Chapter 5

Discussion
Salinity is one of the serious abiotic stresses, which affects the crop productivity severely. Unlike drought, salinity stress is an intricate phenomenon which includes osmotic stress including production of reactive oxygen species (ROS), specific ion effect, nutrient deficiency etc., thereby affecting various physiological and biochemical mechanisms associated with plant growth and development (Sairam et al., 2002; Khan and Panda, 2008). Further, NaCl has been shown to bring about a reduction in the overall growth and productivity of plants by perturbing the functioning of vital components of photosynthesis like PSI, PSII and Rubisco (Apse and Blumwald, 2002; Vaidyanathan et al., 2003). However, in response, plants have developed various combating mechanisms to cope with the deleterious effects of salinity stress. These includes both non-enzymatic and enzymatic defences such as osmolytes (proline, glycine-betaine) and antioxidant enzymatic machinery (CAT, SOD, POX, GR etc.) respectively. However, these responses vary between species to species and plants to plants. The genetic variations among the various crop plants are useful in providing a valuable tool in the selection of cvs with desirable traits (Misra and Dwivedi, 2004), hence it is necessary to screen and observe the genotypic responses for their salinity stress tolerance. Various researchers (Anil et al., 2005; Khan and Panda, 2008; Roychoudhury et al., 2008) emphasized the importance of identifying the traits that impart salt tolerance to rice lines and advocated that it would have significant agronomic consequences.

5.1 Screening of rice cultivars for their NaCl stress tolerance at germination level

The deleterious effects of salinity stress on germination and early seedling growth are well documented and established (Greenway and Munns, 1980; Bewley and Black, 1982; Alam et al., 2002). However, opinions regarding the mode of deleterious effects of salinity on
germination and early seedling growth differ among the researchers (Alam et al., 2002). Some workers describe the effect in terms of osmotic potential (Greenway and Munns, 1980; Bewley and Black, 1982); while others explain it as a toxic effect (Hampson and Simpson, 1990). Some also explain the effect of NaCl on germination and early seedling growth as having both osmotic and toxic components (Katembe et al., 1998; Kumar et al., 2007, 2008).

Seed germination and seedling growth are stages often subject to high mortality rates under salinity stress (Niknam et al., 2004). According to Almansouri et al. (2001) and Okcu et al. (2005) seed germination is usually the most critical stage in seedling establishment, determining the successful crop production. Seed germination and seedling establishment are affected adversely by salinity stress (Jones and Jones, 1989; Sadeghian and Yavari, 2004) with varying responses for species and cvs (Hampson and Simpson, 1990). Salinity usually affects the seed germination by creating an external osmotic pressure that prevents water uptake or due to the toxic effects of Na⁺ and Cl⁻ ions on the germinating seeds (Mansour, 1994; Murillo-Amador et al., 2002; Khajeh-Hosseini et al., 2003). Understanding the responses of plants at these stages is particularly important for elucidating the mechanisms of salt resistance and sensitivity in plants and their survival (Mayer and Poljakoff-Mayber, 1963). Salinity stress affects the germination rate, root and shoot length and biomass production in plant species (Jamil et al., 2005) including rice (Khan and Panda, 2002, 2008; Kumar et al., 2007, 2008; Roychoudhury et al., 2008). These responses are reported to be very important to screen the cvs for their respective salt stress tolerance.

In this study (Table 4.1), the severity of salinity antagonism to the normal growth of plant as indicated by germination percentage, shoot length, root length, root : shoot ratio, fresh and dry weights and relative water content (RWC), indicating that NaCl has a negative influence on the growth and survival of rice seedlings, though, with differential responses among the cvs. Results clearly indicated that high levels of NaCl (more than 50 mM) adversely affected the germination process and germination percentage was decreased gradually with increase in salt stress in all the genotypes (Table 4.1). However, PNL-3 cv was affected by NaCl with a significantly lower extent, and even at as high as 300 mM NaCl concentration it showed 85% germination, reflecting the greater salt tolerance ability of this cv than other cvs. Contrary to this, KJT-3 was affected with highest magnitude and only 25% germination.
was observed at 300 mM NaCl level (Table 4.1). NaCl affected negatively on root length, shoot length and biomass production and effects were more pronounced as the salinity level increased. PNL-3 could withstand high NaCl stress better than other rice cvs and was found therefore, comparatively salt stress tolerant than other cvs.

In general, an increased root : shoot length ratio is usually documented as a general response to salinity, and results indicated that a reduced root-to-shoot ratio may improve salinity tolerance by restricting the flux of toxic ions to the shoot and consequently by delaying the onset of the tolerance threshold (Moya et al., 1999; Dalton et al., 2000; Maggio et al., 2007). The results of present study showed that shoot growth was more retarded by salinity stress than root growth, but with significant variation among the cvs, with highest reduction in cv KJT-3 and lowest in PNL-3. This differential response leads to the disparity in root/shoot ratio among the cvs. Root/shoot length was increased considerably in all the cvs as the salinity increased, however, NaCl stress could not affect the PNL-3 cv with great extent and at 300 mM NaCl the root/shoot ratio (1.16) was observed even less than at the control (1.18). KJT-3 was influenced severely by salinity stress and root : shoot ratio was found a whooping 3.56 at 300 mM NaCl against 1.33 at control. Therefore, the ability to maintain lower root/shoot ratio seems to be a useful indicator relating to salt tolerance nature of PNL-3. The proportion of dry weight allocated to roots increased with increasing NaCl levels, as shown by the responses of root/shoot ratio (Table 4.2). These results are similar to earlier reports, where Theerakulpisut et al. (2005) and Roychoudhury et al. (2008) observed greater reduction in shoot growth than the roots under salinity stress, higher being in sensitive rice cvs, which was therefore, accompanied by comparably higher root : shoot in sensitive cvs than the tolerant ones. Further, Sekmen et al. (2007) reported the parallel observations and reported more growth reduction in shoot than root growth, with more pronounced effects in salt sensitive species of plantain. Like results of the present investigation, root-shoot ratio has been reported to increase under increasing salt stress in tomato by Maggio et al. (2007). Furthermore, similar trends were also evident in soybean and alfalfa (Berstein and Ogata, 1966), cotton (Meloni et al., 2001) and Prosopis alba (Meloni et al., 2004). Results of present investigation are in harmony of all these reports and root/shoot was increased with salt concentration, with lower to higher root/shoot ratio in sensitive to tolerant cvs.
Biomass production was influenced by NaCl stress significantly as the salinity level increased (Table 4.3 and Table 4.4). Highest fresh weight (95 mg plant⁻¹) of shoots was recorded at 300 mM in PNL-3, whereas lowest fresh weight (31 mg plant⁻¹) was observed in KJT-3 shoots. Similarly, the lowest dry weight (9 mg plant⁻¹) of shoots was observed in KJT-3, while maximum was observed in PNL-3 with 17 mg plant⁻¹. Similar trends were followed in fresh and dry weight of roots and PNL-3 emerged as the salt tolerant cv with maximum fresh weight (81 mg plant⁻¹) and dry weight (16 mg plant⁻¹) at 300 mM salt concentrations as compared to 38 and 6 mg fresh weight and dry weight of roots plant⁻¹ of KJT-3 respectively.

Likewise, the RWC was also decreased at a higher rate in KJT-3, whereas other genotypes were not so affected by NaCl stress (Table 4.5). Similar trends were reported by Sairam et al. (2002 and 2005) for wheat genotypes and Sumithra et al. (2006) for mungbean genotypes.

Results of the present investigation are in confirmation of previous studies, in which various researchers (Yeo and Flowers, 1983; Yeo et al., 1990; Garcia et al., 1995; Misra et al., 1997; Dionisio-Sese and Tobita, 1998; Alam et al., 2002; Lee et al., 2003; Zafrar et al., 2004; Anil et al., 2005; Singh et al., 2007) concluded that salt tolerant rice cvs showed lesser effect of NaCl stress, as reflected by greater germination percentage, biomass production and overall better survival rate than salt susceptible rice cvs when irrigated with NaCl-dominated waters. These results made it clear that PNL-3 could withstand salinity stress with comparable ease than other cvs at germination level. On the basis of the results obtained during germination and seedling stages, the cvs were categorized as salt tolerant (PNL-3), moderately-salt tolerant (KR) followed by comparably salt sensitive cv (KJT-3). These three cvs were selected and used for further biochemical and enzymatic analyses to observe their differential behaviour under varying NaCl stress.

5.2 Effect of NaCl stress on biochemical parameters (chlorophyll, protein, proline, lipid peroxidation, phenol, starch and sugars) in rice cultivars:

The salt stress responses are so complex that it affects virtually all the physiological and biochemical processes starting from germination to biomass production, rate of photosynthesis, ionic balance and finally crop yield. NaCl stress affects rice crop worldwide
as it is considered a moderately to sensitive crop towards NaCl stress. In general, rice plants are very sensitive to salinity stress at the young seedling stages and less so at reproductive stages (Lutts et al., 1995), hence improvement in salinity tolerance of rice is likely to be most successful by investigating sensitivity at the most susceptible growth stages (Singh et al., 2007). Therefore, we investigated salinity tolerance of six *indica* type rice genotypes at germination (discussed in previous section, 5.1) and young seedling growth stages in terms of biochemical parameters including chlorophyll pigments, total protein content, starch, reducing and non-reducing sugars lipid peroxidation, free proline accumulation and antioxidant enzyme activities and enzyme expression using native PAGE. All these parameters were greatly influenced by salinity stress; we are discussing the results of these experiments in the following sections.

Chlorophyll (Chi) content is fundamental to understand the plant response to the environment in which it resides. To know if plants are damaged in the photosynthetic metabolism as a consequence of salt stress, we decided to measure the Chi a, Chi b and total Chi content in the leaves of all the three selected rice genotypes under varying levels of NaCl-driven salt stress. Chi contents in this study were markedly reduced due to 7 days of NaCl stress in leaves of rice cvs at vegetative stage. Salt sensitive rice genotype KJT-3 showed significantly higher decline in chlorophyll a, b and total Chi pigments than comparably tolerant genotypes PNL-3 and KR. Indeed, Chi a and b levels were not equally affected by salinity and Chi a was reduced at much higher rate in salt sensitive cv KJT-3 with increasing salinity (Table 4.6 and Fig. 4.1). Similar to the observations obtained from present investigation, Singh et al. (2007) also observed more reduction in Chi a than Chi b in rice cvs under salt stress. Total Chi content was less affected in PNL-3 cv followed by KR and KJT-3 respectively (Fig. 4.1). This loss of Chi content could be associated with the accumulation of Na in the leaves, as evident from the results. According to Choudhary and Choe (1996) decline in photosynthetic rate with increased concentration of NaCl may be associated with decreased pigmentation concentration. These results clearly indicated that salinity stress negatively impact Chi content and therefore, photosynthesis process, which in turn reduce the biomass production, which was evident in present investigation. Thus, more degradation in Chi content can easily be correlated with higher biomass degradation in sensitive rice genotype. Lutts et al. (1996) observed highly significant decrease in Chi concentration with increasing salinity in salt sensitive rice genotypes, while much lesser
affect was recorded in salt tolerant genotypes. Misra et al. (1997) reported that the Chl contents were affected heavily by NaCl stress in salt sensitive rice cv Jaya, while in salt tolerant cv Damodar, more Chl accumulation was observed than the control plants. Similar observations are reported by Faustino et al. (1996); Theerakulpisut et al. (2005) and Singh et al. (2007) reported that total Chl content was reduced more in salt sensitive rice cvs than tolerant ones. Similar genotypic variations, with comparably more Chl content degradation in salt sensitive genotypes than the tolerant ones have been reported in a number of crop plants including pea (Hernandez et al., 1995), mulberry (Kumar et al., 2003), wheat (Sairam et al., 2005) and green gram (Misra and Gupta, 2005). The results of present study are in harmony of these observations and a better Chl stability of salt tolerant cv PNL-3 (relatively lesser break-down of chlorophylls) supported the salt tolerant nature of cv PNL-3.

In the present investigation, overall higher protein content was observed in shoots (including leaves) than roots of all the cvs irrespective of salt concentration (Table 4.7 and Fig. 4.2). However, the effect of salinity level was also more pronounced on shoots of rice genotypes than the roots. The protein contents were increased up to 100 mM NaCl concentration than the control in salt tolerant cvs PNL-3 and KR, however, the protein levels were highly reduced at all levels of salinity from 50 mM to 300 mM NaCl in the cv KJT-3 (Fig. 4.2). Likewise, Agastian et al. (2000) have observed that total soluble proteins increase at low salinity and decrease at high salinity in mulberry. In mungbean seedlings also, Zayed and Zeid (1998) reported reduction in total proteins content under salinity stress. However, various researchers have reported differential genotypic responses between salt sensitive and tolerant cvs in terms of protein content. Similar to trends obtained in present study, Lutts et al. (1996) observed comparably higher reduction in salt sensitive rice cvs than the tolerant ones under NaCl stress in terms of protein content. Misra et al. (1997) also reported that salinity stress resulted in comparably higher decrease in protein content in salt sensitive rice cv than their tolerant counterparts. A higher content of soluble proteins has been observed in salt tolerant cvs of barley, sunflower, finger millet and rice (Ashraf and Harris, 2004). However, contrary to these results, Ahmad and Jhon (2005) concluded that NaCl stress had positive impact on total protein contents in *Pisum sativum* and observed gradual increase in protein contents with increasing salinity.

