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Engineering for biosynthesis of ectoine (2-methyl 4-carboxy tetrahydro pyrimidine) in tobacco chloroplasts leads to accumulation of ectoine and enhanced salinity tolerance

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Abstract

Ectoine is a non-accumulated compatible solute in many halophilic bacteria. Its biosynthesis originates from L-aspartate β-semialdehyde and requires three enzymes: L-2,4-diaminobutyric acid aminotransferase (gene: *ect B*), L-2,4-diaminobutyric acid acetyl transferase (gene: *ect A*) and L-ectoine synthase (gene: *ect C*). Twenty-four transgenic tobacco plants, generated for biosynthesis of ectoine in the chloroplasts, showed no phenotypic abnormality. NMR detected the accumulation of ectoine. Eight out of the 15 transgenic plant lines capable of growth and germination in plant media fortified with 300 mM additional NaCl in the growth room maintained at 22–24 °C were randomly selected for the dual salinity and temperature stress experiments at peak summer temperatures in the greenhouse. Salinity stress was administered for the span of three stress cycles, by placing the plant pots in trays filled with distilled water fortified with 250 mM NaCl for 1 week followed by 1 week of normal watering. Five of the eight lines were capable of growth, flowering and seed setting despite these conditions. These transgenic lines showed significantly higher accumulation of proline and phenol and also higher activities of peroxidase, polyphenol oxidase, catalase and phenylammonia lyase. Decline in the activity of glutamate dehydrogenase after the second stress cycle was evident. In addition, preservation of the activity and integrity of Rubisco, higher rates of photosynthesis and greater membrane stability as compared to SR1 controls and the three less tolerant transgenic lines was also evident. Targeting the biosynthesis of ectoine to chloroplasts confers salinity and temperature stress tolerance on the transgenic plants. It may also be a viable alternative for bulk production of this compound.

Keywords: Ectoine; Transgenic tobacco; Salinity stress; ABA; Oxidative enzymes; Rubisco

1. Introduction

Genetic manipulation of crop plants for enhanced tolerance to abiotic stresses holds great promise for sustainable agriculture [1]. Abiotic stresses have been shown to have a quantitative character and they are controlled by multiple genes [2,3]. However, there are a number of reports documenting single gene transfers that have led to the development of tolerant plants [2,4,5]. Considering the complex metabolic reactions operating in the cell in response to abiotic stresses, there is a need to test possible contributions of other candidate genes and pathways for addressing the problem of salinity stress, which is clearly a multigenic trait. Incorporation of biosynthetic pathways comprising of more than one gene and leading to biosynthesis of a compatible solute is relatively recent and has been shown to confer higher levels of tolerance to several abiotic stresses [4,6–8].

The traditional function of compatible solutes, which is known to be 'osmoregulation' was thought to be best executed by their enhanced accumulation [9]. However, the mechanism of action of the 'compatible solutes' from their mere involvement in

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regulation of cell turgor is now changing in favor of a greater 'regulatory effect' [10,11]. Such a regulatory role is instrumental in explaining the alleviation of salinity and drought stress to a considerable level despite low accumulation of molecules such as ectoine and trehalose [6–8].

Ectoine is one of the most widespread compatible solutes that is synthesized by both halotolerant and halophilic bacteria [12]. The three genes for biosynthesis of this compound are comprised of *ect B*, *ect A* and *ect C* which code for L-2,4-diaminobutyric acid aminotransferase, L-2,4-diaminobutyric acid acetyl transferase and L-ectoine synthase, respectively. Ectoine, as well as its hydroxy derivative, have been shown to protect and stabilize enzymes, proteins, membranes and whole cells against exposure to salt, heating, freezing and desiccation [13]. Consequently, there is now an increased interest in use of ectoine and its derivatives in the biotechnology industry and in several medical applications [13,14]. Based on these reports, and in view of our past experience in synthesizing ectoine in the cytoplasm, albeit at nanomolar concentrations, we decided to target the transcripts coding for the three biosynthetic enzymes to the chloroplast, with an aim to enhance the level of its biosynthesis and accumulation. This approach was considered feasible due to the following reasons: (1) the genes used for biosynthesis of ectoine, in this investigation, were PCR amplified from the prokaryote, *Marinococcus halophilus*. There is ample evidence that genes of prokaryotic origin are best expressed in chloroplasts [15,16]. (2) Aspartate semialdehyde, which is the precursor of ectoine [17], is likely to be present in a much greater amount in the chloroplasts owing to predominance of amino acid biosynthesis in this organelle [15]. (3) In view of increasingly convincing reports about the role of ectoine as an enzyme and protein protector, its accumulation in the chloroplasts, would allow us to investigate its protective effect (if any) on Rubisco. Susceptibility of Rubisco to abiotic stresses is one of the many factors that adversely affect photosynthesis ([15] and references therein). (4) Although the isolation of ectoine by the process of bacterial milking [18] seems feasible at the present time, in view of increasing demand for this compound, it may be essential to develop alternative cost effective strategies for its bulk production. Its biosynthesis in the chloroplast of tobacco appeared to be a viable alternative.

