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TRANSGENIC APPROACH TOWARDS DEVELOPMENT OF COLD STRESS TOLERANT VEGETABLES FOR HIGH ALTITUDE AREAS

Maya Kumari*, Vikas Y Patade and Z Ahmad
*Corresponding author address:
Plant Molecular Biology and Genetic Engineering Lab, Defence Institute of Bio-Energy Research, DRDO, Goraparao, Haldwani-263139, Uttrakhand, India
Email: madhulogin@yahoo.co.in

ABSTRACT

High altitude areas are the most different terrains with cold/freezing as one the major problem which reduces the crop duration, affects quality and productivity as most part of the year is covered with unfavourable climatic conditions not suited for crop growth. Despite continued efforts, traditional breeding gave limited success in imparting crop plants with better freezing tolerance due to very little understanding about the mechanisms that regulate chilling and freezing tolerance. The constraints of conventional breeding can be overcome by application of modern biotechnology tools. Various traits such as biotic stress resistance, quality and storage life have been successfully engineered into vegetable crops and some of them have been commercialized to some extent in different countries. Although the progress in commercialization of transgenic vegetable crops has been relatively slow, transgenic vegetables engineered for cold tolerance will contribute significantly to the high altitude agriculture in near future. In this review article we discuss the effect of cold stress on plants, the mechanism developed by plants to cope with cold stress and also mention about different techniques that can be applied for crop improvement for cold stress in particular. This review also focus on different cold related genes identified so far for development of transgenics for cold tolerance in different crops and DRDO, biotechnology initiatives in identification, isolation, characterization and cloning of cold tolerant genes for developing transgenic vegetables for cold tolerance for high altitude agriculture.

Key words—Transgenic plants, coldstress, high altitude

INTRODUCTION

Food security and food sufficiency has always been a looming problem in Indian context and also in developing and underdeveloped nations where agriculture contributes majorly to the economy. Vegetables play an important role in human nutrition and health. Cultivation of vegetable crops is an integral part of the agricultural economy of many developing countries. Vegetable crop productivity and quality are seriously affected by several biotic and abiotic stresses, which destabilize rural economies in many countries. Cold, drought, heat and salinity are environmental stress factors, which cause major economic loss to agriculture worldwide. All these forms of abiotic stress primarily affect the water relations of a plant on cellular as well as whole plant level causing specific as well as

Dr (Mrs) Maya Kumari, Ph.D

Dr (Mrs) Maya Kumari, Ph.D in Agriculture Botany with specialization in Genetics and Plant breeding, from BHU, Varanasi joined DIBER, (DRDO), Haldwani in Dec, 2008. Since then she has been working on transgenic development for cold tolerance in Capsicum for high altitude cultivation in harsh winter. She is also involved in Jatropha plant improvement for yield enhancement. She has an excellent academic record and secured first position and gold medal in M.Sc from Dr. PDKV, Akola, Maharastra. She was awarded Chinese Govt. Scholarship to visit CAU, Beijing, China in 2005 for a part of her Ph.D research and did her Postdoctoral research in 2008 from CLIMA, UWA, Australia, under Endeavour Research Scholarship by DEST, Australia. She was awarded young women scientist fellowship by DST in 2007 for training in IPR. She has presented and published some interesting research findings in national and international journals. Her major areas of research interest are Jatropha yield improvement, gene cloning, genetic engineering for abiotic stress and phylogenetic studies in Capsicum.