Proline is one of the most important and sought after osmolytes found in plants and related with abiotic stress tolerance. Proline is generally assumed to serve as a physiologically
compatible solute that increases as needed to maintain a favourable osmotic potential between the cell and its surroundings (Pollard and Wyn Jones, 1979). In addition to this, proline is an important osmoprotectant in plants and can protect the photosynthetic machinery against salt induced damage (Sivakumar et al., 2002, Jogeswar et al., 2006). Rapid accumulation of free proline is a typical response to salt stress (Demiral and Turkan, 2005; Parida et al., 2008). When exposed to drought or a high salt content in soil, many plants have been observed to accumulate high amounts of proline, in some cases several times the sum of all other amino acids (Ali et al., 1999; Mansour, 2000). The accumulation of free proline has been studied in a number of taxa subjected to hyperosmotic stress conditions for over 45 years (Jogeswar, 2005).

In the present investigation, the NaCl treatment induced an increase in proline content in shoots and roots of rice cvs irrespective of their salinity tolerance capability. The increase in proline content was positively correlated to the level of salt tolerance. The comparison in terms of proline content in shoots and root tissues amongst the rice cvs used in this study revealed that very high rate of proline accumulation was seen in salt tolerant cv PNL-3 followed by moderately tolerant cv KR and the rate of increase in proline content with salinity was significantly lower than the earlier cvs. Salt tolerant cv PNL-3 accumulated comparably very high amount of proline under 300 mM salinity level both in shoots (7556 µg g⁻¹ DW, Table 4.8 and Fig. 4.3a) and roots (8293 µg g⁻¹ DW, Table 4.8 and Fig. 4.3b) as compared to the salt sensitive cv KJT-3 (2504 µg g⁻¹ DW in shoots and 3237 µg g⁻¹ DW in roots). Results of the present study are in conformity with various reports, stating the steep increase in proline levels under salinity stress in a number of crops including rice (Kavikishor, 1988, 1989; Lutts, et al., 1999; Khan and Panda, 2002; Lin et al., 2002; Hur et al., 2004; Su and Wu, 2004; Demiral and Turkan, 2005), *Pisum sativum* (Ahmad and Jhon, 2005), cactus pear (Silva-Ortega et al., 2008) and cotton (Parida et al., 2008). Further, as in the present study, Sairam et al. (2002, 2005) in wheat, Kumar et al. (2003) in mulberry, Misra and Gupta (2005) in green gram, Jogeswar et al. (2006) in sorghum and Koca et al. (2007) in sesame cvs observed much higher proline accumulation in salt tolerant genotype than their salt sensitive counterparts. Contrary to these results, Vaidyanathan et al. (2003) and Demiral and Turkan (2005) concluded higher proline accumulation salt sensitive rice cvs than salt tolerant cvs under high salinity stress.
However, a large number of researchers have advocated the major role played by the proline in salt stress tolerance and post-stress recovery of the plants. Proline has been found to protect cell membranes of onion against salt injury (Mansour, 1998). Sultana et al. (1999) have suggested that proline accumulation in both salinised leaves and grains of rice plants is implicated in osmotic adjustment to salinity. Proline accumulation under stress conditions may either be caused by induction or activation of enzymes of proline biosynthesis or a decreased proline oxidation to glutathione, decreased utilization of proline in protein synthesis and enhanced protein turnover (Delauney and Verma, 1993). The expression of genes encoding key enzymes of proline synthesis (PSCS, EC 2.7.2.11; PSCR, EC 1.5.1.2) and proline oxidation (proline dehydrogenase- PDH; EC 1.4.3) is controlled by osmotic and salinity stress and proceeds the increase in or decrease in proline concentration in plant tissues (Strizhov et al., 1997). It is suggested that proline may be the major source of energy and nitrogen during immediate post-stress metabolism and the accumulated proline apparently supplies energy for growth and survival thereby inducing salinity tolerance (Ahmad and Jhon, 2005). Present investigations' results supported these hypotheses and we concluded that comparably higher proline accumulation under progressing salt stress in PNL-3 might have played a major role in salt tolerance capability of this cv.

Lipid peroxidation measured as amount of MDA is produced when polyunsaturated fatty acids in the membrane undergo oxidation due to the accumulation of free oxygen radicals. As lipid peroxidation is the symptom mostly ascribed to oxidative damage, it is often used as an indication of membrane damage and leakage under salt stress conditions (Katsuhara et al., 2005; Khan and Panda, 2008). The results presented in Table 4.9 and Fig. 4.4 clearly indicated that peroxidation of lipid membranes was affected highly by NaCl stress in shoots and roots of all the three cvs. Generally, the rate of lipid peroxidation was overall higher in shoots than the roots. Amongst the rice cvs, maximum lipid peroxidation, as suggested by MDA content, was observed in KJT-3 (shoots) and KR (roots). The damage to the lipid membranes was comparatively less severe in the shoots and roots of salt tolerant cv PNL-3, which may be because of better antioxidative machinery, as seen in the results (Table 4.9 and Fig. 4.4). This seems to be important sign of higher oxidative-damage limiting capacity of PNL-3 under salinity stress. The severe damage was observed in KJT-3 while intermediate responses were observed in KR cv. Growth inhibition under salinity in cv KJT-3 is in good correlation with increased lipid peroxidation levels. The results of present study
are in agreement of earlier reports, in which higher lipid peroxidation in sensitive rice cvs than the tolerant one have been reported. Dionisio-Sese and Tobita, (1998); Vaidyanathan et al. (2003); Demiral and Turkan (2005); Singh et al. (2007); and Khan and Panda (2008), have reported greater lipid peroxidation under salinity stress in salt sensitive rice genotypes than their tolerant counterparts. Further, parallel trends have also been reported in pea (Hernandez et al., 1993); wheat (Sairam et al., 2002 and 2005); tomato (Koca et al., 2006) and sesame (Maggio et al., 2007).

Such kind of responses may be attributed to the generation of higher amount of reactive oxygen species or free radicals in salt sensitive cvs than salt tolerant ones under high salt concentration. It is already known that free radical-induced peroxidation of lipid membranes is a reflection of stress-induced damage at the cellular level (Jain et al., 2001). Therefore, the level of MDA, produced during peroxidation of membrane lipids, is often used as an indicator of oxidative damage. The lower level in PNL-3 shoots and roots than of KJT-3 and KR suggests that it may have better protection against oxidative damage under salt stress. The improved protection in PNL-3 may reflect a more efficient antioxidative system as evidenced by a higher activity of enzymes such as SOD, CAT, POX, PPOX and GR (Tables 4.17, 4.18, 4.19, 4.20 and 4.21, respectively).

The amounts of total phenols were increased in shoots of KJT-3 with highest magnitude followed by cv KR with progression of soil salinity (Table 4.10, Fig. 4.5a). However, in the shoots of PNL-3, the phenols were either had no significant increase or even reduced as the soil salinity increased. In the roots, similar kinds of trends were seen and total phenol contents were not significantly affected by NaCl stress in PNL-3 cv (Table 4.10, Fig. 4.5b). The moderately salt tolerant cv KR and salt sensitive cv KJT-3 showed more or less same amount of phenols and positive correlation with salinity level was observed in these cvs. Similar results are reported by Parida et al. (2002), and author reported increase in polyphenol levels in Brugiera parviflora under salt stress.

In general, starch content was higher in shoots than the roots of all the cvs, both under non-saline and varying salinity stress conditions. The starch content increased steeply in PNL-3 shoots up to 150 mM NaCl, with >3 times more starch content than control shoots, however, starch content decreased thereafter (Table 4.11, Fig 4.6a). On the other hand, starch content decreased drastically with increasing salt stress in KJT-3 shoots, while not much difference
in starch content was noticed in KR shoots. But comparison between the cvs in terms of starch content revealed that PNL-3 showed much higher starch content than other two cvs under both non-saline as well as varying saline conditions. In root tissues, the starch content was around 2.5 times higher in PNL-3 than other two cvs under non-saline conditions. Similarly, at the severe salinity stress (300 mM NaCl), starch content was around 4 times more in roots of PNL-3 than KJT-3 and around 1.5 times more than KR (Table 4.11, Fig 4.6b). Rathert (1984) advocated the role of starch as an indicator of salt stress tolerance in various crops including rice, soybean, and cotton. A decrease in salt stress driven starch content has been evidenced by Parida et al. (2002) in Brugiera parviflora. Similar to the results of present investigation, Kafi et al. (2003) observed higher starch content under non-saline conditions in salt tolerant wheat cvs than the sensitive one. Further, they observed higher starch content in salt tolerant wheat genotype Kharchia than sensitive cv Ghods at 300 mM NaCl stress. Dubey and Singh (1999) obtained the similar kind of results and concluded that the starch content was reduced with much higher magnitude in roots of salt sensitive rice cvs than that of salt tolerant ones up to moderate salinity stress (150 mM). However, they did not observed any significant change in starch content in shoots by salinity stress. Starch is an important component of plant tissues and accumulates in leaves as a temporary reserve form of carbon and is the principal component of dry mass accumulated in mature leaves, hence the accumulation of more starch in salt tolerant cv PNL-3 may seen as the protective mechanism during stress conditions.

In the present investigation, significantly higher amount of reducing sugars were found in shoots as well as roots of KR as compared to other two cvs under non-saline as well as saline conditions. In KR shoot tissues, reducing sugars increased with increase in salinity stress up to 100 mM NaCl (55 mg g⁻¹ DW) and decreased afterwards at all the salinity levels, however, at 300 mM NaCl it showed 96% reducing sugar contents of control shoots. On the other hand, reducing sugars were decreased with increasing salinity levels in KJT-3 and PNL-3 cvs, with KJT-3 showed 74% of control and PNL-3 showed 53% reducing sugar contents of control at 300 mM NaCl stress level (Table 4.12, Fig. 4.7a). In roots, reducing sugars were decreased gradually with increasing salt stress in KR and PNL-3, whereas, reducing sugar were increased significantly with salinity up to 200 mM (8.82 mg g⁻¹ DW) in cv KJT-3. Around 155% reducing sugars of control were observed in KJT-3 roots against 51% of KR and 40% in PNL-3 at 300 mM NaCl (Table 4.12, Fig. 4.7b).
Comparably much higher non-reducing sugar levels were observed in PNL-3 shoots under control conditions with around 3 times more than KJT-3 and KR shoots, while in roots also it was higher by around 1.5 and 3.5 times more respectively. In PNL-3 shoot tissues, non-reducing sugars, even though after a slight increase at 50 mM NaCl, decreased gradually under increasing salt stress and at 300 mM NaCl, 70% non-reducing sugars of control were observed. However, at 300 Mm NaCl stress level, the non-reducing sugar levels of PNL-3 shoots were higher than KR by around 4 times, while almost same amount was observed in KJT-3 shoots (Table 4.13, Fig. 4.8a). Contrary to the results obtained in shoots, the non-reducing sugars uniformly decreased with increasing salinity stress in roots of all the cvs. The highest effect was observed on KJT-3 and non-reducing sugars were less than 50% of control in this cv at 300 mM (85 mg g⁻¹ DW) as compared to 63% in KR and 69% in PNL-3 (Table 4.13, Fig. 4.8b).

Similar to the findings of present study, Ashraf and Tufail (1995) determined the total soluble sugar content in five sunflower accessions differing in salt tolerance; the salt tolerant lines had generally greater soluble sugars than the salt sensitive ones. Studies by Dubey and Singh (1999) observed the increase in soluble sugars in rice plants under salinity stress. They observed more increase in the sugar contents in salt sensitive cvs than salt tolerant one. Ashraf (1997) observed increase in reducing sugars in Cenchrus pennisetiformis. Parida et al. (2002) also reported increase in sugar content in Brugiera parviflora under salt stress. Increased accumulation has been reported in many plant species exposed to salinity (e.g. Flowers et al., 1977; Chaillou and Guerrier, 1992). But the pattern of increase in sugar contents has been reported to depend on genotypes and salt concentrations. It is believed that under salinity stress accumulation of sugars along with other compatible solutes may contribute to osmotic adjustment. Parvaiz and Satyawati (2008) advocated the role of soluble carbohydrates in plants in osmoprotection, osmotic adjustment, carbon storage and radical scavenging. The results obtained from present study also confirmed this hypothesis and indicated the correlation between salt tolerance capacity of PNL-3 and higher sugar contents under non-saline as well as saline conditions.
5.3 Effect of NaCl stress on mineral nutrients in rice cultivars:

Ionic balance in the cells is an important phenomenon required for normal growth of plants. Under salt (NaCl) stress conditions the ionic balance gets disturbed and accumulation of Na has been a known fact now. The Na/K ratio is of great importance in cells, as it is significantly affected by high salt concentration in soil. Plants growing under saline conditions accumulate more of sodium and chloride resulting in ionic imbalance, specific ion effects and nutrient deficiency symptoms in plants and also affect plant metabolism by toxic effects by accumulated ions (Agrawal, 1964).

