The Physiological and Molecular Characteristics of Chemically Induced Abiotic Stress Resistant Mutants of Cauliflower (*Brassica oleracea var. botrytis*)

by

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School of Biomedical and Biological Sciences
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The Physiological and Molecular Characteristics of Chemically Induced Abiotic Stress Resistant Mutants of Cauliflower (*Brassica oleracea var. botrytis*)

Fazal Hadi

Abstract

N-nitroso-N-ethyleurea (NEU) and N-nitroso-N-methyleurea (NMU) induced mutants and control plants had been maintained in *in-vitro* condition for 3 years by continuous sub-culturing and screened 2 generations for resistant mutants selection. In this study highly resistant mutants were regenerated and assessed by leaf discs assay for drought, salt and frost resistance to confirm the persistence of mutation over generations of subculture. Assessment was carried out using mannitol (drought stress), NaCl (salt stress) and freezing (frost stress). Cold-acclimated and non-acclimated leaves were assessed for frost resistance. Results confirmed the persistence of mutations in clones with enhanced tolerance levels to stresses over control plants. Response of individual mutants was different for each of the stresses, some mutants were resistant to two stresses whilst others demonstrated multiple resistance and no one mutant was resistant to a single stress. Acclimation at 4 °C appeared good enough to increase frost resistance compared to non-acclimation. Acclimation also tended to emphasis the difference between mutants and some mutants (K18 & K19) showed highly significant increase in frost resistance at -6 °C compared to control. Responses of *in-vitro* and *in-vivo* plants within a clone were correlated.

Molecular and biochemical analysis was carried out with objectives (1) To investigate the presence of CBF/DREB1 and COR15 genes in cauliflower (2) To investigate whether the induced resistance can be attributed to the expression of these genes and proline level. The clones (mutants and control) were analyzed under cold acclimation (4 °C) and non-acclimation (22 °C). Total RNA was isolated after 3 h, 6 h, 24 h and 14 d acclimation. Proteins and free proline were isolated after 14 d acclimation. Under non-acclimation, RNA, protein and proline isolated once at end of experiment. cDNA was produced using RT-PCR, with specific primers the gene was detected only in acclimated clones and no PCR product appeared under non-acclimation. The PCR product was isolated, sequenced, and compared the nucleotides and deduced amino acid sequences with other plants. Very high resemblance (~91%) with *Brassica* species (*BnCBF5/DREB1*, *BrDREB1* and *BjDREB1B*) were found and confirmed the first reporting of the transcription factor *BoCBF/DREB1* in cauliflower. This resemblance was reduced to 67% when compared to other plants, confirms that this sequence is conserved in Brassica. The transcript level increased up to 24 h acclimation and then declined. The response of the mutants was different, some showed PCR product at 3 h while others only after 6 h and 24 h acclimation. Through SDS-PAGE and Western blotting, the COR15a protein was detected with specific antibodies obtained from MSU (USA), and the blots appeared in all clones under cold acclimation correlated with frost resistance but under non-acclimation the COR15a constitutively expressed only in 3 mutants with increased frost resistance that confirms the persistence of mutation.

The genotypes showed positive correlation between *BoCBF/DREB1* expression and frost resistance and this correlation was significant after 24 h and 14 d cold acclimation. The highest R^2 value was found between *BoCBF/DREB1* expression at 14 d and EC% at -6 °C (93.43% of variation accounted for) followed by *BoCBF/DREB1* expression at 24 h and EC% at -6 °C (82.57%). The proline level under acclimation increased about 8 times compared to non-acclimation and demonstrated positive and significant correlation with *BoCBF/DREB1* expression. Proline also showed positive and significant correlation with frost resistance under cold acclimation but very weak under non-acclimation. The effect of cold acclimation on proline and total protein was evaluated and negative correlation was found to be non significant between free proline and total protein content in clones.

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Dedication

I would like to dedicate my thesis to my beloved parents

(Almighty God may bless them)

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Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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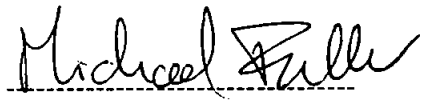
I declare that the work submitted in this thesis is the results of my own investigations except where reference is made to published literature and where assistance is acknowledged.

Candidate



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- **Hadi, F.** Fuller, M. P., Gilpin, M. and Nisr, R.B. Identification and expression analysis of CBF/DREB1 and COR15a in dehydration stress resistant mutants of cauliflower (*Brassica oleraceae* v. *botrytis*), Proceeding, International conference organised by Society of Experimental Biology (SEB), 30th June-3rd July 2010, Prague, Czech Republic, pp 323-324.

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List of Abbreviations

2,4-D:	2,4-dichlorophenoxyacetic acid
ABA:	Abscisic Acid
ANOVA:	Analysis of variances
APS:	Amonium Persulfate
BLAST:	Basic Local Alignment Search Tool
BoCBF:	Brassica oleracea C-repeat binding factor
BSA:	Bovin Serum Albumin
CAMTA:	Calmodulin Binding Transcription Activator
CBF:	C-repeat binding factor
cDNA:	Complementary deoxyribonucleic acid
COR:	Cold regulated
CRT:	C-repeat
DAB:	Diaminobenzidine
dATP:	Deoxyadenosine triphosphate
dCTP:	Deoxycytidine triphosphate
ddH_2O:	Double-distilled water
dGTP:	Deoxyguanosine triphosphate
DNA:	Deoxyribonucleic acid
dNTPs:	Deoxynucleoside Triphosphates
DRE:	Dehydration Responsive Element
DREB:	Dehydration Responsive Element Binding Factor
DTT:	Dithiothreitol
dTTP:	Deoxythymidine triphosphate
EBI:	European Bioinformatics Institute
EC:	Electrical Conductivity
EDTA:	Ethylenediaminetetraacetic acid
EMBL:	European Molecular Biology Laboratory
EMS:	Ethyl Methane Sulfonate
ERD:	Early Responsive Dehydration Gene
EtBr:	Ethidium Bromide
gDNA:	Genomic deoxyribonucleic acid
H_2O_2:	Hydrogen peroxide
HCl:	Hydrogen chloride
HRP:	Horseradish Peroxidase

IAEA:	International Atomic Energy Agency
IBA:	Indole-3-butyric acid
ICE:	Inducer of CBF Expression
$K_2HP O_4$:	Dipotassium Hydrogen Phosphate
kDa:	Kilo Dalton
KH_2PO_4:	Potassium Dihydrogen Phosphate
KPO_4:	Potassium Phosphate
LEA:	Late Embryogenesis Abundant (LEA) proteins
LS:	Linsmair and Skoog
LSD:	Least Significant Difference
LTRE:	Low Temperature Responsive Element
MS:	Murashige and Skoog
MSU:	Michigan State University
mtlD:	Mannitol Dehydrogenase
Na_2HPO_4:	Sodium Hydrogen Phosphate
NaCl:	Sodium chloride
NaH_2PO_4:	Sodium Dihydrogen Phosphate
NaOH:	Sodium Hydroxide
NCBI:	National Center for Biotechnology Information
NEU:	N-nitrose-N-ethylurea
$NiCl_2$:	Nickel Chloride
NMU:	N-nitrose-N-methylurea
OH:	Hydroxyl radicals
P5CS:	D1-pyrroline-5-carboxylate synthase
PAGE:	Polyacrylamide Gel Electrophoresis
PBS:	Phosphate Buffer Saline
PBST:	Phosphate Buffer Saline Tween
PCR:	Polymerase Chain Reaction
RNA:	Ribonucleic acid
ROS:	Reactive Oxygen Species
RT-PCR:	Reverse Transcription-Polymerase Chain Reaction
RWC:	Relative Water Content
SCE:	Sister Chromatid Exchange
SDS:	Sodium Dodecyl Sulfate

SNP:	Single-Nucleotide Polymorphism
TBE:	Tris Borate Ethylenediaminetetraacetic acid
TEMED:	Tetramethylethylenediamine
TILLING:	Targeting Induced Local Lesions in Genomes
UDS:	Unscheduled DNA synthesis
UV:	Ultra Violet

Chapter 1: General Introduction and Literature Review

1.1. The Cauliflower (*Brassica oleracea var.botrytis* L.) plant

Cauliflower is one of the popular vegetable crops originated in the Mediterranean coastal area and reached to the Southeast Asian countries (Lu, 1992; Nonnecke, 1989; Thompson & Kelly, 1957) . Cauliflower is one of the varieties of the highly polymorphic species *Brassica oleracea*. The other varieties are *acephala (*Kale), *capitata (*Cabbage), *gemmifera* (Brussels sprouts), kohlrabi and broccoli (Christopher, 1994). This species belongs to the family Brassicaceae, which is a cosmopolitan family, while mostly found in the northern temperate regions and it has high diversity in Mediterranean areas like Italy. Cauliflower is grown for its white curd, which consists of inflorescence and floral meristems and their interconnecting stem branch tissues (Lee & Graham, 2000).

The Brassica species are associated with each other as described by the triangle of U (U, 1935) in Figure 1. The three diploid *Brassica* species *B. rapa*, *B. nigra* and *B. oleracea* have hybridized in all possible combinations to produce the three allotetraploid (hybrid that has a chromosome set 4 times that of a haploid) species *B. juncea*, *B. napus* and *B. carinata*. The genomes have been named as A, B and C respectively *B. rapa* AA, *B. nigra* BB and *B. oleracea* CC. Therefore the resulting amphidiploids (having a diploid set of chromosomes from each of its parents) cytodemes (a group of individuals differing cytologicaly from other groups, usually in chromosome number) become AABB, AACC and BBCC for *B. juncea*, *B. napus* and *B. carinata*, respectively (Lars & Graham, 2008).

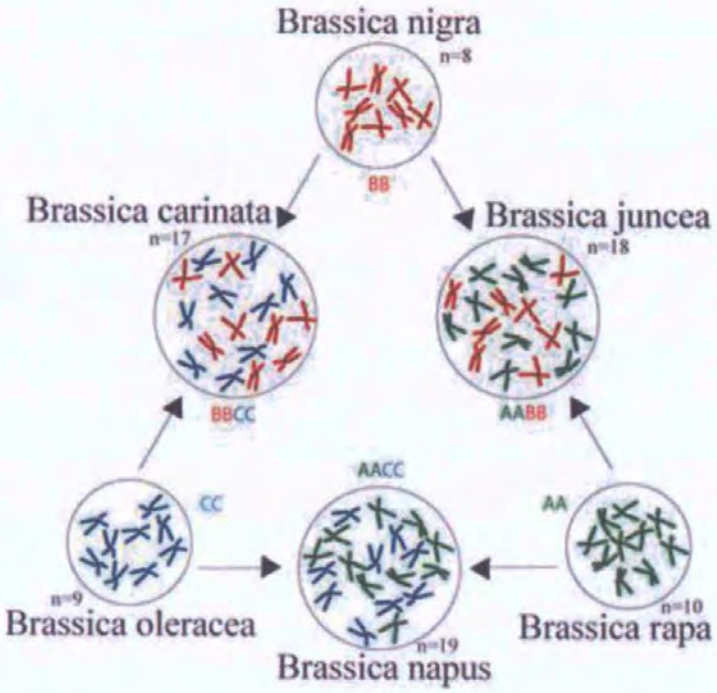


Figure 1. The origin of three amphidiploids species from three diploid Brassicas (Jules, 2009; U, 1935),
AA - 2n=2x=20 - *Brassica rapa (syn. Brassica campestris)* - Turnip, Chinese cabbage
BB - 2n=2x=16 - *Brassica nigra* - Black mustard
CC - 2n=2x=18 - *Brassica oleracea* - Cabbage, kale, broccoli, Brussels sprouts, cauliflower
AABB - 2n=4x=36 -*Brassica juncea* - Indian mustard
AACC - 2n=4x=38 -*Brassica napus* - Rapeseed, rutabaga
BBCC - 2n=4x=34 -*Brassica carinata* - Ethiopian mustard

Cauliflower is a low-calorie vegetable. It is a rich source of vitamins C, K, and A (beta-carotine), and folic acid, fiber, and flavonoids, which gives the cauliflower anti-inflammatory and antioxidant proprieties, as well as it is an important source for animal feed (Tossaint, 1994). Christopher (1994) investigated the dietary anti-mutagenic ability of cauliflower and other varieties of *Brassica oleraceae* and reported them to be a group of potentially cancer preventative vegetables (Table 1), particularly against the bowel, breast, and other female cancers. Cauliflower can also play an important role in protection against arteriosclerosis and offers a high degree of protection from strokes (Pattison et al., 2004). Cauliflower is also a carbohydrate source that is an efficient fuel for energy production (Robert, 2001) as cauliflower dried waste has been reported as a supplementary source of ethanol production and incorporation of dried cauliflower waste in cane molasses at the level of 15 % increased ethanol production by nearly 36 % compared to molasses alone (Dhillon, Bansal & Oberoi, 2007).

Cauliflower requires constant moisture with cool temperatures and frequent use of fertilizers. It should have an uninterrupted growth; and any delay in growth encourages the plants to form a premature small head of no value. To avoid this the soil should be high in organic matter with optimum pH of about 6.5, and for best development of cauliflower a large amount of available nitrogen is required along with minor elements particularly boron and magnesium (John, 1996). Cauliflower curd initiation depends on nitrogen level, temperature, genotype, photoperiod and irradiance. Nitrogen deficiency can prevent curd initiation (Atherton, Hand & Williams, 1987) because the leaf area development is restricted, which affects growth. Temperature is considered as a major factor in curd initiation (Atherton, Hand & Williams, 1987; Sadik, 1967; Salter, 1960) and some varieties stay vegetative under high temperature (Booij, 1987; Haine, 1959). The optimum temperatures for curd initiation in different varities is proposed as 14 °C

for variety Revito (Pearson, Hadley & Wheldon, 1994), 15.5 °C for variety Plana (Wheeler et al., 1995) and 13 °C for cv. White Fox (Hand, 1988). Different genotypes make possible the cultivation of cauliflower over a range of climatic conditions (Nieuwhof, 1969; Wurr, Akehurst & Thomas, 1981.). Investigations show that each variety or genotype has different requirements for curd initiation, that's why it is important to select a suitable variety according to climatic conditions for commercial cultivation. Cauliflower shows variation in responses to photoperiod and reduction in total irradiance can delay curd initiation under warm conditions, and increased irradiance can act as partial substitute for low temperature in accelerating curd initiation (Hand, 1988; Sadik, 1967).

Table 1. Summary of the anti-mutagenic ability of the crude extracts from the *Brassica oleraceae* varieties; Ames (to assess mutagenic potential of chemical compounds using bacterial strains) type assays (Christopher, 1994).

Plant extracted	**Mutagens**	**Percent (%) reduction in the rate of mutagenesis**
Cauliflower	Nitrate + methylurea	78
Cauliflower	Nitrate + aminopyrine	57
Cabbage	Nitrate + sorbic acid	Moderate (not calculable)
Cauliflower	Nitrate + sorbic acid	Moderate (not calculable)
Cabbage	Tryptophan pyrolysate	97
Broccoli	Tryptophan pyrolysate-l	97
Broccoli	Tryptophan pyrolysate-	81
Broccoli	Ethidium bromide	92
Broccoli	2-Aminoanthracene	84
Broccoli	AF-2	0
Broccoli	Oxidized linolenic acid	82
Cabbage	Oxidized linolenic	76
Red cabbage	Oxidized linolenic acid	81
Cauliflower	Oxidized linolenic acid	76
Cabbage	Tryptophan pyrolysate-2	35

1.2 Plant tissue culture

Plant tissue culture refers to growing and multiplication of plant cells, tissues and organs on distinct solid or liquid media under aseptic and controlled condition as shown in Figure 2. Plant tissue culture technology is being widely used for large-scale plant multiplication. The commercial technology is primarily based on micro-propagation, in which rapid proliferation is achieved from minute stem cuttings, auxiliary buds, and to a limited extent from somatic embryos and cell clumps in suspension cultures (Ahloowalia et al., 2002)

1.2.1 Explants Source

Plant tissue cultures are initiated from small pieces (known as explants), taken from any part of the plant. The "explant" is removed surgically from surface sterilized part of plant and then placed on a nutrient medium to initiate the mother culture, which is multiplied repeatedly by sub-culturing (Ahloowalia et al., 2002). The following plant parts are widely used for micro-propagation.

Shoot-tip or meristem-tip: Shoots develop from a small group of cells known as shoot apical meristem. The apical meristem maintains itself, gives rise to new tissues and organs, and communicates signals to the rest of the plant (Medford, 1992). It is the most ''well-liked'' source of explants. The apical meristem of a shoot is the portion lying distal to the youngest leaf primordium (Cutter, 1965), and is about 100 µm in diameter and 250 µm in length (Quak, 1977). Shoot-tip explants are cultured to obtain plants free from viruses. The term "meristem-tip culture" has been suggested to distinguish the large explants from those used in conventional propagation (Bhojwani & Razdan, 1983).

Nodal or auxiliary buds: Consist of a piece of stem with auxiliary bud, when only the auxiliary bud is taken, it is known as "auxiliary bud" culture.

Floral meristem and buds: Floral meristems and buds can generate complete plants.

Other sources of explants: In some plants, leaf discs, intercalary meristems from nodes, small pieces of stems, immature zygotic embryos, anthers, pollen, microspores and nucellus have also been used as explants to initiate cultures.

1.2.2 Cell suspension and callus cultures: A callus is a mass of unorganized cells, which upon transfer to suitable medium is capable of giving rise to shoot-buds and somatic embryos, which then form complete plants. Such calli in liquid media on shakers are used for initiating cell suspensions (Ahloowalia *et al.*, 2002)

1.2.3 Pathways of Cultured Cells and Tissues

Cultured cells and tissues take numerous pathways to generate a complete plant. Among these, the pathways that lead to the production of plants in large numbers are the popular and favoured ones for commercial multiplication. The following terms are used to describe various pathways of cells and tissue in culture (Bhojwani & Razdan, 1983; Pierik, 1989).

1.2.4 Regeneration and organogenesis

In this pathway, groups of cells of the apical meristem in the shoot apex, auxiliary buds, root tips, and floral buds are stimulated to differentiate and grow into shoots and ultimately into complete plants. The explants are cultured on media having comparatively high auxin (2,4-D, 2,4-dichlorophenoxyacetic acid) and form an unorganized mass of cells, called callus. The callus can be further sub-cultured and multiplied. The callus shaken in a liquid medium produces a cell suspension, which can be sub-cultured and multiplied into more liquid cultures. The cell suspensions form cell clumps, which eventually form calli and give rise to plants through organogenesis (Ammirato, 1983). In some cases, explants e.g. leaf-discs and epidermal tissue can also generate plants by direct organogenesis and somatic embryogenesis without intervening

callus formation, e.g. in orchard grass. *Dactylis glomerata* L (Hanning & Conger, 1986).

1.2.5 Somatic embryogenesis

Cells or callus cultures on solid media or in suspension cultures can form embryo-like structures called somatic embryos, which on conversion (germination) produce complete plants. The primary somatic embryos are also capable of producing more embryos through secondary somatic embryogenesis (Ahloowalia *et al.*, 2002). Somatic embryos are produced as adventitious structures directly on explants, from callus and suspension cultures. Somatic embryos hold potential for large-scale clonal propagation of superior genotypes (Mamiya & Sakamoto, 2001; Redenbaugh, Fujii & Slade, 1993) and may be used for commercial plant production and multiplication of parental genotypes in large-scale hybrid seed production (Bajaj, 1995; Cyr, 2000).

The synthetic auxin, 2,4-D is commonly used for embryo induction. In many angiosperms, e.g., carrot (Lee, Cho & Soh, 2001) and alfalfa (McKersie & Bowley, 1993), subculture of cells from 2,4-D containing medium to auxin-free medium is sufficient to induce somatic embryogenesis. The process can be enhanced with the application of osmotic stress, manipulation of medium nutrients, and reducing humidity. A major problem in large-scale production of somatic embryos is culture synchronization. This is achieved through selecting cells or pre-embryonic cell clusters of certain size, and manipulation of light and temperature (McKersie & Bowley, 1993), temporary starvation (Lee, Cho & Soh, 2001) or by adding cell cycle synchronizing chemicals to the medium (Dobrev et al., 2002).

Cytokinins seem to play a key role in cell cycle synchronization (Dobrev *et al.*, 2002) and embryo induction, proliferation and differentiation (Schuller, Kirchner-Ness & Reuther, 2000). Abscisic acid is crucial in all the stages of somatic development, maturation and hardening (Nieves et al., 2001; Schuller, Kirchner-Ness & Reuther, 2000).

1.2.6 Culture Media

The growth of plants in *in-vitro* conditions is mostly dependent on the culture media composition. The major components of most plant tissue culture media are mineral salts and sugar (as carbon source) and water. Other components include growth regulators, organic supplements and gelling agent (Gamborg, Miller & Ojima, 1968; Gamborg & Phillips, 1995). The amount of the various ingredients vary in the medium for different stages of culture and plant species, but the most popularly used are the basic MS (Murashige & Skoog, 1962) and LS (Linsmaier & Skoog, 1965) media.

Usually stock solutions are prepared prior to media; the stock solutions consist of groups of chemicals, e.g. macronutrients, micronutrients, vitamins and plant growth hormones. The inorganic chemicals and vitamins solutions can be combined into a single, 10 X concentrated, stock solution. The stock solutions can then be frozen (Prakash, Hoque & Brinks, 2002)

Different types of media are used for *in vitro* plant culture (Pierik, 1989; Street & Shillito, 1977; Torres, 1989) and ingredients compositions have been formulated for the specific plants and tissues (Conger, 1981; Nitsch & Nitsch, 1969). Some tissues respond much better on solid media while others on liquid media. Depending on the presence or absence of gelling agents, the medium can be solid, semi-solid or liquid. Agar is the

most commonly used gelling agent for preparation of solid and semi-solid media and contributes to the matrix potential, the humidity and affects the availability of water and dissolved substances in the culture containers (Debergh, 1983). It is usually unnecessary to use high purity agar for large-scale micro propagation; cheaper brands of agar have been successfully used for industrial scale micro propagation (Boxus, 1978). A semi-solid medium ensures adequate contact between the plant tissue and the medium. It is beneficial to growth as it allows better diffusion of medium constituents, and is easily removed from plantlets before their transfer to *in vivo* conditions. For these reasons, a semi-solid medium is often preferred over solid medium.

The ratio of auxins to cytokinins in the medium is important; their combination determines the morphogenic response for root or shoot formation. A relative high auxin : cytokinine ratio induces root formation while a high cytokinin : auxin ratio induces shoot production (Skoog & Miller, 1957). Generally, buds could be initiated from callus or cut edges of explants when a high cytokinin : auxin ratio is applied (Gresshoff, 1978; Helenice et al., 2003; Hiroharu et al., 2001; Nathan & Sekhar, 2006), while in some species the addition of cytokinin into medium fails to induce shoot, it is suggested that the accumulation of endogenous auxin or other hormones shows inhibitory effect on organogenesis which could not be reversed by exogenous hormones applied (Khalid, 2003; Street, 1977). For root induction usually a high concentration of auxin is favoured but in some cases exogenous auxin shows inhibitory effects on roots (Guichuan, Jeffery & Elison, 2004; Thomas & Street, 1970). Silver nitrate is another important supplement in the culture medium which is essential for maintaining the callus as well as improves regeneration (Sethi, Basu & Cuha, 1990), while in high concentration silver nitrate causes necroses in culture, even though as high as 15 mgL-1 silver nitrate did not show any negative effect on transformation of *Brassica rapa* (Kuvshinov et al., 1999)

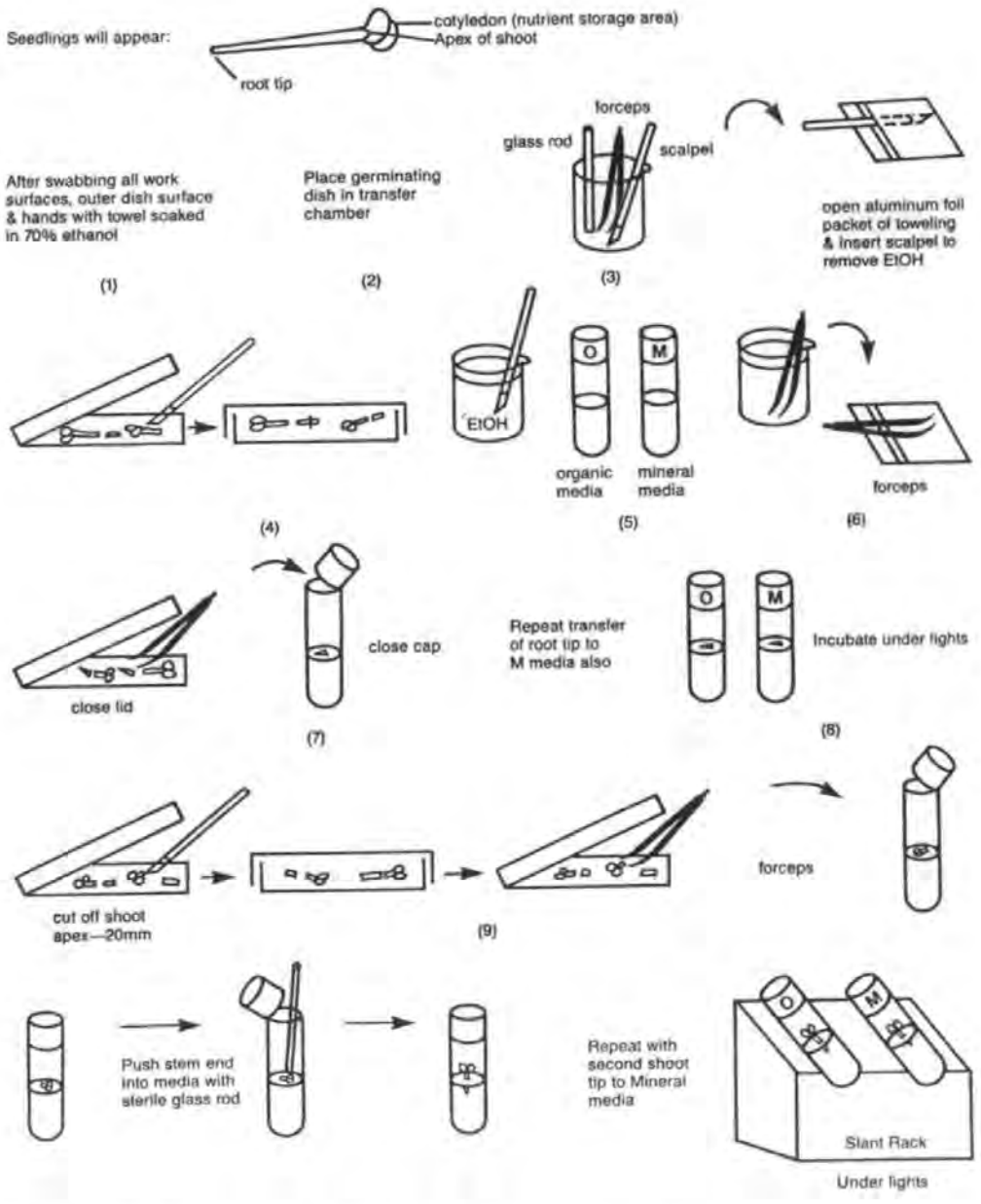


Figure 2: Different steps involved in plant tissue culture.
Source (Mineo, 1990)

1.2.6 Process of micro-propagation

The process of plant micro-propagation aims to produce clones. The process is usually divided into the following stages (Ahloowalia *et al.*, 2002).

Stage 0- pre-propagation step or selection and pre-treatment of suitable plants.

Stage I - initiation of explants - surface sterilization, establishment of mother explants.

Stage II – subculture for multiplication/proliferation of explants.

Stage III – shooting and rooting of the explants.

Stage IV - weaning/hardening.

The cauliflower curd a preinflorescence is an important part of the plant to use for micro-propagation and the use of this curd meristematic tissue for in vitro culture has been reported for micro-propagation (Kieffer, Fuller & Jellings, 1995a). The outermost layer of the curd consists of millions meristems having the capability of producing shoots in *in-vitro* condition (Kieffer, Fuller & Jellings, 1995b). The use of cauliflower curd for micro-propagation is now well established. One of the efficient protocols for clonal propagation of cauliflower has been reported by Kieffer, Fuller & Jellings (1995a). In this protocol the meristematic layer of the curd is removed and partially homogenised to disrupt the meristem clusters and then graded through precision sieves to produce homogenous size-classes. For a single curd, over 400 000 explants of size-class 0.1-0.3 mm can be produced and each explant produces one to three shoots per explant. The number of 'microshoots' produced from one curd within two weeks is over 10 000. The rooting step takes place on a semi-solid medium in the presence of IBA, within 2 weeks over 80% of shoots are rooted. This protocol has the qualities of simplicity, large scale propagation, and high quality propagules, making it superior to conventional methods and a cost effective candidate for an industrial semi-automated system of propagule production (Kieffer, Fuller & Jellings, 1995a).

1.3 Mutation

Mutation means change in the DNA sequence of a genome and mostly caused by radiation, transposons, viruses, and mutagenic chemicals, as well as errors that occur during meiosis or DNA replication (Aminetzach, Macpherson & Petrov, 2005; Bertram, 2000; Burrus & Waldor, 2004). Induction of mutation has become an established method of creating variation within a crop variety. It offers the possibility of inducing desired attributes that either cannot be expressed in nature or have been lost during evolution (Brunner, 1995).

More than 1,700 mutant cultivars of crop plants with significantly improved attributes such as disease and stress resistance, increased yield and improved quality, were released worldwide in the period 1965-95 (Brunner, 1995), and in the past seventy years, more than 2250 varieties have been released worldwide 60% of them released from 1985 onwards. Most mutant varieties were released in China (26.8%), India (11.5%), Russia (9.3%), the Netherlands (7.8%), USA (5.7%) and Japan (5.3%) (Ahloowalia, Maluszynski & Nichterlein, 2004).

In the literature there is a tremendous amount of information available regarding plant abiotic stress resistance and about the different ways to improve resistance in plants, but to date only a limited number of techniques have been successful and traditional plant breeding approaches are showing very limited success (Richards, 1996). The approach of mutagenesis has had some success in agronomic and horticultural crop species (Deane, Fuller & Dix, 1995; Fuller et al., 2006; Mohan, 2010) and some success via genetic modification has been observed but this technology may not readily be operational everywhere in the world due to social-economic limitations (Mohan, 2010).

Mutation induction contributes significantly to plant breeding (Maluszynski et al., 1995; Nichterlein, 2000) and constitutes a valuable strategy to create genetic variability, which in turn reduces the time required to breed new varieties compared with traditional methods (Cornide, 2001). Plant breeding for the improving tolerance against cold and salinity stress by classical methods of selection and crossing is a time consuming and, often, inefficient procedure whilst enhancing the frost and salt tolerance either by direct gene transfer or through DNA mutation is much quicker (Zhang et al., 2000). Also through these methods the cultivar might be improved for a particular trait without disrupting the genotype or breaking desirable gene linkages.

The ability to induce mutations has been a major driving force in genetics (Muller, 1930). Amongst the mutagens that are used to induce mutations, chemical mutagens have become especially popular. Alkylating agents, such as ethyl methane sulfonate (EMS), are most effective, they form adducts with nucleotides, causing them to mispair with their complementary bases, thus introducing base changes after replication (Ashburner, 1990; Haughn & Somerville, 1987). EMS mutagenesis results in high numbers of point mutation without or with a very low level of chromosome breaks which can some times cause aneuploidy, reduced fertility, and dominant lethality. Therefore, the chemical mutagenesis has become the method of choice for genetic studies; this method is popular even with the advent of sophisticated transgenic technologies.

Mutation could be induced by mutagens which may be either physical or chemical, and both have been used in conventional plant breeding programmes as well as in conjunction with *in-vitro* selection methods. The majority of chemicals used to induce mutation in plant cell cultures can be placed in two groups, base analogous and alkaline

agents. Alkaline agents include N-nitrose-N-ethylurea (NEU), N-nitrose-N-methylurea (NMU), alkyl sulphate and nitrogen mustards. NEU or NMU are bio functional agents (Charlotte, 1976) and can induce depurination and depyrimination. Both NEU or NMU have been shown to induce gene mutation (deletion), transition mutation, unscheduled DNA synthesis (UDS), sister chromatid exchange (SCE) and they can also induce DNA-DNA and DNA protein crosslink (IAEA, 1977; Negrutu, 1990). Multiple mutations also occur more frequently in NMU-treated plants. There is great scope for increasing both the frequency and spectrum of mutations in treatments with chemical mutagens through suitable modification of the treatment conditions (Savin et al., 2003). Traditional genetic screens do not readily disclose the underlying mutational process, because geneticists select for phenotypes, and as a result, only a small minority of mutations applying to target gene are examined. This is changing now with the availability of large amounts of DNA sequences from model organisms and the incentives to determine the functions of genes discovered from DNA sequence and reverse-genetic approaches are becoming increasingly important. Among these are genome-wide mutagenesis methods followed by screening within individual gene segments, which is made possible by using polymerase chain reaction PCR (Henikoff & Comai, 2003).

Although PCR-based detection of insertions and deletions is straightforward, detection of point mutations, such as those induced by chemicals, is still challenging, because the PCR amplified fragment does not show any change in the size. However, detection of single-base changes has improved rapidly with advances in single-nucleotide polymorphism (SNP) detection technologies (Kwok, 2001), and this has fuelled the application of new technologies to reverse-genetic mutational screening. One example of SNP detection technology being applied to reverse genetics is TILLING (targeting

induced local lesions in genomes), in which chemical mutagenesis is followed by screening for point mutations (McCallum et al., 2000). TILLING has been streamlined for high output with the use of the CEL1 endonuclease (Colbert et al., 2001), which cleaves at mismatches within hetero duplexes formed between mutant and wild-type strands (Oleykowski et al., 1998). This allows for cleaved fragments to be detected on electrophoretic gels, revealing the mutation and its approximate position in the fragment.

Agrobacterium mediated transformation is mostly used for transgenic plant production but is also being used as an effective mutagen and as a tool for functional genomics in higher plants. Besides the fact that the insertion of T-DNA (transfer DNA) element into a gene can lead to loss or gain of function, ingenious use of a variety of vectors have led to the identification of genes and regulatory elements in *Arabidopsis* and focus has shifted from structural genomics to functional genomics, specifically in plants with the availability of complete genome sequences of several plants. An advantage of using T-DNA as the insertional mutagen as compared to transposons is that the T-DNA insertions do not transpose subsequent to insertion and are chemically and physically stable through multiple generations (Resmi, Anand and Ramamurthy 2005).

1.4 Abiotic stresses

Plants are exposed to various abiotic stresses, such as frost, drought and salinity in the field environment. It is estimated that such stresses can potentially reduce the yield of crop plants by more than 50% (Boyer, 1982; Bray et al., 2000; Shubha & Akhilesh, 2007), and can cause extremely high economic losses. It is accepted that the human population of the world is increasing day by day at an alarming rate and food productivity is decreasing due to various abiotic stresses (Shilpi & Narendra, 2005). The

minimization of these losses is a major area of concern for plant and crop scientists. Since it is often difficult or impossible to eliminate or reduce the stresses themselves, it is important to develop stress tolerant or resistant crop genotypes (Shilpi & Narendra, 2005).

Plant abiotic stress tolerance is a complex trait that involves multiple physiological and biochemical mechanisms coded by numerous genes (Figure 3). However through the growing power of genomic and proteomic tools, progress in understanding abiotic stress resistance is accelerating and with a better understanding comes more effective ways to improve plant tolerance to abiotic stress (Ji, Dai & Hong, 2007b).

Drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are major threats to crops and natural ecosystems as increased salinization of arable land is expected in 30% land loss within the next 25 years, and up to 50% by the year 2050 (Wang, Vinocur & Altman, 2003). Breeding for abiotic stress tolerance in crop plants for food supply and in forest trees (a central component to global ecosystems) is therefore important and should be given high research priority. Research into the molecular mechanisms of stress responses like genetic modification of stress tolerance has shown promising results in agriculturally and ecologically important plants (Wang, Vinocur & Altman, 2003). Results are not always consistent in plants that over-express the genes regulating osmolytes, specific proteins, antioxidants, ion homeostasis, transcriptional factors and membrane composition (Zhang et al., 2000). Generating crops having multi-stress tolerance capability should be priority strategy of future research program (Mittler, 2006) and understanding the genetic and functional basis of multiple stress tolerance will be an important step toward increasing plant productivity (Swindell, 2006).

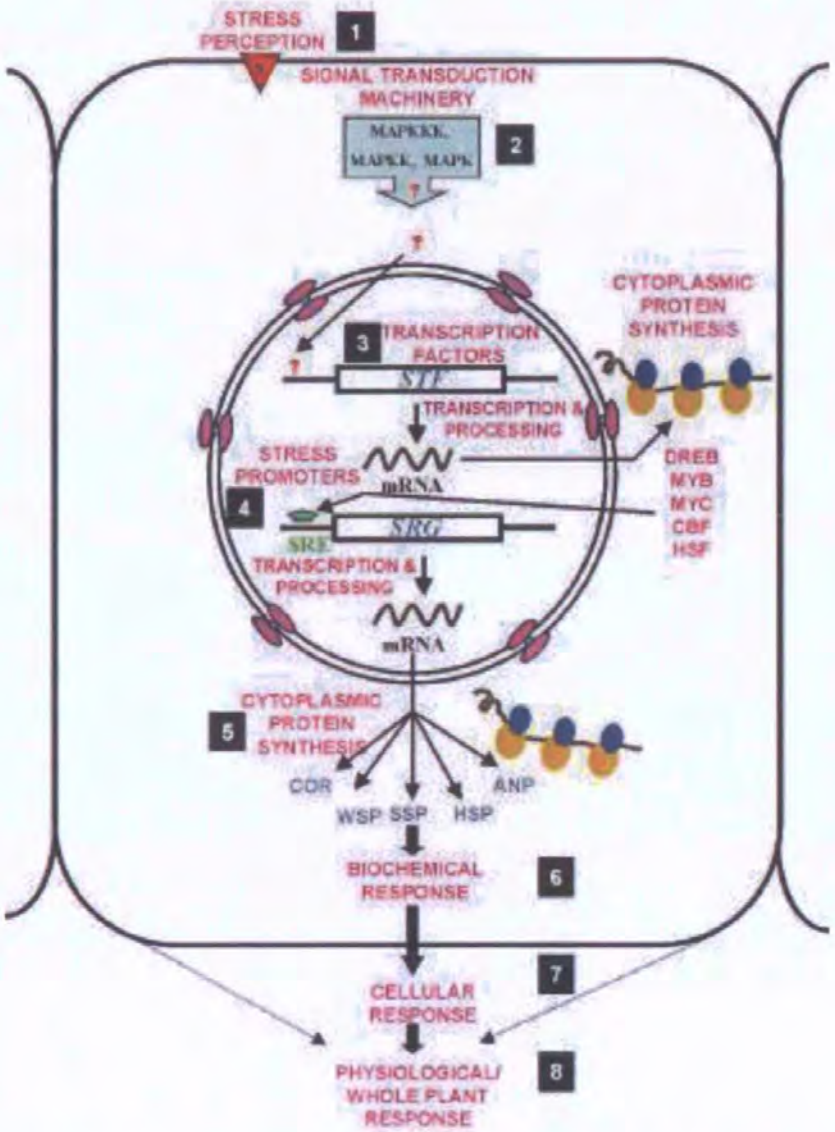


Figure 3: A complex mechanism of abiotic stress response in plants at molecular to cellular and whole plant level (Diagram reproduced from Anil et al (2001): (1) Stress perception (2) stress signal is transduced through the signal transduction machinery, which may involve protein kinases, phosphatases, and Ca2+-binding proteins. (3) stress signal is transduced inside the nucleus where the genes encoding the stress transcription factors (STF; e.g. *dreb, myc, myb, cbf* and *hsf*). The synthesis of transcription factors involve cytoplasmic ribosomes, which means that nucleus–cytoplasmic crosstalk is an important feature in this respect. (4) transcription facors re enter into nucleus and binds to stress responsive elements in promoters (5) Stress responsive genes (*SRG*) are transcribed and translated leading to stress proteins synthesis (6) initiate a biochemical response (7) cellular response and subsequently the (8) physiological and finally the whole plant response. HSP (heat shock proteins), WSP (Water Stress Proteins), ANP (Anaerobic Proteins), SSP (Salt stress Protein).

There are many factors determine how plants respond to environmental stresses as shown in Figure 4. The genotype of the plant, the duration and severity of the stress, and synergistic effect of multiple stresses on failure to compensate for a severe stress can result in plant death (Bray, Julia & Weretilnyk, 2005).

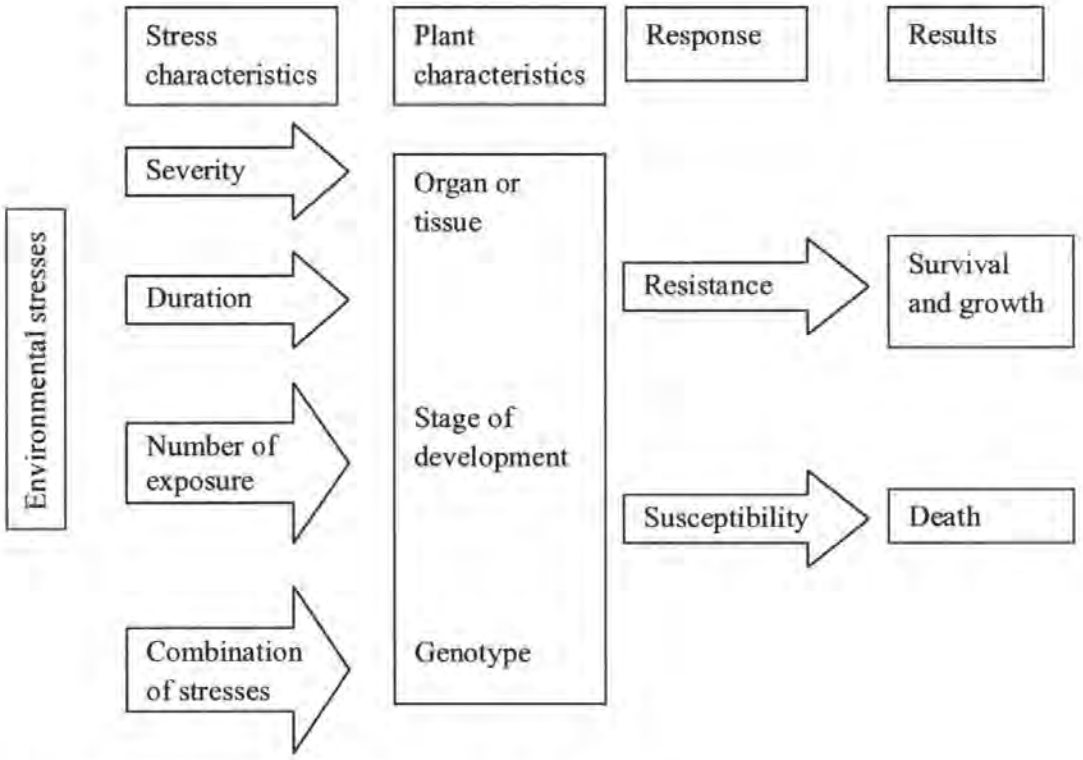


Figure 4: Factors determine how plants respond stresses, modified from Bray, Julia & Weretilnyk (2005).

Abiotic stress tolerance molecular mechanisms are based on the activation and regulation of specific stress-related genes, such genes are involved in the whole series of stress responses, like signaling, transcriptional control, protection of membranes and proteins, and free-radical and toxic-compound scavenging (Wang, Vinocur & Altman, 2003).

Cold, drought and salinity are stressors which due to their wide range occurrence may cause the most fatal economic losses in agriculture. The effects of these stressors have been tackled in various studies ranging from the molecular to the whole plant level, all of these three forms of stress affect the water relations of plants at the cellular as well as entire plant level causing specific and unspecific reactions, and inducing adaptation reactions (Erwin et al., 2007).

Cold (usually low +ve temperatures) induces the expression of C-repeat binding transcription factors (CRBs of which CBF is one), which activate downstream the cold regulated genes and several CRT-binding proteins have been identified which act as transcription factors (Browse & Xin, 2001; Nakashima & Yamaguchi, 2006b). The expression of CRBs is regulated by the transcription factor inducer ICE1 (Inducer of CBF expression1) which is probably negatively controlled by HOS1, a ring finger protein that has been identified as an E3 ubiquitin conjugating enzyme (Erwin *et al.*, 2007; Viswanathan, Zhu & Zhu, 2006).

Transcriptome profiling of about 8,000 genes of *Arabidopsis* showed that multiple regulatory pathways are involved in the cold response and the expression of more than 300 genes was affected by low temperature with increasing transcripts expression for

218 genes and decreasing transcripts expression for 88 genes during 7-day treatments (Fowler & Thomashow, 2002a).

1.4.1 Cold and dehydration stress response

Calcium (Ca) plays a vital role in cold or drought stress signal transduction. Proteins, which sense changes in the cytoplasmic calcium concentrations are the important components of the signal transduction chain, although at which level of the signalling chain the specific responses arise is still an unanswered question (Erwin *et al.*, 2007). It was found through a short term treatment experiment that 30% of the *Arabidopsis* transcriptome responded to cold, salinity and water deficiency treatment in a quite specific way. However only < 5% of the responses were induced by all of the 3 stressors. Even though this rate decreased further to < 0.5% after about 1 day, which indicating a growing tendency for a specific reaction (Kreps et al., 2002b). Cross talk in the signalling pathways is apparent and is characterized by the well-known cross-protections, e.g. frost hardening by drought or salt treatment. Also as well cooperative actions of transcription factors and the occurrence of several different *cis*-acting elements in one promoter exist as shown in Figure 5. (Erwin *et al.*, 2007; Mahajan & Tuteja, 2006).

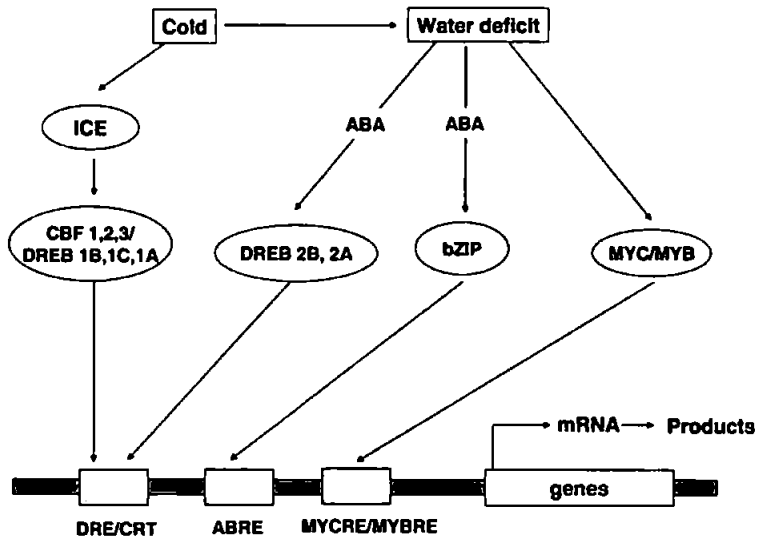


Figure 5: Various signal transduction elements involve in cold and drought response. ICE, transcription induction factor; DREB, bZIP, MYC and MYB, transcription activators and DRE/CRT, ABRE, MYCRE and MYBRE are responsive elements in the promoter(Erwin *et al.*, 2007; Mahajan & Tuteja, 2006).

