A novel approach for raising salt tolerant transgenic plants based on altering stress signalling through Ca^{++}/calmodulin-dependent protein phosphatase calcineurin

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Excess amounts of salts in soil adversely affect growth and development of salt-sensitive plans (i.e. glycophytes). Processes leading to seed germination, seedling growth, flowering and fruit set are negatively influenced by high salt concentrations. Due to high levels of salt stress, the genetic potential of the plant is not utilized to its maximal extent towards tapping the grain yield. Conventionally, it is accepted that a saline soil is one which has a conductivity of 4 ds/m, which translates into nearly 2.56g total dissolved salts in the saturated extract (or if all the salt is NaCl, an ionic concentration of 44 mM). In India, large tracts of land are unsuitable for cultivation due to high salt levels. Furthermore, the problem of excess soil-salinity is getting accentuated due to extension of the irrigation network in cropping systems. There is a great deal of urgency for developing crop varieties which can sustain and set seeds under high salt concentrations. In order to produce salt-tolerant crops by plant genetic engineering methods, it is important to obtain in-depth understanding of the plant responses to high salt concentrations[1–3].

The common salt – NaCl, is often the most abundant salt in saline soils. This compound in higher concentrations causes water deficit, ion toxicity, ion imbalance or a combination of these factors. A major part of the NaCl-induced growth inhibition is caused by excess Na ions. There is a natural tendency in all the cells to accumulate K and exclude Na ions for maintaining favourable K$^+$/Na$^+$ ratios[4]. Presence of higher concentrations of Na$^+$ in the growth medium upsets this balance. Excess Na$^+$ may compete with K$^+$ in membrane transport and when accumulated in the cytoplasm, it inhibits many enzymes[4].

It has been known for a long time that presence of Ca^{++} in the growth medium enhances the selective absorption of K$^+$ by plants at high concentrations of NaCl (ref. 5). Such observations have been made in a large number of plant species. The beneficial effects of adding CaCl$_2$ to the root medium of rice plants which are subjected to NaCl stress are shown in Figure 1 (see Bhushan and Grover[6] for more details on Na/Ca interactions in rice). To explain the mechanism(s) underlying this phenomenon, it is suggested that Ca^{++} sustains K$^+$ transport and K$^+$/Na$^+$ selectivity in Na-challenged plants[4]. However, the mechanistic details of this process are largely unknown.

J. Liu and J-K. Zhu from the University of Arizona, USA, have studied the genetics of the Na$^+$/K$^+$/Ca^{++} interactions employing *Arabidopsis thaliana* plants. These workers isolated a recessive mutant (sos3) in this species which is hypersensitive to Na$^+$. Under salt stress, sos3 plants accumulated more Na$^+$ and retained less K$^+$ than the wild type plants[7]. It was further found that sos3 plants were incapable of growing under low K concentrations. Increased Ca in the culture medium partially suppressed the Na hypersensitivity of sos3 plants and completely suppressed the defect in the K nutrition. Based on extensive data, it was inferred that the *SOS3* gene product is part of a crucial pathway for mediating the beneficial effects of Ca during NaCl stress. Liu and Zhu[8] banked on the positional cloning technique for cloning SOS3 locus which is on chromosome V between two molecular markers namely 'nga 139 and CDPK 9'. These molecular markers were employed as the starting points for identifying the overlapping YAC clones. RELP analysis delimited SOS3 locus to a 120 kilobase region of DNA between the left ends of two YAC clones. A BAC contig was subsequently assembled within this region. Further, a binary cosmid clone which rescued sos3 mutant phenotype upon transformation into sos3 mutant plants was identified. The *SOS3* gene in this complementing cosmid was identified by sequencing candidate genes from the sos3 mutant plants. The transcribed sequence of the *SOS3* gene was determined by sequencing several overlapping cDNAs obtained by library screening and by reverse transcriptase–polymerase chain reaction (RT–PCR). Importantly, the deduced amino acid sequence of the *SOS3* gene has been found to be similar to that of a large number of EF hand calcium-binding proteins. Specifically, the amino acid sequence of the *SOS3* gene product shows its close affinity to calcineurin (CAN) and neuronal calcium sensors (NCS) of animals, which can stimulate protein phosphates or inhibit protein kinases. These versatile proteins participate in some ion transport phenomena in other organisms. In animal cells, CAN plays a key role in diverse cellular functions, including T cell activation, neutrotransmission, neutrophil migration, and Na$^+$ homeostasis.

