

Analysis of developmentally regulated cold tolerance in *Arabidopsis thaliana* and
characterization of cold regulated sulfotransferases in *Triticum aestivum*

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ABSTRACT

Analysis of developmentally regulated cold tolerance in *Arabidopsis thaliana* and characterization of cold regulated sulfotransferases in *Triticum aestivum*

Hetal Patel

The large differences in freezing tolerance that exist between winter and spring wheat cultivars suggest that the regulation of the initiation of reproductive organs and development plays an important role in the potential of the plant to cold acclimate, which is a process of treating the plants at low non-freezing temperatures in order to increase its freezing tolerance ability. Because of the advantages of analyzing in model species, we have studied the changes in the capacity of *Arabidopsis thaliana* to cold acclimate at different stages of development. We have found that freezing tolerance decreases after flowering, with a critical and abrupt drop in the tolerance two days after the opening of the first flowers. Freezing tolerance was increased after flowering by the exogenous application of abscisic acid (ABA) and by sodium nitroprusside (SNP) which produces NO, thus implicating ABA and NO in the signaling pathways involved in cold acclimation.

In addition, we have found that the expression of three wheat sulfotransferases (ST) namely: *TaST2*, *TaST3* and *TaST4* are regulated during cold acclimation. Molecular characterization of *TaST2*, *TaST3* and *TaST4* revealed that the deduced amino acid sequences of all three enzymes contained the conserved regions and amino acids involved in 3'-phosphoadenosine 5'-phosphosulfate (PAPS) binding and catalysis (Varin, 1992

and Marsolais, 1995). All three sulfotransferases were found to be differentially regulated by cold in winter and spring wheat. Wheat EST clone collections were used to sub-clone *TaST2*, *TaST3* and *TaST4* in a bacterial expression vector pQE30. The purified recombinant proteins were tested for purity by SDS-PAGE and various ranges of compounds were tested as possible substrates.

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LIST OF ABBREVIATIONS

ABA	Absciscic acid
aba1	ABA-deficient-1
abi1	ABA-insensitive-1
ADH	Alcohol dehydrogenase
ANOVA	Analysis of variance
AP2	Apetala2
AtST	<i>Arabidopsis thaliana</i> sulfotransferase
bHLH	Basic- Helix loop helix
BnST	<i>Brassica napus</i> sulfotransferase
BRs	Brassinosteroids
CBF	Cor-repeat binding factor
CO	Constans
COR	Cold regulated
CORI-7	Coronatine induced-7
CRT/DRE	C-repeat/dehydration responsive element
CS	Choline-O- sulfate
CST	Choline sulfotransferase
DHEA-ST	Dehydroepiandrosterone sulfotransferase
DMSO	Dimethyl sulfoxide
dsGS	desulfoglucosinolate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetate
EL	Electrolytic leakage
ERD	Early dehydration inducible
ESK1	Eskimo1
F3ST	Flavonol-3-sulfotransferase
FLC	Flowering locus C
GFP	Green Fluorescent Protein
GI	Gigentia
GSs	Glucosinolates

GUS	β-Glucuronidase
HOS	High expression of osmotically responsive genes
HPLC	High performance liquid chromatography
ICE	Inducer of CBF expression
kDA	kilo Daltons
KIN	Cold inducible
LEA	Late embryogenesis-abundant proteins
LT ₅₀	Lethal temperature 50
LTi	Low temperature inducible
LUC	Luciferase
MeJA	Methyl Jasmonate
NaCl	Sodium chloride
NO	Nitric Oxide
NOS	Nitric oxide synthase
12-OHJA	12-hydroxy jasmonic acid
P5CS	Pyrroline-5-carboxylate synthetase
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PLD α	Phospholipase D α
PLMF-1	Periodic leaf movement factor-1
PSK α	Phytosulfokine α
PST	Phenol sulfotransferase
RAB	Responsive to ABA
RD	Responsive to desiccation
RT	Reverse transcription
SFR	Sensitivity to freezing tolerance
SNP	Sodium nitroprusside
ST	Sulfotransferase
TaST	<i>Triticum aestivum</i> sulfotransferase
TaVRT	<i>Triticum aestivum</i> vegetative to reproductive transition

TBADP	Tetrabutylammonium dihydrogen phosphate
TLC	Thin layer chromatography
UV	Ultraviolet
VRN	Vernalization
WCS	wheat cold- specific

INTRODUCTION

Freezing tolerance is the ability of plants to tolerate temperatures below 0 °C. Freezing tolerance in wheat is induced during growth at low, above freezing temperatures, in a process known as cold acclimation. After acclimation freezing tolerance decreases after plants make the transition from vegetative growth to reproductive growth (Fowler *et al*, 1996a, 1996b and Mahfoozi, 2001). The association of the loss of low temperature tolerance with reproductive transition was hypothesized and linked with expression of low temperature tolerant genes and expression of developmental genes (Mahfoozi *et al*, 2001). In view of the loss of low temperature tolerance with reproductive stage transition, studies were initiated in *Arabidopsis thaliana* to investigate the changes in freezing tolerance with respect to developmental changes. *Arabidopsis thaliana*, which is a dicot plant, was selected as a model plant system for our studies as its genome is completely sequenced and smaller than that of wheat. Also *A. thaliana* has a shorter life cycle than wheat and various mutants are available for further studies.

We also found three sulfotransferases (STs) whose expression was changed during cold acclimation in wheat. Sulfotransferases are enzymes that catalyze the transfer of a sulfonate group to an acceptor molecule (reviewed in Falany, 1997). Considerable work has been done on mammalian STs, however in comparison to work in mammalian system there is little known about plant sulfotransferases. The objective of this study was to characterize *TaST2*, *TaST3* and *TaST4* at the molecular and biochemical level. However, the substrates for wheat sulfotransferases were not clearly identified.

Chapter 1

REVIEW OF LITERATURE

Cold Tolerance

Cold acclimation is a process that occurs in plants, when it is exposed to low non-freezing temperatures, that increases its freezing tolerances. There are several cellular and metabolic alterations occurring in the plant during cold acclimation. These changes include: alterations of cell membrane, increase in sugar and other small organic molecules, induction of dehydrin protein expression and changes associated with gene regulation.

Cell membrane alterations:

During cold acclimation the membrane lipid composition of the membrane changes such that the threshold temperature for membrane damage is lowered. Along with an increase in total lipid content the ratio of lipid to protein also increases during cold acclimation. Lipid rearrangements in response to cold acclimation have been demonstrated in *Brassica napus* and lipid diffusion across the plasma membrane of cold acclimated plants was shown to be higher than non-acclimated plants (Tasseva *et al*, 2004). It has been shown that the total percentage of lipid content increases in cold acclimated *Arabidopsis thaliana* and specifically the phospholipid, phosphatidylcholine, was found to increase during cold acclimation (Uemura *et al*, 1995). The change in the structure of the plasma membrane during freezing involves a transition from a lamellar to a H_{II} phase structure (Uemura *et al*, 1995). Formation of the H_{II} phase is an event that

occurs within different lipid bilayers in a cell and is a three dimensional array of inverted cylindrical micelles with water in the central core of each cylinder. The phase transition to H_{II} phase is associated with cellular damage due to cold. Plasma membrane has been observed to form H_{II} phase structures in *A. thaliana* cells and their formation was shown to decrease by cold acclimation of the plant. This decrease is due to changes occurring in the lipid content of the plasma membrane (Uemura *et al*, 1995). In wild type *A. thaliana*, phospholipase D α (PLD α) hydrolyzes phosphatidylcholine and reduces the freezing tolerance of the plant (Welti *et al*, 2002). Plants that were PLD α deficient showed increased freezing tolerance. This shows that lipids such as phosphatidylcholine are involved in increasing cold acclimation of the plant (Welti *et al*, 2002).

Increase in small organic molecules:

Soluble sugars like sucrose, fructose and glucose and the amino acid proline are known to accumulate in many different plants including *A. thaliana* during cold acclimation. These molecules maintain the turgor in dehydrating cells and are thought to play a role in the protection of membranes and proteins under the conditions of low temperature potential (Smirnoff N, 1998). Proline is one of the important molecules that accumulates during environmental stress including low temperature, high salinity and stress. Proline dehydrogenase is an enzyme that breaks down proline to pyrroline-5-carboxylate in a proline catabolism pathway (Nanjo *et al*, 1999). Transgenic plants having an antisense proline dehydrogenase gene showed increased low temperature and salt tolerance compared to wild type plants (Nanjo *et al*, 1999). Resistance to salt stress was higher than the freezing stress in antisense proline dehydrogenase plants. Soluble

sugars like glucose, fructose and sucrose were found to steadily increase until 5 days in *A. thaliana* that were acclimated with illumination as opposed to non-acclimated plants or plants acclimated in the dark. (Wanner *et al*, 1999).

Induction of dehydrin type protein expression:

Dehydrin proteins are a type of late embryogenesis-abundant protein (LEA) that are activated during late embryo maturation and desiccation (Delseny *et al*, 1994). Dehydrin proteins also accumulate during various environmental stresses including low temperature, saline conditions and drought. These proteins vary in molecular weight. They have three different conserved domains in the protein structure. The K-segment or the lysine rich motif, which can be found in all dehydrin type proteins and consists of a repeated amino acid sequence EKKGIMDKIKEKLPG. This segment is normally located near the carboxy terminal region of the protein (Close, 1996). A second type of conserved domain is a stretch of serine residues also known as the S-segment that can be phosphorylated. The third conserved domain is called the Y-segment (DEYGNP), that is found in the amino-terminal region of the protein and is generally present in one to three copies (Close, 1996). Dehydrins are known to play a protective role during dehydration in plants. Nylander *et al* (2001) have studied the accumulation of several dehydrin proteins in *A. thaliana*. Dehydrin-like proteins LTI29, ERD14 and RAB18 showed tissue and cell specific accumulation. *ERD14* was found to accumulate in parenchymal cells, vascular tissues and in root tips. *RAB18* accumulate in stomatal cells and *LTI29* was found in root tip of unstressed *A. thaliana* plant. Under stressed conditions LTI29, ERD14 and RAB18 proteins accumulates in all type of cells and tissues, however there

accumulation was more near the vascular tissues, suggesting their role in osmoregulation (Nylander *et al*, 2001). It has also been demonstrated that ERD14 possesses ion binding capacity which is regulated by phosphorylation. The kinase activity that phosphorylates ERD14 steadily increases after 1 day of cold treatment (Alsheikh *et al*, 2003). Dehydrin WCOR410 in *T. aestivum* was found to stabilize membranes during low temperature acclimation (Danyluk *et al*, 1998).

Changes associated with gene regulation:

There are many changes that occur at the level of gene expression when plants undergo low temperature stress and cold acclimation. The most global approach to study changes in gene expression is microarray-based as it monitors expression of thousands of genes at once and thus can identify the changes associated with various signaling pathways. Several microarray-based experiments have been done to identify pool of genes that are cold regulated (Kreps *et al*, 2002, Vogel *et al*, 2005).

CBF cold acclimation pathway

In *A. thaliana* cold acclimation associated with the CBF/DREB1 pathway is well understood (reviewed by Thomashow, 1999). CBF/DREB1s are cold regulated transcription factors that bind to DNA having C-repeat/dehydration response elements (CRT/DRE). The DNA sequence of the low temperature response element is CCGAC. This family of transcription factors includes CBF1, CBF2, and CBF3 or DREB1b, DREB1c and DREB1a, respectively. Transcripts for CBFs start accumulating within 15 min of transfer to cold temperatures and levels continue to increase up to 2 hours after the

beginning of cold treatment. There is subsequent upregulation of several genes that have CRT/DRE regulatory elements in their promoters. The CBF1 protein and its association with CRT/DRE element was first identified by Stockinger *et al* (1997). Later, Medina *et al* (1999) identified CBF2 and CBF3 proteins from *A. thaliana*. These proteins contain an N-terminal AP2 DNA binding motif and an acidic C-terminal domain that acts as an activator domain (Stockinger *et al*, 1997). The function of the CBF regulon is to protect the cells and tissues against low temperature stress (Stockinger *et al*, 1997, Medina *et al*, 1999). During cold acclimation CBF proteins are known to regulate other genes like *KIN1*, *KIN2/COR6.6*, *COR15A*, *RD17/COR47*, *COR78/RD29a*, *ERD10*, *ERD6* and *ERD9* (reviewed by Thomashow, 1999). CBF2 has been reported to be a negative regulator of *CBF1* and *CBF3* (Novillo *et al*, 2004). *cbf2* mutant plants showed up-regulation of *CBF1*, *CBF3* and other downstream regulated genes like *KIN1*, *COR47*, *COR15A*, *LT178*, *RC11A* and *RC12A*. In addition, *cbf2* mutants showed increased dehydration and salt stress tolerance compared to the wild type plant (Novillo *et al*, 2004).

The fact that *CBF* expression is induced within 15 minutes of cold treatment led to the hypothesis that there is another regulatory factor acting upstream of CBF that induces CBF expression during this short time interval. This factor was called inducer of CBF expression (ICE) (Gilmour *et al*, 1998). Chinnusamy *et al* (2003) have identified the *ICE1* locus by map-based cloning and have proposed that upon cold treatment the ICE1 protein is activated and turns on the transcription of *CBFs*, which in turn activates other cold regulated proteins like dehydrins and *CORs*. In *ice1* mutant plants, the transcription of the *CBF* genes was affected by cold. Induction of *CBF3* by cold was almost abolished, *CBF2* expression was lower at 1hr of cold treatment and the *CBF1*

gene expression was lower after 1 and 3 hr of cold stress in *ice1* mutant plants (Chinnusamy *et al*, 2003). Expression of *RD29A*, *COR15A* and *COR47A* was also lower in this mutant under cold stress conditions. The freezing tolerance of the *ice1* mutant was lower than wild type plants at all the temperatures that were investigated. The cloned *ICE1* protein were used to study recognition and binding to the *CBF3* promoter by electrophoresis mobility shift assay (Chinnusamy *et al*, 2003). The *CBF3* promoter's four potential MYC-recognition sequences were used to study this DNA-protein interaction. Results indicated that all the sequences were identified by the *ICE1* protein but it had higher affinity for the MYC-2 type of recognition sequence. This suggests that *ICE1* is an inducer for CBFs (Chinnusamy *et al*, 2003). *ICE1* protein has bHLH and leucine zipper domains at the C-terminal and an acidic domain and S-domain at the N-terminal region. These domains are also conserved in MYC-related bHLH transcription factors (Chinnusamy *et al*, 2003).

The circadian clock was also shown to affect the expression of the *CBFs* in *A. thaliana* (Fowler *et al*, 2005). *CBFs* transcripts accumulate in response to low temperature in the morning, but are down regulated in the evening (Fowler *et al*, 2005). Promoter fusion experiments with *CBF2::GUS* showed that *CBF2* promoter activity was also regulated by the circadian clock (Fowler *et al*, 2005) however, the biological significance of this regulation still needs to be determined.

Sensitivity to freezing tolerance genes (SFR)

There is a set of genes known as sensitivity to freezing (*SFR*) which are involved in cold acclimation of the plants. Of the several *sfr* mutants isolated by Warren *et al*

(1996), only *sfr6* was found to affect the expression of genes that are CBF regulated (Knight *et al*, 1999). However, the regulation of CBF transcription factors was not impaired in *sfr6* mutants (Knight *et al*, 1999) suggesting that the SFR6 protein acts between CBF transcript accumulation and induction of the CBF regulon (Thomashow, 2001).

High expression of osmotically responsive genes (HOS1)

Ishitani *et al* (1998) identified the *HOS1* by positional cloning in *A. thaliana*. Plants mutant at the *hos1* locus were found to have lower freezing tolerance under non-acclimated conditions than wild type plants. However, two days after cold acclimation both wild type and *hos1-1* plants showed similar cold acclimation. Transcript levels of cold regulated genes like *RD29*, *COR15A*, *KINI*, *ADH* and *COR47* were more strongly induced by cold treatment in *hos1-1* mutant plants than in wild type plants. Induction of *COR47*, *COR15A* and *KINI* transcripts by NaCl and PEG treatment was much lower in *hos1-1* mutants than in wild type plants. Comparison of NaCl, PEG and cold treatment was performed with these genes in wild type and *hos1-1* plants since the salt, osmotic and cold regulatory signaling pathways converge as shown in Figure 1.1 (Shinozaki *et al*, 2003). Induction of *COR15A* and *KINI* was also inhibited by ABA in *hos1-1* mutant plants as compared to wild type plants (Ishitani, 1998). Other osmotically regulated genes like *RAB18*, *RD22*, *RD29B* were found to be upregulated by osmotic stress and to some extent with ABA treatment too, however they were not found to be regulated by cold in *hos1-1* mutant plants. Since *hos1-1* altered expression of cold regulated genes and did not affect the expression of other osmotically regulated genes, it is hypothesized

that HOS1 participates in the cold response pathway before the cold and osmotic regulatory genes converge. Since the expression of cold regulated genes was induced in *hos1-1*, HOS1 is predicted to be a negative regulator of cold induction (Ishitani, 1998). Based on the knowledge of cold regulation in *A. thaliana* the cold regulatory pathway is outlined in Figure 1.1.

Cold regulation in *Arabidopsis thaliana* that is independent of CBF regulation

Several genes like *ESK1*, *HOS9*, and *GI* are known to control cold regulation in *A. thaliana* independently of CBF regulation (Xin and Browse, 1998, Fowler and Thomashow, 2002, Zhu *et al*, 2004, Shuqing *et al*, 2005).