The products of different genes which respond to dehydration stress can be categorised into two groups i.e. functional and regulatory proteins as shown in Figure 6 (Erwin *et al.*, 2007). The functional genes include ones which encode metabolically inactive polypeptides, such as dehydrins, chaperones (including proteases), antifreeze proteins or ice-nucleation active proteins genes for metabolic pathways leading to the synthesis of low molecular osmolytes, radical scavengers or compounds with both functions which increase stress tolerance, whilst the other genes encode regulatory proteins such as transcription factors, protein kinases, phospholipase C or 14-3-3 proteins (Erwin *et al.*, 2007).

Most of the cold up-regulated genes are expressed at a slightly higher level after the cold pulse, but the dehydrins encoding genes are strongly induced by cold (Browse & Xin, 2001). The dehydrins have also been found to act as chaperones that stabilize proteins, membrane structure and vesicles in the abiotic stressed plants (Allagulova et al., 2003a; Koag et al., 2003).

Cellular dehydration by drought or frost stress can induce the expression of genes encoding dehydrins, which also accumulate in seeds during maturation where they are known as Late Embryogenesis Abundant (LEA) proteins. Dehydrins are a group of proteins having wide range of molecular masses from 9 to 200 kDa, they are thermostable and contain a high proportion of glycine and lysine residues (Allagulova *et al.*, 2003a; Erwin *et al.*, 2007). Dehydrins have been found in vascular plants, mosses, ferns, lichens and algae, and their molecular functions are not well understood as they do not catalyze any metabolic reaction (Erwin *et al.*, 2007)

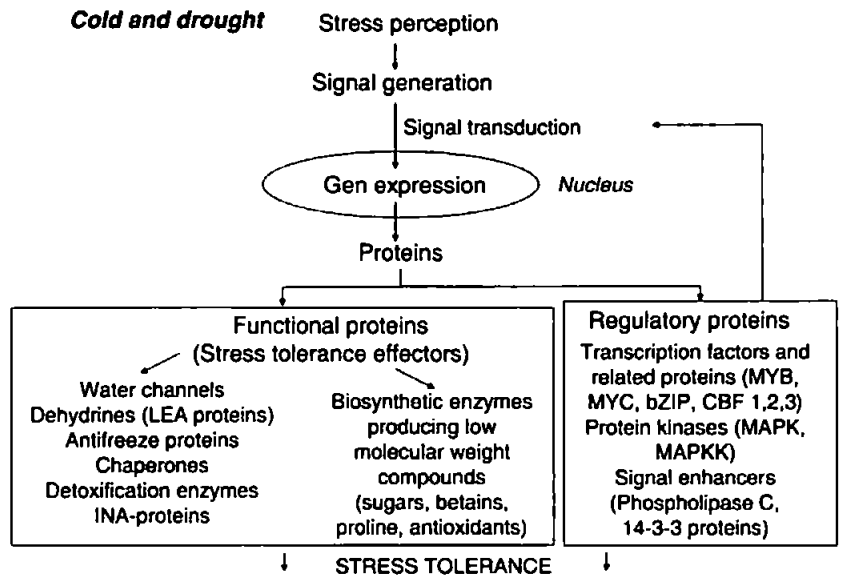


Figure 6: Dehydration Stress tolerance factors produced in plant
Source (Erwin *et al.*, 2007)

Producing drought and frost tolerant crop plants has been undertaken over many years using conventional breeding and targeted gene transformation. To date however the progress has been very small due to the fact that the tolerance or hardiness mechanism is multi-factorial and multi-genic (Bohnert, Nelson & Jensen, 1995; Mittler, 2006; Shinozaki & Yamaguchi-Shinozaki, 2000). Sometime by improving resistance against one stress through gene transfer can also alleviate other stresses and therefore generate plants with a higher stress tolerance than those which have been genetically tailored against a specific stress (Levitt, 1980).

A good example for a kind of a master switch is transcriptional activator CBF (C-repeat binding factor), which binds to the C-repeat/dehydration responsive element (DRE with the core sequence CCGAC) of the promoter of cold and drought-regulated genes (Jaglo-Ottosen et al., 1998b; Kasuga et al., 1999; Stockinger, Gilmour & Thomashow, 1997c). CBF over-expression activates multiple genes (CBF regulon) whose products directly or indirectly enhance multi stress tolerance.

The molecular mechanism of abiotic stress resistance varies depending on the stress type and intensity (Valerie, Seifollah & Mylene, 2009). Some times molecular mechanisms are initiated in response to different stresses in plants and share similar steps in the pathway e.g. drought, salt and freezing stresses disturb the osmotic homeostasis of the plant cell. Moreover in plants there are numerous ways to respond to abiotic stress depending on the growth stage and plant genotype. Given this complexity it is difficult to meet the criteria to declare a genotype as 'resistant' to abiotic stress.

1.4.2 Drought stress

Drought is one of the major limitations to decline in crop productivity. To enhance the plant tolerance towards drought stress, a basic understanding of physiological, biochemical and gene regulatory networks is essential. Understanding of stress signal perception, transduction, and molecular regulatory network is being improved through different genomics tools (Babu & Henry, 2006). These tools expose a number of stress inducible genes and various transcription factors that regulate the drought stress-inducible systems. Translational genomics of drought responsive genes have provided encouraging results using model plants, whilst transgenic crop plants in the field testing for better performance and productivity is still minimal. In addition, the better understanding of the specific roles of various metabolites which are involved in crop stress tolerance mechanism can give rise to a strategy for the metabolic engineering of crop tolerance of drought (Babu & Henry, 2006).

Through transcriptomics and proteomics studies, the activation and regulation of a number of stress inducible genes have been identified, which are usually classified into two major categories on the bases of their involvement in stress tolerance mechanisms. One group is involved in signaling cascades and in transcriptional control, whereas the other group participate in membrane protection, as osmo-protectants, as antioxidants and as reactive oxygen species (ROS) scavengers. Manipulation of genes from both of these groups has been the major target of attempts to produce plants with enhanced stress tolerance (Babu & Henry, 2006).

Modification of plants for enhanced drought tolerance is mostly based on the manipulation of either signalling or transcription factors or genes that directly protect plant cells against water deficit, but a full understanding of the molecular and

biochemical mechanisms for drought stress perception, transduction and tolerance is still a major challenge in plant biology (Babu & Henry, 2006).

The ability of a plant to avoid or repair the membrane damage during dehydration or rehydration processes is essential for the maintenance of membrane integrity, especially for those that embed functional proteins, such as water transporters, which play important role in the regulation of plant water status as well as transport of other metabolites. Some of the mechanisms leading to adaptation to dehydration or rehydration, has been possible by the identification of key genes and emphasis is given to the promising technologies of genetic engineering in crops, using regulatory or functional genes, such as the transfer and expression of transcription factors in modified plants to alter metabolism and increase plant tolerance to drought (Chaves & Oliveira, 2004).

1.4.3 Cell signaling and gene regulation under stresses

Gene expression profiling using micro-arrays or cDNA technology has developed the basic understanding of gene regulatory networks in plants under various stresses (Bray, 2004; Denby & Gehring, 2005; Shinozaki, Yamaguchi & Seki, 2003). There are a number of genes that are early responsive dehydration (erd) genes and responsive to dehydration (rd) genes in the model plant Arabidopsis (Shinozaki & Yamaguchi, 1996). At least four independent regulatory systems for gene expression in response to dehydration stress have been identified, two are abscisic acid (ABA) independent and two are ABA dependent pathways (Shinozaki & Yamaguchi-Shinozaki, 2000).

In the ABA-independent regulatory systems, a cis-acting dehydration responsive element/C-repeat (DRE/CRT), is involved, which was confirmed by over-expression of

the DRE/CRT-binding protein DREB1/CBF in transgenic Arabidopsis plants where changes in the expression of more than 40 stress-inducible genes were identified, which led to the increased freezing, drought and salt tolerance (Maruyama et al., 2004; Seki et al., 2001). Arabidopsis genes CBF3 and ABF3 that function in ABA-independent and ABA dependent stress-response pathways, respectively were tested in transgenic rice (Oh et al., 2005). The over-expression of these genes improved the drought and high salinity tolerance while slightly improving low temperature tolerance (Babu & Henry, 2006; Oh *et al.*, 2005).

1.4.4 Engineering for osmo-protectant accumulation

Osmo-protectants are the small neutral molecules in the cell which at molar concentration are not toxic to the cell, and they play an important role to stabilize proteins and cell membranes against the denaturing effect of stress conditions on cellular functions (Yancey, 1994). Generally, the osmolytes are contained in the cytoplasm of the plant cells and their active accumulation decreases the osmotic potential of the cells and maintains the cell turgidity (Pathan, Brigitte & Subudhi., 2004). Osmoprotectant accumulation, however, does not always lead to osmotic adjustment in cells in response to stress, and they also play a role in other ways, such as the scavenging of ROS, and chaperone-like activities that protect protein structure and metabolic detoxification (Serraj & Sinclair, 2002).

Some important crops lack the capability to synthesize the particular osmoprotectants which are accumulated naturally in stress-tolerant plants. Therefore, a potential strategy for improving the stress tolerance of crop plants could be enhanced by engineering the introduction of osmoprotectant synthesis pathways in the abiotic stress susceptible plants (Rathinasabapathi, 2000). Genetic engineering for the production of osmolytes

such as mannitol, fructans, trehalose and proline etc might increase resistance to dehydration, but the complete mechanisms are still not completely identified through which these osmolytes provide protection to the cell under dehydration stress (Ramanjulu & Bartels, 2002).

1.4.5 Mannitol

Mannitol is an important photosynthetic product in higher plants and some algae, which enhances tolerance to dehydration stress mainly through osmotic adjustment (Loescher et al., 1992). The introduction of a mannitol dehydrogenase (mtlD) gene into wheat showed a substantial increase in dehydration stress tolerance (Abebe et al., 2003). However, there was no significant difference in osmotic adjustment between the mtlD transgenic wheat and control plants, at either the callus or whole-plant level and it is suggested that protective mechanisms other than osmotic adjustment are likely to be involved in the scavenging of hydroxyl radicals (OH) or the stabilization of macromolecules. Another example is the transgenic tobacco, where the mannitol protected thioredoxin, ferredoxin, and glutathione and the thiol-regulated enzyme phosphor ribulo kinase from the effects of OH (Shen, Jensen & Bohnert, 1997).

1.4.6 Raffinose, galactionol, fructan and trehalose

Dehydration stress induce the synthesis of metabolically important carbohydrates in plants to facilitate adaptation of plants under stress conditions (Pattanagul & Madore, 1999) such as raffinose-family oligosaccharides, like raffinose, stachyose and galactinol, play vital roles in the dehydration tolerance in plants. Seven galactinol synthase (GolS)-related genes have been identified in Arabidopsis but their roles in accumulation of galactinol and raffinose in plants under dehydration stress is still not clearly known (Taji et al., 2002).

Over-expression of the AtGolS1 and AtGolS2 genes in Arabidopsis showed enhanced tolerance to drought stress in relation to the galactinol and raffinose accumulation in transgenic plants. The endogenous production of these sugar compounds provided membrane protection and a reduced rate of transpiration, which resulted in dehydration tolerance, thus, galactinol and raffinose act not as osmo-protectants but rather by osmotic adjustment to provide an adaptation to water stress conditions (Taji et al., 2002).

Fructans are poly-fructose molecules, located in the vacuoles and its metabolism plays a significant role in drought- and cold-stress tolerance in plants (Vereyken et al., 2003). These compounds are soluble, and play an important role in osmotic adjustment. Transgenic sugar beet and tobacco plants that were engineered with the bacterial fructan gene showed increased tolerance to drought stress (Pilonsmits et al., 1995; Pilonsmits et al., 1999).

Trehalose (a-D-glucopyranosyl-1,1-a-D-glucopyranoside) is a disaccharide present in many plants that functions as a stress protectant, stabilizing proteins and membranes and protecting them from denaturation (Goddijn & Van, 1999). Transgenic plants that expressed the trehalose biosynthesis genes resulted in the accumulation of trehalose and an elevated level of drought-stress tolerance. Metabolic engineering for the accumulation of trehalose in plants has led phenotypic abnormalities which were noticed in some cases (Avonce et al., 2004; Penna, 2003).

1.4.7 Proline

Proline is thought to play a role as an osmo-regulatory solute in drought and salt stresses and this amino acid could be part of a general adaptation to several environmental stresses like low temperature, nutrient deficiency, exposure to heavy metals and high acidity (Ashton & Desh, 1993). Plants accumulate proline on exposure to cold acclimation (Wanner & Junttila, 1999a) and the proline accumulation under stress was reported for the first time in plant tissues of rye grass (Kemble & MacPherson, 1954).

The dehydration stress damage in plants can be very much reduced with the accumulation of proline. Its accumulation decreases osmotic potential in the cytosol and facilitate water uptake along with other functions like protecting proteins from misfolding and overcoming the toxic effect of ROS (Xiong & Zhu, 2002). The biosynthetic pathway of proline in plants has been well characterized as shown in Figure 7 (Delauney & Verma, 1993; Nanjo et al., 1999) and its involvement in the response to dehydration stress has been demonstrated in transgenic tobacco when proline biosynthesis enzymes genes were over-expressed in transgenic plants (Kavi Kishor et al., 1995; Roosens et al., 2002). And in contrast its suppression in transgenic plants increased sensitivity to water deficit (De, Spreeth & Cress, 2000). Other examples are the transgenic petunia and soybean plants that over-expressed the P5CS gene from Arabidopsis and rice respectively and showed drought tolerance over wild type plants (Simon et al., 2005; Yamada et al., 2005).

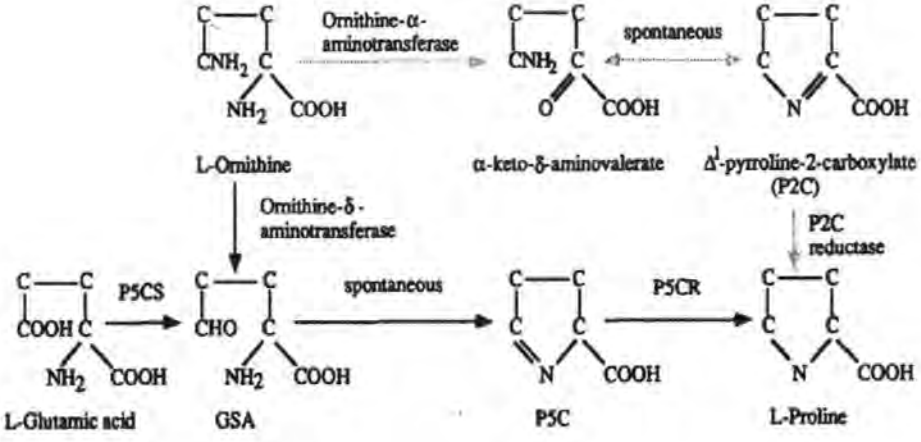


Figure 7: Proline biosyntheses pathways in plants.
The proline is synthesed in plants via P5C and not P2C. P5CS=pyrroline-5-carboxylate synthetase, P5CR= pyrroline-5-carboxylate reductase. (Delauney & Verma, 1993)

1.4.8 Functions of drought-inducible genes

The products of drought stress-inducible genes are classified into two groups (Figure 8). The first group includes proteins that probably function in stress tolerance and known as functional proteins, while the other group include regulatory protein that are involved in further regulation of signal transduction and gene expression that function in stress response (Shinozaki & Yamaguchi, 2007)

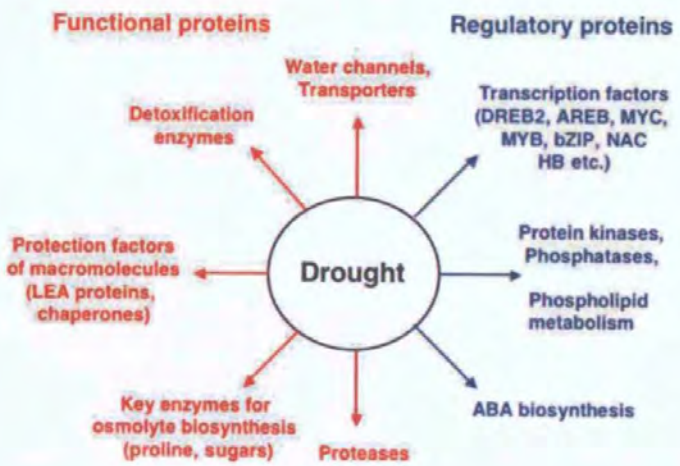


Figure 8: Drought stress-inducible genes products.
Source (Shinozaki & Yamaguchi, 2007)

1.5 Transcriptional factors and abiotic stresses

Transcriptional regulation is essential for plant adaptation to abiotic stresses (Ji, Dai & Hong, 2007a). Many plants can tolerate dehydration stresses by activating transcription of genes that cause biochemical and physiological changes (Steponkus, Uemura & Webb, 1993; Thomashow, 1999). ABRE and DRE/CRT function in ABA dependent and ABA-independent gene expression, respectively, in response to dehydration stress as sown in Figure 9 (Shinozaki & Yamaguchi, 2007).

Transcription factors that belong to the ERF/AP2 family which bind to DRE/CRT elements have been isolated and named as CBF/DREB1 and DREB2 (Yamaguchi & Shinozaki, 2005) and their conserved DNA-binding motif is A/GCCGAC. The CBF/DREB1 genes are induced by cold stress and their products activate the expression of stress responsive genes (Jaglo-Ottosen *et al.*, 1998b; Kasuga *et al.*, 1999; Liu et al., 1998b).

Over-expression of CBF/DREB1 in transgenic plants has increased tolerance to freezing stress, suggesting that the CBF/DREB1 proteins function in the development of cold-stress tolerance without modification (Liu *et al.*, 1998b). A number of CBF/DREB1 target genes have been identified in plants using cDNA and Gene Chip microarrays (Fowler & Thomashow, 2002a; Maruyama *et al.*, 2004; Seki *et al.*, 2001; Vogel et al., 2005b).

The DREB2 genes are induced by dehydration stress and activate expression of other genes that are involved in drought and salt stress tolerance (Liu *et al.*, 1998b; Shinozaki & Yamaguchi, 2007). Such an active form of DREB2 was shown to activate target stress-inducible genes and found improved drought tolerance in transgenic Arabidopsis (Sakuma et al., 2006).

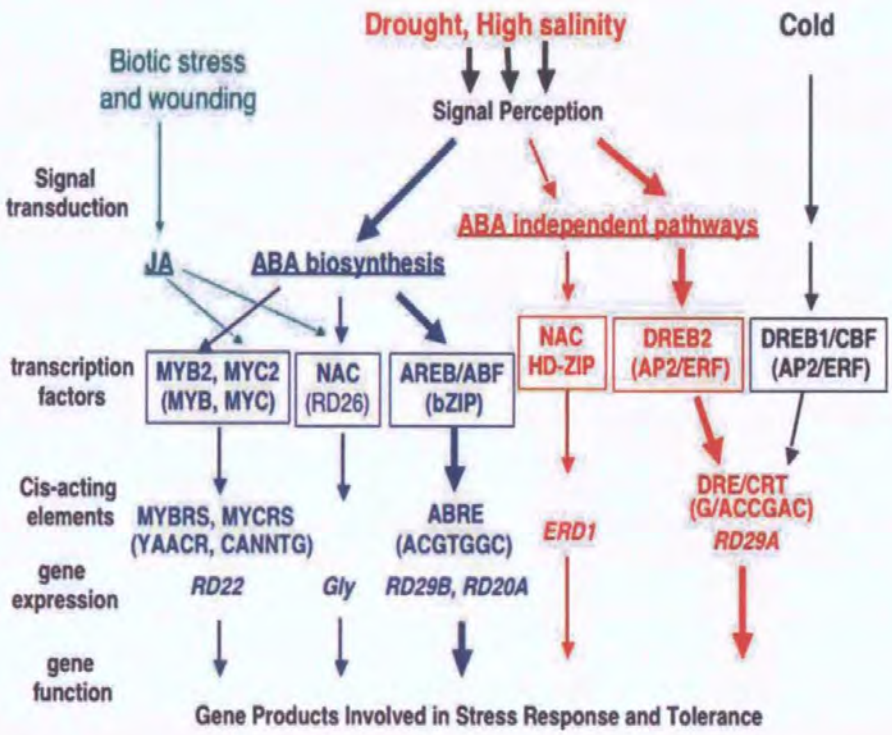


Figure 9: Transcriptional regulatory networks of abiotic stress signals and gene expression.

Six different signal transduction pathways exist in drought, high salinity, and cold stress responses: three are ABA dependent and three are ABA independent. In the ABA dependent pathway, ABRE functions as ABA-responsive element. MYB2 and MYC2 function in ABA-inducible gene expression. In one of the ABA-independent pathways, DRE is mainly involved in the regulation of genes not only by drought and salt but also by cold stress. DREB1/CBFs are involved in cold-responsive gene expression. DREB2s are important transcription factors in drought and high salinity stress-responsive gene expression. Another ABA-independent pathway is controlled by drought and salt, but not by cold. The NAC and HD-ZIP transcription factors are involved in ERD1 gene expression. Source (Shinozaki & Yamaguchi, 2007)

1.5.1 CBF/DREB (C-repeat binding/dehydration responsive element binding factor)

The name 'CBF' originates from the observation that AtCBF is a C-repeat (CRT) binding factor, a binding that results in the activation of genes with a CRT element in their promoter (Stockinger, Gilmour & Thomashow, 1997c; Thomashow, 1999). The DRE/CRT was identified as a *cis*-acting element regulating gene expression in response to dehydration (salt, drought, and cold stresses) in *Arabidopsis* (Yamaguchi & Shinozaki, 1994).

In plants, it is possible for a single transcription factor to control the expression of many target genes through the specific binding of the transcription factor to cis-acting element in the promoters of their respective target genes. This type of a transcription unit is called a "regulon." (Kazuo & Kazuko, 2006). A regulon can also be defined as a set of nonadjacent structural genes that are under the control of a common regulatory gene, the different structural genes of a regulon are located at different sites on a chromosome, or are scattered over several chromosomes as opposed to an operon (Stenish, 1975). Analysis of the expression mechanisms of osmotic stress- and cold stress-responsive genes revealed apparent presence of multiple regulons in Arabidopsis like (DREB1)/C-repeat (CRT)-binding factor (CBF) and DREB2 regulon involved in stress-responsive gene expression (Kazuo & Kazuko, 2006) .

Progress has been made towards understanding the role of CBFs/DREBs as described by various scientists that many plants can tolerate freezing and drought stresses by activating transcription of genes that cause biochemical and physiological changes such as changes in the composition of lipid membranes, increases in soluble protein content, in levels of molecules that can serve as protectants, such as sugars (Tabaei-Aghdaei,

Pearce & Harrison, 2003) and proline (Steponkus, Uemura & Webb, 1993; Thomashow, 1999). Two similar regulatory sequences, called C-repeat (CRT; TGGCCGAC) and dehydration-responsive element (DRE; TACCGACAT) were found in the promoters of such genes (Gilmour et al., 1998b; Liu et al., 1998a; Stockinger, Gilmour & Thomashow, 1997c).

Various investigators have demonstrated through gene fusion studies that the promoters of Arabidopsis COR15a (Baker, Wilhelm & Thomashow, 1994a), COR6.6 (Wang et al., 1995), and COR78 (Horvath, MacLarney & Thomashow, 1993; Yamaguchi & Shinozaki, 1993) genes are induced in response to low temperature. The cold regulatory element that appears to be primarily responsible for this regulation was identified by Yamaguchi and Shinozaki (1994) in RD29A (COR78) promoter. It is a 9-bp element, TACCGACAT, referred to as the DRE (dehydration responsive element). The DRE which has a 5-bp core sequence of CCGAC designed the CRT (C-repeat) which stimulates gene expression in response to low temperature, drought, and high salinity (Baker, Wilhelm & Thomashow, 1994a). The element is also referred to as the LTRE (low temperature regulatory element (Jiang, Iu & Singh, 1996; Nordin, Vhala & Palva, 1993).

Stockinger et al., (1997c) isolated the first cDNA for a protein that binds the CRT/DRE sequence. The designated protein CBF1 (CRT/DRE binding factor 1), has a mass of 24 kDa, a bipartite sequence, an acidic region that potentially serves as an activation domain, in addition it has an AP2 domain, a 60 amino acid motif that has been found in a large number of plant proteins including *Arabidopsis* APETALA2 (Jofuku et al., 1994b), AINTEGUMENTA (Klucher et al., 1996b), and TINY (Wilson et al., 1996a). Oham & Shinshi (1995) have demonstrated that the AP2 domain includes a DNA-

binding region. Such domain is present in over 140 proteins encoded in the Arabidopsis genome. These proteins are classified into five subfamilies based on the amino acid sequences of the AP2 domains (Sakuma et al., 2002b). A recent domain-swap study demonstrated that the N-terminal 115 amino acids are sufficient to both target CBF1 to COR gene promoters and enable binding to CRT/DRE elements, while the C-terminal 98 amino acids are sufficient for transcriptional activation (Wang et al., 2005).

Stockinger et al., (1997c) have reported that the CBF protein binds to the CRT/DRE sequence and activates expression of reporter genes in yeast carrying the CRT/DRE as an upstream regulatory sequence, which leads to confirm that CBF1 is a transcriptional activator that can activate CRT/DRE containing genes and thus was a probable regulator of COR gene expression in *Arabidopsis*. Jaglo *et al.,* (1998b) have demonstrated that constitutive over expression of CBF1 in transgenic Arabidopsis plants resulted in expression of CRT/DRE controlled COR genes without a low temperature stimulus. Thus CBF1 appears to be an important regulator of the cold acclimation response, controlling the level of COR gene expression which in turn promotes freezing tolerance.

Gilmour et al., (1998b) and Shinwari et al., (1998) have realized that CBF1 is a member of a small gene family encoding three closely relate transcriptional activators. The three genes referred to as either CBF1, CBF2 and CBF3 (Gilmour *et al.*, 1998b) or DREB1B, DREB1C and DREB1A respectively (Liu *et al.*, 1998a; Shinwari *et al.*, 1998) are physically linked in direct repeat on chromosome 4 near molecular markers PG11 and m600 (~71cM) (Liu *et al.*, 1998a; Shinwari *et al.*, 1998), and they are unlinked to their target CRT/DRE controlled genes, COR6.6, COR15a, COR47 and COR78 which are located on chromosomes 5,2,1 and 5 respectively (Gilmour *et al.*, 1998b) .

CBF2 and CBF3 proteins like CBF1 can activate expression of genes containing CRT/DRE in their promoter (Gilmour *et al.*, 1998b) , this was realised by a study on reporter genes in yeast that contain the CRT/DRE as an upstream activator sequence, these results indicate that these two family members are also transcriptional activators. Liu et al., (1998a) have shown that over expression of DREB1A/CBF3 in transgenic Arabidopsis plants enhanced both the freezing and drought tolerance in transgenic plants.

Various investigators have explored that transcripts for CBF1, CBF2 and CBF3 can accumulate to detectable levels within 15 min of exposing plants to low temperature (Gilmour *et al.*, 1998b; Jaglo-Ottosen *et al.*, 1998b; Liu *et al.*, 1998a; Medina et al., 1999) demonstrating extreme sensitivity to an environmental stimulus.

There are many published reports addressing that orthologs of *Arabidopsis CBF/DREB1* genes have been found in every higher plant that has sofar been examined (Benedict et al., 2006; Jaglo et al., 2001; Kayal et al., 2006; Nakashima & Yamaguchi, 2006a; Owens et al., 2002; Skinner et al., 2005). Recently a fourth CBF gene (CBF4) from grape (*Vitis riparia*) has been identified. The expression of the CBF4 gene was low at ambient temperature, but enhanced upon exposure to low temperature (4 °C) and this expression was maintained for several days in both young and mature tissue, in contrast to the previously described *Vitis CBF1, CBF2* and *CBF3* (Huogen et al., 2008).

Many researchers have demonstrated that the CBF pathway alone appears sufficient to increase abiotic stress tolerance since constitutive expression of *CBF* genes in transgenic *Arabidopsis* plants induces expression from CRT-containing genes and results in an increase in freezing and drought tolerance without prior stimulus (Gilmour

et al., 2000; Haake et al., 2002; Jaglo-Ottosen *et al.*, 1998b; Kasuga *et al.*, 1999). However, the presence of the CBF pathway is apparently not complete in all plants, either because CBF members are not activated in time or for a sufficiently long period, or they are not active, or the CBF regulon is smaller. For example, over-expression of Arabidopsis AtCBF3 or the tomato LeCBF1, while increasing stress tolerance in transgenic *Arabidopsis*, did not have the same effect in transgenic tomato (Zhang et al., 2004).

Liming *et al.*, (2002) conducted a reporter gene-aided genetic screen in Arabidopsis. They reported that seven allelic mutations in the FIERY2 (FRY2) locus resulted in significant increases in the expression of stress-responsive genes with the DRE/CRT (drought-responsive/C-repeat) cis element but non-DRE/CRT type stress-responsive genes were less affected. These results indicate that the presence of DRE/CRT sequence in regulatory sequences of target genes is essential for the expression of stress responsive genes.

Many researchers have noticed side effects of the over expression of DREB1A, DREB1B, or DREB1C in transgenic Arabidopsis in the form of dwarfism (Gilmour, Fowler & Thomashow, 2004; Gilmour *et al.*, 2000; Kasuga *et al.*, 1999; Liu *et al.*, 1998a). Similarly, the development of dwarf phenotypes was also found in transgenic tomato over expressing Arabidopsis DREB1B, and was prevented by exogenous application of gibberellins (Hsieh et al., 2002a; Hsieh et al., 2002b). This side effect was reduced by findings of Kasuga *et al.*,(2004) they reported improved drought and low-temperature stress tolerance in tobacco with minimized negative effects on growth by constitutive over expression of transgene Arabidopsis DREB1A, via the stress-inducible RD29A promoter.

The first CRT/DRE binding factor was discovered by using a reporter gene in yeast that carried the CRT/DRE element and CBF1 (CRT/DRE binding factor 1), and was initially shown to activate expression of the reporter gene (Stockinger, Gilmour & Thomashow, 1997c) and indicated that the protein, which has an AP2/EREBP DNA binding motif (Riechmann & Meyerowitz, 1998) is a transcriptional activator. Jaglo et al (1998b) reported that over-expression of CBF1 in Arabidopsis activates expression of the entire battery of known CRT/DRE regulated *COR* genes and to enhance whole plant freezing survival without a low temperature stimulus. All of the three *CBF* genes of Arabidopsis are cold-induced and *CBF* transcript levels increases within 15 min of transferring plants to low temperature which followed at approximately 2 h by the transcript accumulation of CRT/DRE-regulated *COR* genes (Gilmour *et al.*, 1998b; Shinwari *et al.*, 1998). The mechanism whereby the *CBF* genes are activated by low temperature is not known but does not appear to involve autoregulation (Gilmour *et al.*, 1998b).

Jonathan et al (2005) indicated the existence of additional cold response pathways which may have important roles in plant life at low temperature. They studied CBF proteins and other transcription factors with roles in cold acclimation, they used the Affymetrix GeneChip containing probe sets for approximately 24000 Arabidopsis genes to define a core set of cold-responsive genes and to determine which genes were targets of CBF2 and six other transcription factors that appeared to be co-ordinately regulated with CBF2, for this investigation. A total of 514 genes were placed in the core set of cold-responsive genes, 302 of which were upregulated and 212 downregulated. Through hierarchical clustering and bioinformatic analysis, they found 84 % genes induced by CBF2 and 8 % were regulated by both CBF2 and ZAT12. They concluded that the large majority (92%) of the most highly induced genes belong to the CBF and ZAT12 regulons.

In Arabidopsis cold acclimation involves action of the CBF pathway (Thomashow, 2001). This pathway comprises the CBF1, CBF2, and CBF3 genes (Gilmour *et al.*, 1998b; Jaglo *et al.*, 2001; Medina *et al.*, 1999), also known as DREB1b, DREB1c, and DREB1a, respectively (Liu *et al.*, 1998a), which encode transcriptional activators that bind to the C-repeat (CRT)/dehydration response element (DRE) regulatory element present in the promoters of COR and other cold responsive genes (Baker, Wilhelm & Thomashow, 1994a; Gilmour *et al.*, 1998b; Stockinger, Gilmour & Thomashow, 1997c; Yamaguchi & Shinozaki, 1994) .

Constitutive expression of the CBF genes can increase the constitutive expression of the CBF regulon, which enhances the freezing tolerance without a low-temperature stimulus (Gilmour, Fowler & Thomashow, 2004; Gilmour *et al.*, 2000; Jaglo-Ottosen *et al.*, 1998b; Liu *et al.*, 1998a) . The freezing tolerance was conferred by the CBF regulon involves the production of cryoprotective polypeptides such as COR15a (Artus et al., 1996a; Steponkus et al., 1998a) and the accumulation of compatible solutes such as sucrose, raffinose, and proline (Gilmour, Fowler & Thomashow, 2004; Gilmour *et al.*, 2000; Nanjo *et al.*, 1999).

Fowler and Thomashow (2002b) investigated and found the expression of about 8000 Arabidopsis genes in response to low temperature, their results indicated that extensive changes occur in the transcriptome during cold acclimation. However, only about 12 % of these genes were assigned to the CBF regulon and at least 28% of the cold-responsive genes were not affected by the expression of CBF transcription factors. It was concluded that cold acclimation is associated with the activation of multiple low temperature regulatory pathways.

1.5.2 AP2- EREBPs

AP2 (APETALA2) and EREBPs (ethylene-responsive element binding proteins) are the members of a family of transcription factors in plants and their unique characteristic is that they have the AP2 DNA-binding domain. They play a variety of roles throughout the life cycle of the plant, from being key regulators of several developmental processes, like floral organ identity determination or control of leaf epidermal cell identity, to forming part of the mechanisms used by plants to respond to various types of biotic and abiotic environmental stresses (Riechmann & Meverowitz, 1998).

The AP2 domain was first identified as a DNA binding domain conserved in a family of tobacco ethylene responsive element binding proteins (EREBPs) (Ohme-Takagi & Shinshi, 1995), and later found, conserved in Arabidopsis APETALA2 (AP2) a gene which is involved in flower development (Jofuku et al., 1994a).

The number of different proteins containing an AP2 like domain in plant species appears to be strikingly large and the number of genetically and biochemically characterized AP2 like domain containing protein is increasing. These include Arabidopsis AINTEGUMENTA (ANT) a gene that regulates flower development and is vital for ovule formation (Elliott et al., 1996; Klucher et al., 1996a), TINY, a gene that suppresses cell proliferation during both vegetative and floral organogenesis (Wilson et al., 1996b) and CBF (Stockinger, Gilmour & Thomashow, 1997a). The AP2 domain is ~ 60 amino acid residue domain found in transcription factor proteins which can bind to DNA.

The APETALA2 (AP2) domain also known as the AP2/ethylene-responsive element–binding factor (ERF) domain or ERF/AP2 domain, which defines a large gene family of

DNA-binding proteins called AP2/ERF. AP2/ERF genes are divided into different classes on the bases of the number of AP2 domains present. One class encodes a protein containing two AP2 domains (Jofuku et al., 1994a) like AINTEGUMENTA (ANT) (Elliott *et al.*, 1996; Klucher *et al.*, 1996a) and Glossy15 (GL15) (Moose & Sisco, 1996). The second class encodes a protein with only one AP2 domain (Ohme-Takagi & Shinshi, 1995) like TINY (Wilson *et al.*, 1996b), AtEBP (Buttner & Singh, 1997), and ABI4 (Finkelstein et al., 1998). A third class of AP2/ERF genes, RAV1 and RAV2 (Kagaya, Ohmiya & Hattori, 1999) encode proteins that have two different DNA-binding domains, AP2 and B3 (Giraudat et al., 1992).

The Arabidopsis AP2 is a well studied gene among the AP2/ ERF family. There are two AP2 domains, and each domain contains 68 amino acids with an 18 amino acids core region that forms an amphipathic a-helix (Allen et al., 1998; Jofuku et al., 1994a). These domains are essential for AP2 function (Jofuku et al., 1994a). In addition to AP2, other genes encoding the AP2 domain have been well studied in Arabidopsis. These include ANT (AP2-like), a gene that regulates floral organ growth (Elliott *et al.*, 1996; Klucher *et al.*, 1996a) and CBF1 (ERF-like).

Following the complete sequencing of the Arabidopsis genome, Riechmann et al., (2000) searched for AP2/ERF in the Arabidopsis genome and found 144 AP2/ERF genes. Sakuma et al., (2002a) classified AP2/ERF genes of Arabidopsis in five classes based on the similarities in their DNA-binding domains: AP2 subfamily (14 genes), RAV subfamily (6 genes), DREB/CBF subfamily (55 genes), ERF subfamily (65 genes), and others (the fifth group; 4 genes).

1.6 Cold acclimation and frost stress tolerance in plants

In many plants, a period of exposure to low non freezing temperatures results in an increased level of freezing tolerance, the phenomenon is known as cold acclimation (Levitt, 1980; Sakai & Larcher, 1987; Smallwood & Bowles, 2002; Thomashow, 1999). Different tissues within an organ respond to cold in different way and express different genes, indicating that the whole plant could display a combination of requirements for cold acclimation not displayed by a single tissue or a cell culture (Pearce et al., 1998). Extensive effort has been made to understand the biochemical and molecular basis of cold acclimation response, and a variety of changes have been exposed that occur during cold acclimation, including alterations in lipid, protein, and carbohydrate composition (Guy, 1990; Steponkus & Lynch, 1989; Thomashow, 1990). While sensing the cold, an influx of calcium into the cytosol is the starting point of signaling pathways (Monroy & Dhindsa, 1995), followed by the regulation of downstream genes as follows: activation of transducers, expression of inducer of CBF (C-repeat binding factor) expression (ICE), induction of CBF genes, and finally expression of downstream COR (cold-regulated) genes (Chinnusamy, Zhu & Zhu, 2006). Even though the whole molecular mechanism of cold acclimation is still not well understood, a specific profile of gene expression has been observed during this process (Chunzhen et al., 2009).

Once it was recognized that changes in gene expression take place through cold acclimation this opened a floodgate of effort by investigators to identify and characterize cold-responsive genes (Guy, Niemi & Brambl, 1985; Pearce *et al.*, 1998; Thomashow, 1999) and through investigations on cold-regulated gene expression in Arabidopsis, a family of transcriptional activators, the CBF/DREB1 was discovered, that have a key role in cold acclimation (Thomashow, 2001). Some cold-responsive genes continue a high expression level until cold temperatures are removed, while

others are only expressed transiently (Tang et al., 2005; Xiong & Fei, 2006). These responses are activated soon after sensing cold (Thomashow, 1999).

Freezing injury in plants largely results from the cellular dehydration that occurs upon ice formation, and the consequent physical damage to cellular membranes (Atici & Nalbantoglu, 2003; Griffith et al., 1997; Thomashow, 2001). The expression of the second group of genes minimize the dehydration injury by up-regulation of cold-regulated (COR), dehydration-responsive, and ice re-crystallization inhibition (IRI) genes to limit ice crystal growth, and regulation of photosynthesis and respiration-related genes which are important in increasing freezing tolerance in important assimilatory mechanisms (Chunzhen et al., 2009).

The freezing tolerance acquired through cold acclimation is not static but varies seasonally and is lost quickly when plants are returned to a warm temperature and it is a photosynthetic activity-demanding process (Griffith & Mcintyre, 1993; Wanner & Junttila, 1999). For successful cold acclimation, moderate to high light conditions are essential, which otherwise expose the plant to photo-inhibition and can lead to formation of reactive oxygen species (ROS) (Foyer, Lelandais & Kunert, 1994; Wanner & Junttila, 1999).

A plants capability to cold-acclimate is a polygenic trait and various physiological and biochemical changes occur during cold acclimation as shown in Figure 10. The most remarkable changes include reduction in growth and tissue water content (Levitt, 1980), increase in abscisic acid (ABA) levels (Chen, Brenner & Li, 1983), changes in membrane lipid composition (Lynch & Steponkus, 1987; Uemura & Steponkus, 1994), the accumulation of compatible osmolytes such as proline, betaine, polyols and soluble

sugars, and increased levels of antioxidants (Dorffling et al., 1997; Kishitani et al., 1994; Koster & Lynch, 1992; Murelli et al., 1995; Nomura et al., 1995; Tao, Oquist & Wingsle, 1998).

Cell membranes are the primary sites of freezing injury and changes in membrane to develop freezing tolerance is critically important, ultra-structural changes in plasma membrane have been observed within 6 h of the cold acclimation in *Arabidopsis* (Ristic & Ashworth, 1993). Variations in membrane lipid composition are correlated with membrane cryostability and have been observed in all investigated plants during cold acclimation (Steponkus, 1984; Uemura et al., 1995; Uemura & Steponkus, 1994).

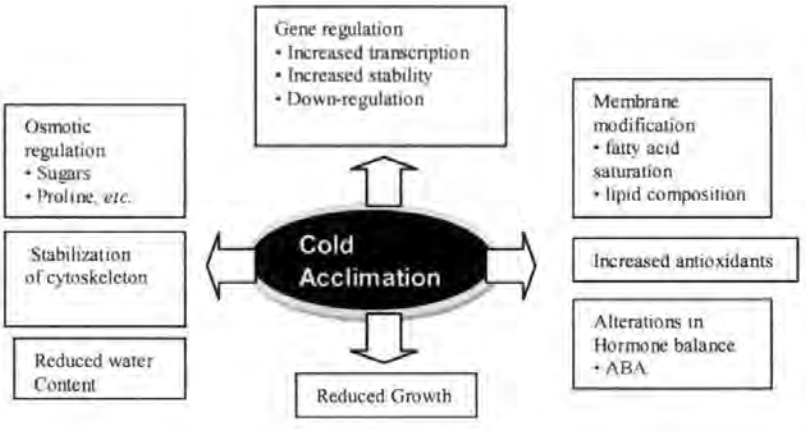


Figure 10: Cold acclimation induces changes in cellular processes.
Different responses are observed while exposing plants to low non-freezing temperatures. Modified from (Xin & Browse, 2000).

1.6.1 Regulation of genes expression in response to low temperature

Cold acclimation involves numerous genes expression either up or down-regulated (Fowler & Thomashow, 2002a; Pearce *et al.*, 1998; Seki et al., 2002; Seki *et al.*, 2001; Xiong, Schumaker & Zhu, 2002). Some of the cold-induced genes are also up-regulated by drought or salt stress (Kreps et al., 2002a; Nuotio, Heino & Palva, 2001; Seki *et al.*, 2002; Seki *et al.*, 2001; Shinozaki, Yamaguchi & Seki, 2003; Thomashow, 1999). The expression of *COR* genes is regulated by the transcription factor DREB/CBF which binds to DRE/CRT in the promoter of COR genes (Ishitani et al., 1997; Shinozaki & Yamaguchi-Shinozaki, 2000). The genes expression profiles show the activation of multiple regulatory pathways during cold acclimation which indicate that cold-induced genes can be members of more than one cold regulon (Fowler & Thomashow, 2002a).

1.6.2 CBF cold response pathway

The cold responsive *CBF/DREB1* genes are induced transiently by cold and their expression regulates COR genes expression (Gilmour et al., 1998a; Liu *et al.*, 1998a; Medina *et al.*, 1999). There are three different cold-inducible *CBF/DREB1* genes, have been identified in Arabidopsis known as *CBF1/DREB1B*, *CBF2/DREB1C* and *CBF3/DREB1A* (Thomashow, 2001). Over-expression of CBF1 conferred freezing-tolerance in Arabidopsis (Pearce, 1999) and the expression of CBFs/DREB1s in transgenic plants has shown the activation of downstream cold-responsive genes even at warm temperatures and improved freezing, drought and salt tolerance (Hsieh *et al.*, 2002b; Jaglo-Ottosen et al., 1998a; Kasuga *et al.*, 1999; Liu *et al.*, 1998a).

The orthologues of Arabidopsis CBF/DREB1 have been identified in other plant species (Jaglo et al., 2001) and suggesting the CBF transcriptional cascade is highly conserved in the plant kingdom during cold stress. The *CBF/DREB1* genes expression related to temperature changes such that the lower the temperature the higher the *CBF*

transcription, but the expression of *CBF* genes becomes desensitized at a given low temperature, and resensitization requires exposure to a higher temperature (Zarka et al., 2003).

The CBF genes expression is apparently repressed by either their own products or the products of their downstream target genes, which ensuring transient and tightly controlled expression of these genes (Chinnusamy et al., 2003; Guo et al., 2002) as shown by Novillo et al. (2004), who found *CBF1/DREB1B* and *CBF3/DREB1A* negatively regulated by the expression of *CBF2/DREB1C*.

The transcript levels for all three *CBF* (CBF 1, 2, 3) genes increases within 15 min exposure to cold temperatures, which is then followed by the induction of CBF mediated COR genes at about 3 h (Xin & Browse, 2000). The expression of *COR* genes is induced by both the *CBF1 (*ABA-independent) pathway and by the bZIP-mediated ABA-dependent pathway (Gilmour & Thomashow, 1991; Mantyla, Lang & Palva, 1995; Nordin, Heino & Tapio, 1991). The constitutively freezing-tolerant mutant *esk1* accumulates high levels of proline but does not constitutively express the *COR* genes (Xin & Browse, 2000).

The existence of a transcription factor designated ICE, which acts at the CBF/DREB promoters upon exposing plants to low temperature; the ICE becomes activated and stimulates transcription of the *CBF/DREB* genes followed by induction of the CBF regulon as shown in Figure 11. (Thomashow, 2001) subsequently increases the freezing tolerance.

