



## MOLECULAR BREEDING AND PHYSIOLOGICAL MECHANISM OF DEVELOPING SALT-TOLERANCE IN RICE PLANT (*Oryza Sativa* L)

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### ABSTRACT

Rice (*Oryza Sativa* L.) is an important staple crop that feeds more than one half of the world's population and is the model crop. Susceptibility or tolerance of rice plants to high salinity is a coordinated action of multiple stress-responsive genes, which also interacts with other components of stress signal transduction pathways. The salinity threshold for rice plants is 3 dS·m<sup>-1</sup> EC (electrical conductivity). Above this threshold, a 12% reduction in rice yield occurs if there is a 1 dS·m<sup>-1</sup>EC increase in salinity. Rice seedlings die at a salt level corresponding to 10 dS·m<sup>-1</sup>, and yield loss can be as high as 90% if the level reaches 3.5 dS·m<sup>-1</sup> during the reproductive stage. One way to address the salinity issue is to develop tolerant rice varieties using conventional, modern breeding techniques, and through genetic engineering by introducing salt-tolerance genes. Despite many attempts using different strategies to improve salinity tolerance in rice, the achievements so far are quite modest. This review aims to discuss challenges that hinder the improvement of salinity stress tolerance in rice as well as potential opportunities for enhancing salinity stress tolerance in rice.

**Keywords:** *Breeding, Physiology, Salinity, Tolerant, Rice.*

### INTRODUCTION

Salinity stress drastically reduces growth and productivity of glycophyte, which is the majority of agricultural products. The term "salinity" represents all the problems of the soil accumulating excessive salts, which can be categorized into sodic (or alkaline) and saline soils (IRRI 2011). Sodic soils having a poor soil structure generally spread over arid and semi-arid regions, retaining high concentrations of Na<sup>+</sup> at the exchangeable site of clay particles in the soil, which shows high pH (greater than 8.5) with a high exchangeable sodium percentage (ESP>15) (IRRI 2011). Saline soils can be generally found in arid regions, estuaries, and coastal fringes, which are dominated by Na<sup>+</sup> ions with electrical conductivity (EC) more than 4 dS/m that corresponds to approximately 40 mMNaCl (IRRI 2011). Moreover, saline soils exhibit ESP of <15 and much lower pH values than the sodic soils (IRRI 2011). With more than 830 million hectares (ha) of salt-affected land globally (FAO, 1988, Munns, 2005). And approximately two million ha of land uncultivable due to excessive salinity added each year (Umali, D.L 2013). (Figure1) salinity is a worldwide predicament and it can occur naturally. However, the human intrusions have disturbed natural ecosystems, by changing the hydrology of the landscape and significantly accelerate the salinization of land and waterways. Irrigation and extensive clearing of vegetation, which bring the

groundwater with soluble salts too, or close to, the soil surface, are the two major human activities that accelerate salinity. Of the 230 million ha of the world's irrigated land, 45 million ha (20%) has been salt-affected (Cominelli et al., 2013). When growing on salt-affected soils, crops must compete with salts in the soils for water as well as to cope with ion toxicification, nutritional disorders and poor soil physical conditions to survive, therefore, their productivity was reduced (Shrivastava and Kumar, 2015; Munns and Tester, 2008 ). By 2050, the world's population is predicted to reach 9.6 billion people and food production needs to increase approximately 70% by 2050 or 44 million metric tons annually to provide sufficient food for this population (FAO. 2009, UN. 2016). This is a challenge because there is very little potential for future expansion of arable lands, whereas environmental stresses affecting crop production are increasing (Tester, M *et al.*, 2010, Cominelli *et al.*, 2013 ) Salinity and drought were listed as the two most serious factors that limit food production worldwide (Munns, 2013). To help sustain the increasing population, crops with enhanced salinity tolerance must be developed to increase productivity on salt-affected lands.