Analysis of Arabidopsis thaliana eskimo-1 mutants

Mutation at the *ESK 1* locus leads to constitutive induction of cold tolerance in *A. thaliana* (Xin and Browse, 1998). *esk1* mutant plants have elevated freezing tolerance with higher proline and soluble sugar concentrations compared to wild type plants. In the freezing tolerance experiment, *esk1* plants showed 100% survival after the plants were exposed to -8°C temperature while all the wild type plants died under same conditions. Cold acclimation of the *esk1* mutants increased their freezing tolerance up to -14.8°C . Transcripts of all the *COR* genes (*COR 6.6*, *COR15A*, *COR47*, *COR78* and *RAB18*) that are CRT/DRE regulated were not affected by the *esk1* mutation and showed regulation identical to wild type plants. This indicates that the higher freezing tolerance of *esk1* plants is independent of the CBF cold regulatory pathway. This suggests the presence of a cold acclimation pathway independent of CBF regulation. The proline content of the

esk1 plant was constitutively high and did not increase with the cold acclimation. The transcript of pyrroline-5-carboxylate synthetase (P5CS), which is involved in proline synthesis is also expressed in non-acclimated wild type plants and its levels increases by 3-fold after two days of cold acclimation, *P5CS* transcripts was 8-fold higher in *esk1* mutants compared to wild type non-acclimated plants. However, *P5CS* transcripts were slightly reduced in *esk1* mutants after two days of cold acclimation. *A. thaliana* proline oxidase (AtPOX) is the enzyme that catalyzes the break down of proline and is normally induced by proline. The transcript levels of this gene were similar in both wild type and *esk1* plants even though *esk1* mutants had elevated proline levels. Also, feeding experiments with proline did not increase *AtPOX* expression in *esk1* plants. Taking everything into consideration, it looks like cold acclimation is not under the control of a single linear signaling pathway, but that there are other parallel or branched pathways that regulate cold acclimation.

Homeodomain transcription factor gene HOS9 in Arabidopsis thaliana

In *A. thaliana* *HOS9* encodes a homeodomain family transcription factor localized in the nucleus as demonstrated by HOS9-GFP fusion protein studies (Zhu *et al*, 2004). Non-acclimated and cold acclimated *hos9-1* plants were more susceptible to low temperature compared to wild type plants. Thus *hos9-1* plants lost their ability to cold acclimate (Zhu *et al*, 2004). Expression of *CBF* transcripts in *hos9-1* plants remained similar to wild type plants after cold treatment. Apart from that, the *HOS9* gene was found to be constitutively expressed in wild type plants and its expression was not altered with cold, ABA or salt treatments. This is different from the *CBFs* gene expression,

which is induced only by cold treatment, thus indicating the involvement of *HOS9* in cold regulation in a CBF independent pathway (Zhu *et al*, 2004). Analysis of the microarray data from experiments with *hos9-1* mutants revealed that expression of at least 140 genes is three fold higher in *hos9-1* plants than wild type plant and thirty-five genes are down regulated by at least two fold. Out of the 140 genes, 41 genes were found to be cold regulated. None of cold regulated genes in *hos9-1* mutant were controlled by the *CBF* gene family. Thus, suggesting *HOS9* is involved in cold regulation in a CBF independent pathway (Zhu *et al*, 2004).

Involvement of GIGANTIA in cold acclimation regulation

GIGANTIA (GI) is a newly discovered gene in *A. thaliana* and encodes a nuclear localized protein (Huq *et al*, 2000). It is involved in several developmental processes like photoperiod-mediated flowering, phytochrome B signaling, circadian clock regulation and carbohydrate metabolism. There was very little known about its involvement in cold regulation until microarray analysis showed that it was upregulated by cold treatment (Fowler and Thomashow, 2002). Recently the *GI* gene was reported to be upregulated by cold but not following saline, ABA or mannitol treatments (Shuqing *et al*, 2005). Regulation of *CBF* transcripts and genes that are induced by CBFs like *KIN1*, *KIN2*, *RD29A* and *COR15A* were not differentially regulated in a *gi-3* mutant as compared to wild type *A. thaliana* plants. These results indicate that *GI* is involved in cold regulation of gene expression independently from the CBF pathway. However, it is still unclear what is the other pathway that regulates cold tolerance in *A. thaliana*. *GI* is known to

regulate carbohydrate metabolism in the plant and thus it remains to be investigated if *GI* regulates freezing tolerance by changing carbohydrate metabolism (Shuqing *et al*, 2005).

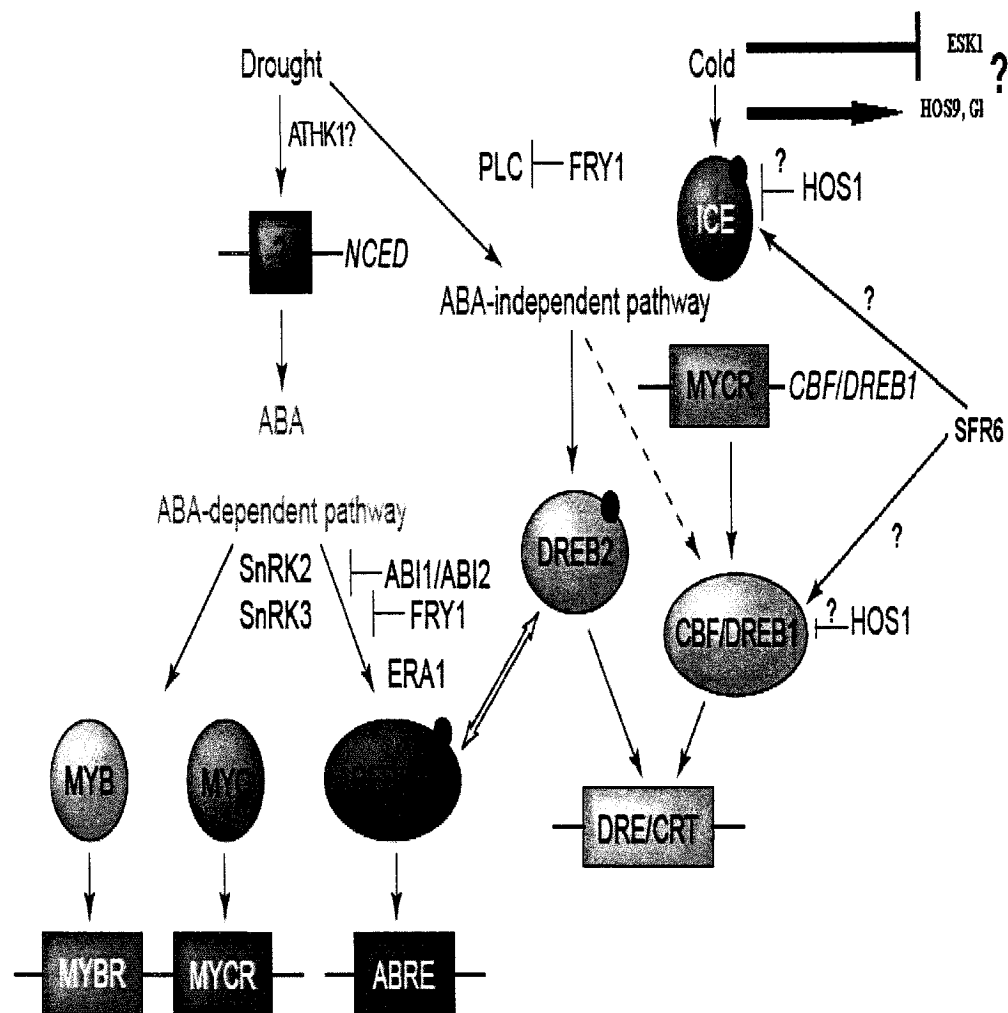


Figure 1.1 The cold regulatory pathway and its convergence with ABA-dependent and ABA-independent pathways. This figure has been taken from (Shinozaki et al, 2003) and modified. Genes in the cold regulatory pathway with unclear functions are marked with a “?”.

Link between cold tolerance and flowering

Various crops show maximum low temperature tolerance during the vegetative phase and as the crop enters the reproductive phase it loses its ability to cold acclimate (Fowler *et al*, 1996a, Fowler *et al* 1996b, Mahfoozi *et al*, 2001). It has also been shown that vernalization and photoperiod regulate the expression of low temperature induced genes through separate pathways, which converge to activate genes controlling plant development (Fowler *et al*, 2001). Mahfoozi *et al* (2001) have demonstrated that wheat has the ability to cold acclimate and reach maximum freezing tolerance when it is in the vegetative state. As soon as the plant enters the reproductive state, it begins to lose its ability to cold acclimate. The three winter wheat varieties used in the study (Norstar, Cheyenne and Warrior) cold acclimated during the vegetative growth of the crop. When plants were deacclimated for several days and re-acclimated, it was found that plants that were in the vegetative phase had the capacity to re-acclimate while the plants that are in the reproductive phase could not cold acclimate. The results of a time course study has shown that low temperature-induced gene expression is developmentally regulated (Fowler *et al*, 1996a, Fowler *et al*, 1996b). The authors have pointed out the association of reproductive transition and low temperature tolerance loss supporting the hypothesis of Fowler *et al* (1996b) that plant response to low temperature acclimation is a function of the degree and duration of expression of low temperature tolerance genes and that developmental genes are the key factors responsible for the duration of their expression (Mahfoozi *et al*, 2001).

Difference in regulation of TaVRT-1 and TaVRT-2 in wheat

Triticum aestivum *vegetative to reproductive transition-1* (*TaVRT-1*) transcripts are upregulated during the vegetative to reproductive transition in wheat (Danyluk *et al*, 2003). *TaVRT-1* is a MADS-box family transcription factor. And in *A. thaliana* these homologous MADS-box proteins like *agamous*, *apetala1*, *apetala3* and *FLC* are associated with the flowering control pathways. *TaVRT-1* was constitutively expressed in spring wheat and was expressed in winter wheat only after 35 days of low temperature treatment (Danyluk *et al*, 2003). The constitutive expression of *TaVRT-1* in the spring cultivar was paralleled by a low constitutive expression of *COR* regulated genes like *wcs19* and *wcs120*. In the winter type wheat the expression of *TaVRT-1* increased as *COR*-regulated genes expression decreased with heading (Danyluk *et al*, 2003). The *TaVRT-1* homologue in *Hordeum vulgare*, *HvBM-5* was also found to be positively associated with heading. There is a positive regulation of *TaVRT-1* expression with flowering in cereals that is regulated by vernalization. Recently, *TaVRT-2* from wheat has been characterized and was also found to be a MADS-box transcription factor (Kane *et al*, 2005). Mode of regulation of *TaVRT-2* transcript was opposite to *TaVRT-1*. *TaVRT-2* transcript was present at a low constitutive level in spring wheat compared to the high constitutive level of the *TaVRT-1* transcript. In winter wheat *TaVRT-2* expression was induced with cold acclimation until 35 days when it started to decline. Protein interaction studies using yeast two hybrid assays showed that *TaVRT-2* interacts with *TaVRT-1* and other MADS box proteins, *TaVRT-2* was also found to interact with the *VRN-2* protein which is a zinc-finger like protein involved in the control of flowering, suggesting *TaVRT-2* is linked to the flowering pathway (Kane *et al*, 2005). It

is still very difficult to place *TaVRT-1* and *TaVRT-2* in a flowering pathway regulated by photoperiod since very little is known about flowering pathways in cereals.

In Arabidopsis FVE links flowering time and cold regulation

Kim *et al* (2004) have demonstrated a genetic link between cold tolerance and flowering through *FVE* in *Arabidopsis*. The *acg1* mutant plants were isolated by a targeted gene approach using a 4-C/DRE-GUS construct. The freezing tolerance of the *acg1* mutant plants was higher than the wild type *A. thaliana*. The *acg1* mutant plants flowered later than wild type plants. *acg1*, which is a null mutation in *FVE* gene, did not affect the flowering time with intermittent cold treatments as compared to wild type plants where the flowering time was increased.

The expression of *COR* genes that are induced by the *CBF* pathway was studied in the *acg1* mutants. The *COR15a* and *COR47* levels were always higher in the *acg1* mutant plants as compared to wild type plants containing 4-C/DRE-GUS at other loci. *CBF* genes were not expressed at 23 °C in the *acg1* mutant, thus the elevated *COR* genes expression at 23 °C in the mutant was independent of the *CBF* regulation. In *acg1* mutant plants, *CBF* expression is altered causing an early *CBF* expression with cold treatment (Kim *et al*, 2004). The *FLC* transcript, which encodes an inhibitor of flowering showed an elevated expression which agrees with the late flowering phenotype of *acg1* mutants. Collective analysis of the data suggests a dual role of *FVE* in flowering time and cold response in *A. thaliana* (Kim *et al*, 2004).

Effect of abscisic acid on plant cold acclimation

Abscisic acid (ABA) is a sesquiterpene, which is synthesized by the farnesyl pyrophosphate pathway (Bennett *et al*, 1984). ABA is a plant growth hormone and inhibits auxin-induced growth. It also promotes seed maturation and dormancy and inhibits seed germination. ABA may also be involved in promoting dormancy in leaves and flower buds in autumn. It also stimulates senescence in plants. It is known to be induced by cold, salt and osmotic stress in plants.

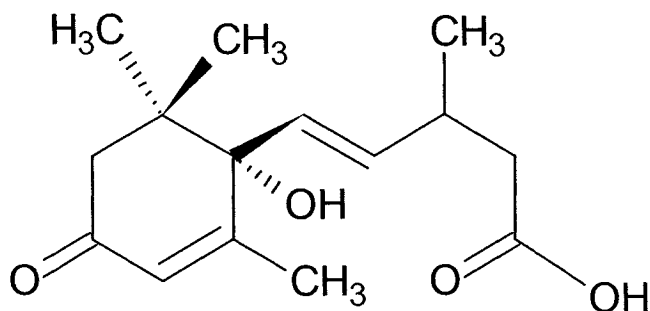


Figure 1.2: Chemical structure of ABA

The exogenous application of ABA was shown to increase freezing tolerance of potato plants (Chen *et al*, 1983). It was also found that the level of ABA increases in plants that are cold acclimated (Thomashow, 1999). *A. thaliana* mutant plants *abal* and *abil* that have altered ABA synthesis and ABA sensitivity, respectively were found to be less freezing tolerant (reviewed by Thomashow, 1999). Cold regulated gene expression of *RAB18* and *LT165* was severely impaired in *abal* and *abil* mutant plants. Thus ABA is thought to be involved in controlling freezing tolerance in plants. The increase in ABA in *A. thaliana* is transient, with a peak 24 hours after cold treatment and a return to basal

level within 2 days even though the plant still shows freezing tolerance for 2 to 3 weeks. ABA increases freezing tolerance by inducing the *COR* genes which are cold regulated through CRT/DRE elements. Ishitani *et al* (1997) tested various *A. thaliana* mutant plants using the *LUC* reporter gene to study the response to ABA, cold and osmotic stress. They isolated various mutant plants that were responsive to cold stress and ABA treatment. The results of their study demonstrated a relationship between the ABA and cold signaling pathways (Ishitani *et al*, 1997). Thus it is clear that ABA has role in inducing cold responsive genes, but its effect still remains to be studied (reviewed by Thomashow, 1999).

Effect of nitric oxide on plants

Nitric Oxide (NO) is a small water and lipid soluble free radical, which acts as a biological messenger in mammals (Kerwin *et al*, 1995). Its target sites are sulfur containing metals and thiol containing proteins. In plants, it has been shown to affect germination, leaf expansion, senescence, programmed cell death and organogenesis. It was also demonstrated that NO is required for the activation of plant cell division (Otvos *et al*, 2005). NO has been shown to break the dormancy during seed germination in *A. thaliana* and *Hordeum vulgare* (Bethke *et al*, 2004). Its effect was found to be concentration dependent; 25 μ M sodiumnitroprusside (SNP), which releases NO, concentration was effective in reducing seed dormancy. Concentrations in the range of 100-250 μ M delayed germination of the seedlings by 1 to 2 days and concentration above 250 μ M affected root growth indicating NO-dependent inhibition of enzyme activities (Bethke *et al*, 2004). It is also known that NO represses flowering in *A. thaliana*. NO

synthase (NOS) is an enzyme involved in NO synthesis. *nos1* mutants flowered earlier than wild type plants whereas the NO over producer mutant *nox1* showed a late flowering phenotype. Treatments with 100 μ M SNP increased the expression of the *FLC* gene, which is a floral repressor in the *A. thaliana* flowering pathway. This indicates that NO affects flowering in *A. thaliana*. It is thought that NO controls flowering through both the photoperiod and the autonomous flowering pathway (He *et al*, 2004).

Sulfotransferases

Sulfotransferases (STs) are enzymes found in bacteria, plants and mammals that can sulfonate hydroxyl groups on a variety of organic molecules. The universal sulfonate donor is 3'-phosphoadenosine 5'-phosphosulfate (PAPS). The general scheme of the sulfation reaction is shown below:

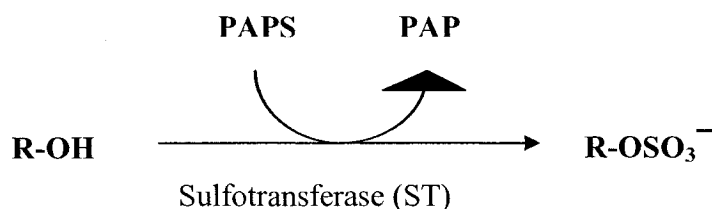


Figure 1.3 General scheme of the sulfoconjugation reaction

Sulfotransferases can be divided into two major groups: Membrane bound sulfotransferases that are known to sulfonate glycoproteins and tyrosine residues in proteins, and soluble sulfotransferases which sulfonate small organic molecules such as; flavonoids, steroids, catecholamines and xenobiotics (Falany, 1997).

Membrane bound plant sulfotransferases

Phytosulfokine α - sulfotransferase

Phytosulfokine α (PSK α) is a sulfated pentapeptide growth factor found both in monocots and dicots. The pentapeptide sequence consists of Tyr (SO₄H)-Ile-Tyr (SO₄H)-Thr-Gln (Yang *et al*, 2000). PSK α was initially isolated from *Asparagus officinalis* L mesophyll cell culture (Matsubayashi and Sakagami, 1996). PSK α has also been identified in conditioned medium derived from maize, rice, carrot and *Zinnia* species

suggesting its universal presence in plants (reviewed in Matsubayashi and Sakagami, 2000). PSK α is known to have mitogenic activity thus promoting cell-proliferation. Deletion of the sulfate group from the two tyrosine residues leads in the loss of its function and reduction in cell growth, suggesting the importance of the sulfate group of the peptide for biological activity in cell-proliferation (reviewed in Matsubayashi and Sakagami 2000). The sulfation of the tyrosine residues is performed by a membrane bound tyrosine protein ST, which is similar to the previously characterized mammalian tyrosine protein ST. The phytosulfokine α - ST is a membrane bound enzyme localized in the Golgi network (Hanai *et al*, 2000).

