Chapter 1

Introduction
1.1 Rice: an important crop:

Cultivated rice (Oryza sativa L.) has been consumed by humans for almost 5000 years (Zhou et al., 2002). It was most probably domesticated in India around 3000 B.C. (Christou, 1994) and its cultivation spread to the north and east to reach China and Indochina as well as Indonesia to the south and Japanese islands (Christou, 1994). During the early middle-ages, the plant was introduced to southern Europe, and in USA, it was first planted in the seventh century.

Rice is one of the world’s most important cereal crops, providing staple food for nearly one-half of the global population (Kathuria et al., 2007), with the global production around 634 million tonnes (Mt) in 2006, occupying a land area of more than 154 million hectares (Mha) with an average production of around 4 t/ha (World Rice Statistics, 2008). At least 114 countries grow rice, and more than 50 have an annual production of 1 lakh t or more. Asian farmers produce about 90% of the total, with two countries, China and India, growing more than half the total crop (World Rice Statistics, 2008). Its importance can be estimated by the fact that the year 2004 was declared as International Year of Rice by the United Nations Food and Agriculture Organization.

Rice is staple food of over 65% population of India (IRRI, 2006). In India, 45 Mha land is under rice cultivation, being the largest in the world. India is second only to China in rice production with more than 136 Mt in 2006 (World Rice Statistics, 2008). In Asia, rice growing countries can be classified on the basis of yield in three categories namely high, medium and low. China, Japan, Iran, Korea and Taiwan comes under first category and produce as high as 6-7 t/ha. India, Pakistan, Malaysia, Indonesia, the Philippines and Afghanistan produce medium yields in the range of 2-4 t/ha. Countries producing less than 2 t/ha come under third category and most remaining Asian countries fall in this category. The
average yield in India is very low (3 t/ha) as compared to some other Asian rice producing countries like China, Korea and Japan. The main constraints for the low yield are dependency on monsoon (floods and droughts), improper irrigation and drainage facilities, overuse of fertilizers and soil related problems including soil salinity and alkalinity.

Further, in the state of Maharashtra the situation is rather more unsatisfactory and the annual production of rice in Maharashtra has dropped down from 4.2 Mt in 2003 to 3.7 Mt from ~1.5 Mha land in 2006 (World Rice Statistics, 2008). Amongst the five categories as given in Table 1.1, based on rice yield in India, Maharashtra comes at third place with an average yield below 2.5 t/ha (http://dacnet.nic.in/Rice/). Soil related problems (including salinity) are the major cause for this reduction in yield.

Table 1.1 Categorisation of rice producing states in India based upon their average yield:

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Category</th>
<th>States</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>High Productivity</td>
<td>Punjab, Andhra Pradesh, Tamil Nadu</td>
</tr>
<tr>
<td>2.</td>
<td>Medium Productivity</td>
<td>Haryana, Uttar Pradesh, Kerala, Karnataka</td>
</tr>
<tr>
<td>3.</td>
<td>Medium-Low Productivity</td>
<td>Maharashtra, Gujarat, Jammu &amp; Kashmir, Tripura and Mizoram</td>
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<tr>
<td>4.</td>
<td>Low Productivity</td>
<td>Bihar, Rajasthan and most of the north-eastern states</td>
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<tr>
<td>5.</td>
<td>Very-Low Productivity</td>
<td>Madhya Pradesh</td>
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</table>

1.1.1 Rice as a model monocot system:

Rice with its relatively small genome size (~ 430 Mb), ease of transformation, well developed and known in detail genetics (Sahi et al., 2006b; Kathuria et al., 2007), availability of a dense physical map and molecular markers (Chen et al., 2002, Wu et al., 2002), full-genome transcription profiling using high density oligonucleotide tiling microarrays (Li et al., 2006) together with its complete genome sequence (Sasaki et al., 2005) is considered as a model monocot system for various biotechnological, metabolic, genetic engineering and functional genomics development studies worldwide (Shimamoto and Kyozuka, 2002; Bajaj and Mohanty, 2005; Hoque et al., 2005; Ge et al., 2006) as Arabidopsis thaliana is considered for dicotyledonous plants. Rice is being used to
understand several fundamental problems of plant physiology, growth and development processes that ranges from elucidation of single gene function to whole metabolic pathway engineering. In addition, rice shares extensive synteny among the other cereals thereby increasing the utility of this system. These together with availability of ~ 28,000 full length cDNAs (The Rice Full Length cDNA Consortium, 2003) a large number of expressed sequence tags and rich forward and reverse genetic resources (Hirochika et al., 2004) has made rice a worthy forerunner among the plants especially among the cereals (Bajaj and Mohanty, 2005).