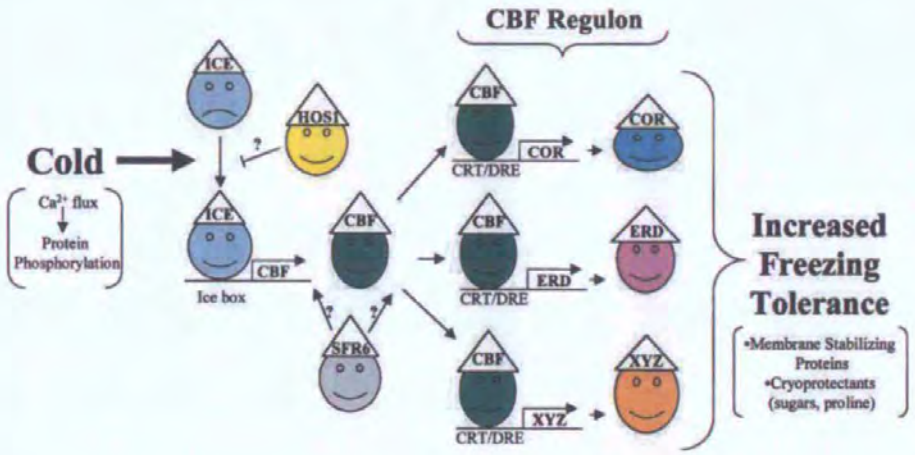


Figure 11: CBF cold acclimation pathway (CBF regulon).
Low temperature leads to rapid induction of the CBF/DREB1 genes followed by the expression of the CBF regulon of CRT/DRE-regulated genes. CBF regulon includes COR, ERD, and presumably non-discovered ("XYZ") cold-regulated genes, increases freezing tolerance in plants. Low temperature either activate the ICE protein or other protein(s) with which it interacts. Such activation involves alterations in protein phosphorylation by a cold-induced influx of calcium. The SFR6 protein appears to act between CBF/DREB1 transcription and induction of the CRT/DRE-regulated genes whereas HOS1 appears to act upstream of CBF transcription (Thomashow, 2001).

Using the PCBF3::LUC bioluminescent genetic screen, Chinnusamy *et al.* (2003) also identified an upstream transcription factor Inducer of CBF Expression1 (ICE1) in Arabidopsis. They investigated that a dominant ice1 mutation blocked the expression of CBF3 and decreased the expression of many CBF-target genes. The ice1 mutant showed impaired chilling tolerance and cold acclimation, while constitutive over expression of ICE1 enhanced the expression of CBFs and constitutively expressed COR genes but activation of CBF expression required cold treatment. These results show that ICE1 is a master switch that controls many cold-responsive CBF-dependent regulons. Probably, ICE1-like bHLH transcription factors may be involved in the regulation of CBF1 and/or CBF2 (VanBuskirk & Thomashow, 2006). It can be considered that regulon biotechnology is hoped in the future to contribute positively to sustainable food production by increasing abiotic stress resistance in plants.

1.6.3 Calcium role in CBF regulon pathway

However, little is known about how the cold signal is perceived and how the CBF genes themselves are regulated (Nancy, 2009). Calcium was thought to be involved in cold acclimation process and a rapid increase in free calcium in the cytoplasm on low temperature exposure is found. This free Ca came from extra cellular and intracellular calcium stores and might be induced the CBF regulon (Knight & Knight, 2000; Knight, Trewavas & Knight, 1996).

The evidence of a link between calcium signalling and cold induction of the CBF pathway was provided with the discovery of calmodulin binding transcription activator (CAMTA) factors which bind to a regulatory element in the CBF2 gene promoter which play a role in controlling the CBF regulon and freezing tolerance (Doherty et al., 2009). The calmodulin is Ca binding protein and CAMTA proteins are calmodulin binding

transcription factors, they play direct role in transduction of low temperature induced cytosolic calcium signals into downstream regulation of gene expression (Doherty et al., 2009; Nancy, 2009).

The peak levels and duration of the calcium influx is altered upon cold acclimation and becoms reduced in amount and prolonged in length, and the expression of certain cold-regulated genes, including *COR* and other *CBF*-targeted genes, appears to involve the action of calcium as a second messenger (Daniel G. Zarka, 2003; Knight, Trewavas & Knight, 1996). Thus when it is shown that cold-induced calcium influx was inhibited using chemical agents, the expression of the *COR* genes was weakened but when the chemical agents were used to raise intracellular calcium levels at warm temperatures, the *COR* gene expression was induced (Daniel G. Zarka, 2003; Knight, Trewavas & Knight, 1996; Tahtiharju et al., 1997).

1.6.4 Half-Life of CBF Transcripts at warm temperatures

The *CBF* transcripts at warm temperatures has a very short half-life, about 7.5 min at warm temperatures, this value is amongst the shortest half life for described plant genes (Daniel G. Zarka, 2003). The CBF genes promoters could become inactive promptly within minutes of transferring plants from low to high temperatures (Daniel G. Zarka, 2003). Similarly, no transcripts were detected 90 min after transferring plants from cold to warm temperatures (Daniel G. Zarka, 2003).

1.6.5 Regulation of the CBF pathway

There are no evident DRE/CRT elements in the promoter regions of *CBFs* genes that indicate that, these genes do not appear to be controlled by auto-regulation (Gilmour *et al.*, 1998a). Factors controlling the cold induced expression of CBFs have been

identified; one example is ICE (inducer of CBF expression) which was identified through mutational screening. On the other hand, *ice1* showed minor effect on cold-induced accumulation of *CBF2* transcripts, which indicate that differences in the activation mechanisms exist within the *CBF/DREB1* family (Chinnusamy et al., 2003). The over-expression of *ICE1* enhances the expression of the *CBF* regulon and improves freezing tolerance in transgenic plants (Chinnusamy et al., 2003).

LOS4 is another gene that encodes DEAD-box RNA helicase, and plays a positive role in CBF expression. In *los4-1* mutant plants the expression of *CBFs*, their downstream target genes and cold acclimation were impaired (Gong et al., 2002). *los4-1* plants were highly sensitive to chilling when exposed to cold in darkness. This could be specifically due to the impaired expression of *CBF2* in *los4-1* plants, as *CBF2* was expressed in wild-type Arabidopsis plants when exposed to cold in darkness (Gong et al., 2002).

It has been recognized that the promoters of the *CBF1/DREB1b*, *CBF2/DREB1c*, and *CBF3/DREB1a* are responsive to low temperatures (Shinwari et al., 1998) and a transcription factor, ICE1 (Inducer of CBF Expression 1) has a role in *CBF* expression (Chinnusamy et al., 2003). As shown that transferring of Arabidopsis plants immediately from 20 °C to 4°C results in the rapid accumulation of *CBF* transcripts (Gilmour *et al.*, 1998a; Liu *et al.*, 1998a; Medina *et al.*, 1999). The *CBF* transcript levels reached a maximum at about 3 h and then significantly declined, but remained elevated over those found in warm grown plants over the course of the 3-week experiment (Daniel G. Zarka, 2003).

The amount of the cold shock affects the *CBF* transcripts levels such as, that when plants were transferred from 20 °C to 10 °C, the *CBF* levels after 2 h were less than the

plants when transferred from 20 °C to 4 °C. Similarly, higher levels of *CBF* transcripts were found in plants transferred from 20 °C to -5 °C when compared with the 20 °C to 4 °C treatment (Daniel G. Zarka, 2003). The threshold temperature for CBF induction was observed 14 °C at which the accumulation of *CBF* transcripts became detectable (Daniel G. Zarka, 2003). As the temperatures continued to drop, the levels of *CBF* transcripts continued to increase along with an increase in the transcript levels of the *CBF* target gene, *COR15a* (Daniel G. Zarka, 2003). Plant cold acclimated at 4 °C for 14 d when transferred to 0 °C or -5 °C, an increase in *CBF* levels observed as well as when subjected to a gradual decrease in temperature from 20 °C to 10 °C, the accumulation of *CBF* transcripts observed but the transcript levels declined upon continued exposure to this temperature (Daniel G. Zarka, 2003). While on renewal of a gradual decrease in temperature, the *CBF* transcripts again increased (Daniel G. Zarka, 2003).

1.6.6 Functions of the CBF Regulon

The fundamental function of the CBF regulon in plant is to protect cells against freezing and other stresses involving dehydration (Thomashow, 2001). There are about six CRT/DRE-controlled genes have been identified which are *KIN1*, *COR6.6/KIN2*, *COR15a*, *COR47/RD17*, *COR78/RD29a*, and *ERD10* (Steponkus *et al.*, 1998a; Thomashow, 2001). The over-expression of *COR15a* gene increases the freezing tolerance by 1 to 2 °C in non-acclimated plants (Artus et al., 1996b; Thomashow, 2001) through decreasing the membrane tendency to form detrimental hexagonal II phase lipids upon freeze-induced dehydration (Steponkus *et al.*, 1998a). Plants over-expressing *CBF* not only increase the levels of COR proteins, but also elevate the levels of proline and total sugars (Gilmour et al., 2000). Such increase in proline and sugars levels occur in a wide variety of plants under cold acclimation, that contribute to the enhancement of freezing tolerance (Thomashow, 2001).

1.6.7 Plant breeding and freezing tolerance

Freezing tolerance in plant is a complex trait with multigenetic inheritance (Thomashow, 1990) and plants vary in inherent freezing tolerance before cold acclimation as well as varying in the potential to acquire freezing tolerance during cold acclimation (Xin & Browse, 2000). Crosses of two potato species with different freezing tolerance established that these traits are controlled by different sets of genes (Stone et al., 1993) and this finding is very important to show that it may be possible to manipulate different aspects of freezing tolerance and combine them to make a significant improvement in freezing tolerance.

Although it is not clear that how many genes are involved in freezing tolerance, progress in mapping of quantitative trait loci has permitted the identification of major loci that have a great effect on freezing tolerance (Galiba et al., 1995; Pan et al., 1994). Mapping of major loci may ultimately lead to the identification of genes contributing to freezing tolerance and the identification of these genes may allow the study of naturally evolved mechanisms of freezing tolerance, while the cloning of these quantitative trait loci could be a huge endeavour (Xin & Browse, 2000).

1.6.8 Freezing injuries in plant

Freezing temperatures induce ice formation in the intercellular spaces and cell walls, because of the higher freezing point of intercellular than cytoplasmic fluid. In addition, the intercellular fluid also contains heterogeneous ice nucleating agents, such as dust and ice-nucleating proteins (Brush, Griffith & Mlynarz, 1994). In the absence of these ice-nucleating agents, pure water remains as a super-cooled liquid until -39 °C, which is the temperature at which water freezes in the absence of any heterogeneous nucleating agents (Xin & Browse, 2000).

The ice-state of water has a much lower water potential than liquid state and this difference increases as temperature decreases (Guy, 1990). So, when ice forms extra-cellularly, water potential outside the cell drops suddenly and consequently, the water from the cytoplasm moves to the outside through the plasma membrane by osmosis, causing cellular dehydration (Xin & Browse, 2000).

The removal of the net amount of water from the cell depends on the initial solute concentration of the cytoplasm and the freezing temperatures. For instance, it has been calculated that freezing to −10 °C causes −11·6 MPa water potential, that removes 90% of the osmotically active water from the cytoplasm. Due to accumulation of the solutes during cold acclimation, the same freezing temperature removes only 80% of cellular water in acclimated plants. As a consequence, freezing injury is mainly caused by cellular dehydration and, that's why freezing, drought and salt stresses share many characteristics (genes) in common (Xin & Browse, 2000).

Dehydration damages cellular functions in different ways. In the case of freezing stress, injury usually involves effects on plasma membrane structure and function (Uemura *et al.*, 1995; Webb, Uemura & Steponkus, 1994). The cell membrane has been considered as the primary site of freezing injury for many years (Levitt, 1980). Freezing-induced destabilization of plasma membrane involves lesions (Uemura *et al.*, 1995; Webb, Uemura & Steponkus, 1994) as demonstrated in non-acclimated protoplasts, where reduction in volume was observed at -5 °C due to invagination of the membrane followed by budding off of endocytotic vesicles and upon rewarming, the melted water was drawn back into the cells, but because of the irreversible loss of plasma membrane, the protoplast bursts before it regained the original volume due to the hydrostatic pressure created by the incoming water. This type of lesion is known as 'expansion-

induced lysis (Webb, Uemura & Steponkus, 1994). In contrast, protoplasts prepared from cold-acclimated leaves did not form endocytotic vesicles (Xin & Browse, 2000).

Certain lipids in non-acclimated plant cell membrane aggregate to form an inverted structure with hexagonal packing symmetry (HexII phase) arranged in cylinders of 20 Å in diameter, which disrupts the bilayer of cell membrane. Thus the plasma membrane becomes permeable to water and solutes upon re-warming and loses osmotic responsiveness (Uemura *et al.*, 1995; Webb, Uemura & Steponkus, 1994; Xin & Browse, 2000). In cold-acclimated cell membranes, freezing injury is associated with 'fracture-jump lesion' due to the localized fusion of the plasma membrane with other cellular membranes, especially the chloroplast envelopes (Webb, Uemura & Steponkus, 1994). It is believed that both of HexII and fracture-jump lesions are created from a common structural intermediate of membranes (Uemura *et al.*, 1995) but still it is not clearly understood why HexII lesions are observed only in non-acclimated tissues whereas fracture-jump lesions are observed exclusively in cold-acclimated tissues. In addition, the temperatures at which fracture-jump lesions are observed vary among plant species (Uemura *et al.*, 1995; Webb, Uemura & Steponkus, 1994) and little is known about the biochemical and molecular basis of this variation (Xin & Browse, 2000).

1.7 Cold regulated gene (COR15)

Plants have evolved various adaptive mechanisms to tolerate abiotic stresses, most higher plants due to their sessile nature, have developed more diverse strategies to acclimatize to abiotic stresses than animals (Seki et al., 2003).

A number of genes are involved to respond to abiotic stress (Thomashow, 1998). COR15 gene is one of these genes and was initially isolated from *Arabidopsis thaliana*.

This gene encodes a 15 kD protein having extensive resemblance with LEA (late embryogenesis abundant protein) in its amino acid sequence (Lin & Thomashow, 1992). LEA genes are specifically up-regulated during the dehydration phase of seed development and are assumed to help assist plants to protect their cells during dehydration.

Cor15 proteins are found in the stromal compartments of the chloroplasts, these proteins are involved in the tolerance mechanisms against dehydration stress in plants. The over expression of the COR15 gene can reduce the susceptibility of membranes to form hexagonal-phase lipids during freezing stress (Steponkus et al., 1998b) and enhance the cold tolerance (Artus *et al.*, 1996b).

A homolog of the COR15 gene (with 82% amino acid similarity) has also been discovered in *A. thaliana* (Wilhelm & Thomashow, 1993). These two homologs are present on the same chromosome but their pattern of expression is different in the response to cold stresses (Wilhelm & Thomashow, 1993). Likewise, two COR15 copies have also been discovered in *Brassica napus*, which are also involved in responses to cold stresses (Weretilnyk et al., 1993), but the evolutionary patterns of this small gene family in plants have not been explored (Dangwei et al., 2008).

Extensive attempts have been made by many workers to explore the regulatory mechanism of the expression of cold regulated gene (Shinozaki, Yamaguchi & Seki, 2003). Their findings have revealed that CBF/DREB transcription factors are the key regulators of cold-regulated (COR) genes expression (Jaglo-Ottosen *et al.*, 1998a; Liu et al., 1998c; Stockinger, Gilmour & Thomashow, 1997b). The over expression of CBF/DREB transcription factors increased the freezing tolerance in Arabidopsis (Jaglo-Ottosen *et al.*, 1998a; Liu *et al.*, 1998c). There might be some other regulatory

pathways which still need to be explored but CBF/DREB is becoming the most commonly found regulatory pathway for freezing tolerance increase.

Certain of the cold-regulated (cor) genes encode polypeptides which remain stable on boiling in aqueous solution and COR15 protein is one of them (Chentao & Michael, 1992). Through immunoblot analyses it was found that Arabidopsis corl5 gene encodes a 14.7-kD cold-regulated polypeptide, which is processed *in-vivo* to about 9 kD polypeptide, a mature protein designated as COR15m, which is soluble, hydrophilic, and in addition, COR15m located in the chloroplasts; particularly it can be detected in soluble protein extracts from chloroplasts (Chentao & Michael, 1992).

Different investigation showed that acclimation plays important role in the freezing tolerance like cold-acclimated spinach and cabbage synthesized proteins that can protect isolated thylakoid membranes against freeze damage in vitro, but non-acclimated plants did not produce such proteins (Hincha, Heber & Schmitt, 1989; Volger & Heber, 1975). The cryoprotective activity of these proteins is high as they are >10,000 times more effective than sucrose in protecting thylakoids against damage by a freeze-thaw cycle (Hincha, Heber & Schmitt, 1989; Volger & Heber, 1975).

Earlier studies have shown that the corl5 gene is expressed in response to low temperature and has a role in freezing tolerance (Hajela et al., 1990). On the other hand, corl5 transcripts also accumulate in response to water stress, certainly, each of the four cor genes studied were induced in response to water deprivation (Hajela *et al.*, 1990). There is a question why the genes involved in cryoprotection might be activated under drought conditions? One possibility relates to the fact that the damaged plant cells in response to a freeze-thaw cycle results largely from the cellular dehydration that occurs during freezing (Levitt, 1980; Steponkus & Lynch, 1989). Accordingly, tolerance to

freezing must include tolerance to water stress. It seems reasonable to guess that freezing and drought tolerance might involve related mechanisms and the activation of related or identical genes (Chentao & Michael, 1992). In fact, water stress has been shown to increase the freezing tolerance of certain cereal (Siminovitch & Cloutier, 1983) and Brassica species (Chentao & Michael, 1992; Cox & Levitt, 1976).

Over-expression of *COR15a*, encodes a polypeptide which is targeted to the chloroplasts, showed increase in freezing tolerance of chloroplasts in vivo and protoplasts in vitro (Artus *et al.*, 1996b). This increase in freezing tolerance was found from the *COR15a* encoded protein which stabilized membranes against freezing injury (Artus *et al.*, 1996b; Steponkus *et al.*, 1998b). There is also evidence that the mature polypeptide of COR15a, directly acts as a cryoprotective protein that decreases the propensity of lipid bilayers to form deleterious hexagonal II phase lipids (Steponkus *et al.*, 1998b).

COR genes have been implicated in the acquisition of low temperature tolerance in wheat, the genes transcript levels peaked at two days of the cold acclimation period and differences among genotypes were most apparent at this time. COR gene expression was highest for the low temperature tolerant and was lowest for the tender genotypes (Seedhabadee et al., 2008). The most rapid changes in low temperature tolerance, as measured by LT50, occur during the initial stages of low temperature acclimation, but plants cannot fully acclimate until temperatures drop well below the threshold induction level (Fowler, 2008).

Moreover, the expression of the entire set of *COR* genes, which includes the *COR47*, *COR6.6*, *COR78* and *COR15* gene pairs (Jaglo-Ottosen *et al.*, 1998a) was made

possible to express by the discovery of the CBF family of transcriptional activators (Gilmour *et al.*, 1998a; Stockinger, Gilmour & Thomashow, 1997b), also known as DREB1 proteins (Liu *et al.*, 1998c; Shinwari *et al.*, 1998). *COR6.6*, *COR15a*, *COR47*, and *COR78*, and presumably other genes yet to be discovered are CRT (C-repeat)/DRE (dehydration responsive element)-regulated *COR* genes, contain CRT/DRE in their promoters which is a cold and dehydration-responsive DNA regulatory element (Baker, Wilhelm & Thomashow, 1994b; Yamaguchi-Shinozaki & Shinozaki, 1994).Transgenic Arabidopsis plants which over-expressed *CBF3* gene at normal growth temperature, that constitutively produced the COR15am protein at levels equal or greater than in cold-acclimated wild-type plants (Gilmour *et al.*, 2000)

1.8 Aim and objectives of thesis

Aim

The aim of this research was the investigation of CBF regulon existence and its relation to abiotic stress resistance in mutants of cauliflower (*Brassica oleracea var. botrytis* L). The mutants had been produced using microshoots treated with the chemical mutagens (NEU & NMU) and then selected for resistance to hydroxyproline (Fuller & Eed, 2003). Hydroxyproline resistance selection was used to obtain high proline accumulating lines, as a strategy for improving abiotic stress tolerance. The free proline accumulation in response to environmental stress has been observed in plants (Deane, Fuller & Dix, 1995).

Objectives

1. To regenerate the previously created clones (Mutants and control)
2. To analyse the clones (Mutants and control) for frost, drought and salt stress resistance for confirmation of mutation stability in mutants.
3. To investigate the presence of CBF regulon pathway in cauliflower and to study its role in cauliflower mutants for abiotic stress resistance

Chapter 2: General Materials and Methods

2.1. Mutagenesis and abiotic stress resistant mutants selection

The January heading Roscoff F1 hybrid cauliflower Medaillon (courtesy of Elsoms Seeds Ltd) was grown in the field of the Seale-Hayne Estate, University of Plymouth, Devon, UK, following good commercial practice (Anon, 1982). The curds were harvested and taken to the laboratory where *in-vitro* micro-shoots were produced in liquid culture according to the method of Kieffer et al., (1995; 2001). This method produces a high volume of single or double curd meristem explants in the size range 300-600 µm.

The mutagenesis was carried out by Fuller & Eed (2003) and the procedure is given here for reference. N-nitroso-N-ethyleurea (NEU) and N-nitroso-N-methylurea (NMU) were used as mutagens. The NEU and NMU were used at 1 and 2.5 mM concentration respectively for 90 minutes in liquid culture, 24 h after the preparation of the microshoots. The mutagens were removed by decanting and washing the microshoots three times in fresh liquid culture medium of Kieffer et al (1995). Approximately 1.5 million microshoots were exposed to the mutagens. All techniques were carried out according to safe working practices established by McCabe et al (1990).

After mutagenesis selection of stress resistant mutants was made by the addition of 3 mM hydroxyproline to the final liquid culture medium and incubated for 3 weeks. Surviving green shoots after selection were removed from liquid medium and sub-cultured onto S23 solid culture medium of Kieffer et al (1995) without hydroxyproline to develop into shoots. Shoots with obvious morphological abnormalities either died or were discarded. Selections were then subjected to a multiplication phase (S23 + Kinetin 2 mgL^{-1} and IBA 1 mgL^{-1}) to produce clones of each selection which were either rooted and regenerated or put into a cold storage (+5 ^{o}C). A population of non-

mutated/selected control clones was also prepared from the same curd materials (Fuller & Eed, 2003). After continuous sub-culturing for a three years period, the clones were re-assessed for abiotic stresses and further selection was made (Fuller et al., 2006). Sub-culturing was carried out after each 3 to 4 month period on S23M medium and maintained in *in-vitro* condition.

2.2 Weaning process

In-vitro clones grown on S23M medium were uprooted and agar from the roots was gently removed by hand. A systemic general fungicide was sprayed on the roots to protect from soil borne pathogenic fungi, and then plants were transferred to pots (6 cm x 6 cm) containing moist compost and kept in a growth chamber at 20 °C with 16 hours light (light intensity 180.8 μmol m^{-2} s^{-1}). After 5 days the lids of the culture pots were perforated using a hot needle to reduce humidity inside the pots and left for 5 days, then lids were taken off and for 5 days regular water checking was carried out. The bases of pots were then perforated with a hot needle and after 5 days the pots were transferred to the green house under shade. After 5 days under shade the plantlets along with compost were transferred to bigger pots (12 cm x 13 cm) containing moist compost and exposed to full natural light in the green house (min 15 °C, 16 h long day photoperiod) and allowed to grow in *in-vivo* conditions. This weaning process demonstrated 100% successful transfer of *in-vitro* clones to *in-vivo* conditions without any damageable symptom observed in a single plant.

2.3 Regeneration and sub culturing of experimental clones

The experimental clones were regenerated and the curds from the mature regenerated plants were subjected to the multiplication medium of Kieffer et al (1995) in order to build up clone numbers for the project experimentation. The *in-vitro* cultures were

maintained in a growth chamber at 23 °C, 16 h photoperiod, 50 μmol^{-1} m^{-2} s^{-1} light intensity.

2.3.1 Media preparation

Cauliflower (*B. oleracea* var. *botrytis* L.) clones were proliferated *in-vitro* clonally through tissue culture. Three different shoot induction media were prepared for proliferation. Different media were prepared to compare their response on shoot induction and growth rate. Media were differentiated on the basis of agar concentration. Proliferation medium S23M (Kieffer, Fuller & Jellings, 1995) was prepared by dissolving the following ingredients in distilled water. MS (Murashige & Skoog, 1962) basal salts 4.4 gL^{-1}, Thiamine 0.4 mgL^{-1}, Adenine sulphate 80 mgL^{-1}, sodium dihydrogen orthophosphate 170 mgL^{-1}, sucrose 30 gL^{-1}, Kinetin 4 mgL^{-1}, indole-3-butyric acid (IBA) 2 mgL^{-1} and agar was added as 7 gL^{-1}, 4 gL^{-1} and 0 gL^{-1} denoted as T1, T2 and T3 respectively. The pH of media was adjusted to 5.8 and autoclaved. 20 ml pot^{-1} of medium was poured into each sterile plastic pot (5 cm x 4 cm) under aseptic conditions in a laminar flow cabinet; a lid was then placed on each pot and allowed to cool overnight at room temperature.

2.3.2 Hormonal stock solution

Stock solutions of indole-3-butyric acid (IBA) 2 mg ml^{-1} and kinetin 1 mg ml^{-1} were prepared. Kinetin was dissolved in few drops of 2M HCl and IBA in 2M NaOH prior to making stock solutions. The volume was then increased to 50 ml with distilled water. From stock solutions 1 ml of IBA and 4 ml of kinetin per litre was transferred to each tube of shoot induction medium.

2.3.3 Explants preparation and inoculation

Cauliflower curds were used as explants materials for proliferation. Curd material was collected from plants in the green-house and were packed in labelled plastic bags and

brought to the laboratory in a cooled insulation box. Curds were cut into small pieces and surface sterilization was carried out with 70% ethanol for 40 seconds and then with 10% bleach for 5 minutes. Surface sterile curds were rinsed 5 times with sterile distilled water for removal of bleach from the surface. Surface sterile pieces were then transferred to a sterile Petri dish and cut into small pieces about 2 to 3 mm across. Each small piece was inoculated into shoot induction media in small and sterile plastic pots and gently pressed in media with curd side facing up. Inoculation was carried out in sterile conditions in laminar flow cabinet. Pots were kept in incubator at 23 °C with 8 h photoperiods and observation was carried out until shoots developed.

2.3.4 Transfer to rooting media and sub-culturing

After a 5 week period, the young shoots were cut midway at an inter-node with a sterile scalpel and transferred to S23M hormone free medium. Pots were kept at 23 °C with 16 h photoperiod in the incubator and were observed until complete rooted plantlets were produced. Sub-culturing of *in-vitro* clones on S23M hormone free media was carried out regularly after each 3 to 4 months period to maintain plant materials for continued experimentation during project work.

2.4 Physiological screening of clones for abiotic stress resistance

In the present study, highly resistant mutants of cauliflower (*Brassica oleracea var. botrytis*) were selected from a mutant population in the plant physiology laboratory of the University of Plymouth, selection was made on the bases of previous screening results of Fuller et al (2006), and reassessed in the present study for abiotic stress (Drought, Frost and Salt stress) resistance under both *in-vitro* as well as *in-vivo* conditions. The genotypes (clones) were designated as mutants (K1, K4, K9, K11, K13, K18, K19, and K21) and control (KC).

2.4.1 Drought stress resistance investigation

Drought resistance assessment of genotypes was carried out by leaf disc assays to compare their resistance potential. For this evaluation 4 g L^{-1} MS medium (Murashige & Skoog, 1962) was dissolved in distilled water with different test concentrations of Mannitol added (0, 150, 250, 350 and 450 mM) denoted by T0, T1, T2, T3 and T4 respectively. The pH of all of the media was adjusted to 5.8 prior to being autoclaved. The sterilized media were poured into sterile Petri dishes under aseptic conditions in a laminar flow cabinet. Leaves from both *in-vivo* (from green house) as well as *in-vitro* clones were tested. Two fully expanded upper leaves from each plant (clone) were collected from the green-house and surface sterilised in 70% ethanol for a few seconds and then in 10% bleach for 10 minutes followed by three rinses with sterile distilled water.

Leaf discs were cut using a 1.0 cm diameter cork borer from the leaf blade areas avoiding the major vascular bundles and leaf discs of each genotype were transferred to a specifically labeled and sterilized petri dish containing sterilized distilled water and allowed to stand overnight at room temperature in order to become turgid. The following day the turgor weight (TW) of each leaf disc was recorded, using a 5 decimal place balance, and then the discs were allocated to each one of the different media contained in petri dishes and incubated for seven days in an incubator at 23 °C with 16 h photoperiod. Three and two divisions replicate petri plates were used for *in-vivo* and *in-vitro* clones respectively for each treatment, and each plate contained three discs which had been individually labeled on the leaf surface (1, 2, 3) using a permanent marker pen during discs preparation. After seven days the weight of each disc was re-measured and noted as the fresh weight (FW). Discs were then freeze dried and the dry weight (DW) of each of the discs recorded. Percent relative water content (RWC) for each disc was

measured using the formula RWC% = (FW-DW) / (TW-DW) x 100. The mean value of replicates discs and then of replicate petri plates was analysed. The total number of petri plates used for *in-vivo* clone screening were = 9 genotypes x 3 rep x 5 treatments = 135, and for *in-vitro* clones screening were = 9 genotypes x 2 rep x 5 treatments = 90.

2.4.2 Frost stress resistance analysis

Frost resistance analysis of the cauliflower (*Brassica oleracea* var. *botrytis* L.) clones (K1, K4, K9, K11, K13, K18, K19, K21 and KC) was carried out to compare the degree of their frost resistance. The electrical conductivity technique described by Fuller et al (2003; 1989) was used for analysis. Both *in-vitro* as well as *in-vivo* clones were tested at different temperatures +1 °C, -2 °C, -4 °C, -6 °C. Four fully expanded upper leaves from each genotype in the greenhouse (*in-vivo*) were excised and transferred to the laboratory in a cooled insulated box containing ice packs. Two leaves were used for assessment as non-acclimated and the other two leaves were acclimated by placing them in an incubator (Snijder scientific) at 4 °C, 8 h photoperiod for 14 d. Previous experimentation in the lab had demonstrated that excised leaves of cauliflower referred the ability to acclimate. Acclimation for *in-vitro* clones were carried out by keeping whole plantlet in the same incubator used for *in-vivo* clones leaves. Ten leaf discs of 1 cm diameter each were cut and placed in boiling tubes. Three replicate tubes were used for each treatment and each genotype. The total tubes used for each of *in-vitro* or *in-vivo* clones analyses were = 9 genotypes x 3 replicates x 2 unacclimated/acclimated x 4 different test temperatures = 216 tubes. Three replicate tubes without leaf discs (blank tubes) were used for monitoring EC contamination.

All the tubes were labeled in test tube racks and put in a freezing chamber (Sanyo) adjusted to +1 °C overnight. The following day a small piece of ice was added to each

tube to ensure ice nucleation and the chamber programmed to fall to temperature of -2 °C, -4 °C, and -6 °C with a hold of two hours at each temperature. Samples were removed at the end of the 2 h hold of each temperature. The +1 °C treatment tubes were removed and transferred to a defrost incubator (Sanyo) running at 4 °C. The other treatments were likewise transferred and allowed to defrost overnight. After defrosting the tubes were moved to the laboratory bench at ambient temperature and 20 ml distilled water added to each tube using an automatic dispenser. The tubes were covered and left overnight at room temperature to allow leaching of electrolytes from cells damaged by the freezing. The following day the post-freezing electrical conductivity (EC) of each solution was measured. All tubes were then autoclaved to rupture all the cells to provide a total leachate measurement. After autoclaving samples were kept for overnight incubation at room temperature and then the post-autoclaving electrical conductivity (EC) was measured for all tubes. Identical procedures were used for analysis of both acclimated as well as non-acclimated leaves. The percent relative electrical conductivity (REC) was calculated to give an indication of the level of cell membrane damage at the different freezing temperatures, with more damaged cells giving higher electrical conductivity. For calculation of percent relative EC the following formula was used as

REC% = Post freezing EC/ Post autoclaving EC x 100

2.4.3 Salt stress resistance evaluation

The clones analysed for drought and frost resistance analysis were also tested for salt resistance. Both *in-vivo* as well as *in-vitro* clones were analysed. Liquid media of three different concentrations of sodium chloride (NaCl) were prepared in distilled water i.e. 0 mM (control), 350 mM and 550 mM (approximately the concentration of sea water) and labelled as T0, T1 & T2 respectively, then 4 gl^{-1} MS salts (Murashige & Skoog, 1962) were added to each of T0, T1 and T2. The pH of all media was adjusted to 5.8, and then autoclaved. Media were poured into sterile Petri dishes under aseptic conditions in a laminar flow cabinet. Three replicate petri dishes were used for each clone under each treatment. Two fully expanded upper leaves were detached from each genotype clone in the green-house (*in-vivo*) and brought to lab in a cooled insulation box. Leaves were surface sterilized with 70% ethanol for a few seconds and then in a solution of 10% bleach for 10 mins followed by 3 rinses with sterile distilled water. Leaf discs of 1 cm diameter were prepared in a laminar flow hood under aseptic conditions and 3 discs/petri dish were floated on each liquid media. Petri dishes were properly labeled and three replicate petri plates were used for each genotype and each treatment and placed in an incubator at 23 °C with 16 h photoperiod. Leaf discs from *in-vitro* clones were prepared direct from pots and analysed in a similar way used for *in-vivo* clones analyses. The total petri dishes used for each of *in-vitro* or *in-vivo* clones analyses were = 9 genotypes x 3 rep x 3 treatments = 81 plates. The effect of salt concentrations on leaf discs was recorded after 3, 5 & 7 days treatments. Change in leaf discs color was used as a score to differentiate resistance strength. Color change of leaf discs was categoriesed as:

A. Dark green (100% greenness)

B. Light green-no white (75% greenness)

C. Half light green half white (50% greenness)

D. Small amount of light green (25% greenness)

E. White. (0% greenness)

2.5 Cold acclimation before RNA and Protein extraction

Four replicate plants of each mutant and control clone were transferred from *in-vitro* conditions to small plastic pots contained compost in growth chamber (Sanyo Fitotron) at 22 °C under a long day photoperiod (16 h) with a light intensity of 180.8 µmol m^{-2} s^{-1} and 52.5% humidity. One set of two replicate were retained in the same growth chamber (non-acclimated) and the other set of two replicates for each clone (young plants with 4-6 true leaves) were transferred to another growth cabinet (Snijder scientific) for 14 days cold acclimation (4 °C with 8 h photoperiod, light intensity 180.8 µmol m^{-2} s^{-1} and 52 % humidity).

To investigate the *CBF/DREB1* gene in cauliflower, the total RNA was isolated from non-acclimated and acclimated clones. To study the effects of cold acclimation length on *CBF/DREB1* genes expression in mutants, the mRNA was isolated from clones at different intervals i.e, 3 h, 6 h, 24 h and 14 d acclimation. At each interval one leaf was detached from each clone and frozen immediately in liquid nitrogen and finaly ground in liquid nitrogen in a pestle and mortar. After the liquid nitrogen evaporated, 100 mg of frozen tissue powder for each sample was weighed in 1.5 ml RNase free micro-centrifuge tubes (Ambion) and stored at -80 °C to prevent RNA degradation before RNA isolation. For the investigation of COR15a and COR6.6 proteins in the genotypes, the proteins were extracted from both non-acclimated and acclimated genotypes (clones) once after 14 days acclimation and then stored at -80 °C until further analysis through sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and Western blotting techniques.

2.5.1 RNA isolation and purification

Total RNA was isolated by following the method of Farrell (1998) and Sambrook et al (1989). Samples (100 mg powder) stored at -80 °C were taken and 500 µl of lysis solution (sigma cat # L8167) added and vortexed immediately and vigorously for at least 30 seconds following the instructions from the kit supplier (Sigma cat # STRN50) , and then incubated at 56 °C for 5 minutes. Samples were centrifuged at 13000 xg for 5 minutes to pellet the cell debris. The supernatant was pippetted into a filtration column (sigma cat # C6866) in a 2-ml collection tube and then centrifuged at 13000 xg for 3 minutes and the flowthrough lysate saved. 500 µl of binding solution (Sigma cat # L8042) was pippeted into the lysate and mixed immediately and thoroughly by pippeting at least 5 times and vortexed briefly. 700 µl of the mixture was pippeted into a binding column (Sigma cat # C6991) in a 2 ml collection tube and centrifuged at 13000 xg for 1 minute to bind RNA. The flow-through liquid in the collection the tube was decanted and tube was cleaned with absorbent paper and returned to the column and repeated for the remaining mixture.

DNase1 (Sigma cat # DNASE10) was used for removal of trace amount of DNA. 80 µl of the DNase1 mixture was added directly onto the centre of the filter inside the binding column the cap closed and incubated at room temperature for 15 minutes. 500 µl of wash solution1 was pippeted into the binding column and centrifuged at 13000 xg for 1 minute. The flowthrough liquid was decanted and the tube cleaned with absorbent paper and continued to the second column wash with ethanol diluted wash solution 2 (Sigma cat # W3261) and centrifuged at 13000 xg for 30 seconds and repeated the third column wash with wash solution 2 and centrifuged similarly. The tube was cleaned by absorbent paper and returned to the column into the clean tube, centrifuged at 13000 xg for 1 minute to dry the column. The column was carefully removed and transferred into

a new 2 ml collection tube and 50 µl of elution solution (sigma cat # E8024) pippeted directly onto the centre of binding matrix inside the column and the cap closed and stood undisturbed for 1 minute and then centrifuged at 13000 xg for 1 minute to elute. The purified RNA in the flow-through eluate was distributed in small aliquots and some were stored at -20 °C for short time and at -80 °C for long term storage.

2.5.2 *BoCBF/DREB1* regulatory gene identification using RT-PCR

Two steps PCR was used for cDNA synthesis and amplification. Total RNA was used as the template for the synthesis of the first strand cDNA using ImProm-II™ Reverse Transcription System (Promega cat # A3800). The reverse transcription reaction mixture was prepared in sterile and nuclease free 1.5 ml microcentrifuge tubes (Ambion) on ice. The total mixture was distributed in aliquots of 15 µl for each cDNA synthesis reaction following the instructions of kit manufacturer. RNA was diluted to equilibrate all the samples as 0.8 µg in nuclease free water (Sigma cat # w1754) and each sample was combined with primer oligo $(dT)_{15}$ (0.5 µg/reaction) for a final volume of 5 µl/RT reaction in 0.2 ml nuclease free PCR tubes (Ambion). Tubes were treated at 70 °C for 5 minutes and immediately chilled in ice for 5 minutes and centrifuged for 10 seconds to separate condensate to maintain the original volume. Tubes were kept on ice until further use. Each final RT reaction volume of 20 µl was prepared by combining 5 µl of RNA and oligo $(dT)_{15}$ primer mixture with 15 µl reverse transcription reaction mixture. All the micropipettes and tips used were sterile and nuclease free for preventing RNA degradation and contamination.

First strand cDNA syntheses was carried out by placing the tubes in the thermal cycler (Perkin Elmer 9700) under the following thermal cycle; Annealing: 25 °C for 5 minutes, Extension: 42 °C for 60 minutes, Inactivation of Reverse Transcriptase: 70 °C for 15

minutes. The first strand cDNA was directly amplified in 50 µl of reaction mixture. The mixture was prepared by adding components in the following ratio following the instructions of PCR master mix (Promega cat # M7502). PCR master mix, 2X (25 µl), forward primer, 10 µM (5.0 µl), reverse primer, 10 µM (5.0 µl), cDNA (5 µl), nuclease-free water (Sigma cat # w1754) was added to make a 50 µl final reaction mixture for each sample. According to the *Brassica juncea* and *B.napus CBF/DREB1* genes the following gene specific (degenerate) primers were used for amplification of cDNA strands. (Forward, 5-AAGAAGTTTCGTGAGACCCGTCAC-3 and Reverse, 5-GGCAAAAGCATACCTTCCGCCAT-3). The 50 µl reaction mixture for each sample was run under the following thermal cycle. Initially denaturation at 94 °C for 3 minutes once, 35 cycles of (denaturation at 94 °C for 1 minute, annealing at 61 °C for 1 minute, extension at 72 °C for 2 minutes) and a final extension at 72 °C for 10 minutes and then 4 °C ∞. The PCR products were analysed by using 0.8 % high melting agarose (Sigma) gel added with EtBr to a final concentration of 0.5 µg/ml, and compared with a PCR marker (Promega G3161) consisting of six DNA fragments with sizes of 50, 150, 300, 500, 750 and 1,000 bp. The bands intensity were measured using Quantity one 4.6.3 Bio-Rad software.

2.5.3 RT-PCR product (cDNA) sequence analysis

The specific RT-PCR products were isolated and purified from the gel slice using the Wizard® SV Gel and PCR Clean-Up System (Promega A9281) and the purified products were stored at 4 °C for a short time and then subjected to sequence analyses (Eurofins MWG Operon, Germany). The sequences were then analysed using Clustal W and Basic Local Alignment Search Tool (BLAST) and then compared with nucleotide and deduced amino acids sequences of *CBF/DREB1* genes from other *Brassica* species by iterative multiple alignments.

2.5.4 Genomic DNA isolation and purification

Genomic DNA was extracted from young leaves of the clones based on the methods of Sambrook et al., (1989) and Bruce and Eric (1993). The harvested leaves were ground into a fine powder in liquid nitrogen, using a mortar and pestle, and 100 mg of the powder transferred to a 1.5 ml micro-centrifuge tube kept on ice and 350 µl lysis solution A and 50 µl of lysis solution B added according to the protocol provided by the kit manufacturer (Sigma cat # G2N10). To dissolve any precipitate, the mixture was incubated at 65 °C for 10 minutes, then 130 µl of precipitation solution was added and mixed completely by inversion and placed on ice for 5 minutes. The samples were centrifuged at 13000 xg for 5 minutes to pellet the cellular debris. The supernatant was pippeted onto a GenElute filtration column into a 2 ml collection tube, centrifuged at 13000xg for 1 minute, the filtration column which removed any cellular debris was then discarded. 700 µl of binding solution was added to the flow-through liquid in the collection tube and mixed thoroughly by inversion. A DNA binding column was prepared following the protocol. Carefully 700 µl of the mixture was pippeted onto the DNA binding column and centrifuged at 13000 xg for 1 minute, flow-through liquid was discarded and the collection tube retained, returned to the column the remaining mixture applied onto the column and centrifugation repeated. The binding column was placed into a fresh 2 ml collection tube and 500 µl of ethanol diluted wash solution (Sigma cat # W3011) applied and centrifuged at 13000 xg for 1 minute. The flow-through liquid was discarded and the collection tube was used for a second wash similarly and centrifuged at the same speed for 3 minutes which dried the column. The DNA binding column was then transferred to a fresh 2 ml collection tube for DNA elution. 100 µl of pre-warmed (65 °C) Elution solution (10 mM Tris, 1 mM EDTA, pH 8.0) was applied to the column and centrifuged at 13000 xg for 5 minutes. The elution process was repeated and the column was prevented from contacting the flow-through

liquid. The eluates were stored in small aliquots at 4 °C for short term and at -20 °C for long term for downstream processing.

2.5.5 Analysis of RNA and DNA purity

The purity and concentration of DNA and RNA was determined by spectrophotometric analysis. The RNA was diluted 10-50 fold in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8) and absorbance was measured using A_{260}/A_{280} ratio procedure (Warburg & Christian, 1942). Nucleic acids have a higher absorbance at 260 nm than at 280 nm and pure DNA and RNA samples have expected A_{260}/A_{280} ratios of $\geq$1.8 and $\geq$2.0 respectively (Maniatis, Fritsch & Sambrook, 1982)

2.5.6 DNA quality confirmation

A gel of 0.8% agarose was prepared in a 250 ml flask by melting 0.4 g of agarose in 50 mL of 1xTBE buffer using a microwave to completely melt the agarose. The solution was allowed to cool for a couple of minutes to about 50 °C (a temperature at which one can hold the flask). Ethidium Bromide (EtBr) was added to the agarose solution to a final concentration of 0.5 µg/ml and mixed before pouring the gel. Using a supplied gel tray and comb, the gel was cast and allowed to solidify for a minimum of 20 min at room temperature. 1xTBE buffer was added to submerge the gel. Samples were prepared in PCR tubes (0.2 ml) by adding 4 µl loading dye (Promega cat # G1881) to 10 µl of sample, mixed then 10 µl total volume of each sample per well loaded. 5 µl of the molecular weight markers of 1kb (Promega cat # G5711) was loaded in a well as a reference ladder. The gel was run at 100 V until the bromophenol blue was about 3/4 through the gel (approx. 1 h). The tray with the agarose gel was carefully removed and taken to the UV transilluminator and the gel examined under UV light to confirm the DNA quality. Presence of a highly resolved high molecular weight band indicated good quality DNA. A photograph was made using the gel documentation system. 1 liter of

1xTBE buffer was prepared by diluting 10xTBE (Tris base 10.8 g, Boric acid 5.5 g, EDTA 4 ml from 0.5 M stock solution, and volume rised to 100 ml with dH_2O).

2.6 Protein extraction and purification

The leaf tissues from the –80 °C freezer were thawed on ice and placed in a mortar and pestle. 2 ml of extraction buffer (Ni et al., 1996) (100 mM potassium phosphate buffer (200 ml of 2 M KPO_4 stock solution was prepared by dissolving K_2HPO_4 63.2 g and KH_2PO_4 5.0 g in dw), pH 7.8, 1 mM DTT, 1 mM EDTA, 1% Triton X-100, 10% glycerol) was added to 1 gram tissues and ground. 1 ml of the liquid grindate was transferred to a microfuge tube (1.5 ml) and placed on ice and the mortar and pestle washed in preparation for the next sample. Centrifugation was carried at 13000 xg for 15 minutes at 4 °C. The supernatant was pippeted into a new microfuge tube (1.5 ml) and centrifuged again for 10 minutes then the clear supernatant was distributed in aliquots and stored at -80 °C until used.

2.6.1 SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis)

SDS-PAGE analysis was carried out with Laemmli's buffers (1970). Vertical 8.6 x 7.7 cm (WxL) polyacrylamide gels were prepared and run in a Biometra Minigel-twin electrophoreses system. Each experiment was repeated 3-4 times. Buffer A (for the resolving gel) (Tris HCl 0.75 M, SDS 0.2% w/v, pH 8.8), buffer B (for the stacking gel) (Tris HCl 0.25 M, SDS 0.2% w/v from 10% stock solution of SDS, pH 6.8), running buffer (Tris HCl 25 mM, SDS 0.1% w/v, pH 8.3) and loading buffer (Tris HCl 0.5 M pH 6.8, SDS 10% w/v, bromophenol blue 0.1%, β-mercaptoethanol 0.5 ml/10 ml, Glycerol 20%) were prepared. 11.41 ml solution for the 12.5% resolving gel was prepared by mixing buffer A 5.2 ml, ddH_2O 1.4 ml, 4.4 ml of 30% (w/v) solution of Acrylamide/Bis-acrylamide (29:1) (Sigma cat # A3574), 380 µl of amonium persulfate

(APS) 10% w/v and 30 µl tetramethylethylenediamine (TEMED). 4.1 ml of 5% stacking gel was prepared by mixing buffer B, 2 ml, ddH2O 1.3 ml, 30% solution of Acrylamide/Bis-acrylamide 0.7 ml, APS 100 µl, and TEMED 15 µl. APS and TEMED were added before pouring the gel. Resolving gel solutions were poured quickly into the gel casting system and space left about same size of combs for the stacking gel below the bottom of the comb. Any bubbles were removed by adding isobutanol to the top of the gel. Isobutanol also prevented the gel from drying out. After 15 minutes, the gel was completely polymerized; the isobutanol was washed away and the gel dried with blotting paper prior to pouring the stacking gel. The comb was added soon after pouring the stacking gel and allowed to polymerize completely for 1 hour. 30 µl of loading buffer was mixed with 40 µl of each sample and boiled in water a bath for 5 minutes then cooled; centrifugation at 1300 xg for 2 minutes was carried out to remove any debris which could cause any blockage of gel pores. 20 µl of the supernatant of each sample mixture was loaded into each well. 6 µl of standard protein marker (Sigma cat # C1992) was loaded as a reference ladder; the marker proteins consisted of 8 bands of different colours and sizes i.e Violet 220, Pink 100, Blue 60, Pink 45, Orange 30, Blue 20, Pink 12 and Blue 8 kDa. The gel was run at 80 volts while the samples reached the resolving gel and then increased to 100 volts for about 2 further hours. The staining potential of staining solution (Coomassie blue 1.25 g, methanol (100%) 400 ml, glacial acetic acid 70 ml, dH_2O 530 ml per liter solution) and staining reagent (Sigma cat # G1041) was compared and the Sigma reagent was found to be better only requiring a ddH_2O for destaining, while other stains required destaining solutions of 100% methanol 400 ml, glacial acetic acid 70 ml, dH_2O 530 ml per litre of solution. After destaining, photographs were made with the gel documentation system and stored for further analysis. A non stained gel was used for protein transfer to nitrocellulose membrane for subsequent Western blotting.