Gallic acid glucoside sulfotransferase

In *Mimosa pudica*, gallic acid β -D-glucopyranosyl-6'-sulfate was identified as the periodic leaf movement factor (PLMF-1) (Schildknecht and Schumacher, 1981). PLMF-1 is involved in leaf closure that is induced by mechanical stimulus (seismonasty). The enzyme that catalyzes the final step of gallic acid glucoside sulfate biosynthesis is called gallic acid glucoside ST. The enzymatic activity was detected in the microsomal preparation of primary, secondary and tertiary pulvini which are the motor organs of the plant (Varin *et al*, 1997). The enzyme was localized on the plasma membrane of sieve tube cells by using immunocytochemistry confirming its association with membrane (Varin *et al*, 1997).

Soluble Sulfotransferases

Soluble sulfotransferases from plants and animals share approximately 25 to 31% amino acid similarity with four conserved regions (Varin *et al*, 1992). The conserved region I consists of the amino acid sequence YPKSG(T/N)W and is located close to the N-terminal region. The conserved region IV is located close to the C-terminal region of the proteins and consists of the amino acids RKXXXGDWKNXFT. Site directed mutagenesis of the flavonol 3-ST from *Flaveria chlorraefolia* revealed that the conserved lysine at position 59 (region I) was involved in catalysis and arginine at position 276 (region IV) was involved in PAPS binding (Marsolais and Varin, 1995). His¹¹⁸ (region II) of the flavonol 3- ST was also suggested to be involved in catalysis (Marsolais and Varin, 1997). These critical amino acids were found to be present in all soluble STs characterized so far (reviewed by Negishi *et al*, 2001).

Plant soluble sulfotransferases

Flavonol sulfotransferases

Flavonoid compounds are known to be involved in the plant defense response to microbial attack and in UV protection (Long *et al*, 1989). Flavonoids are also known to act as signaling molecules in the induction or inhibition of early nodulation genes in Rhizobium (Long *et al*, 1989) and as regulators of polar auxin transport (Faulkner and Rubery, 1992). Flavonol STs were first identified from *Flaveria chloraefolia* and *Flaveria bidentis*. The flavonol 3-ST and flavonol 4'-ST were the first plant STs to be

cloned and characterized (Varin *et al*, 1992). The biological function of flavonol STs still remains unclear although its biochemistry is well understood.

Choline sulfotransferase

Choline sulfotransferase (CST) was detected in *Limonium sativum*. It catalyzes the transfer of a sulfuryl group from PAPS to choline, thus forming choline-O-sulfate (CS) as a final reaction product. CS is found to accumulate in salt stress tolerant plants of the Plumbaginaceae family. CST was isolated *in vitro* from the root extracts of *Limonium sinuatum*. Salinization induced CST in roots, leaves and cell cultures by six fold (Rivoal and Hanson, 1994). These results were consistent with the 2 to 3 fold increase of CS in salt treated plants (Rivoal *et al*, 1994). CST activity was not detected in plants like barley, maize, sunflower and *Brassica* spp. suggesting that CST is restricted in *Limonium* spp.

Glucosinolates sulfotransferases

Glucosinolates (GSs) are hydrophilic thioglucosides present in all members of the Brassicaceae family which includes agriculturally important crops and vegetables like broccoli, cauliflower, cabbage and brussels and an important oil seed crop, *Brassica napus*. It is believed that GSs are involved in sulfur and nitrogen storage and also in providing defense against pathogens and herbivores. GSs are derived from amino acids and are hydrolyzed by a thioglucosidase known as myrosinase releasing glucose and unstable aglycons such as isothiocyanates, organic nitriles and thiocyanates. The final step of GS biosynthesis is catalyzed by sulfotransferases that add a sulfonate group to

desulfoglucosinolates (dsGS). *A. thaliana* dsGS-STs are known as AtST5a, AtST5b and AtST5c (Piotrowski *et al* 2004, Klein *et al*, 2006). Transcript of AtST5a was found to be induced by the phytotoxin coronatine which is a structural homolog of jasmonic acid (JA). AtST5a expression also increased with methyl jasmonate (MeJA), octadecanoids and ethylene treatment. These compounds are involved in the plant wound defense mechanism. AtST5a transcripts were also induced during wounding in the plant (Piotrowski *et al*, 2004).

AtST5a and its closest homologs AtST5b and AtST5c were cloned in an expression vector to further study their substrate specificities. AtST5a preferred tryptophan and phenylalanine derived dsGS, while AtST5b and AtST5c showed substrate specificity for methionine derived long chain dsGS (Piotrowski *et al*, 2004). The K_m values for *A. thaliana* dsGS-STs range from 50 to 100 μ M for dsGS and 25 to 100 μ M for PAPS (Klein *et al*, 2006). The sub-cellular localization studies of *A. thaliana* dsGS-STs was performed using *AtST-GFP* and *GFP-AtST* constructs and were found to be localized in the cytosol (Klein and Papenbrock, 2004; Klein *et al*, 2006).

Steroid sulfotransferases

Brassinosteroids (BRs) are plant specific naturally occurring polyhydroxysteroids. Sulfated BRs have still not been identified in plants. However, feeding experiments with [3 H]-castasterone which is a precursor molecule of brassinosteroid biosynthesis, to mung-bean plants showed that along with a glucoside conjugate, a polar non-glucoside conjugate was formed that could not be released by enzymatic hydrolysis (Yokota *et al*, 1991). Similar experiments were performed with rice seedlings and based on the

chromatographic behavior of the labeled metabolites it was proposed that the non-glucoside conjugates could be sulfated compounds (Yokota *et al*, 1992)

BnST1, BnST2, BnST3 and BnST4 were isolated from *Brassica napus*. When expressed, BnST3 showed substrate specificity for steroid substrates and sulfated brassinosteroids (BRs) (Rouleau *et al*, 1999). BnST3 has highest substrate specificity for 24-epicathasterone. The enzyme could also sulfate 24-epiteasterone but with lesser affinity than 24-epicathasterone, thus suggesting that 24-epicathasterone is its *in vivo* substrate. BnST4 has a broad range of substrate specificities as compared to other plant STs that shows strict specificity towards a substrate (Marsolais *et al*, 2004). BnST4 could also sulfonate of 24-epicathasterone and 24-epitestosterone (Marsolais *et al*, 2004).

BnST1 transcripts were shown to be induced with 10 μ M Salicylic acid. *BnST* genes are also inducible by ethanol and other xenobiotics (Marsolais *et al*, 2004), which suggests that BnSTs might be involved in detoxification. Furthermore, it was also reported that the sulfation of steroid compounds might lead to their inactivation. Transgenic *BnST3* over expression not lead to a brassinosteroid-deficient phenotype suggesting that the steroid ST is not directly involved in brassinosteroid inactivation (Marsolais *et al*, 2004).

Hydroxyjasmonate Sulfotransferase

12-hydroxyjasmonate is also known as tuberonic acid due to its tuber inducing properties. It was first isolated from *Solanum tuberosum* (Gregory, 1956). It is derived from jasmonic acid, which is an important signaling molecule in the plant defense response and in the control of various developmental processes. AtST2a from *A.*

thaliana, catalyses the transfer of a sulfonate group from PAPS to 12-hydroxyjasmonate (Gidda *et al*, 2003).

Plants treated with 12-OHJA showed a 9-fold increase in 12-OHJA-sulfate as compared to untreated plants. The transcript level of *AtST2a* was also increased with 12-OHJA treatments. Transcripts of *AtST2a* were also increased by dark treatment (Gidda, 2001). *A. thaliana AtST2a* antisense plants flowered earlier than wild type plants while the transgenic plants over expressing *AtST2a* flowered late as compared to wild type plants suggesting the involvement of *AtST2a* in the control of flowering (Gidda, 2001). This was the first report showing the involvement of 12-OHJA as a signaling molecule in non-tuber forming plants.

Chapter 2

MATERIALS AND METHODS

Materials

All the chemicals were purchased from Sigma-Aldrich and BioRad. Enzymes used for cloning were from Fermentas and New England BioLabs. *A. thaliana* seeds were obtained from the Arabidopsis Biological Resource Center. [³⁵S] PAPS was purchased from PerkinElmer Life Sciences (Boston, MA, USA).

Plant growth conditions

A. thaliana seeds were soaked in water and stratified at 4 °C for two days before planting and plants were grown in soil in a growth chamber at 22° C with 16 hours of light. For cold acclimation experiments *A. thaliana* plants at different developmental stages were exposed to 4 °C for 7 days with 16 hours of photoperiod.

For the chemical treatments, two days post flowering *A. thaliana* plants, were sprayed daily with 2 or 20 µM abscisic acid (ABA) and 50 or 100 µM sodium nitroprusside (SNP) for 2 min everyday for 7 days along with the cold acclimation at 4 °C, then their freezing tolerance was tested. For control treatments, the plants were sprayed with H₂O.

Detection of freezing tolerance by the electrolytic conductivity assay

Freezing sensitivity of the rosette leaves from *A. thaliana* plant was measured by electrolytic leakage (EL) at sub-zero treatments lowered at the rate of 2 °C per hour. This protocol is modified from Lee *et al.*, (1999). Leaflets were incubated at 0 °C for 1 hour followed by ice formation, which was initiated by the addition of an ice chip in the test-tube. After obtaining the desired temperature the test tubes containing the rosette leaves were immediately transferred to ice and incubated for 15-18 hours. Distilled H₂O (10 ml) was added to each tube and incubated on shaker for 1 hour before measuring the electrical conductivity of the solution. Leaflets were then frozen in liquid nitrogen for 10 minutes and thawed for 2 hours before a second measurement of the electrical conductivity of the solution. For each temperature point six replicates were used.

Percentage value was calculated by dividing initial electrolyte leakage with the complete electrolyte leakage value after freezing the leaflets in liquid nitrogen. LT₅₀ values were extrapolated from the graphs of temperature vs. % EL. Two way ANOVA test was performed on the data to check the significance of cold acclimation versus developmental stages in *A. thaliana*. One way ANOVA was done to test the significance of ABA and SNP treatments on 2 days after flowering plants. Regression studies were done using the % EL values with the selected temperature points that were close to LT₅₀. The means of the % EL values were directly compared using t-test for the important temperature points at three different flowering stages.

Amino acid sequence deduction and alignment

The DNA sequences of TaST2, TaST3 and TaST4 were obtained from the cDNA clones WEF077_J03, L6B008_B08 and L6B015_M01 from the FGAS clone collection. Sequencing of the clones was performed at the Montreal Genome centre. The obtained DNA sequences were translated using the Expert Protein Analysis System (ExPASy, <http://ca.expasy.org/tools/dna.html>) translation tool. All the amino acid sequences for *A. thaliana* STs were obtained from the TAIR (<http://www.arabidopsis.org>) website using the gene IDs listed in Table 4.1. Amino acid sequence alignment was performed using the ClustalW1.8 program. The scores generated were used to create a phylogenetic tree using the same program.

Cloning of TaST2 and TaST4

The coding sequences of TaST2 and TaST4 were amplified from their respective cDNA clones as mentioned above, with *Pfu* DNA polymerase (Fermentas, Burlington, Ontario).

Oligonucleotides used for PCR amplification are

TaST2F- 5' GTCGACGCATGGCCCGTGTAGCGGAG 3'

TaST2R- 5' AAGCTTTTACTGTGGCATGGAGATGCC 3'

TaST4F- 5' GTCGACGCCTTGGCAACCATGGGCAGTAAC 3'

TaST4R- 5' AAGCTTTTTATAGGAAGCCAGGGCACA 3'

The PCR products were digested with *SalI* and *HindIII*, and then ligated to the corresponding sites in the polylinker of pQE30 (Qiagen, Mississauga, Ontario), to express

N-terminal His-tagged recombinant proteins. The constructs were screened using colony PCR followed by restriction enzyme analysis and verified by sequencing.

Cloning of TaST3

TaST3 was PCR amplified using FGAS cDNA clone L6B008_B08. The amplified PCR product was ligated in pGEM-T (Promega, Nepean, Ontario) cloning vector and transformed in to *E. coli*.

Oligonucleotides used for PCR amplification are

TaST3F- 5' GTCGACGCATGGCGCTAGCAAGTGCCC 3'

TaST3R- 5' AAGCTTTTCACAGGAGATCTAGGCCCGATC 3'

Selection of positive colonies was performed on Luria-Bertani nutrient media supplemented with X-gal/IPTG/ampicillin/tetracycline. Selected positive clones were confirmed by restriction enzyme digestion with *Hind*III and *Sal*I and the digested TaST3 insert was purified from agarose gel after electrophoresis and used for ligation in the pQE30 vector. Selected positive clones of TaST3 were confirmed as mentioned previously.

Expression and purification of recombinant TaST2, TaST3, TaST4 proteins

The production of recombinant TaST2, TaST3, TaST4 in *Escherichia coli* cultures ($A_{600} \approx 0.5$) was induced with 1 mM isopropyl β -D-thiogalactopyranoside (IPTG) for 10 h at 18°C. Bacterial cells were collected by centrifugation, resuspended in 50 mM sodium phosphate (pH 8.0), 300mM M NaCl and lysed by sonication. The lysate was centrifuged at 12000 rpm for 10 minutes at 4 °C. The soluble protein fraction was

applied on a nickel-nitrotriacetic acid agarose column (Qiagen), which was pre-equilibrated with the lysis buffer. The matrix was washed twice with a wash buffer containing 50 mM sodium phosphate buffer (pH 6.0), 300 mM NaCl and 50 mM imidazole. The recombinant protein was eluted with the elution buffer containing 50 mM sodium phosphate buffer (pH 6.0), 300 mM NaCl and 250 mM imidazole. Protein concentration was determined by the method of Bradford using the Bio-Rad dye reagent (Mississauga, Ontario) and bovine serum albumin as reference protein (Bradford, 1976).

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting analysis

To verify the solubility and estimate the level of purity of the recombinant protein after chromatography on nickel-agarose, aliquots of the recombinant enzyme were subjected to 12% polyacrylamide gel according to the method of Laemmli (1970). The proteins were visualized by coomassie blue staining. While performing western blotting two identical gels were electrophoresed under the same conditions. One of the gels was used for coomassie blue staining while the other gel was subjected to western blotting following transfer to nitrocellulose membrane. A pool of primary antisera raised against AtST2a, BnST1, and flavonol 3- ST was used to identify the recombinant proteins.

Sulfotransferase assay

The substrates that were used for the enzymatic assays were either dissolved in 50% DMSO or 50% ethanol. Substrate specificity studies were performed using three different substrate concentrations: 1, 10 and 100 μ M. A 50 μ l reaction mixture was used for the assays which contained 50 pmol [35 S] PAPS, approximately 0.25 μ g of Nickel

agarose purified recombinant enzyme TaST2, TaST3 and TaST4 in 25 mM Tris-HCl or 50 mM Phosphate buffer pH 7.5. Reactions were carried out at room temperature for 10 minutes then stopped by the addition of 20 µl of 2.5% acetic. The reaction products were extracted either with water-saturated butanol or in 0.1% tetrabutylammonium dihydrogen phosphate (TBADP) and ethyl acetate (Varin *et al*, 1987). 50 µl organic phase from butanol extraction and 100 µl organic phase from TBADP extraction were mixed with liquid scintillation fluid and counted on a liquid scintillation counter.

Table 2.1 List of compounds used as substrates in sulfotransferase enzyme assays

No.	Groups of the compounds	Substrate list
1.	Alcohol	Cis-3-hexene-1-ol, geraniol, 3-indolemethanol
2.	Flavonol	Kaempferol, quercetin, riboflavin
3.	Phenolic	4-hydroxy cinnamic acid, 5-methoxy tryptophal, Gallic acid, salicylic acid
4.	Plant hormone	Gibberelic acid
5.	Steroid	Homo brassinosteroid, castasterone, ecdysone, Epibrassinolide, β-estradiol
6.	Sterol	Cholesterol
7.	Sugar	N-acetyl glucosamine
8.	Other compounds	Choline, 12-hydroxy jasmonic acid and prostaglandin E ₂

Thin Layer Chromatography

For some substrates we used TLC and autoradiography to visualize the reaction products. One fourth of the reaction mixture was spotted on cellulose TLC plates and allowed to dry at room temperature. The TLCs were migrated using butanol-acetic acid-water (6:2:2) as a solvent system in a TLC chamber for 2-3 hours. The TLC plates were dried at room temperature. After drying, the plates were layered with BioMax XAR (Kodak) film in the darkroom and exposed for 4-10 days at room temperature. The films were developed in an automated developer.

Wheat metabolite extraction used for enzyme assays

The plant material was ground in liquid nitrogen and the metabolites were extracted in 50% methanol (3 ml/gr of tissue). After 15 minutes of incubation the slurry was filtered through a nylon mesh and the samples were centrifuged at 8000 rpm for 10 minutes at 4 °C. The supernatant was flash evaporated to half the volume to remove the methanol and the remaining solution was mixed with one volume of ethyl acetate in a separatory funnel. The ethyl acetate fraction was collected and left for over-night evaporation while the aqueous layer was mixed with butanol. The butanol phase was collected and evaporated in a flash evaporator. The ethyl acetate and butanol fractions were dissolved in a minimum volume of methanol and small aliquots were lyophilized in a speed vac. For enzyme assays the dried samples were dissolved in 50% DMSO.

Reverse transcriptase- Polymerase Chain Reaction (RT-PCR)

For RT-PCR experiments, 2.5 µg of total RNA was treated with 20 U DNase I (Roche) in 20 µl of 0.1 M sodium acetate, 5 mM MgSO₄, pH 5.0 for 10 min at 37°C. DNase I was heat inactivated at 95°C for 5 min. The cDNA was synthesized using Expand reverse transcriptase (Roche) as recommended. Initially, 2 µl of the reaction product was used for PCR with *Taq* DNA polymerase (New England BioLabs). The volumes of cDNA used as template for PCR were adjusted after a preliminary calibration based on the levels of α -*Tub* PCR products of cold treated *Triticum aestivum* samples: for spring wheat cultivar, 2 µl for control, 1.2 µl for 6 h, 1.3 µl for 1 d, 1.3 µl for 3 d, 1 µl for 6 d, 2.1 µl for 14 d and 2 µl for 36 d; for winter wheat cultivar, 2 µl for control, 2 µl for 6 h, 2 µl for 1 d, 2 µl for 3 d, 1.2 µl for 6 d, 1.3 µl for 14 d and 1.3 µl for 36 d. The following thermocycling program was used: initial denaturation at 94 °C for 2 min, followed by the specified number of cycles of 94 °C for 30 sec, 56 °C for 45 sec (for α -*Tub* at 58 °C), and 72 °C for 45 sec, and a final extension at 72 °C for 5 min.