1.1.2 Nutritional value of rice:

Rice is the major nutritional source for about 40% of the world population (Hiei and Komari, 2006). Rice processed to remove only the husk (called brown rice), contains approximately 8% protein and small amounts of fats and is a source of various vitamins such as thiamine, niacin, riboflavin, iron and calcium. But, removal of bran with milling results in white rice that is greatly diminished in nutrients, which makes a risk of vitamin deficiency as a result of lack of thiamine and minerals in the diet, especially in Asian countries where rice forms the major part of the diet, the nutritional values of rice are given in Table 1.2 (Julino, 1985).

<table>
<thead>
<tr>
<th>Nutrients (at 14% moisture)</th>
<th>Brown rice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (%)</td>
<td>7.30</td>
</tr>
<tr>
<td>Fibre (%)</td>
<td>0.80</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>2.20</td>
</tr>
<tr>
<td>Available carbohydrates (%)</td>
<td>74.3</td>
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<tr>
<td>Thiamine (mg/100g)</td>
<td>0.30</td>
</tr>
<tr>
<td>Riboflavin (mg/100g)</td>
<td>0.04</td>
</tr>
<tr>
<td>Niacin (mg/100g)</td>
<td>4.00</td>
</tr>
<tr>
<td>Lysine (g/16g N)</td>
<td>3.80</td>
</tr>
<tr>
<td>Threonine (g/16g N)</td>
<td>3.60</td>
</tr>
<tr>
<td>Methionine + Cysteine (g/16g N)</td>
<td>3.90</td>
</tr>
<tr>
<td>Tryptophan (g/16g N)</td>
<td>1.10</td>
</tr>
<tr>
<td>Iron (mg/100g)</td>
<td>3.00</td>
</tr>
<tr>
<td>Zinc (mg/100g)</td>
<td>2.00</td>
</tr>
</tbody>
</table>
1.1.3 Botanical aspects of rice:
Rice is an anomaly among the domesticated cereals-a tropical C3 grass evolved in semi-aquatic and low radiation habitat (Lafitte et al., 2004). There are 25 species of rice, among them only two have been domesticated namely O. sativa and O. glaberrima (Christou, 1994). These belong to the tribe Oryzeae under the subfamily Pooidae in the grass family Gramineae (Poaceae) (Chang and Vaughan, 1991). The cultivated species are diploid (2n = 24), whereas wild species may also be tetraploid. O. sativa, the Asian rice and O. glaberrima from west Africa, share parallel evolutionary pathways, from perennial wild to annual wild to cultivated annual (Christou, 1994). O. sativa is an annual cereal crop with long leaves bearing an inflorescence (panicle) composed of spikelets with flowers producing the seed or grain. Natural cross pollination in cultivated rice is rare, normally less than 1% (Virmani and Athwal, 1973). Following meiosis, which takes place after development of the flag leaf, the panicle emerges which bears perfect flowers in single flowered spikelets. A flower has six stamens, each composed of an anther on a slender filament and pistil containing one ovule. The style is short, with a feathery bifid stigma. The flower is fully developed and the stigma fully receptive during pollen shedding. At this stage the lemma and plea are forced apart, anther dehiscence and extrusion occur simultaneously, and the lemma and plea close. This stage is reached in the uppermost florets when the panicle is about 50% emerged (Christou, 1994).

