chromatin modification (Ponting, 1996). This is a particular important point in the context of BradiADA2 composing part of a SAGA-like chromatin-modifying complex in Brachypodium. The zinc finger domain of BradiADA2 contains two zinc fingers, which is composed of six conserved cysteine residues that coordinate two zinc ions, flanked by two conserved histidine residues that assist in coordinating the zinc ions. This domain is capable of binding both DNA and proteins (Ponting, 1996). Therefore BradiADA2 may bind BradiCBF1 through this domain, or binds DNA through the zinc finger domain and BradiCBF1 through an alternative domain, or the zinc finger domain interacts with both, analogous to the dual-purpose AP2 domain of BradiCBF1.
In close proximity to the zinc finger domain of BradiADA2 is a Myb-like SANT domain. This domain contains a bHLH motif that may bind to similar motifs in other proteins, in addition to being capable of binding DNA. The bHLH motif is responsible for the dual-purpose ability of the SANT domain; the smaller helix is able to fit against a small helix in the bHLH of an interacting partner, while the larger helix binds DNA. The presence of a SANT is interesting in that these types of domains are known to dimerize (Davis et al., 1990; Aasland et al., 1996). This domain may thus facilitate BradiADA2 homodimerization, adding another layer of complexity and regulation to the Brachypodium SAGA-like complex.

The third major domain found in BradiADA2 is the C-terminal SWIRM domain. SWIRM domains remain largely uncharacterized, although they are typically found in members of the SWI/SNF family of chromatin remodeling complexes (Da et al., 2006). BradiADA2 may thus play a role in directly modifying chromatin or recruiting chromatin-modifying machinery via this domain. At this point, however, this type of domain has only been demonstrated to bind DNA (Da et al., 2006).

In summary, the structures of BradiCBF1 and BradiADA2 proteins are highly conserved across plant species, both monocot and dicot. BradiCBF1 and BradiADA2 both have the characteristics of transcription factors involved in chromatin modification mechanisms.

Temporal and spatial expression analysis of BradiCBF1 and BradiADA2

This section of our project was partially based on the results of Badawi et al. (2007). Badawi et al. focused on the expression of wheat CBF genes at the early stages of cold exposure, which revealed a common peak in expression of CBFs over a roughly 24-hour time period. It should be noted that TaCBF1 was not analyzed in the Badawi study. However, due to the strength of the general trend one can extrapolate the behaviour of TaCBF1 expression in response to cold treatment.

Analysis of BradiCBF1 and BradiADA2 expression demonstrated that the expression of both genes induced by cold treatment, with an earlier peak by BradiCBF1 being observed (Figure 4.3). From this we can speculate that the presence of BradiCBF1 leads to the
downstream expression of BradiADA2, which follows in expression by peaking at 6h following the induction of cold treatment. There are no ICE repeats in the BradiADA2 promoter (data not shown), suggesting that ICE cannot be responsible for activating BradiADA2 expression in response to stimuli as it is in the case of BradiCBF1 (Chinnusamy et al., 2003). BradiCBF1 expression may thus upregulate the expression of BradiADA2 downstream by binding to the CRT/DRE repeats in the BradiADA2 promoter, over a 2-4h time period, which has frequently been observed for other COR genes that are activated by CBF1 (Gilmour et al., 1998).

In Bd21, following the induction of cold treatment the expression of both genes is upregulated in crown tissue (Figure 4.4). Upregulation of BradiCBF1 and BradiADA2 expression following cold treatment were consistently observed in the crown tissue. Crown tissue is the primary site of the vernalization response in plants and is the centre of meristematic tissue in plants. It is the most important to protect, and thus is the primary site of cold acclimation in plants (Watts, 1972; Peacock, 1975; Thomas and Stoddart, 1984; Pearce et al., 1993).

Expression of BradiCBF1 and BradiADA2 in leaf tissue is also upregulated following cold treatment, albeit not nearly to the same degree as was observed in crown tissue. This reflects the relative unimportance of leaf tissue in the cold acclimation process of the plant, as this tissue tends to die off as a result of freezing damage (Steponkus, 1984).