2.6.2 Western blotting (Immunoblot Analysis) for detection of COR15

Protein samples resolved using SDS-PAGE were transferred to nitrocellulose membrane and subjected to immunoblot analysis (Towbin et al., 1979). The nitrocellulose membrane (Sigma) was placed next to the gel and sandwiched between absorbent paper and sponge (- sponge/filter paper/gel/membrane/filter paper/sponge +). All were clamped tightly together after ensuring no bubbles have formed between the membrane and gel. The sandwich was submerged in transfer buffer (Towbin *et al.*, 1979) so that the gel was closest to the negative electrode and an electrical field was applied as 300 mA or 15-20 volts for 90 minutes. Transfer time and voltage depends on protein size, time or voltage is reduced for smaller protein. The negatively charged proteins travel towards the positively charged electrode but the membrane stop them and bind them. Bio-Rad criterion blotter was used for protein transfer. Hybridization for COR15a was performed with specific primary antibodies obtained from the Prof. Michael Thomashow's Lab, (Michigan State University, USA). Each experiment was repeated 3-4 times.

1xTris-glycine transfer buffer preparation (1 Litre)

Tris HCl = 3.05 gm

Glycine = 14.4 gm

Methanol = 200 ml

ddH_2O = 800 ml

pH = 8.3 (adjusted with NaOH)

Tris and Glycine were dissolved first in dH_2O and then methanol was added, for protein larger than 80 kDa, SDS is recommended at a final concentration of 0.1%.

Blocking solution and hybridization: after proteins were transferred to nitrocellulose membrane the membrane was incubated in the blocking solution (PBST + 1.5-3 % BSA) for 1 hour at room temperature with mild shaking. PBST (Phosphate buffer saline

Tween 20) is 80 mM Na_2HPO_4; 20 mM NaH_2PO_4; 10 mMNaCl; 0.1% Tween 20; pH 7.5. The 2% BSA (Bovin serum albumin) was prepared by dissolving 2 g BSA per 100 ml of PBST buffer, mixed well and filtered. PBST (1 tablet in 200 ml water was dissolved. pH 7-7.5 if not, adjusted with NaOH and then autoclaved). After the incubation in blocking solution the membrane was washed with PBST twice for 5 minutes each, followed by incubation with primary antibodies (produced in rabbit) obtained from the Michigan State University, USA. Antibodies were diluted (1:1000) in a total volume of 20 ml PBST and membrane incubation was carried overnight at room temperature with very mild shaking. The following day the membranes were washed three times with PBS while agitating, for 10 min each. The goat anti rabbit IgG conjugated to horseradish peroxidase (abcam) was used as secondary antibody. Membranes were incubated in PBS diluted (1:20,000) secondary antibodies, incubation was carried out for 90 minutes at room temperature with mild shaking on a shaker and then washed 3 times for 10 minutes of each wash with PBS.

Developmental solution: A solution was prepared by dissolving DAB (diaminobenzidine) 0.06 g, $NiCl_2$ 0.03 gm in 100 ml PBST. The chromogenic reaction was initiated by the addition of 100 µl H_2O_2 into solution, the H_2O_2 was added immediately before pouring the solution on to the membrane. Membranes were incubated in developmental solution for about 15 minutes to overnight in the dark, DAB is sensitive to light. Membranes were washed repeatedly with PBS and then digital images were made from the membranes.

2.7 Free-proline (Pro) and protein evaluation in genotypes under cold acclimation

The levels of free-proline and protein in tested clones (K1, K4, K9, K11, K13, K18, K19, K21 and KC) grown under different environmental conditions were analysed. (1)

In growth chamber at (22 °C day night, 16 h photoperiod, light intensity 180.8 µmol $m^{-2}s^{-1}$ and 52.5% relative humidity), (2) Cold acclimated in a growth cabinet at 4 °C, 8 h photoperiod, 180.8 µmol-$m^{-2}.s^{-1}$ and 52% relative humidity, (3) In green house at 15 °C in natural light). Some of the *in-vitro* clones grown on agar media (S23M) at 23 °C in growth cabinet were transferred into plastic pots (6 cm x 6 cm) containing compost and retained in the same cabinet at 23 °C for one week and watered on a daily basis (weaning process). Two replicate pots for each clone were transferred to the green house for six weeks and four replicate pots for each clone were kept in a growth chamber (Sanyo Fitotron) in the lab at 22 °C, 16 h photoperiod. After 4 weeks in the fitotron cabinet, for acclimation process two replicate pots for each clone were transferred into another growth cabinet for a two weeks period adjusted to 4 °C (Fig. 2.1). After a 6 week period the leaves were detached from each clone and frozen promptly in liquid nitrogen and then stored at -80 °C in freezer until further use.

2.7.1 Proline (Pro) extraction and estimation

Proline extraction and biochemical quantification was carried out following the method of Bates, Waldern & Teare (1973). 100 mg powder of frozen leaf tissue was homogenized in 1.5 ml of 3% sulfosalicylic acid in 2 ml tubes. Centrifugation was carried out at 13000 xg for 5 minutes. 300 µl of the supernatant was treated with 2 ml glacial acetic acid and 2 ml acid ninhydrin (1.25 g ninhydrin warmed in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid until dissolved) in test tubes at 100 °C in a boiling water bath for 1 h. The reaction was then ended immediately by dipping the tubes in ice. The reaction mixture was extracted with 1 ml toluene by mixing vigorously for 10-30 seconds. The chromophore containing toluene was pipetted from the aqueous phase, warmed to room temperature and the absorbance was read at 520 nm by spectrophotometer using toluene for a blank. The concentration of proline in different

samples was determined from a standard curve. The reaction for each sample was performed in triplicate.

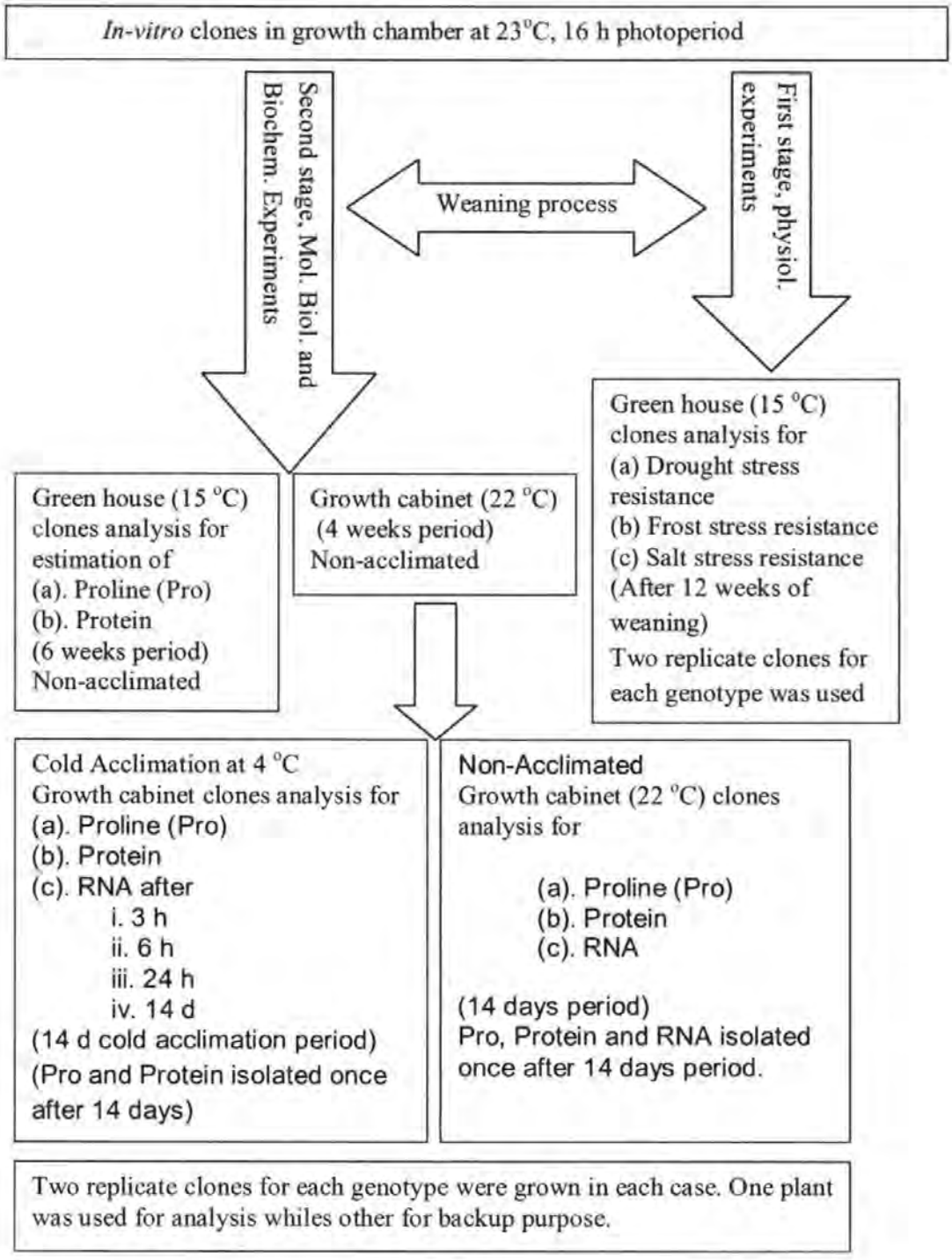
2.7.2 Protein estimation

For total protein isolation the method was used as described earlier for protein extraction (Ni et al., 1996). Protein content estimation was made by the standard method of Clive, Daniel & Steve (1989) based on Bradford (1976). Three replicate aliquots of 100 µl for each sample was mixed with 5 ml of 1:4 diluted Bradford dye reagent (sigma cat # B6916). 100 µl of protein extraction buffer was mixed with dye in similar way used for samples and used as a blank. After 15 minutes, 1 ml of each replicate was transferred to a disposable polystyrene cuvette and the absorbance at 595 nm was recorded using a single beam spectrophotometer.

2.8 Statistical Analysis

The statistical program Minitab 15 was used for the analyses of data. Analysis of variances (ANOVA) was performed and the means were compared using least significant difference (LSD) test. The probability table (Fisher & Frank, 1948) was used for determination of the significance level. Correlation among the different parameters was also investigated using Excel fitting curve and values of the correlation coefficient for different levels of significance investigated according to Fisher and Frank (1948).

2.9 Summary of Experimental approach



Chapter 3: Physiological Characteristics of Mutants

3.1. Introduction

3.1.1. Micro-propagation and sub-culturing of clones

The plant micro-propagation process is used for proliferation of clones of genotypes. The process usually consists of the following steps; selection of the suitable plants, surface sterilization, initiation of explants, establishment of explants, subculture for multiplication of explants, shooting and rooting induction, and the weaning/hardening step to produce complete *in-vivo* plant clones (Ahloowalia *et al.*, 2002). Plant micro-propagation is initiated from small pieces, known as explants. The explant is isolated from surface sterilized part of a plant. The widely used explants are shoot tips or meristem-tips which are the most well-liked explant source (Medford, 1992). Other explant sources like floral meristem and buds, nodal or axillery buds, anthers, pollen and microspores have also been used as explants.

The cultured tissues generate a complete plant through regeneration process through different ways either by callus production or by direct shoot induction. Callus is a mass of unorganized cells which have the potential to produce complete plant upon transfer to suitable media and widely used for plant clonal generation (Bhojwani & Razdan, 1983; Pierik, 1989). The explants are stimulated to differentiate in shoot and roots using specific types of media and grow into a complete plant is known as regeneration and organogenesis. The media having comparatively high auxin (2,4-D, 2,4-dichlorophenoxyacetic acid) form callus which can be further sub-cultured and multiplied for clonal production. In some cases, explants e.g. leaf-discs and epidermal tissue can also generate plants by direct organogenesis and somatic embryogenesis without prevailing callus formation (Hanning & Conger, 1986).

The regeneration and growth of plants in *in-vitro* condition depends on the composition of media, the major components of culture media are mineral salts, sugar and water while growth regulators, organic supplements and gelling agent are other important components including in media (Gamborg, Miller & Ojima, 1968; Gamborg & Phillips, 1995). The quantity of the components vary for plant species and stage of growth, however the most widely used media for plant tissue culture are the basic MS (Murashige & Skoog, 1962a) and LS (Linsmaier & Skoog, 1965) media. Many plant tissues grow better on solid media while others on liquid media, the solid, liquid and semi liquid media formation depends on the absence or presence of gelling agent. Agar is the most commonly used gelling agen (Debergh, 1983).

The growth regulators like auxins to cytokinins in media play important role and their ration in media determines the morphogenic response for root or shoot formation as a relative high cytokinin : auxin ratio induces shoot production while high auxin : cytokinine ratio induces root formation (Skoog & Miller, 1957). Usually high concentration of auxine is favoured for root induction but in some cases exogenous auxin show inhibitory effect on roots (Guichuan, Jeffery & Elison, 2004; Thomas & Street, 1970). Another important supplement in media is silver nitrate which play role in maintaining the callus as well as improves the regeneration (Sethi, Basu & Cuha, 1990), while in high concentration causes necroses, while though as high as 15 mgL-1 silver nitrate did not show any negative effect on *Brassica rapa* culture (Kuvshinov et al., 1999).

3.1.2. Abiotic stress tolerance through mutagenesis

Environmental stresses such as drought, salinity, extreme temperatures, toxic chemicals, and excessive ozone or carbon dioxide, are known as abiotic stresses. These stresses cause reduction in the productivity and causing average yield loss of more than 50% for major agricultural crops (Boyer, 1982; Bray *et al.*, 2000; Shubha & Akhilesh, 2007). It is a major concern for the scientist to reduce the loss of yield but is difficult and about impossible to reduce the stresses. So there is ultimate way to develop the stress tolerant crops.

The change in sequence of genetic material is known as mutation, which has become an established method of inducing variation within a variety of crop. This method contribute significantly to plant breeding (Maluszynski et al., 1995; Nichterlein, 2000) in creating genetic variability, which reduces the time to breed new varieties in comparison with traditional methods (Cornide, 2001). Mutation also offers the possibility of inducing desired attributes that either cannot be expressed in nature or have been lost during evolution (Brunner, 1995). The classical methods of breeding is time consuming and sometime inefficient while through DNA mutation or direct gene transfer the cultivar might be improved for stress resistance without disrupting the genotype and breaking of gene linkages (Zhang et al., 2000).

The mutation detection through traditional genetic screens mostly not exposed the mutation because geneticists select for phenotypes, and sometime only a small mutations within a target gene could not examined. Even though the deletion and insertion detection by PCR is straightforward, but detection of point mutations, mainly inducing by chemicals is challenging, because the PCR amplified fragment does not show any change in the size. Single base variation detection has also been improved

with advances in single-nucleotide polymorphism (SNP) detection technologies (Kwok, 2001). One example of SNP detection technology being applied to reverse genetics is (targeting induced local lesions in genomes), in which chemical mutagenesis is followed by screening for point mutations (McCallum et al., 2000).

Substances that cause mutation known as mutagens, mutagens may be either physical or chemical, and both are in conventional plant breeding. The chemical mutagens that induce mutation in plant cell cultures could be divided into two groups, base analogous and alkaline agents. Alkaline agents include N-nitrose-N-ethylurea (NEU), N-nitrose-N-methylurea (NMU), alkyl sulphate and nitrogen mustards. NEU or NMU are biofunctional agents (Charlotte, 1976) and can induce depurination and depyrimination. Both NEU or NMU have been shown to induce gene mutation (deletion), transition mutation, unscheduled DNA synthesis (UDS), sister chromatid exchange (SCE) and induce DNA-DNA and DNA protein crosslink (IAEA, 1977; Negrutu, 1990). They can also cause mispairing of nucleotides with their complementary bases, so introducing base changes after replication (Ashburner, 1990; Haughn & Somerville, 1987). Multiple mutations also occurred more frequently in NMU-treated plants. There is great scope for increasingly both the frequency and spectrum of mutations in treatments with chemical mutagens through suitable modification of the treatment conditions (Savin et al., 2003).

3.1.3. Dehydration stresses effect on plant

In some cases the plant response to different stresses share similar steps in the mechanism e.g. freezing, drought and salt stresses disturb the osmotic homeostasis of the plant cell and these stresses affect the water relations of plant and causes cellular dehydration and collectively these stresses are known as dehydration stresses (Erwin et

al., 2007). Frost, drought and salinity due to their wide range occurrence may cause the most fatal economic losses in agriculture. It was found through a short term treatments experiment on *Arabidopsis* responded to cold, drought and salt stresses in a quite specific way (Kreps et al., 2002) and a cross talk in the signalling pathways appeared in frost hardening by drought or salt treatment and showed cooperative actions for all of these stresses (Erwin *et al.*, 2007; Mahajan & Tuteja, 2006).

The cell membrane damage occurs during dehydration or rehydration and the capability of plant to avoid or repair this damage to membrane is essential for plant to survive under dehydration stresses. some of the mechanisms leading to adaptation to dehydration, has been possible by the identification and manipulation of key genes and transcription factors to alter metabolism and increase plant tolerance to dehydration (Chaves & Oliveira, 2004). The dehydration stress tolerance in crop could be improved by engineering and manipulation of osmoprotectent syntheses pathway in the susciptable plant (Rathinasabapathi, 2000). Gene manipulation for the production of osmolytes such as mannitol, fructans and proline etc might increase resistance to dehydration (Ramanjulu & Bartels, 2002). Modification of plant for increased dehydration tolerance is mainly based on the manipulation of either signalling or transcription factors or genes that directly protect plant cells against water deficit, but understanding of the molecular and biochemical mechanisms is still a challenge for scientists (Babu & Henry, 2006).

3.1.4. Frost stress injuries in plant

Freezing temperatures damage the plant cells and tissues as well as entire plant, while the cell membrane has been the primary site of freezing injury (Levitt, 1980). The ice formation induces in intercellular spaces and cell wall on exposure to freezing temperature, in some cases the ice nucleating agents such as dust or ice nucleating bacterial proteins further facilitate the ice formation (Brush, Griffith & Mlynarz, 1994).

The freezing temperature destabilize the cell membrane (Uemura et al., 1995) and the water from the cytoplasm moves outside through the plasma membrane by osmosis and that's why the freezing injury is mainly caused by cellular dehydration (Xin & Browse, 2000). Certain lipids in cell membrane form an inverted structure with hexagonal packing symmetry which disrupts the bilayer of cell membrane and the plasma membrane becomes permeable to water and solutes and loses osmotic responsiveness (Uemura *et al.*, 1995; Webb, Uemura & Steponkus, 1994; Xin & Browse, 2000). The solute concentration of the cytoplasm play vital role and the removal of the net amount of water from the cell some time depends on the solute concentration in cytoplasm (Xin & Browse, 2000).

3.1.5. Cold acclimation and frost stress tolerance in plants

Freezing tolerance increases in many plants on exposure to non freezing low temperature for certain period, the process is known as cold acclimation (Levitt, 1980; Sakai & Larcher, 1987; Smallwood & Bowles, 2002; Thomashow, 1999). Cold acclimation is a collective processes and whole plant display a combination of responses in different organs and tissues during cold acclimation which could not display by a single tissue or cell culture (Pearce et al., 1998).

Cold acclimation has provided an avenue for investigation that how plant cells can tolerate freezing and the biochemical changes that occur during cold acclimation include the changes essential for freezing tolerance. However, the biochemical changes are not necessarily all adaptive to freezing stress (Gareth, 2001). Further several plausible reasons for biochemical changes occurring during acclimation were listed (Gareth, 2001; Pearce, 1999). In association with low temperature stress, there may be adaptive responses to other stresses as well, such as attack by snow moulds or ice encasement and developmental responses such as vernilization also entail novel biochemistry and some biochemical changes may be not adaptive (Gareth, 2001). Therefore, while other changes may make zero or negative contribution (Gareth, 2001).

Light also plays an important role in cold acclimation process as moderate to high light is essential for cold acclimation process, in the absence of light, photo-inhibition leads the plant to form reactive oxygen species causing oxidative stress (Foyer, Lelandais & Kunert, 1994; Wanner & Junttila, 1999).

The cold acclimation capability of plant is a quantitative trait involving a large number of genes, and massive reprogramming of gene expression is associated with cold acclimation (Mantas, Pekka & Tapio, 2010). It has been estimated in Arabidopsis that 5-25% of the genes show varied patterns of expression during low temperature (Mantas, Pekka & Tapio, 2010; Robinson & Parkin, 2008). The induction of low temperature responsive genes resulted in a large amount of physiological, metabolic, and biochemical alterations that determine the ultimate level of freezing tolerance achieved during acclimation (Mantas, Pekka & Tapio, 2010). These alterations include the changes in the level of antioxidants, phytohormones, and changes in the level of production of compatible solutes and protective proteins, the proteins including

chaperons and some of other proteins of unknown functions (Aalto, Heino & Palva, 2006; Mantas, Pekka & Tapio, 2010)

In this part of the the study, the N-nitrose-N-ethylurea (NEU) and N-nitrose-N-methylurea (NMU) created mutants were regenerated, sub-cultured the clones after each 3-4 months continuously for the maintenance of the material for the project and clones were screened for frost, drought and salt stress resistance. The frost stress resistance was also investigated under cold acclimation conditions.

3.2 Aim and objectives

Aim

The aim of physiological assessment was to confirm the persistence of abiotic stress resistance tolerance in mutants clones over many clonal generations, which was important to identify the response of each mutant line prior to further molecular and biochemical characterization of mutants.

Objectives

- To regenerate the experimental clones of cauliflower to maintain materials for project work
- To screen the cauliflower *in-vitro* clones (mutants and control) under frost stress
- To analyse *in-vivo* clones (mutants and control) of cauliflower under frost stress
- To screen the cauliflower *in-vitro* clones (mutants and control) under drought stress
- To analyse *in-vivo* clones (mutants and control) of cauliflower under drought stress
- To evaluate the cauliflower *in-vitro* clones (mutants and control) under salt stress
- To screen *in-vivo* clones (mutants and control) of cauliflower under salt stress
- To investigate the effect of cold-acclimation on frost stress resistance in cauliflower clones (mutants and control)
- To investigate multi-stress potential of each mutant

3.3 Materials and methods

3.3.1 Selection of abiotic stress resistant mutant clones.

The clones were maintained in *in-vitro* condition for about three years by continuous sub-culturing and reassessed for selection of stress resistant clones (Fuller et al., 2006). In the current study, the highly resistant mutants from mutated population were selected on the bases of previous screening (Fuller *et al.*, 2006) and the resistant clones were regenerated, propagated and screened once again physiologically for the frost, drought, and salt stress resistance. After stress resistance confirmation through physiological screening, the highly resistant clones were then subjected to molecular and biochemical investigations. For the present study, the selected clones were designated as KC (control) and the mutants K1, K4, K9, K11, K13, K18, K19, and K21.

3.3.2 Regeneration and propagation of clones

For regeneration and propagation of clones, the S23M media (Kieffer, Fuller & Jellings, 1995) was prepared and 20 ml of medium was poured into each sterile plastic pot (5cm x 4cm) under aseptic conditions, and allowed to cool overnight at room temperature. Three different shoot induction media were tested with different agar concentration (T1 = 7g, T2 = 4g, T3 = 0g) and their responses on shoot induction was observed.

Curds were used as explants materials for regeneration and clonal proliferation. In the green house, the explants were collected in labelled plastic bags and brought to laboratory in a cooled insulation box. Curds were cut into small pieces and treated with 70% ethanol for 40 seconds and then with 10% bleach for 5 min for surface sterilization and then washed 5 times with sterilize dH2O in order to remove bleach from the surface. The explants (2-3 mm) were then inoculated into shoot induction media in

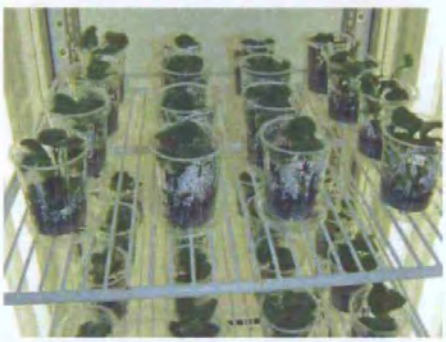
plastic pots. The pots were kept in incubator at 23 °C and 8 h photoperiod and observation was carried out until shoots were produced. The young shoots after 5 weeks period were excised with sterile sharp scalpel and inoculated into hormone free S23M media in plastic pots and allowed at 23 °C with 16 h photoperiod in the incubator. Observed until complete rooted plantlets were regenerated and sub-culturing of these *in-vitro* clones on S23M hormone free media was carried out regularly after each 3 to 4 months for maintenance of the plant materials for continued experimentation during the whole project work. All of the tissue culturing steps were performed in sterile conditions in laminar flow cabinet.

3.3.3 *In-vitro* clones transfer to *in-vivo* conditions

The *in-vitro* clones were grown on S23M medium at 23 °C and 16 h photoperiod light intensity 180.8 µmol m^{-2} s^{-1} were transferred to *in-vivo* conditions through the weaning process (Figure 12) and through this process 100% successful transfer of all clones was obtained. The process in detail has been discussed earlier in chapter 2.

a.

b.



c.

d.

e.

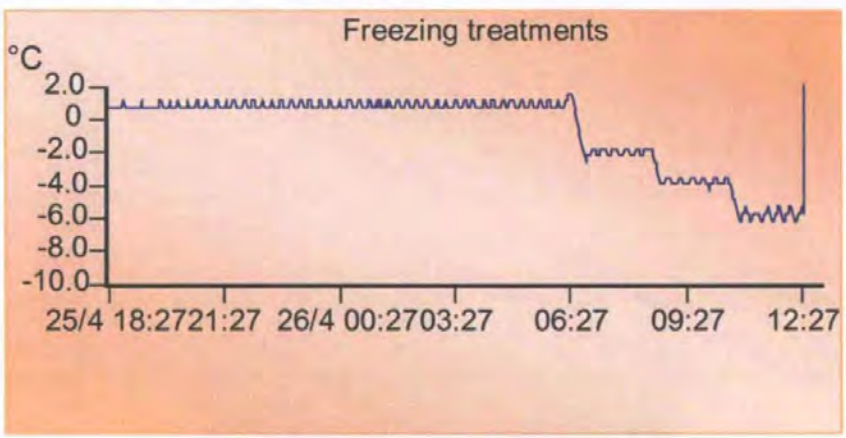
f.

g.

Figure 12: Weaning process: transfer of *in-vitro* clones into *in-vivo* conditions. a. removal of *in-vitro* plants from agar media and fungicide spray on roots, b. *in-vitro* plants transferred to compost, c. plants in growth cabinet, d. plants in green house under shade, e plants transfred into bigger pots in green house, f. young plants, g. mature plants.

3.3.4 Frost stress resistance

Both of *in-vitro* as well as *in-vivo* clones were examined for frost stress resistance at different temperatures i.e. +1 °C, -2 °C, -4 °C, and -6 °C with hold of two hours at each freezing temperature (Figure 13). The leaf discs assay was performed under cold-acclimated and non-acclimated conditions using the electrical conductivity technique described by Fuller et al (2003; 1989). For acclimation process, four fully expanded upper leaves from each genotype in the greenhouse (*In-vivo*) were excised and transferred to the laboratory in insulation box containing ice packs. Two leaves were used as non-acclimated and the other two leaves were acclimated in incubator (Snijder scientific) at 4 °C, 8 h photoperiod for 14 d. In the same incubator the *in-vitro* clones were acclimated as entire plant for 14 d. The percent relative electrical conductivity was measured as REC% = Post freezing EC/ Post autoclaving EC x 100.



a

b

Figure 13: Frost resistance test.
(a). Example of the temperature trace of a frost test at +1, -2, -4, -6 °C to study damage of cell membranes. **(b).** General view of the set-up for measuring electrical conductivity.

3.3.5 Drought stress resistance

Leaf discs assay was carried out for drought stress resistance for both of *in-vivo* and *in-vitro* plants. The basal MS medium (Murashige & Skoog, 1962b) at ration of 4 gl^{-1} was dissolved in dH_2O and added with different concentrations of mannitol as 150, 250, 350 and 450 mM and control media was used without mannitol. Leaf discs were incubated overnight in sterilized dH_2O in labeled an sterilized petri plates at room temperature to become turgid (Figure 14) and after measuring turgor weight (TW), leaf discs were allocated to test media in petri dishes and incubated for seven days at 23 °C with 16 h photoperiod and re-measured the weight (fresh weight FW) then dried the discs for dry weight measurement. Percent relative water content in discs was measured as RWC% = (FW-DW) / (TW-DW) x 100.

Figure 14: Drought resistance test.

3.3.6 Salt stress resistance

Plants were screened for salt (NaCl) resistance using a leaf disc assay. Leaf discs of one centimetre diameter were prepared from the leaves of both *in-vivo* and *in-vitro* clones. Leaf discs from *in-vivo* clones were surface sterilized in 70% ethanol for a few seconds, followed by shaking in 10% bleach solution (sodium hypochlorite) for ~10 minutes followed by three rinses with sterile distilled water. The *in vitro* clones were used direct from culture pots.

Leaf discs were transferred to Petri-dishes containing 20 ml sterile liquid medium (M&S salts at 4 g l–1) supplemented with NaCl at concentrations of 350 and 550 mM. Media without NaCl was used as control media. Leaf discs damage was assessed after 3, 5 and 7 days using a five point score based on the percentage greenness of the leaf discs.

3.4 Results

3.4.1 Regeneration and sub-culturing of experimental clones

Responses to different media was different for the same genotype, the medium with 4 gl^{-1} agar (T2) was found to be best for shoot induction and subsequent growth rate whilst the medium with 7 gl^{-1} agar (T1) was better than the medium without agar (T3) which tended to leave the explants vitrified. The difference in shoot induction and growth was observed after three weeks period but this difference was more clear after 5 days period as shown in Figure 15.

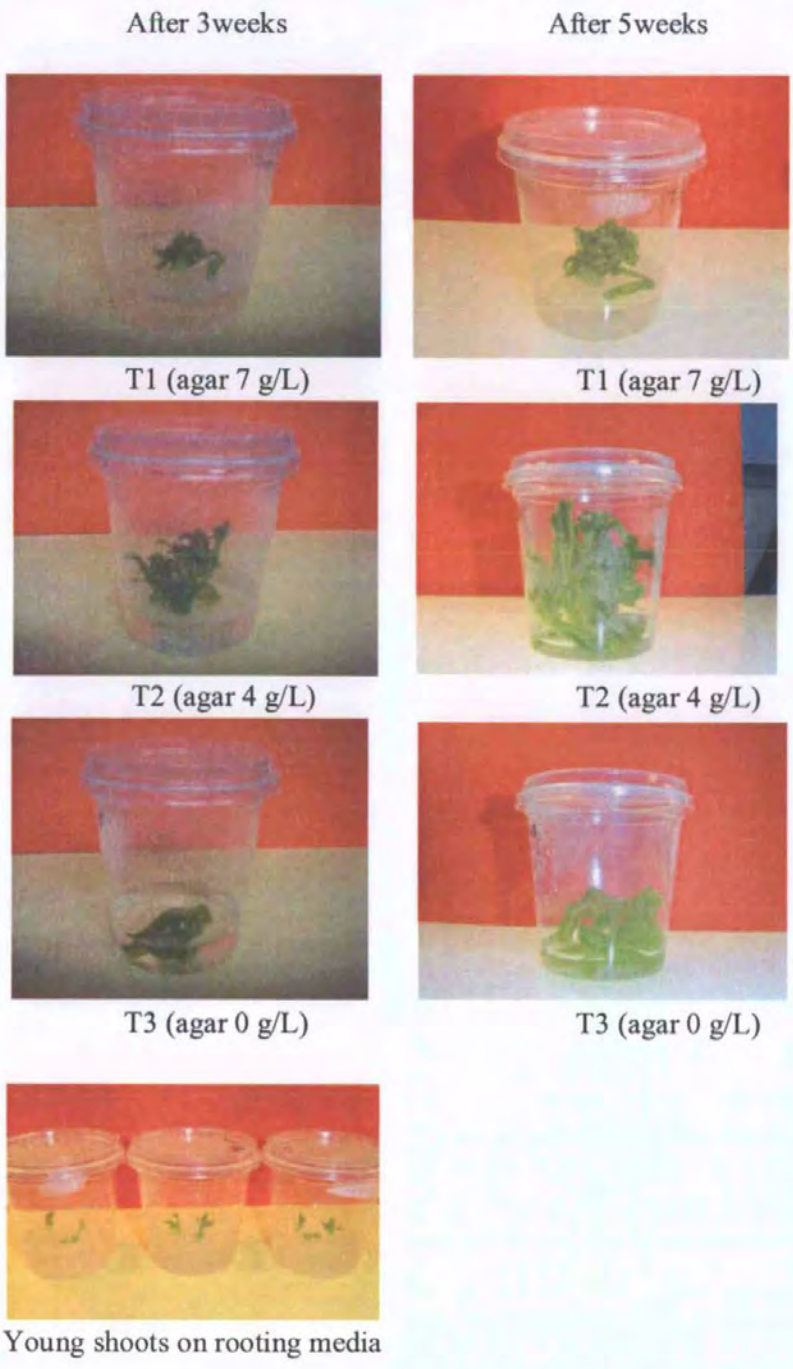


Figure 15: Regeneration response of clone on different media.

3.4.2 Assessment of *in-vivo* and *in-vitro* mutants for frost resistance under cold-acclimation

Cell damage was increased with lowering temperature i.e. -2 °C, -4 °C and -6 °C and highest damage in terms of highest relative electrical conductivity was observed at -6 °C. The response of non-acclimated and acclimated *in-vivo* plants at +1 °C and -2 °C (Figure 16 A and B) were similar and showed less leakage of electrolytes from the cells but at -4 °C and -6 °C (Figure 16 C and D) an increase in electrical conductivity was observed. The acclimation process was very effective in increasing frost resistance in all *in-vivo* genotypes but was more effective in the mutant genotypes and clear differences existed among acclimated mutants at -6 °C with some mutants like K18, K19, K1 and K11 showing high resistance as compared to control clone (Figure 17). Other mutants like K13, K4, K9 and K21 showed moderate resistance as compared to control and the highest resistant mutant was found to be K18 followed by K19 (Figure 17).

The acclimation process was also found to be effective for the increase in frost tolerance in *in-vitro* clones. Figure 18 showed that acclimation process increased tolerance over non-acclimated plants. All the cold acclimated mutants showed more tolerance compared to the control and electrical conductivity increased with lowering the temperature (Figure 18 A-D). All clones in the *in-vivo* state showed higher resistance than in the *in-vitro* state in terms of relative electrical conductivity. One reason for this difference between *in-vivo* and *in-vitro* might be the young and soft *in-vitro* growing in incubator under constant optimum temperature and humidity prior to exposure to freezing, while *in-vivo* plats were mature and grown in glass house environment where plants are constantly challenged with some minor stresses. It can be concluded from these results that K18, K19, K1 and K11 are highly resistant to frost compared to control.

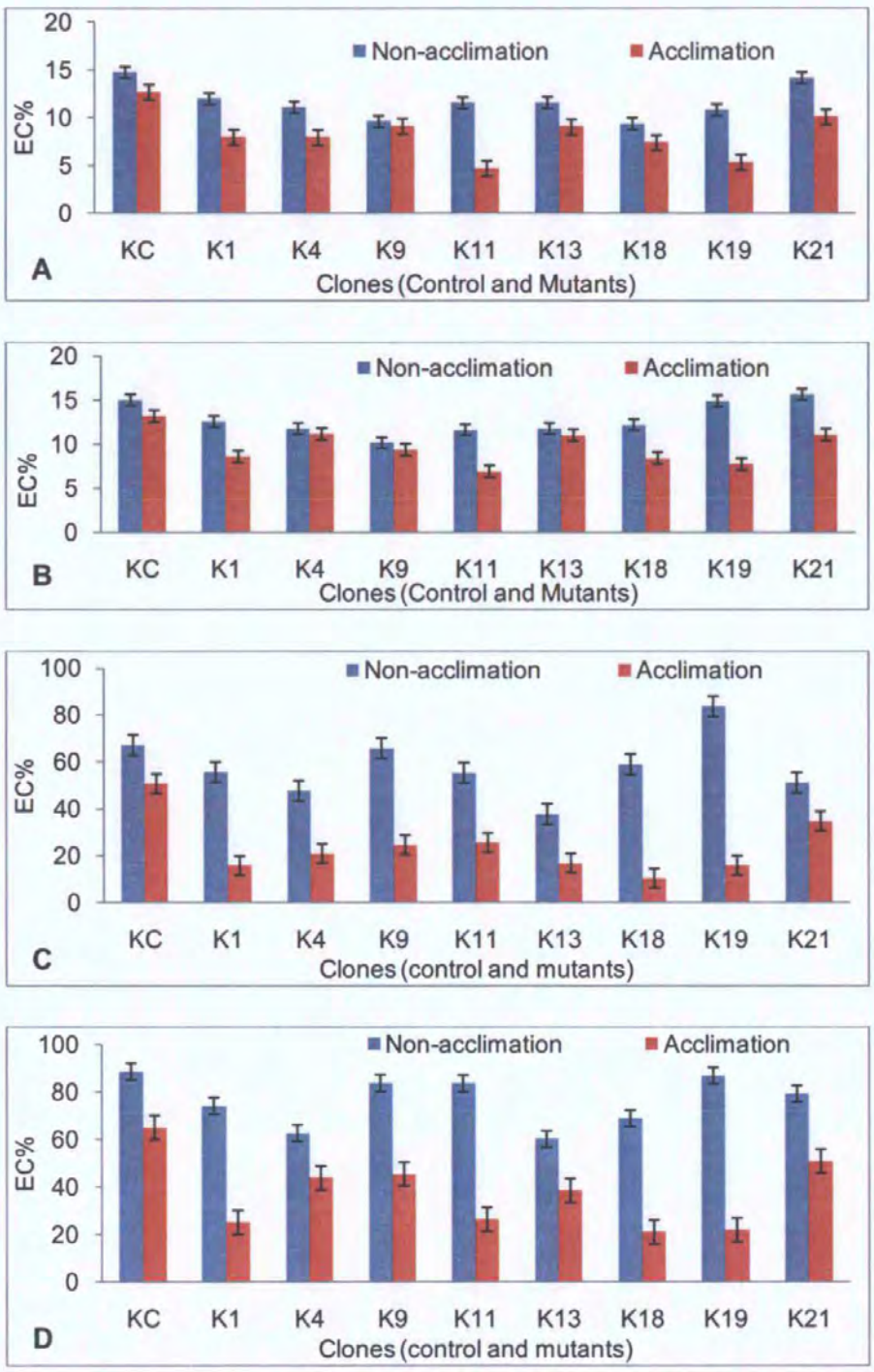


Figure 16: Effect of cold acclimation on leakage from cell membrane of *in-vivo* clones at different freezing temperatures.
(**A**) +1 °C, (**B**) -2 °C, (**C**) -4 °C and (**D**) -6 °C. Lower the electrical conductivity (EC%) means less damage as measured by leakage against freezing temperature. The clone KC is control and K1 - K21 are mutants.

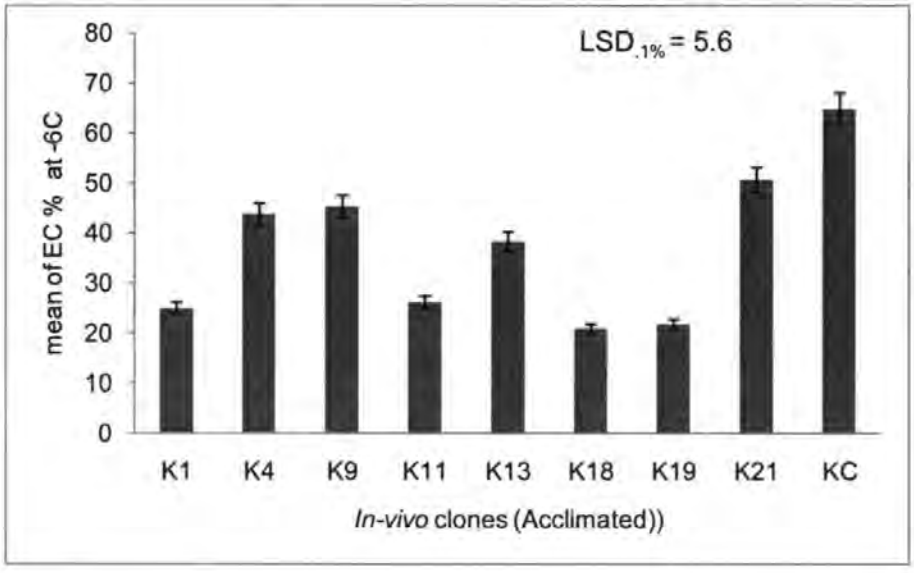


Figure 17: Response of cold acclimated *in-vivo* clones at -6 °C .
Lower the %EC higher the frost resistance. The clone KC is control and K1 - K21 are mutants. The results show that the mutants are significantly different while some of the mutants are highly significant when compared with the control clone.

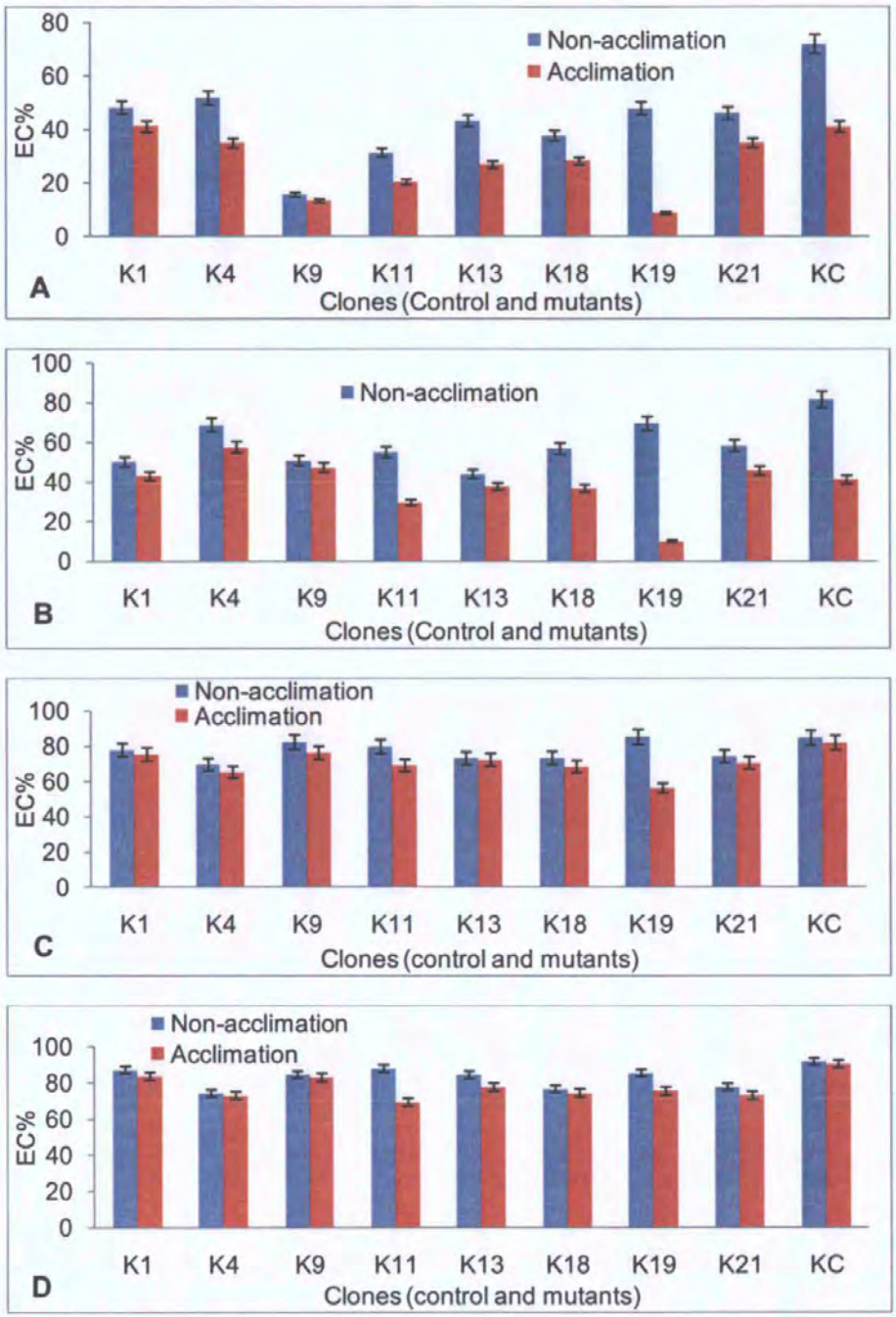


Figure 18: The cold acclimation effect on *in-vitro* clones.
Response of clones at different freezing temperature, (**A**) +1 °C, (**B**) -2 °C, (**C**) -4 °C and (**D**) -6 °C. Lower the electrical conductivity (EC%) means less damage as measured by leakage. The clone KC is control and K1 - K21 are mutants

3.4.3 Evaluation of *in-vivo* and *in-vitro* shoots of mutants for salt resistance

The increase in salt concentration and time of exposure showed a decrease in greenness in both of *in-vivo* (Figure 19A) and *in-vitro* (Figure 19B) clones. After 3 days incubation the differences between genotypes was not obvious but by day 5 differences were clear and on day 7 there was very clear differentiation between mutants and the greenness in *in-vivo* clones reduced with increase in salt concentration from 350 mM to 550mM (Figure 20 A and B). Some clones had also progressed from green to white and the higher concentration of NaCl (550 mM) in liquid media showed clear differences in colour change (Figure 20B) and control clone discs changed from dark green to white after seven days treatments (Figure 20B and 21). Some mutants, e.g. K19, K9, showed a high level of resistance and maintained 83% and 73% greenness respectively at 550 mM NaCl after 7 days (Figure 20B and Figure 21). All the *in-vivo* mutants showed significant difference when compared with the control (Figure 22). Moderately resistant mutants (K13, K11 and K4) showed mix of colours of light green and with some discs white. Control leaf discs showed less than 40% greenness after 5 days treatment at 550 mm NaCl (Figure 22). *In-vitro* grown clones showed the same general response to *in-vivo* clones with increasing salt concentration and exposure time of treatments (Figure 19B) and all the mutants showed higher greenness compared to control (Figure 23A-B). All the mutants showed more resistance than the control clone and among the mutants the response of each mutant was different and the control line changed from green to white after 7 days treatment at 550mM salt (Figure 23B and Figure 21D). These results confirmed that their resistance had been maintained after long time and many sub-cultures of the clones.