1.1.4 Rice varieties:
Most cultivated rice is O. sativa, and even in West Africa O. glaberrima is being gradually replaced by O. sativa due to its better yield and adaptability to local growing conditions (Christou, 1994). Three O. sativa varieties are recognised as distinct ecotypes- indica, japonica and javanica. Indica rice varieties are primarily grown in the tropical, subtropical and temperate regions and occupy 80% of the rice cultivation areas in the world (Swaminathan, 1982; Ayers and Park, 1994). They exhibit profuse tillering and are tall (Chandler, 1979), grain is medium to long with higher amylose content resulting in dry and fluffy cooked rice that shows little disintegration. Most of the indica varieties are grown in the Indian subcontinent, southern China and South America and a large portion of the rice grown in USA. Scented rice varieties are grown primarily in northern India and Pakistan and...
are aromatic and highly valued (Christou, 1994). *Japonica* varieties most likely originated in China and are grown in regions of temperate regions of the world, such as southern Europe, Japan and central and northern China (Christou, 1994). *Japonica* varieties have a lower tillering capability than *indica* and the amylose content of the grain is lower so cooking results in stickier and glossier grains and tends to disintegrate if boiled too long (Chandler, 1979). *Javanica* are primarily grown in Java and Bali, in the terraces of Philippines and the mountainous regions of Madagascar. However, these varieties are gradually disappearing due to rapid spread of modern *indica* varieties.

1.2 Problem of soil salinity and justification for present study:

The accumulation of water-soluble salts in the soil solum or regolith to a level that impact on agricultural production, environmental health, and economic welfare is termed as salinisation. Salt-affected lands occur in practically all climatic regions, from the humid tropics to the Polar Regions covering more than 100 countries of the world with a variety of extents, nature, and properties (Singh and Chatrath, 2001; Rengasamy, 2006). Currently, more than 800 Mha of land throughout the world are salt affected (FAO, 2008). No climatic zone in the world is free from salinisation, although the general perception is focused on arid and semi-arid regions (Rengasamy, 2006). Saline soils can be found at different altitudes, from below sea level (e.g., around the Dead Sea) to mountains rising above 5000 meters, such as the Tibetan Plateau or the Rocky Mountains (Singh and Chatrath, 2001). The occurrence of saline soils is not limited to desert conditions; the problem has been reported in the tropical belts of Africa and Latin America, and even in the polar-regions, particularly Antarctica. Areas affected by soil salinity are not well defined, since detailed maps are available for only a few. Nevertheless, it is considered as a single largest soil toxicity problem in tropical Asia (Greenland, 1984).

Soil salinity is measured by its electrical conductivity and it is expressed with SI unit dS m$^{-1}$ (deci Siemens per meter). According to United States Department of Agriculture (USDA) Salinity laboratory (USSL), a soil is said to be saline if it is having an $EC_e$ is 4 dS m$^{-1}$ or more (USSL, 2005). $EC_e$ is the electrical conductivity of saturated paste extract, that is, of the solution extracted from a soil sample after being mixed with sufficient water to produce
Chapter 1 Introduction

Salt stress is a major abiotic stress problem around the globe especially in arid and semi-arid regions and irrigation area. Salinity affects approximately 7% of the world’s land area, 20% of world’s cultivated land and approximately half of irrigated land is affected with high salt contents (Sairam and Tyagi, 2004; Anil et al., 2005; Sahi et al., 2006a). In Asia alone, 21.5 Mha of land area is thought to be salt affected (of which 12 Mha is due to saline conditions and the remaining 9.5 Mha is due to alkaline/ sodic conditions, with India having 8.6 Mha salt-affected area (including 3.4 Mha sodic soils) which constitutes a major part of problem soils in India (Deosthali and Akmanchi, 2005; Sahi et al., 2006b). In the state of Maharashtra, more than 6 lakh ha land is saline and this area is increasing day by day due to irregular monsoon, faulty water management, irrigation with saline water and excess use of fertilizers (Singh and Chatrath, 2001; Deosthali and Akmanchi, 2005).

In addition, natural boundaries imposed by salinity also limit the caloric and nutritional potential of agricultural production (Yokoi et al., 2002). Increasing salinity of irrigation water along with progressive salinisation of agricultural land is of increasing importance to agriculture, as it limits the distribution of plants in certain natural habitats and induces a wide range of adverse metabolic responses in higher plants (Demiral and Turkan, 2005). The problem of soil salinity is ever increasing owing to: 1) The use of poor quality water for irrigation, 2) Improper drainage in irrigated agro-ecosystems, 3) Entry of seawater during cyclones in coastal areas, and 4) Salt accumulation in the root zone in arid and semi-arid regions due to high evaporative demand and insufficient leaching of ions as the rainfall is inadequate (Chinnusamy and Zhu, 2003).