List of oligonucleotides used for RT- PCR

α -*Tub* F- 5' TGAGGTTTGATGGTGCTCTG 3'

α -*Tub* R- 5' CCTGGTGGCTGGTAGTTGAT 3'

RT-TaST2 F- 5' AAGAAGCTGGAGGTGAACCA 3'

RT-TaST2 R- 5' AATGGATATGCCTCGTGACC 3'

RT-TaST3 F- 5' GATAAAAGATCCGGCCAACA 3'

RT-TaST3 R- 5' CAAGTTTGCATGCTGGTAAAT 3'

RT-TaST4 F- 5' CAGCTTGGGTAAGCTCAAGG 3'

RT-TaST4 R- 5' CTTGGCCAGGACGTTTACAT 3'

Chapter 3

ANALYSIS OF DEVELOPMENTALLY REGULATED FREEZING TOLERANCE IN *ARABIDOPSIS THALIANA*

Freezing tolerance changes with the development in *Arabidopsis thaliana*

It has been demonstrated in wheat that the plants lose their ability to cold acclimate when the plants initiate flowering. We tested the model plant *A. thaliana* also loses its ability to cold acclimate at the same developmental stage. Sensitivity to freezing was measured by the electrolytic leakage (EL) value, in which the conductivity of a solution in which the leaves from the cold treated plants were immersed was measured relative to the conductivity of the solution after cells of the leaf sample were completely disrupted by freezing in liquid nitrogen. A graph of freezing temperature versus % EL for samples taken from *A. thaliana* at different developmental stages was plotted. The lethal temperature₅₀ (LT₅₀) values were extrapolated from the graphs, as the temperature at which the plant loses 50% of its electrolytes. This value is taken as a measure of freezing tolerance of the plant. The pre-flowering non-cold acclimated *A. thaliana*, which were around 15 days old, had a LT₅₀ of -4.9 °C. While, the plants of the same developmental stage that were cold acclimated for seven days had an increased LT₅₀ of -7.3 °C (Figure 3.1). Plants that were in the bolting stage had no significant change in freezing tolerance. LT₅₀ of the non-cold acclimated plants in the bolting stage was -4.7 °C and in plants with seven days of cold acclimation it was calculated to be -7.2 °C (Figure 3.2). However, plants that were in the flowering stage having 2-3 open flowers but no growth of siliques showed a decrease in freezing tolerance. LT₅₀ of the non-cold

acclimated plants in the flowering stage reduced to -3.6°C . Plants at this stage of development that were cold acclimated had an LT_{50} of -4.8°C which was significantly different from non acclimated plants (Figure 3.3). In the post flowering stage that is marked with the presence of 2-4 siliques, no considerable change in freezing tolerance was observed relative to flowering plants. LT_{50} values of the non-cold acclimated and cold acclimated post-flowering plants were -3.3 and -4.8°C (Figure 3.4).

Statistical study of the data was analyzed by two-way ANOVA using a general linear model (GLM) with repeated measures. According to this study, the two different variables being tested were the developmental stage of the plant, with four stages evaluated and cold acclimation. The result of ANOVA are shown in (Table 3.1). The analysis shows that there is a strong significant effect of each of the variables, developmental stage of the plant and acclimation. In addition there is significant interaction effect, which indicates that the capacity to acclimate is significantly different at different stages of development.

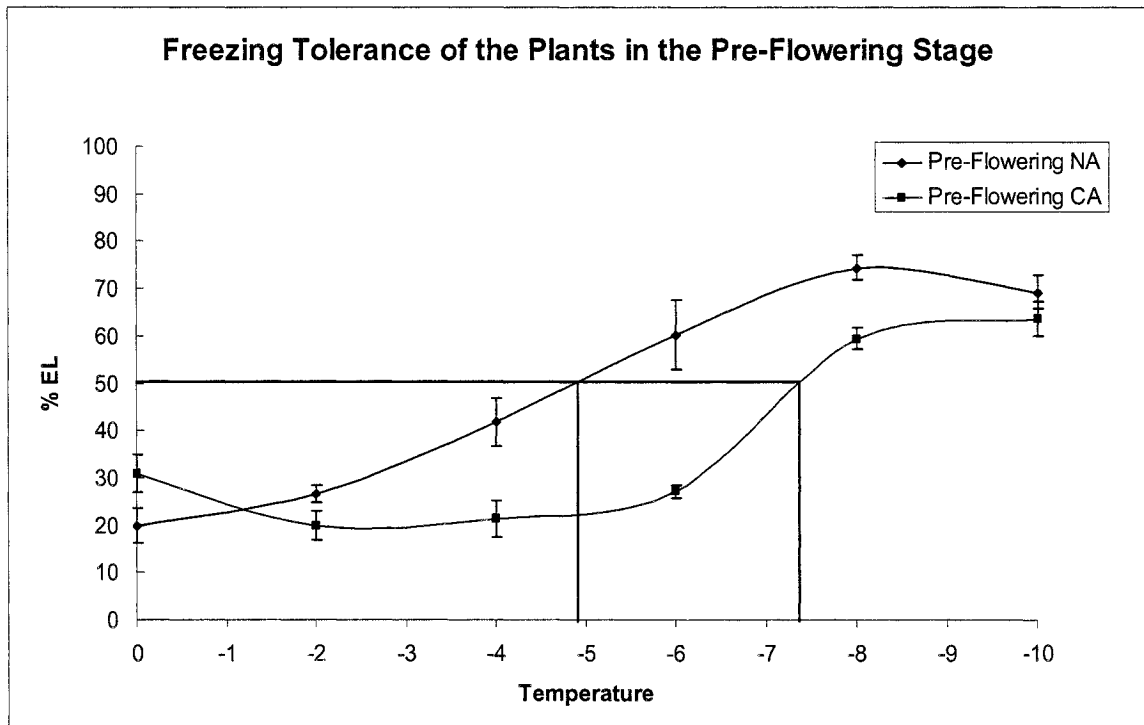


Figure 3.1 Graph demonstrating changes in freezing tolerance of the Pre-flowering plants with non-cold acclimation (NA) and cold acclimation (CA). Each temperature point shows an average of six percentage electrolytic leakage points and the bar represents the standard error. The line that drops from the 50% EL on the temperature axis shows the temperature at which the plant shows 50% of electrolytic leakage from the leaves. Bars are standard deviation (n = 6).

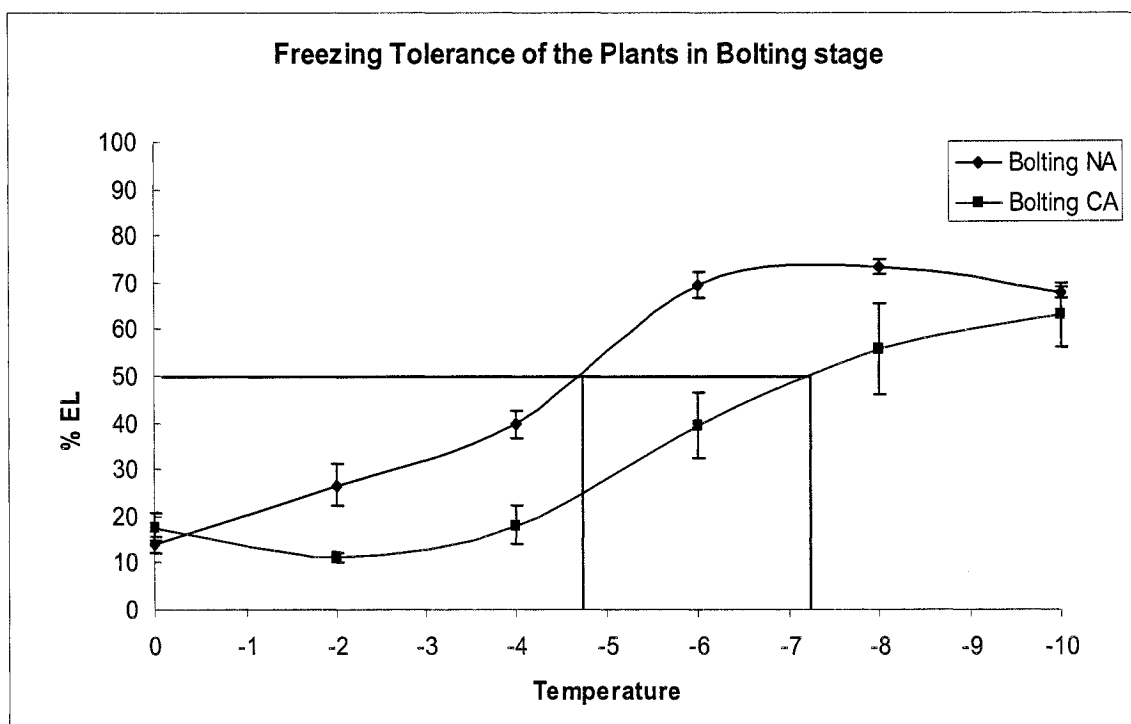


Figure 3.2 Graph demonstrating changes in freezing tolerance of the Bolting plants with non-cold acclimation (NA) and cold acclimation (CA). Each temperature point shows an average of six percentage electrolytic leakage points and the bar represents the standard error. The line that drops from the 50% EL on the temperature axis shows the temperature at which the plant shows 50% of electrolytic leakage from the leaves. Bars represent standard deviation, (n = 6).

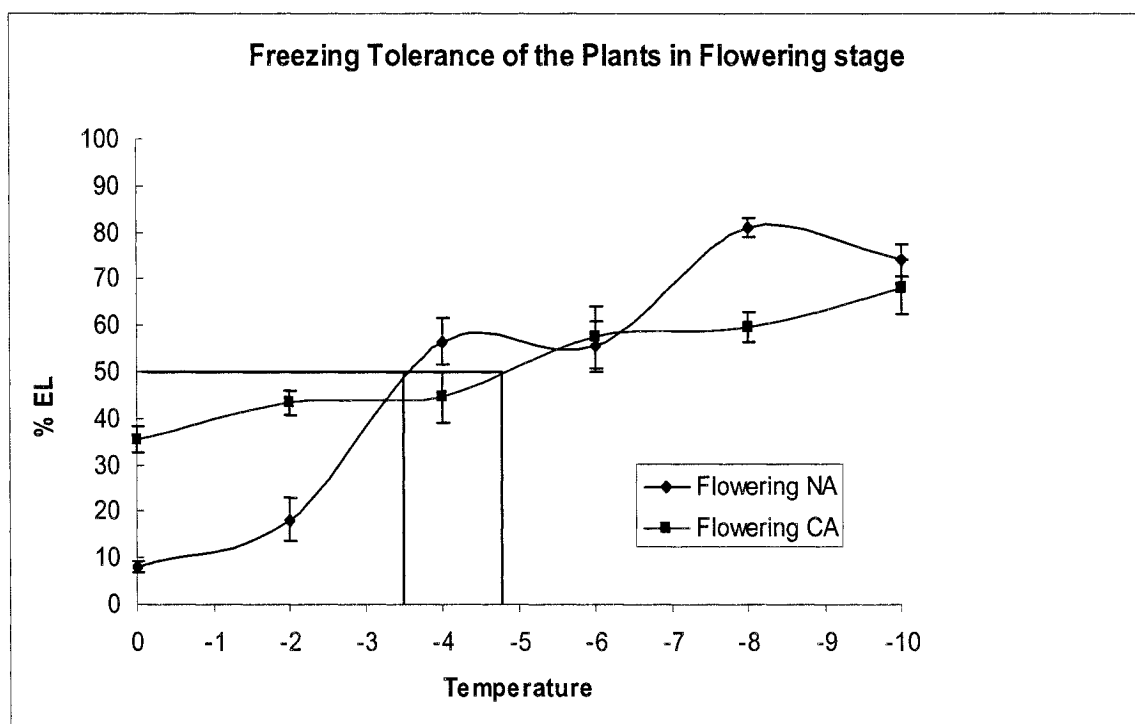


Figure 3.3 Graph demonstrating changes in freezing tolerance of the Flowering plants with non-cold acclimation (NA) and cold acclimation (CA). Each temperature point shows an average of six percentage electrolytic leakage points and the bar represents the standard error. The line that drops from the 50% EL on the temperature axis shows the temperature at which the plant shows 50% of electrolytic leakage from the leaves. Bars represent standard deviation, (n = 6).

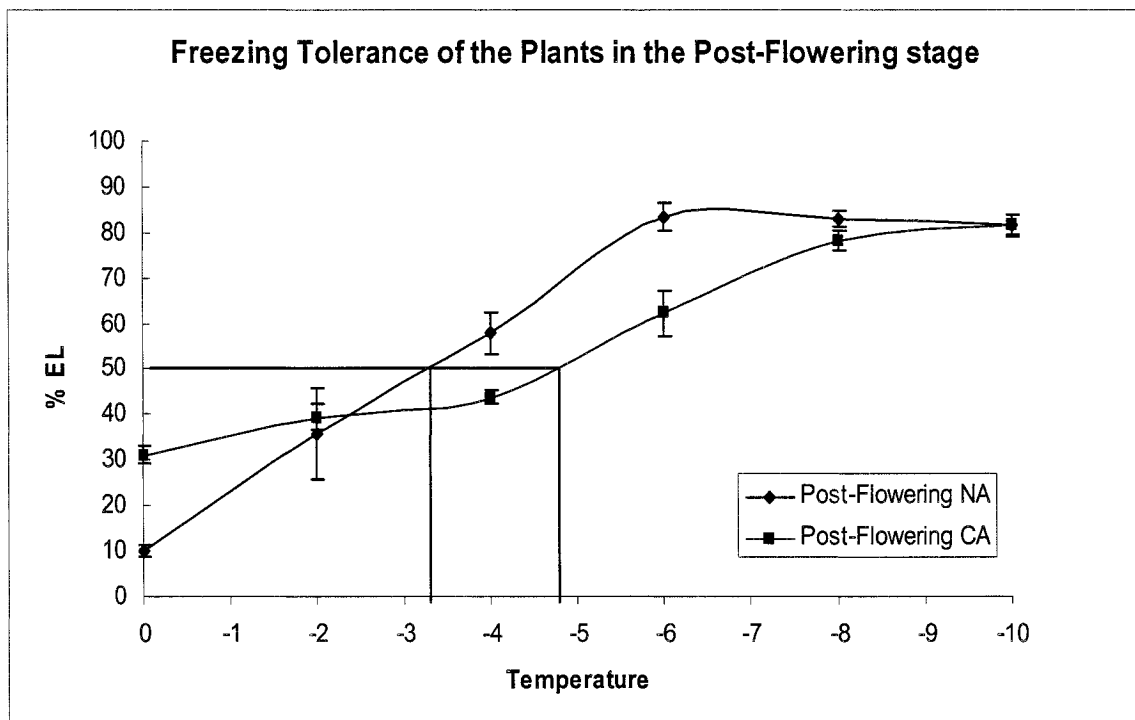


Figure 3.4 Graph demonstrating changes in freezing tolerance of the Post-flowering plants with non-cold acclimation (NA) and cold acclimation (CA). Each temperature point shows an average of six percentage electrolytic leakage points and the bar represents the standard error. The line that drops from the 50% EL on the temperature axis shows the temperature at which the plant shows 50% of electrolytic leakage from the leaves. Bars represent standard deviation, (n = 6).

Source	Type III Sum of square	df	Mean square	F-stat	p-value
Age	7,516.15	3	2,505.38	18.80	0.00
Type of acclimation	1582.30	1	1,582.30	7.14	0.01
Age* type of acclimation	5029.54	3	1,676.51	10.49	0.00

Table 3.1 Two-way ANOVA studies to identify effects between age, type of acclimation and their interaction. Source represents the variables that were studied. Age includes four different developmental stages, and type of acclimation includes non-acclimated and cold-acclimated plants. In column III, df represents degrees of freedom.

Time course study of the flowering plants and freezing tolerance in *Arabidopsis thaliana*

Our preliminary results indicate the reduction in freezing tolerance of the plants after they have reached flowering stage and thus led us to investigate a more precise plant stage that shows a reduction in freezing tolerance. The first experimental plant stage was marked with the presence of 1-2 open flowers and no siliques and was called the first-flowering stage. The LT_{50} of non-acclimated first flowering plants was found to be -4.7°C , while the LT_{50} of the cold acclimated first flowering plants was -7.6°C (Figure 3.5). Two days old after flowering, plants had the presence of more growth of apical flowers on the principal bolt and also considerable growth of lateral bolts with closed flower buds. The LT_{50} of the plants in this stage was -3.6°C with no cold acclimation. And the LT_{50} of the plants in the same stage with cold acclimation was -5.7°C . Plants that were four days old after flowering showed growth of siliques on the first flowering stem and complete opening of flowers from the secondary or lateral bolts. Non-acclimated plants, four days after the first flower opened showed a freezing tolerance of -3.6°C and cold acclimated plants at the same stage showed freezing tolerance of -5.6°C . Other morphological changes and physiological changes that were observed in the cold acclimated plants included incomplete opening of mature flowers and accumulation of anthocyanin-like pigments in the flower buds (Figure 3.6).

Statistical study of the data was analyzed by two-way ANOVA using a general linear model (GLM) with repeated measures. In the study, there are three different flowering stages, namely, first-flowering stage when the first flower was completely open, second flowering stage which is two days after flowering and the third flowering

stage which is four days after flowering. The two different variables in the analysis include developmental stage and cold acclimation. Table 3.2 shows the ANOVA for this experiment. There was a significant effect of the developmental stage and of cold acclimation, however there was not a significant interaction effect. This experiment was different from the first one, which showed a significant development by cold acclimation interaction, in that the first experiment included pre-flowering stages of development. This indicates that there is a critical change in the ability to acclimate between pre-flowering and the first stage of flowering.