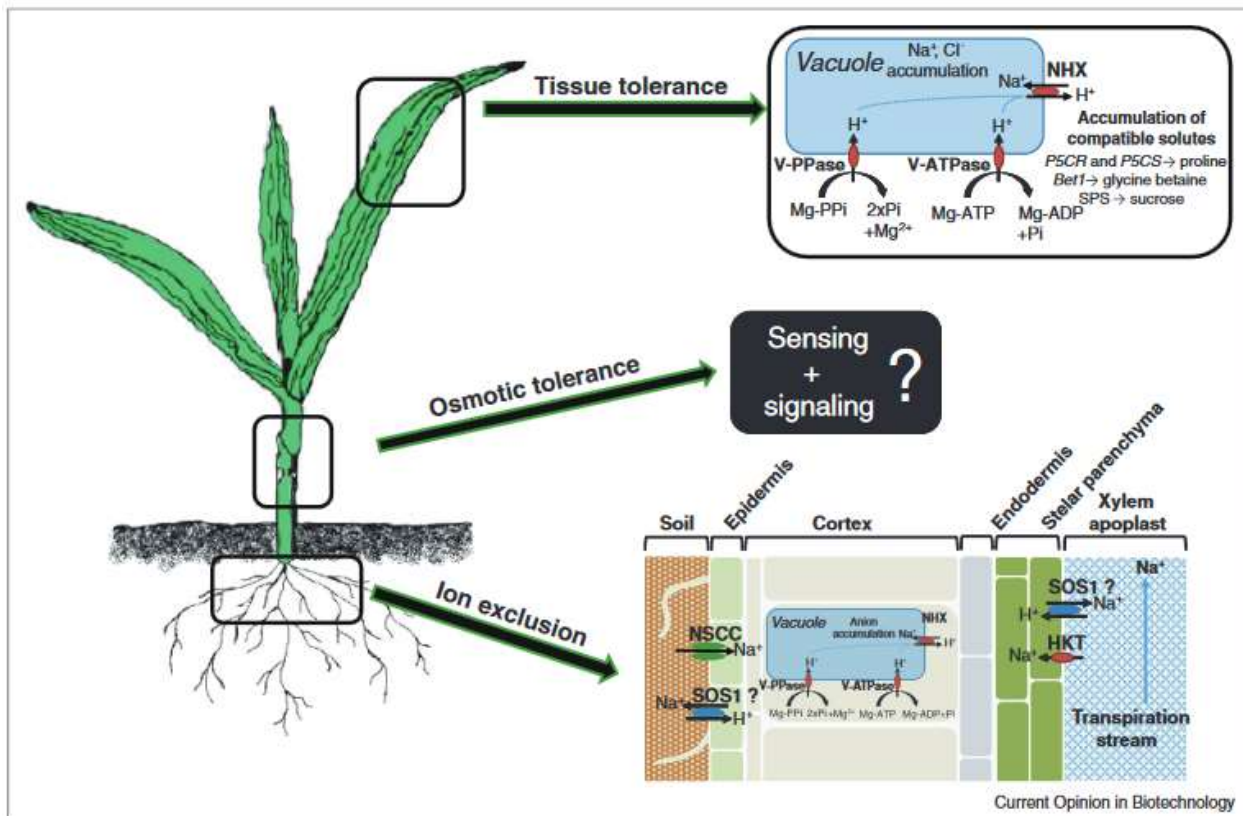
Rice is an economically and scientifically important crop. It is considered as the staple cereal food and a major component of energy resource of the humanity. Rice is a member of the genus *Oryza* in the Family Poaceae. The *Oryza* genus has many

species, of which two—*Oryza sativa* L. and *O. glaberrima* Steud - are cultivated. *Oryza sativa* is most commonly grown in Asia while *O. glaberrima* is native to West and central Africa (Linares, O.F 2002, and Vaughan *et al.*, 2016). Rice is cultivated in 114 of the 193 countries around the world in six continents, Asia, Africa, Australia, Europe, Latin America and North America (Virmani, S.S.; Ilyas-Ahmed). However, rice is very sensitive to salinity stress and is currently listed as the most salt-sensitive cereal crop with a threshold of 3 DSM – 1 for most cultivated varieties USDA 2016, whereas, generally, a soil is only considered saline (salt-affected) if it has an ECE (electrical conductivity of its saturation extract) above 4 DSM – 1 Rengasamy, P 2006 . Even at ECE as low as 3.5 DSM – 1, rice loses about 10% of its yield, and 50% yield loss was recorded for rice at ECE 7.2 DSM – 1. The review will provide an insight into the mechanisms of salt tolerance, screening strategies for salt tolerance and developing salt-tolerant cultivars on rice. It focuses on recent studies on conventional and modern breeding techniques, transgenic approaches. This review also provides comprehensive

information to the scientific community for further efforts in combining conventional and modern approaches to develop salt-tolerant rice plants/cultivars

