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Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine

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Abstract

Glycine betaine is an osmoprotectant found in many organisms, including bacteria and higher plants. The bacterium *Escherichia coli* produces glycine betaine by a two-step pathway where choline dehydrogenase (CDH), encoded by *betA*, oxidizes choline to betaine aldehyde which is further oxidized to glycine betaine by the same enzyme. The second step, conversion of betaine aldehyde into glycine betaine, can also be performed by the second enzyme in the pathway, betaine aldehyde dehydrogenase (BADH), encoded by *betB*. Transformation of tobacco (*Nicotiana tabacum*), a species not accumulating glycine betaine, with the *E. coli* genes for glycine betaine biosynthesis, resulted in transgenic plants accumulating glycine betaine. Plants producing CDH were found to accumulate glycine betaine as did F_1 progeny from crosses between CDH- and BADH-producing lines. Plants producing both CDH and BADH generally accumulated higher amounts of glycine betaine than plants producing CDH alone, as determined by ^{1}H NMR analysis. Transgenic tobacco lines accumulating glycine betaine exhibited increased tolerance to salt stress as measured by biomass production of greenhouse-grown intact plants. Furthermore, experiments conducted with leaf discs from glycine betaine-accumulating plants indicated enhanced recovery from photoinhibition caused by high light and salt stress as well as improved tolerance to photoinhibition under low temperature conditions. In conclusion, introduction of glycine betaine production into tobacco is associated with increased stress tolerance probably partly due to improved protection of the photosynthetic apparatus.

Key words: Transgenic tobacco, BADH, CDH, salt stress, cold stress, photoinhibition, recovery.

Introduction

Glycine betaine (hereafter referred to as betaine) is a common compatible solute in many different organisms, including higher plants (Csonka and Hanson, 1991; Le-Rudulier, 1993; Rhodes and Hanson, 1993). Many plant species accumulating betaine inhabit saline and arid areas and accumulate the compound in response to drought and salinity (Rhodes and Hanson, 1993). However, several plant species, including important crop plants, are incapable of synthesizing betaine. Betaine is thought to protect the plant by acting as an osmolyte maintaining the water balance between the plant cell and the environment (Robinson and Jones, 1986) and by stabilizing macromolecules during cellular dehydration and at high salt concentrations (Incharoensakdi *et al.*, 1986; Murata *et al.*, 1992; Santoro *et al.*, 1992; Mohanty *et al.*, 1993; Papageorgiou and Murata, 1995). Betaine has also been shown to accumulate in response to low and high temperature stress in higher plants, where it might play a role in protecting membranes and/or protein complexes (Yang *et al.*, 1996; Zhao *et al.*, 1992).

Betaine is produced by a two-step oxidation of choline in both eukaryotes and prokaryotes involving either one

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or two enzymes (Ikuta *et al*., 1977; Andresen *et al*., 1988; Hanson *et al*., 1985). In higher plants the biosynthetic pathway has been described for a number of species (Hanson *et al*., 1985; Rhodes and Hanson, 1993) where oxidation of choline into betaine aldehyde is catalysed by a ferredoxin-dependent choline monooxygenase (CMO) (Brouquisse *et al*., 1989), whereas the conversion of betaine aldehyde into betaine is catalysed by a NAD^+-dependent betaine aldehyde dehydrogenase (BADH) (Weigel *et al*., 1986). The activity of these two enzymes is localized to the chloroplast stroma, although a minor BADH activity is also found in the cytoplasm (Weigel *et al*., 1986). In spinach and sugar beet the enzymes responsible for converting choline into betaine have been characterized, and the corresponding genes cloned and sequenced (McCue and Hanson, 1992; Rathinasabapathi *et al*., 1997; Weretilnyk and Hanson, 1990).

Bacteria do not produce choline, but can effectively take up the substrate from the environment via specific uptake systems (Styrvold *et al*., 1986). In *E. coli*, a 62 kDa membrane-associated choline dehydrogenase (CDH) first oxidizes choline into betaine aldehyde. CDH is also able to catalyse the second step, oxidation of betaine aldehyde into betaine (Landfald and Strøm, 1986). This second step of the process is further catalysed by a specific 53 kDa soluble betaine aldehyde dehydrogenase (BADH). The two genes *betA* and *betB*, encoding the CDH and BADH, have been cloned and characterized (Lamark *et al*., 1991).

The accessibility and the simplicity of the betaine biosynthesis pathway in *E. coli* prompted the introduction of the bacterial pathway into tobacco, a higher plant not accumulating betaine, in order to study the effects of betaine accumulation on tolerance to salt and cold stress. The successful introduction of the second enzyme of the *E. coli* betaine pathway, *betB*, into tobacco has been described in a previous study (Holmström *et al*., 1994). The introduction of *betB* is, however, not sufficient for betaine-production as this enzyme only converts betaine aldehyde into betaine and is incapable of oxidizing choline to betaine aldehyde. In a recent study, the *betA* gene, encoding the first enzyme (CDH) of the *E. coli* betaine biosynthesis pathway, was introduced into tobacco (Lilius *et al*., 1996). Transgenic plants expressing *betA* showed improved salt tolerance, however, no data on betaine accumulation and correlation of this to stress tolerance were presented.