Dr. Zakwan Ahmad

Dr. Zakwan Ahmad, M.Sc. (Ag.) and Ph.D. from G.B. Pant University of Agriculture and Technology, Pantnagar, joined DRDO in 1985. Since then he had contributed in the development and identification of varieties/ hybrids of vegetable crops suitable for high altitude areas and development of transgenic in Tomato, Capsicum, Cucumber and pea. He has contributed significantly to the Army and Civil Sector by helping in the socio-economic upliftment of local populace. Dr. Ahmad was Director of Defence Institute of High Altitude Research, Leh, Ladakh from 2004 and 2007 and since Sep, 2007 he is Director of Defence Institute of Bio-Energy Research, Haldwani and Programme Director of DRDO- Army Bio-Diesel Programme. 50 research papers, six chapters in Book and 03 Books are in his credit besides patents / TOTs of products developed. He is the proud recipient of several DRDO awards namely, DRDO Scientist of the Year award–2006, Bioved Scientist of the Year award –2007, Honorary Fellowship (FRSCS) of the Royal Society of Crop Science- 2007. He is the fellow of various professional Societies, Examiner of Universities and Secretary of FS&B of LSRB. He was an expert member for "ladakh Vision 2025" and expert invitee for consultation on Hill Agriculture of National Commission on Farmers, Ministry of Agriculture, Govt. of India. He visited Bejing, China to present research findings on Seabuckthorn in 2005. In 2009 he visited USA as a member delegate, through MNRE to collaborate with US institutions working on Biofuel programme.

Dr. Vikas Patade

Dr. Vikas Patade did his Ph.D. in the field of Biotechnology under BARC-Pune University PhD program and joined DRDO as a Scientist in 2009. He is engaged in plant biotechnological research on Jatropha, Capsicum and Sugarcane. Dr. Patade has to his credit, more than 15 research publications in journals and books published by national and international publishers. His main thrust areas are biochemical and molecular characterization of abiotic stress responses, genetic transformation and chemical seed priming in Capsicum and Jatropha.

unspecific reactions, damages and adaptation reactions (Beck et al, 2007). In high altitude areas cold/ freezing is the major problem. This reduces the crop duration, affects quality and productivity as most part of the year is covered with unfavourable climatic conditions not fit for growing crops. Despite continued efforts, traditional breeding gave limited success in imparting crop plants with better freezing tolerance due to very little understanding about the mechanisms that regulate chilling and freezing tolerance. Marker and mapping technology has enriched diversity analysis in existing germplasm of different crops. It also provides phylogenetic relationship based on which germplasm selection can be made for crop improvement through hybridization and selection. Different techniques that can be applied for crop improvement for cold stress are:

- Germplasm collection and evaluation for novel cold tolerant genes
- Diversity analysis using molecular markers like RAPD, ISSR, SSR, AFLP, etc.
- Gene isolation and characterization for cold tolerant genes through differential display (DD), subtractive subtraction hybridization (SSH), microarray technology etc.
- Transgenic development for cold tolerance
- Molecular mapping of cold related genes using molecular markers

In the past four decades, conventional breeding has contributed significantly for the improvement of vegetable yields, quality, post-harvest life, and resistance to biotic and abiotic stresses. However, there are many constraints in conventional breeding, which can only be

overcome by application of modern biotechnology tools. Various traits such as biotic stress resistance, quality and storage life have been successfully engineered into vegetable crops and some of them have been commercialized. Significant progress has been made to manipulate vegetable crops for abiotic stress tolerance, quality improvement, pharmaceutical and industrial applications. With the advent of molecular genetics and biotechnology, it is now possible to genetically engineer plants to be more tolerant to many environmental adversities, including low temperature. Genetic engineering and tissue culture technology has been used effectively to isolate, clone and characterize useful genes for their further use to develop transgenics. Effective regeneration and transformation systems are the prerequisite for successful genetic transformation (Sharma et al, 2005). Still today the most widely and cost effective method of transformation is *Agrobacterium* mediated marker free method. The transgenics can be either released directly after testing as essentially derived variety as the cultivars generally chosen are best ruling cultivar/ varieties with specific defect, which needs biotech attention. The transgenics can also be used as non-recurrent parent in further breeding program to transfer the gene in any desirable background without inviting biosafety concerns. Presently development of transgenics and moreover it commercialization is under hammer in lot of countries owing to its unpredicted effect on environment and health in long term, which nothing but time can answer. Till date there is no single report of commercialisation of any abiotic stress tolerant transgenic crop. Although the progress in commercialization of transgenic vegetable crops has been relatively slow, transgenic vegetables engineered for cold tolerance will contribute significantly to the high altitude agriculture in near future.