### Salt tolerance mechanism in rice

Salinity tolerance is a multifaceted trait and it is often accompanied by other stresses like high temperatures and flooding. Over the years, plants have developed certain mechanisms to cope with high salinity levels. A variety of cell signaling cascades and metabolic pathways involving several transcription factors are involved (Samiullah *et al.*, 2016). These mechanisms can be classified into three main categories : firstly, osmotic tolerance, which is regulated by long-distance signals that reduces hoot growth and is triggered before shoot  $\text{Na}^+$  accumulation; secondly, ion exclusion, where  $\text{Na}^+$  and  $\text{Cl}^-$  transport processes in roots reduce the accumulation of toxic concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  within leaves; and finally tissue tolerance, where high salt concentrations are found in leaves but are compartmentalized at the cellular and intracellular level especially in the vacuole. Stuart *et al.*, 2014) (figure 2)

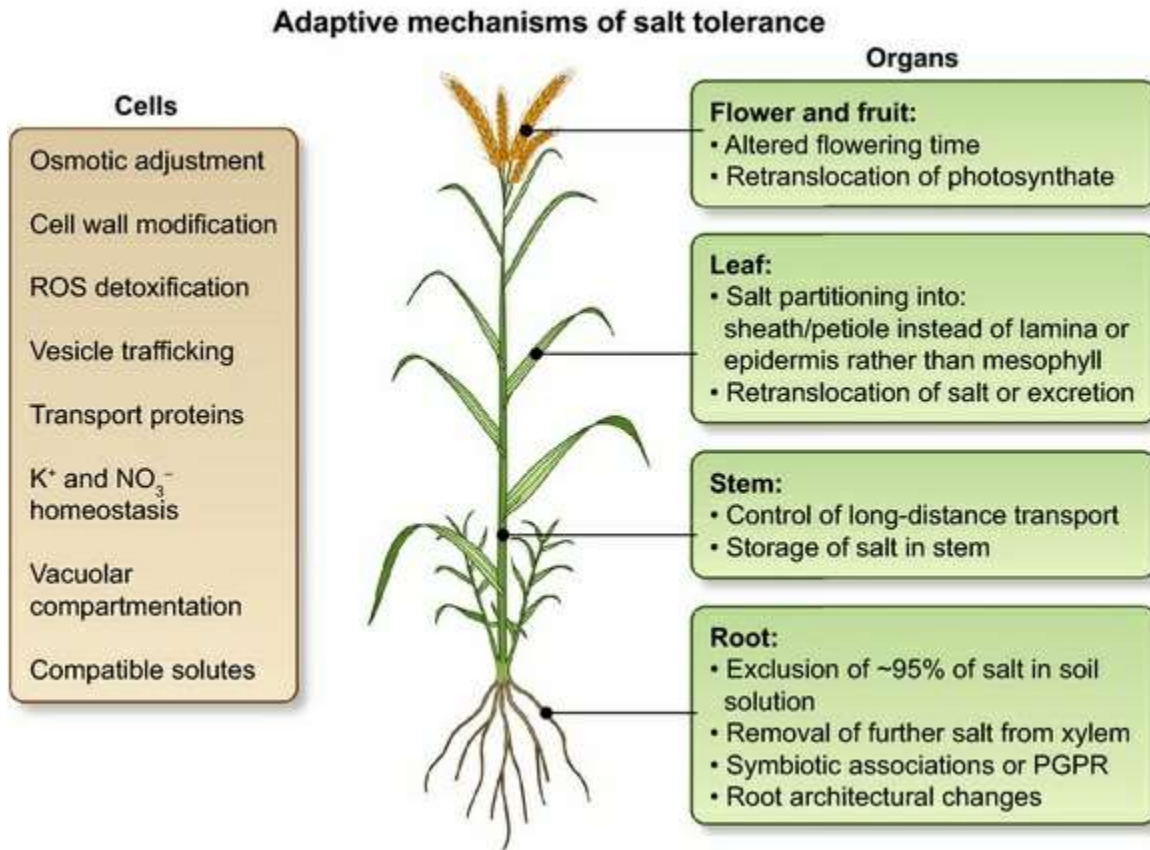


The three main mechanisms of salinity tolerance in a crop plant. Tissue tolerance, where high salt concentrations are found in leaves but are compartmentalized at the cellular and intracellular level (especially in the vacuole), a process involving ion transporters, proton pumps, and synthesis of

compatible solutes. Osmotic tolerance, which is related to minimizing the effects on the reduction of shoot growth, and may be related to as yet unknown sensing and signaling mechanisms. Ion exclusion, where  $\text{Na}^+$  and  $\text{Cl}^-$  transport processes, predominantly in roots, prevent the accumulation of

toxic concentrations of Na<sup>+</sup> and Cl<sup>-</sup> within leaves. Mechanisms may include retrieval of Na<sup>+</sup> from the xylem, compartmentation of ions in vacuoles of cortical cells and/or (figure3)

efflux of ions back to the soil (Source Journal of Current opinion in biotechnology)



(Source: Rana Munns Matthew Gilliam 2015)

Adaptive mechanisms of salt tolerance. On the left are listed the cellular functions that would apply to all cells within the plant. On the right are the functions of specific tissues or organs. Exclusion of at least 95% (19/20) of salt in the soil solution is needed as plants transpire 20 times more water than they retain (Munns, 2005). Most of these functions are explained in the text. Omitted for space, and lack of recent advances, is the limitation that Cl<sup>-</sup> can impose on growth through its antagonistic accumulation against the nitrogen form NO<sub>3</sub><sup>-</sup> (NO<sub>3</sub><sup>-</sup> homeostasis) (Henderson *et al.