A clear picture on CAN involvement in Na$^+$-responses has emerged from the study of yeast cells. In the yeast *Saccharomyces cerevisiae*, Na$^+$ homoeostasis is achieved by the coordinated regulation of plasma membrane influx and efflux systems[9]. Na$^+$ enters the yeast cell through K$^+$ uptake system. Under Na$^+$ stress, the K$^+$ uptake system is converted into a high affinity mode of K$^+$ transport that results in higher K$^+$/Na$^+$ discrimination, thereby reducing the influx of Na$^+$. The expression of this high affinity state depends on the *TRK*1 gene encoding a putative K$^+$ transporter. Na$^+$ efflux is mediated by the P-type ATPase encoded by *ENA*1, an essential gene for NaCl tolerance. Complementation of NaCl-sensitive yeast mutants led to the isolation of *CNB*1 gene which encodes for the regulatory subunit (CNB) of the Ca^{++}/calmodulin dependent protein phosphatase CAN. Cells deficient in CNB accumulated Li$^+$ (Li$^+$ is a toxic analogue of Na$^+$) due to

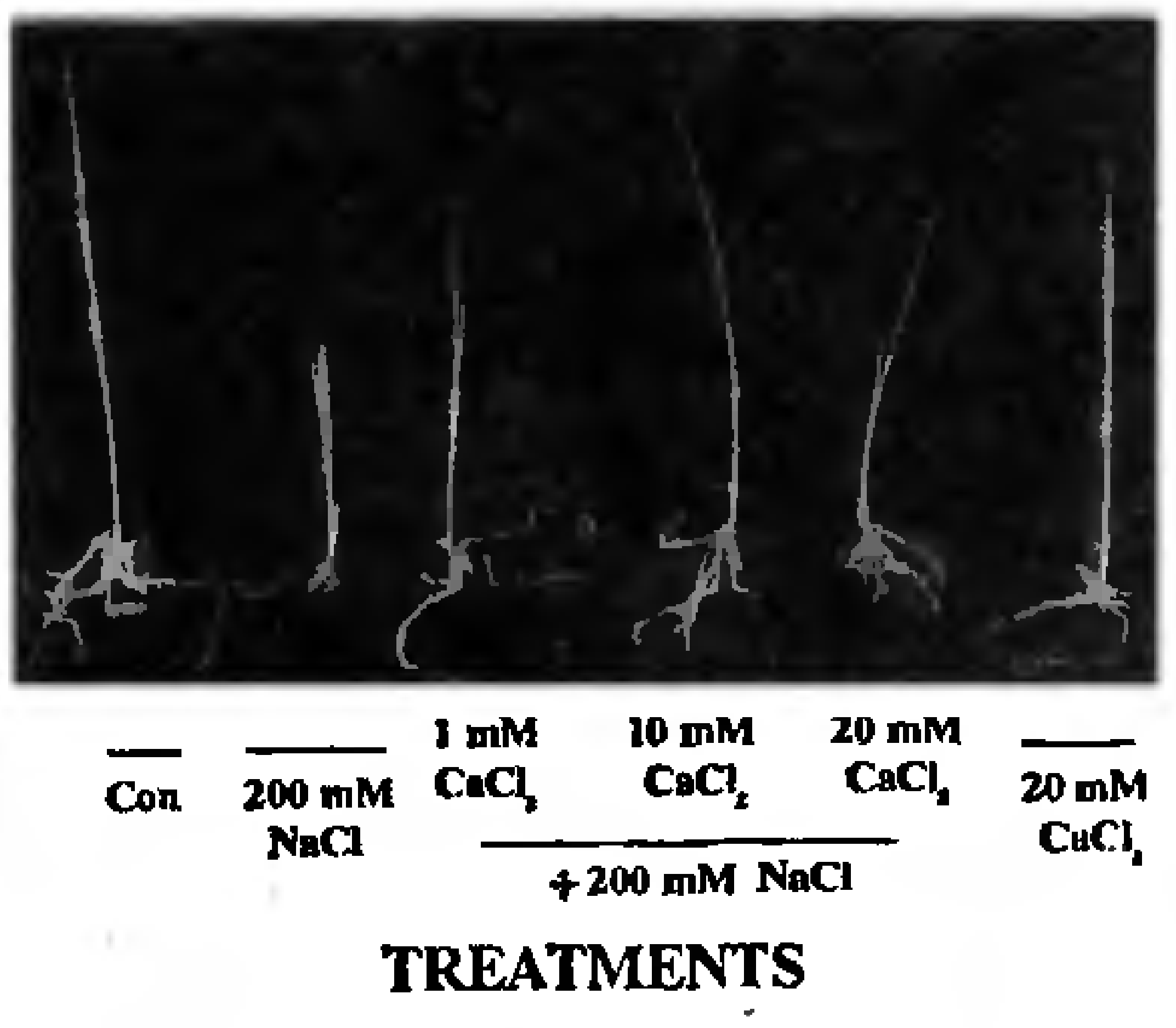

Figure 1. Ameliorating effect of $CaCl_2$ on NaCl-induced injury in rice. Treatment of rice seedlings to 200 mM NaCl reduced the seedling growth. Inclusion of varying concentrations of $CaCl_2$ from 1 to 20 mM had a remarkable effect in mitigating the adverse effect of NaCl (Pareek, A. and Grover, A., unpublished).

reduced expression of *ENA*1, which is responsible for Na^+ efflux as stated above. In addition, it was found that the K^+ transport system of cnb1 mutant cells was not converted to the high affinity state that facilitates better discrimination of K^+ over Na^+.

If CAN coordinates gene expression and activity of ion transporters to facilitate ion homoeotasis, can overexpression of this protein improve salt tolerance in plants? CAN holozyme in yeast is a heterodimer consisting of a catalytic subunit (CAN A or CNA) and a regulatory subunit (CAN B or CNB). The regulatory subunit binds up to four molecules of free Ca^{++}, stimulating the protein phosphatase activity of the CNA. CNB complex that is inactive in the absence of Ca^{++}. Recently, Pardo *et al.*[10] co-expressed catalytic and regulatory subunits of yeast CAN in transgenic tobacco plants and reconstituted a constitutively-activated phosphatase *in vivo*. Importantly, several different transgenic lines that expressed activated CAN also exhibited substantial NaCl tolerance and this trait was linked to the genetic inheritance of the CAN transgenes. It was further seen in this study that the enhanced capacity of plants expressing CAN to survive NaCl shock was similar when evaluation was conducted on seedlings in tissue culture conditions or plants in hydroponic culture. From the above work, it was concluded that in plants, like in yeast, a Ca^{++} and calmodulin-dependent CAN pathway regulates determinants of salt tolerance required for stress tolerance.

