Theor Appl Genet (2002) 106:51–57
DOI 10.1007/s00122-002-1063-5

A. Mohanty · H. Kathuria · A. Ferjani
A. Sakamoto · P. Mohanty · N. Murata · A.K. Tyagi

Transgenics of an elite *indica* rice variety Pusa Basmati 1 harbouring the *codA* gene are highly tolerant to salt stress

Received: 24 January 2002 / Accepted: 28 March 2002 / Published online: 4 September 2002

Abstract Transgenic lines of *indica* rice were generated by *Agrobacterium*-mediated transformation with the choline oxidase (*codA*) gene from *Arthrobacter globiformis*. Choline oxidase catalyses conversion of choline to glycine betaine. Glycine betaine is known to provide tolerance against a variety of stresses. Molecular analyses of seven independent transgenic lines as performed by Southern, Northern and Western hybridization revealed integration and expression of the transgene as well as inheritance in the progeny plants. A good correlation was observed between levels of mRNA and protein accumulation, and a significant amount of choline oxidase product, i.e. glycine betaine, accumulated in R0 as well as R1 plants. Mendelian as well as non-Mendelian segregation patterns were obtained in the progeny plants. Challenge studies performed with R1 plants by exposure to salt stress (0.15 M NaCl) for 1 week, followed by a recovery period, revealed that in some cases more than 50% of the transgenic plants could survive salt stress and set seed whereas wild-type plants failed to recover.

Keywords Abiotic stress tolerance · Glycine betaine · *Indica* rice · Transgenics

Introduction

Increased tolerance against various osmotic stresses is one of the major objectives of plant biotechnology (Holmberg and Bülow 1998; Tyagi et al. 1999; Sakamoto and Murata 2001). Among various osmotic abiotic stresses such as drought, salinity and freezing, salinity is the most important factor limiting crop productivity. According to the United Nations Environment Program, nearly 20% of the World's agricultural lands are salt-stressed, especially in countries like India, Pakistan, sub-Saharan Africa and Australia where approximately one-third of the land may be adversely affected. Worldwide, about 10 million ha of irrigated land is abandoned because of excess salt deposition each year (Nelson et al. 1998). Higher plants from several families (e.g. Chenopodiaceae, Asteraceae) have evolved mechanisms such as the accumulation of osmoprotectants including amino acids, ammonium compounds and polyols/sugars to protect themselves under stress conditions. Glycine betaine, a quaternary ammonium compound, is a compatible solute which accumulates under periods of stress in higher plants (Robinson and Jones 1986; Rhodes and Hanson 1993; Yeo 1998; Sakamoto and Murata 2000, 2001; Tyagi and Mohanty 2000), animals (Garcia-Perez and Burg 1991) as well as bacteria (Csonka 1989). This is known to provide tolerance to the cells under stress by stabilizing the quaternary structure of the complex proteins and adjusting the osmotic potential in their cytoplasm to maintain water content. In photosynthesis it stabilizes both PSII complexes (Papageorgiou and Murata 1995), as well as Rubisco, at a high concentration of NaCl and at extreme temperatures (Gorham 1995). A two-step enzymatic process accomplishes production of glycine betaine in plants. The first step involves conversion of choline to betaine aldehyde by choline monoxygenase, a stromal enzyme with a Rieske-type [2Fe-2S] centre (Brouquisse et al. 1989), and the second step involves betaine aldehyde dehydrogenase, a nuclear-encoded chloroplast stromal enzyme, which converts it to glycine betaine (Weigel et al. 1986). However, an endogenous supply of choline is also important as shown by Nuccio et al. (1998) with transgenic tobacco plants expressing genes encoding choline monoxygenase enzyme. Earlier, transgenic *Arabidopsis*

Communicated by J.W. Snape

A. Mohanty · H. Kathuria · A.K. Tyagi (✉)
Department of Plant Molecular Biology,
University of Delhi South Campus, New Delhi-110021, India
e-mail: akhileshtyagi@hotmail.com
Fax: +91-11-4675095, Fax: +91-11-4109450