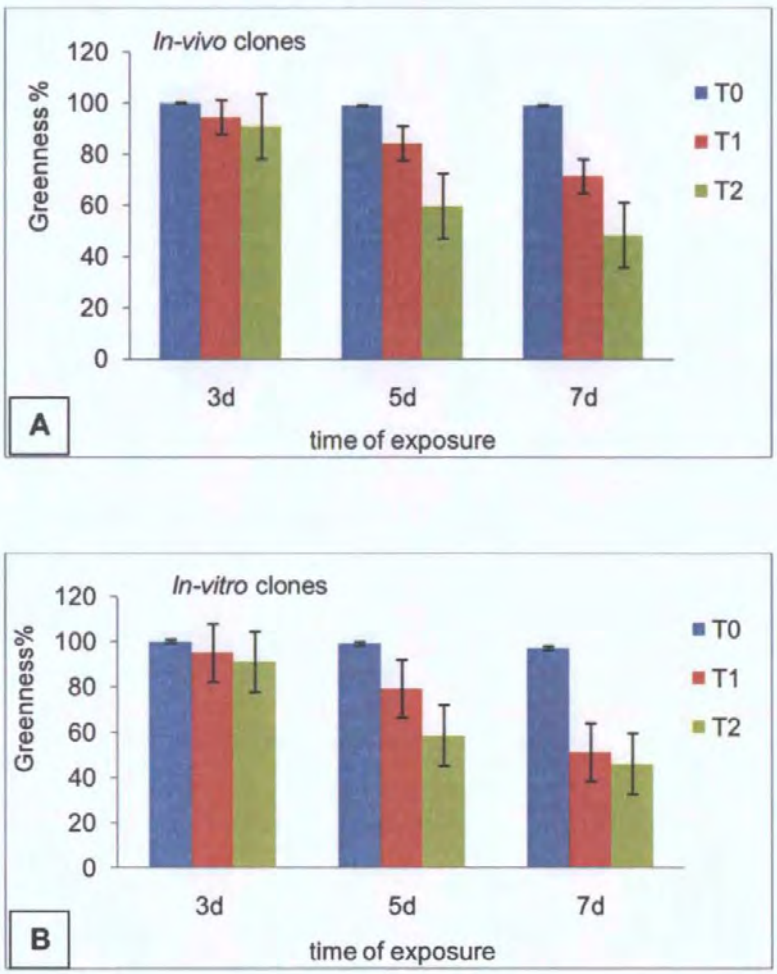


Figure 19: Effect of salt treatments on greenness of leaf discs.
Salt stress tolerance of clones after 3, 5 and 7 days treatments with different concentrations of salt (T0= without salt, T1= 350 mM NaCl, T2= 550mM NaCl): *In-vivo* clones (**A**) and *in-vitro* clones (**B**), the values represent mean of all clones.

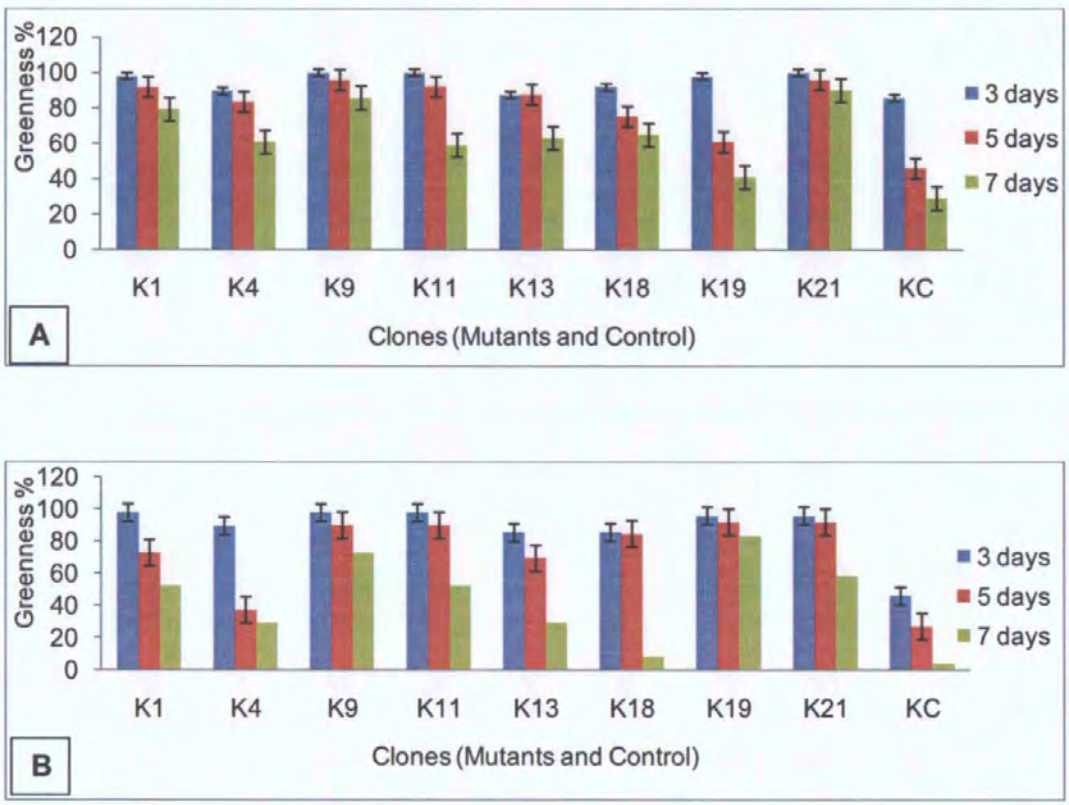


Figure 20: The response of *in-vivo* clones at different NaCl concentrations.
The response of clones at 350 mM (**A**) and 550 mM salt in media (**B**) after 3, 5 and 7 days treatments. KC is control and K1-K21 are mutants.

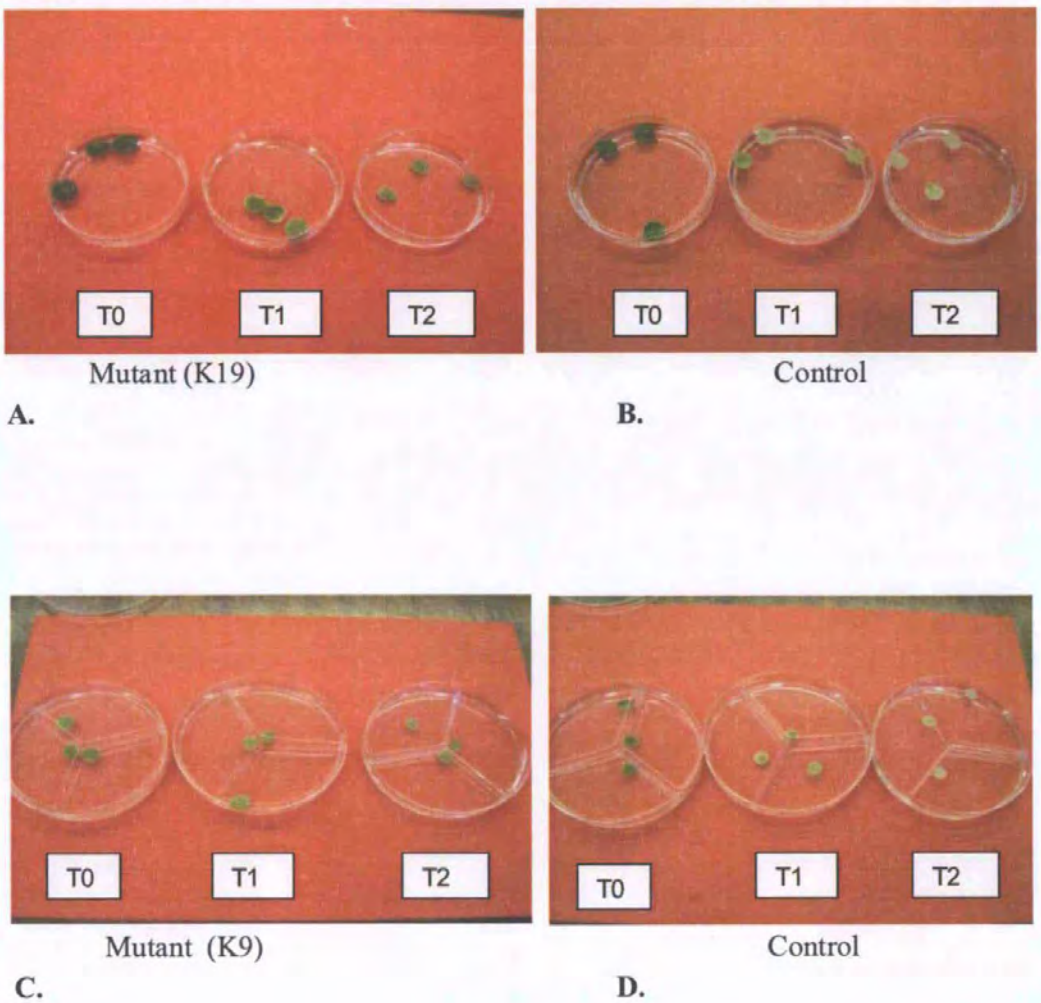


Figure 21: The effect of NaCl concentrations on leaf discs after 7 days treatments. **A** and **B**.are *in-vivo* klones, **C** and **D** are *in-vitro* klones. T0 = Control media without NaCl, T1 = 350 mM NaCl in media, T2 = 550 mM NaCl in media, Greenness indicate resistance.

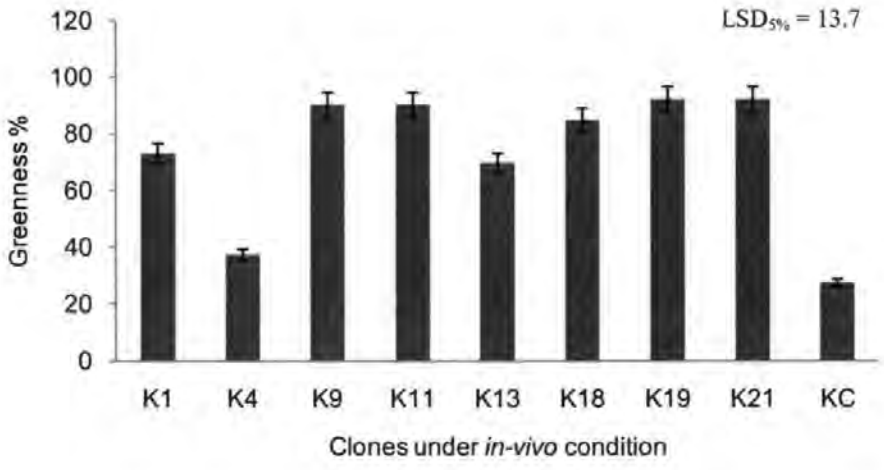


Figure 22: Response of *in-vivo* clones to higher salt concentration (550mM) after 5 days treatment.
KC is control and K1 - K21 are mutants. Higher greenness indicates salt resistance.

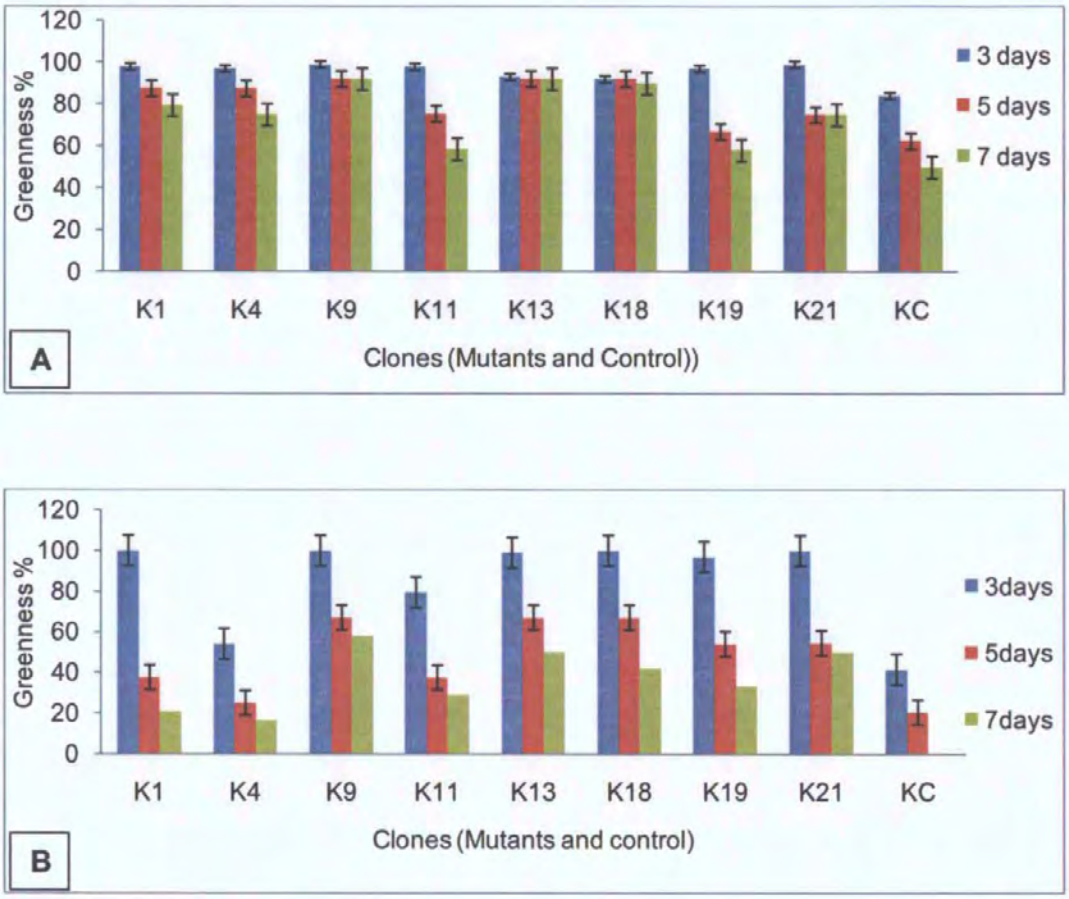


Figure 23: Response of *in-vitro* clones at different salt concentrations.
The individual genotype response of *in-vitro* clones at 350 mM (**A**) and 550 mM NaCl in media (**B**), after 3, 5 and 7 days of treatments. KC is control and K1-K21 are mutants.

3.4.4 Assessment of *in-vivo* and *in-vitro* mutants for drought resistance

Results showed clear differences in leaf disc relative water contents of *in-vivo* clones after treatment with different mannintol concentrations and the relative water content was reduced while increasing the mannitol concentration showing the dehydration effect of the treatments, and there was differentiation between the genotypes (Figure 24). The mutant genotypes maintained more water even at 450 mM mannitol as compared to control plant indicating drought resistance (Figure 25). All the mutant genotypes showed more than 50% RWC at 450 mM while control plant maintained less than 50% RWC at 450 mM mannitol (Figure 25). The highly resistant mutants K21, K1, K19 and K18 showed 73%, 69%, 62% and 57% respectively at 450 mM (Figure 25). The *in-vitro* plants showed similar response to *in-vivo* plants with increasing mannitol concentration reducing the water contents of leaf discs (Figure 26). This decrease in water contents was very obvious between 150 mM and 350 mM but decreased somewhat at 450 mM (Figure 26). Figure 27 showed that even at the highest concentration of mannitol (450 mM) some mutants like K1, K19, and K21 maintained higher water content compared to the control (KC). It can be concluded that mutants like K1, K19, and K21 were highly resistant to mannitol induced drought as compared to control plant.

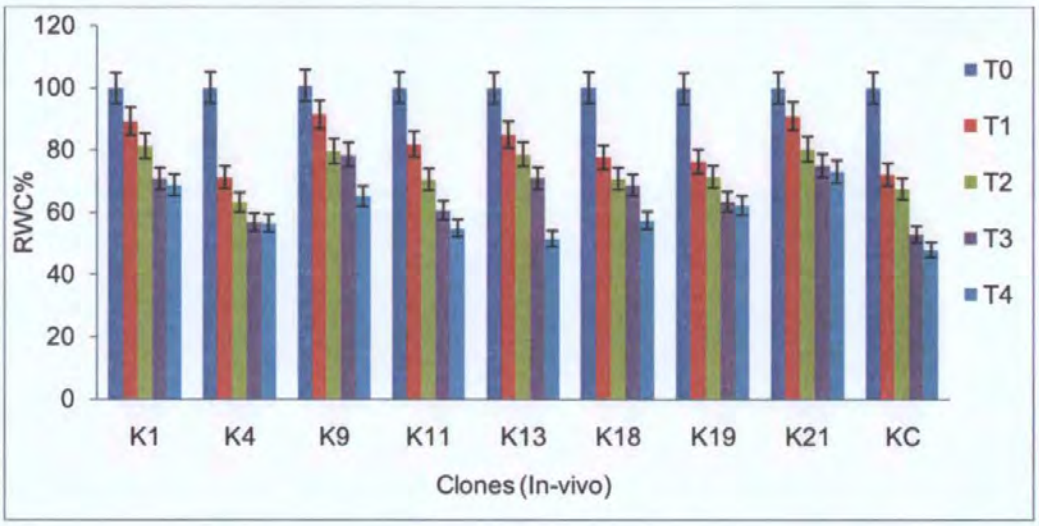


Figure 24: The relative water content (RWC%) in leaf tissues of *in-vivo* clones after treatments with mannitol at different concentrations.
T0= control without mannitol, T1= 150 mM, T2= 250 mM, T3= 350 mM and T4= 450 mM mannitol in media. High RWC% indicates more resistant to drought. KC is control and K1 - K21 are mutants.

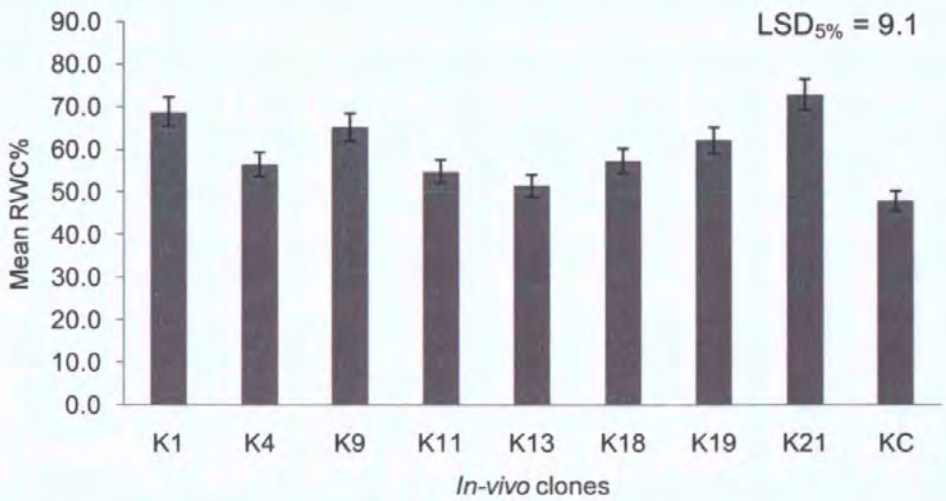


Figure 25: Response of leaves tissues of *in-vivo* clones to 450 mM mannitol in media. KC is control and K1 - K21 are mutants. High %RWC more resistant to drought.

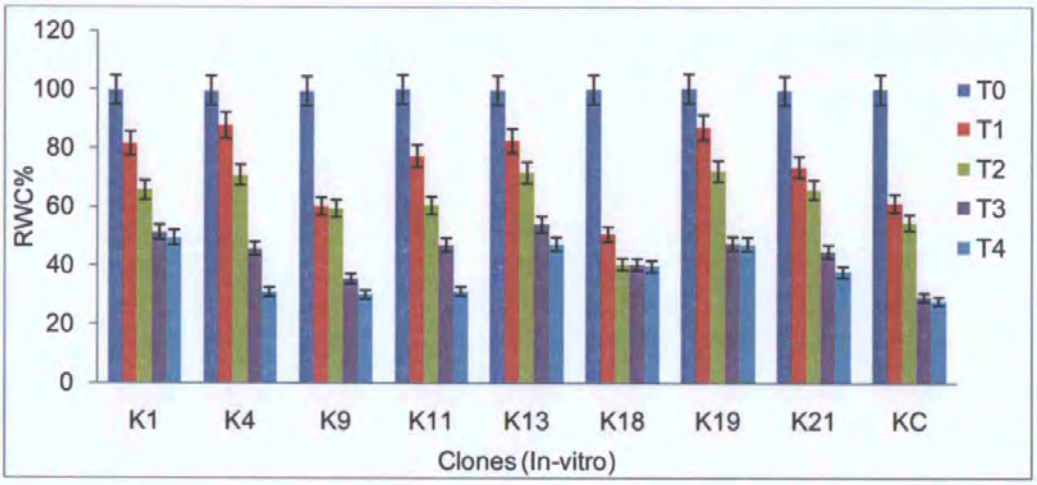


Figure 26: The relative water content (RWC%) in leaf discs of *in-vitro* clones at different concentrations of mannitol in media.
T0= control without mannitol, T1= 150 mM, T2= 250 mM, T3= 350 mM and T4= 450 mM mannitol in media. High RWC% indicates more resistant to drought. KC is control and K1 - K21 are mutants.

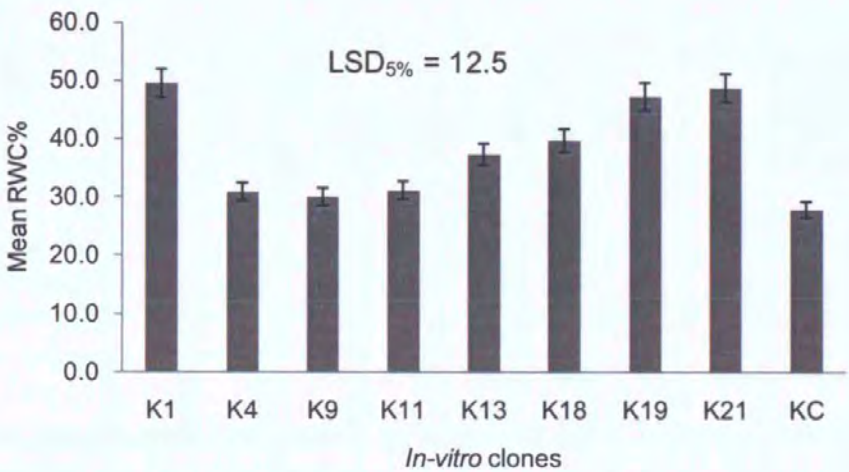


Figure 27: Response of leaf discs of *in-vitro* clones at 450 mM mannitol in media. High the RWC, more resistant to drought. K1 to K21 are mutants and KC is control.

3.4.5 Correlations between *in-vitro* and *in-vivo* clones for drought, salt and frost resistance

The leaf discs either from *in-vivo* or from *in-vitro* clones, both showed damage on exposure to drought, salt and frost stresses. Overall, the *in-vivo* grown clones showed higher level of resistance over *in-vitro* clones. There was a significant positive linear correlation between the *in-vitro* and *in-vivo* clones for drought stress resistance (Figure 28) and salt stresses resistance (Figure 29). For frost stress resistance a positive correlation was found between *in-vitro* and *in-vivo* clones but the relation was not significant (Figure 30) which might be due to the higher susceptibility of soft and fragile leaves of *in-vitro* clones to lower temperature.

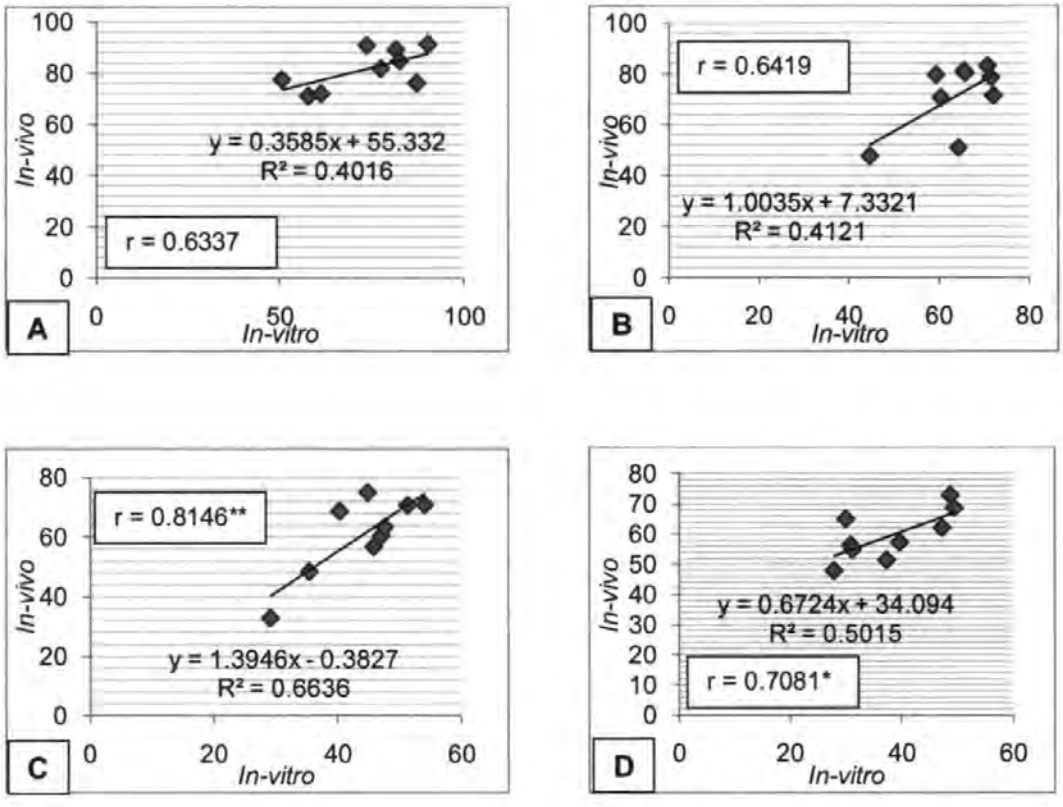


Figure 28: Correlation of relative water content (RWC %) between *in-vivo* and *in-vitro* clones.
In the presence of different mannitol concentrations in media (**A**) 150 mM, (**B**) 250 mM, (**C**) 350 mM, (**D**) 450 mM mannitol in media.

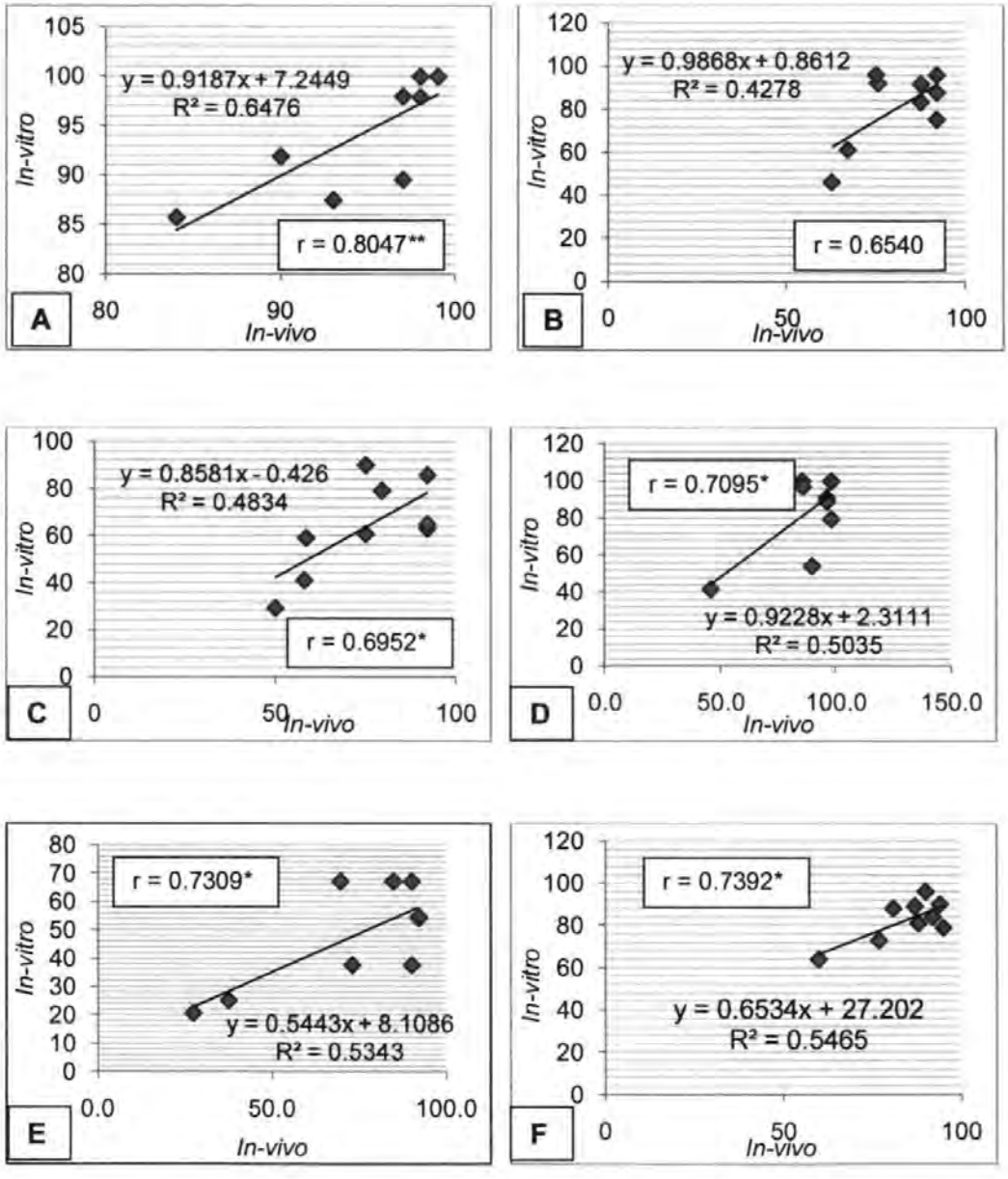


Figure 29: Correlation of greenness % between *in-vivo* and *in-vitro* clones.
In the presence of 350 mM NaCl in media after 3 days (**A**) 5 days (**B**) 7 days (**C**), and in the presence of 550 mm NaCl in media after 3 days (**D**) 5 days (**E**) 7 days (**F**) of treatments.

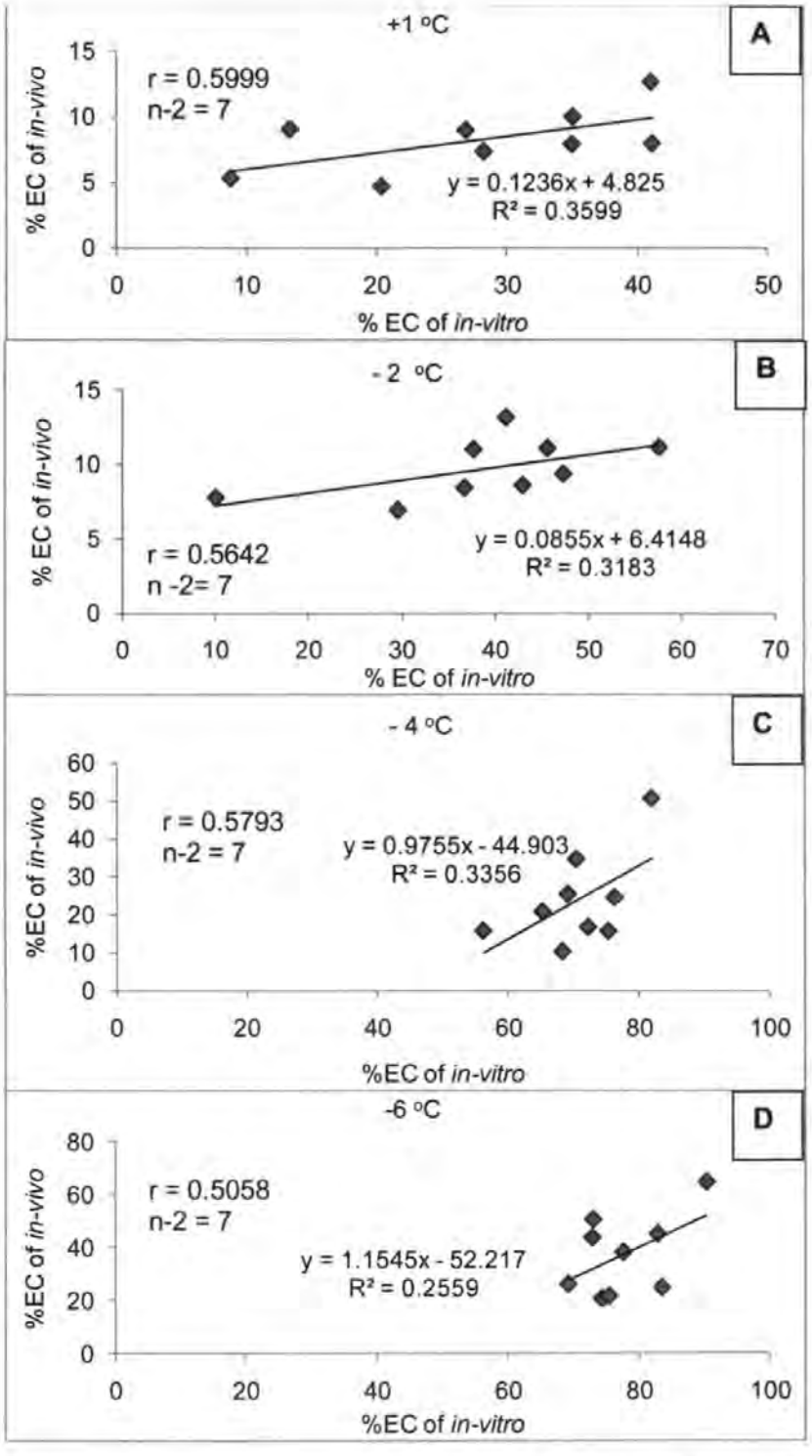


Figure 30: The frost resistance correlation between *in-vivo* and *in-vitro* acclimated klones at different test temperatures.
(A) +1 °C **(B)** -2 °C **(C)** -4 °C **(D)** -6 °C

3.4.6 Correlation of proline with salt and drought resistance in clones

The proline level was measured in *in-vivo* clones and correlated with greenness% (salt stress) and relative water content % (drought stress) of clones leaf discs. A positive and significant correlation was found between greenness% and proline level in clones (Figure 31), and similarly a positive found in relative water content % and proline level in clones (Figure 32).

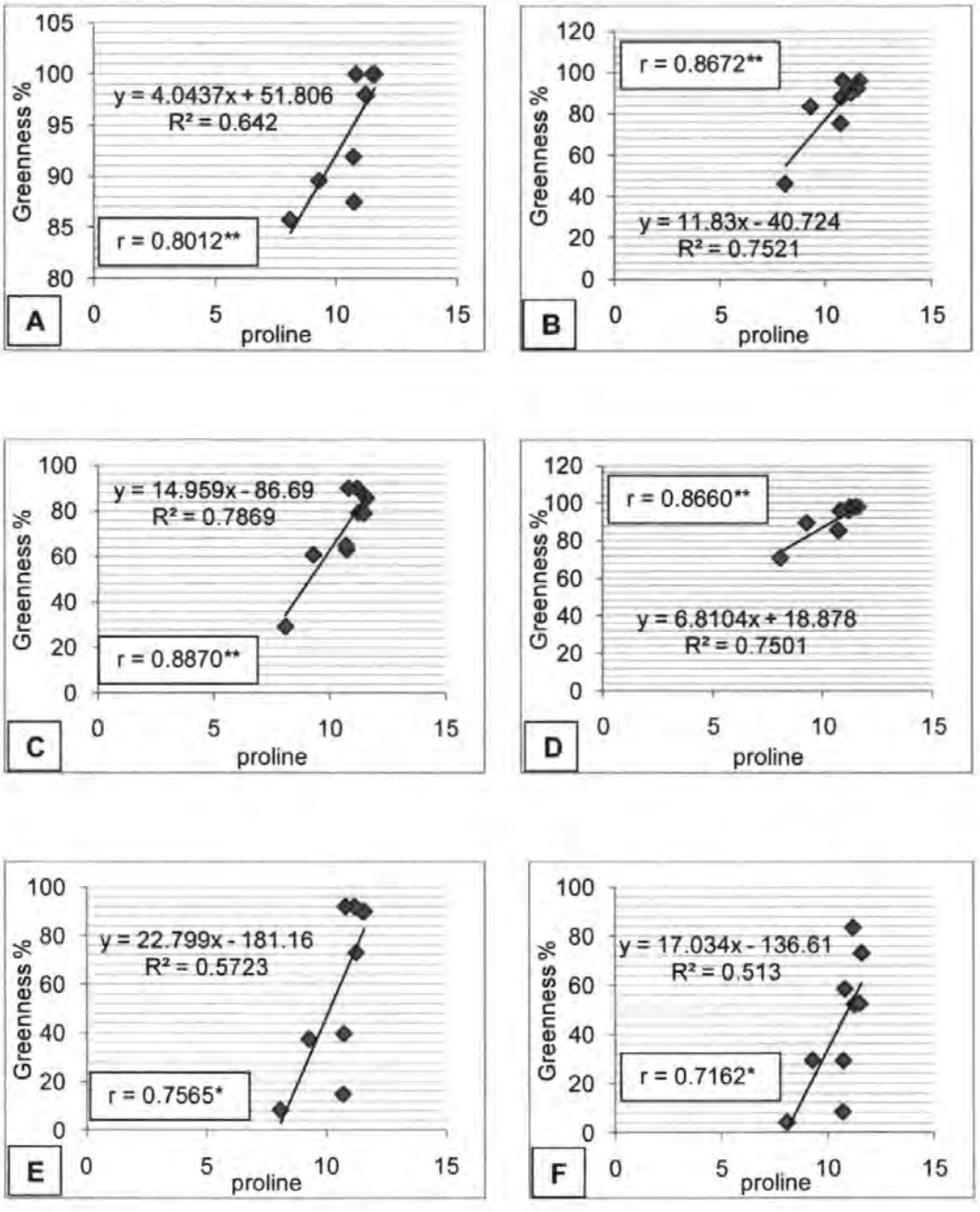


Figure 31: Correlation between salt tolerance and proline level in *in-vivo* clones
In the presence of 350 mM NaCl in medium after 3 days (**A**), 5 days (**B**) 7 days (**C**) and in the presence of 550 mM NaCl after 3 days (**D**) 5 days (**E**) and 7 days (**F**) of treatments.

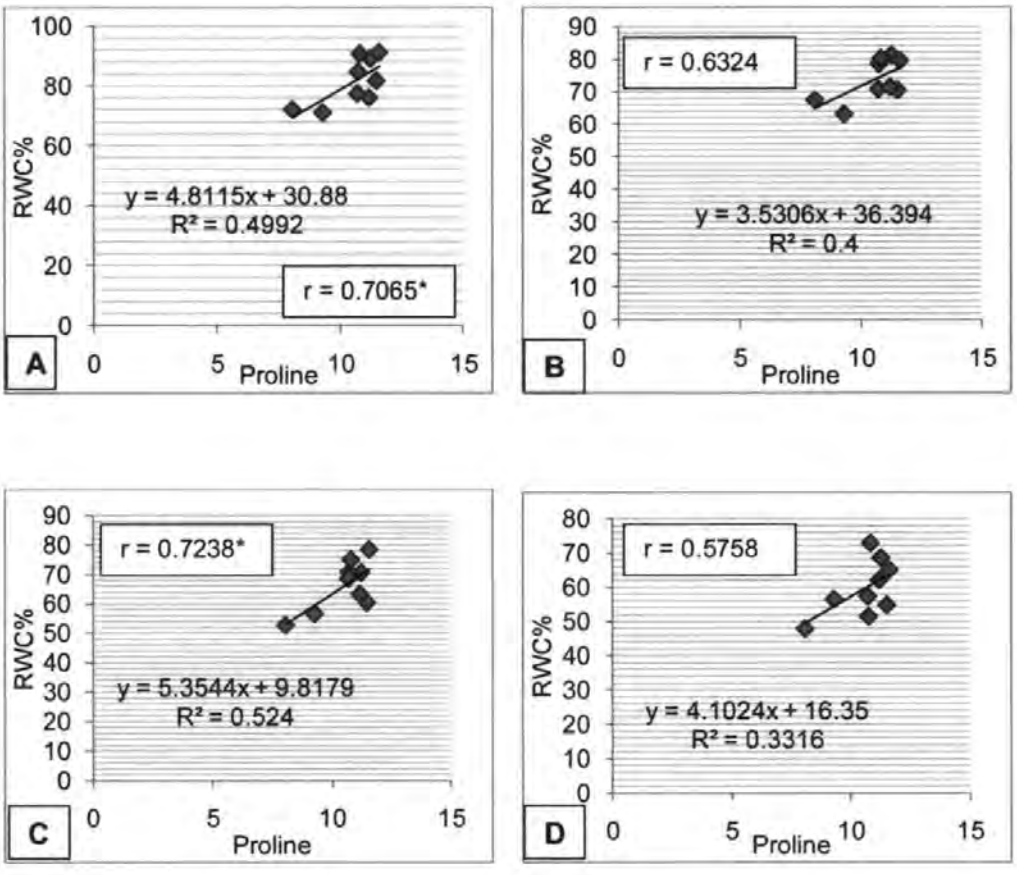


Figure 32: Correlation between water content % and free proline level in *in-vivo* clones RWC% in leaf discs under different concentrations of manitol in media. (**A**) 150 mM (**B**) 250 mM (**C**) 350 mM (**D**) 450 mM mannitol.

3.4.7 Multi-stress resistance summary of mutants

The summarize data of clones resistance to different stresses are presented in Table 2. All of the mutants except a few showed higher resistance over control for all of the three frost, drought, and salt stresses, which clearly demonstrated the existence and stability of the chemically induced mutations through many sub-culturing in *in-vitro* conditions over time. The cold-acclimation process was found highly effective for frost resistance over non-acclimated clones and even in the control clone the level of resistance enhanced over non-acclimated (Table 2). Among the mutants the response of individual mutant was different for each of the stresses, some mutants were resistant to double stresses like K4, K13 and K18. Other mutants like K1, K9, K11, K19 and K21 were triple stress resistant (Table 2).

Table 2. Summary of the resistance to multi-stresses

	Frost resistance			
Clones (K)	Acclimated	Non-acclimated	Drought resistance	Salt resistance
K1	***	*	**	*
K4	***	**	*	-
K9	**	*	**	**
K11	***	*	*	*
K13	***	**	*	-
K18	***	**	*	-
K19	***	-	**	***
K21	**	*	***	*
KC	*	-	-	-

KC is control and K1 - K21 are mutants.

Highly resistance (***)
Resistant (**)
Moderate (*)
Sensitive (-)

Frost resistance on the bases of % relative electrical conductivity at -6 °C:

Acclimated	Non-acclimated
<45 (***)	<55 (***)
45-60 (**)	55-70 (**)
60-75 (*)	70-85 (*)
>75 (-)	>85 (-)

Drought resistance bases on % relative water content at 450mM mannitol for 7 d treatment.
<50 (-)
50-60 (*)
60-70 (**)
>70 (***)

Salt resistance on the bases of % greenness at 550 mM NaCl for 7 d treatment
<50 (-)
50-60 (*)
60-75 (**)
>75 (***)

3.4.8 Correlations among frost, drought, and salt stress resistance in mutants

The stress resistance responses of mutants were examined by correlation for the relationships between the responses to all of the three stresses resistance. There was a positive correlation between drought and salt stress resistance (Figure 33A) as well as the correlation between drought or salt stress resistance with frost resistance of non-acclimated mutants were also positive as shown in Figure 33B and 33C respectively. While very weak or no relationship between drought or salt stress resistance with frost resistance of cold acclimated mutants were found (Figure 34A & B).

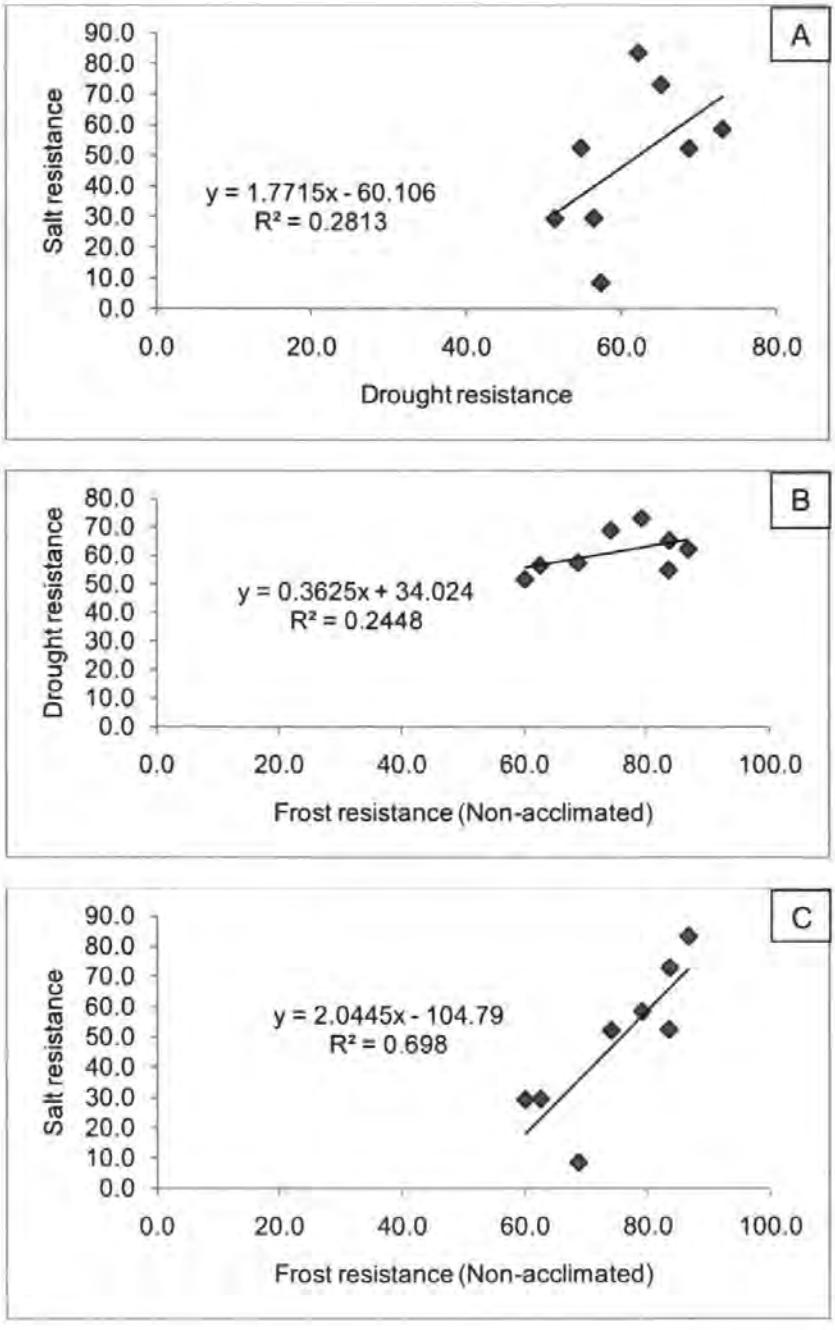


Figure 33: Correlation among Non-acclimated frost, drought and salt resistance. **(A)** Between salt and droght resistance, **(B)** Between drought and frost resistance, **(C)** Between salt and frost resistance.

