Seabuckthorn (*Hippophae salicifolia* L.) plant: As source donor of cold tolerant genes for improving high altitude agriculture during cold stress

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**Abstract:** Cold stress is a major environmental factor limiting the geographical locations suitable for growing plants and periodically account for significant losses in plant productivity. Cold stress cause losses worth hundreds of million dollars each year due to reduction in crop productivity and crop failure that also threaten the sustainability of agricultural industry. It is now well known that the stress signal is first perceived at the membrane level by the receptors and then transduced in the cell to switch on the stress responsive genes for mediating stress tolerance. Understanding the mechanism of stress tolerance along with a plethora of genes involved in stress signaling network is important for crop improvement. Modern biotechnology has the tools to develop cold tolerant varieties, which enhance the productivity and profitability of farming. The present article examines the use of cold tolerant genes of Seabuckthorn plant for the development of cold tolerant transgenics in vegetables crops. Such crops may not only bring additional areas under cultivation but also help in optimizing productivity in high altitude and remote areas without any additional cost.

**Key words:** Abiotic stress, Cold stress, Cold tolerant genes, High altitude agriculture, Glycerol-3-phosphate acyl transferase gene, Seabuckthorn plant, *Hippophae salicifolia*

**Introduction**

Global requirement for food, fuel and fodder is now essentially need to be fulfilled by a comparable increase in yield, or by cultivating land not previously suitable for agricultural production such as high altitude regions. Nature has imposed certain limitations in these regions, namely abiotic stress factors like cold, frost, drought, salinity and limited radiation and water.

Conventional breeding methods have met with limited success in improving the freezing tolerance of important vegetables. Biotechnology offers new strategies that can be used to develop transgensics crop plants with improved tolerance to chilling stress. A number of genes have been isolated and characterized that are responsive to chilling stress. The molecular tool makes it possible to select directly at the gene level without waiting for the phenotypic manifestation. Therefore it is important to use most appropriate tools that help in reaching the goals. The designed genotype should be better than the available ones and must reach the farmers. Transgenic technologies have opened up many exciting possibilities to improve products with added value with application in food, agriculture, animal husbandry, environment, medicine and industry (Dunwell, 2000). It also offers uncommon opportunities for improvement in genetic potential of plants and animals by introduction or removal of gene or genes that regulate a specific trait. The conventional breeding methods can be complimented by an array of bio-technological tools can be used to augment vegetable production by saving time and resources. The potential outcome is in the form of development of specific vegetable varieties that are more resistant to biotic and abiotic stresses, enhanced nutritional level of food items, enhanced shelf life of perishable farm produce, conversion of organic waste into bio-fuels.

**Abiotic stresses at high altitude:** A number of abnormal environmental parameters such as cold, chilling, drought, salinity, high temperature, anoxia, high light intensity and nutrient imbalance, collectively called abiotic stresses, negatively affect the processes associated with biomass production and grain yield in plants (Wang et al., 2003). And with the increasing world population, as predicted to an approximately 9 billion by 2050, there will be a definite shortage of food unless world food production rises by ~ 70% (Alan, 2003). This dramatic demand of sustained food production is needed to be achieved at a time when the world agriculture is facing the challenge of global climate change. Recent assessments suggest global climate change to increase crop yield at high and mid latitudes and reduce yield at lower latitude (Jan et al., 2009). It is also considered likely that frost and freezes may become more frequent in some areas and less frequent in other areas creating both windfalls and challenge for agriculture and natural flora and fauna (Alan, 2003). The abiotic stresses affect the plant cell in various aspects, disturbing their normal morphological built-up, physiological processes and molecular parameters. Numerous changes occur in plants some of which are built up of reactive oxygen species, changes in plasma membrane structure, up/down regulation of molecular modules etc (Levitt, 1972). Abiotic stresses lead to dehydration or osmotic stress through reduced...
availability of water for vital cellular functions and maintenance of turgor pressure. Stomata closure, reduced supply of CO₂ and slower rate of biochemical reactions during prolonged periods of dehydration, high light intensity, low temperature all lead to high production of reactive oxygen intermediates (ROI) in chloroplasts causing irreversible damage to cell and photo inhibition also (Arora et al., 2002). The high influxes and absorption of UV-B radiation affects terrestrial plants through damage to DNA directly or indirectly via formation of free radicals; damage to membranes by peroxidation of unsaturated fatty acids, PS II, phytohormones and even symbiotic relationship of plants with microorganisms. A number of secondary metabolites such as flavonoids, tannins and lignins are increased at elevate levels of UV-B radiation which screen UV-B and protect the cellular components against the UV-B damage. The ability of a plant to scavenge toxic oxygen species is considered to be critical for abiotic stress tolerance. Hence, the genes for superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase all contribute to tolerance of abiotic stresses (Fig. 1).