EFFECT OF COLD STRESS ON PLANTS

Low-temperature stress includes two different processes, chilling and freezing. Chilling stress (0–10°C) causes membrane leakiness and inhibition of photosynthetic processes, whereas freezing stress (below 0°C) leads to cellular dehydration caused by the formation of ice crystals in the extracellular space (Zhang et al, 2004; Ensminger et al, 2006; Verslues et al, 2006). Freezing temperatures are responsible for more crop losses worldwide than any other single cause and may be a significant factor influencing plant distribution (Li et al, 2005). As the temperature decreases ice formation begins on the epidermal surface, followed by ice formation within the plant tissues. At about –1°C, the extra-cytoplasm, where the concentration of the solution is the lowest, begins to freeze and as a result withdraws water from the cytoplasm, dehydrating the cells and withering the cell membranes (Steponkus and Webb 1992). The secondary stress effect is damage caused by reactive oxygen species (ROS), which arises from imbalance between their generation and scavenging (Randy, 1995). Low temperature affects plants by causing dehydration of the cells and tissues due to crystallization of the cellular water (Beck et al, 2004). Plants have developed a variety of defences against low temperatures.

1. There is shrinkage of protoplast because of movement of water outside the cell and extracellular ice formation.
2. Membrane lipid composition and membrane viscosity changes owing to disintegration of membrane bilayer by freeze dehydration. This causes membrane leakage (Verlues et al, 2005)
3. There is reduction of metabolic activity to minimum or nil in extreme conditions.
4. The expression of Cold regulated genes (COR) increases (Thomashow, 1999).
5. There is accumulation of compatible solutes e.g. sucrose and proline, which acts as osmoprotectants or cryoprotectants.
6. Hydrophilic proteins such as dehydrins accumulate in plant parts during cold exposure.
7. Chilling induces photoinhibition affecting photosynthetic machinery and efficiency (Liu et al, 2001).
8. Low temperature promotes free radical formation and oxidative stress by delayed dissipation of photosynthetic energy (Vogg et al, 1998). There is increase in level of

radical scavengers to remove ROS (singlet oxygen, hydrogen peroxide, hydroxyl radicals (Veranova et al, 2002).

9. Levels of enzymatic antioxidants {Superoxide Dismutase (SOD), Ascorbate Peroxidase (APX), Glutathione Reductase (GR), Di-Hydro Ascorbate Reductase (DHAR)} and non-enzymatic antioxidant metabolites (alpha-tocopherol, carotenoids, ascorbate, and glutathione) also increases.
10. Ionic homeostasis takes place to maintain normal metabolic reactions. Plant cells maintain high K^+ (100-200mM) and lower Na^+ (<1mM) levels in the cytoplasm.

MECHANISMS DEVELOPED TO COPE WITH COLD STRESS

To cope with unfavourable environmental conditions plants undergo certain physiological adjustments to adapt and acclimatize in due course of time to cold stress.

Acclimatization: Most temperate plants can acquire tolerance to freezing temperature by a prior exposure to low non-freezing temperature, a process known as cold acclimatization (Browse and Xin, 2001). Plants sensitive to chilling temperature are incapable of cold acclimation. Different studies support the fact that cold regulated gene expression is critical in plants for both chilling tolerance (Hsieh *et al*. 2002) and cold acclimation (Thomashow 1999; Tamminen, 2001). Cold responsive genes encode numerous proteins such as enzymes involved in respiration and metabolism of carbohydrates, lipids, phenylpropanoids and antioxidants: molecular chaperones, antifreeze proteins, and others with a presumed function in tolerance to dehydration caused by freezing (Thomashow 1999). During cold acclimatization there is change in the gene expression in plants resulting in increased tolerance (Steponkus et al. 1993). Certain COR (cold regulated) genes might have role in freezing tolerance. These COR genes were identified, isolated and characterized to establish their role in cold stress. Number of COR genes have been identified in *Arabidopsis thaliana* (Gilmour et al, 1998; Liu et al, 2004).