In the present investigation, the sodium content was increased in all the cvs with increase in salinity stress. The sodium accumulation rate was more in shoots than the roots under salt stress in all the genotypes (Table 4.14, 4.15 and 4.16). In the roots of cv PNL-3 the accumulation was lowest amongst the three genotypes at 300 mM NaCl (7.2 mg g^{-1} DW) against 4.7 mg g^{-1} DW at control (Table 4.16). However, sodium accumulation was highest in shoots of PNL-3 at high salinity level than the other two cvs. The potassium (K) content was highly affected by salt stress. Potassium content was decreased significantly at a much higher rate in roots and shoots of KJT-3, than KR and PNL-3. The reduction in potassium levels was lowest in both shoots and roots of cv PNL-3. Sodium/potassium ratio was less in both shoots and roots of PNL-3 as compared to KJT-3. The Na/K ration was even below one at 300 mM NaCl stress in roots of PNL-3. These results suggested that PNL-3 was less affected by high salinity stress as high salinity is reported to inhibit the efficiency of translocation (Iqbal and Ashraf, 2007).

Salinity induced increase in sodium and depletion of potassium contents have been reported previously in a number of crop species including mungbean (Zayed and Zeid, 1998), wheat (Sairam et al., 2002; Mandhania et al., 2006) and rice (Nguyen et al., 2005; Singh et al., 2007; Khan and Panda, 2008; Roshandel and Flowers, 2008). Less accumulation of Na and more accumulation of K are general phenomena in the salt tolerant varieties of rice as suggested by various researchers (Vaidyanathan et al., 2003; Lee et al., 2003; Singh et al., 2007; Khan and Panda, 2008; Roshandel and Flowers, 2008). Similar trends were observed by Anil et al. (2005) as they recorded more depletion in K ions and more accumulation in Na contents in salt sensitive varieties under salinity stress. Further El-Baz et al. (2003) also reported the similar trends in cucumber genotypes. The results obtained from present...
investigation are in harmony of these conclusions and we recorded less depletion of K ions in salt tolerant cv PNL-3. The results evidenced that the Na exclusion was not present in salt tolerant cv PNL-3; however it accumulated K ions in order to maintain Na/K ratio and ion homeostasis (Table 4.14, 4.15 and 4.16). It has been suggested that the plant’s tolerance response is characterized by distinctly lower sodium/potassium ratio, which may be used to predict tolerance or salinity in crop varieties (Joshi et al., 1979; Mandhania et al., 2006; Singh et al., 2007; Khan and Panda, 2008; Roshandel and Flowers, 2008).

Amongst the other mineral ions, CI contents increased more significantly with increasing salt concentrations in salt sensitive cv KJT-3 (Table 4.14) than salt tolerant cv PNL-3 (Table 4.16), while intermediate results were seen in cv KR (Table 4.15). Similar results have been reported earlier in pea by Hernandez et al. (1999) and in sorghum by Jogeswar et al. (2006). Gibbs et al. (1989) suggested that the elements provided in salt water were generally the major components for osmotic adjustment in salt-treated plants. The accumulation of ions such as Na and CI in the roots and shoots of rice genotypes in this investigation supports this idea.

It is evident that beneficial effects of Ca on salt stressed plants are associated with the maintenance of cell membrane integrity that may reduce Na absorption and favour K absorption (Epstein, 1998; Iqbal and Ashraf, 2007). The results of present investigation are in agreement of this and Ca contents gradually increased in shoots and roots of PNL-3 with increasing soil salinity, while contrasting trends were evidenced in shoots as well as roots of KJT-3 with linear reduction in Ca content with increasing salinity level. These results clearly indicated that accumulation of Ca under salinity stress provided a protective mechanism to combat salt stress in PNL-3. Jogeswar et al. (2006) also reported the similar trends and observed higher Ca content in tolerant sorghum cv than the sensitive one under short term salinity stress. Further, Anil et al. (2005) advocated the role of Ca in altering the ion selectivity of uptake by plants and in enhancing the salt tolerance in plant species. The Mg content was slightly reduced by salinity stress in shoots of KJT-3, while it was increased under the salt stress in shoots of KR and PNL-3 (Table 4.14, 4.15 and 4.16). Similarly the Mg content was reduced in roots also of the cv. KJT-3, however, while it was gradually increased in roots of KR. However, in case of PNL-3 roots, the Mg content was enhanced up 100 mM NaCl and it was same as to control up to 200 mM, while a slight
reduction was observed in Mg content. Zayed and Zeid (1998) reported the similar patterns and observed the reduction in Mg content in shoots under soil salinity but an increase was seen in roots in mungbean seedlings.

However, contrasting trends were seen in case of P content, and its amount was considerably increased under salinity stress in shoots and roots of KJT-3, whereas, in KR and PNL-3, after a slight increase in P content at lower salt concentration, a linear decrease was noticed under high salinity stress (Table 4.14, 4.15 and 4.16). Hu et al. (2006) reported lower amounts of P in salt-stressed wheat compared to the crop grown under non-saline conditions.

Amongst other nutrients, N, Fe and Zn all were decreased with increasing soil salinity in shoots and roots of KJT-3 and KR, except Fe content, which was significantly increased in KR, both in shoots and roots. In the salt tolerant cv PNL-3, amongst these nutrients, no significant affect was seen on N contents in shoots however, it was reduced in roots. Other nutrients such as Fe and Zn were also reduced in PNL-3 under salinity stress. However, the rate of reduction in these nutrients was lower in PNL-3 than in other two cvs.

These results clearly revealed that PNL-3, a salt tolerant cv has comparatively better machinery in terms of maintaining ionic concentrations and their homeostasis to sustain salinity stress tolerance than the other cvs.

5.4 Effect of NaCl stress on activity of antioxidant enzymes (SOD, CAT, POX, PPOX and GR) in rice cultivars:

Salinity stress limiting photosynthesis can increase oxygen-induced cellular damage due to increased ROS generation (Alscher et al., 2002; Mittler, 2002). Therefore, salt stress resistance may depend, at least in part, on the enhancement of the antioxidative defence system, which includes antioxidant compounds and several antioxidative enzymes. In the present study, the responses of SOD, CAT, POX, PPOX and GR enzyme activities suggest that oxidative stress and resulting activation of enzymatic machinery is an important component of salt stress and its tolerance in rice plants.

The aim of the present study was to study the comparative enzymatic ROS scavenging mechanisms operational in rice cvs with differential salt stress tolerance. This may be helpful to shed the light on the key components employed by salt-tolerant cvs to combat
salinity induced oxidative stress. This in turn may be helpful in the identification of candidate genes for salt-stress tolerance of important crop plants. Thus the present work reports activities of important antioxidant enzymes separately from shoots and roots in the rice cvs PNL-3, KR and KJT-3 to NaCl stress. Under normal conditions, there is a balance between the production of reactive oxygen species (ROS) and their removal in plants, but this balance is sensitive to and destroyed by salt stress which consequently results in accumulation of ROS. Increasing evidence exists that excessive ROS may initiate membrane lipid peroxidation, weaken membrane lipid unsaturation, trigger membrane protein polymerization, and result in an increase in membrane permeability (Chen, 1991; Hernandez et al., 2000; Khan and Panda, 2008). ROS can also cause dysfunction of proteins, attack nucleic acids, lead to DNA destruction and mutation (Imlay and Linn, 1998), disrupt DNA copy and transcription (Byrd et al., 1990), and finally cause cell death and formation of heat shock like proteins. Therefore, to scavenge ROS, plants have evolved specific defence tactics involving both enzymatic such as superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT), as well as non-enzymatic like proline, ascorbic acid and glutathione.

As described above, salt stress is detrimental to normal functioning of plants and may cause membrane damage, and stimulate formation of different ROS such as superoxide, hydrogen peroxide and hydroxyl radicals. Among ROS, superoxide is converted by SOD enzyme into $\text{H}_2\text{O}_2$, which is further scavenged by CAT and POXes (Demiral and Turkan 2005). Peroxidases are also reported to be involved not only in scavenging the $\text{H}_2\text{O}_2$ produced in chloroplasts but also in growth and developmental processes (Dionisio-Sese and Tobita 1998). The results of many studies (Singha and Choudhury, 1990; Lin and Kao, 2000; Vaidyanathan et al. 2003; Demiral and Turkan, 2005; Kumar et al. 2007) suggested that the resistance to salt stress is usually correlated with a more efficient antioxidative system, with high enzymatic activities and better biomass production in salt tolerant cvs of rice than sensitive ones. Increase in enzymatic activities has also been reported in wheat under salt stress with comparatively higher activities in tolerant genotypes by Sairam et al. (2002, 2005, 2006). The results of present study are in conformity of these reports and conclude that the better antioxidant enzymatic machinery may be correlated with salt tolerance ability of the tolerant rice genotypes.

In the present investigation, all the antioxidant enzymes (SOD, CAT, POX and PPOX) were significantly increased with increasing salinity concentrations in PNL-3. SOD is one of the
ubiquitous enzymes in aerobic organisms and plays a key role in cellular defence mechanisms against ROS. Its activity modulates the relative amounts of $\mathrm{O}_2^{-}$ and $\mathrm{H}_2\mathrm{O}_2$, the two Haber–Weiss reaction substrates and decreases the risk of $\mathrm{OH}^-$ radical formation, which is highly reactive and may cause severe damage to membranes, protein and DNA (Bowler et al., 1992). In the present study, SOD activity was considerably higher in PNL-3 than other two cvs, both under control as well as saline conditions (Table 4.17 and Fig. 4.9). The SOD activity was increased steeply in roots and shoots with salinity level in PNL-3 and showed maximum activity at 300 mM NaCl followed by KR and KJT-3. However, SOD activity was decreased gradually with increasing soil salinity level in KJT-3 and KR roots, suggesting that the salt-tolerant genotype has a better $\mathrm{O}_2^{-}$ radical scavenging ability. It has been shown that salinity increases SOD activity in salt-tolerant rice cvs and decreases it in salt-sensitive ones (Dionisio-Sese and Tobita, 1998; Singh et al., 2007; Khan and Panda, 2008). In addition, Badawi et al. (2004) reported that transgenic tobacco plants over-expressing Cu/Zn-SOD showed enhanced tolerance to salt and water stresses as well as polyethylene glycol-induced stress.

The product of SOD activity is $\mathrm{H}_2\mathrm{O}_2$, which is still toxic and must be eliminated by conversion to $\mathrm{H}_2\mathrm{O}$ in subsequent reactions. In plants, a number of enzymes regulate $\mathrm{H}_2\mathrm{O}_2$ intracellular levels, but CATs and POXes are considered the most important. Results given in Table 4.18 and Fig. 4.10 revealed that overall higher CAT activity was found in roots and shoots of PNL-3 cv, irrespective of their saline or non-saline environment. The CAT activity was elevated with increasing salinity levels both in shoots and roots of PNL-3, while rate of increase in CAT activity with salinity was to lesser extent in KJT-3. Like these two enzymes, the activities of POX and PPOX were also positively raised very high with progression in salinity level in shoots and roots of the rice cv PNL-3. The POX activity was also increased in KR and KJT-3 cvs but the rate of increase was much lesser than PNL-3 (Table 4.19 and Fig. 4.11). In PNL-3, the POX activity was about 3 times more at 300 mM NaCl stress than at control. Similar to the results of present investigation, Roychoudhury et al. (2008) observed steep increase in POX activity in response to salt stress in rice cvs. Increased total peroxidase activity in response to salinity reflects the changing mechanical properties of the cell wall, which in turn could be related to the salt adaptation process (Sancho et al. 1996; Roychoudhury et al., 2008). The PPOX activity was elevated in roots and shoots of PNL-3, the increase in activity from control to 300 mM NaCl level was
recorded more than 3 times in roots and shoots (Table 4.20 and Fig. 4.12). However, the PPOX enzyme activities were slightly decreased in roots of KR and KJT-3, with more affect on KJT-3.