Using a slightly different approach, it was possible to demonstrate that introduction of the gene *codA*, encoding a choline oxidase from the soil bacterium *Arthrobacter globiformis*, into *Arabidopsis thaliana* increased tolerance to both salt and chilling stress (Hayashi *et al*., 1997). Furthermore, the transgenic plants exhibited an increased tolerance to photoinhibition (Hayashi *et al*., 1997). Similarly, it was shown that accumulation of betaine in cyanobacterial cells, transformed with *codA*, improved the capacity of these cells to grow at low temperatures (Deshnium *et al*., 1997). The improved chilling resistance was found to be due to enhanced capacity to recover from photoinhibition as well as enhanced membrane protection.

In this study, the accumulation of betaine in transgenic tobacco expressing the two *E. coli* genes *betA* and *betB* is demonstrated. It is shown that transgenic lines expressing only *betA* accumulate betaine, although accumulation is increased 2–3-fold in transgenic plants producing both enzymes. These betaine-synthesizing transgenic lines exhibit increased tolerance to salt stress. Furthermore, production of betaine enhanced tolerance to photoinhibition at low temperature and improved the capacity of photosystem II (PSII) to recover from photoinhibition caused by salt stress and high light.

Materials and methods

Plant material, culture conditions, transformation and crossings

Nicotiana tabacum cv. Samsun was employed in all experiments. Greenhouse plants were grown in soil at 22 °C under 16 h of light at 150 μmol m^{-2} s^{-1}. For axenic growth, seeds were surface-sterilized in 7.5% sodium hypochlorite solution containing 0.1% Tween 20 and thereafter rinsed in sterile distilled water. Sterilized seeds were plated in 24 well tissue culture plates containing 1 ml of solidified MS medium (Murashige and Skoog, 1962), supplemented with 2% sucrose (MS-2). Plates were thereafter transferred to a growth chamber at 22 °C using 16 h of light at 250 μmol m^{-2} s^{-1}. Following germination the seedlings were grown for 2–3 weeks until used in experiments.

For *Agrobacterium tumefaciens*-mediated transformation, leaf discs were isolated from axenically grown *N. tabacum*. Transformation procedures and tissue culture conditions were essentially as described previously (Mandal *et al*., 1993), but with the following modifications. In addition to higher concentration of kanamycin (200 mg l^{-1}) the selective shoot induction medium was supplemented with 0.2 mg l^{-1} of gibberellic acid (GA_3). Shoots of approximately 5 cm in length were subsequently transferred to root induction medium containing 4.5 mg l^{-1} indole-3-butyric acid (IBA). After a 4–6 weeks subculture approximately 50 rooted plantlets were transferred to the greenhouse for seed production.

Transgenic tobacco producing both CDH and BADH were obtained by fertilizing emasculated BADH-producing plants with pollen from CDH-producing lines. Double transgenic lines were selected by germination and growth on MS-2 medium supplemented with 20 mM choline.

For determination of salt stress tolerance at the whole plant level, T_1 seeds were germinated and grown axenically on solidified selective MS-2 medium and thereafter transferred to soil in the greenhouse. After 1 week of growth in the greenhouse, plants were subjected to salt stress for 2 weeks by watering twice a week with 200 ml 200 mM of NaCl in 0.1% RIKA-medium (Weibulls, Sweden). With the exception of NaCl, control plants were watered in the same manner. Plants were harvested after 2 weeks and the fresh weight was measured separately for each plant.

Plants used for physiological studies were germinated *in vitro*

and thereafter transferred to soil pots and grown in the greenhouse for 3–4 weeks.

Plasmid construction

The treatment of DNA was carried out according to established laboratory routines. *E. coli* strain DH5α (Hanahan, 1983) was used for plasmid amplifications. The light-stimulated promoter from the *Arabidopsis* gene *RbcS1A*, encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco), was used to regulate expression of the CDH gene (*betA*) from *E. coli* (Andresen *et al.*, 1988).