Physiological adjustment: Plants vary greatly in their ability to withstand low temperature stress. Cold stress signal transduction is a complex process. As an adaptation strategy during cold acclimatization many biochemical and physiological changes take place. Many physiological changes like tissue break down and senescence occur due to cold stress. Upon exposure to low temperature, temperature sensors in the cell membrane generates stress signals which are transmitted and amplified through multiple steps that include sensors like Ca^{2+} permeable channels, histidine kinases, receptor kinases and phospholipases. The massage eventually reaches the nucleus and regulators of gene expression called transcription factors, which act as master switches to regulate the expression of groups of genes, resulting in the increase of proteins and other organic molecules that protect the cell from freezing damage. Subsequently, cytoskeleton reorganization and cytosolic Ca^{2+} influx takes place (review Solanke and Sharma, 2008). Increase in cytosolic Ca^{2+} is sensed by CDPKs (Calcium dependent protein kinase), phosphatase and MAPKs (Mitogen activated protein kinase), which transduce the signals to switch on transcriptional cascades. Photosynthetic apparatus have also been thought to be responsible for low temperature perception and signal transduction. Many cold induced pathways are activated to protect plants from deleterious effects of cold stress, but till date, most studied pathway is ICE-CBF-COR signaling pathway. Cold stress signalling has certain pathways common with other abiotic and biotic stress signalling, which suggest cross talks among these. The plants response to cold acclimation is quite complex and diverse. Hence the actual biochemical and physiological changes are still poorly understood at the molecular level.

TRANSGENICS FOR COLD STRESS TOLERANCE

When plants are exposed to environmental stress, they undergo physiological and biochemical adaptations (Choi et al, 2002; Xiong et al, 2002). Plants acclimate to environmental stresses by activating cascades or network events starting with stress perception and ending with the expression of many effector genes (Shinozaki

Table 1. Different cold related genes studied so far:

Genes	**Source**	**Reference**
Transcription factors		
DREB1B/CBF1, DREB1C/CBF2, DREB1A/CBF3	*Arabidopsis*	Yamaguchi-Shinozaki and Shinozaki 1994; Liu et al, 1998;
GhDREB1	*Gosypium hirsutum*	Shan et al, 2007
Ethylene-responsive element-binding proteins (EREBP)	*Capsicum annuum*	Hwang et al, 2005
Interleukin 1-alpha Converting Enzyme (ICE1)	*Arabidopsis*	Benedict, et al, 2006
GIGANTEA	*Arabidopsis*	Cao et al, 2005
ABF3(ABRE binding factor)	*Arabidopsis*	Enkhchimeg et al, 2005
Dehydrin genes		
Cadhn	*Capsicum annuum*	Chung et al, 2003
ThCAP	*Tamarix hispida*	Li et al, 2009
Zinc finger proteins		
SCOF-1 (C_2H_2 type Zinc finger protein)	*Glycin max*	Kim et al, 2001
STZ (Salt tolerant Zinc finger protein)	*Arabidopsis*	(Sakamoto et al, 2004)
ZTP2	*Petunia sp.*	Sugano et al, 2003
OSISAP1 (*Oryza sativa* sub sp. *indica* stress associated protein), ZFP245	*Oryza sativa*	Mukhopadhyay et al, 2004; Huang et al, 2005
H2-type zinc ring finger protein	Citrus relative *Poncirus trifoliata*	Sahin-Cevik and Moore, 2006
RDCP1 (Ring domain containing protein)	*Capsicum annum*	Kim et al, 2007
Others		
FeSOD (Iron-Superoxide Dismutase)	*Nicotiana tabacum*	Van Camp et al, 1996
AFP (Antifreeze protein)	Winter flounder	Cheng and Merz, 1997
Osmyb4	*Oryza sativa*	Candida et al, 2004
Osmotin	*Nicotiana tabacum*	Larosa et al, 1992