As mentioned previously[3], tolerance to abiotic stresses is mediated by multiple genes and for pyramiding useful genes it is important to search novel candidate genes. Calcineurin emerges as one such important gene which can be a candidate for pyramiding. More importantly, it has recently been shown that application of regulatory genes (such as transcriptional factor genes) which can influence the whole cascade of genetic alterations is a more rewarding approach for raising abiotic stress tolerant crops[11]. Calcineurin is a focal component of the Ca^{++}-dependent signal transduction pathway and recent success with this component may lead to a new dimension of genes for stress tolerance. Further work on calcineurin must address the following points: (i) Is this pathway universal for explaining $Na^+/K^+/Ca^{++}$ interactive responses for diverse crops? (ii) Is there a limit to which Ca^{++} mitigates Na^+-induced injury and what metabolically determines this limit? (iii) To what extent do the accompanying anions influence the balance/ratios of cations in the cell? (iv) How do the laboratory conditions of altering $Na^+/K^+/Ca^{++}$ match with the field-conditions where there are multi ions species which are under constant dynamism? (v) Can the results of such experiments be extrapolated to the performance of crops at critical stages like flowering, etc.?

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The debate on the dawn of multicellular life on earth

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Few episodes in the long march of time on earth have been under such prolonged debate as the bouts of explosion of life during early Cambrian or their extinction during late Cretaceous. In spite of considerable interdisciplinary research to close or narrow down many of the controversial issues concerning them, a consensus has been eluding the scientists. Currently, the controversy is over the earliest appearance of multicellular organisms (metazoans) on earth. Palaeontologists had, for long time, settled down to the view that evolution of life proceeded very slowly during the Precambrian times (4500–570 m.y ago) even though life, as unicellular forms, had begun as early as