A DNA fragment comprising the first 1727 bp upstream of the ATG in the *Arabidopsis RbcS1A* promoter was amplified by PCR from the plasmid pGSFR401 (Krebbers *et al.*, 1988). In order to locate the product of the fused gene to the cytoplasm the transit peptide from the original gene was excluded. The synthetic 5′ oligonucleotide primer used in the amplification includes the existing *Eco*RI site in the beginning of the promoter fragment while the 3′ primer introduced a *Cla*I site in front of the start codon ATG by substitution of bases positioned at −1 and −3. The coding sequence of *betA* is 1668 base pairs long corresponding to a polypeptide of 556 amino acids. Amplification of the gene was performed by PCR amplification from the plasmid pFF323 (Andresen *et al.*, 1988) in two steps. The original *betA* gene was reported to start with the prokaryotic start codon TTG, and in order to enable correct translation in an eukaryotic system the start codon ATG was added by a 5′ oligonucleotide primer also incorporating a *Cla*I restriction site. To eliminate any possibility of immature termination of the transcript, a putative poly-A signal, AATAA, located in the first part of the *betA* gene was modified as follows. The 3′ oligonucleotide primer used in the amplification of the first part of *betA* was designed to eliminate the AATAA, at position 217–221 in the coding sequence, by changing the triplet AA$\underline{\text{T}}$AA to AA$\underline{\text{C}}$AA, conserving the amino acid asparagine at position 73. However, there is no useful restriction site, around the AATAA sequence, facilitating a ligation between this shorter primary PCR fragment with the second part of the coding sequence. A *Not*I site was therefore created at position 236–243 by changing the sequence of the primer from GCGG$\underline{\text{A}}$CGC to GCGG$\underline{\text{C}}$CGC, conserving the glycine at position 80. In the second step the larger part of the *betA* gene was amplified with a 5′ *Not*I site and a 3′ *Xba*I site, the latter introduced after the original stop codon. This PCR fragment was inserted behind the first part resulting in the construct *pRbcS1A-betA*. The polyadenylation signal from the 3′ end of the T-DNA nopaline synthase (*nos*) gene was amplified and the entire construct was lifted into the vector pDE1001 (Denecke *et al.*, 1992). This resulted in the binary plant transformation vector pKOH45, harbouring between the T-DNA derived left and right borders *pRbcS1A-betA-3′nos* and as a selective marker the chimeric kanamycin-resistance gene p*nos-neo-3′ocs*. Before plant transformation, the vector was transferred into *Agrobacterium tumefaciens* (C58C1Rif ^{R}) containing the non-oncogenic Ti-plasmid pGV2260 (Deblaere *et al.*, 1985) by triparental mating (Fraley *et al.*, 1983).

Production of polyclonal antibodies against CDH

A PCR amplified *betA* fragment of 1700 bp was digested with *Xba*1 and *Ssp*1. The resulting 591 bp fragment, representing the most C-terminal 197 amino acids of the CDH enzyme, was cloned into the *E. coli* expression vector pMAL-c (Biolabs) digested with *Xba*1 and *Stu*1. Transformed *E. coli* were cultivated according to the vendor, and production of the hybrid protein was induced with IPTG. Preparative SDS-PAGE was used to separate the proteins and the band corresponding to the hybrid protein was cut out from the gel. After freeze-drying the gel pieces were pulverized and injected into two rabbits. The antiserum obtained was tested against bacterial CDH and shown to be specific for this enzyme.

Protein extraction

For crude protein extracts plant tissue of 100–200 mg fresh weight were ground in a 1.5 ml microcentrifuge tube with a glass rod on ice in 100 μl of cold extraction buffer (50 mM TRIS-HCl pH 7.2, 250 mM sucrose, 5 mM EDTA, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM β-mercaptoethanol, 1 mM PMSF, 30 μM pepstatin, 50 μM leupeptin, and 10 μM aprotinin). Plant debris was removed by two centrifugations at 10 000 *g* for 10 min at 4 °C. Protein concentration of the supernatant was determined by the dye-binding assay of Bradford (Bradford, 1976).

SDS-PAGE and immunological techniques

Separation of proteins by SDS-PAGE was performed according to Laemmli (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose filters and filters were briefly stained with Poinceau red (0.5 g Poinceau Red + 5% acetic acid in 100 ml H_2O) to confirm equal loading and successful transfer. Filters were subsequently blocked with 5% fat-free dry milk powder in TBS for 2 h, washed (TBS + 0.5% Tween 20) and incubated with antiserum either raised against the bacterial CDH or BADH diluted 1 : 1000 in TBS. Primary antibodies were detected by secondary antibodies coupled to an alkaline phosphatase giving a colour reaction when incubated with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP).

Determination of glycine betaine concentration by ^{1}H-NMR

Plant samples for betaine determination were extracted as described previously (Jones *et al.*, 1986). The ^{1}H NMR spectra (400 MHz) were recorded at 30 °C on a Bruker DRX 400 instrument. Three hundred pulses were given, pulse repetition time was 5.4 s and the r.f. pulse angle 30°. The dried fractions were dissolved in D_2O (0.7 ml). 1 mg 3-(trimethylsilyl)-propane-sulphonic acid sodium salt (DSS) was used as an internal reference and for quantification.

