**Biotechnological Approaches for the Development of Salt and Cold Tolerance in Crop Plant**

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3. **Introduction**

The primary target of genomics is identification and characterization of genes and gene products. Starting point of genomics may be a whole genome sequence, from which gene and protein structures can be predicted by computational and bioinformatics approaches or genes can be identified step starting from identification of a phenotype by induction and detection of mutation, construction of a genetic map demarcating the position of the character, physical location, identification of the gene, cloning and characterization of the gene or genome sequence by sequencing followed by expression analysis to correlate the phenotypic expression. In genomics, Transcriptomics encompasses a number of technologies developed to enable the genome-wide analysis of gene expression patterns at the level of the m-RNA population is called transcriptome. These are high throughput technologies designed to measure global changes in gene expression profiles in different tissue or in response to different conditions or treatments. The detection of groups of genes that show altered expression patterns under specific experimental conditions or at particular developmental stages provides a means of identifying likely multi gene clusters involved in a particular response or developmental stage. Thus, the technology is one of the most powerful tools for gene discovery. However, it has wider application in the development of Tolerance crop. In Transcriptomics some technologies are used for the analysis of gene expression like Expressed sequence tag collections (EST), Serial analysis of gene expression (SAGE), Microarrays and Gene chips and massively parallel signature sequencing. This chapter will consider a variety of way in which productivity of crop plants can be improved by enhancing their ability to tolerant salt and cold stresses. This chapter will also look at the number of example of how crop productivity can be genetically enhanced through this technology.

1. **Tolerance to salt stress**

Tolerance to salt stress, also known as salinity tolerance, is the ability of an organism, particularly plants, to survive and grow under conditions of elevated soil salinity. High levels of salt in the soil can be detrimental to plants, as it disrupts water uptake and ion balance, leading to cellular damage and reduced growth. Salt stress is a significant abiotic stress factor that affects agriculture, particularly in regions with poor drainage or where irrigation water has a high salt content.

Improving salt stress tolerance in crops is of great importance for sustainable agriculture in salt-affected regions. Plant breeding programs and biotechnological approaches, such as genetic engineering and marker-assisted selection, are being employed to develop salt-tolerant crop varieties that can thrive in saline soils and contribute to food security in challenging environments.

Salt tolerance is the difference in how different genotypes of a species respond to the same tissue salt concentration in terms of various life processes. There is a lot of evidence to suggest that genotypes can tolerate different amounts of salt in their tissues. However, halophytes' cellular and enzyme functions are just as salt-sensitive as glycophytes'. When saline fields are reclaimed by applying gypsum, the majority of crops are planted there, but yields are often low in the first three to four years. Therefore, boosting crops' resistance to salt may significantly aid in raising food output for India's expanding population. Therefore, in addition to lowering the need for input in the form of chemical amendment, the growth of salt-tolerant types can play a significant role in the rehabilitation of such areas.

**2.1 Salt tolerance mechanism at physiological levels**

Salt tolerance mechanisms at the physiological level in plants involve various adaptations that help maintain cellular homeostasis, water balance, and overall plant function under high salinity conditions. These mechanisms aim to minimize the toxic effects of excess salts (sodium and chloride ions) and osmotic stress caused by the imbalance of water and ions. Salt-tolerant plants transport excess salts to specific cellular compartments, such as the vacuoles, where they are sequestered and stored, reducing their harmful effects on essential cellular processes. This compartmentalization prevents the accumulation of toxic ions in the cytoplasm and maintains ion homeostasis. High salt concentrations in the soil create an osmotic imbalance, leading to water loss from the plant cells. Salt-tolerant plants counteract this osmotic stress by accumulating compatible solutes or osmolytes, such as proline, betaine, and soluble sugars, which help maintain cellular turgor and retain water within the cells. Salt stress can lead to the generation of reactive oxygen species (ROS), causing oxidative damage to cellular components. Salt-tolerant plants possess a robust antioxidant defense system, including enzymes like superoxide dismutase, catalase, and peroxidases, which scavenge ROS and protect cells from oxidative stress. Some salt-tolerant plants can transport potassium (K+) ions preferentially over sodium ions into the root cells. This helps maintain a favorable potassium-to-sodium ratio, which is crucial for cellular function and osmotic balance. Plant hormones, such as abscisic acid (ABA), play a role in regulating responses to salt stress. ABA helps in stomatal closure to reduce water loss and initiates the expression of stress-responsive genes.