2. Materials and methods

2.1. Bacterial strains and growth conditions

M. halophilus DSM 20408$^{\Gamma}$ was grown aerobically at 37 °C in LB medium, containing 0.3% (w/v) artificial sea salt and 3.7% (v/v) NaCl.

2.2. Nucleic acid manipulations and designs of the final 'constructs'

PIJT117 [19] vector having the Cauliflower Mosaic Virus (CaMV) double 35S promoter, the CaMV polyadenylation signal and pea rbcS sequence as the transit peptide was used for engineering the intermediate 'constructs' for the ectoine biosynthetic pathway. This plasmid was appropriately modified to incorporate the different plant promoters as well as the PCR amplified sequences for the three genes of the ectoine pathway. Individual genes were PCR amplified from the DNA extracted from *M. halophilus* DSM 20408$^{\Gamma}$ according to the method of Gustinich et al. [20].

2.2.1. Design of intermediate constructs

Ect A: Primers for *ect A* were designed as follows. Primer I with the *Pst*I site was, GAGctgcagTACAGGTAGCCCTCT. Primer II with the *Bam*H1 site was, GTAAggatccTCCTTAGGGATGTTATC. The PCR amplified fragment was cloned in PGEM-T-easy vector (Promega, Madison, WI) and several clones were sequenced completely. The appropriate re-cleaved fragment was then cloned into the PIJT117 vector in *Pst*I and *Bam*H1 sites. The resulting '*ect A* cassette' comprised of the CaMV double 35S promoter, and the CaMV polyadenylation signal in addition to *ect A* (Fig. 1a).

Ect B: The PIJT117 vector was first modified to incorporate the rice phospholipase D (PLD) promoter (accession number of c-DNA is AB001920, with promoter sequence from 1 to 1904). This fragment was originally cloned in pBluescript. PIJT117 was first cleaved with *Sal*I, blunt ended and religated to destroy the *Sal*I cloning site. It was then restricted with *Sac*I and *Hin*dIII to cleave the double 35S promoter. The linearized plasmid was blunt ended and dephosphorylated. Phospholipase D promoter was cleaved from pBluescript, with *Kpn*I and *Eco*R1 and blunt ended. The PLD promoter fragment was ligated with the linearized blunt ended PIJT117 plasmid in place of the double 35S promoter. This new plasmid was named PIJT117-PLD. The PLD fragment that was ligated using the above strategy, had a useful *Sal*I site at the 5′ end of the promoter fragment that originated from the pBluescript. *Ect B* fragment was PCR amplified from DNA of *M. halophilus*, with the following primers: primer I: CAAagatctTGTCATGTATTATGTAAA, with in built *Bgl*II site; primer II: CGATCgaattcTTTTTATCCAATTAG, with the inbuilt *Eco*RI site. The PCR amplified fragment was cloned in PGEM-T-easy vector and several clones were sequenced completely. One of the appropriate clones was re-cleaved with *Bgl*II and *Eco*R1 and was cloned into the *Bam*H1–*Eco*R1 cloning site of the PIJT117-PLD plasmid. The polyadenylation signal of the Cauliflower Mosaic Virus, originally, present in the PIJT117 plasmid and that was carried over in the PIJT117-PLD plasmid was used as the poly A signal (Fig. 1b).

Ect C: To prevent the possibility of recombination in planta, *ect C* was cloned with a different promoter as well as polyadenylation signal, in the suitably modified PIJT117 plasmid, which was restricted with *Eco*R1 and *Xho*I, blunt ended and religated. The new plasmid, devoid of the polyadenylation signal of CaMV, was named PIJT117-2. PIJT117-2 was also restricted with *Sac*I and *Hin*dIII, to cleave off the CaMV double 35S promoter. The linearized PIJT117-2 was blunt ended and dephosphorylated. This fragment was then