Measurements of chlorophyll a *fluorescence*

Chlorophyll fluorescence was measured with a Plant Stress Meter (Biomonitor, Umeå) as described previously (Öquist and Wass, 1985). All the measurements were taken from the two youngest fully expanded leaves from plants of the same age and developmental stage. Leaves were kept in darkness for 30 min before recording the fluorescence parameters.

Measurements of photosynthesis

CO_2 uptake was measured under ambient greenhouse conditions from the two youngest fully expanded leaves of each plant with an infrared gas-analyser (Li-Cor Ltd, Nebraska, US). Oxygen evolution under saturating carbon dioxide and light was measured from leaf discs of fully expanded leaves with an oxygen electrode (Hansatech Ltd, Norfolk, UK).

Results

Transformation of betA *to tobacco*

Tobacco (cv. Samsun) was transformed with the chimeric *betA* gene from *E. coli*, regulated by the *Arabidopsis RbcS1A* promoter (Krebbers *et al.*, 1988). Fifty independent transformants were selected on kanamycin-containing media. Resistant plantlets were subjected to a primary screening by Western blot analysis using antiserum raised against CDH to demonstrate the production of the polypeptide (Fig. 1). Most kanamycin-resistant plants transformed with the *betA* gene were also found to produce detectable amounts of CDH. The CDH positive plants chosen for further experiments had a relatively high CDH-production, as deduced from Western blot analysis, and contained one insert as concluded from segregation pattern (3 : 1) of the kanamycin resistance marker as well as production of the CDH protein, in the F_1 generation.

Resistance of transgenic plants to toxic levels of choline and betaine aldehyde

In order to assess if CDH produced in transgenic tobacco retained enzymatic activity, the ability of the plants to survive toxic concentrations of choline (Lilius *et al.*, 1996) and betaine aldehyde (Holmström *et al.*, 1994) was characterized. Concentrations of 20 mM of choline in the growth medium reduced the growth yield of wild-type tobacco, measured by fresh-weight, to 30% of control plants grown on MS-2 medium. Transgenic lines producing CDH did not show any reduction in fresh weight at this choline concentration (Fig. 2). In a similar experiment plants were exposed to 10 mM betaine aldehyde which is completely inhibitory to germination of wild-type seeds (Holmström *et al.*, 1994). In contrast, CDH transformants were able to germinate and grow although growth was severely affected (Fig. 3). The BADH-producing transgenic tobacco plants were more resistant to this betaine aldehyde concentration than CDH-producing plants, suggesting that BADH might be more effective in converting betaine aldehyde into betaine. A further growth improvement was observed in the F_1 progeny from crosses between lines producing CDH and BADH (Fig. 3).

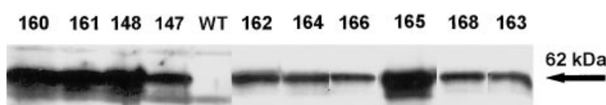

Fig. 1. Characterization of the CDH polypeptide, in transgenic betaine-producing tobacco, by SDS-PAGE and Western analysis. A polyclonal antiserum raised against the C-terminus of the CDH polypeptide was used for detection of the 62 kDa CDH polypeptide (arrow) in the transgenic plants. Numbers above figure indicates different transgenic lines, WT = non-transformed control plant.

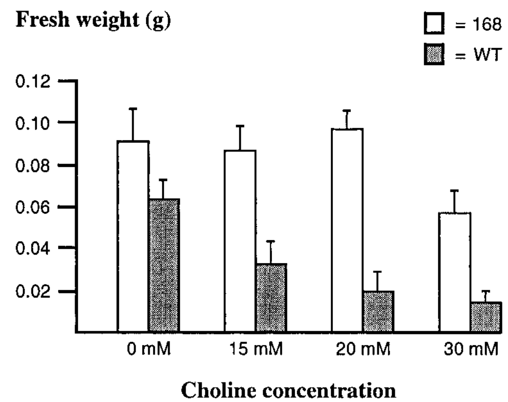

Fig. 2. Growth of transgenic *betA*-expressing tobacco on choline. *In vitro* plants were propagated on MS-2 medium supplemented with various concentrations of choline for 28 d and their fresh weight measured. Error bars represent standard deviation based on the growth of 30 plants in two independent experiments. WT = non-transformed control plant. 168 = transformed line 168 producing CDH as deduced from Western blot analysis.