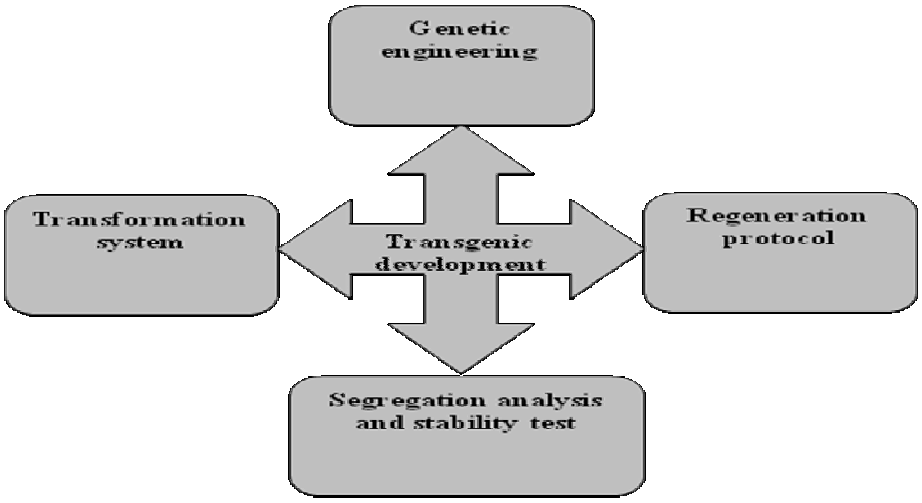
et al, 2003; Shou et al, 2004). There are different pathways leading to cold tolerance. These pathways are under the control of several enzymes the genes for which are under the control of different transcription factors. A number of studies have demonstrated that overexpression of cold-induced genes can confer cold tolerance to transgenic plants (Puhakainen et al, 2004; Shou et al, 2004; Zhu et al, 2004). During the past decade, a family of transcription factors known as dehydration-responsive element binding proteins (DREBs) has been identified in *Arabidopsis*, and a number of DREB-like proteins have been isolated from other plant species (Guo et al, 2002; Shen et al, 2003). These factors interact with cold- and dehydration-responsive elements (DREs) and enhance tolerance to freezing, drought and high salinity in plants (Jaglo et al, 2001; Kasuga et al, 2004). DRE elements contain the conserved CCGAC core sequence, which is sufficient to induce gene transcription under cold stress and is present in the promoters of many cold-inducible genes (Stockinger et al, 1997; Kim et al, 2002; Narusaka et al, 2003). Expression of DREB1 genes is strongly induced by low-temperature stress, whereas expression of DREB2 genes is

Table 2. Status of transgenic research for cold stress

Crops	Gene	Reference
Rice	Glycine betaine, BADH (Betaine Aldehyde dehydrogenase); trehalose (*otsA* and *otsB*)	Garg et al, 2002
Rice	Cod A (Cytocine deaminase gene)	Van Camp, et al, 1994
Alfalfa	SOD	McKerise, 1996
Tobacco	DREB1A	Kasuga et al, 2004
Rice, Tomato, Capsicum, Canola	CBF1	Lee et al, 2004; Hsieh et al, 2002; Jaglo et al, 2001
Rice	OsDREB	Yamaguchi-Shinozaki and Shinozaki, 2005
Carrot, Tobacco, Potato	AFP	Worrall et al, 1998; Li et al, 2005,
Apple	*Osmyb4*	Pasquali et al, 2008
Arabidopsis	Galactinol synthase	Taji et al, 2002
Rice	CDPK7	Saijo et al, 2001
Rice	*OsMYB3R-2*	Ma et al, 2009
Rice	*OsbHLH1*	Wang et al, 2003
Rice	Glutamine synthetase (*GS2*)	Kozaki et al, 1992
Maize, Alfalfa, Tea	*MnSOD*	McKersie, 1993; Karnodle and Scandalios, 1996; Vyas and Kumar, 2005
Rice	Calreticulin (CRT)	Li et al, 2003
Tomato	*dhn24*(dehydrin gene)	Glodek et al, 2008
Lettuce	*ABF3*	Enkhchimeg et al, 2005
Tomato	*osmotin*	Randhawa et al, 2009
Olive tree	*Osmotin*	D'Angeli and Altamura, 2007
Summer squash	*Cbf1*	Shah et al, 2008
Papaya	*Cbf1 and 3*	Dhekney et al, 2007
Tobacco	Omega-3-fatty acid desaturase gene (FAD7)	Khodakovskaya et al, 2006
Populus	*ThCAP* (Cold acclimation protein)	Guo et al, 2009