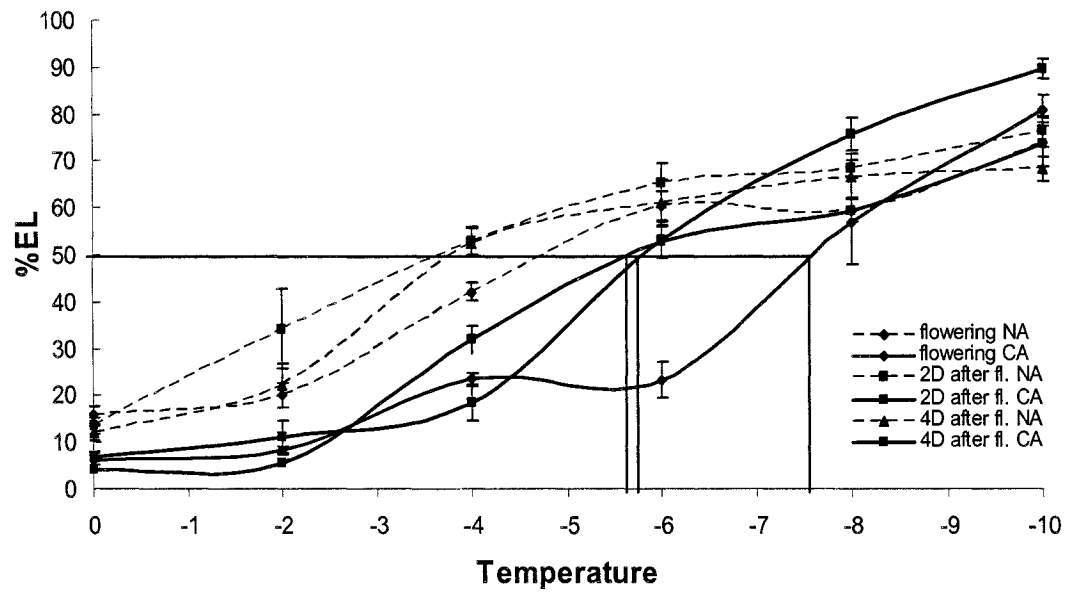


Figure 3.5 Shows the changes taking place in freezing tolerance between the flowering stage, 2 days after flowering and 4 days after flowering plants that were non-acclimated (NA) and cold acclimated (CA). Bars represent standard deviation, (n = 6).




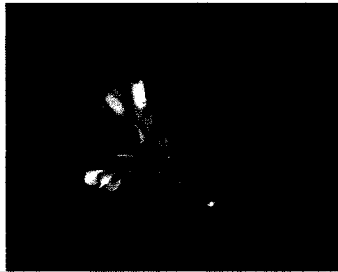

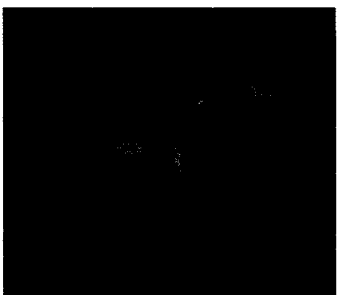
	Non-Acclimated	Seven days cold acclimated
1 st Flowering stage		
2 Days after flowering		
4 Days after flowering		

Figure 3.6 Changes occurring in *A. thaliana* during different flowering stages of the plant. Images were taken under the magnifying microscope.

Source	Type III Sum of square	df	Mean square	F-stat	p-value
Flowering stages	2212.49	2	1106.24	10.36	0.00
Type of acclimation	5624.79	1	5624.79	19.41	0.01
Flowering stages* type of acclimation	93.73	2	46.86	0.47	0.62

Table 3.2 Two-way ANOVA studies to identify effects between different flowering stages, type of acclimation and their interaction. Source represents the variables that are studied. Flowering stages represents three different developmental stages, and type of acclimation includes non-acclimated and cold-acclimated plants. In column III, df represents degrees of freedom.

It is important to consider the marked differences in freezing tolerance. I used two statistical measures to show these differences, namely linear regression analysis and directly comparing the means of critical temperature points near LT_{50} . The repeated measurement in these experiments is electrolyte leakage and the LT_{50} is taken as the intercept of the line for 50% EL.

Linear regression is a good measurement for studying two adjacent temperature points and thus validates the LT_{50} temperatures of the different flowering stages. The critical temperature points -6 and -8 °C were compared in the three different flowering stages by linear regression. A line was drawn using the % EL values obtained from the analysis. The graphs (Figure 3.7) show the equation of the line ($y = mx + b$) and the R^2 values. In the equation x and y are the values from X and Y intercept, m is slope of the line and b is the Y intercept value when $x = 0$. R^2 is the coefficient that measures the fit of the regression line to the data. Higher the R^2 value, greater is the linear relationship. The LT_{50} temperatures obtained after the regression analysis were - 7.2 °C, -6.2 °C and - 6.4 °C for the first flowering stages, two days after flowering and four days after flowering stage, respectively.

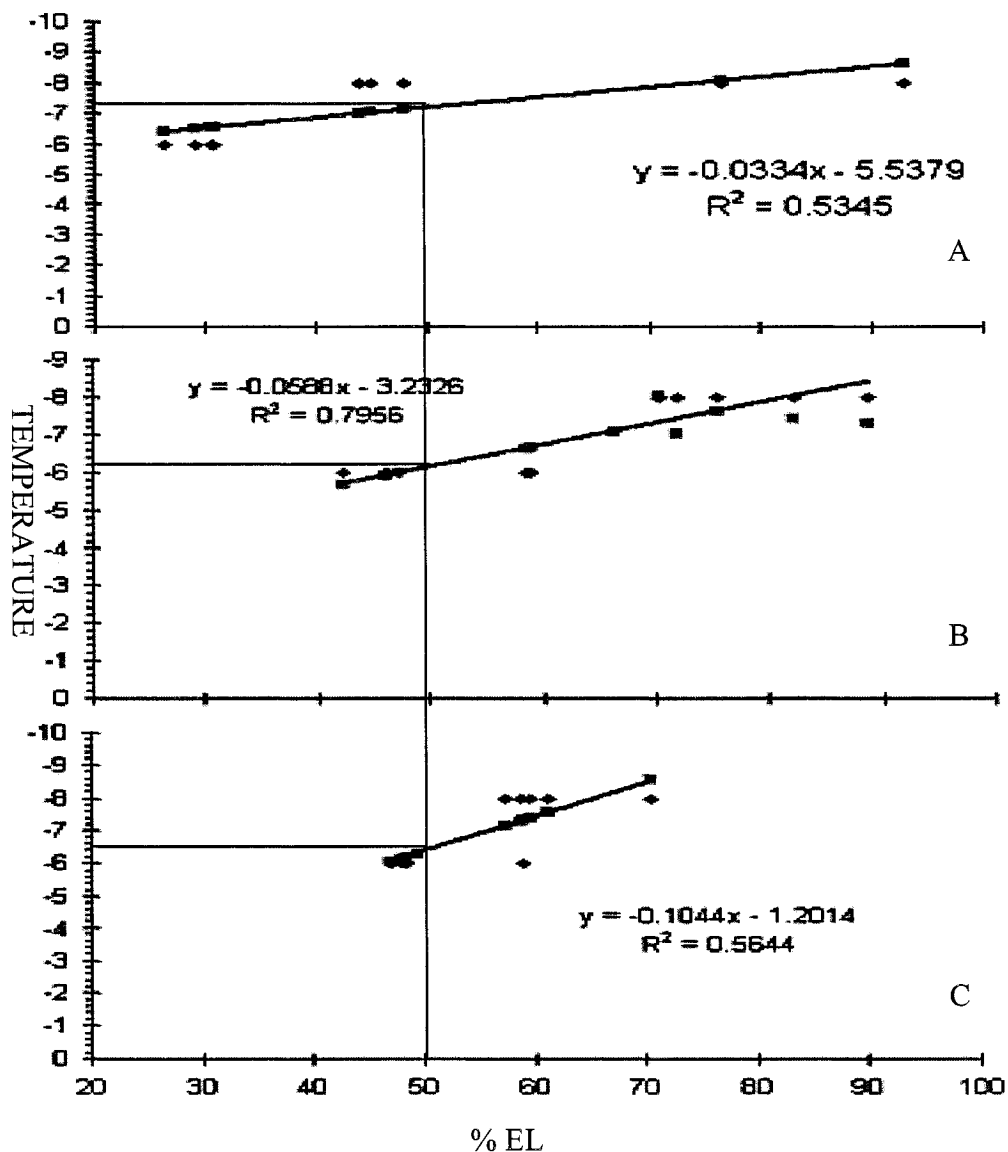


Figure 3.7 Graphs representing regression analysis for the two important temperature points of three different flowering stages with cold acclimation. (A) Represents 1st flowering stage [-7.2], (B) represents two days after flowering [-6.2] and (C) represents four days after flowering plants [-6.4]. Blue diamonds represents calculated % EL and the purple squares represents expected % EL. R^2 is known as coefficient. The equation in each graph is represented as $y = mx + b$. Values in the square brackets indicate LT_{50} values obtained after regression and extrapolated from the graph.

To test the significance for the differences in freezing tolerance of the plants at different developmental stages, I compared electrolyte leakage at the two temperatures closest to the LT_{50} for the plants. This was done using a *t*-test. The % EL means at -6 °C for the 1st flowering stage and 2 days after flowering are very different and this is confirmed by the *t*-test (at $p = 0.05$), the means of the same flowering stages at -8 °C are still different but are not highly significant, $p = 0.08$ (Table 3.4).

	1 st Flowering stage	2 days after flowering	Sig (t-test)
-6 °C	23.43	53.17	0.00025
-8 °C	57.11	75.58	0.08855

Table 3.3 t-test to study significance of the mean electrolyte leakage at 1st flowering stage and 2 days after flowering stage, at two temperatures close to LT_{50} , ($n = 6$).

To confirm the importance of the change in the ability to cold acclimate between flowering and two days after flowering, I also compared electrolyte leakage at the later two flowering stages, 2 days after flowering and 4 days after flowering. The results of *t*-test shown in Table 3.4 indicate the % EL are not significantly different and are validated by a very high *p*-value, *p* = 0.953, for the comparison at -6 °C, the temperature closest to the LT₅₀ for these samples. The means are significantly different at lower temperature, -8 °C though this temperature is significantly below the LT₅₀.

	2 days after flowering	4 days after flowering	Sig (t-test)
-4 °C	23.07	31.7	0.08712
-6 °C	53.17	52.88	0.95389
-8 °C	75.58	59.37	0.00473

Table 3.4 *t*-test to study significance in the mean electrolyte leakage at 2 days after flowering and 4 days after flowering stage, at two temperatures close to LT₅₀, (n = 6).

Effect of freezing tolerance on the plants that are treated with ABA and SNP

In order to investigate the effect of growth regulators on freezing tolerance of *A. thaliana* that are two days old after flowering, we carried out treatments with ABA and SNP, which produces nitric oxide after the treatment. ABA is a plant growth regulator that is known to increase freezing tolerance of plants. Plants that were in the flowering stage were sprayed with ABA and SNP during the cold acclimation period of seven days and were tested for freezing tolerance by electrolytic leakage test. The LT₅₀ of the plants treated with 2 µM and 20 µM ABA increased to – 6.7 and –7.0 °C, respectively (Figure 3.8) as compared to non-treated plants that has an LT₅₀ -5.5 °C. For SNP the freezing tolerance was found to be concentration dependent. A treatment with 50 µM SNP increased the LT₅₀ of the plants to – 6.8 °C while 100 µM SNP concentration had no effect on freezing tolerance. The LT₅₀ of the plants treated with 100 µM SNP was –5.1 °C, which was found to be similar to the untreated plants (–5.5 °C) (Figure 3.9). The concentrations used for SNP treatment were same as those used by He *et al* (2004).

The statistical analysis of the freezing tolerance data obtained by ABA and SNP treatments used a similar general linear model with repeated measure. The p-values for the ABA treatment are statistically significant (Table 3.6). ANOVA analysis shows that SNP treatments are similarly significant (Table 3.7).

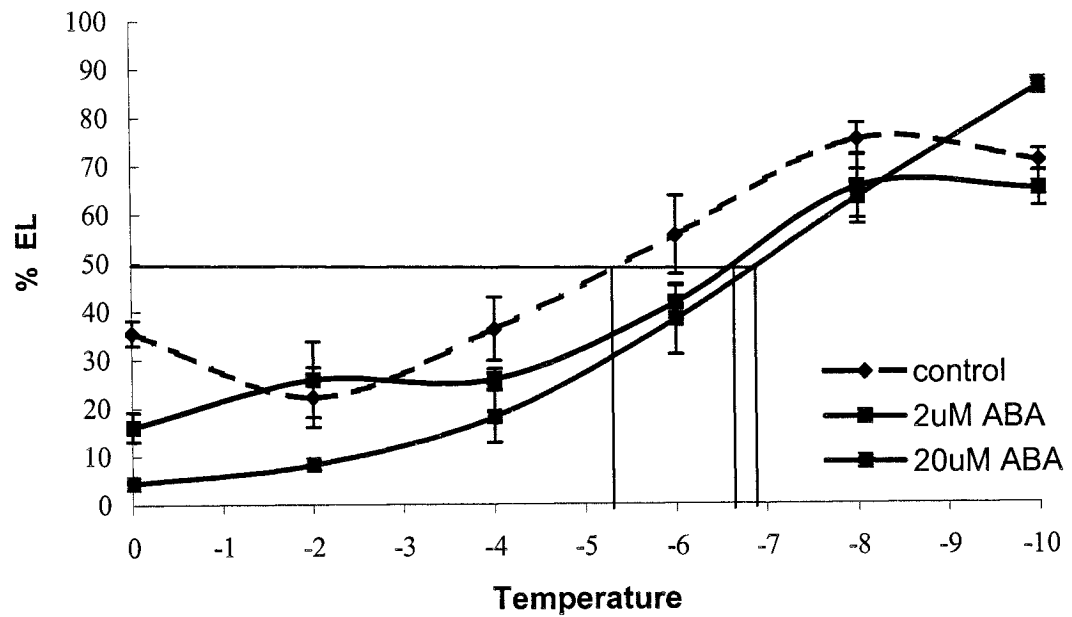


Figure 3.8 Freezing tolerance changes in *A. thaliana* plants that were two days old after flowering and were sprayed with ABA and cold acclimated for 7 days before performing electrolytic leakage test. Control plants were sprayed with water. Bars represent standard deviation, (n = 6).

Source	Type III Sum of square	df	Mean square	F-stat	p-value
ABA	3173.61	2	1586.80	8.63	0.00

Table 3.6 ANOVA studies to identify the significance of ABA treatment on *A. thaliana* that were two days old after flowering and were cold acclimated. Source represents the variable that was studied. In column III, df represents degrees of freedom.

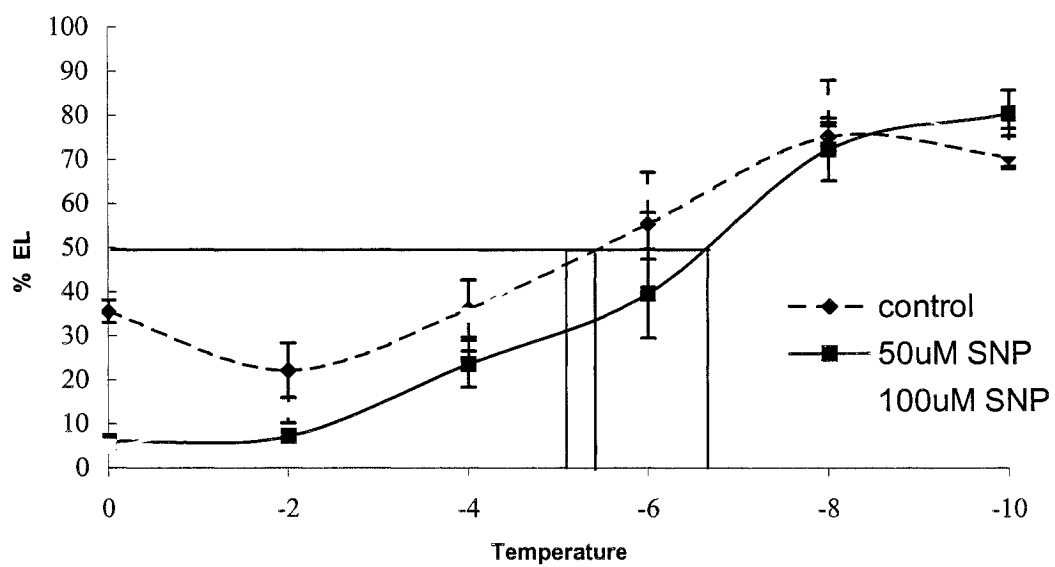


Figure 3.9 Freezing tolerance changes in *A. thaliana* plants that were two days old after flowering and were sprayed with SNP and cold acclimated for 7 days before performing electrolytic leakage test. Control plants were sprayed with water. Bars represent standard deviation, (n = 6).

Source	Type III Sum of square	df	Mean square	F-stat	p-value
SNP	2208.99	2	1104.49	5.58	0.006

Table 3.7 ANOVA studies to identify the significance of SNP treatment on *A. thaliana* that were two days old after flowering and were cold acclimated. Source represents the variable that was studied. In column III, df represents degrees of freedom.

Discussion

A. thaliana, showed an increase in low temperature tolerance to around -7.0°C during the vegetative phase after seven days of cold acclimation at 4°C . Flowering *A. thaliana* loses its ability to cold acclimate at 4°C : a decrease of around 2°C is observed in low temperature tolerance. This research was carried in order to investigate a drop in low temperature tolerance of the plant in the transition from vegetative to reproductive phase. Several studies have been carried on wheat, which demonstrate a sudden plunge in low temperature tolerance with a change from vegetative to reproductive phase of the plant (Mahfoozi *et al* 2001). We found that the interaction between different developmental stages x type of acclimation is significant ($p = 0.05$).