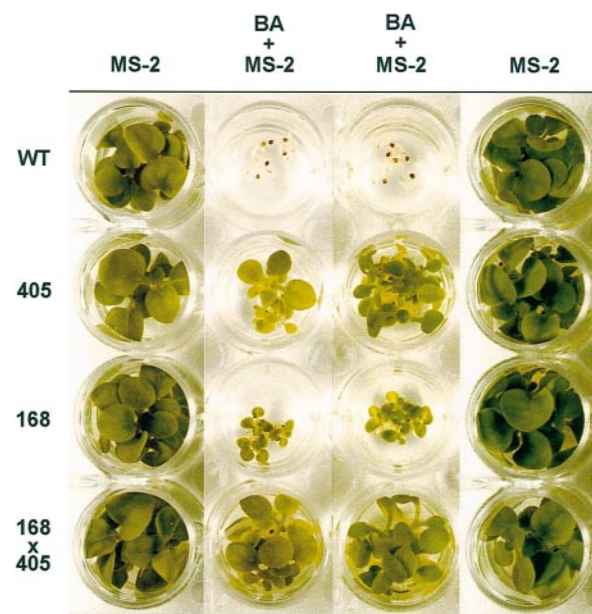

Fig. 3. Growth of transgenic tobacco, producing CDH, BADH or both proteins, in presence of toxic concentrations of betaine aldehyde (10 mM). Seeds were germinated and plantlets grown on MS-2 medium supplemented with 0 or 10 mM of betaine aldehyde. Betaine aldehyde (BA), non-transformed control plant (WT), *betB*-transformed line (405), *betA*-transformed line (168), and a *betA* × *betB* (168 × 405) double transgenic line.

Betaine accumulation in transgenic tobacco

To determine if transgenic plants producing CDH also synthesized betaine, the betaine concentration in the leaf tissue was measured by ^1H NMR (Jones *et al.*, 1986). The level of betaine found was 35 ± 15 nmol g^{-1} FW based on measurements from six seedlings from the CDH-producing transgenic line 168, whereas no accumulation of betaine could be found in non-transformed wild-type

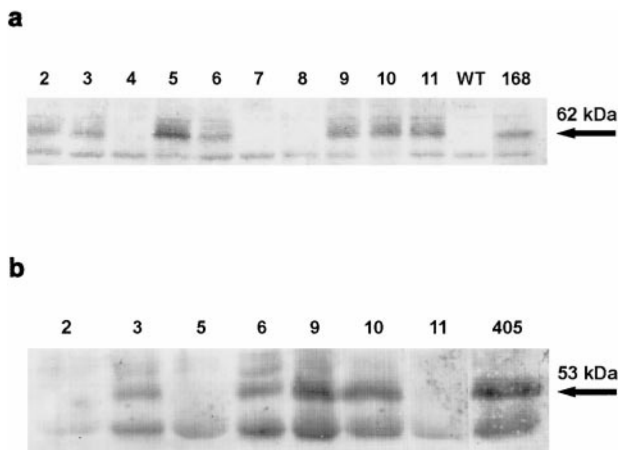

Fig. 4. (a, b) Production of CDH (a) and BADH (b) in F_1 progeny from a cross between CDH- and BADH-producing transgenic tobacco. Proteins were extracted from leaves of the independent F_1 progeny plants, separated by SDS-PAGE and subsequently electrophoretically transferred to nitrocellulose filters. Filters were thereafter incubated with antiserum raised either against the CDH (a) or BADH (b) polypeptide. Progeny plants producing both proteins were used in further experiments. Lanes 2–11 show different F_1 plants while 168 (CDH) and 405 (BADH) indicate parental lines used in the cross.

plants or transgenic lines producing BADH only (<1 nmol g^{-1} FW). To test the effect of the complete biosynthesis pathway on betaine-production, CDH- and BADH-producing lines were crossed. Crosses were made by taking pollen from CDH plants and fertilizing emasculated BADH producing lines. F_1 progeny plants were thereafter tested for production of CDH and BADH by antisera raised either against CDH or BADH. Seven F_1 seedlings were found to produce CDH (Fig. 4a) and four out of these seven CDH-positive plants were also found to produce BADH (Fig. 4b). Betaine accumulation was determined by ^{1}H NMR in the four F_1 plants producing detectable amounts of both CDH and BADH. Accumulation of betaine was found to be about 2- to 3-fold higher (66 ± 18 nmol g^{-1} FW) than in plants only producing CDH.

Effect of betaine-production on salt stress tolerance

Both wild-type plants (Fig. 5a, c) and betaine-producing transgenic lines (Fig. 5b, c) became severely inhibited in growth when watered with 200 mM NaCl as compared to non-stressed controls. However, the transgenic lines were clearly less affected (23 ± 4% of non-stressed fresh weight) by the stress compared to wild-type plants