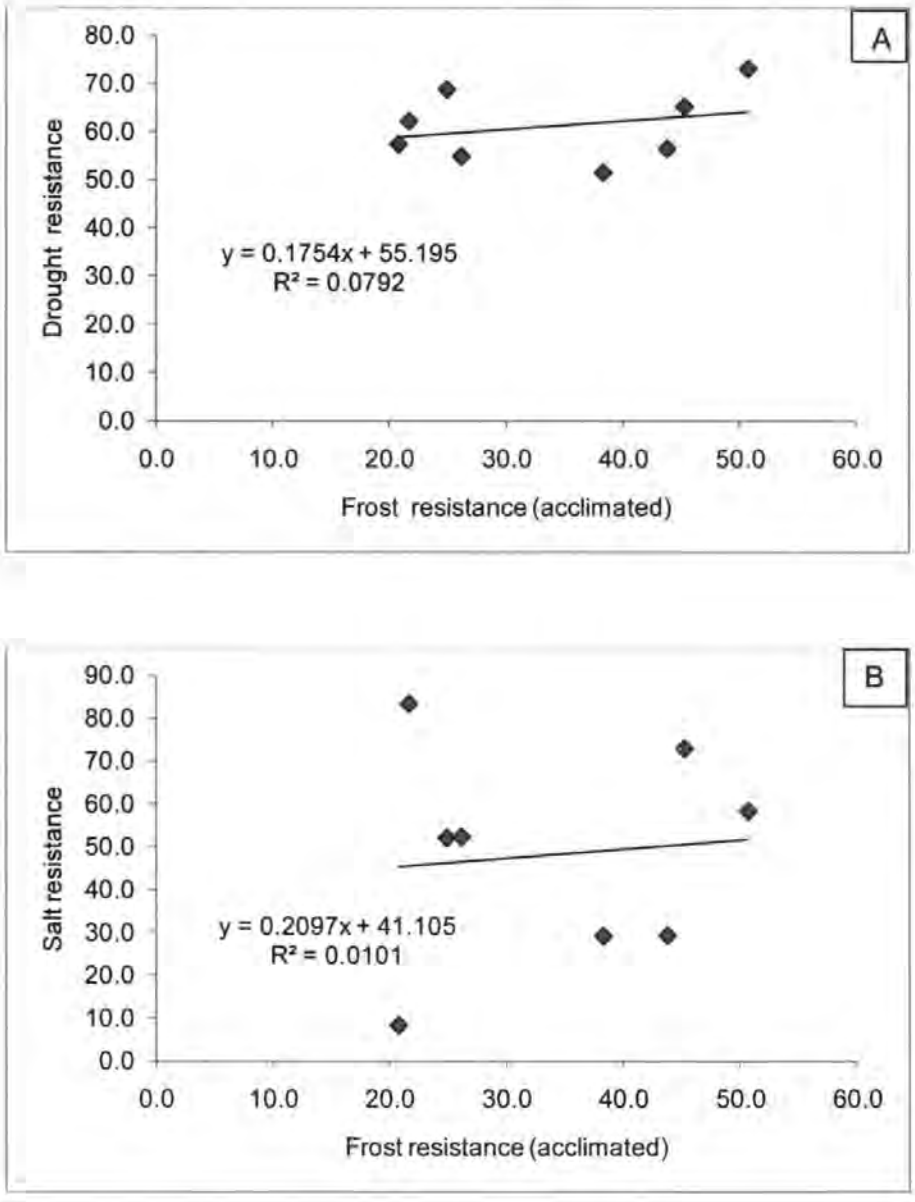


Figure 34: Correlation among acclimated frost with drought and salt resistance.
(A) Between drought and frost resistance of cold acclimated mutants, (B) Between salt and frost resistance of cold acclimated mutants.

3.5 Discussion

The results clearly demonstrated altered abiotic stress resistance in chemically (NEU & NMU) induced mutants of cauliflower compared to control plants. This confirmed the persistence of the mutations over-long time storage and many sub-cultures of the clones. In addition, the *in-vivo* forms of these mutants correlated positively with *in-vitro* screening of resistance, indicating a stability of the mutation after regeneration. The mutants used in this investigation had been created by chemical mutagenesis and the results show that this approach is successful in producing mutant lines with improved resistance. These findings suggest that the NEU and NMU could be used in plant breeding programs for *Brassica oleraceae* (IAEA, 1977; Negrutu, 1990).

The simple leaf disc assay refined in this investigation was found to successfully differentiate the control and mutant clones for salt, drought and frost stress resistance and the selection process used in this investigation clearly show that this type of selection in cauliflower is very useful to generate abiotic stress resistant genotypes like in other *Brassica* species (Ashraf & Harris, 2004; Ashraf & McNeilly, 2004; Fuller et al., 2006).

The Electrical Conductivity Test (EC) was confirmed as a useful test and cold acclimation effectively reduced the EC and indicated increase in frost resistance, confirming previous findings (Fuller et al., 2006; Jianhua et al., 2007; Thomashow, 1999).

In all tests there was no significant leakage of electrolytes from leaf discs treated at -2 °C , while there was a sharp increase in leakage at -4 and -6 °C. This suggests *B. oleraceae* is constitutively resistant to -2 °C. The mutants showed significant variation in EC values were differentiated at both -4 °C and -6 °C. Cold acclimation at 4 °C with 8 h photoperiod for 14 d appeared good enough to reduce leakage of electrolytes and activate cold acclimation responses in *Brassica olearacea*. Low EC% due to no or less leakage of electrolytes is described in term of the stability of cell membranes. An increased leakage of electrolytes reflects the damage to cell membranes (Maheshwari et al., 1999; Srinivisan et al., 1996). Guy (2003) found that the ability of higher plants to acclimate and tolerate freezing stress is a complex quantitative trait and the product of activities of not one but a sizable suite of genes 200-2000. It is possible that the mutants produced here carry the same or similar mutations.

It was assumed that the electrical conductivity of the leachate is directly proportional to the extent of damage of leaf discs caused by low temperature. There was wide range of EC values among low temperature treated leaf discs either unacclimated or acclimated genotypes. This is due to variations in leaf discs thickness and lack of complete homogeneity of the discs, and necessitated the calculation of relative electrical conductivity.Even with the use of relative conductivity, there is variation and therefore replication and randomisation are important in experimental designs.

Generally cauliflower can withstand light frost (-2 °C) and the results showed that there are possibilities to increase frost resistance in *Brassica olearcea* by exposing to low temperatures (cold acclimation). Genetic diversity also exist in genotypes of *B. oleracea* after acclimation (Fuller, Gout & Tapsell, 1989). The variation in frost resistance and

low temperature induced acclimation may be exploited for selection and breeding programme for frost prone areas.

The results clearly showed differentiation in mutants for salt resistance and this difference was very prominent after 7 days of salt treatments. All of the mutants showed higher resistance compared to the control clone. These results confirm the previous findings of Fuller et al (2006) who reported 80% damage for control population and significant degree of resistance with less than 50% damage for selected population. Kingsbury et al (1984) reported that sensitive species were more impaired by salt stress than resistant one due to reduced photosynthesis and a greater osmotic shock. Munns et al (2002) also observed that salinity reduces the ability of plants to take up water. Salt stress leads to both an osmotic stress which can be like freezing stress but also to sodium poisoning as potassium channels cannot distinguish between Na and K and excess Na uptake is toxic.

A technique used previously by Fuller et all 2006 for salt stress resistance screening of clones was followed in the present study and a similar response was found that confirms the effectiveness of the technique. The screening technique effectively discriminated the clone (having different genetic make up) in term of greenness scoring of leaf discs floated in a saline liquid media.

Leaf discs of control clones lost their greenness (chlorophyll) resulting in a bleaching effect under salt stress. It might be suggested that leaf discs of control clone lost chlorophyll as a symptom of salt stress injury or that the plasmalemma is damaged and the cell contents leak out and the cell dies. Gibon et al (2000) hypothesised that the loss of chlorophyll was a result of stress induced senescence and Huang and Redman (1995)

proposed the death of leaves due to the build up of Na in tissues which would prevent the supply of other nutrients to leaves leading to the death of tissues.

Different selection methods in Brassicas have been used for salt tolerance by using different concentration of NaCl e.g. Jain et al (1991) performed *in-vitro* selection for salt tolrence in *Brassica juncea* using cotyledon explants, callus and cell suspension cultures in Petri dishes containing M&S agar media supplemented with 0, 0.25, 0.50, 0.75, 1.0 and 1.25% (w/v) NaCl.

The clones found to exhibit salt resistance might have some osmo-protective or specific ion toxicity resistance mechanisms. Osmo-protective mechanism for salt resistance depends upon the genetic make up of plants (Moghaieb, Saneoka & Fujita, 2004)and specific ion toxicities depend upon adaptation to sodium toxicity (Kingsburry & Epstein, 1986).

Fuller et al (2006) regarded cauliflower *in-vivo* having the damage of greenness of leaf discs less than 50% showing significant degree of resistance. Following this criteria at day 7 of NaCl treatment, the *in-vivo* mutants K1, K9, K11, K19, and K21 showed less than 50% loss of colour and therefore showed salt resistance, while others showed a colour change of 50% or more and were classified as sensitive to NaCl.

The results of the drought resistance tests demonstrated a clear variation in relative water content (RWC) at different concentrations of mannitol and RWC decreased with an increase in mannitol concentration.Also the response of each clone was different at each mannitol concentration. Some mutants such as K1, K9 and K21 showed about 70% RWC even in the presence of high 450 mM mannitol in the media with no symptoms of

necrosis. Chandler and Thorpe (1987) also reported similarly that mannitol up to 440 mM concentration was not toxic in the screening medium and all unselected replicate explants remained green and healthy. The present findings confirmed the safe use of mannitol with *B. oleraceae* indicating it was a suitable stressor for induced drought stress resistance screening of cauliflower leaf discs.

Mannitol is an important photosynthetic product in higher plants and some algae, which can enhance tolerance to dehydration stress mainly through osmotic adjustment (Loescher et al., 1992) and many crop genotypes have been screened for drought resistance using mannitol induced drought e.g *in-vitro* screening of Prunus accessions (Rajasheker et al., 1995), legumes (Grezesiak et al., 1996), sugar beet (Sadighian & Yavari, 2004).

Relative water content (RWC) is suggested as a sound index of water status in plant tissues (Diaz-Perez, Shackel & Sutter, 1995). In the present investigation the mechanisms leading to clonal variation on the basis of RWC are unknown but one might be osmotic adjustment allowing uptake of water from the mannitol supplemented media. Osmotic adjustment in plants under stress has been reported in Brassica species (Chandler & Thorpe, 1987; Kumar et al., 1984), in sorghum (Blum & Sallivan, 1986) and in wheat (Moinuddin et al., 2005). Cell wall elasticity may also be the cause for variable RWC (Kumar & Elston, 1992) and both osmotic adjustment and cell wall elasticity might have adaptive mechanisms to drought stress.

Osmotic effects are similar in Frost, Salt (NaCl) and Drought (Mannitol) stress but specific ion toxicities are specific to Salt stress. Specific ion toxicities are due to sodium and chlorine accumulation in a tissue to damaging levels and damage is visible as a

foliar chlorosis and necroses (Ferguson & Grattan, 2005). Leaf discs greenness scoring for salt resistance was based on the magnitude of foliar chlorosis and necrosis, the damage specific to salt stress resulting poor or no relationship to frost or drought stress, as in frost stress electrolytes leakage (EC%) and in drought stress relative water content (RWC%) was measured instead of leaf discs greenness score. In the present work, improved resistance to drought, salinity and sub-zero temperatures was demonstrated.

3.6 Conclusions

The physiological analysis of *in-vivo* and *in-vitro* clones (mutants and control) for frost, drought and salt stress resistance indicated that the mutations were expressed both in *in-vivo* as well as in *in-vitro* clones after many clonal generations and the mutants maintained higher resistance over control plants. This fulfilled the aim of the first part of this investigation and confirmed that the second aim, molecular and biochemical analysis could be pursued.

Chapter 4: Molecular and Biochemical Characteristics of Mutants

4.1 Introduction

4.1.1 Cold acclimation and the CBF regulon

The exposure of many plants for a period to non freezing low temperature increases their freezing tolerance and this phenomenon is known as cold acclimation (Thomashow, 1999). Cold acclimation plays an important role in freezing tolerance as shown by early research that cold-acclimated cabbage and spinach synthesized proteins protect thylakoid membranes against freeze damage but such proteins were not found in non-acclimated plants (Hincha, Heber & Schmitt, 1989; Volger & Heber, 1975). The cryoprotective activity of these proteins was 10,000 times higher than sucrose in protecting thylakoids against freeze-thaw damage (Hincha, Heber & Schmitt, 1989; Volger & Heber, 1975).

The cold acclimation process is a polygenic trait and a variety of physiological and biochemical changes take place during acclimation. These include changes in membrane lipid composition (Lynch & Steponkus, 1987; Uemura & Steponkus, 1994), the increase in production of compatible osmolytes such as proline, and soluble sugars, as well as increased levels of antioxidants (Dorffling et al., 1997; Kishitani et al., 1994; Koster & Lynch, 1992b; Murelli et al., 1995; Nomura et al., 1995; Tao, Oquist & Wingsle, 1998), abscisic acid (Chen, Brenner & Li, 1983), reduction in growth and water content in tissues (Levitt, 1980). Even though the whole molecular mechanism is still not well understood, various specific profiles of gene expression has been observed during cold acclimation (Chunzhen et al., 2009).

In many higher plants the existence of a transcription factor ICE (Inducer of CBF expression), acts at the promoter of CBF/DREB (C-repeat/dehydration responsive

element binding factor) genes upon exposure of plants to low temperature. The ICE stimulates the transcription of *CBF/DREB* genes followed by induction of the CBF regulon and subsequently freezing tolerance is increased. Eukaryotic regulon is a set of co-expressed genes that share a similar expression profile across multiple temporal, spatial, genetic and environmental conditions and under the common transcriptional regulations (Jaglo-Ottosen et al., 1998a; Wieslawa & Eve, 2008). In higher plants the model CBF regulon is shown in Figure 35.

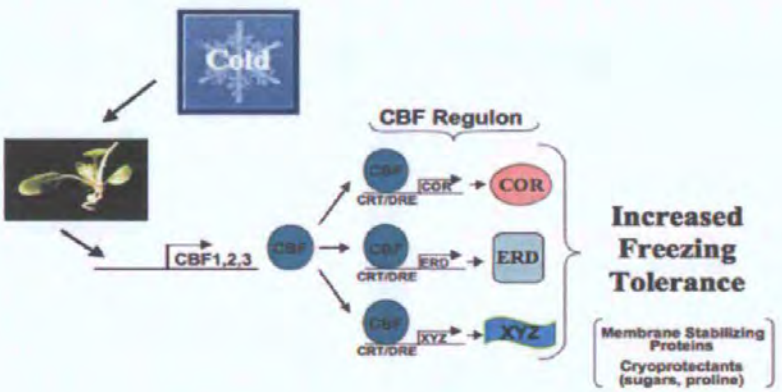


Figure 35:CBF regulon of model Arabidopsis plant:
Low temperature leads to rapid induction of the *CBF* genes (*CBF1, 2,* and *3*), which in turn results in expression of the CBF regulon of the CRT/DRE-regulated genes. Action of the CBF regulon, which includes *COR, ERD,* (*e*arly-*r*esponsive to *d*ehydration) and yet to be discovered *("XYZ")* cold-regulated genes. Source: Michael F. Thomashow www.prl.msu.edu/Publications/ thomashow m.html

The constitutive expression of CBF regulon in some transgenic studies occurs when the CBF genes are expressed constantly and this enhances the freezing tolerance without a low-temperature stimulus (Gilmour, Fowler & Thomashow, 2004; Gilmour et al., 2000; Jaglo-Ottosen et al., 1998b; Liu et al., 1998a). The freezing tolerance in plants conferred by the CBF regulon has been shown to involve the production of cryoprotective polypeptides such as COR15a (Artus et al., 1996a; Steponkus et al., 1998b) and the accumulation of compatible solutes such as sucrose, raffinose, and proline (Gilmour, Fowler & Thomashow, 2004; Gilmour *et al.*, 2000; Nanjo et al., 1999).

4.1.2 Transcription factors (CBFs/DREBs)

Transcription factors known as C-repeat binding factors (CBFs) (Gilmour et al., 1998b; Stockinger, Gilmour & Thomashow, 1997b) or dehydration-responsive element binding factors (DREBs) (Liu *et al.*, 1998a; Shinwari et al., 1998) have been identified, and regulate the expression of cold and dehydration stress responsive genes in plants. These transcription factors bind to two analogous regulatory sequences, known as C-repeat (CRT; TGGCCGAC) and dehydration-responsive element (DRE; TACCGACAT) which have been identified in the promoters of cold and dehydration stress regulated genes (Gilmour et al., 1998a; Liu *et al.*, 1998a; Stockinger, Gilmour & Thomashow, 1997c). CRT/DRE elements contain the highly conserved core 5-bp sequence of CCGAC, which is able to induce transcription under low temperature, drought and high salinity (Baker, Wilhelm & Thomashow, 1994; Yamaguchi & Shinozaki, 1994). This transcriptional regulation of genes is essential for plant adaptation to abiotic stresses (Ji, Dai & Hong, 2007).

It has been demonstrated earlier that the CBF pathway appears to be sufficient to increase abiotic stress tolerance in plants since constitutive expression of *CBF* genes in

transgenic plants induces expression of CRT-containing genes and results in an increase in freezing and drought tolerance without prior stimulus (Gilmour *et al.*, 2000; Haake et al., 2002; Jaglo-Ottosen *et al.*, 1998b; Kasuga et al., 1999). However, the presence of the CBF pathway is apparently not complete in all plants, either because CBF members are not activated in time or for a sufficiently long period, or they are not active, or the CBF regulon is smaller. For instance, over-expression of AtCBF3 or the tomato LeCBF1, increases stress tolerance in transgenic *Arabidopsis*, but does not have the same effect in transgenic tomato (Zhang et al., 2004).

The mechanism whereby the *CBF* genes are activated by low temperature or dehydration is not well known but does not appear to involve autoregulation (Gilmour *et al.*, 1998a). The role of CBF as transcriptional activator in the expression of CRT/DRE containing genes was confirmed by the findings of Stockinger et al., (1997c), where they reported that the CBF protein binds to the promoter of the reporter genes in yeast carrying the CRT/DRE sequence in the promoter. The constitutive over expression of CBF1 in transgenic plants resulted in expression of CRT/DRE controlled COR genes without acclimation which further confirmed the role of CBF as the transcription activator (Jaglo *et al.*, (1998b).

The transcripts of *CBF* genes have a very short half-life, of about 7.5 minutes at warm temperatures, which is amongst the shortest half life for the plant genes thus far described (Daniel G. Zarka, 2003). "The decay rate of transcripts in plants appears to be similar to those observed in other multicellular eukaryotes and half-lives range from 1 h for unstable messages, to several days for stable transcripts, the average being several hours"(Rodrigo, Gustavo and Pamela 1999). The promoters of the CBF genes become inactive promptly within minutes of transferring plants from low to high

temperatures, with no transcripts detected in plants after 90 minutes of transferring plants from cold to warm temperatures (Daniel G. Zarka, 2003). The transcript levels of CBF genes increase within 15 minutes of exposing plants to low temperature, followed by the transcript accumulation of the CRT/DRE regulated COR genes at approximately 2 h (Gilmour *et al.*, 1998a; Shinwari *et al.*, 1998). The COR gene expression is regulated by the CBF/DREB transcription factor which binds to CRT/DRE element in the promoter of COR genes (Ishitani et al., 1997; Shinozaki & Yamaguchi-Shinozaki, 2000).

4.1.3 CBF regulation

There are no evident CRT/DRE sequence in the promoter of *CBFs* genes, which shows that CBF genes do not appear to be controlled by auto-regulation (Gilmour *et al.*, 1998b). Different factors controlling the expression of cold induced CBF genes have been identified, such as ICE (inducer of CBF expression), which was identified through mutational screening. *ICE* encodes a transcriptional activator, which binds to the *CBF* promoter to start its expression, as negative mutation of *ICE1*, *ice1*, almost completely abolished *CBF3* transcript accumulation even in the presence of low temperatures. On the other hand, *ice1* showed insignificant effect on *CBF2* transcripts, which suggests the existence of differences in the activation mechanisms within the *CBF/DREB* family (Chinnusamy et al., 2003). The over-expression of ICE1 transcription activator enhances the expression of the *CBF* regulon and subsequently increased the freezing tolerance in transgenic plants (Chinnusamy et al., 2003). Another transcription factor known as *LOS4* that encodes DEAD-box RNA helicase, which plays a positive role in CBF expression as in *los4-1* mutant plants the expression of *CBFs*, and other downstream target genes were reduced (Gong et al., 2002) and *los4-1* plants were highly sensitive to low temperature, that could be due to the impaired expression of *CBF* (Gong et al., 2002).

The expression of CBF genes is repressed by either their own or the products of their downstream target genes, which ensuring controlled expression of these genes (Chinnusamy et al., 2003; Guo et al., 2002) as shown by Novillo et al. (2004), who reported the negative regulation of CBF1/DREB1B and CBF3/DREB1A by the expression of CBF2/DREB1C.

4.1.4 Cold regulated genes (COR15 gene)

A number of CRT/DRE controlled cold regulated COR genes have been identified including *COR6.6*, *COR15a*, *COR47*, and *COR78* (Steponkus *et al.*, 1998b; Thomashow, 2001). The products of these genes help in increasing cold or dehydration stress tolerance, for instance, the over-expression of *COR15a* gene increases the freezing tolerance by 1 to 2 °C in non-acclimated plants (Artus et al., 1996b; Thomashow, 2001) through decreasing the membrane tendency to form detrimental hexagonal II phase lipids upon freeze-induced dehydration (Steponkus *et al.*, 1998b). The COR15 gene was identified as an important low temperature induced, cold regulated gene in Arabidopsis and was designated as COR15a (Lin & Thomashow, 1992). The mature COR15 protein is designated as COR15m, and is hydrophilic, soluble, and can be detected in soluble protein extracts from chloroplasts (Chentao & Michael, 1992). The COR15 gene encodes a 15 kD protein having extensive resemblance with LEA proteins (late embryogenesis abundant) in its amino acid sequence (Lin & Thomashow, 1992). LEA genes are up-regulated in seeds during the dehydration phase and help to protect embryo cells during dehydration.

Some of the cold-regulated genes encode polypeptides, which are boiling stable and COR15 is one of these (Chentao & Michael, 1992). The COR15 proteins are involved in the dehydration tolerance process in plants and are found in the stromal

compartments of the chloroplasts. Over expression of the COR15 gene reduces the susceptibility of membranes to form hexagonal-phase lipids during freezing stress (Steponkus et al., 1998a) and subsequently enhances cold tolerance in plants (Artus *et al.*, 1996b).

Many early researchers tried to identify the regulatory mechanism of cold regulated genes expression and all found that the CBF/DREB transcription factors are the key regulators controlling their expression (Jaglo-Ottosen *et al.*, 1998a; Liu et al., 1998b; Stockinger, Gilmour & Thomashow, 1997a). However, there may be other regulatory pathways that need to be explored but it is accepted that the CBF/DREB regulatory mechanism is the most important regulatory pathway for cold regulated genes expression to increase freezing tolerance.

The COR15 gene plays a particular role in freezing tolerance but also is induced in response to water stress (Hajela et al., 1990). There is a common cellular dehydration in both freezing and water stress, and cell damage from dehydration occurs (Levitt, 1980; Steponkus & Lynch, 1989). So, it is rational to assume that freezing and drought tolerance involve associated mechanisms and identical genes activation during these stresses is expected (Chentao & Michael, 1992). This has been shown by different workers with certain cereal (Siminovitch & Cloutier, 1983) and Brassica species (Chentao & Michael, 1992; Cox & Levitt, 1976).

4.1.5 CBF expression and level of free proline (Pro)

Different mechanisms in plant cells at the biochemical and molecular level are involved to facilitate plants survival in adverse environments. One of the most familiar mechanisms in higher plants is the synthesis and accumulation of various low molecular

metabolites, that serve as "compatible solutes". These solutes include proline (free amino acid), mannitol, sorbitol, trehalose and these compounds play crucial roles in cellular response to dehydration stresses (Bohnert, Nelson & Jensen, 1995; Misra, Biswal & Misra, 2002; Misra et al., 1990).

The first time proline accumulation under stress conditions was observed in tissues of rye grass (Kemble & MacPherson, 1954) and proline was considered mostly to play a vital role in balancing of osmotic stress produces by drought or salt stress. Subsequent investigations showed that the accumulation of proline may be a part of general adaptation to other abiotic stresses as well, including, low temperature, nutrient deficiency, exposure to heavy metals and high acidity (Ashton & Desh, 1993). Low temperature stress increases proline accumulation in plants (Wanner & Junttila, 1999a) and its accumulation contributes to improved freezing tolerance, which was confirmed from various freezing-tolerant mutants of *Arabidopsis* that accumulated proline (Xin & Browse, 1998).

The enzyme D1-pyrroline-5-carboxylate synthase (P5CS), which is the proline biosynthetic enzyme has an essential role in the proline level determinination in plants (Yoshiba et al., 1997). The transcript level of *P5CS* was observed to be increased in Arabidopsis in response to low temperature (Xin & Browse, 1998). Further investigations were carried out to find the relationship of proline transcript level and CBF3 expression and found approximately 4-fold higher *P5CS* transcript levels in *CBF3*-expressing non-acclimated plants when compared with non-acclimated plants (Gilmour *et al.*, 2000).

CBF over-expression not only increases the levels of COR proteins, but also increases the proline and total sugars, and this increase of proline and sugars occur under cold acclimation in plants, that contribute to the development of freezing tolerance (Gilmour *et al.*, 2000; Thomashow, 2001). Proline, COR proteins, and sugars biosynthesis are regulated co-ordinately and the *CBF3* gene play an essential role in this regulation (Gilmour *et al.*, 2000).

The present investigation looked at the molecular and biochemical evidence of the existence of the CBF regulon in mutants of *Brassica oleracea var. botrytis*. The investigation was carried out for the identification and expression pattern of *CBF/DREB1* and *COR15* genes in genotypes and also the proline estimation under cold acclimated and non acclimated conditions.

4.2 Aim and objectives

Aim

The aim of this study was to investigate the presence of CBF regulon pathway in mutants of *Brassica oleracea var. botrytis* and to investigate the response of different mutants under cold acclimation.

Objectives

1. To optimize a PCR protocol for CBF/DREB1 gene detection
2. To investigate the presence of CBF regulon in cauliflower (BoCBF)
3. To investigate the effect of cold acclimation on BoCBF/DREB1 expression
4. To identify the BoCBF/DREB1 gene and to determine its sequence homology
5. To investigate the effect of cold acclimation on COR15 gene in cauliflower
6. To evaluate the effect of cold acclimation on proline level in mutants
7. To investigate the correlation of BoCBF/DREB1 expression with frost resistance
8. To investigate the correlation of BoCBF/DREB1 expression with proline production
9. To investigate the correlation between proline production with frost resistance under cold acclimation
10. To investigate the relationship of proline and total protein level under cold acclimation

4.3 Materials and methods

4.3.1 Plant materials and growth conditions

The *in-vitro* mutant clones of *Brassica oleracea var. botrytis* were first transferred to *in-vivo* conditions through the weaning process and then grown under cold-acclimating and non-acclimating conditions (Figure 36). For cold acclimation, the clones were grown in a growth chamber for 14 d at 4 °C, 8 h photoperiod, 180.8 µmol m^{-2} s^{-1} light intensity and 52 % humidity. Non-acclimated clones were grown in two groups, in a growth chamber (22 °C with 16 h photoperiod and with same light intensity and humidity as provided during cold acclimation) and in a green house with a minimum temperature of 15 °C and 16 h photoperiod natural light.

a.

b.

c.

Figure 36: Cold acclimation process and plants growth conditions.
(a) Acclimation in growth cabinet (4 °C, 8 h photoperiod) **(b)** Non-acclimated clones in growth chamber (22 °C, 16 h Photoperiod) **(c)** Non-acclimated clones grown in green house (15 °C, 16 h light).

4.3.2 RNA extraction

For RNA extraction leaf tissues were used. From non-acclimated clones leaves were detached after 14 d and from cold acclimated clones leaves were detached after 3 h, 6 h, 24 h and 14 d of cold acclimation and immediately frozen in liquid nitrogen to avoid RNA degradation. The tissues were then ground in liquid nitrogen and 100 mg of frozen tissue powder for each sample was weighed into 1.5 ml RNase free micro centrifuge tube (Ambion) and stored at -80 °C before RNA isolation. Leaf tissues samples (100 mg) were taken from -80 °C and 500 µl lysis solution (sigma cat # L8167) added and vortexed immediately and vigorously for at least 30 seconds and then incubated at 56 °C for 5 minutes. Following the instructions from the kit manufacturer (Sigma cat # STRN50) the total RNA was extracted from each clone and the purified RNA in flow through eluate was distributed in small aliquots and stored at -20 °C for a short time and at -80 °C for long term storage.

4.3.3 Identification of *BoCBF/DREB1* gene

Total RNA was used as the template for the synthesis of first strand cDNA using ImProm-II™ Reverse Transcription (RT) System (Promega cat # A3800) following the instruction from the kit manufacturer. Each RNA sample (0.8 µg/reaction) was diluted with nuclease free water (Sigma w1754) and primer oligos $(dT)_{15}$ (0.5 µg/reaction) were combined for a final volume of 5 µl/RT reaction in a 0.2 ml nuclease free PCR tube (Ambion). A final volume of 20 µl of RT mixture for each sample was prepared by combining 5 µl of RNA + oligo $(dT)_{15}$ primer mixture with 15 µl RT reaction mixture. The first strand cDNA was synthesized in the thermal cycler (Perkin Elmer 9700) under the thermal cycle i.e. Annealing: 25 °C for 5 min, Extension: 42 °C for 60 min, Inactivation of Reverse Transcriptase: 70 °C for 15 min. The amplification of first

strand cDNA was made in a 50 µl PCR reaction of 25 µl PCR master mix 2X (Promega cat # M7502), 5 µl (10 µM) of each forward and backward primers, 5 µl of first strand cDNA, and added nuclease-free water (Sigma cat # w1754) to make 50 µl of final reaction mixture for each sample. The gene specific (degenerate) forward and backward primers (For, 5-AAGAAGTTTCGTGAGACCCGTCAC-3 and Rev, 5-GGCAAAAGCATA CCTTCCGCCAT-3) were used for amplification of cDNA strand of *BoCBF/DREB1* gene under the thermal cycle as given. Initial denaturation at 94 °C for 3 min once, denaturation at 94 °C for 1 min, annealing at 61 °C for 1 min, extension at 72 °C for 2 min (35 cycles)) and then a final extension at 72 °C for 10 min followed by 4 °C ∞. The PCR products were run on a 0.8 % high melting agarose (Sigma) gel and than visualized under UV light and photographs taken with the gel documentation system. The PCR products were compared with PCR marker ladder (Promega cat # G3161) and band intensities were semi-quantitatively measured using Quantity one 4.6.3 Bio-Rad software.

4.3.4 cDNA sequencing

The DNA fragment of interest excised in a minimum volume of agarose gel using a clean and sharp scalpel. Each slice was then transferred to 1.5 ml microcentrifuge tube and isolation of the DNA fragment was carried out by using the ® SV Gel and PCR Clean-Up System (Promega A9281). The weight of the tube was noted before and after addition of slice. Membrane binding solution was added to the tube at a ratio of 10 µl/10 mg of agarose gel slice, mixed very gently and incubated at 61 °C until the gel slice dissolved completely. This was centrifuged briefly at room temperature and DNA was purified with an SV mini-column following the instruction of the kit manufacturer and the purified DNA was subjected to sequencing by Eurofins MWG Operon (Germany).

Some of the purified DNA was stored at 4 °C for a short time and at -20 °C for longer storage..

4.3.5 Multiple alignment of sequences

Multiple nucleotide sequence alignment and deduced amino acids sequences of *BoCBF/DREB1* comparison with other cold induced genes sequences was carried out using ClustalW 2. EMBL-EBI (Larkin et al., 2007) and BLAST (NCBI). Following this a phylogenic tree was constructed using this sequence information.

4.3.6 Genomic DNA isolation

Leaves from each clone were ground into a fine powder in liquid nitrogen, using a mortar and pestle, and 100 mg of the powder transferred to a 1.5 ml microcentrifugse tube on ice and 350 µl lysis solution A and 50 µl of lysis solution B were added according to the protocol provided by the kit manufacturer (Sigma G2N10) based on the methods of Sambrook et al., (1989) and Bruce and Eric (1993).

4.3.7 Protein extraction and SDS-PAGE

Leaf tissues from –80 °C were thawed on ice, placed in a mortar and pestle and then 2 ml extraction buffer (Ni et al., 1996) (100 mM potassium phosphate, 1 mM DTT, 1 mM EDTA, 1% Triton X-100, 10% glycerol, pH 7.8) was added to 1 g of tissues and ground. 1 ml of the liquid grindate was transferred to microfuge tube (1.5 ml) on ice. The supernatant was centrifuged twice at 13000xg for 15 and 10 min respectively at 4 °C. The clear supernatant containing proteins was separated using SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Laemmli, 1970) and visualized by using coomassie blue staining solution. Unstained gels were used for Western blotting and the proteins were transferred to a nitrocellulose membrane by electro blotting at 15 V for 90 min. 1 L of 1XTris-glycine transfer buffer was prepared by dissolving Tris

HCl 3.05 gm, Glycine 14.4 gm, adding methanol 200 ml, ddH_2O 800 ml, pH 8.3 adjusted with NaOH (Towbin et al., 1979).

4.3.8 Western Blot Analysis for COR15

After transferring the proteins on to the nitrocellulose membrane, the membrane was incubated in blocking solution (PBST + 2% BSA) for 1 hour at room temperature with mild shaking. After incubation, the membrane was washed with PBST twice for 5 min each, followed by incubation with primary antibodies obtained from the Dr. Thomashow's lab, Michigan State University, USA. Primary antibodies were diluted (1:1000) in a total volume of 20 ml PBST and the membrane was incubated at room temperature overnight with very mild shaking. The membranes were washed 3 times with PBS while agitating, for 10 min each. The membrane was then incubated in goat anti rabbit IgG horseradish peroxidase conjugated secondary antibody (PBS diluted 1:20,000) (Abcam) for 90 min at room temperature with mild shaking to detect primary antibodies attached to the desired protein (COR15). The membranes were washed 3 times with PBS for 10 minutes of each and then incubated in developmental solution (DAB 0.06 g, $NiCl_2$ 0.03 gm in 100 ml PBST) for about 15 min. The membranes were washed repeatedly with PBS, and blots were observed and digital images made.

4.3.9 Proline and protein estimation

The leaves were powdered in liquid nitrogen and stored at -80 °C. The Proline was extracted from frozen powders and estimated following the method of Bates et al, (1973). 100 mg powders were homogenized in 1.5 ml of 3% sulfosalicylic acid in 2 ml tubes andcentrifuged at 13000xg for 5 minutes in room temperature. 300 µl of the supernatant was incubated for 1 h with 2 ml glacial acetic acid and 2 ml acid ninhydrin in test tubes at 100 °C in a water bath. The tubes were then immediately dipped in ice.

The reaction mixture was extracted with 1 ml toluene by mixing vigorously for 10-30 seconds. The chromophore containing toluene was pipetteed into a fresh tube, warmed to room temperature and its absorbance noted at 520 nm by spectrophotometer using toluene as a blank. The proline concentration in samples was determined from a pre-determined standard curve. The reaction for each sample was performed in triplicate. The proteins were extracted as described earlier (Ni et al., 1996) and protein estimation was made by the standard method of Clive, Daniel & Steve (1989) based on Bradford (1976). Three replicate aliquots of 100 µl for each sample were mixed with 5 ml of Bradford dye (1:4 diluted) reagent (Sigma cat # B6916). 100 µl of protein extraction buffer was mixed with and used as a blank. A 1 ml sample mixture was transferred to a disposable polystyrene cuvette and the absorbance recorded at 595 nm using a spectrophotometer.

4.4 Results

4.4.1 Identification and expression of *BoCBF/DREB1* gene under cold acclimation.

The PCR conditions were optimized empirically by adjusting annealing temperature (Figure 37). Through RT-PCR with gene specific primers, the *CBF/DREB1* gene of *B.oleracea var. botrytis* was identified and examined for its expression pattern under cold acclimation.

Among the mutants there was apparent differences in expression level observed under cold acclimation treatments. After 3 h cold acclimation only K1, K11, K13, K18 and K19 showed PCR product (Figure 38a), after 6 h, two more clones K21 and KC also showed the PCR product (Figure 38b), after 24 h acclimation, all the clones except K9 showed the product (Figure 39a), while after 14 d, only very weak bands were present for all clones (Figure 39b).

Cold acclimation increased the expression level of *BoCBF/DREB1* transcript up to 24 h cold acclimation (Figure 39a), whilst the PCR product bands were not clear to observe at day 14 but still very weak bands were appeared there (Figure 39b) indicating that the transcript level had declined.

In non-acclimated condition no PCR product was found in all clones either mutants or control as shown in Figure 40. The analysis was repeated 3-5 times for confirmation.

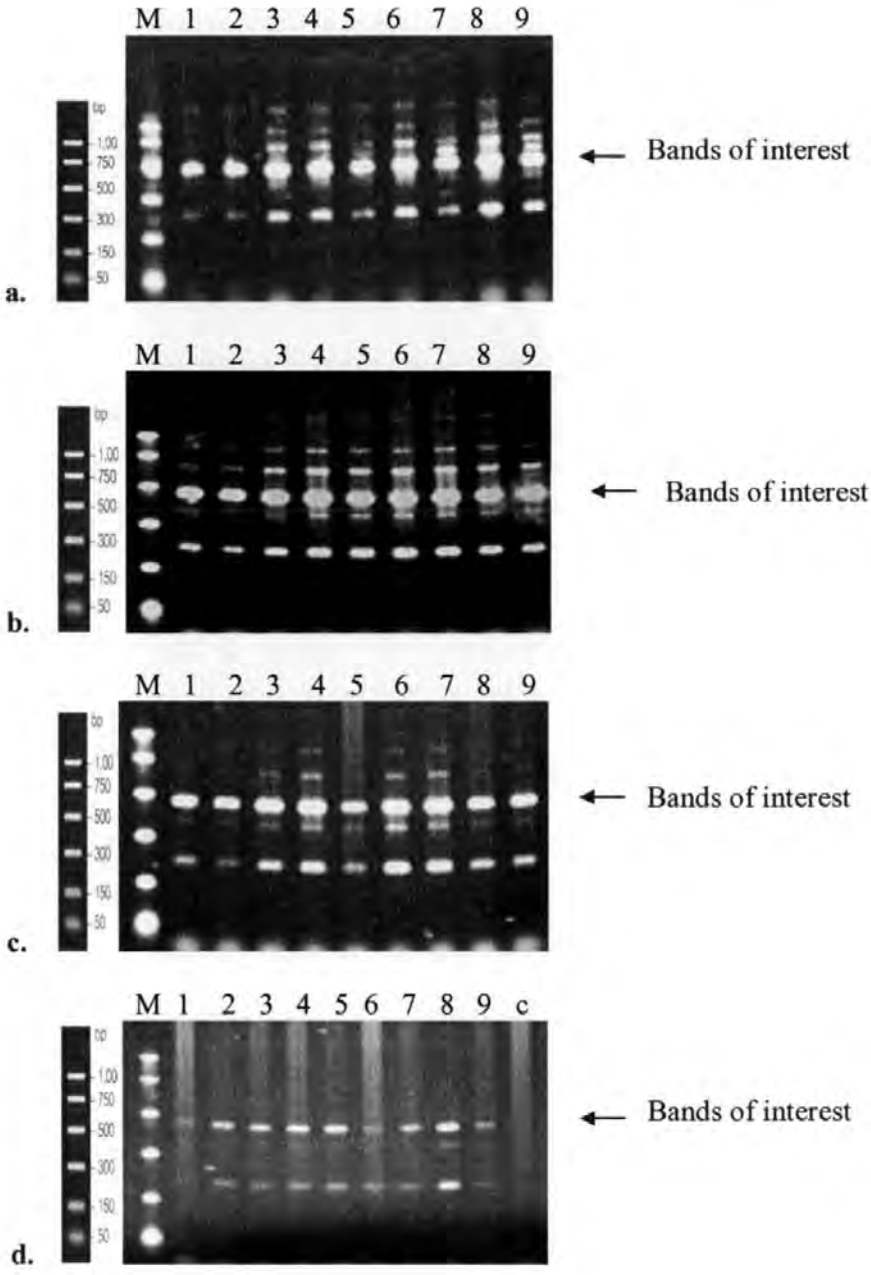


Figure 37: PCR optimization:
Annealing temperature is 5, 4, 3, and 2 degrees less than melting temperature in Fig. a, b, c, and d respectively. M = Marker, lane 9 = Control clone, lanes 1-8 = mutant clones. 1 = K1, 2 = K4, 3 = K9, 4 = K11, 5 = K13, 6 = K18, 7 = K19, 8 = K21, 9 = KC, K = Klone (clone), KC = Control plant clone, c = reaction without template gDNA.

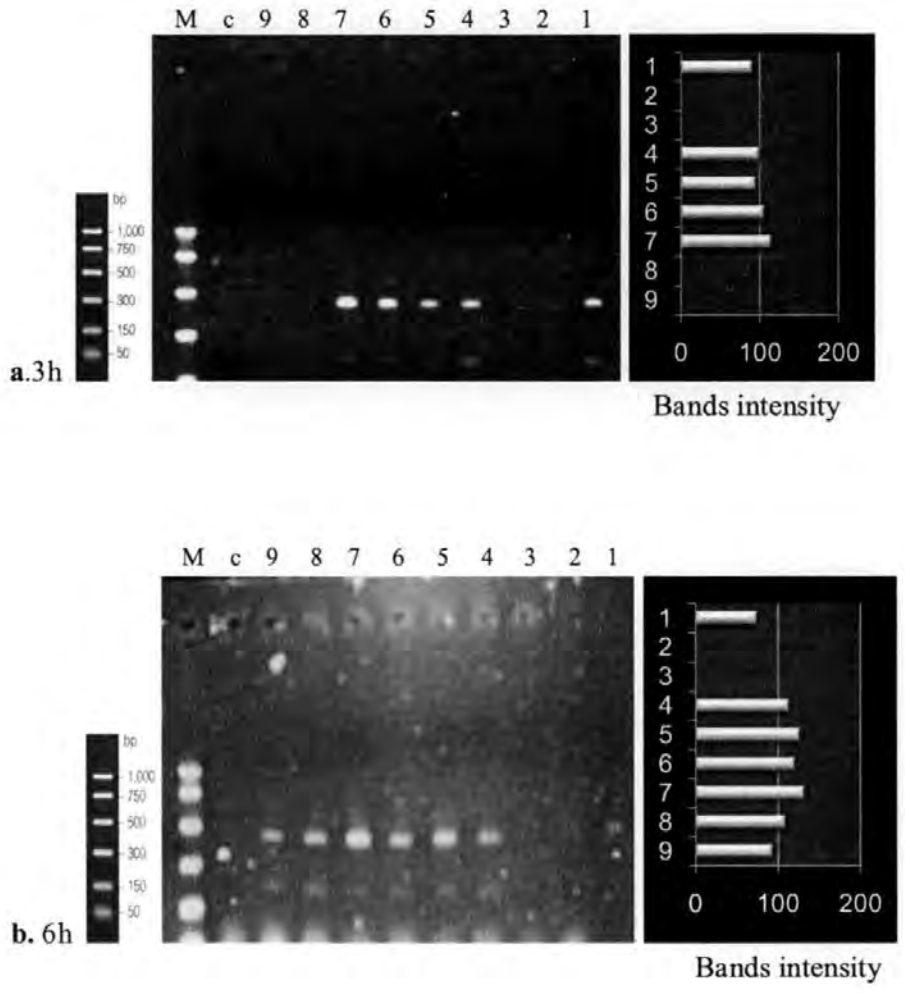


Figure 38: RT-PCR product and band intensity of CBF/DREB1 after 3 and 6 h acclimation.
(a). after 3 h and **(b).** after 6 h cold acclimation. M = Marker, lane 9 = control clone, lane 1-8 = mutant clones, 1 (K1), 2 (K4), 3 (K9), 4 (K11), 5 (K13), 6 (K18), 7 (K19), 8 (K21), 9 (KC), K = Klone (clone), KC = Control wild type clone, c = reaction without template RNA. Band intensities were measured with Quantity one 4.6.3 Bio-Rad software.