induced by dehydration, indicating that two independent families of DREBs function as trans-acting factors in two separate signal-transduction pathways under low-temperature and dehydration conditions (Shinozaki et al, 2003; Sakuma et al, 2006). It has also been suggested that low temperature could trigger the expression of DREB1 transcription factors, which play a key role in cold tolerance, on the basis of the observation that ectopic expression of AtDREB1A and AtDREB1B induces the transcription of genes containing the DRE promoter element and enhances cold tolerance of transgenic plants (Gilmour et al, 2000; Hsieh et al, 2002).

The CBF genes represent one of the most significant discoveries in the field of low temperature adaptation and signal transduction. Using CBF genes various plants have been transformed for cold tolerance such as canola contain this gene and were able to survive



freezing temperature as much as 4-5°C lower than the non transgenic controls (Jaglo et al, 2001). Tomato plants have also been successfully engineered using the CBF genes to achieve chilling tolerance (Hsieh et al, 2002). Over expression of rice Osmyb4 gene in *Arabidopsis* lead to increased chilling and freezing tolerance (Candida et al, 2004). There is strong evidence that the decrease in fatty acids saturation upon exposure to low temperature contributes to cold tolerance in plants. In tobacco a broad-spectrum desaturase gene from a cyanobacterium (Ishizaki-Nishizawa et al, 1996) was introduced which lead to low temperature tolerance of transgenic plants. Expression of a plant phosphatase (At PP2CA) in transgenic *Arabidopsis thaliana* accelerates cold acclimation and increased freezing tolerance. Transgenic plants expressing a constitutively active kinase NPK1 were found more tolerant to chilling and other abiotic stresses (Kovtun et al, 2000). Other transcription factors, like ABI3 and SCOF-1 have been used to successfully increase cold tolerance in transgenic plants (Tamminen et al, 2001*).* Transcription factor genes ABI3, CBF1 and 3, DREB1A, DREB1 and DREB2 from *A. thaliana,* osmolyte biosynthesis genes from *Arthrobacter globiformis* and AFP (anti freeze protein) from *A. thaliana* are some other important genes which were found to play significant role under cold stress in plants.

Frost-hardy plants produce colligative cryoprotectants such as sucrose and proline to reduce the dehydration of cells (Wallis et al, 1997). Several classes of proteins have been found to be associated with cold tolerance in plants and animals, some of which are implicated in other key stress responses, such as tolerances to drought and salinity. However, some cold-hardy plants produce specific antifreeze proteins (Urrutia et al, 1992; Griffith et al, 1994). Antifreeze proteins (AFPs) have been described in fish and insects (Griffith and Ewart, 1995), which provides frost tolerance to organisms. These proteins reduce frost injury by thermal hysteresis and inhibition of ice recrystallization. Thermal hysteresis is the process of lowering the freezing point of a liquid below the melting point. Antifreeze proteins bring about thermal hysteresis by inhibiting ice crystal growth through adsorption to the surface of the crystal (Davies and Sykes, 1997; Yeh and Feeney, 1996). Once the ice crystals melt, ice recrystallization (growth of larger ice crystals at the expense of smaller ones) is inhibited by the adsorption of AFPs to the crystal surface. AFPs with similar properties have also been isolated from cold acclimated plants. Although plant AFPs exhibit weak thermal hysteresis, they are efficient in inhibiting ice recrystallization. A gene, which encodes a protein proven to inhibit the recrystallization of ice, has been isolated from carrots (Worrall et al, 1998). Antifreeze proteins have exceptional properties and when applied, these properties could have great benefits. The expression of carrot antifreeze genes in plants is a potential approach to increase the frost resistance. Raffinose family oligosaccharides (RFO) accumulating during seed development are thought to play a role in the desiccation tolerance