**Cold stress and signal transduction cascade:** Among other abiotic stresses, low temperature stress is an important factor limiting the production and geographic distribution of many horticultural species. Even in areas considered suitable for the cultivation of given species, decreases in yields and crop failures frequently occur as a result of aberrant chilling temperature. Plants vary greatly in their ability to withstand low temperature stress. As an adaptation strategy, most plants native to the temperature climates develop chilling tolerance after prior exposure to cold, non-freezing temperatures, a phenomenon called acclimatization (Xin and Browse, 2000). Many biochemical and physiological changes are known to occur during cold acclimation. With the onset of low temperature, putative temperature sensors at the cellular membrane generates stress signals which are transmitted and amplified through many steps that include Ca²⁺ signaling and a stepwise kinase/phosphatase chain reaction termed the kinase cascade (Fig. 2). The message 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. The plants response to cold acclimation is clearly complex and diverse, and the actual biochemical and physiological changes are still poorly understood at the molecular level (Knight and Knight, 2001).

**Mode of action of cold tolerant genes during cold stress:** When plants are exposed to freezing temperature, ice crystals are formed within the extra cellular spaces. The growth of the ice crystals is closely related to the degree of freezing damage, which causes freeze-induced cell dehydration and formation of several different lesions in the plasma membrane most often near the chloroplast envelope in the cell. In non-acclimated leaves of herbaceous species, these lesions are the result of lamellar-to-hexagonal II phase transitions in the molecular structure of the plasma membrane (Tomokazu et al., 2008). Freeze-induced formation of the hexagonal II phase disrupts both the physical continuity and semipermeable characteristics of the plasma membrane such that the cell becomes leaky and flaccid frequently results in loss of crops and significant loss of revenue for farmer. Under cold stress, there are several molecular and genetic changes that occur in plants for cold acclimation that make a plant freeze tolerant. During cold stress several genes expressed such as; genes controlling osmotic factors and other protectants; late embryogenesis abundant (LEA)-related genes; oxidative stress related genes; hormone regulating genes; genes encoding for molecular chaperones; genes encoding proton pumps, antiporters and ion transporters and others various regulatory genes that improved chilling tolerance of the plant (Yamaguchi-Shinozakai and Shinozakai, 2006; Thomashow, 1999).

Thomashow (1998) group recently provided the first direct evidence that expression of COR (cold related) genes are functionally involved in cold acclimation (Gilmour et al., 1998). Studies using NMR have shown that COR15A alters the intrinsic curvature of the inner membrane of the chloroplast envelope and thereby alters the phase transition of the membrane which decreases the propensity for freeze induced lesions. Yeh et al. (2000) also shown that antifreeze protein genes such as CLP, GLP, and TLP binds to an ice nucleator through hydrogen bonds prevents growth of ice-crystals and also reduces freezing damage. In addition, the gene for dehydrin5 (Dhn5) induced upon cold acclimation was shown to be present in membranes adjacent to apical meristem and not able to supply water to form ice crystal in extra cellular spaces (Danyluk, 1994). Another cold inducible gene, bit 101 was cloned and it was shown to be present in vascular transition zone of crown and closely related to survival of plant during winter. Transformation of Arabidopsis with the Cod A gene for choline oxidase enhances freezing tolerance of plant (Sakamoto, 2000; Seki et al., 2001). Ectopic expression of ABI3