These physiological mechanisms collectively contribute to the salt tolerance of plants, enabling them to adapt and survive in saline environments. Understanding and harnessing these mechanisms through breeding and biotechnological approaches can lead to the development of salt-tolerant crop varieties that can thrive in salt-affected soils and contribute to sustainable agriculture in challenging environments.

As significant sections of irrigated land are affected by the buildup of salt, salt tolerance is increasingly becoming a major goal for crop improvement. Additionally, the need for land has necessitated thinking about the possibilities of cultivating crops in more salty circumstances with lower quality water. Osmotic stress is caused by salty circumstances, which inhibit water intake by roots and water outflow from cells. However, by impeding protein synthesis, photosynthesis, and enzymes that are vulnerable, the buildup of Na+ and Cl ions in the cytoplasm may potentially have direct harmful effects (Estan, M.T. et al., 2005). As a result, methods for engineering water stress tolerance through the generation of suitable solutes may offer defence against the osmotic effect of salinity but not against ion toxicity. There might also be a need for further strategies to reduce the hazardous effects of particular ions. By contrasting the responses to salt stress of plants that are susceptible to high salt circumstances (glycophytes) with those of plants that can tolerate high salt conditions (halophytes), strategies for engineering salt tolerance have been devised. Typically, glycopytes accumulate osmoprotectants in response to salt stress, whereas halophytes use particular methods to counteract the harmful effects of Na+ and Cl- ions. However, it is more typical for plants to regulate Na+ ions out of the cell, whereas other cells with big vacuoles may act as sinks for the buildup of excess sodium through transport into the vacuole. Some halophytes actually excrete salt through specialised glands on their leaf surfaces. Intense research efforts have concentrated on understanding the physiological basis of tolerance in higher plants (Cuartero, J., et al., 2008; Afzal et al., 2022a and 2022b). Tolerance to salt stress is a complex process at both the entire plant level and the cellular level. For the selection of high tolerant cultivars, certain physiological markers that are extremely specialised for saline conditions were identified and used often. These factors included low Na+/K+ ratio, preferential Na+ accumulation in older leaves, strong Cl- uptake, low K+ uptake, and Na+ transport to shoot (Sharma and Goyal, 2003).

One way to improve salt tolerance is to mimic the mechanisms halophytes utilise to move Na+ ions out of the cytoplasm. It is vital to take into account the mechanisms of ion transport out of the cytoplasm in order to put this into practise. The initial transgenic studies looked at Na+ ion transport into the vacuole. This transport requires energy input because it is working against a concentration gradient. To do this, a proton pump that transports H+ ions in the opposite direction is coupled to the transport protein. It is well known that the vacuolar Na+/H+ antiport protein AtNHX of Arabidopsis couples to proton pumps such AVP1, a vacuolar H+ translocating pyrophosphatase. AtNHX1 and AVP1 have been compared as a rotating door and an energy source for the door, respectively. Therefore, to improve the flow of traffic over the membrane, one may either add additional doors or give the current doors more energy to spin more quickly. The first method, transformation with the Arabidopsis AtNHX1 antiport protein gene, was successful in engineering salt tolerance in tomato plants (Yokoi et al., 2002). NaCl caused a rise in AtNHX1 steady state transcript levels, indicating that osmotic stress is more common than ionic stress in the up-regulation of AtNHX1 transcripts (Zhu 2002). To increase salt tolerance, NHX antiporters have been highly expressed (Wu et al., 2004). When salt tolerance has been studied, the Arabidopsis gene has also been introduced into maize and wheat plants, resulting in better grain yields and improved salt tolerance. For instance, rice modified with the OsNHX1 gene demonstrated enhanced growth and buildup of biomass when exposed to salt stress. (Wu *et al.,* 2005, Chen *et al.,* 2007).

Recent studies have looked into the consequences of adding a plasma membrane Na+/H+ antiporter to expel Na+ ions from plant cells. In transgenic Arabidopsis, the AtSOS1 gene was overexpressed, which increased salt tolerance in callus cultures and decreased Na+ ion levels in the xylem of transgenic plants (shi et al., 2003). Seed germination and seedling salt tolerance were both increased by Fission Yeast SOD2 gene expression in Arabidopsis (Zhao et al., 2006). Rice that has been modified to have the E. coli nhaA plasma membrane Na+/H+ antiporter grew and produced more under salt- and drought-stressed conditions (Wu et al., 2005). It's interesting to note that nhaA expression also led to higher proline levels in the transgenic rice, which suggests that improved osmoregulation may result from proline production being activated in plat.