A. Ferjani · A. Sakamoto · N. Murata
National Institute for Basic Biology, Okazaki 444-8585, Japan

P. Mohanty
School of Life Sciences, Jawaharlal Nehru University, New Delhi, India

plants were obtained with the *codA* gene and it was shown to provide tolerance, not only against salt stress (Hayashi et al. 1997), but also against low temperature, freezing, high temperature as well as light stress (Alia et al. 1998, 1999; Sakamoto et al. 2000). The same group reported transformation of *japonica* rice with the *codA* gene (Sakamoto et al. 1998). For this purpose, the gene construct originally used for *Arabidopsis* transformation was modified by incorporating a rice *RbcS* transit peptide for chloroplast targeting (chl-COD). The other construct (cyt-COD) would result in accumulation of glycine betaine in the cytosol. PSII activity of both types of transgenic plants was tolerant to low temperature as well as salt stress. The potential of the gene, therefore, needs to be evaluated in other crops, as engineering of complex traits for abiotic stress resistance would be of great value (Christou 1994; Holmberg and Bülow 1998; Bajaj et al. 1999; Grover et al. 1999; McNeil et al. 1999; Sakamoto and Murata 2000, 2001; Tyagi and Mohanty 2000).

Rice is a salt-sensitive cereal crop and it cannot produce glycine betaine in response to abiotic stress. *Indica* rice contributes 80% of total rice production (Christou 1994). Earlier, we have reported an efficient protocol for generation of *Agrobacterium*-mediated transgenic *indica* rice of an elite variety Pusa Basmati 1 (Mohanty et al. 1999, 2000). In the present study, we report *Agrobacterium*-mediated transformation of Pusa Basmati 1 with the *codA* gene. A large number of morphologically normal and fertile transgenic plants was obtained. Detailed molecular, biochemical, genetic and physiological analyses were carried out with selected R0 as well as R1 transgenic plants.

Materials and methods

Agrobacterium-mediated transformation of Pusa Basmati 1

Agrobacterium-mediated transformation was carried out as described earlier (Mohanty et al. 1999, 2000). Briefly, scutella-derived calli were used for co-cultivation with *Agrobacterium tumefaciens* strain EHA101 (pGAH/*codA*, Hayashi et al. 1997). After removing excess bacterial cells, calli were selected on callusing medium containing hygromycin (50 mg/l) and cefotaxime (250 mg/l) or timentin (125 mg/l). It was observed that for removing EHA101 (pGAH/*codA*) cells, timentin was more effective. Further, calli growing on selection medium were excised and transferred to fresh selection medium. Proliferating calli were transferred to regeneration medium and incubated at 26 ± 2 °C, 16-h photoperiod for 2–3 weeks. Regenerated plants of 2–3 cm height were transferred to MSBH medium (Mohanty et al. 1999) for root formation. Rooted plants were transferred to pots containing a mixture of soil and soilrite (1:1) and were incubated in a growth chamber or a green house operating at 24–28 °C, 14–16 h light at 100–125 µmol m^{-2} s^{-1} and 70–75% relative humidity. The plants were regularly supplied with rice growth medium (Mohanty et al. 1999) and were grown to maturity.

Molecular analysis

Genomic DNA isolation and Southern hybridization

Genomic DNA isolation and Southern hybridization were performed as described earlier (Sambrook et al. 1989; Mohanty et al. 1999). Briefly, 5 µg of genomic DNA was digested with *Eco*R1 and resolved on a 1.2% agarose gel. The probe used was a 1.2-kb *Bam*H1/*Sac*I fragment of pGAH/*codA* encompassing the coding sequence of the *codA* gene. The probe was labeled with α-^{32}P [dATP] using a Megaprime labeling kit (Amersham Pharmacia Biotech UK limited, England). Hybridization was carried out at 42 °C in a solution containing 50% formamide, 5% SSC [20 × SSC (saline sodium citrate, 175.3 g l^{-1} of NaCl; 88.2 g l^{-1} of sodium citrate; pH 7.0)], 1 × Denhardt's solution, 10% dextran sulphate and denatured herring sperm DNA (200 µg/ml) for 24 h. Filters were washed at high stringency by gradually reducing the salt from 5% SSC and 0.1% SDS to 0.1% SSC and 0.1% SDS. The filters were wrapped in cling film and exposed to X-ray film for a suitable period before developing.