The data given in Table 4.21 and Fig. 4.13 showed that in general, highest GR activity was observed in both shoots and roots of PNL-3 at control as well as progressing soil salinity levels. High salinity stress significantly reduced GR activity in salt sensitive cv (KJT-3) and salt moderately-tolerant cv KR. On the other hand, contrasting observations were noticed in PNL-3, as activity was increased markedly due to salt stress both in shoots and roots (Table 4.21 and Fig. 4.13). The reduction in activity of GR in comparably salt sensitive cvs suggests a decreased GSH turnover rate. Several authors investigating antioxidant responses of salt-tolerant and salt-sensitive cvs of various crops have suggested that the salt tolerance character is related to increased GR activity in salt-tolerant cvs (Hernandez et al. 2000; Sudhakar et al. 2001; Meloni et al. 2003; Sairam et al. 2005).

Results of present study clearly indicated that the antioxidative enzymatic mechanism was more active in PNL-3, as evidenced by higher enzyme activities. Higher enzymatic activities might have played a role in scavenging the ROS species under high salinity stress and would have provided the salt stress tolerance to the plants of cv PNL-3. Various studies have shown that salt tolerance may be improved if the free radicals formed during the accompanying activated/ reactive oxygen damage are scavenged by an enhanced antioxidative defence system (Alscher et al., 2002; Shigeoka et al., 2002). There are enough evidences that the alleviation of oxidative damage and increased tolerance to salinity and other environmental stresses is often correlated with an efficient antioxidative system (Scandalios, 1993; Hasegawa et al., 2000b; Bor et al., 2003; Demiral and Turkan, 2005). Dionisio-Sese and Tobita (1998) studied the activities of SOD and POX enzymes under NaCl stress in leaves of four cvs of rice differing in their salt stress tolerance. In addition, PPOX is also considered to have a role in scavenging ROS (Saiedian et al., 2007). These results have indicated that salt tolerance capacity of salt-tolerant species is closely related with the maintenance of specific activity of antioxidant enzymes studied.

Further, Vaidyanathan et al. (2003) have found higher activities of CAT and SOD in rice varieties under salt stress conditions than control. They observed more pronounced increase in enzyme activities in salt tolerant rice cv Pokkali, while these enzyme activities were either
reduced or did not showed any notably increase. A sharp increase in activities of SOD and CAT enzymes under NaCl stress has been reported by Nguyen et al. (2005). El-Baz et al. (2003) reported that POX activity was sharply enhanced by salinity stress in cucumber, while similar observations have been reported by Sreenivasula et al. (1999) in foxtail millet. Similarly Koca et al. (2006) observed higher SOD and POX activities in salt tolerant tomato genotypes than the salt sensitive genotypes. Contrary results have been reported by Khan and Panda (2002) in roots of rice and observed a gradual decrease in CAT and SOD activities with increasing salinity stress. It is a general and widely accepted view that SOD activity (both constitutive and induced) of salt tolerant cvs would substantially higher in comparison to sensitive ones. Higher SOD activity has been reported in salt tolerant genotypes of cotton (Gossette et al. 1994), barley (Acar et al. 2001), tomato (Shalata and Tal 1998) and wild beet (Bor et al. 2003) than their respective sensitive counterparts. The results of present investigation are in harmony with these observations and we can conclude that SOD has a major role to play in salt tolerance ability of rice genotypes.

Furthermore, comparatively higher enzymatic activities in salt tolerant cvs than susceptible ones have been reported in wheat (Sairam et al., 1997, 1998, 2002), Pisum sativum (Hernandez et al., 2000) and maize (Neto et al., 2006), suggesting that higher antioxidant enzyme activity have a role in imparting tolerance to these cvs against environmental stresses. In this background higher SOD, CAT, POX, GR and PPOX activities in PNL-3 under increasing salinity stress signifies its relative tolerance to salinity stress, while KJT-3 was inferior on this count.

5.5 Effect of NaCl stress on isozymatic activity patterns of SOD, CAT and POX using native PAGE) in rice cultivars

5.5.1 Effect of NaCl stress on isozymatic activity pattern of SOD

The SODs can be divided into three classes based on the metals present in active site: Cu/Zn-, Mn-, or Fe-SOD (Lee et al., 2001). In the present experiment, we observed all the three types of SODs. The results given in Plate 5a, clearly suggested that at least four clear SOD isoforms were observed in native PAGE gel after soluble proteins from shoots were electrophoresed followed by staining using inhibitors. These isoforms identified as one Mn-SOD, two CuZn-SODs, named II and I in order of increasing migration, and one Fe-SOD
on the basis of assays performed in the presence of selective inhibitors. All three bands have been reported earlier in rice cvs (Singh et al., 2007). The Fe-SOD band was quite faint, in contrast CuZn and Mn-SOD bands were very clear. No significant effect of NaCl stress ranging from 100 – 200 mM seen on CuZn SOD I and Fe-SOD activities of KJT-3 and KR shoot tissues. However, an increase in activity (as indicated by staining intensity) was noticed in Mn-SOD and CuZn-SOD II at 100 mM NaCl stress against the control shoot tissues of KJT-3, but these activities reduced a bit at 200 mM NaCl. On the other hand, the band intensity of CuZn SOD II increased under increasing salt stress in shoot tissues of PNL-3. These results support the data obtained in terms of total SOD activity (Table 4.17), where maximum increase in SOD activity was observed in shoots of PNL-3 genotype than other cvs under salt stress. The enhancement of total SOD activities appeared to be due to preferential induction of specific SOD isozymes such as Cu/Zn-SOD I and II. The present study’s results are in harmony of the earlier results, where salt stress caused an increase in Cu/Zn-SOD II activity, both in leaves as well as roots of rice genotype, as revealed by the native activity-gel (Lee et al., 2001). However, they could not detect any Fe-SOD isoform in activity-gel. Similar results are also reported by Fadzilla et al. (1997) and Singh et al. (2007) as they observed increase in Cu/Zn-SOD under salt stress in shoot tissues of rice. Further, similar increase in SOD activity was observed in response of salt stress in common bean (Jebara et al., 2004) and in response to water deficit in white clover (Chang-Quan and Rui-Chang, 2008).

5.5.2 Effect of NaCl stress on isozymatic activity pattern of CAT

The CAT zymogram (Plate 5b) revealed two isoforms, one small band CAT I, and one prominent major band CAT II. The CAT I did not show any significant variation in activation under the influence of progressing NaCl stress. On the other hand, clear effects of salinity stress seen on CAT II isozyme. CAT II activity was increased up to 100 mM NaCl stress, however, the activity was lower at 200 mM NaCl stress as compared at 100 Mm NaCl stressed shoots, as the band intensity appeared to decline in response to this salinity stress level. CAT isozymes activated under progressive salt stress in shoots of comparably salt tolerant cvs (Plate 5b). Amongst these two comparably salt tolerant cvs, the enhancement of activity in CAT II was significantly higher in PNL-3 than KR.

Similar to the observations made in present work, Jogeswar et al. (2006) have reported more increase in CAT isozymes under salt stress in tolerant cv than sensitive one. Varying results
have been reported however in rice, where Tsai et al. (2004) noticed lower CAT activity under salt stress, on the other hand Lin and Kao (2000) reported no change in CAT activity in rice under salt stress. Further, higher CAT activity was noticed in tobacco (Bueno et al. 1998) and mulberry (Sudhakar et al. 2001) in salt stressed plants than control plants. These results clearly indicated that antioxidant enzyme activation varies among crop species and in the present case higher CAT activity in salt tolerant cv PNL-3, as revealed both by spectrophotometric analyses as well as by in-gel assays, may have a significant role in its salt tolerant nature.

5.5.3 Effect of NaCl stress on isozymatic activity pattern of POX

The peroxidases (POX) are associated with such biochemical and physiological processes as growth, cell formation, fruit development, ethylene biosynthesis, as well as the response to various stresses (Matamoros et al., 2003). POX are homoproteins of approximately 50 kDa that are present as multiple isozymes in plant tissues; but their functions and subcellular localisation are unknown (Matamoros et al., 2003). In the present study, the POX activity staining on native PAGE revealed at least five isoforms (POX I-V) in shoots of KR and PNL-3; however, POX IV and POX V were not detected in KJT-3, neither in non-saline conditions nor under salt stress varying from 100 to 200 mM NaCl (Plate 5c). Similar to present results, five isoforms were reported in common bean plants grown under salt stress conditions (Jebara et al., 2005).

Two isoforms (POX IV and POX V) seems to be correlated with the salt tolerant nature of cvs KR and PNL-3, as these bands appeared exclusively under salt stress and not seen in non-saline conditions as well as in salt sensitive cv KJT-3 under both saline and non-saline conditions. These results clearly indicated the exclusive salt stress responsive gene expression in salt tolerant cvs of rice, namely PNL-3 and KR. In addition, the intensities of POX I, POX II, and POX III bands sharply increased in KR and PNL-3 under increasing salt stress, with significantly higher increase in the later. In contrast, no significant change in intensities of these bands noticed in KJT-3. This transient increase in POX activity and isozymatic expression clearly indicated that POX plays an important role in antioxidant defence mechanism of rice to detoxify the ROS, as suggested earlier by Oidaira et al. (2000) and Srivalli et al. (2003). Similar results were reported by Lee et al. (2001) with increase in POX activity was noticed under increasing salt stress in rice tissues. Higher POX isozymic activities are reported in comparably salt tolerant genotypes of foxtail millet (Sreenivasulu et
al., 1999) and cucumber (El-Baz et al., 2003) and related the high POX activity to the salt adaptation process of these crops. However, contradicting results have been reported by Rashed et al. (1994) and they observed the occurrence of differential response in the decrease of intensity in salt tolerant genotypes under salt stress in wheat. Increase in POX activity was also observed under salt stress in common bean, and the salt tolerance capacity of the plants was attributed to high POX activity and its ability to detoxify H$_2$O$_2$ (Jebara et al., 2005).

The results of antioxidant enzymes activity and in-gel assays using native-PAGE done in the present investigation, confirmed the earlier conclusions by various researchers (Singha and Choudhury, 1990; Lin and Kao, 2000; Vaidyanathan et al., 2003; Demiral and Turkan, 2005; Kumar et al., 2007; Khan and Panda, 2008) that the resistance to salt stress is usually correlated with a more efficient antioxidative system, with high enzymatic activities and better biomass production in salt tolerant cvs of rice than sensitive ones.

5.6 Effect of NaCl stress on electrophoretic (SDS-PAGE) banding patterns of proteins in rice cultivars:

Salt stress condition caused marked changes in protein profiles of roots and shoots of rice seedlings. At least 33 proteins were altered prominently in all the three cvs both in shoots and roots, amongst these many proteins synthesized might be de novo while others were either up or down -accumulated under the influence of salt stress (Plate 6). KJT-3 did not show any new band under salinity stress of both the levels. However, the intensity of three bands of 17.3, 21 and 55.6 kDa molecular weight was decreased as the salinity increase whereas; up-accumulation of 29 and 39 kDa was observed under the influence of increasing salinity stress in shoots of KJT-3 (Plate 6a). These results clearly indicated that some proteins were suppressed while a fewer proteins were over-expressed by soil salinity in shoots of genotype KJT-3. A new band of 32 kDa in the shoots of PNL-3 was seen (Plate 6a) besides, the increase in expression level of 18.6, 77, 80.7, 89.4, 93.8 kDa polypeptides. A salt stress inducible protein of 32 kDa has been reported by Chen and Plant (1999) in tomato roots.
Furthermore, in KR, a moderately salt tolerant genotype a band of 154.5 kDa was seen in control shoots but this band was disappeared at both the salinity levels. In addition, the intensity of the 89.6 kDa was decreased at 200 mM while a bands of 18.6 kDa, 60.1 was seen at 100 mM but was eventually disappeared at 200 mM. The intensity of a band of 41.6 kDa band was increased at 100 mM but decreased at 200 mM as compared to the control.

Salinity stress is reported to triggers the expression of several osmo-responsive proteins in rice tissues and a correlation has been found between greater accumulations of these stress proteins in halotolerant, compared to salt sensitive rice cvs (Chourey et al. 2003). The proteins that accumulate under salt stress conditions may provide a storage form of nitrogen that is re-utilized in post-stress recovery (Singh et al. 1987) and also play a role in osmotic adjustments. These proteins may be synthesized either de novo in response to salinity stress or may be present constitutively at low concentration and increase when plants are exposed to salt stress (Pareek et al. 1997), as in the present investigation, de novo proteins were seen in salt tolerant genotypes PNL-3 and KR besides increase in the concentration of a number of proteins under increasing salinity stress. As in the case of shoots, no new bands were seen in roots of KJT-3, however, the band intensity of polypeptides of 19.9, 35.6, 54.4 and 122.6 kDa was increased as compared to the control; on the other hand, the band intensity of 113 kDa protein was increased at 100 mM NaCl but decreased at 200 mM stress (Plate 6b).