*, 2014) and the differential capacity and sensitivity of different cell types and tissues to accumulate Na<sup>+</sup> and Cl<sup>-</sup>; for example, NaCl accumulation within photosynthetic cells incurs a larger cost than accumulation in root cortical cells (Conn & Gilliam, 2010). Major progress has been made in breeding salt-tolerant high-yielding rice varieties for various inland saline, coastal saline, and alkaline soils of fragile ecosystems many salt-tolerant rice have been developed using both conventional and molecular approach.

### Conventional breeding

Conventional breeding or traditional method of breeding has been used for the production of salt-tolerant rice varieties through two basic steps (Bresghello *et al.*, 2013). This method of breeding has been used for the production of salt-tolerant rice varieties through two basic steps. The first is to generate/obtain a breeding population that is highly variable for salt tolerance, and the second involves a selection among the segregating progeny for individuals that combine most of the parent's useful traits and a high degree of salt tolerance. By using several methods such as introduction, hybridization, and mutagenesis for generation of highly variable salt tolerant breeding populations, considerable success has been obtained in raising rice genotypes with improved tolerance towards salinity. Lee *et al.*, 2003; Yen *et al.*, 2011). Attempts to improve salt tolerance in rice by conventional breeding, however, have experienced difficulties. One of the reasons is that it is difficult to establish the physiological characters

which best define salt tolerant genotypes. These traits are controlled by numerous genes that generate a continuous variation, the so-called “quantitative trait loci” (QTL) (Fita *et al.*, 2005). In addition, a limited parental resource for breeding also contributes to the limited success of breeding programs for enhanced rice salinity stress tolerance. Another complication that needs to be considered when breeding for salinity tolerance in rice is that different rice varieties have varied levels of salinity tolerance at different growth stages. Rice is comparatively tolerant to salinity stress during germination, active tillering, and towards maturity, but is sensitive during early seedling and reproductive stages. Moreover, salinity tolerance at the seedling and reproductive stages are only weakly, if at all, associated. It has been reported that salinity tolerance at the seedling stage is independent of salinity tolerance at the flowering/reproductive stage (Jena *et al.*, 2008), for example, CN499-160-13-6 genotype has been confirmed as susceptible at the seedling stage, but tolerant at the flowering stage (Gorantla *et al.*, 2005). Due to the variation in sensitivity to salinity during the life cycle, evaluation of salinity tolerance in rice is complex (Waziri *et al.*, 2016).