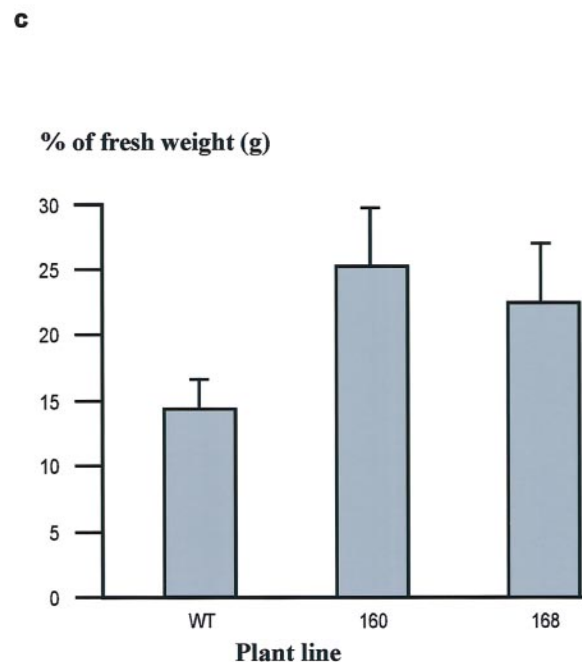

Fig. 5. (a, b, c) Enhanced salt stress tolerance in greenhouse-grown CDH-producing tobacco. Seeds were germinated *in vitro* and seedlings were thereafter transferred to greenhouse and grown in soil pots for 1 week. Plants were subsequently subjected to salt-stress by watering twice a week with 200 ml 200 mM of NaCl in 0.1% RIKA-medium (Weibulls, Sweden). After 2 weeks of stress treatment the non-transformed control tobacco (a, c) and two independent transgenic CDH-producing lines 160, shown in (b, c), and 168 (c) were harvested and fresh weight was measured (c). The first panel (a) shows non-transformed control tobacco at the time of harvest, the upper row non-stressed (WT ns) and the lower row salt-stressed (WT NaCl) control plants. The second panel (b) demonstrates glycine betaine producing transgenic tobacco at the same time, the upper row non-stressed (168 ns) and the lower row salt-stressed plants (168 NaCl) expressing the *E. coli* gene *betA*. The effect of salt stress on biomass accumulation (fresh weight as percentage of that in non-stressed plants) is depicted in (c). Error bars represent standard deviation from six plants from each line.

(14 ± 3.1% of non-stressed fresh weight), indicating a protective role for betaine during salt stress.

Measurements of photosynthesis under non-stressing conditions

There were no significant differences in the photosynthesis of transgenic and wild-type plants when measured as CO_2 uptake at ambient conditions or as O_2 evolution under saturating light and carbon dioxide (results not shown).

Decreased susceptibility to photoinhibition during salt stress and low temperature in transgenic betaine-producing tobacco

Earlier studies have suggested a protective role for betaine on photosystem II (PSII) at high salt concentrations (Papageorgiou and Murata, 1995). To examine if transgenic tobacco producing betaine showed any difference in PSII protection during salt stress, chlorophyll fluorescence was measured from leaf discs exposed to 200 mM NaCl for 7 h at low light (30 μmol m^{-2} s^{-1}) and high light (390 μmol m^{-2} s^{-1}), respectively. The ratio of variable to maximal fluorescence (F_v/F_m) is indicative of the potential photochemical efficiency of PSII (Krause and Weis, 1991) and represents a measure of the functional status of the oxygen evolving complex. Neither wild-type nor transgenic plants showed any photoinhibition by salt stress at low light indicating that the salt treatment by itself did not affect the PSII (data not shown). However, at high light conditions and salt stress the transgenic lines producing betaine showed about 25% photoinhibition while wild-type plants had an inhibition of around 35%.

Furthermore, when photoinhibited leaf discs were let to recover at room temperature (20 °C) in H_2O in low light conditions (30 μmol m^{-2} s^{-1}), transgenic lines producing betaine recovered faster than non-transformed wild-type plants (Fig. 6). After 14 h of recovery the F_v/F_m of the leaf discs from betaine-producing transgenic plants remained at the initial 25% photoinhibition, while discs from control plants showed a further decrease to about 40% of the original non-stressed F_v/F_m values (Fig. 6). After 23 h the wild-type tobacco had recovered to 70% of the initial F_v/F_m while the plants producing both CDH and BADH performed notably better with a recovery improved to more than 95% of the initial value. However, there was no statistically significant difference between CDH-producing lines and CDH- and BADH-producing lines although double transgenic lines showed a tendency to recover faster. To ensure that the recovery treatments as such did not affect the fluorescence characteristics, leaf discs punched from non-stressed plants were incubated for 23 h in water and low light. No changes in F_v/F_m were detected due to this treatment (data not shown).