![Fig. 1: Cold acclimation induces many cellular changes in plants.](image)
gene also enhances freezing tolerance in response to ABA and low temperature in *Arabidopsis thaliana* (Tamminen, 2001). Glycerol-3-phosphate acyltransferase (GPAT) gene (Gupta et al., 2009) encoded a protein that increases the unsaturation of fatty acids in phosphatidylglycerol from leaves and improved chilling tolerance (Na et al., 2007). Besides genes, several transcription factors such as SCOF-1 and osCDPK7 are also involved in the induction of cold tolerant genes. Over expression of a transcription factor (CBF1) that binds specifically to the “CRT/DRE element” in the promoter of cold inducible gene (Stockinger...
et al., 1997; Chen et al., 2002). DREB genes encode transcription activators that function in drought, high salt and cold responsive gene expression (Dubozet, 2003). Research has been conducted in over expressing a fusion of DRE-containing promoter from a dehydration induced gene (rd29A) with a DREB gene in Arabidopsis that resulted in tolerance of plants to freezing, water stress and salinity (Agrawal et al., 2006; Kasuga et al. 1999). In short, there are numerous genes now available that can help tolerate cold. Yu and Griffith (1999) cloned an antifreeze gene from winter ryegrass.

**Seabuckthorn: An ideal cold tolerant model plant for cold tolerant gene isolation:** The primary objective of our research is to identify and isolate cold inducible genes that contribute towards freeze tolerance. To accomplish our objective, we have to investigate and select an extremely freeze tolerant species to be used for isolating cold tolerant gene(s). There are several species, which grow very widely in cold Himalayan region such as seabuckthorn, Ephedra, Cornus sericea that can survive even at –196°C (Parsons, 1991) and we believe that investigation on one of the most freeze tolerant organism will help us to identify genes that facilitate survival of many horticultural crops at low temperature. To accomplish our objective, we have selected Seabuckthorn as model plant for isolating cold tolerant gene(s) (Fig. 3). Seabuckthorn (Hippophae salicifolia L.) is a hardy, deciduous shrub of the mountainous region of China and Russia with wide but fragmented distribution in Eurasia between 27 and 69°N latitude and 7°W and 122°E longitude. Seabuckthorn (Hippophae salicifolia L.) plant belongs to Eleagnaceae family. The plant grows naturally in sandy soil at an altitude of 1200-4500 meters (4000-14,000 feet) in cold climates. It can withstand temperatures from -43 °C to +40°C and is considered to be drought resistant (Lu, 1992; Yao and Tigerstedt, 1995). It is an ideal plant for soil erosion control, land reclamation, wildlife habitat enhancement and farm stand protection (Schroeder et al., 1996). The name Hippophae means shiny horses and refers to the good coat development by horses feeding off the plant. Seabuckthorn has turned out to be useful because it withstands severe weather and grows huge root system in poor soil (and fixes nitrogen in the soil).

Seabuckthorn has been reported to contain more than 190 compounds in the seeds; pulp, fruit and juice. These compounds include fat-soluble vitamins (A, K, E), 22 fatty acids, 42 lipids, organic acids, amino acids, carbohydrates, vitamin C, vitamin B1, vitamin B2, folic acid, tocopherols, and flavonoids, phenols, terpenes and tannins. It also contains 20 mineral elements. Many of the substances that are found in Seabuckthorn are known to have beneficial effects on health. The high level of unsaturated fatty acids (i.e. linolenic acid, linoleic acids) makes Seabuckthorn seeds appropriate for decrease of risk heart disease. Antioxidants (Vitamin A, C, E) also got to reduce the risk of heart disease by preventing the oxidation of LDL cholesterol. It is proposed that the linolenic acid present in the Seabuckthorn may have useful role in treating disorders in which the immune response is hyper stimulated i.e. rheumatoid, arthritis, psoriasis multiple sclerosis and systemic lupus. Vitamin C has been shown to improve immune status in humans. Since the 1950’s many medicinal preparations of Seabuckthorn from both wild and cultivated materials, has been clinically used to treat radiation damage, burns oral inflammation and gastric ulcers in China and former Soviet Republics. It is an ingredient in sun block-Hippophae oil has UV-blocking activity as well as aid in promoting regeneration of tissues. It has many diverse uses such as fodder, nutritious foods, drugs and skin care products. The Seabuckthorn is highly stress-resistant as it contains vitamin C, E, β-carotene, phytosterols, unsaturated fatty acids and flavonoids and it also serves as an anti-oxidant that slows the ageing process and improves memory.

