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Enhanced NaCl Stress Tolerance in Transgenic Tobacco Expressing Bacterial Choline Dehydrogenase

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The intracellular accumulation of osmoprotectants, such as glycine betaine and other low molecular weight compounds, is a well investigated response of environmental stress occurring in a wide range of organisms. By introducing the bacterial *betA* gene, encoding choline dehydrogenase (CDH), into tobacco both a salt and choline resistant phenotype was achieved. As measured by dried weights, there was an 80% increase in salt tolerance between the transgenic and wild-type plants at 300 mM NaCl.

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Many plants live in conditions where growth may be dramatically impaired by environmental factors such as drought, salinity or cold temperatures[1]. Plants have evolved to protect themselves against these abiotic stress factors that lead to cellular dehydration. A common cellular adaptative mechanism is the accumulation of osmotically active, low molecular weight, nontoxic compounds[2]. Collectively referred to as osmoprotectants, these compounds benefit stressed cells in two ways: (i) by acting as a cytoplasmic osmolyte, thereby facilitating water uptake and retention[3] and (ii) by protecting and stabilizing macromolecules from damage induced by high salt levels[2,4]. The most widely known osmoprotectants are sugars, alcohols, proline and quaternary ammonium compounds. The quaternary ammonium glycine betaine is a very effective osmolyte. It accumulates during water stress, induced primarily by high salinity but also by drought and low temperatures[5,6] in bacteria, cyanobacteria and certain higher plants. Glycine betaine synthesis and accumulation in plants have been best characterized in members of two families, *Chenopodiaceae* and *Poaceae*[7,8]. Several commercially important crops such as potato, rice, tomato, and tobacco[3,8] do not accumulate glycine betaine. This has motivated us to introduce a glycine betaine biosynthetic pathway in such species to increase their stress tolerance and crop yield.

Glycine betaine biosynthetic pathways are similar in higher plants and microorganisms. In both cases, betaine is derived from a two step oxidation of choline to glycine betaine via the unstable intermediate, betaine aldehyde[9]. In the conversion of choline to betaine aldehyde, spinach uses the ferredoxin dependent choline monooxygenase[10] while *Escherichia coli* use the membrane linked choline dehydrogenase[11,12] (CDH). The second step in the glycine betaine biosynthetic pathway is catalysed by the NAD^+ dependent betaine aldehyde dehydrogenase (BADH), which exhibits strong similarities in plants and bacteria[13]. These include structural homologies and the ability of both sugar beet and bacterial BADH to produce glycine betaine from betaine aldehyde in transgenic tobacco[13–15]. Bacterial CDH is the most useful enzyme for introducing this pathway into new species since it is able to catalyze, not only the oxidation of choline to betaine aldehyde, but also the second step to glycine betaine. Bacterial CDH works independent of soluble cofactors[11,12], a favorable trait to avoid interference with other metabolic pathways in a transgenic organism. In this study, we have introduced the *E. coli betA* gene, encoding CDH, into tobacco (*Nicotiana tabaccum* cv. SR1) in order to enhance stress tolerance by intracellular accumulation of glycine betaine. Tobacco was selected as host species because it lacks both enzymatic steps in the glycine betaine biosynthetic pathway[8]. Tobacco was transformed with a Ti-plasmid–based vector harboring the *betA*