In both crown and leaf tissue BradiCBF1 is upregulated considerably more than BradiADA2. This may reflects the fact that CBF1 is a regulator of many downstream COR genes (Thomashow, 2001) and thus many more CBF1 proteins relative to ADA2 proteins are required to activate the entire downstream COR gene regulon.

The interaction of BradiCBF1-BradiADA2 in planta

It is known that AtCBF1 and AtADA2a interact with one another via their N-termini in vitro (Mao et al., 2006). These two proteins were able to interact when the EYFP tags were located downstream (C-terminally located) of the proteins. The tags are fused to the proteins in such a way that they are far away from interaction sites, cannot interfere with
binding. Lack of interaction for the other orientations may be due to steric hindrance, and/or preventing proper folding of the interaction domains. When tags are both upstream of genes, they are close to the interaction sites and likely interfere with binding; when tags are in opposite orientations, regardless of which gene, the two halves of EYFP are held too far apart to interact. Thus, the location and/or orientation of interaction sites to one another, may affect the assay.

It is important to consider the effect of the YN tag being larger than the YC tag. In the cases where YN is fused to CBF1 it may block the binding of ADA2 to the closely located AP2 binding site. The YN tag, when fused to ADA2 upstream, may interfere with binding of CBF1 to the ZZ/SANT domain region, which is also N-terminally located. When fused upstream of the gene of interest the YN tag may also interfere with proper folding of interaction sites in ADA2; may affect folding of SWIRM domain when downstream, but this domain is not required for ADA2-CBF1 interaction.

It was hypothesized that location and orientation affected fluorescence for Arabidopsis FIE and MEA interacting pair. The strongest EYFP signal was produced when either YN or YC were fused downstream of either FTA or FTB. In opposite orientations fluorescence was weaker, whether EYFP halves were upstream or downstream of interactors. No fluorescence when EYFP halves were both downstream of interactors. In the case of this study, however, no weaker interaction was observed for any other interaction for either for the Brachypodium or Arabidopsis pairs.

The location of the interaction sites of CBF1 and ADA2 may also play a part in determining whether EYFP reconstitution can occur. CBF1 and ADA2 interact via N-terminally located domains (Mao et al., 2006). When the EYFP halves are fused to the interacting proteins in the same orientation, they come in close proximity to one another and EYFP is able to reform. When the EYFP halves are fused to the interacting proteins in opposite orientations to one another, they may be too far away to interact, and the proteins/linkers may not be not flexible enough for EYFP to reconstitute.

The rate of transformed cells observed in this study was relatively low (~10%) when compared to 90% success rate in Brachi-Drori et al. (2004). There are several possible reasons for this to occur. For the interaction to occur in a cell, two different Agrobacterium cells with each vector must both infect the cell. Additionally, an
Agrobacterium cell harbouring the p19 suppressor vector must also infect the cell. Therefore, the seemingly low odds that all three necessary vectors are present in a Nicotiana epidermal cell at precisely the same time may be responsible for the low rate of transformation when using this strain of Agrobacterium for the infiltration assay. Additionally, in order to express protein and produce the interaction in the nucleus, the cell must remain extremely healthy, with the cell wall intact. This presents another challenge in observing a positive result.

In summary, our study has demonstrated that AtCBF1 and AtADA2a interacted in planta, and that BradiCBF1 and BradiADA2 interact in planta (Figure 4.6; Figure 4.7). Both complexes localized to the nuclei of Nicotiana epidermal cells. While we have demonstrated that BradiADA2 interacts with the BradiCBF1 in vivo, and that AtCBF1 and AtADA2a interact both in vitro (Stockinger et al., 2001) and in vivo (this study) the exact mechanism of interaction remains unclear. While Mao et al. (2006) have demonstrated in vitro that AtCBF1 interacts with AtADA2a/2b via its AP2 domain, precisely through which domain ADA2 interacts with CBF1 is currently unknown.
Chapter 6: Concluding statements

6.1 Concluding statements

We have obtained data that suggest the presence of a SAGA-like complex within *Brachypodium distachyon*. Orthologs of both CBF1 and ADA2 have been isolated and characterized from *Brachypodium*. Sequence analysis has demonstrated that BradiCBF1 and BradiADA2 feature protein- and DNA-interaction domains that are highly similar to the domains of their counterparts in other plant species, such as wheat, rice and *Arabidopsis thaliana*.