**Genes responsible for stability of plasma membrane during cold stress:** The plasma membrane is considered to be the primary site of injuries because of freezes induced dehydration during freezing injury (Steponkus et al., 1998). It is necessary that plasma membrane increases its cryostability during freeze thaw excursions. During cold acclimation under normal and artificial conditions there are compositional, structural and functional changes occur in plasma membrane many, if not all, of which contribute to increased stability of the membrane under freezing conditions. Cold acclimation results in many changes in lipid composition of plasma membrane suggesting that these changes are associated with occurrence of specific freeze induced lesions associated with the plasma membrane (Nishida and Murata, 1996; Tomokazu et al., 2008). The most notable changes in lipid composition is an increase in proportion of phospholipids which is observed in a wide range of plants systems from monocots to dicots and from herbs to woody plants. The increase in phospholipids in plasma membrane during cold acclimation occurs at the early stage of cold acclimation when a decrease in cerebrosides occurs gradually throughout the acclimation process. The increase in phospholipids is primarily a result of increase in proportion of unsaturated molecular species of phosphatidyl choline and phosphatidyl ethanolamine, two phospholipids classes in the plasma membrane. In addition there is a decrease in proportion of cerebrosides over a wide range of plants. Also, certain cold responsive plasma membrane proteins affect cold acclimation process to increase freezing tolerance. The outer membrane lipoprotein like protein (lipocalin like protein from Arabidopsis-AtLCN) is a protein that increases substantially during cold acclimation cold induced expression of a lipocalin like protein occurs both in monocots and dicots, suggesting a cryoprotective role of LPCP under freeze induced dehydration. AtLCN seems somehow to change the cryobehaviour of plasma membrane under freeze induced osmotic dehydration such that plasma membrane minimizes the probability of formation of endocytic vesicles. There is possibility that AtLCN may affect cryostability of plasma membrane through an interaction with lipid components of plasma membrane. Another cold responsive plasma protein ERD14 (Early responsive to dehydration protein 14) seems to affect cryobehaviour of plasma membrane differently, although the mechanism is not clear. It is

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Fig. 4: CLUSTAL multiple alignment of the deduced amino acid sequence of GPAT gene (Gupta et al., 2009)
hypothesized that ERD14 may minimize the penetration of ice from outside protoplasm by altering characteristics of plasma membrane during freezing. In any case the plasma membrane is the primary site of injury on subjecting plant cells to cold stress. Several genes for change in membrane components are identified which on presence in plant genome may strengthen the cryostability of plasma membrane such as COR15a (Steponkus et al., 1998); dehydrin ERD10; glutathione-S-transferase; phospholipase D delta; carbonic anhydrase; glycerol-3-phosphate acyltransferase (Gupta et al., 2009) and more many.

Role of glycerol-3-phosphate acyltransferase (GPAT) in cold tolerance: The biosynthesis of phosphatidylglycerol represents a central pathway in lipid metabolism in all organisms (Xu et al., 2006). Acyl-CoA: glycerol-3-phosphate acyltransferase (GPAT) catalyzes the first step during de novo synthesis of triacylglycerol (Cao et al., 2006) and, therefore, it is a potential site for triacylglycerol synthesis regulation (Roy et al., 2006). Glycerolipids form bilayers and provide the necessary background for the functioning of membrane proteins. The physical properties of glycerolipids depends on the degree of unsaturation of the fatty acids that are esterified to the glycerol backbone of the lipids and, consequently, the molecular motion of these glycerolipids is affected by alterations in the extent of unsaturation of fatty acids.

Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the transfer of an acyl group from an acyl donor to the sn-1 position of glycerol 3-phosphate. The plant cell contains three types of GPAT, which are located in the chloroplasts, mitochondria and cytoplasm, respectively. The enzyme in chloroplasts is soluble and uses acyl-(acyl-carrier protein) as the acyl donor, whereas the enzymes in the mitochondria and the cytoplasm are bound to membranes and use acyl-CoA as the acyl donor. cDNAs for GPAT of chloroplasts have been cloned from several plants such as rice (Chen et al., 1998), vicia faba (Liu et al., 1999), pea (Weber et al., 1991) and many more. The amino acid sequences deduced from the nucleotide sequences of cDNAs indicate that the product of translation is a precursor of about 460 amino acid residues, which consists of a leader sequence of about 70 amino acid residues and a mature protein of about 400 residues, with a molecular mass of about 42 kDa. Genetic engineering of the unsaturation of fatty acids has been achieved by manipulation of the cDNA for the GPAT found in chloroplasts and has allowed modification of the ability of tobacco to tolerate chilling temperatures (Murata and Tasaka, 1997). Moon et al. (1995) have demonstrated that chilling tolerance is affected by the levels of unsaturated membrane lipids in tobacco plants that had been transformed with the cDNA for glycerol-3-phosphate acyltransferase. It proves that unsaturation of fatty acids of phosphatidylglycerol in thylakoid membranes stabilizes the photosynthetic machinery against low-temperature photoinhibition by accelerating the recovery of the photosystem II protein complex (Yan et al., 2008). In addition to cold tolerance, GPAT gene also has other roles such as enhancement of seed oil content; tapetum differentiation and male fertility (Zhifu et al., 2003); morphological and metabolic changes in transgenic wheat with altered glycerol-3-phosphate acyltransferase and synthesis of suberin in seed coat and root of Arabidopsis that act as pathogen barriers and function in the control of water and solute transport.