In order to improve the vacuole's proton pumping potential and subsequently its capacity to transport sodium, it has been proposed to overexpress the gene encoding AVP1, which was first tested in Arabidopsis (Gaxiola et al., 2001). Since the changed ion balance allowed the plants to retain more water, this has improved the experimental plants' resistance to both drought and salt. Similar outcomes have been attained by overexpressing the homologues from Triticum aestivum (TVP1) and Thellungiella halophila (TsVP) in tobacco and Arabidopsis, respectively (Gao et al., 2006; Brini et al., 2007).

**2.2 QTL mapping for salt tolerance**

Therefore, a QTL is a region of a chromosome where there is thought to be a fair chance that functionally distinct alleles would segregate and have a big impact on a quantitative characteristic. A statistical analysis of molecular marker and phenotypic data from a large segregating population is necessary for QTL mapping in order to identify the markers where allelic polymorphism correlates with the quantitative trait phenotype.

Quantitative trait loci are chromosomal regions that are related with specific quantitative traits, such as salt tolerance, and can be found using molecular technology. The QTL for salt damage at early embryo stage in rice has only been documented by a few number of investigators (Prasad et al., 2000).

1. **Tolerance to cold stress**

The capacity of various plants to tolerate cold and freezing temperatures varies greatly (Fig. 1). The majority of tropical plants can not withstand frigid temperatures. On the other hand, depending on the species, any temperature plants can tolerate a range of subfreezing temperatures from -5 to -300C. Even lower stand temperatures are frequently encountered by plants from colder climates. It is well recognised that if plants first experience a period of cold acclimation, at a low but non-freezing temperature, they will be better equipped to handle cold or freezing stress. For instance, wheat plants growing at typical mild temperatures die from freezing at -50C, but after a time of acclimatisation to the cold, when the plant develops at temperatures below 100C, they can survive freezing temperatures as low as -200C.

Different plants have different tolerances to cold or freezing temperatures, and plant breeders have been selectively breeding for this feature for ages. However, traditional breeding has not significantly improved the major crop species' ability to withstand cold over the past 20 years, which has led to the quest for molecular remedies to this issue.



**Figure: 1 Cold stress response in Plant**

Studying the mechanisms that some plant species use to survive freezing has been one strategy. Plants release a number of cold-induced proteins during the acclimatisation stage that are thought to contribute to the development of cold resistance. In various plant species, about 50 cold-induced proteins have been discovered. These can be divided into a small number of categories, but they all have the quality of being very hydrophilic in common. Numerous them also feature repeating themes in their comparatively straightforward amino acid compositions. These families include proteins known as late embryogenesis abundant (LAE) proteins, which seem to act as a barrier against damage during seed desiccation. According to their patterns of expression, a class of genes known as cold responsive genes encodes additional groups of proteins. Although the precise role of these cold-induced genes is unknown, it has been hypothesised that they may directly increase freezing tolerance by reducing the potentially harmful consequences of dehydration brought on by freezing. Therefore, a potential method for targeted engineering of cold or freezing stress tolerance is the overexpression or ectopic production of this cold-induced protein.

There are various examples of transgenic plants expressing cold-induced proteins. For instance, the freezing endurance of chloroplast frozen in situ ad or protoplast frozen in vitro was increased by constitutive expression of the small, hydrophilic, chloroplast specific COR protein COR 15a in Arabidopsis. The survival of frozen plants, however, is unaffected by COR 15a expression. This observation can be explained by the fact that all of the cold-induced proteins are necessary to fully protect the cell and that they may be directed towards variously vulnerable cell components. It follows that many COR genes would need to be added to a transgenic crop in order to significantly improve cold tolerance.

Following the discovery that several different cold tolerance-related genes contain a similar regulatory element in their promoter—the C-repeat (CRT) element/dehydration responsive element (DRE)/low temperature response element (LTRE)—one solution to the problem of engineering a multigene trait has emerged. It has also been discovered that the transcription factor CBF1 binds to the CRT/DRE/LTRE region, promoting the expression of the genes that make up the COR regulon. In order to induce this complete collection of COR cold tolerance genes, the technique is to overexpress the CBF1 gene. There have been created transgenic Arabidopsis plants containing a 35S promoter, CBF1 gene construct. These plants have been proven to be freezing tolerant without prior cold acclimation and to express a number of COR genes. Transgenic plants overexpressing the COR15a protein, used as a control, were discovered to be less freeze resistant than the CBF1 plants. In a comparable experiment, it was shown that various stress responses are interconnected. Plants with increased tolerance for drought, salt, and frost were produced when the expression of a CBFF1 homologue, the DRE binding protein DREB1A, was regulated by a stress-induced promoter in transgenic Arabidopsis.