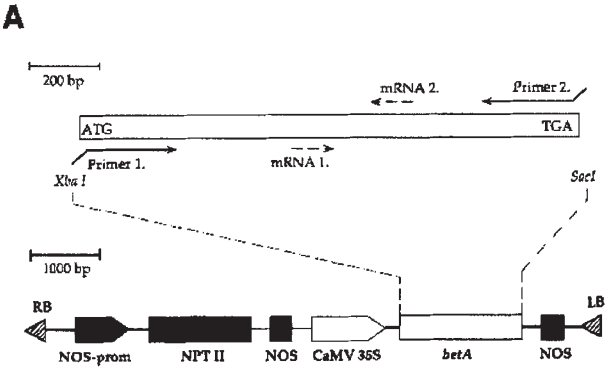

FIGURE 1. (A) Construction of the Ti-expression vector containing the bacterial *betA* gene. The *betA* gene was derived from *E. coli* using PCR assisted cloning. The *betA* gene encoding CDH was placed under control of the CaMV 35S promoter and transcription termination was achieved by the NOS region. The approximate positions of the PCR primers are indicated by solid arrows, while the broken arrows indicate the position of the primers used for *betA* mRNA transcription analysis. Right and left border of the T-DNA are indicated by RB and LB, respectively. NOS-prom, NPT II and NOS are abbreviations for the nopaline synthase promoter, the neomycine phosphotransferase II gene and the nopaline synthase 3′ transcription termination region. (B) The primers used to amplify and clone the bacterial *betA* gene. The 5′ overhangs including restriction enzyme sites are shown in small letters, while the hybridizing portions of the primers are shown in capital letters. The "*" indicates the site of the point mutation, that changed the base thymine into adenine. This was done in order to select a more suitable plant transcription initiation codon, ATG.

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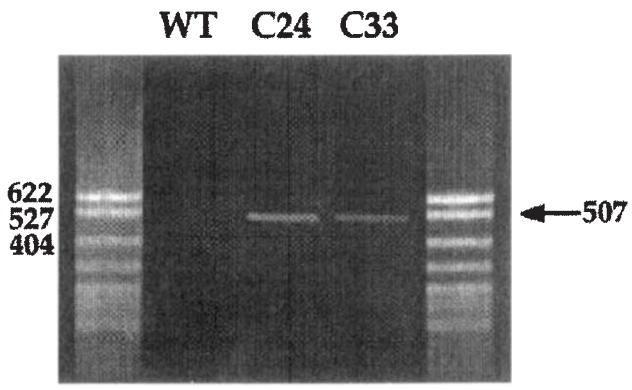

FIGURE 2. Transcription analysis of the *betA* gene by detecting mRNA levels in plant leaves homogenates. The mRNA was purified by oligo(DT)-cellulose chromatography and amplified with *rTth* polymerase yielding detectable amounts of cDNA. The cDNA corresponds to a 506 bp middle section of the *betA* gene, encoding CDH. Wild type tobacco was used as a negative control.

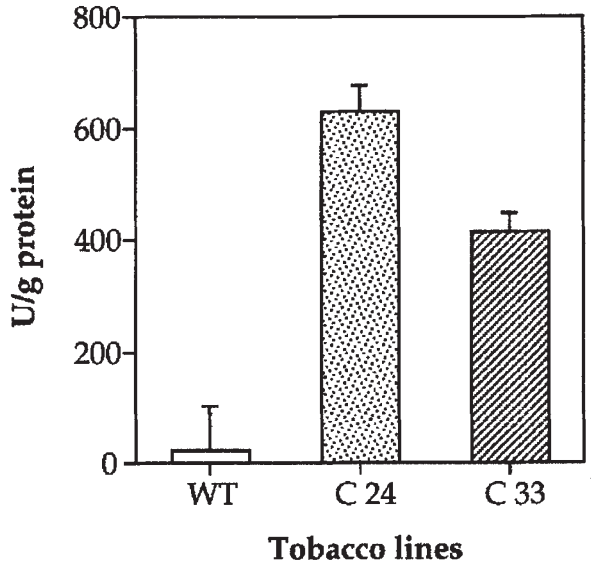

FIGURE 3. Analysis of the CDH activity in transgenic tobacco plants expressing the CDH enzyme. Membrane proteins were extracted from 10 weeks old plants. The membrane extracts of wild type (WT) and transgenic tobacco lines (C 24 and C 33) were incubated with carbon 14 labeled choline at 37°C for 60 minutes. The data represents means of nine plants. Error bars respond to the standard deviation. The experiment was repeated three times.

gene under control of the CaMV 35S promoter using *Agrobacterium tumefaciens*. Our aims were to determine: (i) if the *betA* gene was correctly transcribed; (ii) if bacterial CDH was enzymatically active in tobacco and (iii) if transgenic tobacco expressing CDH exhibited enhanced tolerance against NaCl.