Comparing the protein profiles in the control and salt-treated roots revealed that in PNL-3, new bands of 37 and 116.7 kDa were induced by salinity stress, both at 100 as well as 200 mM level (Plate 6b). In addition, salinity stress over-expressed proteins of 38.2, 53.4, 75, 67.8 kDa which were also presented in non-saline roots of PNL-3, besides one polypeptide, the intensity of which was increased at 100 mM as compared to control but was reduced at 200 mM NaCl stress. Contrary to these observations, one band of 72 kDa was present at the control (non-saline) but was disappeared at both the salinity stress levels. This disappearance of polypeptides may suggest the negative effect of salinity stress on protein/gene synthetic machinery as advocated earlier by Rani et al. (2007).

In the moderately salt tolerant rice genotype KR, new bands of 34, 37 and 43 kDa were appeared de novo under the salinity stress (Plate 6b), in addition to this, the expression levels of two bands of 54.5 and 75 kDa was increased at 100 and 200 mM NaCl, besides two proteins, whose expression levels were increased at 100 mM NaCl but decreased at 200 as
compared to the controlled plants. A number of workers have reported alterations in protein profiles in various crops under salinity stress. Wimmer et al. (2003) observed induction of 25 kDa polypeptide while an increase in level of 33 kDa protein was seen in wheat under combined stress of salt and boron. Sousa et al. (2004) reported that cowpea seedlings subjected to NaCl stress showed increased concentration of 9 proteins, decreased concentration of one and de novo synthesis of one 21.2 kDa protein. Kim et al. (2003) found an increase in the concentration or expression level of a 33.8 kDa protein in rice under salinity stress and identified it as a salt inducible protein. Present investigation’s results are in conformity of these claims and suggest that 34 kDa protein may serve as a salt stress-induced protein which may be correlated with the salt stress tolerance nature of KR genotype. It is imperative that these stress proteins may have a role in providing plants the ability to cross-adapt.

The results clearly indicated that more polypeptides were either induced de novo or their expression level was increased under salt stress in comparably salt tolerant rice genotypes than the sensitive one, which may play a protective role in combating high salt stress.

5.7 Indirect organogenesis and plant regeneration in *indica* rice cv KJT-3:

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; Visarada et al., 2002; Martinez-Trujillo, 2003; Ge et al., 2006) which limit the success in the application of genetic transformation techniques for *indica* rice improvement. Even within the *indica* group, there are significant variations in the in vitro culture response among different genotypes (Peng and Hodges, 1989).

Embryogenic callus production with high regeneration capacity is a pre-requisite for highly efficient transformation of rice. Even though *indica* subspecies is the most widely cultivated type of rice worldwide, the magnitude of genetic manipulations is very less as compared to its *japonica* counterpart. The main reason behind this is that *indica* rice appears as recalcitrant or say more specific than *japonica* rice to tissue culture conditions in *Agrobacterium*-mediated transformation (Ge et al. 2006). Embryogenic callus induction depends upon the interaction between the genotypes and culture conditions. Despite the
availability of a plethora of protocols for rice tissue culture, no procedure appears to be universally adaptable when a new genotype is to be considered for in vitro manipulation. Though regeneration rather than callus induction is limiting in most of the indica rice cvs; introduction of foreign gene, selection and multiplication of only the transformed sectors depends upon the embryogenic potential of callus in in-vitro cultures (Visarada et al., 2002). Hence, development of methods for embryogenic callus production, its antibiotic selection and indirect regeneration is of great importance.

Though various explants have been used for callus induction in rice including immature embryos (Chand and Saharawat, 2001), mature embryos (scutellum) (Khanna and Raina, 1998), roots (Mukhopadhyay 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). However, among the several types of explants, scutellum-derived callus has been found to be the most amenable to transformation (Tyagi et al. 2007), in addition to its year-round availability.

Plant growth regulators (PGRs) have an important role in callus cultures and various effects of PGRs have been investigated by a number of researchers (Rueb et al., 1994; Marassi et al., 1996; Zhang et al., 1996). Auxin, mainly 2, 4-D is used most frequently with little variation (2 to 3 mg l⁻¹) 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; Tyagi et al., 2007). In the present study, we observed that calli initiated from scutella of germinating seeds had embryogenic potential. We tried various auxins such as IAA, IBA, NAA and cytokinins like BAP and Kn for callus induction; however, amongst these, no PGR could induce callus from mature embryos (Plate 7; data not shown). Only 2,4-D was observed as sole PGR suitable for callus induction. Firstly, the optimum concentration of 2,4-D was standardized for callus induction by using 2,4-D from 0 to 4 mg/l (Plate 8 and Fig. 4.14): 2 mg l⁻¹ 2,4-D was most suitable for optimal callus induction. The results of present investigation are in conformity of the use of 2,4-D (with little variation of 2 to 3 mg l⁻¹) for optimal callus induction and proliferation from mature seeds as reported by various researchers (Visarada and Sarma 2002; Saharan et al. 2004; Lin and Zhang 2005; Ge et al. 2006).

However, MS media fortified with 2 mg l⁻¹ 2,4-D without any nutritional supplement such as proline, casein hydrolysate and glutamine, responded poorly in terms of embryogenic nature.
of callus, and only around 10% of callus cultures showed the embryogenic-like callus, while rest were non-embryogenic-like (Table 4.22). Therefore, in addition to this, effects of proline and casein hydrolysate were investigated for improvement of induced callus in terms of its embryogenic-like nature and fresh weight. Interesting results were observed when proline and casein hydrolysate (enzymatic and not acidic) were used, as high rate of embryogenic-like callus induction was observed (CIM II and III, Table 4.22). Both these nutritional supplements have been reported to work as a source of amino acids and addition of these supplements to the callusing media proved to enhance the production of embryogenic type callus in indica rice (Lin and Zhang 2005; Zaidi et al. 2006; Kant et al., 2007; Tyagi et al. 2007). The results obtained from present work confirmed the findings of these investigations.

Moreover, use of maltose in place of sucrose in addition to glucose and tryptophan did not show any significant effect on embryogenic-like callus induction frequency. The results presented in Table 4.22, clearly indicated that CIM II (MS basal, 2 mg l⁻¹ 2,4-D, 500 mg l⁻¹ proline, 500 mg l⁻¹ casein hydrolysate, 30 g l⁻¹ sucrose, 7 g l⁻¹ agar, pH 5.8) was most suitable media for embryogenic-like callus induction and growth. It induced hard, dry, compact, whitish-yellow embryogenic-like callus from mature dehusked seeds (Plate 8).

Similar to the present findings, the use of proline and casein hydrolysate for embryogenic-like callus induction from indica rice, used for Agrobacterium-mediated transformation has been reported by a number of researchers (Saharan et al. 2004; Ge et al. 2006; Kant et al. 2007). Both these kinds of calluses (embryogenic-like and non-embryogenic-like) were however tested for their comparable suitability and response for Agrobacterium-mediated transformation, and each showed different response (data not shown). Since, the non-embryogenic-like calli were watery, in large clumps and was much more intricate on its surface than embryogenic-like callus (Plate 8c), the inoculated Agrobacterium tended to over-proliferate during and after co-cultivation. In addition, non-embryogenic-like callus appeared to be less resistant to Agrobacterium infection than the embryogenic-like type callus and tended to be damaged more easily. This resulted into difficulty in removing the inoculated Agrobacterium from the intricate surface of non-embryogenic-like callus. In contrary, embryogenic-like callus was simple and round in its form and the inoculated Agrobacterium could be easily removed after co-cultivation. Since, these preliminary
experiments showed that embryogenic-like callus produced more transformant (GUS+ calli) (data not shown), the embryogenic-like callus was chosen for subsequent experiments. These results confirm the similar observations and conclusions reported previously by Hiei et al. (1994) and Terada et al. (2004).

Effects of Kn in addition of IAA and NAA were observed on shoot regeneration through callus, though IAA was found unsuitable and optimization of shoot induction media was done by using various combinations of Kn and NAA (Table 4.23). RM III was found most suitable for shoot organogenesis through embryogenic-like callus in indica rice cv KJT-3 with more than 75% cultures showing green shoots at an average of 4-5 shoots per callus. Organogenesis took place within 3-4 weeks after inoculation (Plate 9). Well-developed shoots after 3-4 weeks of inoculation produced vigorous roots upon their transfer to ½ MS media without any PGR (Plate 9). These rooted plantlets were transferred to plastic cups containing soil and gradually acclimated to natural conditions, as described in section 3.7.8 and 3.9.7. The regenerated plants did not show any detectable variation in both morphological and growth characteristics compared with the parent plant.

5.8 Apical shoot meristem derived multiple shoot formation and plant regeneration in indica rice cv KJT-3:

Agrobacterium-mediated transformation has greatly facilitated the widespread application of transformation in japonica rice because of establishment of high efficiency transformation system (Hiei et al. 1994). This technique has been widely used not only to introduce genes of interest into the rice genome (Komari et al., 1998; Lin et al., 2002; Sallaud et al., 2003) but also as a common means of testing gene function by enhancing or inhibiting expression of target genes (Kobayashi et al., 2001; Nagasaki et al., 2001; Mori et al., 2002). However, there are few successful reports of transformation of indica rice, that too either with low transformation efficiency (Aldemita and Hodges 1996; Garg et al., 2002; Wang et al., 2002), or success only with very specific genotypes (Forkan et al., 2004). Several reports have reported transformation of indica rice using embryogenic-like callus as a plant material (Aldemita and Hodges, 1996; Wang et al., 2002; Forkan et al., 2004), however, lack of robust and widely applicable methods for subculture and regeneration have been the main
limitation, in addition to time consuming nature of long-term callus phases during transformation process (Bregitzer and Tonks, 2003; Yookongkaew et al., 2007).

During the earlier studies using apical shoot meristems for co-cultivation with *Agrobacteria*, stable transformation was not seen (Hiei et al., 1994); probably because the apex was not dissected to expose the meristem region. This failure to obtain transformants may be because of lack of dissection, since leaf primordia covering the shoot apex may have prevented *Agrobacterium* from reaching the meristem. By contrast, when the shoots were wounded to allow access to the meristem, stable rice transformants were obtained using *Agrobacterium* and shoot apices (Park et al., 1996), where transgene expression was seen in the R₁ and R₂ generations. During the past decade, scientists have successfully manipulated the shoot apical meristems from seedlings of maize, oat, sorghum, millet, wheat, and barley in an effort to develop a less genotype-dependent and efficient cereal regeneration system that can be maintained in vitro for long periods of time without the need for cryopreservation (Sticklen and Oraby, 2005). Furthermore, apical meristem regeneration systems were used to stably transform maize, wheat, rice, oat, barley, sorghum, and millet. Overall, the shoot apical meristem is considered as a most appropriate explant for genetic manipulation of cereal crops because it is easily cultured in vitro, quickly regenerable, competent to genetic transformation, produces plants genetically identical to the parent, and can be sustained in vitro for long periods of time (Sticklen and Oraby, 2005).

Therefore, a system was established in which shoot apical meristem as an alternative material for *Agrobacterium*-co-cultivation for desired gene transfer in indica rice (*Oryza sativa* ssp. *indica* cv. Karjat-3). However, there is always a fear of getting chimera, when transgenic plants are recovered directly from shoot apical meristem (Zhong et al., 1996). A feasible technique for avoiding this phenomenon is to multiply transgenic meristem cells by treatment with plant growth regulator (Zhong et al., 1996). Therefore, we have tried the same and got the multiple shoots from the co-cultivated apical shoot meristems. Multiple shoots were obtained through apical shoot meristems post co-cultivation and putative transgenic plants were regenerated from them under stringent antibiotic selection pressure. TDZ with varying concentrations from 0 to 10 mg l⁻¹ was used for multiple shoot induction and growth from apical shoot meristems. Amongst these concentrations, the shoots regenerated on 6 mg l⁻¹ TDZ were larger and healthier than those on 4, 8 and 10 mg l⁻¹ TDZ. Shoots regenerated on 4, 8 or 10 mg l⁻¹ TDZ were usually small and weak. Therefore, 6 mg
1^st TDZ was selected as an appropriate concentration for the multiple shoot regeneration system and was further used in Agrobacterium-mediated transformation of KJT-3, using apical shoot meristem for co-cultivation. A maximum of 20 shoots per explant were observed on this media.

Thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea: TDZ) is reported to facilitate multiple shoot proliferation of many plant species (Huetteman and Preece, 1993; Lu, 1993; Murthy and Saxena, 1998; Srivatanakul et al., 2000; Sharma et al., 2004; Yookongkaew et al., 2007). TDZ was reported better than BA for inducing shoot regeneration of rice (O. sativa ssp. indica cv. Jaunala) from embryogenic callus (Gairi and Rashid, 2004). It is reported to be less susceptible to plant-degrading enzymes than endogenous cytokinins (Mok et al., 1987). TDZ may be involved in reprogramming and expression of the competent cells necessary for them to undergo differentiation and development (Yookongkaew et al., 2007). Multiple shoot formation (up to 28 shoots per explant) through apical shoot meristems by using TDZ as a sole PGR is reported in a number of indica rice genotypes by Yookongkaew et al. (2007) and they reported successful Agrobacterium-mediated transformation of apical shoot meristems and multiple shoot regeneration using 4 mg l^-1 TDZ. The results of present work are in harmony to these reports and we have successfully achieved a high number of shoots (up to 20 shoots per explant) and subsequent plant regeneration through these shoots. The plants were transferred to greenhouse conditions and none of the regenerated plants show any detectable phenotypic variation.