Northern analysis

RNA was isolated from green leaves essentially as described by Logemann et al. (1987) with minor modifications. RNA samples (20 µg) were run on a 1.2% agarose-formaldehyde gel using 1 × MOPS buffer (3[N-Morpholino]propanesulfonic acid, 20 mM of MOPS, pH 7.0; 2 mM of sodium acetate; 1 mM of EDTA, pH 8.0) and were transferred to Hybond-C membrane using 20 × SSC. The pre-hybridization solution contains 50% formamide (deionized); 5 × SSC; 50 mM of sodium phosphate buffer, pH 6.5; 250 µg ml^{-1} of herring sperm DNA (denatured); 10 × Denhardt's solution and the hybridization solution contains an additional 0.2 vol of 50% dextran sulphate (final concentration of dextran sulphate is 10%). Other steps such as preparation of a radiolabeled probe and its use, hybridization time and temperature, washing of the blot and exposure to X-ray film, essentially remained the same as for Southern analysis.

Western analysis

Western analysis was performed according to Arakawa et al. (1997) with suitable modifications. Clarified extract was prepared from leaf tissue (500 mg) from 6-month-old R0 plants and 1-month-old R1 plants. An aliquot of 5 µl of clarified extract was used for protein estimation (Bradford 1976). A total of 30 µg of total soluble protein was separated on 10% sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) at 25 mA, constant current, for 60–90 min in Tris-glycine-SDS buffer (25 mM of Tris; 250 mM of glycine, pH 8.3; 0.1% SDS). The separated proteins were blotted onto a PVDF membrane pre-wetted in 100% methanol and then equilibrated in protein transfer buffer, using a Hoeffer electroblotter at a 100 mA current overnight. Subsequently, the membrane was incubated in blocking solution [5% non-fat dry milk prepared in TBST buffer (10 mM of Tris, pH 7.5; 500 mM of NaCl; 0.05% Tween 20)] for 1 h at 40 rpm and room temperature. The membrane was given 3-washes with TBST buffer for 5 min each. The blot was incubated in TBST buffer containing 1% non-fat dry milk and a 1:3000 dilution of anti-rabbit antisera of choline oxidase (Hayashi et al. 1997) for 1 h at RT at 40 rpm. The membrane was washed with TBST buffer three times for 5 min each at 40 rpm and room temperature. The blot was probed with a secondary antibody (anti-rabbit HRP 1:7000 dilution) in TBST buffer containing 1% non-fat dry milk for 1 h at room temperature at 40 rpm, followed by 3-washes with TBST for 5 min each at 40 rpm and room temperature. The blot was developed using the ECL Plus chemiluminiscence kit from Amersham Pharmacia Biotech UK limited, England, as per manufacturer's instructions.

Salt stress studies

R1 seeds of the transgenic lines were grown on hygromycin selection medium. The resistant seedlings were transferred to rice growth medium (Mohanty et al. 1999) after they were 1-week-old under selection. Only hygromycin-resistant seedlings were assayed for salt sensitivity. Seedlings were grown on rice growth

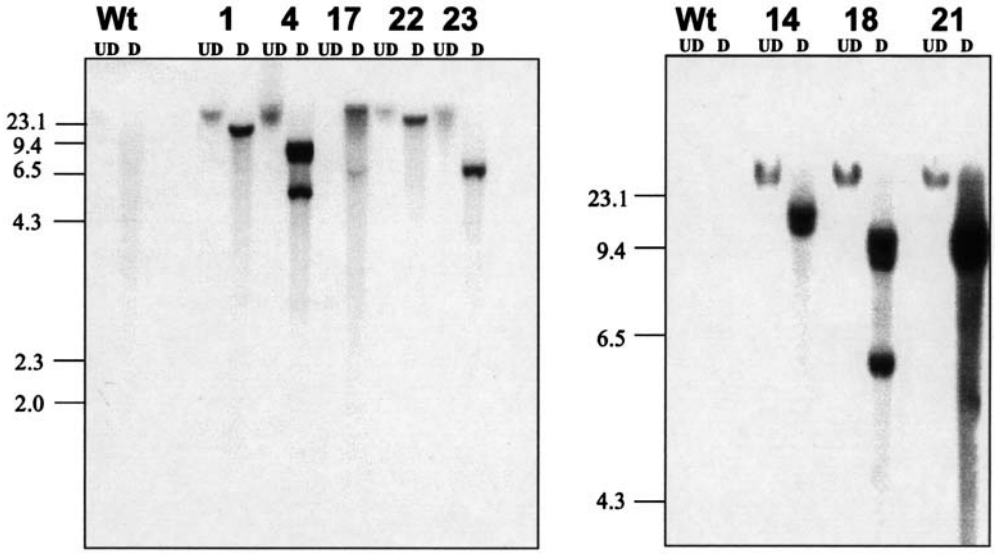

Fig. 1 Southern analysis of the R0 generation of PB1/*codA* transgenic rice plants. Undigested DNA (UD) or genomic DNA (5 µg) digested with *Eco*RI (D) was resolved on the gel. *Wt* represents the wild-type plant and *numbers* represent the identity of transgenic lines