**3.1 Transcriptional regulation by low temperature signaling pathway**

The transcriptional regulation of genes in response to low temperature, or cold, is mediated by a complex signaling pathway known as the cold signaling pathway. This pathway allows plants to sense changes in temperature and activate specific transcription factors and other regulatory proteins that control the expression of cold-responsive genes. The activation of these genes helps the plant acclimate to cold conditions and enhances its cold tolerance.

The promoters of some genes that are susceptible to cold contain the CRT sequence element, which is bound by transcription factors from the CBF family and activates transcription. Therefore, over-expression of one CBF gene causes the production of several genes that are susceptible to cold.

However, not all cold-induced genes have the CCGAC element in their promoter, despite the fact that low temperature-induced gene expression, controlled by the CRT element, appears to be highly preserved in plants. Figure 2 depicts the pathway of low temperature-induced gene expression. This pathway demonstrates how a streamlined signal transduction cascade controls a stress-induced gene's promoter elements. This diagram makes it obvious that the CRT element responds to two signalling channels. These signal transduction pathways, which are ABA independent, are ICE1 and ICE-like protein. The CBF gene is activated by ABA independent signalling. The basic zipper proteins AREB/ABF and the MYC/MYB transcription factors, which cooperatively bind to the MYCR/MYBR regions found in several ABA responsive genes, are two further sets of transcription factors that ABA also promotes the activation.



**Fig: 2 Signal transduction pathway for the regulation of cold stress response gene**

**Expression**

**3.2 Cold responsive gene regulation**

Cold-responsive gene regulation involves the activation or repression of specific genes in response to low temperature (cold) stress in plants. This regulatory process is crucial for plants to adapt and survive under cold conditions and involves various molecular mechanisms. The process begins with the perception of low temperature by temperature sensors or receptors in the plant cells. The mechanisms of temperature sensing are not fully understood, but changes in membrane fluidity and protein conformation are believed to play a role.

Cold stress triggers a cascade of signaling events that ultimately lead to the activation of specific transcription factors. One of the key transcription factor families involved in cold response is the CBF/DREB1 (C-repeat-binding factor/dehydration-responsive element-binding protein 1) family. These transcription factors become activated upon exposure to low temperature and bind to specific DNA sequences called C-repeat/dehydration-responsive elements (CRT/DRE) in the promoters of cold-responsive genes.

Once activated, CBF/DREB1 transcription factors bind to CRT/DRE elements in the promoters of cold-responsive genes. This binding activates the transcription of these genes, initiating the synthesis of cold-responsive gene products. The activated transcription factors, such as CBF/DREB1, induce the expression of a set of cold-responsive genes. These genes encode various cold tolerance proteins, including Cold-Regulated (COR) proteins, Dehydrins, and Late Embryogenesis Abundant (LEA) proteins. The products of cold-responsive genes, such as COR proteins, Dehydrins, and LEA proteins, are essential for protecting plant cells from cold-induced damage. They stabilize cellular structures, prevent ice crystal formation, and maintain cellular function under cold stress.

The promoter sequences of various cold-induced COR genes have been characterised and compared. A common regulatory region called the C-repeat (CRT) or low temperature response element (LTRE), which is five nucleotides long and has a consensus sequence of CCGAC, is shared by the promoters of numerous distinct COR genes. This feature, known as the dehydration responsive element (DRE), had already been connected to drought resistance. The CBF transcription factor CBF1 binds to the CRT/LTRE/DRE. The nuclear localization sequence, DNA binding domain, and an acidic region that may be involved in interactions with other proteins are all depicted in the structure of CBF1 in Figure 3. Cold acclimation causes CBF1 expression, which then causes the expression of the COR genes. The COR regulon has been used to refer to this collection of genes that share a common regulation mechanism.

*CBF1* is a member of a small gene family; *CBF2* and *CBF3* and also transcription factors, and expression of all three *CBF* genes is induced rapidly by low temperatures. In addition, *CBF3* over expression results in several biochemical changes associated with cold acclimation, such as elevated levels of compatible osmolyte, proline and soluble sugar.

However, not all cold-induced genes have the CCGAC element in their promoters, despite the fact that low temperature-induced gene expression, mediated by the CRT element, appears to be highly preserved in plants. Plants appear to have additional low temperature gene expression pathways that are not regulated by CRT/CBF, and the sequence element CCGAAA has been shown to confer low temperature inducibility on some genes. 