5.9 Production of transgenic plants (T_0) of rice cv. KJT-3 and their molecular characterisation:

Studies have indicated that in rice, efficient transformation and subsequent regeneration using Agrobacterium-mediated methods are dependent on several factors. Numerous factors, including choice of explant, hormonal composition of the medium used, nutritional supplements, culture conditions prior to and during inoculation, duration of co-cultivation with Agrobacterium, virulence of Agrobacterium strain, concentration and composition of the bacteriostatic agent used, duration of selection and concentration of antibiotic selection marker, genotype of plants and various conditions of tissue culture including a robust system
of plant regeneration are of critical importance (Aldemita and Hodges, 1996; Hiei et al.,
1997; Mohanty et al., 1999; Yookongkaew et al., 2007).

Already about 40 different genotypes of indica, japonica and javanica rice have been
transformed using Agrobacterium approach (Kathuria et al., 2007). Numerous factors,
including Ti plasmid type (Hiei et al., 1994; Cheng et al., 1998), bacterial strains with broad
host range (Hiei et al., 1994, 1997; Aldemita and Hodges, 1996; Dong et al., 1996), culture
conditions prior to and during inoculation (Aldemita and Hodges, 1996; Mohanty et al.,
1999) and activation of T-DNA transfer process by exogenously added acetosyringone (Hiei
et al., 1994; Kumria et al., 2001; Forkan et al., 2004; Kumar et al., 2005), selection marker
genes and selective agents (Hiei et al., 1997; Yookongkaew et al., 2007), genotype of plants,
types and ages of tissues inoculated (Aldemita and Hodges, 1996; Mohanty et al., 1999) and
various conditions of tissue culture including a robust system of plant regeneration are of
critical importance. The genotypic influence is often overcome by modifying the nutrient
medium (Rachmawati et al., 2004; Lin and Zhang, 2005) or transformation conditions, since
the same nutrient medium is not ideal for all the varieties (Sridevi et al., 2005; Ge et al.,
2006).

Aim of the present study was to optimize Agrobacterium-mediated transformation efficiency
in indica rice cv KJT-3 by using a P5CS gene for developing salt stress resistant plants. We
have standardized reproducible methods for Agrobacterium-mediated transformation using
the pCAMBIA1301 plasmid bearing the proline biosynthetic pathway gene P5CS-F129A
gene with chimeric hygromycin phosphotransferase (hpt) and β-glucuronidase (uidA) as a
reporter gene by using embryogenic-like callus and apical shoot meristem for infection and
cocultivation. Agrobacterium-mediated transformation of rice is advantageous because of
various reasons including the transfer of DNA pieces with defined ends with minimal
rearrangements, the transfer of relatively large segments of DNA, the integration of small
numbers of copies of genes into plant chromosomes and high quality and fertility of
transgenic plants (Hiei et al., 1997).

The results of present investigation showed that CaMV 35S promoter was useful for rice
transformation. Hiei et al. (1994) and Pipatpanukul et al. (2004) used the same promoter for
rice transformation, while Jogeswar (2005) used it for transformation of sorghum and
reported high level of gene expressions of their respective genes. However, contrasting to
the results obtained in the present study. Hauptmann et al. (1988) demonstrated that the
CaMV35S promoter sequence was less effective in cereal cells than in dicot cells. Vector
(pCAMBIA1301) used in the present study, have been proved ideal for rice transformation
(Ilag et al., 2000; Chern et al., 2001; Saharan et al., 2004). Further, the same promoter has
been used for transformation of tobacco plants with P5CS gene (Yamchi et al., 2007).
The most widely used selectable markers in monocot transformation are the genes encoding
hygromycin phosphotransferase (hpt), phosphinothricin acetyltransferase (pat or bar) and
neomycin phosphotransferase (nptII). Use of these marker genes under the control of
constitutive promoters such as 35S promoter from cauliflower mosaic virus, or the ubiquitin
promoter from maize, works efficiently for selection of Agrobacterium-transformed cells
(Cheng et al., 2004). Hygromycin B is widely used as very useful selective marker gene
post-co-cultivation for antibiotic selection (Gritz et al., 1983; Staben et al., 1989; Ortiz et
al., 1996). Hygromycin B is an aminoglycoside antibiotic produced by Streptomyces
hygroscopicus, which kills bacteria, fungi and higher eukaryotic cells by inhibiting protein
synthesis. It also interferes with translocation and to cause mistranslation at the 70S
ribosome. It is the most widely used antibiotic selection agent, as it kills the non-
transformed cells more quickly than kanamycin and only resistant or in other words,
transformed cells survives. This kind of selection is referred to as negative selection
(Shrawat and Lorz 2006). Thus, finding the threshold limit of hygromycin B concentration
for survival of the calluses and apical shoot meristems will help immensely prior to
transformation work, as this concentration of selective agents are needed to avoid
development of undesirable numbers of the escapes, post-transformation. In the present
investigation, hygromycin B showed significant effect on percent survival of rice calli. The
survival of the calli was 100% in the control medium (without antibiotic). As the
concentration of hygromycin B increased in the medium, the percent survival of the calli
decreased. At 15 to 20 mg/l hygromycin B, the callus tissues gradually started turning brown
after 2-3 days of inoculation on selection medium and after 10 days of selection, calluses
were observed completely dark reddish-brown or blackish and 100% inhibition of calli or
cell death was noticed in presence of 20 mg l⁻¹ of hygromycin B, suggesting this as suitable
concentration for selecting the putative transformants for future transformation programmes
of this genotype. On the other hand apical shoot meristems could survive up to 25-30 mg l⁻¹
hygromycin B and therefore, 30 mg l⁻¹ concentration was used for selection. Various
researchers have used 50 mg l\(^{-1}\) hygromycin B for selecting the putative transformants of different indica type rice genotypes (Sridevi et al. 2005; Kant et al. 2007; Nandakumar et al. 2007). On the other hand there a few reports, where hygromycin B has been used as a selective agent below this concentration (Pipatpanukul et al. 2004; Tyagi et al. 2007). Therefore, the findings of present study are significant as we could employ a significantly lower concentration of hygromycin B to completely inhibit callus growth in ‘KJT-3’, useful for its genetic engineering practices.

In order to eliminate *Agrobacterium tumefaciens* after co-cultivation, the use of antibiotics in culture medium is required. In the present investigation, the susceptibility of callus and apical shoot meristematic tissues was standardized for cefotaxime. Survival of the calli was 100% up to 250 mg l\(^{-1}\) of cefotaxime, however, above this level drastic changes were observed and calli were turned brown and subsequently all the callus tissues were observed to be dead on 10 days after inoculation. The reduced regeneration capacity is in agreement with the results obtained by Pipatpanukul et al. (2004), who reported that cefotaxime over 250 mg l\(^{-1}\) played an inhibitory effect of callus growth and regeneration of *indica* rice cv. ‘RD6’. Therefore, the present results clearly suggest that cefotaxime at 250 mg l\(^{-1}\) for both the explants and hygromycin B at 20 and 30 mg l\(^{-1}\) for callus and apical shoot meristems respectively may be used for this cultivar for transformation studies.

The addition of acetosyringone (AS) in pre-culture as well as in co-cultivation medium has been reported to induce vir genes, extend host range of some *Agrobacterium* strains and found essential for rice transformation (Godwin et al., 1991; Hiei et al., 1994; Saharan et al., 2004). In the present investigation, the inclusion of AS (100 \(\mu\)M) during pre-culture and co-cultivation was found to be essential for transformation (Table 4.24) and this observation is consistent with a number of previous reports of rice transformation (Hiei et al., 1994; Mohanty et al., 1999; Kumria et al., 2001; Forkan et al., 2004; Kumar et al., 2005; Tyagi et al., 2007). These researchers observed that the level of transient expression of GUS, a marker enzyme after co-cultivation was either undetectable or extremely low when AS was omitted. Although, improvement in the transient expression levels of GUS does not necessarily promise in better stable transformation (Shen et al., 1993; Maximova et al., 1998). However, most studies have shown that conditions leading to enhanced transient expression do result in a higher number of transformed plants (Kondo et al., 2000; Niu et al., 2000; Suzuki and Nakano, 2002). The results of the present work also show improved
rates of stable transformation using acetosyringone and the other procedures that gives optimum transient expression. In addition, we have observed the addition of AS to pre-culture medium (bacterial suspension 1 h prior to infection) is important for efficient gene transfer as reported earlier by Aldemita and Hodges (1996). However, some researchers have reported successful transformation without adding phenolic compounds such as AS (Raineri et al., 1990; Yookongkaew et al., 2007). Thus, requirements of transformation vary from plant to plant.

Bacterial cell densities ranging from 0.2 to 1.0 were used to infect the plant tissues; however, cell densities below 0.4 did not show any GUS expression in the tissues post-co-cultivation. Highest GUS expression percentage was observed at 0.6, both in the calluses as well as in apical shoot meristems. Higher bacterial density resulted into over-growth of bacteria and it adversely affected the explants' growth and subsequent plant regeneration. In the present study, it was observed that the lower concentration (O.D of 0.6) of *Agrobacterium* culture suspension reduced the browning of calli after co-cultivation possibly because of reduced damage to explants during infection. So, the optimal concentration of agrobacterium culture most suitable for transformation of rice tissues was noticed to be 0.6 OD. Similar results have been reported by Kumar et al. (2005) for *Agrobacterium*-mediated genetic transformation of *indica* rice genotypes. This has resulted in optimum genetic transformation (35 % GUS expression levels in apical shoots while it was 38% in calli) (Table 4.25).

Generally, co-cultivation times for *indica* rice transformation used have varied from 2 to 5 days (Hiei et al., 1997; Forkan et al., 2004; Hoque et al., 2005; Tyagi et al., 2007). However, in this study it was found that 3 days was optimal for rice transformation (Table 4.26). Although calluses, which were co-cultivated for more than 3 days, showed GUS activity, they were adversely affected by over growth of *Agrobacterium* and subsequently died. Same co-cultivation time has been reported by a number of researchers for efficient *indica* rice transformation using callus (Hoque et al., 2005; Nandakumar et al., 2007) as well as apical shoot meristem (Yookongkaew et al., 2007) as target materials.

Reporter genes have been used as convenient markers to confirm foreign gene insertion in plants. Commonly used reporters include genes encoding β-glucuronidase (GUS), chloramphenicol acetyl transferase (CAT), green fluorescent protein (GFP), and luciferase
Amongst these reporter systems, GUS expression analysis is used most widely and worldwide as a reporter gene for genetic transformation of indica rice (Ignacimuthu and Arockiasamay, 2006; Zaidi et al., 2006; Yookongkaew et al., 2007; Tyagi et al., 2007). In the present study, the Agrobacterium-mediated transformation system for embryogenic-like callus as well as apical shoot meristems was optimized by using GUS as a reporter. Histochemical staining using X-gluc showed uniformly high levels of GUS expression.

On the basis of results obtained from studies for optimization of various parameters for efficient transformation of indica rice genotype KJT-3, an efficient and reproducible method for transformation using mature embryo-derived embryogenic-like callus was standardized (Flow Chart 4.1 and Plate 12). We could achieve around 1% transformation efficiency by using embryogenic-like callus of indica rice cv (Table 4.27). KJT-3 varying transformation frequencies have been reported in indica rice cvs- Zhang et al. (1997): 9 to13%; Khanna and Raina (1999): 4.4%; Khanna and Raina (2002): 9%; Zaidi et al. (2006): 7 to 8.3 and Nandakumar et al. (2007): 0.9 to 5.2%. The low efficiency of indica rice transformation in a number of these previous reports is due to possible toxicity of antibiotics to callus growth (Rashid et al., 1996; Khanna and Raina, 1999) and have suggested withholding use of antibiotics on regeneration media. However, in the present study, pressure of hygromycin was maintained up to regeneration medium, resulted in the selective proliferation of resistant calli with transgene. The high efficiency transformation observed here correlates that regeneration of non-transformants was restricted thereby less competition for regeneration of positive transformants.