When studying the precise stage of plant development at which there is the loss of the ability to acquire low temperature tolerance we found that two days after opening of the first flower bud is critical. Wheat changes its freezing tolerance when it is in the heading stage, which is before pollination. The statistical comparison of freezing tolerance between 2 days and 4 days after the opening of the first flower showed a significant effect of different flowering stages and also a significant effect of acclimation was observed on the flowering stages, $p = 0.01$ (Table 3.2). However, it did not show a significant interaction with the flowering stage and type of acclimation ($p = 0.62$). This is likely because the cold acclimation response of *A. thaliana* at 2 days after flowering and 4 days after flowering stage was reduced and was similar. Two out of the three flowering stages tested showed decreased low temperature acclimation and freezing tolerance. At the 1st flowering stage the difference between the non-acclimated and cold-acclimated plants is 3°C and the difference in 2 days after flowering and 4 days after

flowering is of 2 °C. This loss of freezing tolerance with cold acclimation in the last two flowering stages could be the reason for non-significant interaction between flowering stage and type of cold acclimation. At the 1st flowering stage the plants had no growth of siliques and had many unopened secondary flower buds. Also when the plants were cold acclimated we observed accumulation of reddish pigment in the flower buds, which is most likely anthocyanin (Figure 3.6), which is a primary pigment to be produced during abiotic stress. However, to confirm the accumulation of anthocyanin we would need to carry out quantitative measurement by HPLC. Statistical analysis was done on the three different flowering stages with the EL values of the temperatures that are close to LT₅₀. The linear regression analysis (R^2) validated the LT₅₀ values for the three flowering stages. The measure of LT₅₀ was not repeated, so statistical analysis of this number was not possible, therefore we used repeated measurement of % EL values near the LT₅₀ to compare the three flowering stages at the two critical temperature points close to LT₅₀ with Student's *t*-test. The significant difference of EL at -6 °C between the 1st flowering stage and 2 days after flowering is due to the differences in the LT₅₀ values at these stages (Table 3.4). While the means are not significant at 2 days after flowering and 4 days after flowering at -4 and -6 °C (Table 3.5) since the freezing tolerance of *A. thaliana* at these two stages is similar.

There are no experiments found with respect to freezing tolerance studies that have demonstrated use of chemicals like ABA and SNP to revert the drop in freezing tolerance of the flowering plants. In our studies, we used ABA and SNP to treat the flowering plants by spraying the chemical evenly on all the plants for one week when the plants were at 4 °C. ABA is a phytohormone that is known to accumulate during cold

acclimation and the exogenous application of ABA was reported to increase the freezing tolerance of *Solanum commersonii* (Chen *et al*, 1983). This led to hypothesis that ABA plays a role in the cold acclimation process of the plants. We studied the effect of ABA treatment when combined with cold acclimation and showed that ABA could reverse the loss of the ability of the plant to cold acclimate at 2 days after flowering.

Similar analysis was done with *A. thaliana* treated with SNP, and showed that SNP increases freezing tolerance of the flowering plants. Sodium Nitropruside is a chemical that releases NO. NO treated *A. thaliana* seedlings showed delayed in flowering and increase in vegetative growth of the plant (He *et al*, 2004). In addition NO has also been reported to be induced by plants in response to biotic and abiotic stress. We showed that the increase in freezing tolerance of the plant that was concentration dependent, at 50 μ M treatment the freezing tolerance increased but was not affected at 100 μ M (Figure 3.9).

Future Work

While investigating the freezing tolerance of the ABA and SNP treated plants during seven days of cold acclimation, an experiment performed with non-acclimated plants to know if the effects of SNP and ABA are cold acclimation dependent. It would be important to identify the genes that are regulated during the drop in freezing tolerance at the flowering stage of the plant. This could help in the characterization of cold acclimation and the characterization of the signaling pathways that are associated with the interaction of cold acclimation and developmental changes.

Chapter 4

PHYLOGENETIC ANALYSIS OF *TaST2*, *TaST3* AND *TaST4* WITH *AtSTs*

Comparison *TaST* 2, 3 and 4 protein sequences with flavonol 3-ST

The protein sequence deduced from *TaST2*, *TaST3* and *TaST4* cDNAs were initially aligned with the flavonol 3-ST to identify the conserved domains present in cytosolic STs. Two highly conserved domains were recognized in the three ST proteins, which are required for the binding of PAPS which is the sulfonate donor. It includes the PKSGTTWL motif located near the N-terminus region of the protein and RKGXXGDWKN close to the carboxy terminus region (Figure 4.1), the length of *TaST2*, *TaST3* and *TaST4* protein sequences are 350, 352 and 334 amino acids, respectively.

Comparison of *TaST* 2, 3 and 4 with *Arabidopsis thaliana* sulfotransferases

Since *A. thaliana* is a model plant organism and its complete genome is known, we compared its seventeen full-length sulfotransferases with the three wheat sulfotransferases (Figure 4.2, Table 4.1). Out of 18 ST genes of *A. thaliana*, one gene is represented with partial cDNA sequence (At3g51210). Its amino acid sequence represents a carboxy terminal region of plant sulfotransferases with a missing amino-terminal region. Using the Clustal W program, a phylogenetic tree was created with the seventeen protein sequences of *A. thaliana* and the three *T. aestivum* sulfotransferase (Figure 4.3).

TaST2 was found to have a maximum of 40% identity and 57% protein similarity with At2g03770 from *A. thaliana*, while TaST3 was closest to At3g45070 with 41% identity and 56% protein similarity and TaST4 had 36% identity

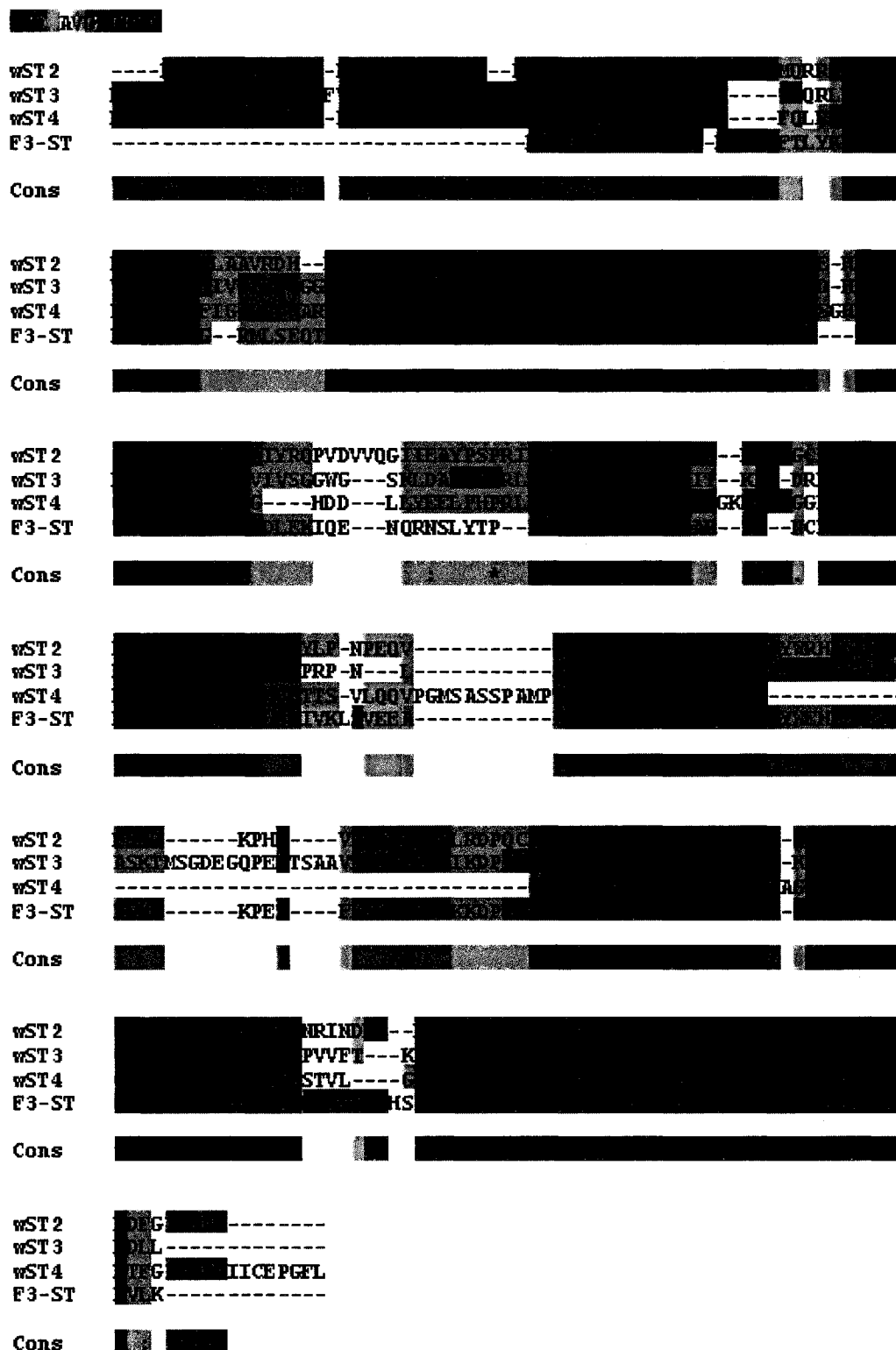


Figure 4.1 Alignment of three wheat sulfotransferase with flavonol 3-ST using an amino acid sequence alignment program (BLOSUM62, 1993). The degree of similarity is based upon the color scale mentioned on top of the image.

Table 4.1 Accession numbers for *Arabidopsis thaliana* sulfotransferases, their amino acids sequence length and their known substrates.

Sr. No.	TIAR number	Arabidopsis ST Designation	Amino acid length	Referred to as	Substrates Specificity
1	At5g43690	MQD19.2	331	AtST7	
2	At4g26280	T25K17.90	314	AtST9	
3	At2g27570	F10A12.24	273	AtST3c	
4	At3g45070	T14D3.10	323	AtST3a	Flavonol (Gidda SK 2001)
5	At3g45080	T14D3.20	329	AtST3b	
6	At1g28170	F3H9.17	326	AtST10	
7	At1g13420	T6J4.16	331	AtST4b	
8	At1g13430	T6J4.24	351	AtST4c	
9	At2g14920	T26I20.8	333	AtST4a	Epibrassinolide (Boyd, 2001)
10	At2g03750	F19B11.20	351	AtST6	Alcohols (Boyd, 2001)
11	At2g03760	F19B11.21	326	AtST1	Brassinosteroids (Marsolais, 2000)
12	At2g03770	F19B11.22	324	AtST8	
13	At5g07000	MOJ9.17	347	AtST2b	
14	At5g07010	MOJ9.18	359	AtST2a	12-hydroxy jasmonate (Gidda, 2001)
15	At1g74100	F2P9.3	338	AtST5a	Desulfo glucosinolates (Piotrowski, 2004)
16	At1g18590	F25I16.7	346	AtST5c	Desulfo glucosinolates (Piotrowski, 2004)
17	At1g74090	F2P9.4	350	AtST5b	Desulfo glucosinolates (Piotrowski, 2004)
18	At3g51210	F24M12.250	-	-	-

At1g18590 -----MESKTINDVVVSES-----NHELA---SSSPSEFEKNQ-----KHYQEIIATLP
 At1g74090 -----MESETLTAKATITTTTLP SHDET--KTESTEFEKNQ-----KRYQDLISTFP
 At1g74100 -----MESKTT-----QNGSE--VVELTEFEKTQ-----KKYQDFIATLP
 At5g07000 -----MAIPSFMSMCHKPELLKEGKS---EGQEEGLS-----YEFQEMLDSLP
 At5g07010 MATSSMSKSI PMAIPSFMSMCHKLELLKEGKTRDVPKAEDEGLS-----CEFQEMLDSLP
 At2g03760 -----MSSSSS--VPAYLGDEDLT-----QETRALISSLP
 At2g03770 -----MTKSETT--LSKLLVESELV-----QECELLSSLP
 At1g13430 -----MDEKD--ILRNLREEEEEEEEENQSEETKILISSLP
 At2g14920 -----MDEKD--RPKNLRREEEEK---PSEETKILISSLP
 At1g13420 -----MGEKD--IPRNLREEEEEEEEENQSEETKSLISSLP
 At1g28170 -----MDETK--IPKKLQSDDEE-----NISLISSLP
 At3g45070 -----MEMNLRIEDLN-----EETKTLISSLP
 At3g45080 -----MDEKK--ITMNVNRNDELS-----EESKTLISSLP
 At2g27570 -----MDDKK--MAVNLRNDDLS-----EETKTLISSLP
 At4g26280 -----ME--KWMNLRDEDLT-----EETKTLISSLS
 At5g43690 -----MAGKSD--LPVIARDDNLS-----EETKTLISSLP
 At2g03750 -MFTFFTILSLCFKSWEQIIITMEASKEAHH--LPNYMKDDNVS-----QETKNLITSLP
 TaST3 -----MSLACALAGGPAAVKDVFDVSRHDPPLPLP---KKDHSGGDGDGD
 TaST4 -----MGSNDALTAGPVTEKEKEGAS--APAATTTP---HANIP EIMSSLP
 TaST2 -----MARVAEQKLQPSCLGDALTARNADNLTTEL---IPSLPVEKRLVP

↓

At1g18590 HKDGWRPKDPFVEYGGHWWLQPLLEGLLHAQKF--FKARPNIFFVCSYPKGTGTWIKALT
 At1g74090 HEKGWRPKPELIEYGGYWLPSPSLEGCIIHAQEF--FQARPSDFLVCSYPKGTGTWIKALT
 At1g74100 KSKGWRPDEILTQYGGHWWQECLEGLFHAKDH--FEARPTDFLVCSYPKGTGTWIKALT
 At5g07000 KERGRRN-RYLYLFQGFRCQAKEIQAITSFQKH--FQSLPDIVVLATIPKSGTTWIKALT
 At5g07010 KERGWRT-RYLYLFQGFRCQAKEIQAIMSFQKH--FQSLPDIVVLATIPKSGTTWIKALT
 At2g03760 KEKGWLV-SEIYEFQGLWHTQAILQGILICQKR--FEAKDSIIILVTNPKSGTTWIKALV
 At2g03770 RDRSVFA-EYLYQYQGFWYPPNLEGLVLYSQKH--FQARDSDIVLASIPKSGTTWIKSLV
 At1g13430 WEIDYLG-NKLFKYQGYWYYEDVLQISPNIHSS--FQPQETDIVVASFYKSGTTWIKALT
 At2g14920 WEIDYLG-NKLFNYEGYWYSEDILQISPNIHSG--FQPQETDIILASFYKSGTTWIKALT
 At1g13420 SDIDCSG-TKLYKYQGCWYDKDILQAILNFNKN--FQPQETDIIVASF PKSGTTWIKALT
 At1g28170 FDVDFDS-TKLFKYQGCWYDDKTLQGVLFNFORG--FEPQDTIIIIASF PKSGTTWIKALT
 At3g45070 SDKDFTG-KTICKYQGCWYTHNVLQAVLNFKQS--FKPQDTIIIVASF PKCGTTWIKALT
 At3g45080 SDKNSTG-VNVCKYQGCWYTPPILQGVLFNFKN--FKPQDTIIIVASF PKCGTTWIKALT
 At2g27570 SDKDSTG-INVCKYQGCWYTHHFLQAVLNFKN--FKPQDTIIIVASF PKCGTTWIKALT
 At4g26280 SEKGYLGRNLCKYQGSWYYNLFQGVLFNFORG--FKPQDTIIIVASF PKSGTLWLKALT
 At5g43690 TYQDSH--VKLCKYQGCWYYHNTLQAVINYQRN--FQPQDTIIILASF PKSGTTWIKALS
 At2g03750 SDKDFMG-YGLYNYKGCWYYPNTLQAVLDVQKH--FKPRDTIIILASLPKGGTTWIKSLI
 TaST3 ALTSNPM-TGQRLYQGTWVMEGWIPGIVQIQNGGGFTPRRGDIVLASFPKCGTTWIKALA
 TaST4 TAAGFPP-FQLRRYGGFWLTDRYLEFAGAFHAREKPRPTDVLASF PKSGTTWIKALG
 TaST2 PPAD----MQRRQYRGYWFPEWHL SALAAVRDH--FEKPTDIFLVSCP KSGTTWIKSLA

At1g18590 FAIANRSKFDVSTN-----ELLKRNPHFVPIEIDFPFFPSVDVLKDEGN-----T
 At1g74090 FAIANRSRFDSSN-----ELLKRNPHFVPIEIDFPFFPEVDVLKDKGN-----T
 At1g74100 YAIVNRSRYDDAAN-----ELLKRNPHFVPIEIDFAFYPTVDVLQDRKN-----P
 At5g07000 FTILTRHRFDPVSSSSDHELLTSNPHDLVPFFEYKLYANGNVPDLSGLAS-----PR
 At5g07010 FTILNRHRFDPVASTN--HELFTSNPHDLVPFFEYKLYANGDVPDLSGLAS-----PR
 At2g03760 FALLNRHKF-PVSSSGN--HELLVTNPHLLVPFLEG-VYYESPDFDFSSL-----PSPR
 At2g03770 FALIHRQEF-QTPLVS--HELLDNPHLTLVTFIEG-FHLHTQDT-----SPR
 At1g13430 FALVQRSKH-SLEDH--HELLSHNPHEIVPYLELDLYLNSSKPDLT KFLSSSSSSSPR
 At2g14920 FALVQRSKH-SLEDH--QHELLHNPHEIVPNLELDLYLKSSKPDLT KFLSSSSSS--SPR
 At1g13420 FALAQRSKH-TSDN---HELLTHNPHELVPYLELDLYLKSSKPDLT KLPSS-----SPR
 At1g28170 VALLERSKQ-KHSSD--DELLLDNPHGLVPFLELRFLTETSKPDLT SISS-----PR
 At3g45070 FALLHRSKQ-PSHDD--DELLSNNPHVLVPYFEIDLYLRSENPDLT KTF-SSS-----PR
 At3g45080 FALVRSKH-PSHDD--HELLSDNPHVLSPLEMYLYLCSENPDLT KTF-SSS-----SR
 At2g27570 FSLVHRSKH-PSHDH--HELLSNNPHVL-----F-SSS-----PR
 At4g26280 VALFERTKN-PSHDDPMSHELLSNNPHNLLS-----SSS-----PR
 At5g43690 VAIVERSKQ-PFDDDLPTHHELLSDNPHGIVPFFEFDMYLKTSTPDLT KFTSS-----PR
 At2g03750 FAVVHREKY-RGTPQ--THELLQNPHDLVPFLEVELYANSQIPDLAKYSS-----PM
 TaST3 FATMARGAHP-AAPD--HELLRHTPHDCVPFMEVIVSG---GWGSKLDALPS-----PR
 TaST4 FCALNRVAHPPSAGSG--HELLHCGSPHDLVRFVEGHDD-----LLYEELPH-----PR
 TaST2 FATVHRHVHPP-SSQE--HELLHQNPHGCVKFIHAIYRQPVDDVQGIIEAYPS-----PR