**Fig: 3 Number of cold response genes contain the *DRE* element in their promoters that is bound by transcription of the *CBF* family that activates the transcription overexpression of a single *CBF* gene induces the several cold responsive gens. NLS (Nuclear localization signal)**

Numerous genes that are induced by various conditions have been discovered through genetic engineering and molecular research. Numerous functional protein-coding stress inducible genes have been employed to increase stress tolerance. Several reviews have been published for the stress tolerance (Christensen and Feldmann 2007; Umezawa *et al.* 2006, Valliyodan and Nguyen 2006, Ahmed 2021). Transgenic plants that can withstand low temperatures have been created using a variety of transcriptional activators, including DREB1/CBF (Kasuga et al. 1999; Liu et al. 1998; kreps, 2002) that trigger the stress sensitive genes (Zhang, 2003). In many plant species, including rice (Dubouzet et al., 2003; Ito et al., 2006), pepper (Hwang et al., 2005), chickpea (mantra et al., 2007), and potato (Rensink et al., 2005), the DREB/CBF genes have been successfully exploited to develop low temperature stress tolerance.

A crucial first step is to look for genes related to cold tolerance. Numerous experiments have been conducted to determine how well plants can withstand cold stress. The C-repeat element and low temperature responsive element are common regulatory elements found in the promoters of all genes associated to cold tolerance. (Table 1) provides a list of genes and transcription factors that are enhancing cold tolerance in various plants.

**Table 1 List of plant genes conferring increased cold tolerance.**

|  |  |  |  |
| --- | --- | --- | --- |
| **Genes** | **Plant** | **Mode of action** | **References** |
| *SICZFP1* | *Arabidopsis* | Regulating cold responsive gene | Zhang, X., *et al* (2010) |
| *OrbHLH001* | *Arabidopsis* | Involved in metabolic regulation or ionic homeostasis pathways in stress | Li,F., *et al* (2010) |
| *Osmyb4* | *Osteosermum ecklonis, apple, Arabidopsis* | Transcription factor | Laura, M., *et al* (2010) |
| *OsLTP* | *Phalaenopsis amabilis* | Increased accumulation of total soluble sugar, proline, antioxidant superoxide dismutase | Qin,X., *et al* (2011) |
| *OsSPX1* | *Arabidopsis and tobacco* | Accumulation of proline and sugar | Zhao,L., *et al* (2009) |
| *OsDREB1D* | *Arabidopsis* | Transcription factor | Zhang,Y., *et al*(2009) |
| *OsiSAP8* | *Tobacco* | Cytoplasmic zinc finger protein that is involved in the signal transduction | Kanneganti, V., and Gupta, A.K.,(2008) |
| *OsDREB1F* | *Arabidopsis* | Transcription factor | Wang,Q., *et al* (2008) |
| *Cat* | *wheat* | Use for the catalase | Matsumura *et al*. (2002) |
| *GS2* | *Rice* | glutamine synthase | Hoshida *et al*. (2000) |
| *P35S-ZFP245* | *Rice* | Accumulation of proline, activation of the pyrroline5 arboxylatesyntetase and proline transporter genes, and enhancement of the ROS-scavenging enzymes | Huang.J., *et al*. (2009) |
| *OsP5CS2* | *Rice* | Accumulation of proline | Hur,J., *et al* (2004) |

1. **Conclusion:**

In this chapter we studied that the effect of environmental conditions such as cold and salinity on crop plants and also highlighted the importance of both stress in determining the large annual fluctuations in crop yield. Genomics, the study of structure and function of the genome is, therefore, a subject that extends genetics from mere phenotype analysis to holistic analysis of the whole genome. The science of genomics has immense implications in every sphere of biological science from evolution to enhance crop yield. In fact it is expanding so rapidly and generating new information every moment that a new data is generated and reported almost every minute.

The fundamental biology of plants under abiotic stress conditions is also understood via molecular genetics. The descriptive power of molecular analysis with the crop grown under stress circumstances, however, may be greatly increased using a novel method derived from functional genomics. The goal of molecular mapping is to create a map with a fine enough scale to identify the precise position of the genes crucial for defining key agronomic features.

In some cases, single-gene mechanisms for tolerating specific stresses can be deployed (e.g. salt stress and cold stress). The overall conclusion from this chapter is that, functional genomics play the key role for the identification of stress responsive gene, by which we can improve the quality and quantity of crop.

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