Amongst various salts including chlorides and sulphates of sodium, potassium and calcium, NaCl is the most important salt and the main contributor for high salinity, an important environmental factor that limits distribution and productivity of major crops as it affects almost all plant functions (Greenway and Munns, 1980; Borsani et al., 2003; Sahi et al., 2006a and 2006b).

Due to ever increasing population, global food production will need to increase by 38% by 2025 and by 57% by 2050 (Wild, 2003, Rengasamy, 2006) if food supply to the growing
world population is to be maintained at current levels. Most of the suitable land has been cultivated and expansion into new areas to increase food production is rarely possible or desirable. The aim, therefore, should be an increase in yield per unit of land rather than in the area cultivated. More efforts are needed to improve productivity as more lands are becoming degraded. Increased salt tolerance of crops including rice is needed to sustain food production in many regions of the world (Munns et al., 2006).

Salinity adversely affects quantity and quality of crop produce (Gepstein et al., 2006; Sahi et al., 2006b). The yield of rice especially Asian rice (sativa) is very susceptible to salinity (Kapp, 1947; Pearson, 1959; Akbar et al., 1972; Flowers and Yeo, 1981; Grover and Pental, 2003; Lee, 2003; Anil et al., 2005; Theerakulpisut et al., 2005; Munns and Tester, 2008). In India and especially in Maharashtra state soil salinity is a major stress that reduces the rice productivity up to a great extent. There are some natural salt tolerant varieties in the state but their yield is very low and they are not considered as quality rice varieties. Therefore, there is an utmost need to produce high quality rice genotypes with high productivity, which can withstand soil salinity, for the state of Maharashtra. One of the important ways to produce salt tolerant variety is the production of transgenics of high yielding and high quality rice genotypes, which can tolerate salt stress by using gene(s) of interest. Therefore, the present study was undertaken to study the differential responses of various local rice cultivars towards salinity stress tolerance and to produce transgenics containing proline biosynthetic pathway gene ($\Delta^1$-Pyrroline-5-carboxylase synthase: P5CS) for combating salt stress.

1.3 Combating salt stress through genetic engineering approach:

Conventional breeding programmes have been employed since last 2-3 decades to produce salt tolerant crops across the globe, but a limited success has been achieved so far, hence the scientist have shifted their interests towards genetic engineering aspects to solve this problem, and there are some really successful cases in a number of crops including tobacco (Nicotiana tabaccum), rice (Oryza sativa), wheat (Triticum aestivum) and Arabidopsis thaliana. Over the next 7 to 8 years, global rice production is predicted to be static, which will result in a shortfall of about 130 Mt (Khush, 2001; Brookes and Barfoot, 2003). To meet this demand conventional breeding programmes supplemented with recent
biotechnological tools to produce rice varieties with higher yield potential, durable resistance to diseases and insects and tolerance to abiotic stresses are needed (Bajaj and Mohanty, 2005). Transgenic approach towards producing salt stress crops is increasing at a great pace. Transgenic research provides much needed flexibility in manipulation of crops by altering the expression levels of native genes or by incorporating alien genes for a desired trait, in a relatively shorter time. In the past one decade of research, production of salt-stress tolerant transgenic plants by genetic engineering has been claimed in over 100 research publications (Grover et al., 2003; Sahi et al., 2006a).