When plants were exposed to low temperature (4 °C) and light (150 μmol m^{-2} s^{-1}) both transgenic lines and

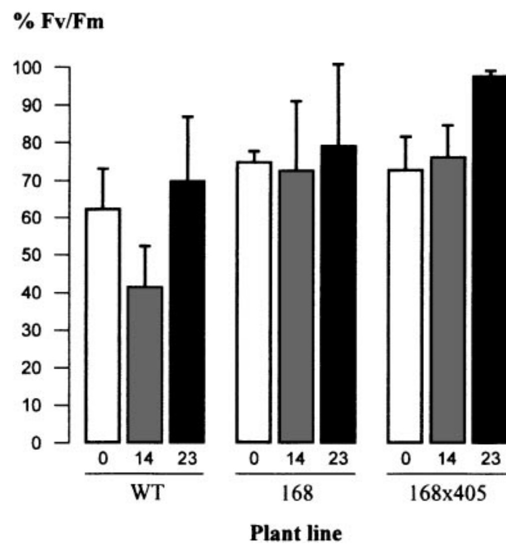

Fig. 6. Photoinhibition and recovery from photoinhibition imposed by salt stress and high light. Leaf discs of 3.5 cm in diameter (with 10 cm^2 area) were taken from greenhouse-grown plants and exposed to 200 mM of NaCl under high light (390 μmol m^{-2} s^{-1}) for 7 h. Discs were then placed in water for 14 h or 23 h (30 μmol m^{-2} s^{-1}) for recovery. F_v/F_m was measured with a Plant Stress Meter (Biomonitor) after 7 h of stress, indicated in the figure by 0 h, after 14 h and 23 h of recovery indicated by 14 h and 23 h, respectively. F_v/F_m was plotted as a percentage compared to unstressed plants from the same lines or wild type (F_v/F_m of unstressed plants was 0.793 ± 0.01, in CDH-producing plants 0.789 ± 0.02, and 0.790 ± 0.02 in BADH-producing plants, respectively). Standard errors are indicated. The results are means of at least 15 measurements of at least three different plants. Non-transformed wild-type tobacco (WT), a *betA* (168) transformant and F_1 progeny from a cross between *betA* (168) and *betB* (405) lines.

wild-type plants showed reduction in PSII efficiency. The reduction in F_v/F_m was 20.5 ± 5% in wild-type plants whereas transgenic lines showed a reduction of 11.5 ± 4% for CDH transformants and 11.5 ± 2.4% for lines producing CDH and BADH, respectively (Fig. 7). No difference was seen in the net photosynthesis at ambient conditions for the transgenic lines and wild-type plants used. The difference between control plants and betaine accumulating transgenic tobacco is statistically significant at the risk level of 1% as analysed by Students *t*-test. No significant difference was observed between CDH transformants and crossings between CDH and BADH plants.

Discussion

The introduction and biological function of BADH in transgenic tobacco, an enzyme involved in betaine biosynthesis in *E. coli*, was described in a previous study (Holmström *et al.*, 1994). This enzyme can, however, only catalyse the second step in the betaine biosynthesis pathway, converting betaine aldehyde into betaine, but is incapable of catalysing the first step of the pathway, oxidation of choline into betaine aldehyde. In this study, the introduction of the first enzyme of the pathway, CDH, into BADH-producing lines, thereby completing

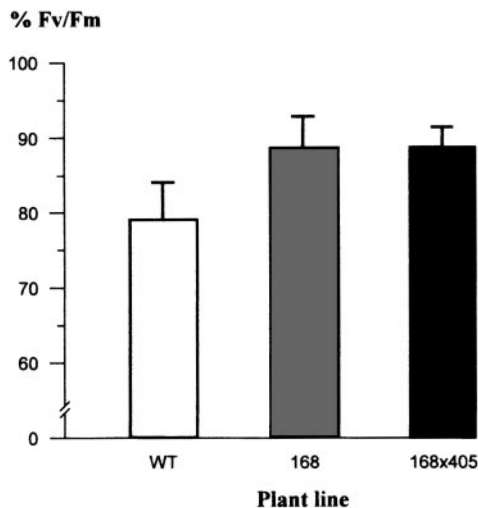

Fig. 7. F_v/F_m in plants exposed to white light and low temperature (4 °C). Leaf discs 3.5 cm in diameter were taken from greenhouse-grown plants and exposed to 150 μmol m^{-2} s^{-1} at 4 °C for 1 h, leading to a reduction in PSII efficiency in both transgenic and control plants. F_v/F_m was measured by a Plant Stress Meter (Biomonitor). Leaf discs were dark-adapted for 30 min before the measurements either at room temperature (control leaf discs) or at 4 °C (treated leaf discs). F_v/F_m was plotted as a percentage compared to unstressed plants from the same lines or wild type. (F_v/F_m of unstressed plants was 0.793 ± 0.03, in *betA* plants 0.788 ± 0.03, and 0.789 ± 0.02 in *betB* plants, respectively). The results are means of at least 15 measurements from at least three different plants. Standard errors are indicated. Non-transformed wild-type tobacco (WT), a *betA* (168) transformant and F_1 progeny from a cross between *betA* (168) and *betB* (405) lines.

the *E. coli* biosynthesis pathway in transgenic tobacco has been described. The double transgenic lines were compared, in their ability to produce betaine and in their stress resistance, to lines producing CDH only.