### Modern Breeding techniques

#### QTL

Hundreds of QTLs have been identified for salinity tolerance in economically important crop plants but quite a few have been confirmed for enhancing salinity tolerance in the field. Among all these crops, rice has received attention for improving salinity tolerance using modern breeding techniques. For example, (Thompson *et al.*, 2010). Fine mapped the Saltol QTL in rice genome and identified QTLs associated with salt tolerance using Recombinant Inbred Lines (RILs) derived from IR29 and Pokkali. Its contribution to phenotypic variation was 43% for seedling shoot Na-K ratio (Bonilla *et al.*, 2002). Identification of molecular markers tightly linked to salt-tolerant genes can serve as landmarks for the physical localization of such genes facilitating marker-assisted selection (MAS). Many QTLs have been identified in rice for salinity tolerance contributing traits and the majority of them have been reported on chromosome 1. Major one of them are saltol Gregorio *et al.*, 2002; Bonilla *et al.*, 1998), QnA for Na uptake (Flowers, *et al.*, 2000), QTL for Na<sup>+</sup> uptake, K<sup>+</sup> concentration and Na /K ratio (Koyama *et al.*, 2001); SKC1 or OsHKT8, RNTQ1, SDS1 (Lin *et al.*, 2003, Renet *et al.*, 2005); Na<sup>+</sup> and Cl<sup>-</sup> transport in stem Ammar, (2004) and qST1 (Lee *et al.*, 2005). There are more reports of other QTLs on other chromosomes for the contributing traits and have been reported on Chromosomes 3, 4, 10 and 12 (Glenn, 1997); chromosomes 4, 6 and 9 (Flowers *et al.*, 2000); chromosomes 4, 6 and 9 Koyama *et al.*, (2001); Chromosomes 4, 6, 7 and 9 Lin *et al.*, (2004); Chromosomes 2, 3, 8, and 9 Ammar, (2004); Chromosomes 3 Lee *et al.*, (2006) and Chromosome 8 and 10 Islam *et al.*, 2006). Molecular markers based approach (MAS)

has enormous potential to be used as a reliable tool to the breeders for the effective, fool-proof and early generation screening. Major bottlenecks of the utilization of the MAS for salinity across the population and comparing the different QTLs are the parental specificity and different screening protocols under different conditions.

#### Cloning of the salt tolerance gene of rice

SKC1: Ren. *et al.*, (2005) isolated the *SKC1*, a major QTL for shoot K<sup>+</sup> content, by map-based cloning, and found that it encoded a member of HKT-type transporters. They compared the *SKC1* nucleotide sequences between a salt-tolerant *indica* Nona Bokra and a susceptible elite *japonica* Koshihikari allele and found six nucleotide substitutions in the coding region which led to four amino-acid changes. Electrophysiological analysis showed that the protein encoded by *SKC1* was specific transport proteins, but not transported directly for K<sup>+</sup>, while the variation of the content of K<sup>+</sup> was caused by the competition of Na<sup>+</sup>. The analysis indicated that the protein located on the cell membrane. The report showed that the above ground of rice (leaf, stem) would accumulate a large number of Na<sup>+</sup> in salt stress condition, while *SKC1* could make the excess Na<sup>+</sup> flow back to the root, which would reduce the toxicity of Na<sup>+</sup> and enhance the salt tolerance of rice. There would be a wide application prospect of *SKC1* in the molecular breeding of salt tolerance (Ren. *et al.*, 2005). DST: A drought and salt tolerance (DST) mutant derived from a japonica cultivar Zhonghua 11 with ethyl methanesulfonate (EMS) treatment was identified, and the *DST* was cloned by the map-based cloning. *DST* encoded a previously unknown zinc finger transcription factor that negatively regulated stomatal closure by direct modulation of genes related to H<sub>2</sub>O<sub>2</sub> homeostasis, which identified a novel pathway for the signal transduction of DST-mediated H<sub>2</sub>O<sub>2</sub>-induced stomatal closure. The assessment of the two amino acid substitutions (N69, A162) in the *dst* mutant showed that the N69 of *DST* was required for transcriptional activation. As a negative regulator, *DST* could directly down-regulated the expression of genes related to H<sub>2</sub>O<sub>2</sub> metabolism when it lost the function, making the ability to remove H<sub>2</sub>O<sub>2</sub> decrease, the accumulation of H<sub>2</sub>O<sub>2</sub> in guard cell increase, then increases the stomata closure and reduces the water evaporation, consequently enhanced the salt tolerance of rice. Moreover, they found that the down-regulation of *DST* did not affect rice grain yield, which facilitated molecular breeding efforts to improve drought and salt tolerance in staple crops (Huang *et al.*, 2009).