Salt-stress response is shown to encompass large number of genes (Yang and Yen, 2002). These genes are linked to different pathways and processes such as stress perception and signalling, leading to molecular, biochemical, cellular, physiological and morphological adaptations to finally the whole-plant response (Flowers, 2004; Bartels and Sunker, 2005; Chinnusamy et al., 2005, Vinocur and Altman, 2005; Sekmen et al., 2007; Hussain et al., 2008). Different stress regulated genes may have cumulative or exclusive roles in salt tolerance (Sahi et al., 2006a). A number of biochemical entities of plants are considered to be salt-stress-tolerance effectors such as enzymes that catalyse rate limiting steps in the biosynthesis of compatible osmolytes, and proteins that protect membrane integrity and control osmotic and/or ion homeostasis and reactive oxygen species (Singla-Pareek et al., 2003; Sottosanto et al., 2004; Taji et al., 2004). Numerous reports in the literature have shown improvement of salt tolerance via genetic engineering. Various genes employed in such studies have been isolated from a number of organisms, ranging from prokaryotic organisms such as E. coli to halophytes or glycophytes. These genes can be classified into five groups according to their functions: i) synthesis of osmolytes, ii) protection of cell integrity, iii) oxidative stress, iv) ion homeostasis, and v) transcription factor

1.3.1 Proline accumulation, biosynthesis and its role in combating salt stress:

There are many cellular mechanisms by which plants ameliorate the effects of environmental stresses; for instance accumulation of compatible osmolytes such as proline is one such phenomenon. Kemble and McPherson (1954) first time observed the free proline accumulation in response to osmotic stress in rye. Very high accumulation of cellular proline (up to 80% of the amino acid pool under stress and 5% under normal conditions)
either due to *de novo* synthesis or decreased degradation under a variety of stress conditions such as salt and drought has been documented in many plant species (Kavi Kishor, 1988; Kohl *et al*., 1991; Delauney and Verma, 1993; Bohnert and Jensen, 1996; Schat *et al*., 1997). The level of proline accumulation varies from plant to plant and can be 100 times greater than in the control situation (Verbruggen and Hermans, 2008).

Proline acts as osmo-protectant, and plays important role in osmotic balancing, protection of sub-cellular structures, enzymes and in increasing cellular osmolarity (turgor pressure) that provide the turgor, necessary for cell expansion (Yokoi *et al*., 2002; Matysik *et al*., 2002; Sairam and Tyagi, 2004). Proline is the only osmolytes which have been shown to scavenge singlet oxygen and free radicals including hydroxyl ions and hence stabilize proteins, DNA as well as membrane (Matysik, *et al*., 2002). Proline is also reported to reduce the enzyme denaturation caused due to heat, NaCl and other stresses. In addition, proline acts as a source of carbon, nitrogen and energy during recovery from stresses (Kavi Kishor *et al*., 2005).

In higher plants, proline is synthesized by two pathways first via arginine/ornithine and second via glutamate. Radioisotope labelling reveals that proline gets synthesized mainly via glutamate pathway under stress conditions. In this pathway glutamate is catalyzed to glutamatic-γ-glutamyl kinase (GSA) by pyrroline 5-carboxylate synthetase (*P5CS*) in plants and other eukaryotes. Pyrroline 5-carboxylate (*P5C*) is then reduced to proline by *P5C* reductase (*P5CR*) (Kavi Kishor *et al*., 2005).

Although proline is known to confer osmotic tolerance during stress conditions, its specific role during plant growth is not completely clear. Genes encoding most of the enzymes associated with the synthesis and degradation of proline were cloned and partially characterized, but the factors regulating the expression of these enzymes are largely unidentified. In the last decade, several attempts were made to increase the level of proline accumulation in plants by transferring the genes associated with the biosynthetic pathway. Tolerance to abiotic stress, especially to salt and improved plant growth was observed in a variety of transgenics that were engineered for overproduction of proline (Kavi Kishor *et al*., 1995; Zhu *et al*., 1998; Hong *et al*., 2000; Sawahel and Hassan, 2002; Roosens *et al*., 2002; Han and Hwang, 2003; Su and Wu, 2004; Yamchi *et al*., 2007).
1.3.2 Proline biosynthetic pathway gene P5CS:
The 2417 bp sequence contains a single major open reading frame (ORF) that encodes a polypeptide of 73.2 kDa. P5CS is a novel bifunctional enzyme (EC 2.7.2.11/ 1.2.1.41) that catalyses the first two steps of proline biosynthesis in plants. This gene has been used a number of times for various plants to combat salinity stresses including tobacco (Kavikishor et al., 1995; Zheng et al., 1995), rice (Zhu et al., 1998; Annop and Gupta, 2003; Hur et al., 2004; Su and Wu, 2004), carrot (Han and Hwang, 2003), latrix (Deidre et al., 2005) and wheat (Vendruscolo et al., 2007). P5CS is a proven gene sequence that increases the rate of proline accumulation under salinity stress. Therefore, it was proposed to introduce this gene into a local high yielding but salt susceptible rice cv Karjat-3 and to produce transgenic plants with over-expression of this gene and subsequently higher salinity stress tolerance.