The expression of both BradiCBF1 and BradiADA2 is induced by cold temperatures and follow a similar expression trend following the initiation of cold treatment. This suggests that the two corresponding proteins are very likely present in the cell at the same time in response to cold treatment.

Most importantly, this study has demonstrated that both *Arabidopsis* and *Brachypodium* CBF1 and ADA2 proteins interact *in planta*. This is the first time that CBF1 and ADA2 proteins from any species have been shown to interact in an *in vivo* setting. Additionally, the pairs interacted with the EYFP fragments in the same location and orientation to the respective genes for both species. This suggests that the two pairs interact in a similar manner, which is supported by the sequence analysis.

Our research has demonstrated that *Brachypodium* presents itself as a more appropriate model for the SAGA-mediated cold response mechanism than *Arabidopsis*. *Arabidopsis* has two ADA2 genes and is the only known plant species with two ADA2 genes. AtADA2a and AtADA2b both function as part of the SAGA complex, but have distinct functions (Hark *et al.*, 2009), complicating the characterization of the general cold response mechanism of plants. The use of *Brachypodium* simplifies this research, due to the fact that it is both a monocot and that it only has one ADA2 gene.

This report suggests that the functions of CBF1 and ADA2 among different species are highly conserved. Now that we have shown that CBF1 and ADA2 shown to interact *in planta*, our findings strongly support our main hypothesis that a SAGA-like complex exists...
in *Brachypodium distachyon*, and that it may play a crucial role in mediating the cold acclimation response in cereal crop plants.

**6.2 Future directions**

*In vitro* confirmation of the BradiCBF1-BradiADA2 interaction

While the interaction between AtADA2a and AtCBF1 has been confirmed *in vitro* and now also *in planta*, the interaction between BradiCBF1 and BradiADA2 has only been shown *in planta*. To support this interaction data effort will be deployed to test for the interaction between BradiCBF1 and BradiADA2 interact *in vitro*. Expression vectors containing his-tagged and FLAG-tagged BradiCBF1 and BradiADA2 are currently being developed. The proteins will be purified using an FPLC system (Bio Rad). The interaction between both proteins following the purifications will be confirmed via anti-FLAG and anti-his antibodies. Pull-down assays using anti-FLAG and anti-His antibodies will be used to confirm the interaction between both proteins following the purifications.

Analysis of potential dimer interactions in BiFC assay

Investigating transcription factor dimerization may provide useful information about how the SAGA complex can perform different functions, as well as create a better picture of how the *Brachypodium distachyon* SAGA-like complex is formed. BLAST and alignment analysis revealed the presence of a basic-helix-loop-helix domain in BradiADA2 similar to those found in Myb-family proteins, which dimerize via this domain (Davis *et al.*, 1990). The BiFC assay can again be used to determine if BradiADA2 dimerizes *in planta* by co-infiltrating two ADA2 constructs with complementary halves of EYFP.

Preliminary BiFC assay results between CBF1 constructs with oppositely oriented EYFP tags have been positive. Initial results have shown what may be fluorescent nuclei
(data not shown). The unusual mechanism of AP2-mediated protein binding may provide the clue for the mechanism of CBF1-CBF1 dimer interactions.

**Testing BradiADA2-AtCBF1/AtADA2a-BradiCBF1 interactions**

It is possible then that performing the BiFC assay with BradiADA2 and AtCBF1, and vice versa, we will observe a positive interaction. This experiment will be performed using the constructs generated for this study to confirm that their respective functions of ADA2 and CBF1 were highly conserved during evolution.