#### Transgene

The transgenic approach uses recombinant DNA techniques to create plants with new and desirable characteristics. This approach has been successfully applied in producing rice salt tolerant plants by introducing new genes and testing against

salinity. Transgenic plants overexpressing OsPP1a show enhanced tolerance to high salt treatment, and SnRK1A, OsNAC5, and OsNAC6 are up-regulated in these transgenic plants Liao *et al.*, (2016). Constitutive overexpression of the vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene (OsNHX1) from the rice landrace (Pokkali) was used to enhance the level of salt tolerance in transgenic rice plants Amin *et al.*, (2016). Overexpression of OsNHX1 in rice is also associated with the altered shoot and root accumulations of Na<sup>+</sup> and K<sup>+</sup> improved biomass production and improved germination (Chen *et al.*, 2007). (Wang *et al.*, 2016). Li *et al.*, (2016) demonstrated that SIDP361 has high potential as a tool for genetically improving salt tolerance in rice. Sahoo *et al.*, (2014) reported the use of OsSUV3. Rice SUV3 functions as DNA and RNA helicase and provides salinity stress tolerance by maintaining photosynthesis and antioxidant machinery. Nath *et al.*, (2016) suggested that PDH45 may be involved similar salt tolerance mechanism in rice transgenic plants in response to salinity stress, sustaining least Na<sup>+</sup> in the shoot. Salinity induces the production of reactive oxygen species, which can be removed enzymatically, such as ascorbate peroxidase, catalase, SOD, and glutathione reductase (Lin and Pu, 2010; Das *et al.*, 2014, Liao *et al.*, (2016). Development of stress tolerant rice varieties with high yield is one of the mandates of the International Rice Research Institute and the national rice research programs in Asia. Care must, therefore, be taken in the choice of a promoter used to drive the expression of salt tolerance transgenes, so as not to disrupt or negatively affect the plant phenotype.

### Haploid and Double Haploid breeding

Double Haploids (DH) are plants that have undergone chromosome duplication from haploid plants. The production of haploids and DHs through gametic embryogenesis is the most effective way for the development of complete homozygous lines from heterozygous parents in comparison with the conventional breeding methods that employ several generations of selfing for getting homozygous plants. DH technique is well established in a range of economically important crop species, including major cereals. Wedzony *et al.*, (2009). Haploid breeding is another useful tool for breeding. Double haploid (DH) lines are generated either through another culture (AC) or chromosome elimination methods. DH lines are excellent materials for rice breeding and genetic research because of their homozygosity and uniformity. (Prasad *et al.*, 2000). Detected 7 QTLs for seedling traits under salt stress using a double haploid population derived from a cross between IR64 and Azucena and a QTL for root length had 18.9% contribution to phenotypic variation. Gong *et al.*, (2002) identified genes underlying a total of 8 QTLs for salt tolerance using a double haploid population derived from a cross between indica variety ZYQ8 and japonica variety JX17. DH breeding saves times to obtain the homozygous state as well as for evaluating the yield and other quantitative traits