1.3.3 Mutated version of P5CS- (P5CS-F129A):
\( \Delta^1\)-Pyroline-5-carboxylase synthase (P5CS) is subject to feedback inhibition by proline. Therefore, Hong et al. (2000) used site-directed mutagenesis to replace the Phe residue at position 129 in P5CS from Vigna aconitifolia with an Ala residue. The mutated enzyme (P5CS-F129A) was no longer subject to feedback inhibition. Plants that expressed P5CS-F129A accumulated about twice as much proline as those that expressed the wild type P5CS.

This difference was further accentuated in plants treated with NaCl. The elevated levels of proline significantly improved the salt tolerance of transgenic seedlings. Increased levels of proline also reduced the production of free radicals, as measured in terms of MDA production. These findings indicated that, in addition to serving as an osmolyte, proline might play a role in reducing the oxidative stress that is brought on by osmotic stress. Therefore, we decided to use P5CS-F129A gene for improving salinity stress tolerance in rice (Oryza sativa L. subsp. indica) cultivar KJT-3, grown widely in Maharashtra.

1.3.4 In vitro regeneration and genetic transformation in indica type rice:
Development of tissue culture methods for whole plant regeneration are extremely important and a prerequisite for genetic transformation studies. The successful application of available genetic transformation methods in rice is possible when efficient and reproducible plant regeneration system is available for the particular cultivar. Regeneration from callus cultures of cereals was considered difficult when compared to other plant species. A regeneration
IntroductioM

protocol is pre-requisite for the transformation of a particular plant either Agrobacterium-mediated or by using direct insertion method such as microprojectile bombardment (Biolistic gene gun). Embryogenic callus is considered the best organ for genetic transformation. From a functional point of view, the most important characteristic of callus is that this unorganised, undifferentiated and continued proliferating tissue has the potentiality to develop into organised organs such as roots, shoots and embryoids, which form the plants (Aditya et al., 2004).

Callus induction and indirect plant regeneration process in rice tissue culture depends on a number of factors, such as genotype of the donor plant, the type and physiological status of the explants, the composition and concentration of the basal salt and the organic components and plant growth regulators in the culture medium (Ge et al., 2006). Compared with japonica rice, indica rice is less responsive to callus induction as well as regeneration efficiency (Abe and Futsuhara, 1984, 1986; Reddy et al., 1985, Kavi Kishor and Reddy, 1986, Mikami and Kinoshita, 1988; Martinez-Trujillo, 2003; Ge et al., 2006). Even within the indica group, there are significant variations in the in vitro culture response among different genotypes (Peng and Hodges, 1989). Even though, various explants have been used in earlier studies for callus induction in rice including immature embryos (Zhang et al., 1996; Chand and Saharawat, 2001), mature embryos (scutellum) (Khanna and Raina, 1998), roots (Mukhopadhyaya et al., 1997), anther (Sugimoto et al., 1999), mature endosperm (Bajaj, 1991), stem base (Finch et al., 1992) and young coleoptiles (Oinam and Kothari, 1993), but currently mature embryo or seed/ scutellum is used widely for callus induction because of its availability throughout the year and its callus induction response. Auxin, mainly 2,4-D is used most frequently with little variation (2 to 3 mg/l) for optimized callus induction and proliferation from mature seeds (Rashid et al., 2000, 2001; Visarada and Sarma, 2002; Saharan et al., 2004; Lin and Zhang, 2005; Ge et al., 2006). For genetic transformation and further biotechnological developments and tolerance to various stresses, biotic and abiotic, an optimised tissue culture system for callus formation and regeneration is must. Therefore, amongst the selected cultivars, KJT-3, which is a recent promising, high yielding (5-6 t/ha), early maturity (110-115 days) and insect resistant, high quality variety widely cultivated in the state (http://agricoop.nic.in/dacdivision/seed/Paddy/PaddyKarjat-3.htm) but found highly salt sensitive, as evident from our results, was selected for

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Agrobacterium-mediated transformation using P5CS-F129A gene. Therefore, we have optimized the various conditions for obtaining compact, hard embryogenic-like calli and subsequently shoot organogenesis and plant regeneration from callus from indica rice genotype KJT-3.