We can postulate, therefore, that changes in the unsaturation of fatty acids should affect various functions of the membrane-bound proteins, such as the photochemical and electron-transport reactions in thylakoid membranes, and the import and export of metabolites and proteins across the plasma membrane. However, the contribution of the unsaturation of fatty acids to cold tolerance has not been obvious, since acclimatization to low temperature induces not only desaturation of fatty acids of membrane lipids but also a number of other metabolic modifications. To determine whether the unsaturation of fatty acids contributes to the ability to tolerate low temperature, it is necessary to alter the extent of unsaturation of fatty acids of glycerolipids exclusively by manipulation of genes for fatty-acyids desaturase, thereby minimizing effects on any other metabolic processes (Norio and Hajime, 1995).

Isolation and cloning of GPAT gene from seabuckthorn plant: We have designed degenerate primers from the conserved regions of the known GPAT gene sequences present in the database. RT-PCR of RNA isolated from sea buckthorn leaves resulted in the amplification of cDNA fragment of GPAT gene. This cDNA fragment was cloned at Smal site of pUC-19 vector. Sequence analysis revealed that, GPAT (689 bp, Acc No: EU081817) shows 97% homology with the Lycopersicon esculentum at nucleotide level and 93% homology with the Capsicum annuum at protein level (Gupta et al., 2009) (Fig. 4). cDNA sequence of isolated GPAT gene will be used for cloning in suitable plant transformation vector for genetic transformation of vegetable crops. The successful transformed crops can then fruit well and give higher yield. Transformed crops also produce vegetable under extreme cold conditioned in order to support logistics of defense forces deployed in high mountain regions of Central Himalayas with respect to fresh vegetable. In addition, due to severe cold during winter season in high altitude areas, there is mono cropping pattern of selected crops. Army deployed in these areas and the inhabitants of the region need vegetables regularly, which are transported from distant places involving heavy expenditure. Also there is remarkable nutrient loss in the transportation. Vegetable crop transformed with cold tolerant gene will involving one time investment and help arrangement of the availability of nutrient rich fresh food even during the winter months through local production when it is not possible to get fresh vegetables due to road breaches. This study is likely to provide new approach in biotechnological to prevent the damage to crops due to unexpected / expected cold snaps.

Conclusion

The high altitude regions have a very harsh climate and a short agriculture season. Only a few vegetable crops viz., radish,
turnip, potato, cabbage and some leafy vegetables are grown traditionally, in these areas. The climatic and geographic differentiation segregates the region from rest of the world. Therefore, the agro-techniques for the vegetable production being employed elsewhere are not suitable for these conditions and specific agro-techniques and vegetable varieties are required for these regions. Lots of works has already been done to identify the suitable varieties/hybrids and cultivation practices for these regions. The transgenic technology is highly precise and beneficial for high altitude farming. The major limitation is the complexity of tolerance to abiotic stresses that is normally dependent on a number of physiological traits, each under multigenic control. The increased wealth of knowledge that is being acquired by means of genomic and other molecular biology studies and the cloned genes will certainly contribute to the development of tolerant genotypes. The successful integration of the cold tolerance genes into high value vegetable crops would increase productivity and production in high altitude regions. The transgenics having cold resistance gene may not only bring additional areas under cultivation but also help in optimizing productivity in high altitude and remote areas without any additional cost will enable or extend the cultivation of crops in abiotic stress, which are presently not under cultivation or are less productive.

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