Figure 4.2 (page 1 of 3)


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At1g18590 PAVYANSAYHRKGVGDWNYLTPEMVARIDGLMEEKFKG-TGFLSSKS-----
At1g74090 PGVYANSAYHRKGVGDWNYLTPEMAARIDGLMEEKFKG-TGLLEHGK-----
At1g74100 PAVYANSAYHRKGVGDWANYLTPEMAARIDGLVEEKFKD-TGLLQHDN-----
At5g07000 --NYENRFLERKGEVSDLVNYLSPSQVERISALVDDKLAG-SGLTFRLS-----
At5g07010 --NFENRFLERKGEVSDWVNYLSPSQVERISALVDDKLG-SGLTFRLS-----
At2g03760 --GIETKTFERKGEIGGWRDTLSESLAEEDRTIEEKFKG-SGLKFSS-----
At2g03770 --GMETKAFFERKGEIGGWRDTLPLLAEEIDKTTKEKLIG-SDFRFFC-----
At1g13430 ---VDYKTYHRKGVGDWKSMTSEMVNKIDMIVEEKLKG-SGLKF-----
At2g14920 ---VDFKSYHRKGVGDWKSMTPEMVDKIDMIEEKLKG-SGLKF-----
At1g13420 --GVSFKSFERKGEVGDWKSMTPEMENKIDMIVEEKLQG-SGLKL-----
At1g28170 --GADHKIFERKGEVGDSKNHLTPEMEKIIDMITEEFEG-SDLKF-----
At3g45070 --GRENKMFERKGEVGDWKNYLTPEMENKIDMIIQEKLN-SGLKF-----
At3g45080 --GRENKTFERKGEVGDWKNYLTPEMENKIDMIIQEKLN-SGLKF-----
At2g27570 --GRDNKTFERKGEVGDWKNYLTPEMENKIDMIIQEKLN-SGLKF-----
At4g26280 --GIEYKNHERKGIVGDWKNHLTPEMGSKIDMIMKEKLKDYSEVWFENAL-----
At5g43690 --NYDYKSYERKGEVGDWKNHLTPEMENKIDMIEEKLKG-SDLKF-----
At2g03750 --GVDSQVFERKGEVGDWKNHLTPQMAKTHDEIIDYRLGD-SGLIFQ-----
TaST3    ---YPRESYHRRGGVGDWMNHMTPEMAHRFDALLQSKFHG-SGLDLL-----
TaST4    ---IKNEAFERSGTVGDWSSCMTPEMAAQILDGIVAEALRG-STLTFGPLAPPIICEPGFL
TaST2    P--MMNDSFERKGVAGDWINHMTPEMAARIDAIVEQALQG-TGFDFGISMPQ-----

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Figure 4.2 Amino acid sequence alignment of three wheat sulfotransferases with the seventeen *Arabidopsis thaliana* sulfotransferases. The boxes indicate the conserved domains found in soluble sulfotransferases of eukaryotes. Arrows indicate critical amino acid residues for PAPS binding and catalysis. The alignment was performed using the ClustalW1.8 program.

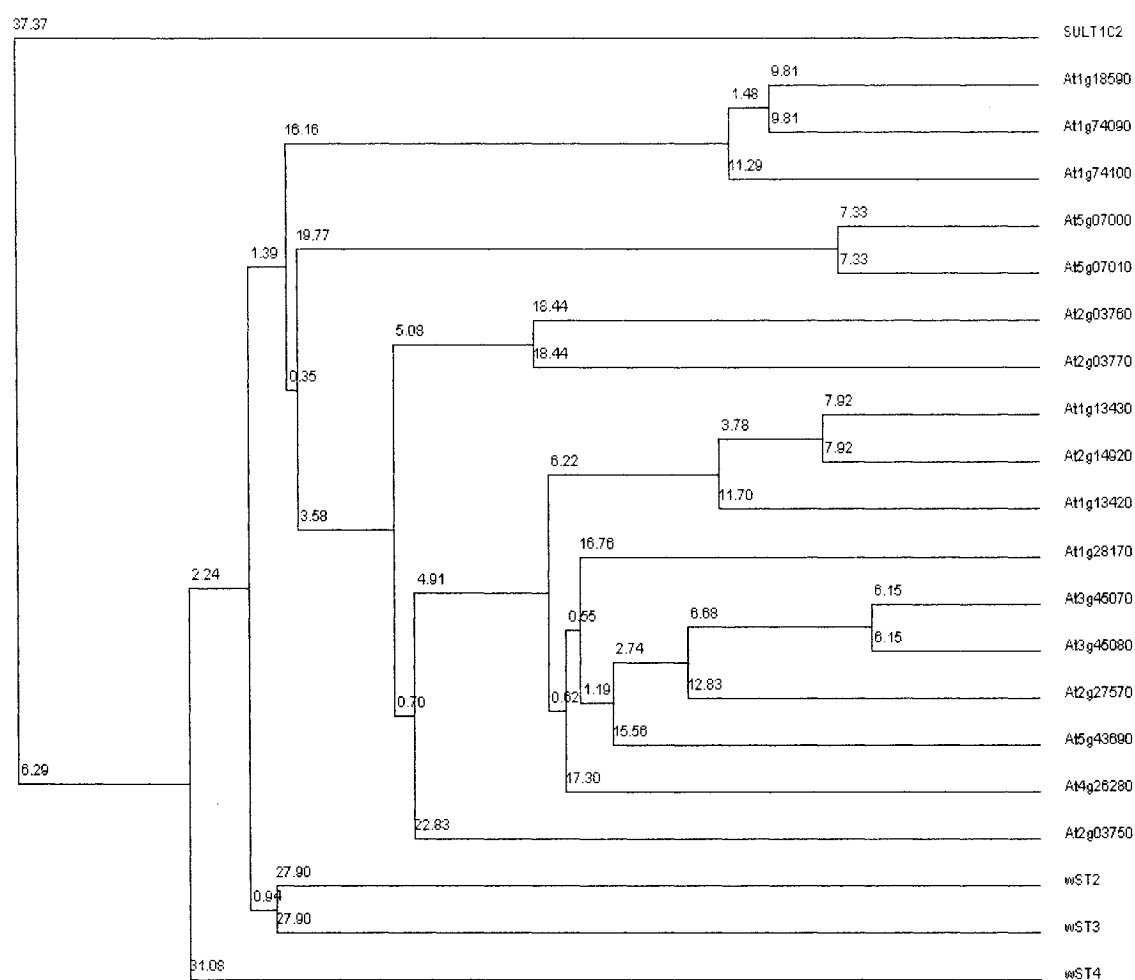


Figure 4.3 Phylogenetic tree of *Arabidopsis thaliana* STs, wST2, 3 and represents TaST2, TaST3 and TaST4, respectively. Human ST SULT1C2 was used as an out-group. Numbers indicate the distances generated using the ClustalW1.8 program during amino acid sequence alignment.

and 51% similarity with At3g45070. In the parsimony analysis of the three *T. aestivum* STs with the seventeen *A. thaliana* STs, the *T. aestivum* sulfotransferase TaST2 and TaST3 formed a clade distinct from *A. thaliana* ST. The protein identity between TaST2 and TaST3 is 43%, while TaST4 is 37% and 38% identical to TaST3 and TaST4. Out of 18 ST genes of *A. thaliana*, one gene is represented with partial cDNA sequence (At3g51210). Its amino acid sequence represents a carboxy terminal region of plant sulfotransferases with a missing amino-terminal region.

Chapter 5

MOLECULAR AND BIOCHEMICAL INVESTIGATION OF *TaST2*, *TaST3* AND *TaST4*

***TaST2*, *TaST3* and *TaST4* induction and regulation studies**

In order to study cold regulation in wheat a microarray experiment was performed and the genes that were induced by cold treatment were selected for further studies. The gene bank accession numbers of the cDNA clones used for *TaST2*, *TaST3* and *TaST4* were CV773316, CK213074 and CK214938, respectively.

The expression of *TaST2*, *TaST3* and *TaST4* was investigated by RT-PCR and was found to be induced at least two fold (Figures 5.1 and 5.2). The time course study was done on winter wheat cultivar Clair and spring wheat cultivar Quantam. In winter wheat *TaST2* was induced by 6 h of cold treatment while its induction was not detected in spring wheat cultivar Quantam by cold treatment until 36 days. In the winter wheat cultivar Clair, *TaST3* was strongly induced by 1 day of cold acclimation followed by a slight decline in mRNA levels, though levels remained higher than that in non-cold acclimated controls until day 36. In the spring wheat cultivar, *TaST3* is induced only at 6 hours while at later time points transcripts were not detected. A different induction pattern was observed with *TaST4* that was only induced after 36 days of cold treatment in spring wheat and no significant expression was seen in the winter wheat until 36 days of cold treatment (Figures 5.1 and 5.2). The experiments were repeated at least two times to confirm the results.

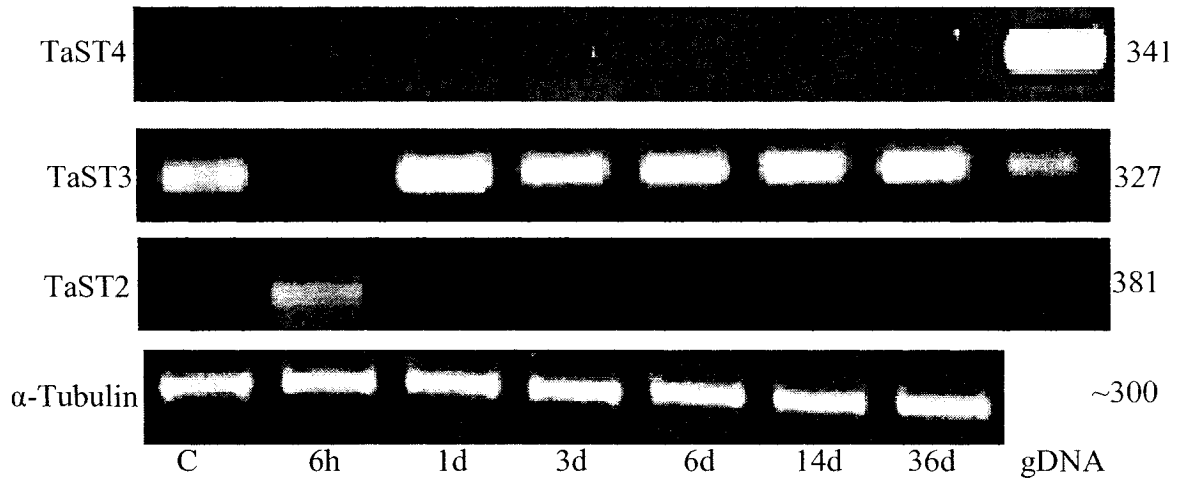


Figure 5.1 Cold induction studies of *TaST2*, *TaST3* and *TaST4* in winter wheat cultivar Clair by RT-PCR. Time points included in the study are 6 hours, 1, 3, 6, 14 and 36 days of cold acclimation. C is for non-cold treated control plants. gDNA is genomic DNA. Numbers on the right indicate length of the amplified RT-PCR fragment. A total of 2.5 µg RNA was reverse transcribed and amplified using *TaST2*, *TaST3* and *TaST4* forward and reverse primers. The RT products were also amplified with α-Tubulin specific primers to confirm that equal amounts of RT product were used in each amplification. All the amplified products were separated by electrophoresis in ethidium bromide-stained agarose gels.

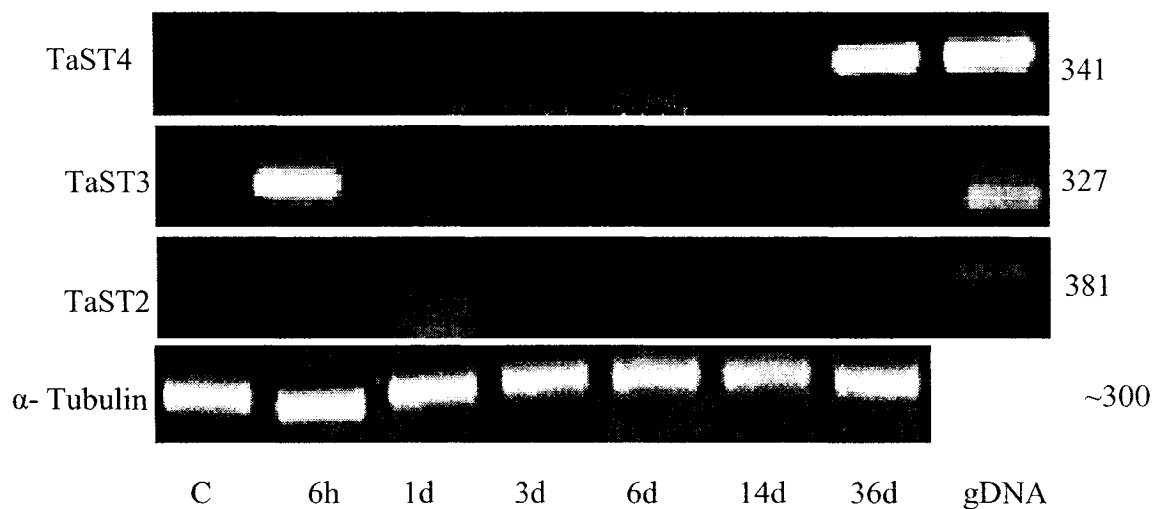


Figure 5.2 Cold induction studies of *TaST2*, *TaST3* and *TaST4* in spring wheat cultivar Quantam by RT-PCR. Time points included in the study are 6 hours, 1, 3, 6, 14 and 36 days of cold acclimation. C is from control samples of non-cold treated plants. gDNA is amplification from genomic DNA. Numbers on the right indicate lengths of the amplified DNA fragment. A total of 2.5 μ g RNA was reverse transcribed and amplified using *TaST2*, *TaST3* and *TaST4* forward and reverse primers. The RT products were also amplified with α -Tubulin specific primers to confirm that equal amounts of RT product were used in each amplification. All the amplified products were separated by electrophoresis in ethidium bromide-stained agarose gels.

Cloning, protein purification and substrate identification of TaST2, TaST3 and TaST4

Five *TaST2* clones were selected for restriction enzyme analysis and confirmed after colony PCR screening. Two out of five were confirmed positive *TaST2* clones. Similar procedures were performed for *TaST3* and *TaST4*. Two out of five and three out of five clones were confirmed for *TaST3* and *TaST4*, respectively. Sequencing of the selected clones was done to confirm the junctions of the vector pQE30 and the *TaST2*, *TaST3* and *TaST4* cDNAs. The purified proteins were separated by electrophoresis using polyacrylamide gel to check their relative purity. Also, western blot analysis was performed to confirm the induction and presence of sulfotransferases by using a pool of different primary antibodies (Figures 5.3 to 5.8). Out of the several different types of compounds tested as potential substrates (Table 2.1 in materials and methods), none were accepted by the enzymes TaST2, TaST3 and TaST4.

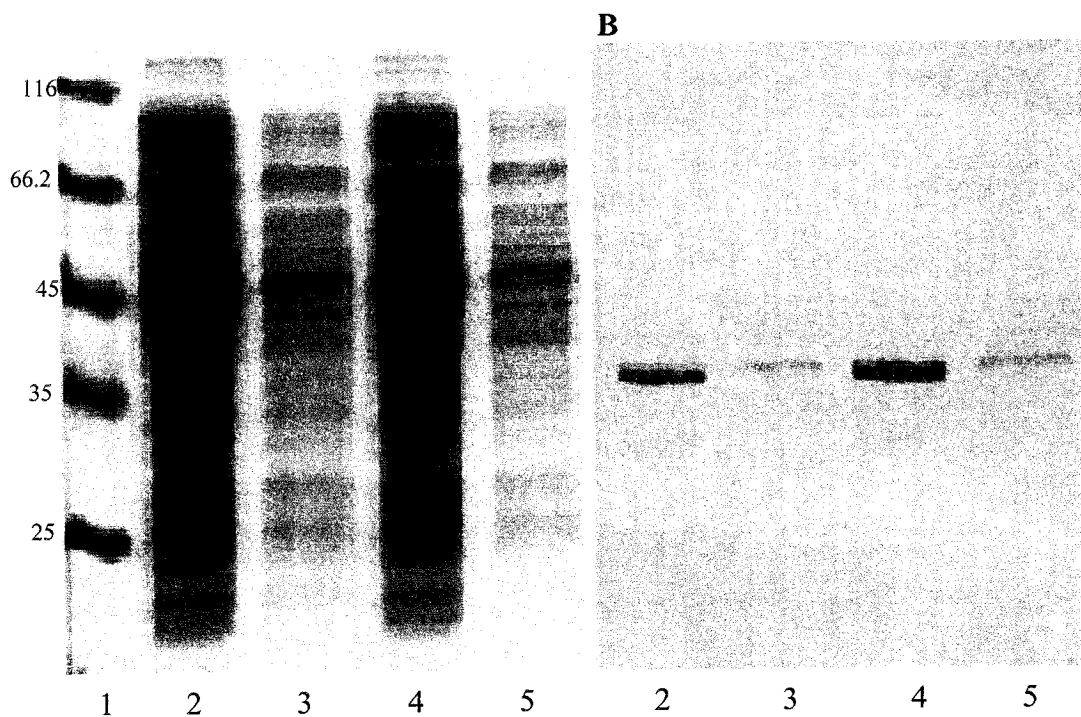


Figure 5.3 Recombinant TaST2 induction studies performed to demonstrate level of recombinant protein induction. Panel A shows SDS-PAGE stained with commassie blue and panel B shows western blotting of an identical SDS-PAGE gel. Figure shows total protein from an uninduced (2), total protein from induced (4), soluble protein from uninduced (3) and soluble protein from induced (5) cultures of *E.coli* harboring *TaST2*. Lane 1 in panel A is protein molecular weight markers in kDa.