**Analysis of BiFC interactions following cold treatment**

CBF1 and ADA2 are believed to work together to enact the expression of COR genes. While it has been shown that their upstream activators (ICE in the case of CBF1) function in response to cold, it is also a possibility that the interaction between CBF1 and ADA2 is modulated by cold exposure. Although these proteins have been shown to interact for both *Arabidopsis* and *Brachypodium*, the interaction may be more efficient in the presence of cold, whether cold treatment increases the expression levels of the genes, stabilizes the proteins in order to increase the likelihood of the interaction occurring or alters the conformation of the proteins in a way that promotes binding.

**Identifying the BradiADA2 domain that facilitates BradiCBF1-BradiADA2 interaction**

It is now known that AtADA2a and AtCBF1 interact *in vitro* and *in vivo*, and that BradiCBF1 and BradiADA2 interact *in vivo*. Pull-down assays using deletion mutants of AtCBF1 and AtGCN5 have determined that AtCBF1 interacts with AtADA2a/2b via an AP2 domain, and that AtADA2a/2b interact with AtGCN5 via both a ZZ-rich domain and a SANT domain (Mao *et al.*, 2006). However, it is not yet known through which domain the ADA2
protein interacts with the AP2 domain of CBF1. The next step is to create a series of BradiADA2 deletion mutants for in vitro pull-down analysis to identify this domain.

**Onion biolistic BiFC assay**

The BiFC assay will also be performed using onion (*Allium cepa*) via the use of a helium-powered Biolistics PDS-1000/He gun (Bio Rad). In addition to confirming the interaction in a separate in vivo setting, this experiment has the advantage of using a monocot. To expedite the experiment the interacting pairs that have been confirmed by the *Nicotiana* assay will be used. Preliminary studies have already optimized the expression of the pSAT4(A)-EYFP positive control vector in onion epidermal cells. Fluorescent nuclei have been observed in cells bombarded with AtCBF1-YC and AtADA2a-YN plasmid.

**Expression analysis of COR genes in Brachypodium winter accession**

Both the temporal and spatial expression will be performed comparing Bd21 to Bd29-1, a winter accession of *Brachypodium distachyon*. Analysis of BradiCBF1 and BradiADA2 expression in *Brachypodium* Bd21 and Bd29-1 root tissue will also be examined.

**Characterization of the BradiADA2 overexpression mutant**

Once the overexpression mutant has been genotyped and validated it will be used for a variety of assays. The first is phenotypic analysis; the seed and the tissues throughout the growth cycle of the plant will be observed and compared to that of wild-type Bd21. This will tell us if the overexpression of BradiADA2 impairs or increases the freezing tolerance capacities of the plant. The second is a freezing tolerance assay; adult transgenic plants will be exposed to temperatures ranging from -5°C to -13°C to determine at which temperature freezing stress becomes lethal to the plant. When compared to data from freezing tolerance
assays performed on wild type *Brachypodium* cultivars we can determine if BradiADA2 overexpression impairs or increased cold tolerance capacities of the plant. Thirdly, mass spectrometric analysis on proteins that co-purify with BradiADA2-GFP. Lastly, mass spectrometric analysis will be performed on proteins that co-purify with BradiADA2-GFP.

**Development of the BradiADA2 knockdown mutant**

In order to further elucidate the function of BradiADA2 a knockdown transgenic line of Bd21 will be developed. The T-DNA vector will operate via a hairpin mechanism, where a short fragment of BradiADA2 will be incorporated in a mirrored fashion. The resulting transcript will fold in on itself and form a hairpin structure that will be broken down into small siRNA fragments via the plant’s natural silencing mechanism (PTGS). This plant will also be utilized for freezing tolerance assays and transcriptomic analyses, similarly to the BradiADA2 overexpression mutant line.

**Next-generation sequencing analysis of COR genes**

Both transgenic *Brachypodium* lines will be used to perform transcriptomic assays to measure the level of histone acetylation on COR genes. Next generation sequencing will also be applied to this mutant. Whole transcriptome shotgun sequencing, also known as RNA-seq, will be performed to analyze differential gene expression across the entire *Brachypodium* mutant line genome.