Results

Cloning of a *betA* gene–containing vector. The *bet* coding region (6493 bp) of *E. coli* harbors four different genes[12], *betA* encoding CDH, *betB* encoding BADH, *betT* encoding a choline transport protein, and *betI* encoding a small repressor protein. Polymerase chain reaction (PCR) was used to isolate the *betA* gene from the *E. coli* genome. The primers used for PCR were designed to contain restriction enzyme sites, so that the *betA* gene could be expressed both in bacterial and plant hosts (Fig. 1). To ensure sufficient expression in plants the bacterial initiation codon of the *betA* gene was changed from TTG to ATG, a more frequently used translation initiation codon in tobacco. The amplified *betA* gene was inserted into pUC 19 and transformed into *E. coli*. Several CDH positive clones were obtained, and two clones were selected and transferred to the plant expression vector pBI121:2 using XbaI and SacI (Fig. 1). The resultant vectors, pCDH2 and pCDH3, were then transformed into tobacco.

Transcription and activity analysis of the transgenic material. PCR was used to screen for *betA*–positive plants. DNA was extracted from 10 randomly chosen kanamycin–resistant transgenic lines of each construct. The extracted DNA was amplified using PCR primers 1 and 2 (Fig. 1). Approximately 90% of the transformed plants gave rise to the 1700 base pairs full length *betA* fragment. Several PCR positive plants were also visually assessed for choline tolerance in growth experiments. The two best choline tolerant lines, C 24 and C 33, were chosen for further studies. PolyA$^+$ mRNA was isolated from leaves of these plant lines. mRNA was detected using two polynucleotide primers (mRNA 1 and 2). This *betA* mRNA region was amplified using *rTth* polymerase[16]. The transgenic tobacco plants, C 24 and C 33, gave rise to the expected cDNA fragment, whereas the control lane containing wild type tobacco did not (Fig. 2).

CDH activity, in plants with detectable *betA* mRNA levels, was determined by using ^{14}C-labelled choline as substrate[11]. When soluble protein fractions of the different clones were analyzed no detectable CDH activity was observed either in the transgenic lines or in the wild-type line. CDH is associated to the cell membranes in *E. coli*[12], indicating the possibility of similar localization in transgenic tobacco. The tobacco membranes from the plants were therefore treated with Triton X-100 to dissociate CDH. The solubilized membrane fractions of the transgenic clones, C 24 and C 33, showed considerably higher CDH activity than wild-type tobacco. The C 24 line gave at least a six-fold, and the C 33 line a 4.5-fold, increase in CDH activity compared to the wild-type (Fig. 3). To test the necessity of adding an exogenous electron acceptor, the activity was measured with and without phenazine methosulphate. No differences in activity were observed indicating that the amount of the endogenous electron acceptor was sufficient.

Expression of the *betA* gene in tobacco results in choline resistance. Although choline is ubiquitous in higher organisms, tobacco growth was severely retarded at external concentrations higher than 10 mM (Fig. 4A). The toxic influence of choline at 30 mM reduced the plant yield of wild-type tobacco to less than 10% of that of nontreated plants. Plants expressing CDH in the cytoplasm might therefore be able to accept higher exogenous concentrations of choline than the wild-type. This hypothesis was tested by growing transgenic (C 24, C 33) and wild-type plants on MS medium containing varying concentrations (0 to 30 mM) of choline (Fig. 4A). At high choline concentrations (20 and 30 mM) transgenic plants are more resistant (Fig. 4A). An improvement of four- and three-fold in choline tolerance was observed in lines C 24 and C 33, respectively. The enhanced resistance of the transgenic lines, C 24 and C 33, could also be correlated to the activity levels, since C 24 exhibits both higher CDH activity and greater tolerance towards choline (Figs. 3 and 4A). The effect of the *betA* gene in the transgenic lines was only marginal at 10 mM choline, which could be expected because the degree of inhibition was low at this concentration.