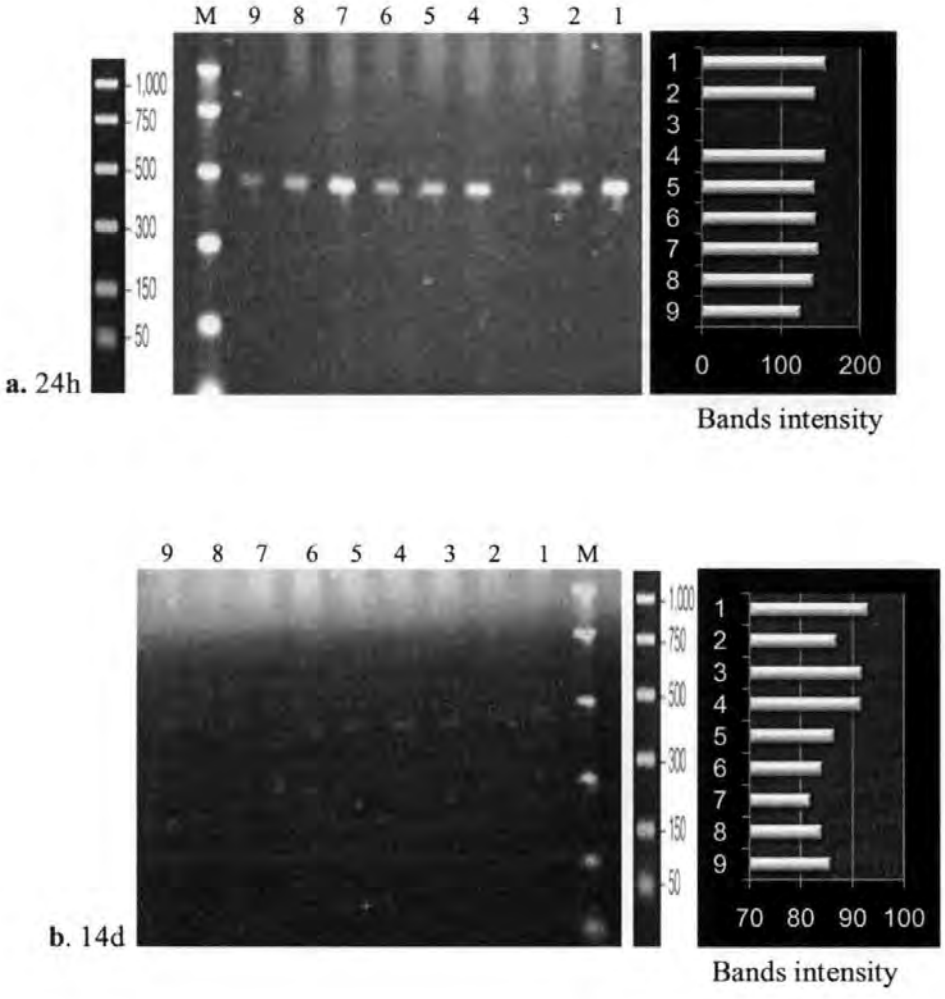


Figure 39: RT-PCR product and bands intensity of CBF/DREB1 after 24 h and 14 d acclimation.
(a) after 24 h and **(b)** after 14 days cold acclimation. M = Marker, lane 9 = Control clone, lane 1-8 = mutant clones, 1 = K1, 2 = K4, 3 = K9, 4 = K11, 5 = K13, 6 = K18, 7 = K19, 8 = K21, 9 = KC, K = Klone (clone), KC = Control plant clone. The bands intensity was measured with Quantity one 4.6.3 Bio-Rad software.

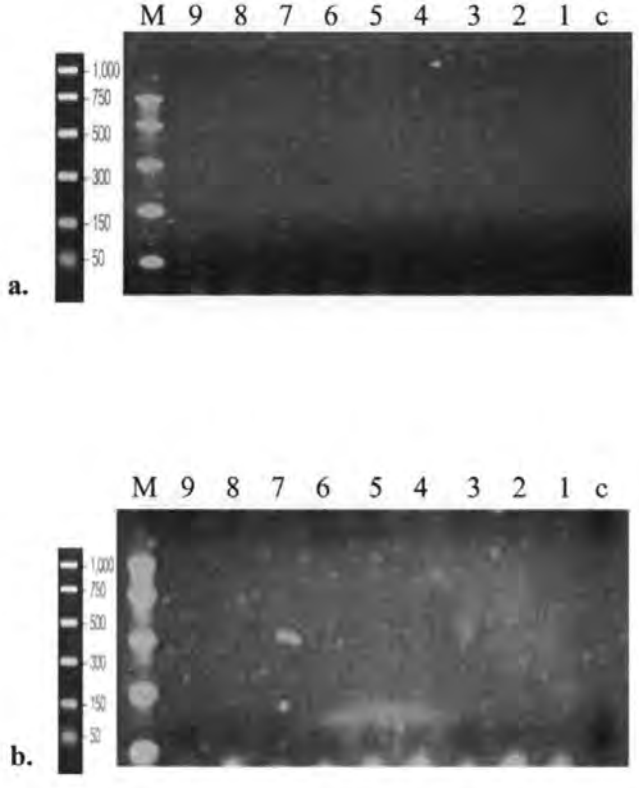


Figure 40: Non-acclimated genotypes RT-PCR.
All of the genotypes either mutants or control clones showed no products (bands) of CBF/DREB1. **a** and **b** are the repetition of the experiment for confirmation. M = Marker, lane 9 = control clone, lane 1-8 = mutant clones, 1 (K1), 2 (K4), 3 (K9), 4 (K11), 5 (K13), 6 (K18), 7 (K19), 8 (K21), 9 (KC), K = Klone (clone), KC = Control wild type clone, c = reaction without template RNA.

4.4.2 Isolation and cDNA sequence alignment of *BoCBF/DREB1* gene

The nucleotide sequence of cDNA isolated from *Brassica oleracea var. botrytis* was compared with *CBF/DREB1* gene sequences reported for other species of *Brassica*. The results in Figure 41 showed significant resemblances with up to 91% sequence consensus found with sequences from *Brassica napus BnCBF5/DREB1* (GenBank: AF499031.1, 879 bp gene, (Gao et al., 2002)), *Brassica juncea BjDREB1B* (GenBank: EU136731.1, 838 bp gene (Cong et al., 2008)) and *Brassica rapa subsp. Pekinensis* (GeneBank: EU924266.1, 645 bp gene) (Wang, Shen & Li, Unpublished). The % consensus was calculated by the number of identical nucleotides in all sequences / total nucleotides sequence isolated than multiplied by 100 = 375/412 x 100 = 91% consensus. These multiple sequence alignments were made using ClustalW 2. EMBL-EBI (Larkin et al., 2007) and BLAST (NCBI).

```
BrDREB1      114 TCGGAAGAAGTTTCGGGAGACGCGTCACCCAATTTACAGAGGAGTACGTCTGAGAAACTC 173
BjDREB1B     121 TCGGAAGAAGTTCCGGGAGACGCGTCACCCAATTTACAGAGGAGTTCGTCTGAGAAACTC 180
BoCBF/DREB1    1 ------------------------------------------------GAGA-ACTC   8
BnCBF5       298 TCGGAAGAAGTTTCGGGAGACGCGTCACCCAATTTACAGAGGAGTTCGTCAGAGACACTC 357
Consensus                                                        **** ****

BrDREB1      174 AGGTAAGTGGGTGTGTGAGGTGAGGGAGCCAAACAAAAAGTCTAGGATTTGGCTCGGTAC 233
BjDREB1B     181 AGGTAAGTGGGTGTGTGAAGTGAGGGAGCCAAACAAGAAATCTAGGATTTGGCTCGGTAC 240
BoCBF/DREB1    9 AGGTAAGTGGGTGTGTGAGGTGAGAGAGCCAAACAAGAAATCCAGGATTTGGCTCGGTAC  68
BnCBF5       358 AGGTAAGTGGGTGTGCGAGGTGAGAGAGCCAAACAAGAAATCCAGGATTTGGCTCGGAAC 417
Consensus        *************** ** ***** ********** ** ** ************** **

BrDREB1      234 TTTCCTAACCGCCGAGATCGCAGCTCGTGCTCACGACGTCGCCGCCATAGCCCTCCGTGG 293
BjDREB1B     241 TTTCCTAACCGCCGAGATCGCAGCTCGTGCTCACGACGTCGCCGCCATAGCCCTCCGCGG 300
BoCBF/DREB1   69 TTTCCTAACCGCCGAGATCGCAGCTCGTGCTCACGACGTCGCCGCCATAGCCCTCCGTGG 128
BnCBF5       418 TTTCCTAACCGCCGAGATCGCAGCTCGTGCTCACGACGTCGCCGCCATAGCCCTCCGTGG 477
Consensus        ************************************************************ **

BrDREB1      294 CAAATCCGCCTGCCTCAATTTCGCCGACTCGGCTTGGCGGCTCCGTATCCCGGAGACAAC 353
BjDREB1B     301 CAAATCAGCTTGTCTCAATTTTGCTGACTCGGCTTGGCGGCTCCGTATCCCGGAGACAAC 360
BoCBF/DREB1  129 CAAATCCGCCTGCCTCAATTTCGCCGACTCGGCTTGGCGGCTCCGTATCCCGGAGACAAC 188
BnCBF5       478 CAAATCCGCCTGCCTCAATTTCGCCGACTCGGCTTGGCGGCTCCGTATCCCGGAGACAAC 537
Consensus        ****** ** ** ******** ** ********************************

BrDREB1      354 ATGCCCCAAGGATATCCAGAAGGCGGCTGCTGAAGCCGCGGTGGCTTTTCAGGCTGAGAT 413
BjDREB1B     361 ATGCCCCAAGGAGATTCAGAAGGCGGCTGCTGAAGCCGCCTTGGCTTTTCAGGCTGAGAT 420
BoCBF/DREB1  189 ATGCCCCAAGGATATCCAGAAGGCGGCTGCTGAAGCCGCGGTGGCTTTTCAGGCTGAGAT 248
BnCBF5       538 ATGCCCCAAGGATATCCAGAAGGCGGCTGCTGAAGCCGCGGTGGCTTTTCAGGCTGAGAT 597
Consensus        ************ ** ************************  ******************

BrDREB1      414 AAATGATACGACGAAGGATCATGGCTTGGACGTGGAGGAGACGATCGTGGAGGCTATTTT 473
BjDREB1B     421 AAATAATACGACGACGGATCATGGCCTGGACATGGAGGAGACGATCGTGGAGGCTATTTT 480
BoCBF/DREB1  249 AAATGATACGACGACGGATCATGGCCTGGACGTGGAGGAGACGATCGTGGAGGCTATTTT 308
BnCBF5       598 AAATGATACGACGACGGATCATGGCCTGGACGTGGAGGAGACGATCGTGGAGGCTATTTT 657
Consensus        **** ********* ********** ***** ***************************

BrDREB1      474 TACGGAGGAAAACAGCGATGGGTTTTATATGGACGAGGAGGAGTCCATGTTCGGGATGCC 533
BjDREB1B     481 CACGGAGGAAAACAACGATGTGTTTTATATGGACGAGGAG---TCCATGTTAGAGATGCC 537
BoCBF/DREB1  309 TACGGAGGAAAACAACGATGGGTTTTATATGGACGAGGAGGAGTCCATGTTCGGGATGCC 368
BnCBF5       658 TACGGAGGAAAACAACGATGGGTTTTATATGGACGAGGAGGAGTCCATGTTCGGGATGCC 717
Consensus         ************* ***** ******************   ******** * ******

BrDREB1      534 GACCTTGTTGGCTAGCATGGCCGAAGGGATGCTTTTGCCGCCACCGTCCGTACAATTCGA 593
BjDREB1B     538 GGCCTTGTTGGCTAGTATGGCGGAAGGAATGCTTTTGCCGCCGCCGTCCGTACATTTCGG 597
BoCBF/DREB1  369 GTCCTTGTTGGCTAGCATGGCGGAAGGTTGCCTTTTGCCATGCT---------------- 412
BnCBF5       718 GTCCTTGTTGGCTAGCATGGCGGAAGGGATGCTTTTGCCGCCACCGTCGGTACGATTCGA 777
Consensus        * ************* ***** *****    ********
```

Figure 41: Nucleotide sequences (cDNA) alignment.
Alignments were made using ClustalW2 EMBL-EBI (Larkin *et al.*, 2007). Consensus symbols denoted as: "*" means that the nucleotides in that column are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed, "." means that semi-conserved substitutions are observed. The AP2 region is indicated by overline. *BrDREB1* from *Brassica rapa subsp. pekinensis*, *BjDREB1B* from *Brassica juncea*, *BoCBF/DREB1* sequence isolated from *Brassica oleracea v. botrytis*, *BnCBF5* from *Brassica napus*.

4.4.3 Amino acid sequence alignment and phylogenetic analysis of BoCBF/DREB1

The multiple sequence alignment and comparison of the deduced amino acids sequence of *Brassica oleracea var.botrytis BoCBF/DREB1* with other cold induced protein sequences in Brassicas is presented in Figure 42 and 43. The *BoCBF/DREB1* protein showed 90% amino acid consensus with other proteins from the members of Brassica species (Figure 42) such as *Brassica napus BnCBF5/DREB1* (AAM18958), *Brassica rapa subsp. Pekinensis BrDREB1* (ACL12046) and *Brassica juncea BjDREB1B* (ABX00639). There was no sequence found in the genebank for *B.nigra* and *B.carinata* for comparision.

When the *BoCBF/DREB1* amino acids sequence was compared in a broad way with members of the Brassicacea family, then amino acid sequence consensus reduced to 67% (Figure 43) which indicated that the sequence isolated from the *B.oleracea var. botrytis* is highly conserved in Brassica species, more than in the Brassicaceae family. The multiple sequence alignment was made using clustalW2 EMBL-EBI (Larkin *et al.*, 2007).

In order to investigate the phylogenetic relationship of *BoCBF/DREB1* with other plants, a phylogenetic tree was constructed (Figure 44). This shows the relationships with other higher plants. Tree was made using clustalW2 EMBL-EBI (Larkin *et al.*, 2007).

```
BoCBF/DREB1  ------------------------------------------------------------ENSG  4
BnCBF5       MTSFSAFSEMMGSENESPA-LSGEYCPTLAASCPKKPAGRKKFRETRHPIYRGVRQRHSG  59
BrDREB1      MTSFSTFSEMLGSEYESPT-LSGEYCPTLAASCPKKPAGRKKFRETRHPIYRGVRLRNSG  59
BjDREB1B     MTSFSTFSEMLGSEYESPVTLGGEYCPTLAASCPKKPAGRKKFRETRHPIYRGVRLRNSG  60
Consensus                                                          .:**

                                                                   +++++
BoCBF/DREB1  KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC  64
BnCBF5       KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC  119
BrDREB1      KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC  119
BjDREB1B     KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC  120
Consensus    ************************************************************

BoCBF/DREB1  PKDIQKAAAEAAVAFQAEINDTTTDHGLDVEETIVEAIFTEENNDGFYMDEEESMFGMPS  124
BnCBF5       PKDIQKAAAEAAVAFQAEINDTTTDHGLDVEETIVEAIFTEENNDGFYMDEEESMFGMPS  179
BrDREB1      PKDIQKAAAEAAVAFQAEINDTTKDHGLDVEETIVEAIFTEENSDGFYMDEEESMFGMPT  179
BjDREB1B     PKEIQKAAAEAALAFQAEINNTTTDHGLDMEETIVEAIFTEENNDVFYMDEE-SMLEMPA  179
Consensus    **:*********:*******:**.*****:************.* ****** **: **:

BoCBF/DREB1  LLASMAEGCLLPC---------------------- 137
BnCBF5       LLASMAEGMLLPPPSVRFEHXYDFDGDAXVSLWSY 214
BrDREB1      LLASMAEGMLLPPPSVQFEYNYDFDGDTDVSLWSY 214
BjDREB1B     LLASMAEGMLLPPPSVHFGHNYDFDGDADVSLWSY 214
Consensus    ******** ***
```

Figure 42: Multiple sequence alignment and comparison of the deduced amino acids sequence of *BoCBF/DREB1* with protein sequences of other Brassica species.
Brassica napus BnCBF5/DREB1 (GeneBank,AAM18958)(Gao *et al.*, 2002), *Brassica juncea BjDREB1B* (ABX00639)(Cong *et al.*, 2008), *Brassica rapa subsp. Pekinensis DREB1* (ACL12046)(Wang, Shen & Li, Unpublished), *Bo. Brassica oleracea*. The symbol "*" indicate the consensus and the DNA (CRT/DRE) binding AP2 domain is indicated by a thick overline and + indicate the CBF signature motif DSAWR and ":" indicate conserved substitutions, "." indicate semi-conserved substitutions. Percent consensus among the Brassica species was calculated and found to be 90% homology for 137 amino acids sequence of *BoCBF/DREB1*. The % consensus calculated as the number of "*"/total amino acid sequence which is 123 aa and multiplied by 100 = 123/137 x 100 = 90%. So it is like CBF2 but not CBF1 or 3. The dot line shows the missing amino acids of the full length gene sequence because of the isolation of the partial sequence of the gene from *B.oleracea v. botrytis*.

```
TaCBF          MNSFSAFAEMFGSEYESPDTIGADYCPTLATSCPKKPAGRKKFRETRHPIYRGVRRRNSG 60
EsDREB1        MNSFSAFAEMFGSEYESPVTVGGDYCPTLATSCPKKPAGRKKFRETRHPIYRGVRRRNSG 60
BoCBF/DREB1    ------------------------------------------------------ENSG 4
BnCBF5         MTSFSAFSEMMGSENESPA-LSGEYCPTLAASCPKKPAGRKKFRETRHPIYRGVRQRHSG 59
RsCBF1         MTSFSTFSELLGSEHESPVTLGEEYCPKLAASCPKKPAGRKKFRETRHPIYRGVRLRNSG 60
BrDREB1        MTSFSTFSEMLGSEYESPT-LSGEYCPTLAASCPKKPAGRKKFRETRHPIYRGVRLRNSG 59
BjDREB1B       MTSFSTFSEMLGSEYESPVTLGGEYCPTLAASCPKKPAGRKKFRETRHPIYRGVRLRNSG 60
AtCBF2         MNSCSAFSEMFGSDYESPVSSGGDYSPKLATSCPKKPAGRKKFRETRHPIYRGVRQRNSG 60
ApCBF          MSSFSAFSEMFGSDYESMISSVGDYSPTLATSCPKKPAGRKKFRETRHPVYRGVRQRNSG 60
Consensus                                                          .:***

                                                                  +++++
TaCBF          KWVCEVREPNKKSRIWLGTFPTAEMAARAHDVAAIALRGRSACLNFADSAWRLRIPESTC 120
EsDREB1        KWVCEVREPNKKSRIWLGTFPTAEMAARAHDVAAIALRGRSACLNFADSAWRLRIPESTC 120
BoCBF/DREB1    KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 64
BnCBF5         KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 119
RsCBF1         KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 120
BrDREB1        KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 119
BjDREB1B       KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 120
AtCBF2         KWVCELREPNKKTRIWLGTFQTAEMAARAHDVAAIALRGRSACLNFADSAWRLRIPESTC 120
ApCBF          KWVSELREPNKKTRIWLGTFQTAEMAARAHDVAAIALRGRSACLNFADSVWRLRIPESAC 120
Consensus      ***.*:******:******* ***:**************:*********.*******::*

TaCBF          AKDIQKAAAEAAVAFQAE-MSDTTT-DHGLDMEETIVEAIVNEE-QSGGFYMDEE-AMFG 176
EsDREB1        AKDIQKAAAEAAVAFQAE-MSDTMTSDHGLDMEETTVEVIVTEEEQSEGFYMDEE-AMFG 178
BoCBF/DREB1    PKDIQKAAAEAAVAFQAE-INDTTT-DHGLDVEETIVEAIFTEE-NNDGFYMDEEESMFG 121
BnCBF5         PKDIQKAAAEAAVAFQAE-INDTTT-DHGLDVEETIVEAIFTEE-NNDGFYMDEEESMFG 176
RsCBF1         PKDIQKAAAEAAVAFQAE-INDTTT-DHGLDVEETIVEAIFTEE-NSDGFYMDEEESMFG 177
BrDREB1        PKDIQKAAAEAAVAFQAE-INDTTK-DHGLDVEETIVEAIFTEE-NSDGFYMDEEESMFG 176
BjDREB1B       PKEIQKAAAEAALAFQAE-INNTTT-DHGLDMEETIVEAIFTEE-NNDVFYMDEE-SMLE 176
AtCBF2         AKEIQKAAAEAALNFQDE-MCHMTTDAHGLDMEETLVEAIYTPEQSQDAFYMDEE-AMLG 178
ApCBF          AKDIQKAAAEAALAFQNELMSDTATTDHGLDMEETLVEAIVTAEQ-IDTFYIDEE-TMFG 178
Consensus      .*:*********: ** * : .  .  ****:*** **.* . *     **:*** :*:

TaCBF          MPRLLANMAEGMLLPPPSVQWGQNYDCDGDADVSLWSY 214
EsDREB1        MPRLLANMAEGMLLPPPSVQWGHNYDCDGDADVSLWSY 216
BoCBF/DREB1    MPSLLASMAEGCLLPC---------------------- 137
BnCBF5         MPSLLASMAEGMLLPPPSVRFEHXYDFDGDAXVSLWSY 214
RsCBF1         MPTLLASMAEGMLLPPPSVQFGHTYDFDGDADVSLWSY 215
BrDREB1        MPTLLASMAEGMLLPPPSVQFEYNYDFDGDTDVSLWSY 214
BjDREB1B       MPALLASMAEGMLLPPPSVHFGHNYDFDGDADVSLWSY 214
AtCBF2         MSSLLDNMAEGMLLPSPSVQWNYTFDVEGDDDVSLWSY 216
ApCBF          MPSLMANMAEGMLLPLPSIQWINNYDVEGDADMPLWSY 216
Consensus      *. *: .**** ***
```

Figure 43: Multiple alignment of the *BoCBF/DREB1* deduced amino acids sequence with members of Brassicacea family.
Brassica oleracea v. botrytis BoCBF/DREB1, *Brassica napus BnCBF5/DREB1* (GenBank: AAM18958.1, 214 aa) (Gao *et al.*, 2002), *Brassica juncea BjDREB1B* (GenBank: ABX00639.1, 214 aa) (Cong *et al.*, 2008), *Brassica rapa subsp. Pekinensis DREB1* (GenBank: ACL12046.1, 214 aa) (Wang, Shen & Li, Unpublished), *Raphanus sativus RsCBF1* (GenBank: ACX48435.1, 215 aa) (Li & Gao, Unpublished), *Thlaspi arvense TaCBF* (GenBank: ABV82985.1, 214 aa) (Zhou et al., 2007), *Eutrema salsugineum EsDREB1*(GenBank: AAS00621.1, 216 aa) (Feng & Zhang, unpublished) and *Arabidopsis thaliana AtCBF2* (GenBank: ABV27090.1, 216 aa) (Lin et al., 2008). Different symbols such as + indicate the CBF signature motif DSAWR,"*" indicate the identical nucleotides in all sequences. ":" indicate conserved substitutions, "." indicate semi-conserved substitutions and CRT/DRE binding AP2 domain is indicated by oveline. The % consensus in the members of Brassicacea family was calculated as the number of "*"/total nucleotides sequence which is 137 aa and multiplied by 100 = 91/137 x 100 = 67%.

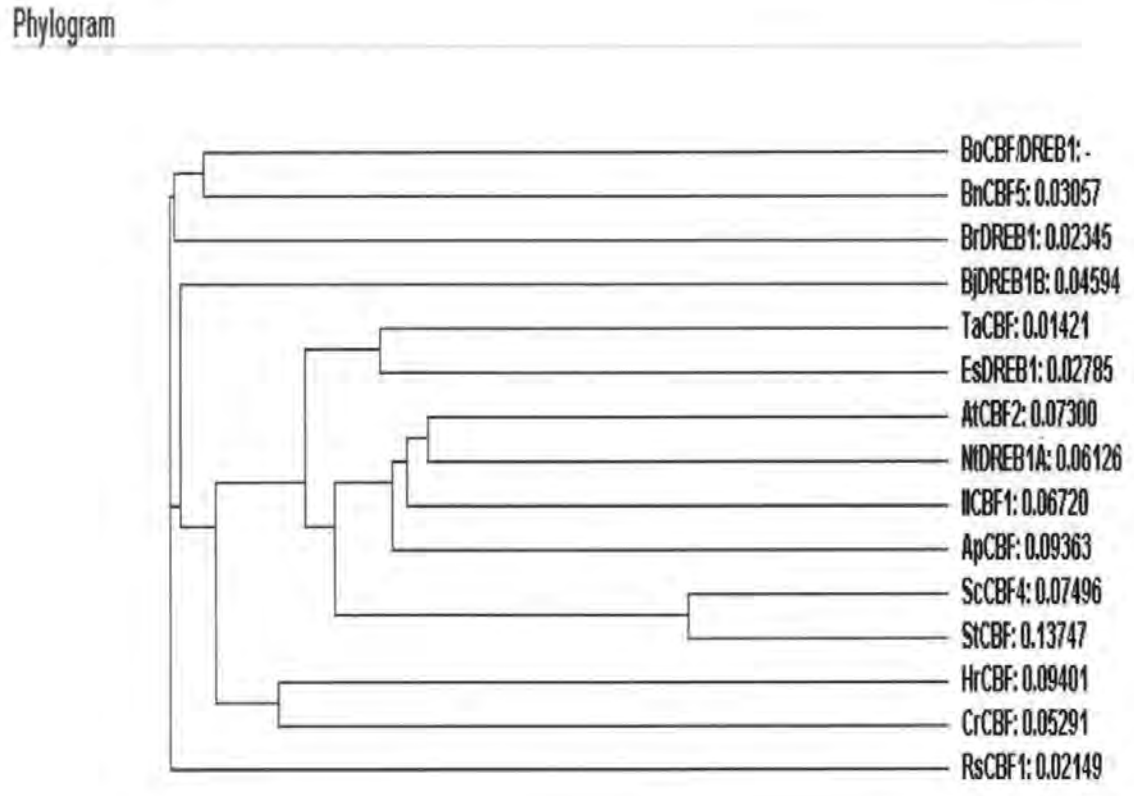


Figure 44: Phylogenic relation of the BoCBF/DREB1 proteins.
The phylogram is based on the alignment of amino acids sequence of *Brassica oleracea v. botrytis BoCBF/DREB1* and the following proteins from the members of Brassicacea and other families. *Brassica napus BnCBF5/DREB1*(AAM18958), *Brassica rapa subsp. Pekinensis BrDREB1*(ACL12046), *Brassica juncea BjDREB1B* (ABX00639), *Thlaspi arvense TaCBF* (ABV82985, Brassicacea) *Eutrema salsugineum EsDREB1*(AAS00621, Brassicacea), *Arabidopsis thaliana AtCBF2* (ABV27090, Brassicacea), *Nicotiana tabacum NtDREB1*(ACE73693, Solanacea), *Iris lacteal IlCBF* (ACK58683, Iridaceae), *Arabis pumila ApCBF* (ABA42927, Brassicacea), *Solanum commersonii ScCBF4* (ACB45084), *Solanum tuberosum StCBF* (ACJ26757), *Hippophae rhamnoides HrCBF* (ABS30426, Elaeagnaceae), *Catharanthus roseus CrCBF*(ABI85277, Apocynaceae), and *Raphanus sativus RsCBF1* (ACX48435, Brassicacea). The values show tree graph distances. The tree was constructed with ClustalW2 EMBL-EBI(Larkin *et al.*, 2007).

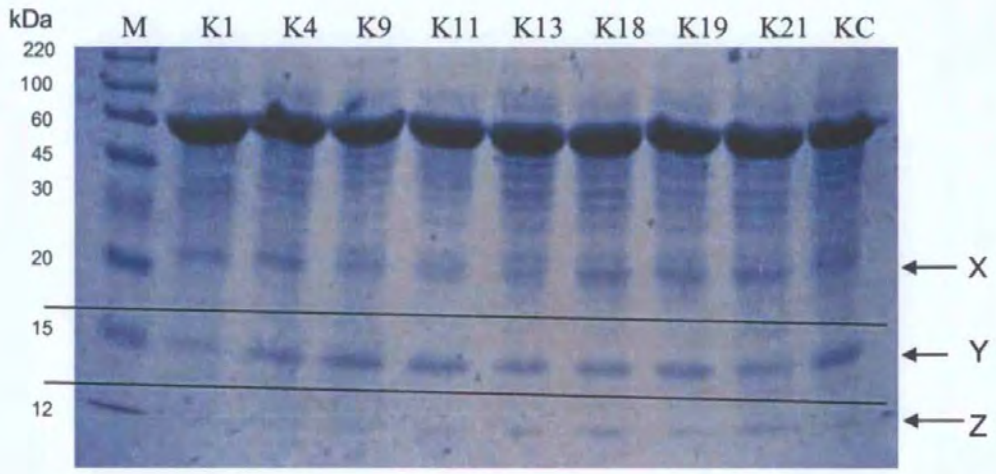
4.4.4 Detection of *COR15* gene in mutants of *Brassica oleracea var. botrytis*

Total soluble protein was extracted from each genotype (mutants and control) grown under acclimated and non-acclimated conditions. The effect of cold acclimation on COR15 gene expression in each genotype was investigated. Sodium dodecyl sulfate polyacylamide gel electrophoresis (SDS-PAGE) was used for protein fractionation and then proteins were transferred to nitrocellulose membrane for detection of COR15 by Western blot technique.

Under acclimation the SDS-PAGE analysis showed similar protein band patterns in all clones and no differences were observed (Figure 45a). Under non-acclimated conditions (Figure 45b) the clones showed differences in bands pattern when compared with acclimated, with the bands designated as Y in Figure 45a and 45b clearly demonstrating the difference between acclimated and non-acclimated genotypes. Lanes # 1 and 5 (K1 and K13 mutants) showed missing bands designated as Y (Figure 45b) but it is interesting that on acclimation these missing bands reappeared as shown in Figure 45a. The marker protein ladder indicates that the molecular weight of band Y is about 15kDa, which is similar in size to COR15.

The Western blot results confirmed the presence of COR15 protein and this was repeated three times to verify the results (Figure 46). The results clearly demonstrated the influence of cold acclimation process on the expression of *COR15* gene, where all the genotypes showed the presence of COR15 protein (Figure 46). In non-acclimated conditions, only mutant clones # 1, 2 and 8 (K1, K4 and K21) showed clear blots while in other clones no clear blots were observed (Figure 47). This suggests constitutive expression of COR15 in these clones. These three clones showed higher frost resistance compared to other mutants except K13 and K18 where the response was about similar (Figure 48).

a.



b.

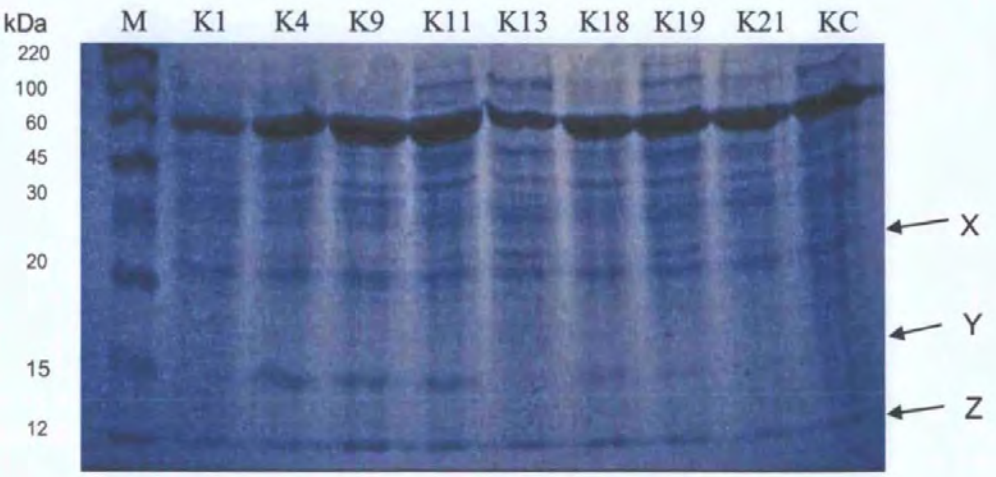


Figure 45: The SDS-PAGE analysis of genotypes.
a. Cold acclimation. **b**. Non-acclimation. M = Marker, lane 9 = Control klone (Clone), lane 1-8 = mutant klones, K = Klone genotype (Clone), lane 1 is K1, 2 = K4, 3 = K9, 4 = K11, 5 = K13, 6 = K18, 7 = K19, 8 = K21, 9 = KC, KC = Control wild type klone.

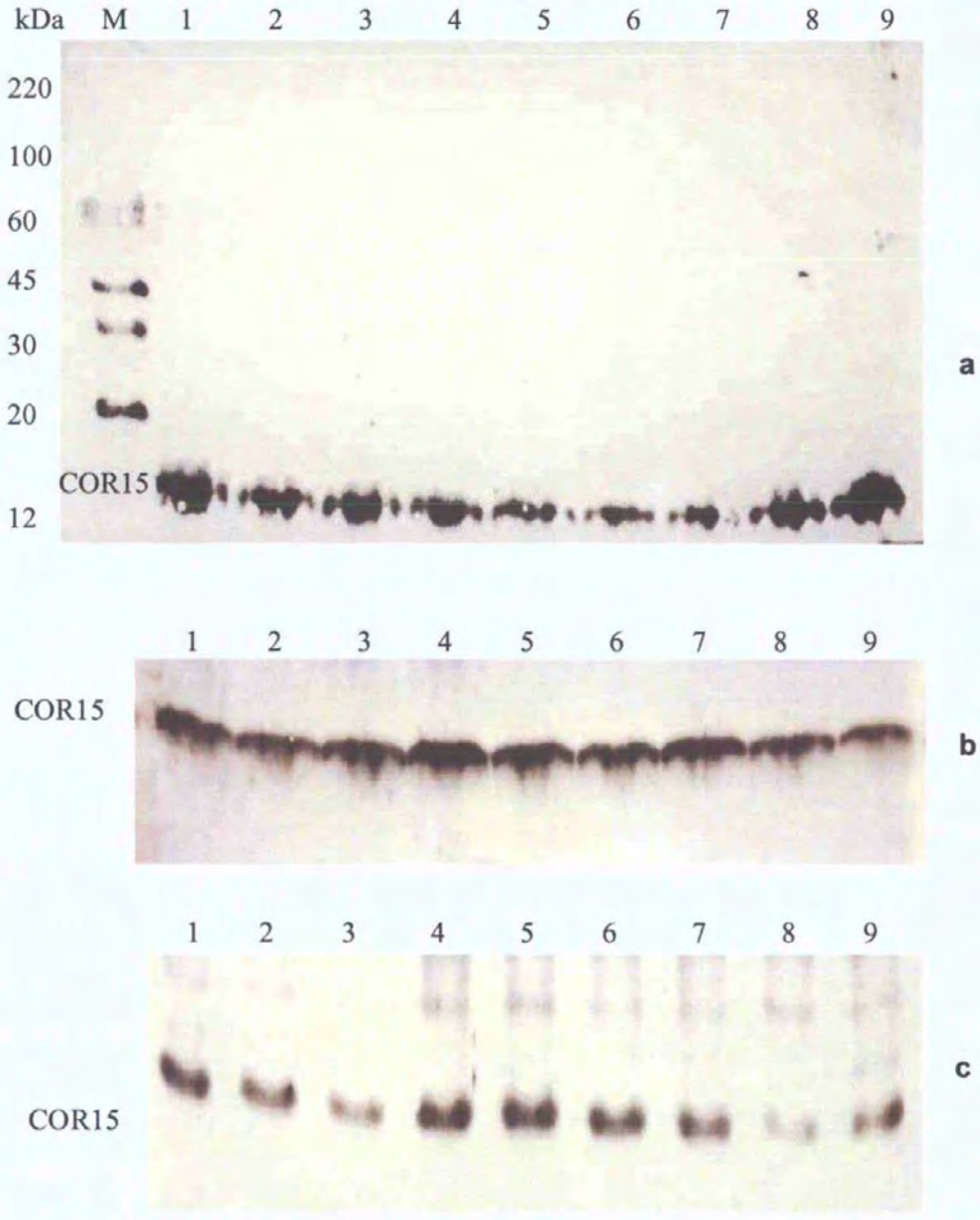


Figure 46: Western blot analysis for the detection of COR15 protein under cold acclimation.

a, b and **c** are the repetition of the same experiment for the verification. M = Marker, K = genotype Klone (Clone), lane 9 = control klone, lane 1-8 = mutant klones, lane 1 is K1, 2 (K4), 3 (K9), 4 (K11), 5 (K13), 6 (K18), 7 (K19), 8 (K21), 9 (KC), KC = Control wild type klone.

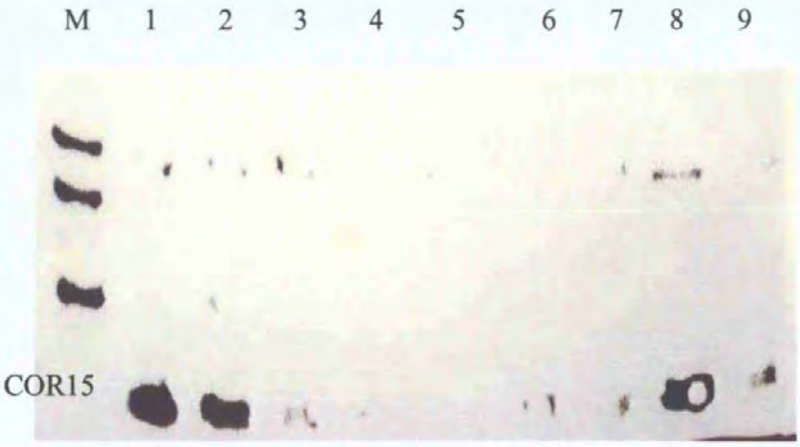


Figure 47: Western blot analysis for the detection of COR15 in genotypes under non-acclimated condition.
The lane 1, 2, and 8 show the blots while no clear blots were appeared in the rest of the genotypes clones. The lane 1, 2 and 8 are mutants K1, K4 and K21 respectively, which shows constitutive expression of COR15. K = genotype Klone (Clone), Lane 3 = K9, 4 = K11, 5 = K13, 6 = K18, 7 = K19, and lane 9 = KC, KC = Control wild type klone.

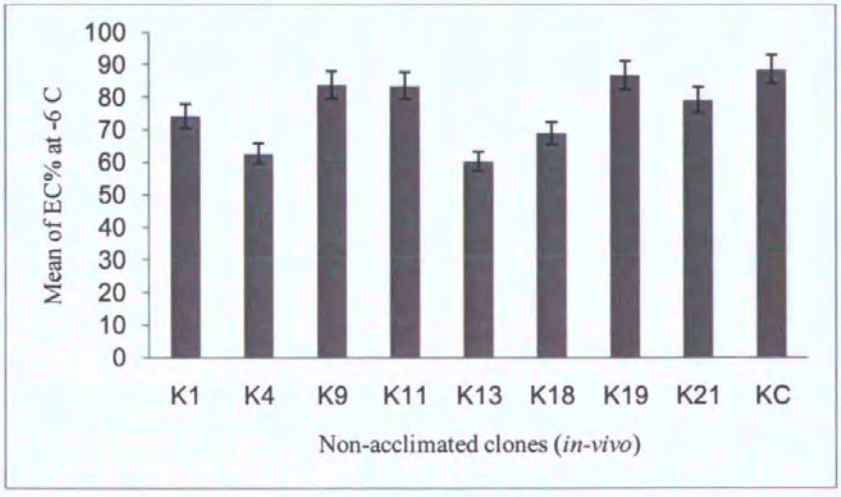


Figure 48: Frost resistance in non-acclimated clones.
K1, K4 andK21 where COR15 expressed without cold acclimation.

4.4.5 The effect of cold acclimation on proline and protein level

The cold acclimation increased the proline production in clones while the total protein level was found to be reduced over non-acclimated clones as shown in Figure 49 and Figure 50 respectively. The correlation between proline and protein under cold acclimation was found negative and non significant (Figure 51).

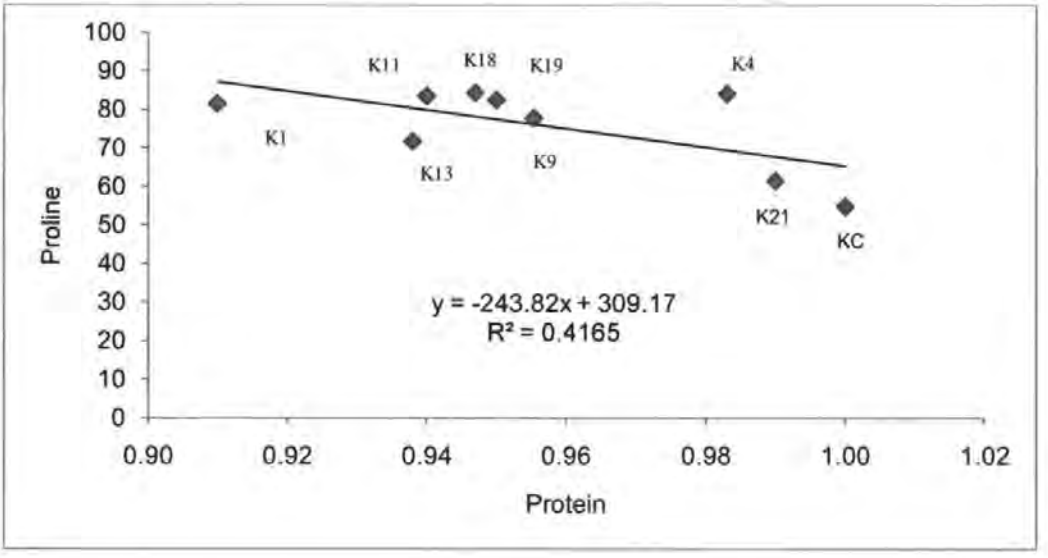


Figure 51: Correlation between free proline and total protein level after 14 d cold acclimation.
KC is control and K1-K21 are mutants

4.4.6 Correlation between frost resistance and CBF expression

The cold-acclimation induced the CBF expression in clones, as well as increased frost resistance (see chapter 3). The relationship between parameters for the clones under cold acclimation was investigated by correlation. There was a positive correlation between frost resistance and CBF expression, with increased CBF expression decreased the % relative electrical conductivity at all test temperatures. This correlation was significant after 14 d (Figure 52) and after 24 h (Figure 53) cold acclimation, but after 6 h (Figure 54) and 3 h (Figure 55) cold acclimation the correlation was also positive but was not significant. The highest R2 value was found between CBF expression at 14 d and EC% at -6 °C (93.43% of variation accounted for) and the second highest between CBF expression at 24 h and EC% at -6 °C (82.57% of variation accounted for).

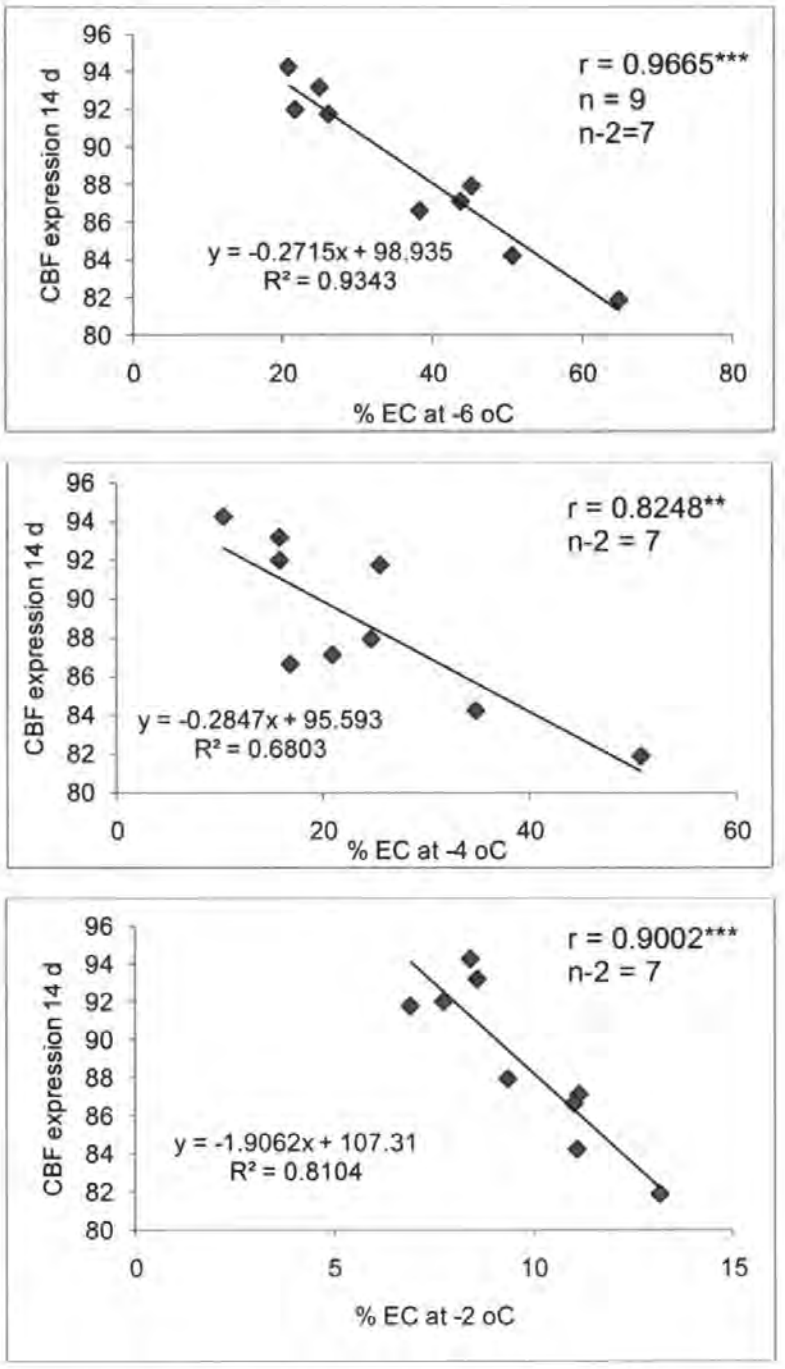


Figure 52: Correlation between frost resistance and CBF expression on 14 d cold-acclimation.
(A) Electrical conductivity (%) at -6 °C, **(B)** Electrical conductivity (%) at -4 °C, **(C)** Electrical conductivity (%) at -2 °C . Lower the % EC, higher the frost resistance.