Among various explants used, scutellum-derived embryogenic calli are the material of choice for efficient transformation of rice (Hiei et al., 1994; Kant et al., 2007). However, long callus cultures during the transformation process enhance the chances of somaclonal variations. Therefore, in the present study, in addition to embryogenic-like calli, we have also used apical shoot meristems for transformation to reduce the time period required to get transgenic plants. For using apical shoot meristems as a target material, two methods may be used to obtain transgenic plants by transfer of DNA into the shoot apical meristem (Yookongkaew et al., 2007). One is that transgene progeny may be directly produced from the meristem cells followed by development of a partially transgenic reproductive organ. Primary transformants produced this way will always be chimeric. The other is to multiply transgenic apical meristem cells, which can be reprogrammed into the developmental stage...
under *in vitro* conditions (Zhong et al. 1996). Thus, manipulation of transgenic meristem cells by treatment with growth regulator to induce multiple shoot regeneration from the shoot meristem could generate more stable transformants. TDZ, a phenylurea-type cytokinin, has been reported to facilitate multiple shoot proliferation in many plants (Gairi and Rashid 2004; Goldman et al. 2003; Srivatanakul et al. 2000). Results of the present investigation are in agreement of these reports, and TDZ was observed to be a sole hormone to induce multiple shoots from apical shoot meristems and it has resulted into a very high (~1.8%) rate of transformation in KJT-3 rice genotype, using apical shoot meristems as the explants for co-cultivation (Plate 13 and Table 4.28). In conclusion, we have effectively accomplished multiple shoot regeneration from shoot apical meristem in *indica* rice and no somaclonal variation was observed in transgenic plants. This protocol may therefore serve as an efficient system for rapid multiplication of rice plants of interest along with for *Agrobacterium*-mediated transformation (described in Flow Chart 4.2). Establishment of an easy, rapid, and widely applicable transformation system for rice is very important for crop improvement and for study of gene function. By using stringent antibiotic selection, multiple shoots were obtained from selective medium and transgenic plants could be generated. It was shown by histochemical GUS expression, PCR products and Southern blot analysis that the *P5CS* gene was present in the genome of the T0 transgenic plants developed by us (Plate 14).

The results obtained from the studies of *Agrobacterium*-mediated transformation of *indica* rice cv. KJT-3 via callus and apical shoot meristems clearly indicated that the later requires much lesser time than the earlier, as it does not require long callus production cycle. Total time required for obtaining transformed plants growing in green house conditions, regenerated through apical shoot meristems takes at least 5-6 weeks lesser to regeneration of transgenics via mature embryo-derived callus. However, in case of apical shoot meristem mediated transformation, the antibiotic selection was done on 30 mg l\(^{-1}\) hygromycin B as compared to 20 mg l\(^{-1}\) hygromycin B for callus mediated transformation. Further, when calluses were transferred to shoot regeneration media, after two cycles of two weeks each on antibiotic selection, use of 20 g l\(^{-1}\) sorbitol was observed inevitable to overcome osmotic stress in calluses, however, in apical shoot meristems, no such supplementation was required. The results presented in Table 4.27 and 4.28, clearly indicated that the rate of
transformation was about 2 times higher in case of apical shoot meristems used as target tissues for transformation than calli.

T-DNA was shown to be stably maintained in transformed (T₀) rice plants. PCR analysis was consistent with genomic integration of P5CS-F129A (Plate 14a). The stable gene insertion and establishment was further confirmed by southern hybridisation, which showed stable transformation of the gene at T₀ level (Plate 14b). P5CS stable insertion was also confirmed by following the histochemical GUS analysis in T₁ generation plant tissues (Plate 15a). The insertion was further confirmed by PCR products obtained from DNA amplification of T₁ plants using hptll specific primers and electrophoresed on agarose gel electrophoresis (Plate 15b). PCR products and Southern blot hybridisation are most widely used techniques for confirmation of transgene integration into host genome. Various researchers have used both these analyses for confirmation of P5CS gene in a number of crop species including rice (Zhu et al., 1998; Anoop and Gupta, 2003; Su and Wu, 2004); citrus rootstock (Molinari et al., 2004); hybrid larch (Deirdre et al., 2005); pea (Najafi et al., 2005); potato (Hmida-Sayari et al., 2005), wheat (Vendruscolo et al., 2007).

5.10 Differential response in terms of proline content between non-transgenics and transgenic (T₀) plants of indica rice cv KJT-3:

Striking differences were seen in terms of free proline level between the non-transformed and PCR positive T₀ transformed KJT-3 plants, however with variations amongst the transgenics. In general, all the putative transformed T₀ plants showed higher proline content compared to the non-transformed in vitro grown plant. However, no considerable variation was observed in terms of proline level between the transgenics produced via callus and apical shoot meristems (Fig. 4.15). Amongst all the 5 transgenic plants, T₀ line 5 showed maximum proline content (4320 µg g⁻¹ DW), which is around 4 times more than non-transformed line (1150 µg g⁻¹ DW) followed by line 2 with 4225 µg proline g⁻¹ DW of tissues. These results, confirmed the insertion and functional expression of P5CS-F129A gene into KJT-3 genome.

Similar to the results obtained in the present investigation, the primary P5CS-transgenic wheat plants showed much higher (more than ten times higher) proline content than their wild type counterparts under non-stress conditions (Sawahel and Hassan, 2002). Han and
Hwang (2003) reported up to 6 time higher proline content in callus and leaves of P5CS-transgenic carrot. Primary transgenics of citrus rootstock Carrizo citrange plants over-expressing P5CS gene showed 120 μmol proline g⁻¹ FW as compared to 17.7 μmol g⁻¹ FW in non-transgenics. Similar results have been reported in case of P5CS-transgenic plants of potato by Hmida-Sayari et al. (2005). Further, Najafi et al. (2005) observed about 4-fold increase in proline content in transgenic pea plants carrying P5CS gene against the non-transgenic pea plants. More recently, Yamchi et al. (2007) observed that there was 26 times more proline production in tobacco plants transformed with P5CS gene as compared to non-transgenic tobacco plants. Results of present work are in harmony with these reports and confirmed the integration of mothbean P5CS-F129A gene into the genome of indica rice cv KJT-3 and its functional expression. The variation among the transgenic lines in terms of proline content may be attributed to the integration position of this gene and transcription level (Yamchi et al., 2007).

5.11 Molecular characterisation and salt tolerance evaluation of transgenic (T\textsubscript{1}) plants of indica rice cv KJT-3:

From the results of segregation analysis of the transformed KJT-3 plants using hygromycin selection for T\textsubscript{1} progeny, it was evident that the transformants showed stable integration and inheritance of introduced gene to the next generation. The T\textsubscript{1} progenies exhibited both, Mendelian as well as non-Mendelian segregation ratios in terms of hygromycin resistance of the transgenics (Table 4.29). Most of the lines exhibited hygromycin resistance and sensitivity ratio as 3 : 1 (except line T\textsubscript{1-1}). Similar results have been reported by Mohanty et al. (2002) in indica rice cv Pusa Basmati-1, transformed with choline dehydrogenase gene (coda), and they reported both Mendelian as well as non-Mendelian segregation ratios on the basis of hygromycin resistance and sensitivities in the transgenics. Further, Anoop and Gupta (2003) also reported similar results in transformed progenies of indica rice cv IR50 with mothbean P5CS-transgene. Sridevi et al. (2005) transformed a non-basmati indica type rice cv White Poonni with a chitinase gene, and the authors used hygromycin-containing medium to study the segregation pattern in T\textsubscript{1} progeny. They reported the hygromycin resistance and sensitivity ratio of 3 : 1 in four transgenic lines and 15 : 1 in one such line.
Furthermore, Sawahel and Hassan (2002) reported Mendelian ratio of 3 : 1 for kanamycin resistance in the wheat plants transformed with *Vigna P5CS*.

Tissue culture-grown hygromycin resistant T₁ seedlings when assayed for GUS expression, the indigenous blue colour developed in the root tissues made it clear that *P5CS129A* gene was stably inserted in KJT-3 genome and was inherited to the next generation (Plate 15a). PCR amplification of *hptII* gene using gene specific primers also confirmed the stable establishment of the gene into the genome of rice genotype KJT-3. The PCR products, electrophoresed on agarose gel clearly showed the stable insertion of the gene (Plate 15b). Therefore, the results of hygromycin selection, GUS assay and PCR analysis, clearly indicated the presence of *P5CS-F129A* gene in the second generation transformants.

In the context of recommendation of the ‘Task Force on Agricultural Biotechnology’, committee chaired by Prof. M. S. Swaminathan that genetic engineering of rice should be confined to non-basmati type rice varieties (Task Force on Agricultural Biotechnology, 2004), it has become important to identify elite, non-basmati *indica* varieties to take-up for genetic engineering (Sridevi et al., 2005). Though the transformation frequency for *indica* rice genotypes remains low, the present investigation have shown that ‘Karjat-3’ is amenable for *Agrobacterium*-mediated transformation using both callus as well as shoot apex as target material for co-cultivation.

Results of the present investigation clearly showed that *P5CS* transgene expression confers increased tolerance to transgenic plants for salinity stress. Generally, all the transgenic lines tested of KJT-3 at T₁ level showed better plant growth, biomass production than the non-transformed control plants under salt stress driven by 150 mM NaCl (Table 4.30 and Plate 15c). It was evident from these results that *P5CS*-transgenic plants produced significantly more proline (4 to 5-fold more than no-transgenics) and protected the transgenic plants from damages due to salt stress treatments; on the other hand, the control plants could not tolerate the same extent of stress. The enhanced salt tolerance of *P5CS*-transgenic KJT-3 plants was associated with higher proline accumulation and lower lipid peroxidation levels (Table 4.31, 4.32 respectively), while the inability of non-transformed plants to tolerate NaCl stress may be due to lower level of proline, higher magnitude of free radical production as suggested by higher MDA content under salt stress conditions. These results
have proved the successful insertion and functional expression of P5CS-F129A gene into the genome of rice cv KJT-3.

Earlier, various researchers have reported higher proline accumulation and subsequent abiotic stress tolerance of transgenic plants over-expressing P5CS genes. These includes salt stress tolerance in a range of crop species consisting of rice (Zhu et al., 1998; Anoop and Gupta, 2003; Su and Wu, 2004), tobacco (Kavi Kishor et al., 1995; Zhang et al., 1995; Yamchi et al., 2007), pea (Najafi et al., 2005), wheat (Sawahel and Hassan, 2002), carrot (Han and Hwang, 2003) and potato (Hmida-Sayari et al., 2005); drought stress tolerance in Carrizo citrange (Molinari et al., 2004) and wheat (Vendruscolo et al., 2007); freezing stress tolerance in tobacco (Parvanova et al., 2004; Konstantinova et al., 2002), lettuce (Pileggi et al., 2001, 2002) and petunias (Yamada et al., 2005).

Enhanced proline accumulation and better growth performances of rice genotypes, both indica and japonica are attributed to the over-expression of P5CS transgene into their genome (Zhu et al., 1998; Anoop and Gupta, 2003; Su and Wu, 2004). The extent of proline accumulation was as much as 26 times in transgenic indica rice cv IR50 than their non-transgenic counterpart under non-saline condition, while it was further enhanced by 3 times at 100 mM NaCl conditions (Anoop and Gupta, 2003). Similarly, Su and Wu (2004) observed proline content in transgenic rice cv. Kenfong plants harbouring P5CS gene was higher by 315% than the control ones, which was further accentuated with upto 216% increase under 3 days of 200 mM NaCl stress. Coming to the over-expression of mutagenic version of P5CS: P5CS-F129A, transgenic tobacco plants expressing this gene accumulated about 2-fold more proline than the plants expressing V. acontifolia wild type P5CS. This difference was further increased in plants treated with 200 mM NaCl (Hong et al., 2004). The same mutagenic P5CS have been used by Pileggi et al. (2001) and Pileggi (2002) for transformation of lettuce and they observed that this gene conferred osmotic tolerance induced by freezing, high temperature and high saline conditions to the transgenics. Further, Molinari et al. (2004) used this gene under the control of constitutive promoter 35S and reported enhanced drought tolerance of the resulting transgenic plants of Carrizo citrange by over-producing proline. The findings of present investigation are in harmony of these reports as we observed steep increase in proline levels under salt stress conditions in transgenic plants obtained by introduction of V. acontifolia mutated gene-P5CS-F129A under the control of 35S promoter. Proline content was increased due to salt
stress conditions both in non-transgenic as well as P5CS-transgenic plants; however with a
significant variation in the extent of increase with 152% increase in the earlier while 302%
to 352% increase in case of the latter (Table 4.31). Such a response to salt stress in
transgenic plants indicated that T-DNA is integrated in the chromosome, which leads to its
efficient transcription.