Transgenic tobacco exhibited enhanced salt stress tolerance. The growth of tobacco plants is strongly inhibited by NaCl concentrations higher than 100 mM (Fig. 4B). To deter-

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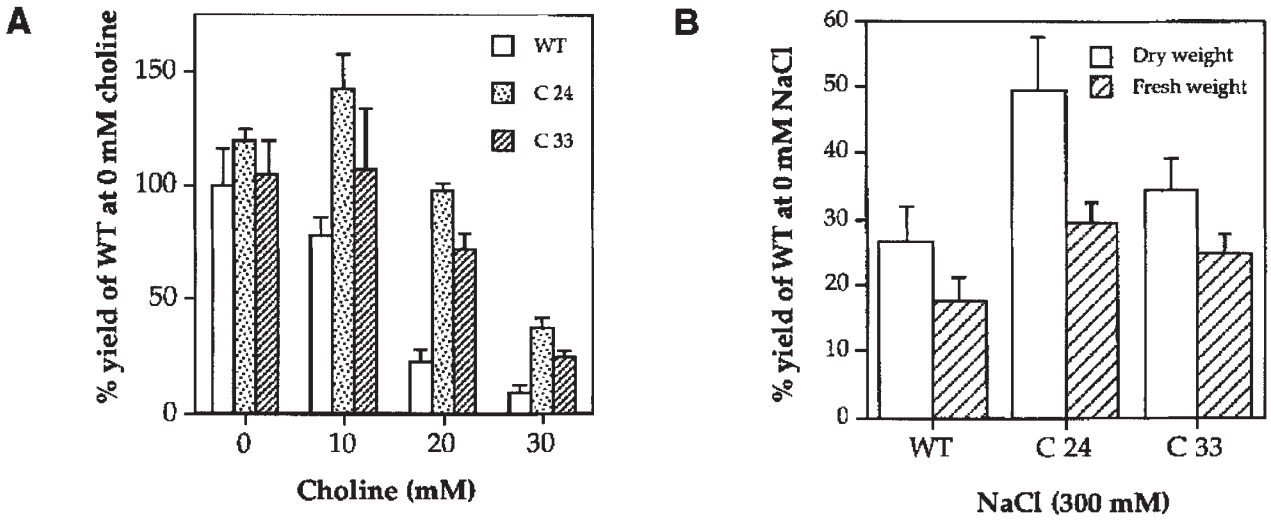

FIGURE 4. Tobacco growth experiments in MS media supplemented with varying concentrations of choline and NaCl. (A) Comparison of the choline toxic effect between wild type and two transgenic lines. A total of 9 plants of the wild type, the C 24 clone and the C 33 clone were grown at each choline concentration (0, 10, 20 and 30 mM). Each data point corresponds to the mean value calculated from nine plants and the error bars correspond to the standard deviation. (B) Growth experiment displaying enhanced NaCl tolerance in transgenic tobacco expressing CDH when grown in MS media containing 300 mM NaCl. Each data point corresponds to the mean value calculated from 10 plants and the error bars correspond to the standard deviation.

Both experiments were repeated twice and the results are presented as mean values of percent relative change in yield compared to the wild type control. The yield was measured as dried weight in the choline tolerance experiments, and as both fresh and dried weight in salt experiments. The data were subjected to statistical analysis utilizing one–way analysis of variance (ANOVA).

mine the NaCl concentration at which CDH expressed in tobacco affected the growth, we exposed 10–day–old plants to various NaCl concentrations (0 to 400 mM) for 30 days. The salt tolerance was assessed by comparing the final dried and fresh weights of CDH lines with that of a wild-type tobacco line. As shown in Figure 4B the NaCl tolerance of transgenic lines, C 24 and C 33, was enhanced particularly at concentrations from 200 to 300 mM NaCl. The difference in salt tolerance between the C 24 and wild-type line, as measured by the dried weights, was >80% ($P<0.01$). These differences could also be directly correlated to the enzymatic CDH activity of the different tobacco lines since the C 24 line exhibited both highest activity, highest NaCl- and choline-tolerance (Figs. 3 and 4). Furthermore, at 300 mM NaCl wild-type tobacco plants exhibited dehydration of lower leaves and necrosis while C 24 and C 33 plants appeared to be much less affected (Fig. 5). All plants, both transgenic and wild type, were severely injured at 400 mM NaCl.