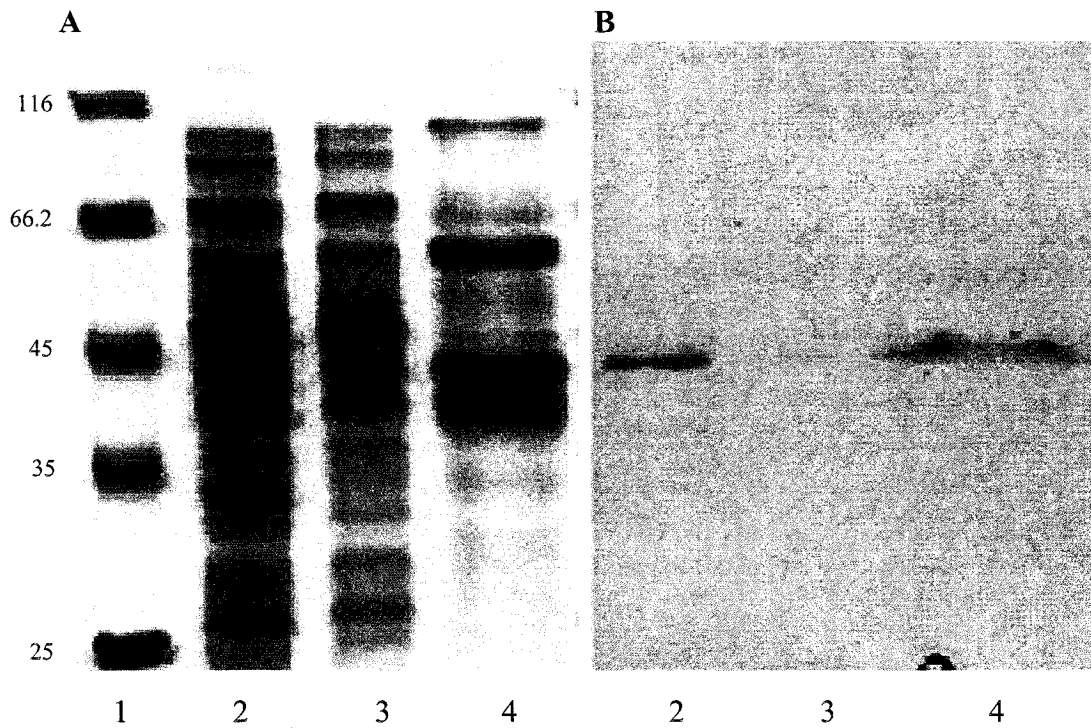


Figure 5.4 Stages of protein purification of recombinant TaST2. Panel A shows SDS-PAGE stained with commassie blue and panel B shows western blotting of an identical SDS-PAGE gel. Lane 2 contains total protein from *E.coli* cells. Lanes 3 shows soluble fraction of protein during purification and lanes 4 show purified protein. Lane 1 in panel A is protein molecular weight markers in kDa.

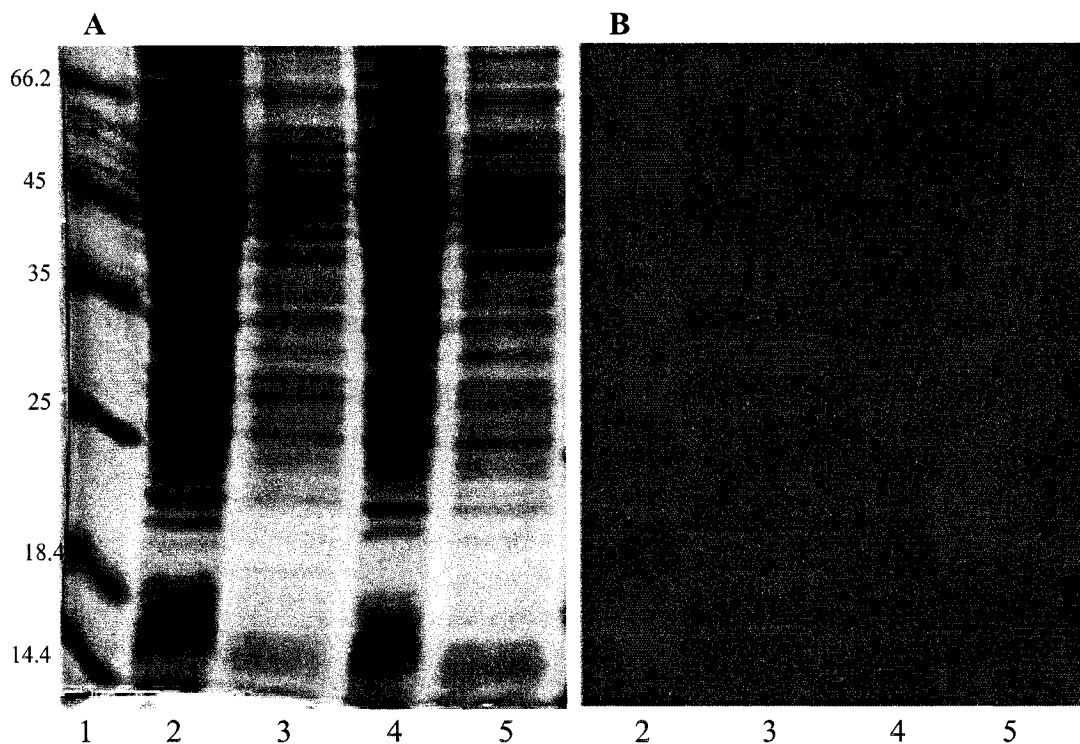


Figure 5.5 Recombinant TaST3 induction studies performed to demonstrate level of recombinant protein induction. Panel A shows SDS-PAGE stained with commassie blue and panel B shows western blotting of an identical SDS-PAGE gel. Figure shows total protein from an uninduced (2), total protein from induced (4), soluble protein from uninduced (3) and soluble protein from induced (5) cultures of *E.coli* harboring *TaST3*. Lane 1 in panel A is protein molecular weight markers in kDa.

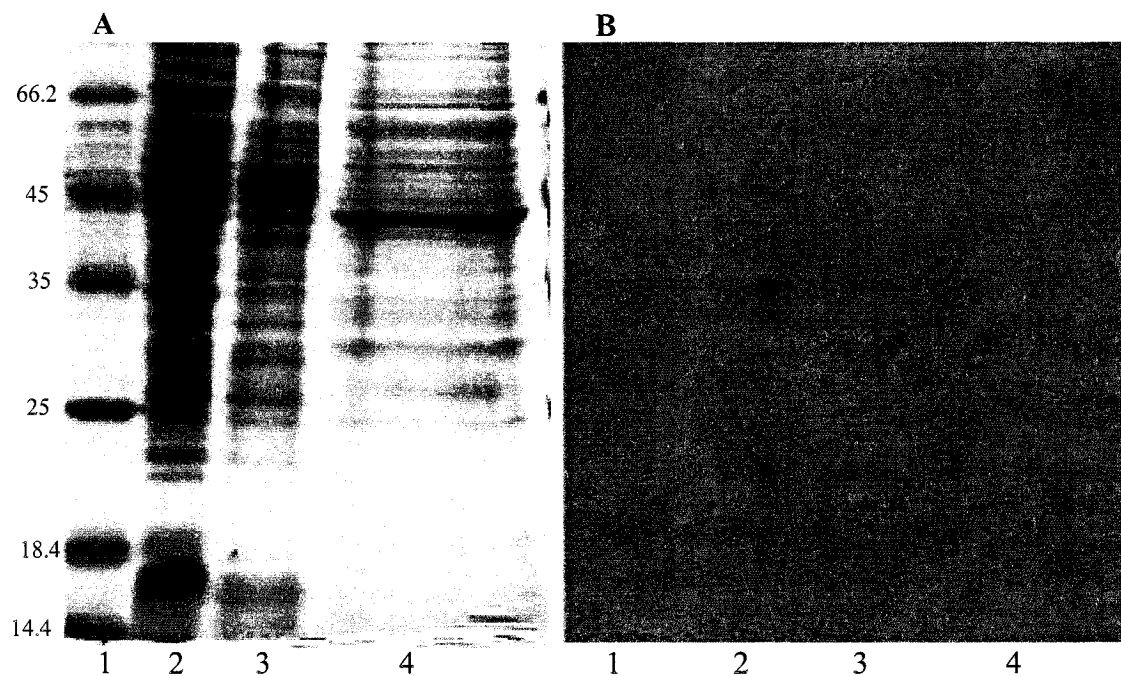


Figure 5.6 Stages of protein purification of TaST3. Panel A shows SDS-PAGE stained with commassie blue and panel B shows western blotting of an identical SDS-PAGE gel. Lane 2 contains total protein from *E.coli* cells. Lanes 3 shows soluble fraction of protein during purification and lanes 4 show purified protein. Lane 1 in panel A is protein molecular weight markers in kDa.

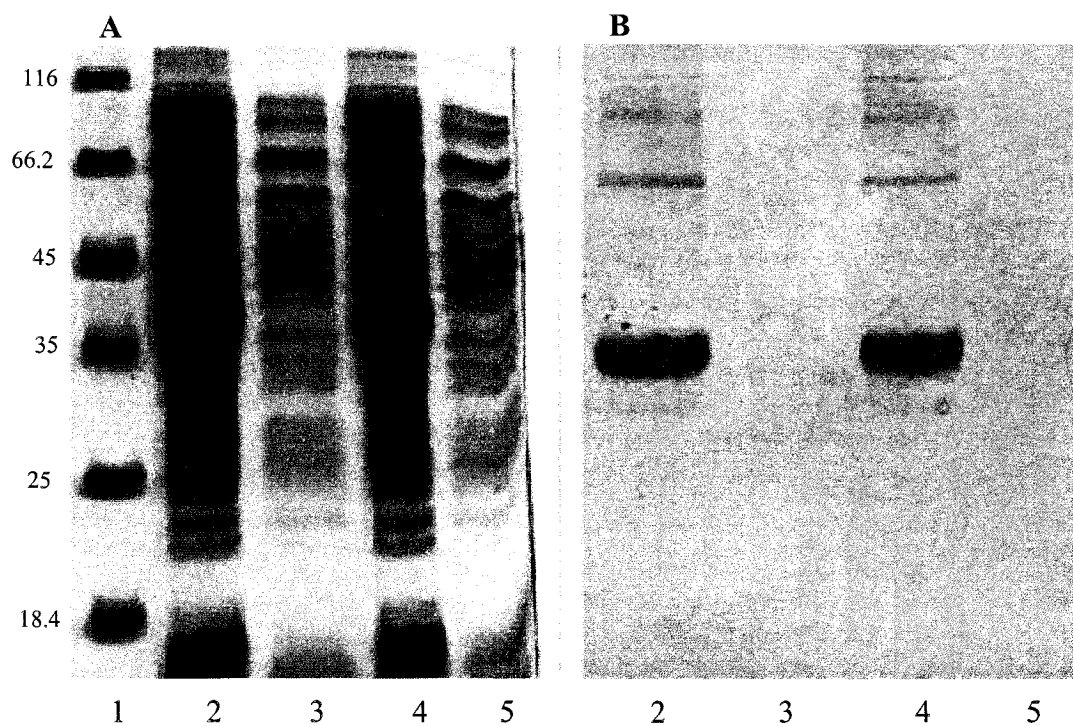


Figure 5.7 Recombinant TaST4 induction studies performed to demonstrate level of recombinant protein induction. Panel A shows SDS-PAGE stained with commassie blue and panel B shows western blotting of an identical SDS-PAGE gel. Figure shows total protein from an uninduced (2), total protein from induced (4), soluble protein from uninduced (3) and soluble protein from induced (5) cultures of *E.coli* harboring *TaST4*. Lane 1 in panel A is protein molecular weight markers in kDa.

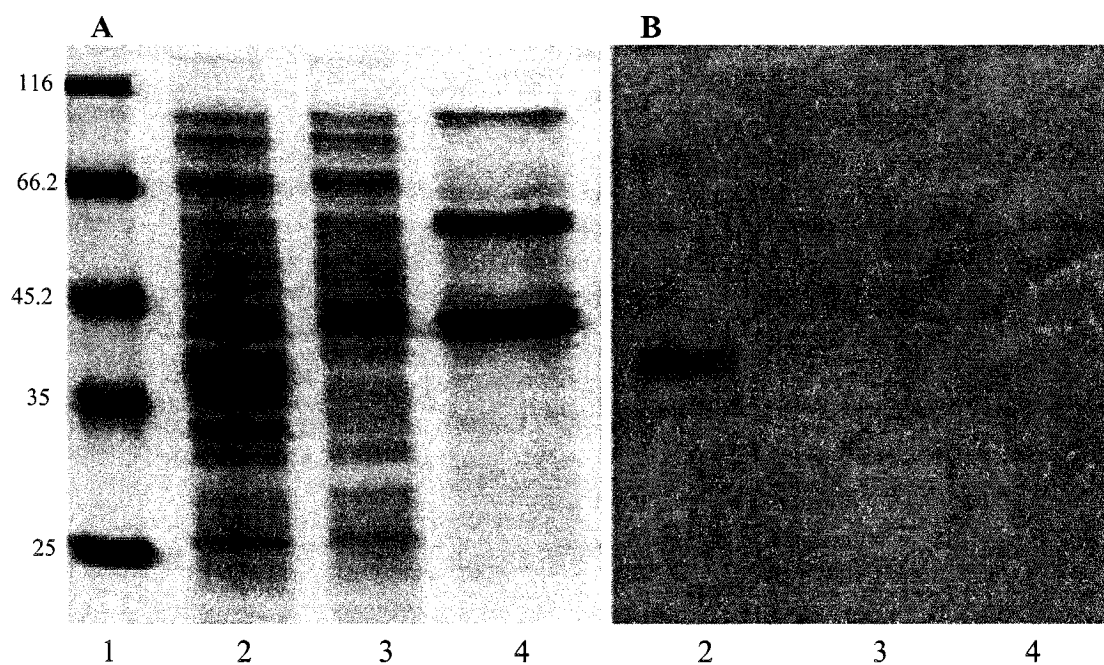


Figure 5.8 Stages of protein purification of TaST4. Panel A shows SDS-PAGE stained with commassie blue and panel B shows western blotting of an identical SDS-PAGE gel. Lane 2 contains total protein from *E. coli* cells. Lanes 3 shows soluble fraction of protein during purification and lanes 4 show purified protein. Lane 1 in panel A is protein molecular weight markers in kDa.

Discussion

To date there are no reports of sulfotransferases being cold regulated, this is the first report of plant sulfotransferases whose expression is altered during cold acclimation. To further characterize the regulation of *TaST2*, *TaST3* and *TaST4*, it is important that other abiotic stresses and hormone treatments including NaCl, drought and ABA be studied since cold, salt stress and ABA regulatory pathways are known to converge. However, the substrates of *TaST2*, *TaST3* and *TaST4* are not clearly identified.

TaVRT-1 and *TaVRT-2*, which showed vernalization and photoperiod requirement in wheat, were differentially regulated in spring and winter wheat (Danyluk *et al*, 2003 and Kane *et al*, 2005). *TaVRT1/Vrn1* is induced after 35 days of cold acclimation while *TaVRT-2* is induced with cold acclimation in winter wheat and repressed after 42 days of cold acclimation (Kane *et al*, 2005). It is believed that *TaVRT1* and *TaVRT2* expression affect the transition from vegetative to reproductive phase and are affected by cold acclimation (Kane *et al*, 2005). Induction of *TaST2* by cold treatment only at 6 hours in winter wheat and no induction in spring wheat suggest that *TaST2* expression is related to cold tolerance or the transition from vegetative growth to reproductive growth, since these are major differences between spring and winter wheat. However, *TaST3*, which is highly induced after 1 day of cold treatment in winter wheat and remains constitutively high until 36 days of treatment in winter wheat also showed induction in spring wheat but only at one time point, 6 hours. This suggests a differential regulation of *TaST3* in winter and spring wheat, which may play a role in freezing tolerance. Finally, *TaST4* was not found to be induced by cold in winter wheat but was induced in spring wheat only after 36 days of cold acclimation suggesting that it is probably not associated with cold stress

tolerance and may have a different biological function. There were 65 genes reported to be differentially regulated in winter and spring wheat at at least one time point (1hr, 6 days and 36 days) of cold acclimation (Gulick *et al*, 2005). Wheat sulfotransferases were not assayed in that study. Like *TaVRT-2*, which is induced with cold acclimation in winter wheat and repressed as it achieves cold acclimation after 42 days, *TaST3* is also induced with cold acclimation until 36 days. To confirm its cold regulation like *TaVRT-2*, a further time point study of transcript regulation is required.

Recombinant TaST2, TaST3 and TaST4 protein products from *E. coli* XL1 Blue cells were purified using Ni-agarose column and separated by electrophoresis on an acrylamide gel to check their purity. Western blotting was performed using a pool of anti-AtST2a, anti-BnST1 and anti-flavonol 3-ST polyclonal antibodies. TaST2, which was found in the total protein extract, the soluble fraction and in the Ni-Agarose purified fraction was detected by this pool of anti-ST antibodies suggesting the presence of an epitope similar to one of the previously characterized proteins used to produce the polyclonal antibodies (Figures 5.3 and 5.4). The pool of anti-ST antibodies did not recognize TaST3 (Figures 5.5 and 5.6) in any of the samples tested. The Identification of TaST2 with interspecies anti-ST antibodies could be due to higher epitope similarities as compared to TaST3. A positive signal is observed for TaST4 only in the total protein extract and is not seen in soluble and purified protein preparations (Figures 5.7 and 5.8). The probable reason could be the insolubility of TaST4 in *E. coli*. While performing the substrate specificity studies TaST2, TaST3 and TaST4 did not demonstrate enzyme activity with any of the substrates tested which included steroids, phenols, alcohols, flavonols, plant hormones, sterols, sugars and hydroxyjasmonates. However, the fact that

no activity was observed with the positive control suggests that a problem occurred in the assay itself. Substrate specificity studies will have to be redone in the future to elucidate the substrate preference of the recombinant wheat STs.

Future Work

Cold regulation studies that involve further time points would be helpful in understanding the role of TaST2, TaST3 and TaST4 in cold regulation. The strong differences between winter and spring wheat shown suggest the results should be confirmed and tested in additional cultivars. Clear substrate identification would also help in understanding the biological function of these enzymes in relation to cold stress. Their regulation should also be studied in response to other stress such as; drought and saline.

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