In addition to proline enhancement, another important parameter to check the level of
stress-induced damage at the cellular level is lipid peroxidation measured as the MDA
content (Parvanova et al., 2004). As a consequence of ROS, lipid peroxidation can lead to
cellular membrane rupture in plants submitted to stress (Hernandez et al., 2000) as
discussed earlier. As expected, there were significant differences between the non-
transgenic and transgenic plants of KJT-3 in terms of MDA content under 7 days of 150
mM NaCl stress (Table 4.32). At 150 mM NaCl stress, non-transgenic plants showed
around 210% increase in MDA content as compared to the non-transgenic plants growing
under non-saline conditions. However, any of the transgenic line did not show significant
enhancement in MDA content under NaCl stress in comparison with non-transformed
plants growing under non-saline conditions and MDA content was increased in the range
of 101% to 131% as compared to 210% increase in MDA content in non-transgenic plants
due to salt stress. These results are in agreement with those obtained by Chen and Li
(2002), who showed that a high concentration of proline in suspension cells avoided lipid
peroxidation on membranes. Also, it has been reported that higher proline accumulation in
P5CS-transformed tobacco plants reduced free radical levels measured by MDA content in
response to osmotic stress (Parvanova et al., 2004; Vendruscolo et al., 2007). The oxygen
singlet quenching capacity of proline seems to be based on its ability to form a charge-
transfer complex due to low ionization potential (Alia et al., 2001). Proline is also
suggested to relieve the oxidative burden from the glutathione system (Sharma and Dietz,
2006). The transformed plants presented low MDA values that could be translated in a
higher maintenance of cellular integrity and basic physiological processes as we have
discussed earlier.

As described earlier that as much as one-half of the irrigated areas of the world are affected
by high salinity. Therefore, there is a high interest to improve plant osmotolerance in
agriculture. This has been achieved by the genetic manipulation of osmolytes,
transcriptions factors and more recently of the cytokinin hormone (Seki et al. 2007; Rivero
et al., 2007). Severe salinity stress causes detrimental changes in cellular components. A wide range of metabolites, called compatible osmolytes gets accumulated under salt stress include amino acids (e.g. proline), quaternary and other amines (e.g. glycine-betaine and polyamines) and a variety of sugars and sugar alcohols (e.g. mannitol and trehalose). Accumulation of compatible solutes results in an increase in cellular osmolarity that can drive influx of water or reduce the efflux. This provides the turgor, necessary for cell expansion. Under osmotic or dehydration stress conditions, membrane integrity must be maintained to prevent protein denaturation.

The enhancement of proline and glycine betaine synthesis in target plants has received more attention (Rontein et al., 2002). Proline accumulation is reported in eubacteria, protozoa, marine invertebrates and plants in response to various stresses (Verbruggen and Hermans, 2008). However, high proline levels are not always correlated with osmotolerance and mutants displaying higher proline accumulation can be salt hypersensitive (Lui and Zhu, 1997). But majority of the investigations, worldwide, evidenced and correlated higher proline accumulation with enhanced tolerance to various abiotic stresses. In plants, proline accumulation has been reported to occur after salt, drought, high/ low temperatures, heavy metal, pathogen infection, anaerobiosis, nutrient deficiency, atmospheric pollution and UV irradiation (Saradhi et al., 1995; Hare et al., 1997; Siripornadulsil et al., 2002).

Proline has been proposed to function as molecular chaperone stabilizing the structure of proteins, and its accumulation can provide a way to buffer cytosolic pH and to balance cell redox status (Verbruggen and Hermans, 2008). Chadalavada et al., (1994) reported the protection of structural and functional integrity of M4 lactate dehydrogenase by proline, while reduction in enzyme denaturation due to heat and NaCl stress has been shown in vitro by Hamilton and Heckathorn (2001). Sivakumar et al. (2000) reported that salt stress reduced the carboxylase activity of Rubisco and enhanced the oxygenase activity; however, oxygenase activity was suppressed by proline even at 50 mM NaCl. Hamilton and Heckathorn (2001) found that while complex-I is protected by antioxidants and small heat shock proteins, complex-II is protected by proline and betaine under NaCl stress. These findings potentiate our view, that proline plays a critical role in protecting photosynthetic activity under stress conditions. In addition, proline acts as a source of carbon, nitrogen and energy during and recovery from stress (Alia et al., 1991; Zhang et al., 1997a).
Proline is also involved in protecting thylakoid membranes against free radical-induced photodamage (Sivakumar et al., 2000). Proline biosynthesis from glutamate would regenerate NADP⁺ that is needed to support the oxidative steps of the pentose phosphate pathway operating in the nodules (Fahrendorf et al., 1995). High concentrations of NADP⁺ are necessary for pentose phosphate pathway for regeneration of NADPH and to supply ribose-5-phosphate for the synthesis of purines. Therefore, proline accumulated under stress conditions might serve as a sink for excess reductants providing the NAD⁺ and NADP⁺ necessary for maintenance of respiratory and photosynthetic processes. While proline synthesis generates NADP⁺, its degradation produces NADPH. Thus, a cycle of proline synthesis and its degradation is essential for buffering cellular redox potential in the cytosol as well as in plastids. Redox cycling is also important in plant antioxidant defence mechanisms under stress conditions (Hare et al., 1998). Among various compatible solutes, proline is the only molecule that has been shown to protect plants against singlet oxygen and free radical induced damages (Alia et al., 1997). Since proline can act as a singlet oxygen quencher (Alia and Pardhasaradhi, 1993), and as a scavenger of OH radicals, it is able to stabilize proteins, DNA as well as membranes (Smirnoff and Cumbes, 1989; Alia et al., 1991; Sivakumar et al., 2000; Hamilton and Heckathorn, 2001; Matysik et al., 2002). Hydroxy-radical scavenging activity was measured for sorbitol, mannitol, myo-inositol and proline and it was found that proline is an effective hydroxy radical scavenger (Smirnoff and Cumbes, 1989; Alia et al., 1997). Thus, proline is not only an important molecule in redox signalling, but also an effective quencher of reactive oxygen species formed under salt, metal and dehydration stress conditions in all plants, including algae (Alia and Pardhasaradhi, 1991). Also, activities of the various enzymes including CAT, POX and PPOX were promoted by proline in vivo as noticed by Paleg et al. (1984). However, the ability of proline to activate the enzymes may suggest a limited conformational change. Thus, it appears that proline has many roles to play during salt, drought, cold, and metal stresses and also during the release of these stresses in plants.

Proline metabolism pathway genes have been of choice for producing transgenics to combat various abiotic stresses such as salt and drought stress. The work for over-expression of P5CS gene and subsequent proline accumulation in transgenics was started in early nineties by the group of Desh Pal Singh Verma (Hu et al., 1992; Kavi Kishor et al. 1995) at Ohio State University, USA. Tobacco plants over-expressing mothbean P5CS gene, coding for the
first enzyme of proline biosynthesis under the activity of a constitutive promoter, synthesized 10 to 18-folds more proline than control plants and were better salt stress tolerant. Over-production of proline enhanced root biomass and also plants tolerated 200 mM NaCl stress in the glasshouse conditions. Surprisingly the osmotic potential of P5CS transgenics was not lower than in control plants. Since proline production increased several folds in transgenics, it suggests that the activity of P5CS in the pathway is the rate-limiting step. Exogenous supply of nitrogen further enhanced proline production in transgenic tobacco (Kavi Kishor et al., 1995). Studies using purified P5CS enzyme indicated that Vigna P5CS is feedback inhibited by 50% by 5 mM proline in vitro (Hu et al., 1992). Substrate nitrogen as well as the end-product of the pathway, i.e. proline, thus control the activity of the enzyme P5CS. A clear correlation exists between the induction of the gene encoding for Δ1-pyrroline-5-carboxylate synthetase (P5CS) and the accumulation of proline in Arabidopsis thaliana under osmotic stress (Savoure et al., 1995). It appeared that the feedback regulation of P5CS is lost in plants under stress conditions. Transgenic tobacco plants expressing a wild-type form of V. aconitifolia P5CS and a mutated form of the enzyme (P5CS-F129A), whose feedback inhibition by proline was removed by site-directed mutagenesis were used to compare proline levels (Kavi Kishor et al., 1995; Zhang et al., 1995; Verma, 1999). Removal of feed-back inhibition of P5CS resulted in higher proline accumulation and protection of plants from osmotic stress (Hong et al., 2000). Elevated levels of proline caused by over-expression of P5CS in transgenic rice conferred enhanced tolerance to salt stress (Zhu et al., 1998). Similarly, P5CS gene was introduced into wheat using Agrobacterium-mediated gene transfer via indirect pollen system (Sawahel and Hassan 2002). Salinity test of these transgenic wheat plants indicated that over-production of proline results in increased tolerance to salt stress. Also, introduction of P5CS gene via Agrobacterium into carrot resulted in enhanced salt tolerance (Han and Hwang, 2003). Vigna P5CS gene was also transferred into the green microalga Chlamydomonas reinhardtii, where it was over-expressed (Sriripornadulasi et al., 2002). It was shown that transgenic algae expressing the mothbean P5CS gene had 80 % higher free-proline levels than wild-type cells. These transgenics grew more rapidly in toxic cadmium concentrations (100 μM) and bound fourfold more cadmium than wild-type cells. Recently, Vendruscolo et al. (2007) and Yamchi et al. (2007) introduced P5CS gene in wheat and tobacco respectively and observed higher proline content in transgenic plants and in turn improved drought tolerance.
to both crops in addition to enhanced salt tolerance in tobacco due to better protection against oxidative stress.

On the contrary, \textit{P5CS} antisense Arabidopsis lines that were impaired in their capacity to synthesize proline were hypersensitive to osmotic stress (Nanjo \textit{et al.} 1999a). \textit{P5CS} antisense lines showed morphological abnormalities of epidermal and parenchymatous cells, underlying the role of proline as major constituent of cell wall proteins (Nanjo \textit{et al.} 1999a). Similarly, \textit{P5CS1} Arabidopsis insertion mutant showed reduced salt tolerance (Szekely \textit{et al.} 2008). Furthermore analysis of Arabidopsis \textit{P5CS} insertion mutants confirmed a role in vivo for proline in ROS scavenging, which was first postulated by Smirnoff and Cumbes in (1989). Enzymes of the ROS-scavenging glutathione-ascorbate cycle showed significantly lower activities in the \textit{P5CS1} mutants compared to wild type under salt stress suggesting that proline accumulation is implicated in the control of either stability or activity of enzymes in the glutathione-ascorbate cycle (Szekely \textit{et al.}, 2008).

Various researchers have also manipulated proline biosynthesis under stress conditions by using \textit{P5CR} gene. La Rosa \textit{et al.} (1991) reported enhanced \textit{P5CR} activity by 50-folds in response to the expression of soybean \textit{P5CR} gene in transgenic tobacco plants. However, enhanced \textit{P5CR} activity in transgenic tobacco plants did not yield significant increase in proline levels. Thus, these transgenic results confirmed that enhanced \textit{in vivo} activity of \textit{P5CR} is limited by the lack of substrate P5C. Further, soybean plants were transformed with \textit{P5CR} gene construct in an antisense direction controlled by an inducible heat shock promoter (IHSP) by De Ronde \textit{et al.} (2000). Reduction of \textit{P5CR} gene expression in antisense lines of soybean plants resulted in a decline in proline as well as protein synthesis and these antisense lines of transgenic soybeans did not withstand the osmotic stress due to decline in proline biosynthesis and accumulation. Low proline synthesis and accumulation in the transgenics resulted in lower seed production than in control plants, indicating that the antisense \textit{P5CR} gene also negatively influenced seed production in soybean (De Ronde \textit{et al.}, 2000). More recently, Simon-Sarkadi \textit{et al.} (2006) over-expressed \textit{P5CR} gene in soybean plants and resulting transgenic lines showed higher proline content than the wild types and observed enhanced drought stress at supra-optimal temperatures. These investigations in addition to the present study clearly indicated that proline biosynthetic pathway manipulations using genetic engineering tools is of tremendous use and have great potential to produce salt tolerant crops.
Results of the present investigation revealed that indica rice cv KJT-3 is amenable to Agrobacterium-mediated transformation using the vector pCAMBIA 1301 harbouring a mothbean P5CS-F129A gene under the control of CaMV 35S promoter. The results clearly showed the successful insertion of this gene in primary (T₀) generation and its inheritance to the progeny plants (T₁) as revealed by GUS expression, PCR products and Southern analysis. The gene was functionally expressed in T₀ plants as indicated by higher proline accumulation in transgenics as compared with non-transgenic plants produced through tissue culture. The testing of salt stress tolerance of transgenic plants of T₁ generation revealed the better growth performance, biomass production, higher proline accumulation and lower rate of lipid peroxidation in comparison with the non-transgenic plants under 150 mM NaCl stress. These results made it clear that this gene was inserted stably in KJT-3 genome.

However, the field trials are necessary to finally release the transgenic salt tolerant plants of this rice cv and for this purpose, the large scale production of KJT-3 transgenics is prerequisite. Therefore large-scale production of transgenics, further confirmation of gene integration in further generations and their salt stress tolerance testing in field conditions and to release the transgenic plants are our future plans.