To verify the dual biological functionality of the bacterial CDH enzyme, i.e. to carry out both enzymatic steps in the betaine biosynthesis in whole plants, the following experiments were conducted. First it was shown that CDH positive plants were able to metabolize choline. Transgenic tobacco did not show any reduction in growth on 20 mM choline whereas wild-type tobacco was severely inhibited at this choline concentration (fresh weight was less than 30% of non-stressed control plants grown on MS-2). This suggests that production of CDH in transgenic tobacco generates an active enzyme capable of oxidizing choline. To verify enzymatic activity for the second oxidizing step, the conversion of betaine aldehyde into betaine, CDH-producing tobacco seeds were sown on 10 mM of betaine aldehyde. At this concentration the seeds were able to germinate and seedlings capable to grow, although the growth was severely inhibited. In contrast, wild-type plants were unable to germinate (Fig. 3). In a previous study it was demonstrated that, in *E. coli*, CDH by itself can synthesize betaine from choline (Landfald and Strøm, 1986), this was now confirmed also for transgenic tobacco by ^{1}H NMR analysis. Furthermore, the accumulation of betaine found in CDH-producing tobacco was sufficient to provide enhanced tolerance to salt stress as seen from whole plant studies (Fig. 5). The same result was obtained previously (Lilius *et al.*, 1996), by introducing the *E. coli* CDH into tobacco in a similar study.

As betaine aldehyde is very toxic to the cell it seems reasonable to assume that the existence of both CDH and BADH in *E. coli* is to ensure efficient oxidation of this toxic intermediate into the non-toxic betaine. In order to test whether expression of both *betA* and *betB* in transgenic tobacco would increase betaine-production, CDH-producing plants accumulating betaine were crossed with non-accumulating BADH-producing plants. F_1 progeny producing both enzymes in the same transgenic line exhibited 2- to 3-fold higher accumulation of betaine compared to transgenic lines expressing CDH only. There was, however, no clear evidence for improved stress protection in the double transgenic lines over the CDH-positive lines. This suggests that the betaine levels obtained in CDH-producing plants are sufficient to improve stress tolerance in transgenic tobacco. Yet, the betaine concentrations obtained are only a fraction of the levels in many natural betaine accumulators under stress (Rhodes and Hanson, 1993). This may be due to stringent control of choline biosynthesis in non-accumulators (as suggested by Nuccio *et al.*, 1998). In conclusion, the present results show that by introducing the *E. coli* betaine biosynthesis pathway into tobacco it is possible to produce betaine and improve the stress tolerance in a higher plant which normally does not produce this compatible solute.

Recently the possible negative effects of betaine application in non-accumulating plants species such as rapeseed (*Brassica rapa* L.) have been pointed out (Gibon *et al.*, 1997). In contrast, it was not possible to detect any negative effect on germination, growth or seed-production nor any phenotypic alterations in transgenic tobacco expressing CDH, BADH or both together under normal growth conditions, in the absence of stress. Neither was it possible to show any differences in gas exchange nor in oxygen evolution of the transgenic lines as compared to wild-type plants when grown under greenhouse conditions. However, the betaine concentrations were much lower in transgenic tobacco used in this study (40–80 nmol g^{-1} FW) than those found in the rapeseed plants (30–1000 μmol g^{-1} DW) (Gibon *et al.*, 1997). In this study it is shown that synthesis of CDH and BADH confers no negative effects in transgenic tobacco, but that production of the enzymes is favourable under stress. Nevertheless, if production of betaine would show any unwanted growth effects these could be circumvented by using inducible promoters to provide stress-dependent expression of the *bet* genes.

The betaine concentration found in transgenic tobacco

is most likely not high enough to generate an osmotic adjustment of the cytosol. The increased tolerance towards salt and low temperature stress is therefore probably due to other effects than osmoregulation. The protection by betaine could involve stabilization of particular protein complexes and membranes. In support of this is the finding that PSII seems to be protected at stress conditions by the elevated levels of betaine in the transgenic plants. A similar result was obtained by producing betaine in *Arabidopsis* chloroplasts (Hayashi *et al.*, 1997). Interestingly, in transgenic tobacco expressing *betA* or both *betA* and *betB*, salinity did not affect PSII activity of leaf discs as such, but only in combination with high light intensity. Thus the protection by betaine might include protection from photoinhibition as the plants grew under moderate light during the salt treatment. In conclusion, relatively low concentrations of betaine can improve stress tolerance in photosynthesizing organisms, possibly by protecting protein complexes involved in the photosynthetic processes.

It has been suggested previously (McCue and Hanson, 1990) that genetic engineering of osmotolerance in plants could be achieved by producing betaine in non-accumulators. Recent independent studies (Lilius *et al.*, 1996; Hayashi *et al.*, 1997) and this work have confirmed this hypothesis at least on a laboratory scale. Further increase in betaine production could be achieved by engineering choline production (as suggested by Nuccio *et al.*, 1998). Thus, betaine-production in non-accumulating plants appears to be a promising approach to enhance crop tolerance to different abiotic stresses.

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