of seeds. Sugar analysis showed that drought, high salinity and cold treated Arabidopsis plants accumulate a large amount of raffinose and galactinol, but not stachyose. Raffinose and galactinol were not detected in unstressed plants. This suggests that raffinose and galactinol are involved in tolerance to drought, high salinity and cold stresses. Galactinol synthase (GolS) catalyses the first step in the biosynthesis of RFO from UDP-galactose. Three stress-responsive GolS genes (AtGolS1, 2 and 3) were identified among seven Arabidopsis GolS genes. AtGolS3 was induced by cold stress but not by drought or salt stress. Stress inducible galactinol synthase seems to play a key role in the accumulation of galactinol and raffinose under abiotic stress conditions, and galactinol and raffinose may function as osmoprotectants in drought and cold stress tolerance of plants.

STEPS TOWARDS TRANSGENIC DEVELOPMENT FOR COLD STRESS:

Transgenic development for cold stress mainly comprises of four major steps: - genetic engineering, establishment of regeneration protocol, transformation system and finally segregation analysis and stable gene integration and expression.

Genetic engineering mainly comprises of following steps:

- Identification of source of gene
- Isolation and full length cloning of gene
- Functional characterization of the cloned gene
- Development and evaluation of gene construct in suitable vector system

Tissue culture towards transgenic development comprises of:

- Selection of suitable explants showing regeneration potential
- Optimization of culture conditions and media composition for development of complete plantlet *in vitro*
- Selection of suitable dose of antibiotics through sensitivity tests
- Hardening technique

Transformation system comprises of:

- Selection of suitable transformation system like direct or indirect
- Molecular confirmation of gene integration and copy number
- Selection of positive plants

Segregation analysis comprises of:

- Molecular and genetic characterization transgenic plants using gene specific DNA markers for stable and efficient gene expression
- Identification of homozygous lines for the trait of interest.
- Stability analyses for the trait under study over generations and location.
- Identification of homozygous and homogeneous progenies from a stable line and its multiplication for different trials.
- Morphological, biochemical and physiological analysis of transgenic lines.
- Toxicological evaluation for bio-safety assessment including food and environmental safety (Following DBT and GEAC guidelines).
- Evaluation of transgenic plants for their effectiveness in alleviating the biotic or abiotic stresses and field performance
- Transfer of genes to elite cultivars by conventional breeding methods if required
- Commercialization of genetically engineered crop.

DRDO BIOTECH INITIATIVES FOR CROP IMPROVEMENT FOR HIGH ALTITUDE AREAS

Different transgenics have been developed so far in different crops for different biotic and abiotic stresses. Here our major focus will be on high altitude problem, which is mainly due to very low temperature prevailing for most part of the year. This is viewed as the most important abiotic stress factors damaging crop production in high altitude areas. In cold areas growing season is of short duration and is available only in summer season for about 100-120 days. Biotechnology has the potential to enhance the productivity and also extend the cropping period in high altitude areas where vegetable cultivation is impossible in severe winter months. This will not only help the troops but also the farmers ensuring food security and profitability and also catering to the local population demand in such areas which is not

easily accessible by rest part of the country through transport due to difficult topography and climate. In this direction DIBER, a DRDO R&D lab is active towards the development of transgenic vegetables for high altitude areas.

One of the major advantages of this modern technology is that it knows no barriers, genetic or environmental. Gene can be transferred to any crop from any source irrespective of genus or species barrier. The only question is stable integration and desirable expression, which needs to be evaluated extensibly. Inspite of lot of work going on in this direction till today there in no transgenic reported to have been released for cold tolerance. However work is going in this direction in different countries and through literature we come to know of current status of transgenics for cold stress.