compared to normal self-pollinating methods Martinez *et al.*, (2002). Development by using conventional breeding can take 8 – 10 year to obtain a promising salinity- tolerant line. However, by using DH culture, the period could be shortened to just 3 years. The cultivar, which is the first DH derived line from indica-indica cross has been released in the Philippines and India Bonilla *et al.*, (2002). Lee *et al.*, (2003) produced salt-tolerant DHs rice using anther culture techniques with different genotypes in six F1 hybrids obtained by back-cross or a three-way cross between indica and japonica differing in salt tolerance. It was found that the efficiency of callus induction and plant regeneration was decreased by NaCl concentration and salt tolerance of donor variety, whereas induction in japonicas was higher than those in indicas. The percentages of callus induction in Gyehwa 5 (japonica, tolerant) and IR61633-B-2-2-1 (japonica, sensitive) were 21.1% and 13.5% on agar medium containing 0.3% NaCl, respectively. In four F1 hybrids, the frequencies of high salt-tolerant DHs were 21.4% and 8.9% in 0.3% NaCl medium and the control, respectively. Therefore, the high frequency of salt-tolerant DHs could be selected in the callus induction medium (0.3% NaCl) and in the combinations crossed with salt-tolerant japonica as the third parent. F1 anther culture has become an effective tool to attain homozygosity of recombinants within the shortest possible time. The technique also offers the opportunity to screen haploid materials at the early stage of tissue culture. Rahman *et al.*, (2010) developed DH lines from the crosses involving salt-tolerant IRRI-derived lines using anther culture and in a field study; one line AC-1 was promising for cultivation in saline areas of Bangladesh. This approach was used to develop salt-tolerant homozygous recombinants from diverse cross combinations, which led to the identification of the promising rice varieties IR51500-AC-17, IR51485-AC-1, IR51500-AC11-1, and AC6534-4 for salinity, AC6533-3 for sodicity, and AC6534-1 for dual tolerance (Singh *et al.*, 2002, Mishra *et al.*, 1998)

### Tissue Culture Approach

Plant tissue culture techniques provide a promising and feasible approach to develop salt-tolerant crop plants. Haploid culture, double haploid, somaclonal variation, and in vitro-induced mutagenesis has been used to create variability to improve salinity tolerance in crop plants. Cell and tissue culture techniques have been used to obtain salt-tolerant plants through in vitro culture approaches: selection of mutant cell lines from cultured cells and subsequent plant regeneration Zair *et al.*, 2003; Gandonou *et al.*, 2006; Lu *et al.*, 2007; Queirs *et al.*, (2007) and in vitro screening of plant germplasm for salt tolerance (Arzani and Mirodjagh 1999; Dziadczyk *et al.*, 2003; Lee *et al.*, 2003b; Wheatley *et al.*, 2003; Dasgupta *et al.*, 2008). Cereal tissue culture is of great economic importance for selection of improved cell lines under in vitro condition.



### Somaclonal variation

Somaclonal variation refers to the variation seen in plants that originated through plant tissue culture. It is particularly common in plants regenerated from callus tissue. The amount of variation that can be expected under in vitro condition may vary with the clone, age of the clone, and use of selection pressure applied to single cells for salinity stress. Somaclonal variations are stable and occur at high frequencies. Novel gene mutations may result during the tissue culture process. It can be performed in vegetative, sexually or asexually propagated plants. This approach reduces the time required for the release of a new variety compared to mutation breeding and has been used in breeding programs (Lu *et al.*, 2007)

### Conclusions

Salt tolerance is a very complex trait, both at the physiological and at the genetic levels, and is also very influenced by other environmental factors acting on the plant at the same time. In addition, the genetic control of salt stress differs in different stages of the plant's life cycle: tolerance at the adult stage does not necessarily correlate with tolerance at the seedling and juvenile stages, or to the ability to germinate in the presence of salts. Yamaguchi *et al.*, 2005, Flower T. J 2004). Rice, for example, is much more affected in grain filling than in vegetative growth by the presence of salt in the soil. To complicate matters further, it is very difficult to design field trials to test the agronomic performance of improved salt-tolerant varieties, as the salt concentration in soils is very variable, and is complicated by the presence of additional pollutants and inland water intrusion. Plant genomes need to be very plastic, a feature required to cope with a variable environment that requires a constant adjustment of the plant's metabolism. It is therefore essential to test newly developed stress-tolerant varieties to multiple stresses in laboratory conditions, and the importance of carrying out extensive field studies in a large range of conditions that assess tolerance as absolute yield increases cannot be over-emphasized (Mitler .R. 2006).

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