**Further characterization of the *Brachypodium* SAGA-like complex**

A third crucial component in the *Brachypodium* SAGA-like complex that has not been discussed in great detail is the histone acetyltransferase GCN5. Characterizing the presence and the function of a *Brachypodium* ortholog of GCN5 would further strengthen the
existence of the *Brachypodium* SAGA complex. Stockinger *et al.* (2001) have demonstrated that GST-tagged AtCBF1 is able to interact with AtADA2a and with AtGCN5. *In planta* BiFC interaction assays would be performed in the same vein as the CBF1-ADA2 interactions, to confirm the interaction of putative BradiGCN5 with BradiCBF1 and with BradiADA2 *in planta*. The contribution of putative *Brachypodium* SPT orthologs to the SAGA-like complex is also a point of interest.

### 6.3 Contributions to science

Characterizing the roles of BradiCBF1 and BradiADA2 in the cold response mechanism of cereal crops has made several valuable contributions to plant research:

- Orthologs of CBF1 and ADA2 exist in *Brachypodium distachyon*

- BradiCBF1 and BradiADA2 are cold-inducible and function at the primary site of cold acclimation

- We have demonstrated that BradiADA2 is a COR gene

- This study is the first to demonstrate that CBF1 and ADA2 interact *in planta*

- The results of this study strongly suggest that a SAGA-like complex exists in *Brachypodium*

- This complex may mediate the plant’s cold response mechanism

- The characterization of a SAGA-like complex in *Brachypodium* opens up future avenues of molecular study in improving the cold acclimation abilities of cereal crop
plants

- By characterizing the cold response mechanism in *Brachypodium*, we will further strengthen its position as a model system for crop research
Chapter 7: References


Da, G. et al. (2005). Structure and function of the SWIRM domain, a conserved protein module found in chromatin regulatory complexes. PNAS. 103, 2057-2062.


Appendix

Gene-YFP N-terminus

BradiADA2-YFP N-terminus

AAGGTAGACCCTGCCAAAAAGTAGATACCTGTTTTATGATATGGTATGAAAGCCTGGT
AGC AAT GAG GAG GCT CCA ACT GTT AAG GGC GAA TTC GAC CCA GCT
Ttc ttg tac aaa gtg gtg ata ggg ccc ggg atc ctg atg gtg agc
aag ggc gag gag ctg ttcacccgggtggtgcccatctttgctgagctgg

BradiCBF1-YFP N-terminus

CATTGACCCCTCCCTCGATGCGGCGAGGTTGCCTCGGCAGAGAGTGCGGAGAGAGACG
ATGACGGCGAG GTC AAC CTA TGG AGC TAC AAG GGC GAA TTC GAC CCA
GCT Ttc ttg tac aaa gtg gtg ata ggg ccc ggg atc ctg atg gtg
agc aag ggc gag gag ctg ttcaccgggttggtgccttaccccttgctgagctgg

AtADA2a-YFP N-terminus

ATAGCTTCTTCAAAAGTAGACCGGAGTAAAGTACGACAGATATATGATATGCTGGTCAT
AAG GGA ATA GGT GAC TCA ACA AAG GGC GAA TTC GAC CCA GCT Ttc
Ttg tac aaa gtg gtg ata ggg ccc ggg atc ctg atg gtg agc aag
ggc gag gag ctg ttcaccgggttggtgcccatctttgctgagctgg

AtCBF1-YFP N-terminus

TGTGGATAATATGGCTGAAGGCTATTTTACCAGCCCGTCTGTTCAATGGAATCAT
AATTAGAC GGC GAA GGA GAT GGT GAC GTG TCG CTT TGG AGT TAC
AAG GGC GAA TTC GAC CCA GCT Ttc ttg tac aaa gtg gtg ata ggg
ccc ggg atc ctg atg gtg agc aag ggc gag gag ctg ttc acc
ggggtgggtgcccatctttgctgagctgg
Gene-YFP C-terminus