Discussion

Different plant organs respond differently to osmotic stress. Differential inhibition of shoot versus root growth in response to osmotic stress is well established. In this study the *betA* gene was placed under the control of the strong CaMV 35S promoter which is activated in all cell types but at varying levels[17]. The localization of glycine betaine is probably essential. In plants, choline monooxygenase has been shown to be localized in the chloroplast stroma. However, in transgenic plants, where expression of bacterial BADH was localized to the chloroplasts, less resistance towards betaine aldehyde was observed than in plants expressing BADH in the cytoplasm[15]. Thus, it will be important to determine the level and influence of *betA* expression in various cell types and organelles.

As irrigation has been more frequently used all over the world soil salinity has become a widespread problem[3]. Several genes induced in response to salt stress in plants have been identified including *RAB* genes, *salT*, and osmotin, but the

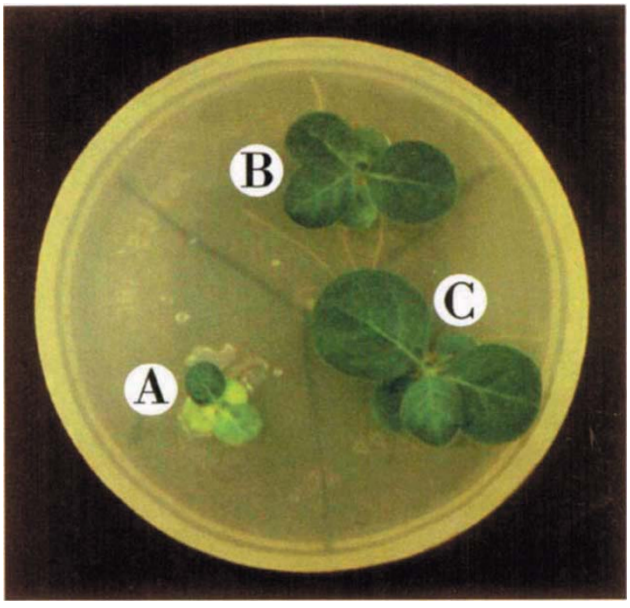

FIGURE 5. Transgenic plants, C 24 and C 33, and a wild type control grown for 4 weeks in MS-media supplemented with 300 mM NaCl. Wild type tobacco is indicated by A, C 33 by B, and C 24 by C.

functions of these genes remain largely unclear[18]. Breeding strategies that involve the expression of osmoprotectants occurring in other salt–tolerant organisms have been applied in order to overcome this growth inhibiting factor. Metabolically engineered plants carrying *E. coli* mannitol-1-phosphate dehydrogenase have been produced, but their ability to withstand high NaCl concentrations requires very high concentrations of mannitol (100 mM), which can have deleterious effects on plant metabolism[19]. Glycine betaine is an extremely efficient osmoprotectant[20], and low concentrations of glycine betaine, <1 mM, give substantial protective effects in plants[21]. Choline is ubiquitous in eukaryotes and the biosyn-

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thesis of choline is under feedback control[3]. Thus, if part of the choline pool is redirected to the introduced glycine betaine producing pathway, further choline uptake or production could be stimulated to replenish the substrate for essential choline metabolic pathways, such as production of phosphatidyl choline.

The intermediate in the CDH biosynthesis of glycine betaine, betaine aldehyde, is toxic to tobacco at concentrations above 4 mM[15]. Transgenic tobacco expressing CDH exhibits growth characteristics similar to those of the wild type control during unstressed conditions (Fig. 4A), implying that the internal betaine aldehyde concentrations do not reach those levels. However, we are currently performing NMR studies on the transgenic plants to investigate the metabolic pathways of choline.

Experimental Protocol

Recombinant DNA techniques and bacterial strains. Plasmid procedures and PCR were performed using standard protocols[22,23]. *Escherichia coli* strain TG1 (F′*traD36lacI*^{q}*Δ[lacZ]M15proA*$^+$*B*$^+$*/supE[hsdM-mcrB]5* [$r_K^-m_K^-$McrB$^-$]*thi[lac-proAB][lac-pro]*) was cultured and transformed, using standard techniques[24]. Restriction enzymes were used according to the manufacturers' recommendations (GIBCO BRL, Labdesign). *Agrobacterium tumefaciens* strain LBA 4404 (ref. 23) was cultured in LB medium (Tryptone 10 g/l, Yeast extract 5g/l, NaCl 10 g/l) and transformed with the aid of *E. coli* HB 101, harboring conjugative plasmid pRK 2013 (ref. 25). Extraction of genomic tobacco DNA for PCR analysis was performed as described[26].