DIBER a DRDO establishment at Haldwani, has taken up the work on development of transgenics for cold tolerance through isolation of cold tolerant genes from highly tolerant high altitude plant species, its full length cloning and transformation into high value, high demand vegetable crops like capsicum, tomato and cucumber to cultivate them in higher altitude areas for the fresh requirement of armed forces and local population. At present the work is carried out to develop transgenics in tomato, capsicum and cucumber. Efforts are also on way to clone cold tolerant genes from seabuckthorn and lepidium plants, which grows at high altitude areas. The genes, which are being used for developing cold tolerant vegetables, are described below:

Osmotin gene*:* Low molecular weight osmolytes such as proline, betaines, amines and sugar alcohol accumulation is an important mechanism underlying adaptation to cold stress. Osmotin is a basic 24 KD protein that was originally identified in tobacco for salt tolerance. The synthesis of osmotin is induced by ABA that accumulates in response to osmotic stress and subsequently plays a pivotal role in osmotic adjustment. It is known that expression of osmotin gene induces proline accumulation in unstressed and stressed plants and imparts tolerance to both salinity and drought stress. The gene has been transferred through *Agrobacterium* in tomato. Transgenic plants are being tested in high altitude areas under controlled environments.

Mannitol-1 phosphate Dehydrogenase (mtlD): Mannitol-1 phosphate dehydrogenase (mtlD) gene encodes for enzyme that synthesize osmoprotectants and enhance their expression in transgenic plants leading to maintenance of osmotic potential upon exposure to cold stress. mtlD gene has been isolated from *E. coli* K-12 strain and code for an enzyme called mannitrol-1 phosphate dehydrogenase involved in mannitol synthesis. Accumulation of mannitol leads to tolerance at cellular level by adjustment of the cytosolic osmotic potential, a situation when the concentration of electrolytes is lower in the cytosol than in the vacuole. Cucumber has recently been transformed with *mtlD* gene and the transgenic plants are under further evaluation.

Glyceraldehydes phosphate Acetyl Transferase (GPAT) gene*:* The gene is responsible for the unsaturation of the fatty acids present in the plant cell wall, which give the cold tolerance to the plants during cold stress. Efforts are on way to clone this gene from seabuckthorn using differential display approach.

CBF1 gene: It is a transcription factor responsible for regulating the expression of many cold tolerant genes during cold stress in plants. First reported in *Arabidopsis thaliana* thereafter reported in many crop species and CBF families have been identified in many species like, rice, barley, cotton etc., expressing under cold stress when transformed in *Arabidopsis.* The expression of these genes changes the membrane lipid composition and osmolyte concentration leading to cold tolerance in transgenic plants. At DIBER, Haldwani, effort has been made towards the development of marker free transgenic *Capsicum annum* in two cultivars, Yolo Wonder and California Wonder, using CBF1 gene from Arabidopsis. Regeneration protocol is one of the major requirements for *in vitro* transformation. In capsicum regeneration is a problem hence different media combinations were tried to standardize a protocol for regeneration from different explants. Regeneration was not obtained through callus as they culminated into false leaf

like structure and never regenerated into plantlet. Shoot tip regeneration was successful and hence it was used as explants for transformation work. *Agrobacterium* mediated gene transfer with two-vector system one for nptII (kanamycin resistance gene) and other for CBF1 (cold responsive gene) in pCAMBIA plasmid vector was done through co-cultivation method. The transgenic plants obtained are now under evaluation for segregation analysis and gene stability expression. Cold assay under multilocation trial will be done further. These lines will further be used in breeding programme as donor parents for introgressing cold tolerance in elite but cold sensitive germplasm.

Conclusion:

High altitude regions face very harsh climate and a short crop-growing season. Few vegetable crops viz, radish, turnip, potato, cabbage and some leafy vegetables are mainly grown in these areas. Therefore there is a need for agro-techniques suitable for vegetable production to meet the requirement of high altitude areas. Work is going on in this direction to develop suitable varieties/ hybrids through conventional breeding for these regions. Conventional breeding alone is not effective for developing abiotic stress tolerance in sensitive crops. There is a need of advanced technology to overcome this limitation. Biotechnology can be viewed as a potential answer. Different techniques have been in use to identify genes for cold stress from cold tolerant species, which are then isolated, cloned and characterized to be used further for transgenic development. Transgenic developed should be marker free mainly due to biosafety issues. Hence, transgenic technology is needed to aid traditional breeding approach for introducing cold tolerance genes from any source into high value vegetable crops. This may help in increasing productivity and production of commercially important crops in high altitude regions also help in bringing additional areas under cultivation which are presently unfit for cultivation or are less productive.

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