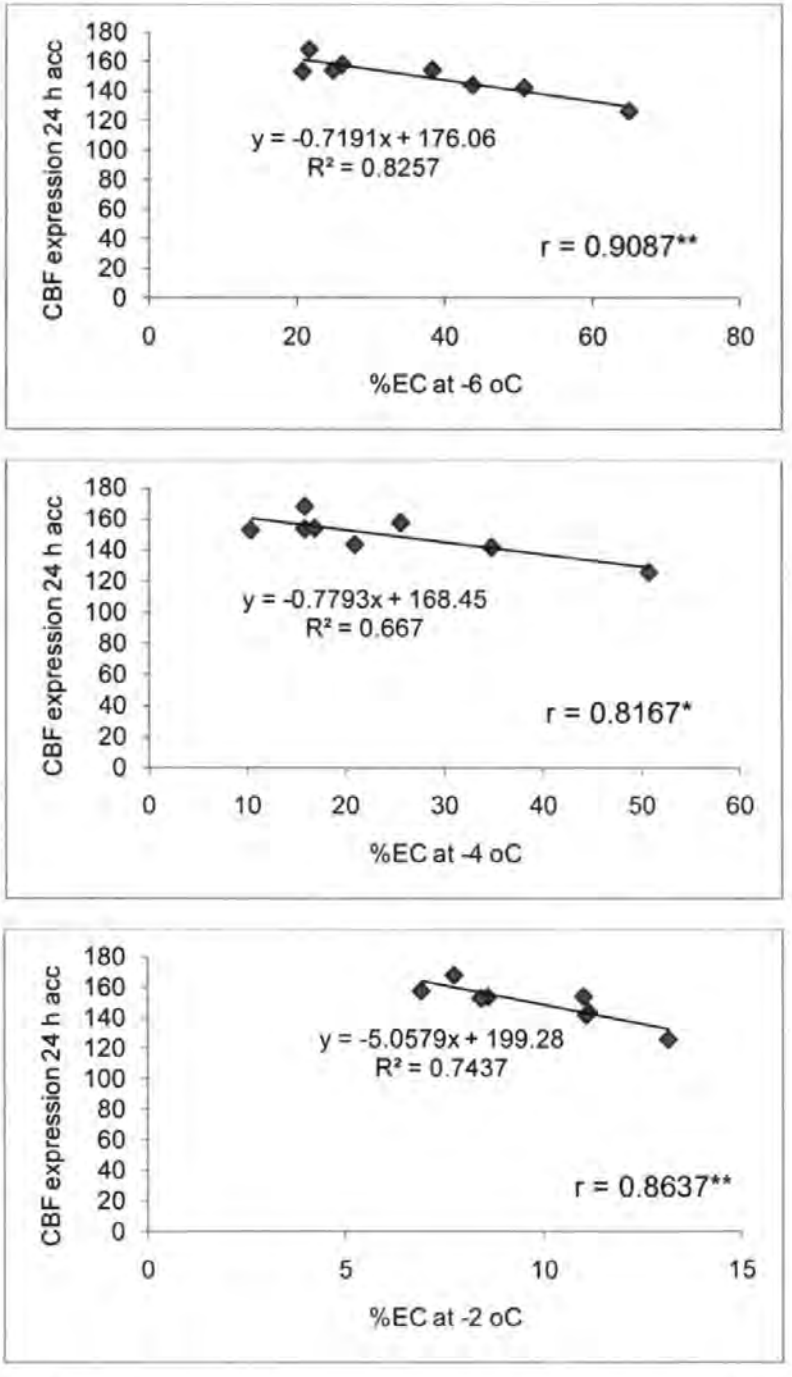


Figure 53: Relationship of frost resistance and CBF expression after 24 h cold-acclimation.
(A) Electrical conductivity (%) at -6 °C, (B) Electrical conductivity (%) at -4 °C, (C) Electrical conductivity (%) at -2 °C .

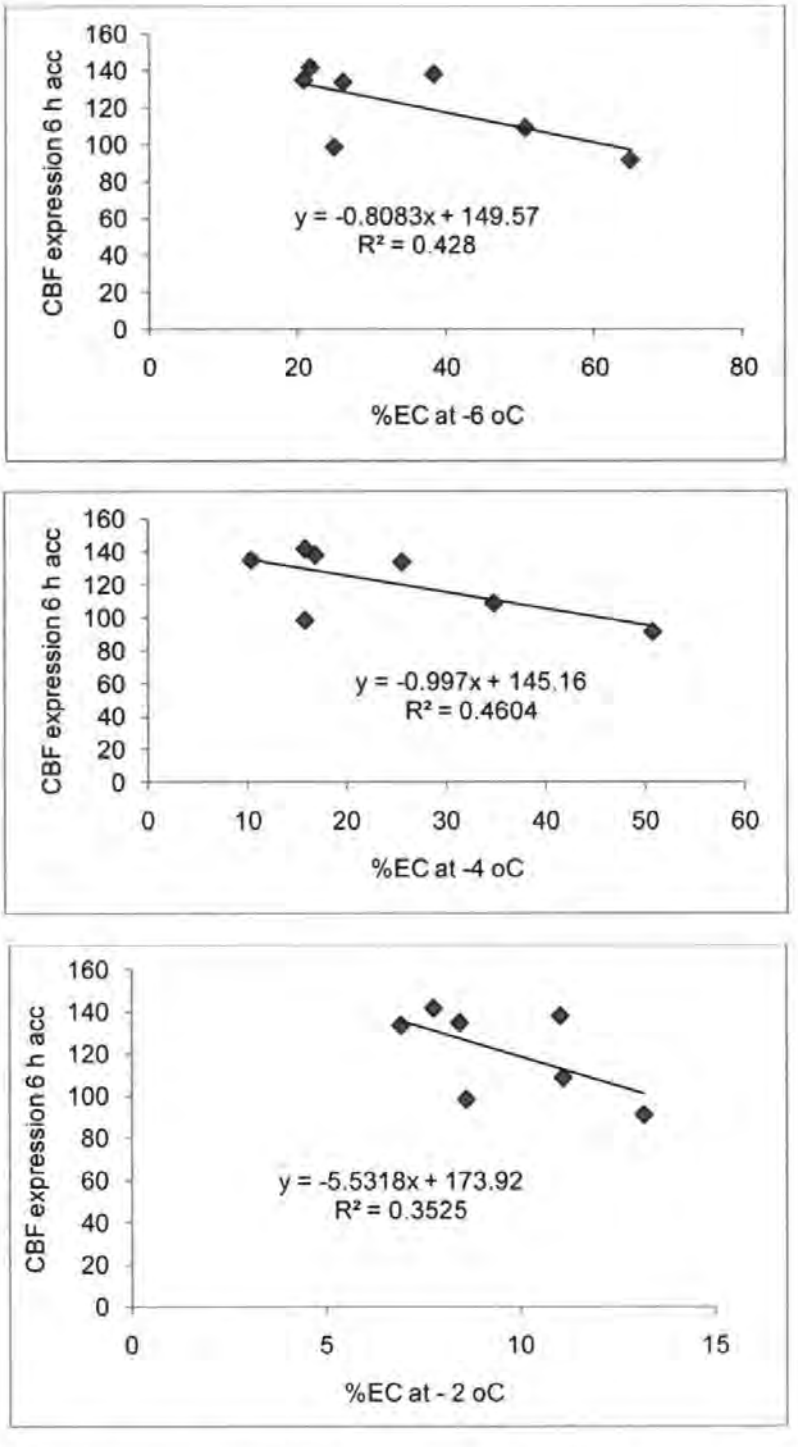


Figure 54: Correlation between frost resistance and CBF expression after 6 h cold-acclimation.
(A) Electrical conductivity (%) at -6 °C, (B) Electrical conductivity (%) at -4 °C, (C) Electrical conductivity (%) at -2 °C .

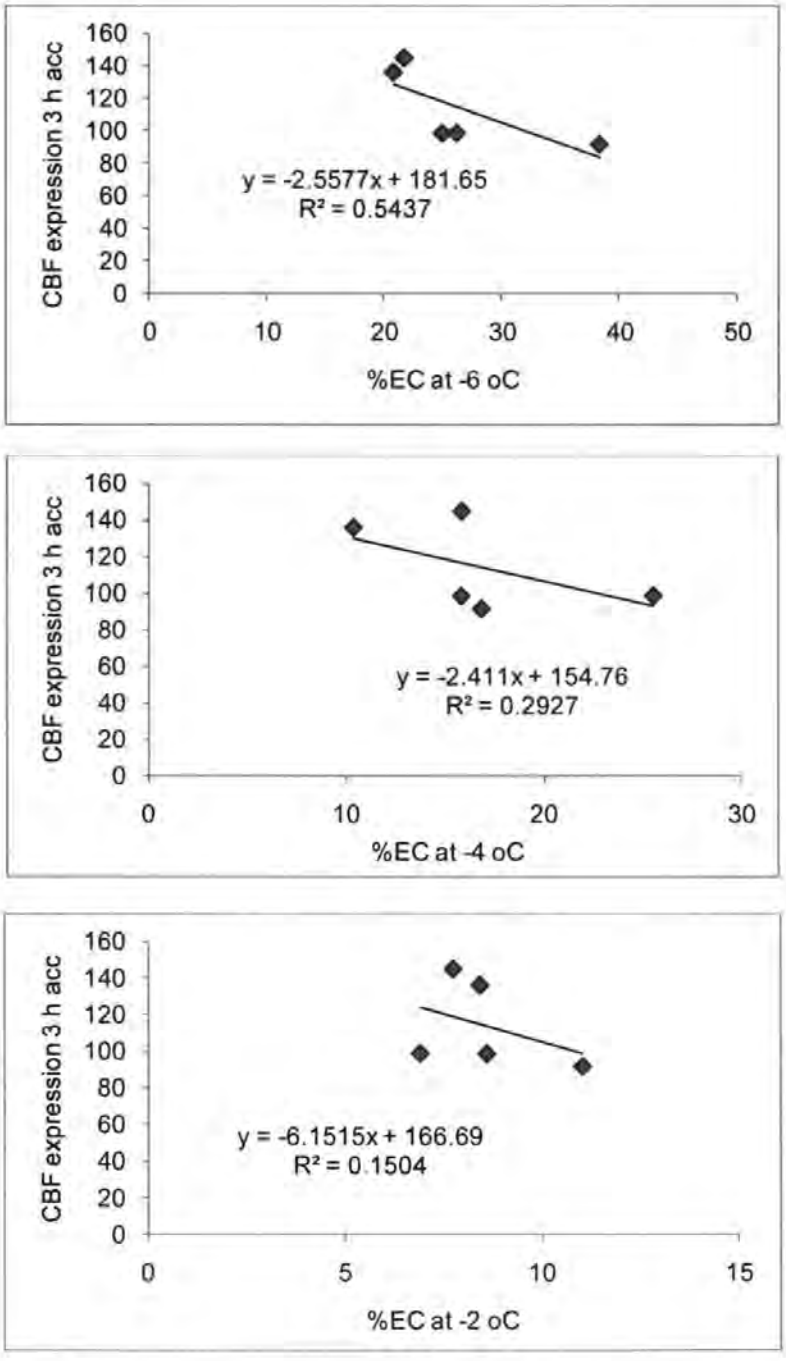


Figure 55: Correlation between frost resistance and CBF expression after 3 h cold-acclimation.
(A) Electrical conductivity (%) at -6 °C, (B) Electrical conductivity (%) at -4 °C, (C) Electrical conductivity (%) at -2 °C .

4.4.7 Relation between CBF expression and proline production

Cold acclimation increased the proline production and also induced the CBF expression. The results indicate that the increase in CBF expression in mutant clones was correlated with increased proline production. A significant positive correlation was found between the CBF expression and proline production under cold acclimation (Figure 56).

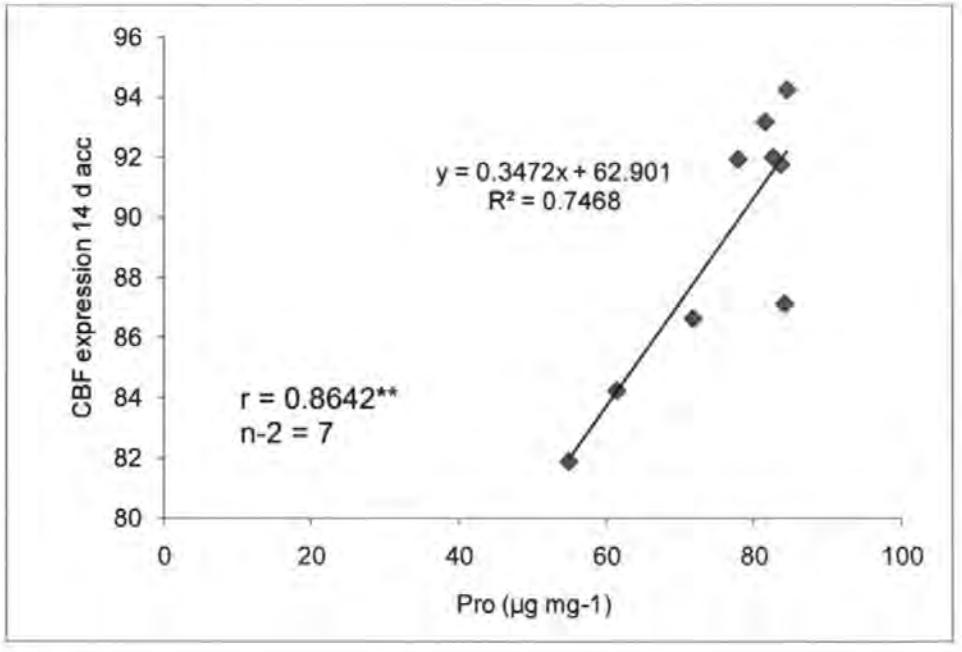


Figure 56: Correlation between CBF expression and Proline production after 14 d cold acclimation

4.4.8 Correlation between proline production and frost resistance

The clones on exposure to cold acclimation increased the proline production and showed decrease in relative electrical conductivity (%EC), which indicates a positive correlation between frost resistance and proline production. The positive correlation was found to be significant between proline and frost resistance under cold acclimation at all of the test freezing temperatures i.e. -6 °C, -4 °C and -2 °C (Figure 57). Non-acclimated clones showed very weak relation between proline and frost resistance (Figure 58).

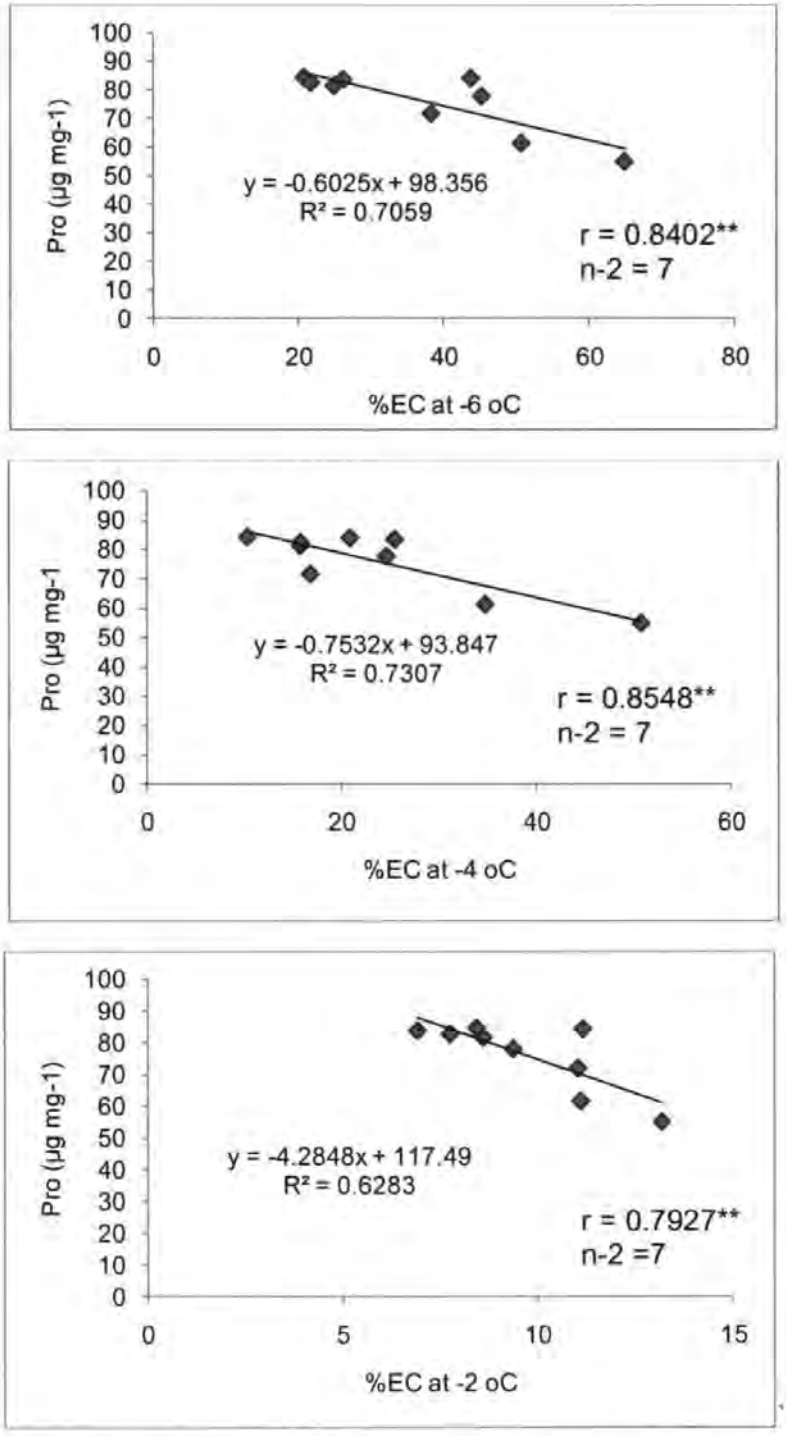
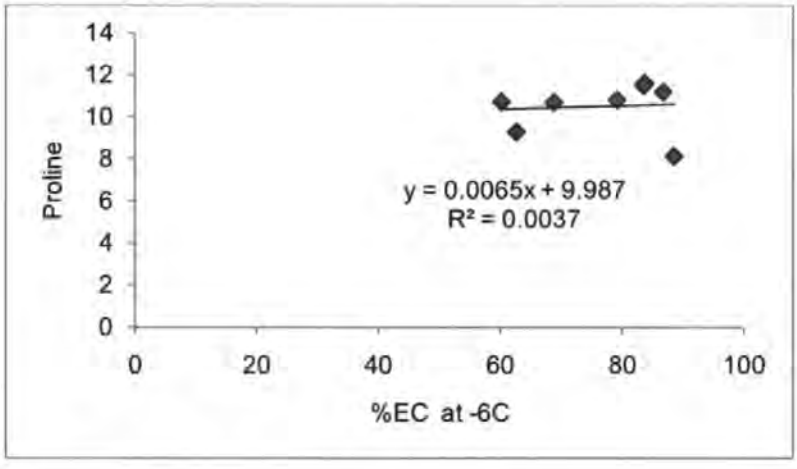
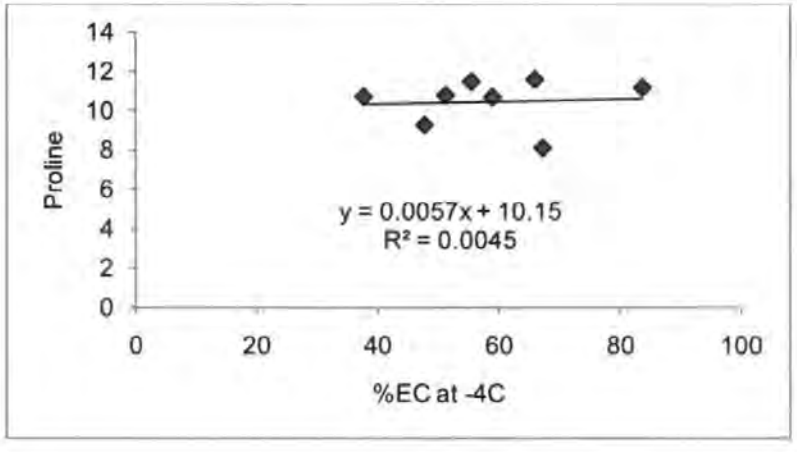


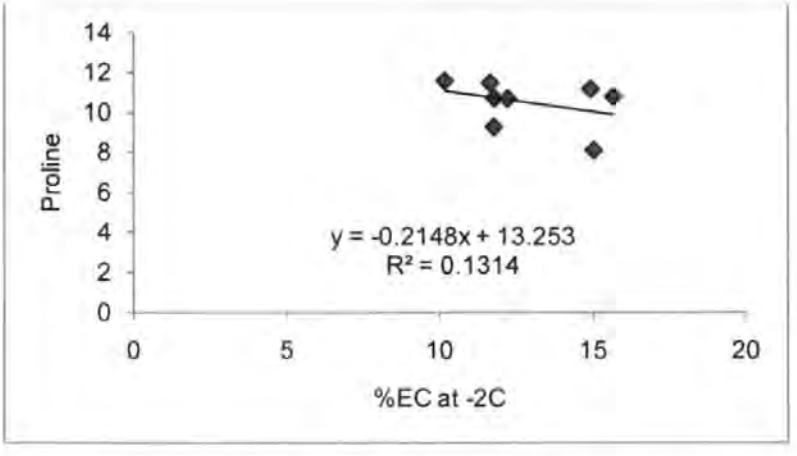
Figure 57: Correlation between frost resistance and proline production in 14 d cold acclimated clones.
(A) electrical conductivity (%) at -6 °C, (B) electrical conductivity (%) at -4 °C, (C) electrical conductivity (%) at -2 °C. Lower the %EC higher the frost resistance.



a



b



c

Figure 58: Correlation between proline level and EC% in *in-vivo* clones under non-acclimated condition,

The clones grown at 22 °C. (A) electrical conductivity (%) at -6 °C, (B) electrical conductivity (%) at -4 °C, (C) electrical conductivity (%) at -2 °C. Lower the %EC higher the frost resistance.

4.5 Discussion

In the present study, a part of the CBF regulon pathway was investigated in *Brassica oleracea var. botrytis* and identified the *CBF/DREB1* like gene, the *COR15a* gene, as well as demonstrating higher levels of free proline under cold-acclimation condition. These results confirmed the existence of the CBF regulon in cauliflower (*B. oleracea var. botrytis*) for the first time. The results also confirmed the induction of CBF regulon by cold acclimation as reported earlier by other scientists (Chentao et al., 1990; Kume et al., 2005; Pearce, 1999; Pino et al., 2008; Thomashow, 1999). The gene has been ascribed a Bo prefix to stand for *Brassica oleraceae*.

The results demonstrate that length of cold-acclimation period plays an important role in the expression level of the *BoCBF/DREB1* gene and acclimation also differentiates the transcript level in genotypes.After 3 h cold acclimation the transcript appeared in few mutant genotypes and after 6 h the number of genotypes increased and increased further up to a maximum at 24 h cold acclimation and then the transcript level declined at 14 d acclimation. All of the non-acclimated genotypes, either mutants or control, showed no RT-PCR product for *BoCBF/DREB1* gene. The DREB1 specifically induces by cold stress (Javad, Sasan & Hassan, 2009) and similar pattern of increase in transcript level with increase in length of cold acclimation has been reported by many scientists (Cong *et al.*, 2008; Novillo et al., 2003; Yong et al., 2006).

For identification and isolation of *BoCBF/DREB1* gene in the present investigation, the RT-PCR was used instead of the more common northern blotting technique to avoid cross-hybridization. Furthermore during the last 5-6 years the RT-PCR technique has been used by many scientists for isolation of cDNA sequences of CBF/DREB genes and has successfully led to sequence identification (Kume *et al.*, 2005; Yong *et al.*, 2006).

Expression analysis for CBF/DREB1 gene in different genotypes was carried out on the basis of band intensity, this method is not suitable for precise evaluation of mRNA quantification, but has a crude ability to compare the gene expression patterns among the genotypes. Mutants and controls showed clear variation among genotypes in the present analysis. These patterns could have been confirmed by quantitative PCR but unfortunately this was not possible within the constraints of the experimental phase of this study.

In this investigation, the cDNA partial gene sequences were isolated from both mutant and control clones.On comparison using bioinformatics tools, their sequences were identical, which shows that in this part of gene sequence no mutation has occurred. Since there is evidence for differences in expression pattern then this suggests that the mutation might be in an upstream position or regulator gene like ICE (Inducer of CBF expression1) or might be in the promoter region of the CBF/DREB1 gene that induces the expression of *BoCBF/DREB1*. ICE1 is a master switch involved in the regulation of CBF genes (VanBuskirk & Thomashow, 2006). Mutations in ICE1 could affect the CBF transcript accumulation under cold acclimation since over-expression of *ICE1*enhances the expression of the *CBF* (Chinnusamy et al., 2003). Further investigation is suggested firstly to identify the remaining *BoCBF/DREB1* sequence and to investigate the upstream DNA in this pathway for mutations.

The present investigation demonstrated that the sequenced part of the *BoCBF/DREB1* gene has no intron as the PCR product from gDNA and RT-PCR product (cDNA) shows similar size of the fragment and this sequence is therefore an exon. Further study

is suggested on the basis of present results for isolation of the whole *BoCBF/DREB1* gene to investigate whether the whole gene is exon or not.

Comparison of the isolated partial sequence by bioinformatics tools indicates that this partial sequence of *Brassica oleracea BoCBF/DREB1* gene shows highly similarity (91%) with other *Brassica* species *CBF/DREB* genes such as *B.rapa DREB1*, *B.juncea BjDREB1* and *B.napus CBF5* (Fig. 41).This confirms that this gene is in the genome of *Brassica oleracea*. Further confirmation was carried out by deduced amino acid sequence comparison.

Deduced amino acid sequence of the *BoCBF/DREB1* partial sequence in comparison with other *Brassica* species showed 90% homology and showed identical conserved AP2 domain as shown in Fig. 42. The AP2 domain may play a crucial role in recognition of DNA binding sequence in the promoter of cold responsive genes (Liu et al., 1998a; Sakuma et al., 2002). This homology reduced to 67% when compared with plants other than Brassicas.

Among the six member *Brassica* species in the triangle of U (U, 1935), the sequence from *B. oleracea* showed high resemblance with the species *B. napus, B. juncea*, and *B. rapa*. For the remaining two species, *B. nigra* and *B. carinata*, no CBF genes have been reported in the literature. All these resemblances confirm that this sequence is highly conserved in *Brassicas* and confirms that this is an important mechanism for regulating cold hardness in plants.

Through immunoblot analysis, the COR15a protein was detected for the first time in *Brassica oleracea*. The results demonstrated the expression of a COR15a gene in all clones under cold acclimation which correlated with higher frost resistance over non acclimated plants. Earlier investigations in the literature show that the COR15 gene is expressed in response to low temperature and correlates with enhanced freezing tolerance (Hajela et al., 1990; Katsuhiro et al., 2007). Non acclimated clones showed no blots for COR15 gene, with the exception of three mutants K1, K4 and K21 where apparent constitutive expression of COR15 is demonstrated even under non-acclimated conditions. This might indicate the presence of other CBF genes which induced the COR15 gene expression because in these mutants no RT-PCR bands appear under non-acclimated conditions with the primers used for the *BoCBF/DREB1* gene sequence isolated. Previous studies show that expression/overexpression of *CBF* increases the levels of COR proteins (Gilmour et al., 2000) and CBF/DREB transcription factors are the key regulators for expression of the COR genes (Stockinger et al 1997, Jaglo-Ottsen et al 1998, Liu et al 1998). So this suggests that there might be other CBF genes in cauliflower. These three mutants K1, K4 and K21 where COR15 is expressed, showed better frost resistance than other mutants K9, K11, K19 and KC under non-acclimated condition. Seedhabadee et al (2008) also reported COR gene expression in tolerant genotypes compared to susceptible genotypes.

The mutants K1, K4 and K21 showed blots for COR15a gene under both conditions of acclimation and non acclimation, but the frost resistance was higher in acclimated mutants compared to non-acclimated.The recent findings of Polashock et al (2010) support the present results, they investigated that transgenic plants over-expressing the CBF gene exhibited induced expression of the Arabidopsis cold-regulated (COR) genes

COR78 and COR6.6, under non-acclimating conditions. They also showed enhanced freezing tolerance in the transgenic plants under non acclimating conditions but not to the level of acclimated control plants.

The other interesting thing observed here is that the frost resistance level of mutants K13 and K18 is also similar to K1, K4, and K21 under non-acclimated condition, while COR15 blots not appear in K13 and K18 under non-acclimated condition. This suggests that mutants K13 and K18 have the presence of CBF and COR genes other than *BoCBF/DREB1* and COR15a, and there might be some upstream mutation which might enhance the frost resistance in these mutants under non-acclimated condition. These results suggest further investigation to analyse these two mutants (K13 and K18) for detection of other CBF and COR genes under non-acclimated conditions. In CBF regulon, the induction of ICE (Inducer of CBF) regulates CBF expression which then controls downstream regulation of COR genes (Chinnusamy, Zhu & Zhu, 2006; Monroy & Dhindsa, 1995) but the whole molecular mechanism of cold acclimation is still not well defined (Chunzhen et al., 2009).

Cold acclimation showed an effect on the production of free proline in relation to total protein content in all of genotypes tested but not significant. A negative correlation in proline level with total protein contents in cold acclimated clones was demonstrated. These results agree with the findings of Debnath (2008) who reported similar increases in proline content with a decrease in protein under dehydration stress. Many other scientists have also reported increase in proline during cold acclimation in Arabidopsis and other plants species (Alberdi et al., 1993; Fuller et al., 2006; Koster & Lynch, 1992a; McKown, Kuroki & Warren, 1996; Misra, Biswal & Misra, 2002; Wanner & Junttila, 1999b). The present results demonstrate increase in frost resistance with raise in free proline level in the clones and shows positive correlation. CBF expression was

shown to increase the level of proline and frost resistance under cold-acclimation in Arabidopsis (Gilmour et al., 2000). Furthermore studies have shown that an increase in free proline show enhanced resistance in plants to abiotic stresses such as salt and freezing (Ashraf & McNeilly, 2004; Dorffling, Dorffling & Lesselich, 1993; Fuller et al., 2006; Kueh & Bright, 1981; Tantau & Dorffling, 1991).

4.6 Conclusions

This work was the first reported evidence of *CBF/DREB1* and *COR15* genes in *Brassica oleraceae*. These genes were identified and demonstrated the major influence of cold acclimation on expression of these genes. Cold acclimation also influenced proline production. These results also verified the existence of mutations in the clones, as the constitutive expression of *COR15* gene was observed in three genotypes even under non-acclimated conditions. The clones demonstrated positive correlation in CBF expression vs frost resistance in cold acclimated plans, CBF expression vs proline production, and the proline level vs frost resistance under cold acclimation and demonstrated negative correlation between proline and total protein in cold acclimated plant.

Chapter 5: General Discussion

5.1 Mutagenesis as a tool to enhance abiotic stress resistance in cauliflower

The appearance of the success of mutagenesis in the first phase of this project confirmed that this is a promising method of producing mutant lines with better stress resistance in *B. oleraceae*. Mutation breeding can have many advantages as well, for instance mutants with multiple traits can be identified compared to transgenic approach where a single trait can be introduced in the crop. Also the chances of survival of mutant varieties are also much higher (Mohan, 2010). Another important use of induced mutagenesis is to build up mutant lines to identify trait specific genes and to set up gene and sequence databases for functional genomics study (Mohan, 2010).

There are shortcomings of the method of mutagenesis because it is random and sometimes desirable mutations are associated with undesirable effects and some times the frequency of desirable mutations are very low. The technique can be considered as a blind technique where specific genes in the genome are not targeted and mutations can be induced any where in the genome. For this reason large populations are used to screen for mutant lines for abiotic stress resistance e.g.thirty one resistant cauliflower shoots were recovered from more than six thousands explants mutagenised that showed a mutation frequency of about 0.52% for the abiotic stress resistance (Deane, Fuller & Dix, 1995).

The tissue culture techniques were used in the present investigation for regeneration, sub-culturing and for screening of the clones. The *in-vitro* culture techniques in combination with induced mutation can speed up breeding programmes such through the generation of variability and through selection to the clonal multiplication of the desired genotypes.

The present findings clearly show that a short selection process with relatively high selection pressure can produce functional and stable mutants in a short time. The physiological screening of cauliflower clones under freezing temperatures and under elevated salt and mannitol concentrations using leaf discs resistance assays provided several advantages over the use of intact plants. These screening processes took a short time and provided excellent chances of repeatability for confirmation, and the processes took place under well controlled experimental conditions in a small space. Many researchers have reported similar techniques as powerful tools for investigation of plant responses to different abiotic and biotic stresses (Baraka & Audran, 1997; Fuller *et al.*, 2006; Marcin, 1999; Vijayan, Chakraborti & Ghosh, 2003).

All the mutants produced more proline over control clones both under cold-acclimation and non-acclimation conditions. This increase in proline level in mutants may possibly be resulting from increased activity of the enzymes involved in the proline synthesis, or might be possibly due to inhibition of enzymes involved in the degradation of proline, a hypothesis supported by Aspinall and Paleg (1981). The results presented here support the hypothesis that proline acts as a protective compound during dehydration stress and similar observations in other species led to the suggestion that proline can be used as a metabolic marker for specific screening or selection (Bhaskaran et al., 1985; Martinz et al., 1996).

The evidence from the present investigation suggests that through chemically induced mutagenesis, mutant lines of cauliflower can be produced which demonstrate correlated resistance to more than one abiotic stress. Some mutants were resistant to all of the three analysed abiotic stresses i.e. frost, drought and salt stress, while some of the others were resistant to salt and drought or drought and frost or salt and frost. No one mutant

was resistant to only a single stress. Similar pattern of multi stress resistance in some mutants was also observed by Fuller et al (2006), and the present findings confirms their findings and suggest that there is a common resistance mechanism involved in resistance to multi stresses in a single mutant. The results from physiological screening of mutants for frost, drought and salt resistance also provide evidence to support the molecular evidence that many genes are involved in the up-regulation of resistance to abiotic stress (Guy, 2003; Pearce, 1999; Vinocur & Altman, 2005).

Many plants on exposure to non freezing low temperature increases their freezing tolerance (Thomashow, 1999) and in the present investigation cold acclimation significantly increased the frost resistance compared to non-acclimation. In the light of all the evidence obtained from physiological screening, it was hypothesised that there is a group of genes where they are expressing to resist multi-stresses in each mutant. On the basis of this hypotheses, the CBF regulon was investigated which is a group of co-expressed genes under cold acclimation (Wieslawa & Eve, 2008). Before this investigation no one had reported this regulon pathway in cauliflower or other *Brassica oleraceae* sub species. Molecular study was carried out for investigation of the presence of CBF regulon pathway in cauliflower and on the basis of the present results it is confirmed that the CBF regulon exists in cauliflower where the CBF/DREB1, COR15a genes and higher level of proline was found under cold acclimation conditions. Thomashow(2001) reported that the CBF regulon includes COR and ERD genes, membrane stabilizing proteins and cryoprotectents such as proline.

5.2 *BoCBF/DREB1* expression and frost resistance in mutants under cold acclimation

In the present investigation cold acclimation always increased the frost resistance and the *BoCBF/DREB1* gene was expressed in clones only under cold acclimation and therefore showed a positive correlation between *BoCBF/DREB1* transcript level and frost resistance. Several of the low temperature responsive genes contain in their promoter regions one or more copies of a cis element C-repeat/low temperature responsive element/dehydration responsive element (CRT/LTRE/DRE) (Baker, Wilhelm & Thomashow, 1994; Nordin, Vhala & Palva, 1993; Yamaguchi-Shinozaki & Shinozaki, 1994) with a core sequence of CCGAC, which is the binding site for the cold specific transcriptional activators CBFs/DREBs (Stockinger, Gilmour & Thomashow, 1997) that induce the expression of cold responsive genes and subsequently enhance cold/frost resistance in plants (Mantas, Pekka & Tapio, 2010).

The level of expression of the *BoCBF/DREB1* was not constant at all times during cold acclimation. The transcript level in mutants increased up to 24 h cold acclimation where the values were maximum and then declined, and this demonstrated that the beginning of cold acclimation highly increases the expression of *BoCBF/DREB1* gene while cold acclimation for longer time losses its potentional to maintain the expression level of *BoCBF/DREB1* gene high. Huogen et al (2008) reported similar pattern of CBF expression in Vitis plants under cold acclimation and also reported that the transcript was maintained for several days under cold acclimation.

Cold acclimation also induced frost resistance in control plants in comparison with control plants grown under non acclimated conditions. This evidence showed that cold acclimation works to increase the frost resistance in cauliflower. Similarly cauliflower is

known to acclimate in the field (Fuller, Gout & Tapsell, 1989).The mutants highly increased the frost resistance compared to the control under cold acclimation even though the transcript of *BoCBF/DREB1* appeared in control plant under cold acclimation; its transcript level was very low as compared to the transcript levels in the mutants. This evidence showed the expression of *BoCBF/DREB1* gene in non mutant cauliflower (control) under cold acclimation but the mutagenesis had increased the level of expression of this gene and ultimately enhanced frost resistance in the mutants over control plant.

Many researchers have reported increased frost tolerance with over-expression of CBF/DREB1 gene in plants (Gilmour et al., 2000; Liu et al., 1998). Constitutive over-expression of the *CBF1/DREB1b* or *CBF3/DREB1a* genes in transgenic Arabidopsis plants induced the expression of cold-responsive CRT/DRE-containing genes without a low-temperature stimulus and non-acclimated transgenic plants were more freezing tolerant than non-acclimated control plants (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Liu *et al.*, 1998). It has been concluded that the "CBF regulon" includes those genes which have roles in cold acclimation (Thomashow, 2001) and previous investigations report that orthologs of *Arabidopsis CBF/DREB1* genes have been found in many higher plants (Benedict et al., 2006; Jaglo et al., 2001; Kayal et al., 2006; Nakashima & Yamaguchi, 2006; Owens et al., 2002; Skinner et al., 2005).

5.3 Expression of *BoCBF/DREB1* and proline production under cold acclimation

The level of proline increases during cold acclimation in Arabidopsis and other plants (Alberdi et al., 1993; Koster & Lynch, 1992; McKown, Kuroki & Warren, 1996; Wanner & Junttila, 1999b). In Arabidopsis plants an increase in proline accumulation

under cold acclimation occurred by the genes which were regulated by the CBF activators, and over-expression of CBF3 resulted in elevated levels of proline in non-acclimated plants (Gilmour *et al.*, 2000).

In the present investigation proline was always produced in clones under both situations of either cold acclimation or non acclimation conditions. Cold acclimation increased the proline level about 8 fold higher than non acclimated clones. One reason here for the significant increase in proline level under cold acclimation might be the expression of *BoCBF/DREB1* gene in cauliflower mutants under cold acclimation because there was no transcript observed for BoCBF/DREB gene in non acclimated clones. These results also indicate that proline production in cauliflower is not only under the control of *BoCBF/DREB1* because the proline produced in non acclimated clones without expression of *BoCBF/DREB1* gene but the level of proline was lower under non acclimated condition. The results indicate a possible role of *BoCBF/DREB1* in the proline production because a positive and significant correlation was found in *BoCBF/DREB1* transcript level and proline production but this correlation was only under cold acclimation.

The mutants always produced higher level of proline with higher transcript level of *BoCBF/DREB1* when compared to control. This increase in proline and *BoCBF/DREB1* transcript in mutants might be due to mutagenesis that has induced over-expression of the *BoCBF/DREB1* in mutants over control plant. Over-expression of CBF has been reported to elevate the levels of proline and total sugars (Gilmour et al., 2000) and such increase in proline levels occur in a wide variety of plants under cold acclimation (Thomashow, 2001).

In the present findings it is speculated that the positive correlation between proline and *BoCBF/DREB1* gene expression might be due to the over-production of an enzyme in the proline cycle such as D1-pyrroline-5-carboxylate synthase (P5CS), which has a key role in determining proline levels in plants (Yoshiba et al., 1997).

P5CS transcript levels have been reported increased in in Arabidopsis in response to low temperature (Xin & Browse, 1998b), and there has been a high interest to determine whether *P5CS* transcript levels could elevated in *CBF* over-expressing plants. Northern analysis revealed approximately a 4-fold higher *P5CS* transcript levels in non-acclimated *CBF* expressing plants than non-acclimated controls and these were about equal to those found in plants which were cold treated for one day (Gilmour *et al.*, 2000). The transcript level of *P5CS* in 7 days cold-acclimated *CBF* expressing plants were 2- 3-fold higher than in cold-acclimated control plants (Gilmour *et al.*, 2000). Thus it appears that CBF binds to the promoters of genes which are directly involved in the proline synthesis (Strizhov et al., 1997) which seems to contribute to the increase in the level of proline.

5.4 Role of free proline and COR15a protein in frost resistance under cold acclimation

In the present investigation the cold acclimation played a vital role in proline production and increased the level of proline in mutants over non-acclimated mutants and subsequently the acclimated mutants demonstrated higher frost resistance than non-acclimated. There was no or very weak correlation between proline production and frost resistance in mutants under non-acclimated conditions, but when the mutants were exposed to cold acclimation, a positive and significant correlation between proline production and frost resistance was obtained. Proline plays an essential role in freezing

tolerance (Xin & Browse, 2000) and many researchers have reported increased proline levels in a wide variety of plants under cold acclimation, correlated with the enhancement of freezing tolerance (Bohnert, Nelson & Jensen, 1995; Gilmour, Fowler & Thomashow, 2004; Gilmour *et al.*, 2000; Misra, Biswal & Misra, 2002; Misra et al., 1990; Nanjo et al., 1999; Thomashow, 2001). The present results also agree with the findings of Fuller et al (2006) who reported that elevated proline can improve resistance some-times but is not always essential for improved resistance to abiotic stress.

The mutants always showed higher proline level and frost resistance compared to control plants but the difference between mutants and control plants become very clear when the plants cold acclimated. These findings demonstrated that the mutagenesis positively increased the proline level, this increases might be due to mutation, that could induce over-expression of proline biosynthetic enzyme P5CS as discussed earlier in the previous section 5.3. These results demonstrated the influential role of cold acclimation in production of proline in cauliflower mutants and subsequently enhanced frost resistance. There is further molecular and genetic evidence that proline contributes to improved freezing tolerance from various freezing-tolerant mutants of *Arabidopsis* that accumulated proline even in the absence of low temperature treatment (Xin & Browse, 1998a). The proline content was increased by 10-fold during two days of cold acclimation at 4 °C in wild-type Arabidopsis, and in the eskimo1 (frost tolerant) gene mutant the proline level was 30-fold higher than wild-type plants under non-acclimated conditions (Xin & Browse, 1998a).

Previous literature have shown that the COR15 gene is expressed in response to low temperature and has a role in freezing tolerance (Hajela et al., 1990) and two COR15

copies have been discovered in *Brassica napus* involved in cold stress response (Weretilnyk et al., 1993). In this investigation the response of clones under cold acclimation and non-acclimated conditions were different for the expression of COR15 . All of the clones expressed the COR15 under cold acclimation but when the clones were grown without cold acclimation, no product of COR15 appeared except in the three mutants where COR15 was expressed constitutively without cold acclimation. The frost resistance in cold acclimated clones where COR15 was strongly expressed was higher than non acclimated clones. This increase in frost resistance might be due to the COR15 protein which can reduce the susceptibility of membranes to form hexagonal-phase lipids and enhance the freezing tolerance by membrane protection (Artus et al., 1996; Steponkus et al., 1998).

In the mutants where COR15 was constitutively expressed, the frost resistance was better among the clones when non-acclimated but this level of frost resistance in constitutively expressed mutants was not similar to that of mutants under cold acclimation. These results confirm the recent findings of Polashock et al (2010) who reported constitutive expression of COR genes in transgenic Arabidopsis under non-acclimated condition but frost resistance in cold acclimated plants was higher. The demonstration of constitutive expression in non acclimated mutants might indicate some upstream mutation in the transcription factor regions that control the expression of COR15a. Earlier reports shows that constitutive expression of ICE1 enhanced the expression of CBFs and constitutively expressed COR genes (2003). Jaglo et al (1998) have also reported similar constitutive expression of CRT/DRE controlled COR15 gene without cold acclimation in Arabidopsis.

5.5 Conclusions

Chemical induced mutagenesis is a promising approach compared with traditional plant breeding approaches and showed highly potential to improve multiple abiotic stresses resistance in cauliflower (*Brassica oleracea var. botrytis*). This approach has many advantages such as low cost, rapid, simple, requires minimal use of chemicals, can improve multiple traits simultaneously and mutated varieties have more chances of survival in the environment.The mutants appear to retain abiotic stress resistance for a long time such as in the present investigation where the resistance in mutants was retained over a 3-4 years period without any phenotypic damage to the mutants.

The *in-vitro* and *in-vivo* screening of cauliflower for abiotic stress resistance using leaf discs is an efficient method to differentiate the genotypes for their potential of abiotic stress resistance and to identify frost, drought and salt resistant genotypes within a limited time and space.

Cold acclimation induced the expression of CBF regulon and the present investigation reported the presence of the CBF/DREB1 regulon in cauliflower for the first time. This regulon was confirmed by identification of CBF/DREB1 partial gene sequence designated as *BoCBF/DREB1* and its expression pattern determined under cold acclimation. The target genes in this regulon are COR genes and COR15 gene product (protein) was identified and correlated with higher frost resistance. The other important molecules in this regulon pathway are enzymes producing cryoprotentents such as proline and sugars and in this investigation the presence of higher level of proline under cold acclimation was reported. The genotypes showed positive correlation between CBF expression and frost resistance, between CBF expression and free proline production, between proline and frost resistance, while negative correlation found between free proline production and total protein content. All these indicate the positive role of

BoCBF/DREB1, COR15 and proline in increased frost tolerance in clones of *Brassica oleracea var. botrytis*.

5.6 Further research recommendations

Cold acclimation induces the expression of numerous genes to enhance frost resistance in many plants. The findings from the present investigation is not the answer for the whole mechanism of frost resistance in cauliflower but this study for the first time report the presence of CBF regulon pathway in cauliflower. This investigation provides a base for further investigations to explore the molecular mechanism and the gene expression in cauliflower during cold acclimation that has increased frost resistance under cold acclimation. The micro-array technique is recommended for the exploration of whole genes expression during cold acclimation that has increased frost resistance in cauliflower. But due to limitation of time and finance, the micro-array technique was not performed in this project to identify how many genes are expressed in the cauliflower mutants under cold acclimation, and this can open the door for further research to enhance frost tolerance in cauliflower.

Dehydration stress resistance is a complex mechanism and numerous genes are evolved in response to dehydration stresses (such as frost, drought and salt stress) and more investigations are required to explore and understand the resistance mechanism to enhance resistance in cauliflower plant. In the present investigation a number of mutants were screened physiologically for frost, drought and salt stress resistance but molecular and biochemical investigation was carried out for frost stress resistance only because of limited time. These mutants along with frost resistance also showed variable resistance to drought and salt stress, further investigation to explore the molecular mechanisms for this drought and salt stress resistance is recommended for these mutants.

5.7 Future perspectives

Explanation of the mechanisms of the cold acclimation and development of freezing tolerance is of vital importance for the design of novel crop varities to allow use of marginal lands for agricultural production. A concerted effort through physiology, molecular biology and biochemistry is required to understand the complex quantitative trait of low temperature and freezing tolerance (Mantas, Pekka & Tapio, 2010). In the coming years a major challenge will be the shortage in food production for the increasing population of the world (Royal Society Report, 2009) . The development of crops with increased environmental stress tolerance will greatly help in this regard, as abiotic stresses (such as frost, drought and salt) limit the geographical locations where crops can be grown and cause significant losses in plant productivity on an annual basis (Thomashow, 2001). There is the possibility of using the Arabidopsis CBF/DREB genes, or homologs from other plants, to optimize the expression of CBF regulons in agriculturally important crops to enhance freezing, drought, and salt tolerance (Thomashow, 2001). One example is the over-expression of the Arabidopsis CBF genes in canola (*Brassica napus*) that increased the expression level of target CRT/DRE-regulated genes and increased freezing tolerance in both non-acclimated and cold-acclimated plants (Jaglo-Ottosen et al., 2000). Further investigations and achievements in this area will not only be exciting and a profound scientific achievements, but will greatly aid efforts in agriculture to continue providing food to feed the world (Thomashow, 2001).

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