BradiADA2-YFP C-terminus

AAGGTAGACCTGCCAAAGTAGATACTGTATTTATGATATGGTGATGAAAAAG CTC GGT
AGC AAT GAG GAG GCT CCA ACT GTT AAG GGC GAA TTC GAC CCA GCT
Ttc ttg tac aua gtt gtt ata ggg ccc ggg atc ctg GGC AGC TCG
CAG CTC

BradiCBF1-YFP C-terminus

CATTGACCCCTCCTCCGGATGCGCGAGTTCGTCGCCAGGAGGTGCGCCGGAGGAGACC
ATGACGGCGAG GTC AAC CTA TGG AGC TAC AAG GGC GAA TTC GAC CCA
GCT Ttc ttg tac aua gtt gtt ata ggg ccc ggg atc ctg GGC AGC
TCG CAG CTC

AtADA2a-YFP C-terminus

ATAGCTTCTCTCAAAAGTAGAAGCCGAGGATAAAGTAGACAGAGATATGATATGCTGGTCAT
AAG GGA ATA GGT GAC TCA ACA AAG GGC GAA TTC GAC CCA GCT Ttc
Ttg tac aua gtt gtt ata ggg ccc ggg atc ctg GGC AGC TCG CAG
CTC

AtCBF1-YFP C-terminus

TGTGGATAATATGGCTGAAGCCATGCTTTTACCGCGCGCTGTTCAATGGGAATCAT
AATTATGAC GGC GAA GGA GAT GGT GAC GTG TCG CTT TGG AGT TAC
AAG GGC GAA TTC GAC CCA GCT Ttc ttg tac aua gtt gtt ata ggg
ccc ggg atc ctg GGC AGC TCG CAG CTC
YFP N-terminus-Gene

N-Terminus YFP-BradiCBF1

Atc gag gac TCC GGA CTC AGA TCT ATC ACA AGT TTg tac aaa aaa
gca ggc tcc gaa ttc gcc ctt CTG CTC GAG CCA CTC TTC ATG GAC
CTC GGT GCT CTC AGC AGC GAC TAC TCG TCA GGG AGG CCG TCT CCG
GTGACGCGGAGCAGGGACGCCGCGCTCTCAGCTACATGACTGTATCCT

N-Terminus YFP-BradiADA2

Atc gag gac TCC GGA CTC AGA TCT ATC ACA AGT TTg tac aaa aaa
gca ggc tcc gaa ttc gcc ctt TTT GAG CTA GGG TTT GGG CCC GCC
GCG ATG GGC CGG TCT CGC GGA GTG CCC AAT CCT GCC GAC GAC GAC
CCAAACACACAGGTGGTTCTGTCTAGTCCTCTTCGTCTCCCTCTGCTATTCCT

N-Terminus YFP-AtCBF1

Atc gag gac TCC GGA CTC AGA TCT ATC ACA AGT TTg tac aaa aaa
gca ggc tcc gaa ttc gcc ctt CCA GTT TCT TGA AAC AGA GTA CTC
TGA TCA ATG AAC TCA TTT TCA GCT TTT TCT GAA ATG TTT GGC TCC
GATTACGAGCCTCAAGGGGAGATTATTGTCCGACGTGCGGCACGAGT

N-Terminus YFP-AtADA2a

Atc gag gac TCC GGA CTC AGA TCT ATC ACA AGT TTg tac aaa aaa
gca ggc tcc gaa ttc gcc ctt ATG GGT CGT TCG AAA CTA GCT TCT
CGTCTGTGAGGAAGACCTGAATCCAGGAAAATCAAAAAAGGAAAAGATATCATTTGGG
Appendix I. BiFC vector sequences. All BiFC vectors were sequenced to confirm that each gene was in frame with its tag. Shaded text: gene of interest; uppercase text: entry clone DNA; underlined text: att site; double-underlined text: ATG start site; lowercase text: destination vector DNA; bold text: tag gene.
Appendix II. Overexpression vector sequence. The vectors were sequenced to confirm that BradiADA2 was in frame with its GFP tag. Shaded text: gene of interest; uppercase text: entry clone DNA; underlined text: att site; double-underlined text: ATG start site; lowercase text: destination vector DNA; bold text: tag gene.