DNA-constructions. The PCR-primers were designed (Biomolecular Centre, University of Lund) with homology to the 5′- and 3′-end of the choline dehydrogenase gene except for the start codon, which was altered from TTG to ATG. The primers XbaI, ClaI and SmaI restriction sites at the 5′-end and SacI and EcoRV sites at the 3′-end. These sites were used to clone into pUC 19 and pBin121:2 (Fig. 1). 10 different clones were tested for CDH activity, and the two with the highest expression were cloned into pBI121:2 yielding $pCDH_2$ and $pCDH_3$.

Plant material. *Nicotiana tabaccum SR.* plants were exposed to 16 h light at 25°C and were either kept in soil or in sterile jars on MS-media[27]. The tobacco plants were transformed by *Agrobacterium*-mediated gene transfer[28]. All experiments were performed using stable transformants of the F_1 generation.

mRNA isolation. 100 mg leaf material from transgenic and wild type plants grown for two weeks were used for mRNA extraction by Quickprep mRNA purification kit™(Pharmacia). 10 μg mRNA was extracted and used with *rTth* polymerase™ to amplify parts of the *betA* mRNA according to the manufacturer (Perkin Elmer).

Protein extracts. Leaf and stem material was homogenized in 100 ml H-buffer containing 50 mM MOPS, 5 mM EDTA, 330 mM sucrose, 2 g/l casein (boiled for 10 min.) and 1 mM phenyl methyl sulfonyl fluroid at pH 7.5. The extract was centrifuged 10 minutes at 1000 g and the supernatant was filtered through a nylon filter (mesh=50 μm). To pellet the cell membranes the supernatant was centrifuged 60 minutes at 50,000 g. The soluble protein fractions were removed for activity studies. The pellet was resuspended in 2 ml H-buffer containing 1% Triton X-100 to dissociate CDH from the membranes, and the activity was measured.

Enzyme assay. The activities of choline dehydrogenase in the membrane and soluble protein fractions were determined according to Landfald et al.[11] with some minor modifications. A reaction mixture containing 900 μl dissolved membrane, or soluble protein, in H-buffer and 100 μl 32 mM [^{14}C]choline (specific activity 55 μCi/mmol) was used to determine the activity of choline dehydrogenase in the protein extracts. The reaction mixture was incubated for 1 h at 37°C and 125 μl were applied to columns (0.4 by 4.5 cm) packed with Dowex-50x4-200 on H^+ form (Sigma). The columns were eluted with 0.4 M NaCl in a 50 mM phosphate buffer. The amounts of reaction products (glycine betaine and betaine aldehyde) in the fractions were determined by liquid scintillation counting (LKB, Sweden). CDH from *E. coli* and choline oxidase from *Arthrobacter globiformis* (Sigma) were used as a positive controls.

Growth experiments. To assess the choline tolerance, seeds were germinated in sterile containers containing 100 ml MS-medium[27] and choline concentrations ranging from 0 to 30 mM. The seedlings were grown for 30 days before harvest. The fresh weight was measured and the plants were dried in 45°C overnight to determined the dried weight. Nine seedlings of the wild type, the C 24 clone and the C 33 clone were grown on each choline concentration (0, 10, 20 and 30 mM). To determine the NaCl tolerance, seeds were germinated in sterile containers containing 100 ml MS-medium. The seedlings were transferred to MS-medium, supplemented with NaCl in varying concentrations (0 to 400 mM) after 10 days. These seedlings were grown for 30 days. The plants were harvested, the fresh weight was measured and then dehydrated at 45°C overnight to determine the dried weight. Ten seedlings of the wild type, the C 24 clone and the C 33 clone were grown at each NaCl concentration. The experiments were repeated twice and the results are presented as mean values of percent change in yield relative to the wild type plants, grown without addition of neither choline nor salt. The yield was measured as both dried and fresh weight in all stress tolerance experiments. Wild type tobacco and vector transformed control plants exhibit comparable growth characteristics when grown at elevated NaCl concentrations[19].

Acknowledgments

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