Although callus is the most widely used organ for transformation of rice, worldwide, there are some limitations associated with the use of callus for transformation such as low rate of transformation and subsequent regeneration of transformants and success with specific genotypes only. Recovery of transgenic plants through indirect organogenesis is quite difficult because cells accessible for gene transfer may not be suitable for plant regeneration (Komari et al. 1998), which demands development of alternative simple approaches for transformation of plants; especially indica rice. Therefore, we have envisaged to establish a system in which shoot apical meristem were used for gene transfer in indica rice cv KJT-3 and subsequently multiple shoot regeneration from them.

Keeping these points in mind, we have successfully attempted the Agrobacterium-mediated genetic transformation of a high yielding rice genotype, KJT-3 using a proline biosynthetic pathway gene construct: pCAMBIA-P5CSF129A under CaMV35S promoter and to study the salt stress tolerance and proline accumulation under salt stress of transformed plants.

Present study was carried out mainly with following objectives:

1. To screen promising high quality rice cvs, widely cultivated in the state of Maharashtra-Bhurarata (BR), Kalarata (KR), Karjat-3 (KJT3), Panvel-1 (PNL1), Panvel-2 (PNL2) and Panvel-3 (PNL3) for salt (NaCl) stress tolerance during seedling and vegetative growth stages. Based on these results, to select three cvs: susceptible, moderately tolerant and tolerant and to carry out further studies in these selected cvs.

2. To screen the three selected rice varieties for their behaviour in terms of mineral nutrients such as K, Na, Cl, Mg, Ca, N, P, Zn, and Fe under salt stress conditions.

3. To understand the physiological and biochemical mechanism of salt tolerance by studying chlorophyll contents, total soluble proteins, proline, lipid peroxidation, starch, polyphenols, sugars and various antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), polyphenol oxidase (PPOX) and glutathione reductase (GR) in these selected rice genotypes under salt stress.
4. To study the electrophoretic patterns of SOD, CAT and POX isoenzymes using native-PAGE to see concerned gene expression in all the tree rice cvs under varying salt stress.

5. To separate proteins from roots and shoots (including leaves) of all the three selected rice cultivars by using SDS-PAGE electrophoresis, and try to get biochemical markers in the form of specific polypeptides for salt stress and its tolerance.

6. To establish a reproducible and efficient protocol for optimum hard, compact embryogenic-like callus production, sensitivity of calluses for antibiotics such as hygromycin and cefotaxime, indirect organogenesis and plant regeneration for cultivar KJT-3, which is a recent promising, high yielding (5-6 t/ha), early maturity (110-115 days) and insect resistant, high quality variety widely cultivated in the state but found highly salt sensitive, as evident from our results.

7. To optimize the various conditions and parameters and establishment of a simple and reproducible method for multiple shoot regeneration from shoot apical meristem and subsequent plant regeneration of KJT-3.

8. To optimize the conditions for genetic transformation using embryogenic-like callus as well as apical shoot meristems of rice cv. KJT-3 by Agrobacterium tumefaciens LBA 4404 strain containing the construct pCAMBIA-1301 P5CS-F129A and to obtain putative transformed rice plants.


10. To select the transformants in the antibiotic selection medium and to raise the T₀ populations (primary transformants).

11. To characterize the genetic transformants at the molecular level using PCR (polymerase chain reaction) and Southern analysis.

12. To determine the comparative proline content in primary transgenic plants and in vitro grown non-transgenic plants.

13. To evaluate transgenic rice plants for their salt stress tolerance in terms of growth performance, biomass production, proline accumulation and lipid peroxidation under saline conditions.