medium for another 2 weeks at the end of which they were transferred to rice growth medium having 150 mM of NaCl. This salt stress was given for 1 week after which seedlings were allowed to grow on normal rice growth medium for another 3 weeks. They were subsequently transferred to soil for further growth and seed harvesting.

Analysis of glycine betaine content by NMR

The levels of glycine betaine and choline were determined in leaves of unstressed mature plants by NMR spectrometric analysis as described by Sakamoto et al. (1998). The analysis was performed in duplicate.

Genetic analysis

For genetic analysis of hygromycin resistance in the progeny, seeds of R0 and R1 plants were de-husked and sterilized, and were inoculated on MSB medium for 2 days (Mohanty et al. 1999). Germinated seedlings were transferred to MSB medium containing 50 mg l^{-1} of hygromycin. Hygromycin resistance/sensitivity was scored after 7 days.

Results

Rice transformation

From six independent transformation experiments with EHA101 (pGAH/*codA*), a total of 53 transgenic plants were obtained. Some of these plants were analyzed in detail, grown to maturity in the greenhouse and seeds collected.

Characterization of R0 plants

A total of eight independent transgenic plants were analyzed by Southern hybridization using the *codA* gene as a probe. Both single as well as multi-copy insertions were revealed (Fig. 1).

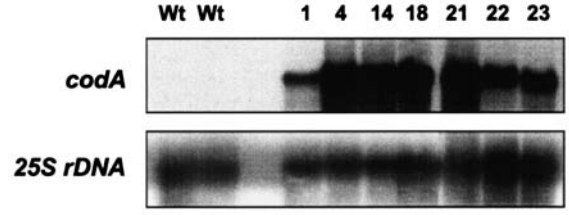

Fig. 2 Northern analysis of the PB1/*codA* gene in the leaves of R0 transgenic plants. The lower panel depicts RNA samples probed with 25S *rDNA* as a loading control. *Wt* represents the wild-type plant and the *numbers* represent the identity of transgenic lines

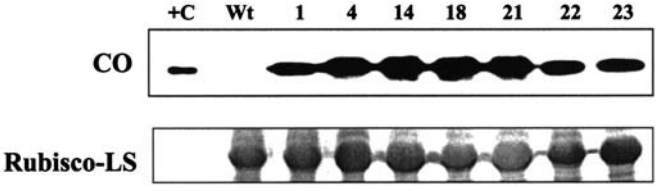

Fig. 3 Western analysis of R0 generation PB1/*codA* transformed rice lines. +C depicts choline oxidase from *Arthrobacter globiformis* as the positive control. *Wt* represents wild-type plants as a negative control. Numbers on the top depict the identity of transgenic lines. The CO panel shows a band of choline oxidase and the lower panel depicts a Rubisco large subunit as a loading control

Expression of the codA gene in transgenic plants

Northern-blot analysis of RNA samples from leaves of seven representative plants revealed variable but significant levels of the *codA* transcript. Transgenic lines 4, 14, 18 and 21 were found to express the transcript at very high levels, followed by transgenic lines 22 and 23. Transgenic line 1 was found to have low expression of the *codA* transcript. No hybridization was detected in wild-type plants (Fig. 2).

Western analysis with choline oxidase antiserum revealed the presence of a 64-kDa protein which corresponds to the choline oxidase protein (Fig. 3). No such

Table 1 Segregation analysis of hygromycin resistance in R1 progeny of PB1/*codA* transgenic rice plants

Plant identity	Copy no.	No. of seeds inoculated	No. of germinated seeds transferred to hygromycin	No. of seeds resistant to hygromycin	No. of seeds sensitive to hygromycin	χ^2 value
1	1	67	67	36	31	0.373 (1:1)[b]
4	5	69	66	31	35	0.242 (1:1)[b]
14	2	74	69	55	14	0.814 (3:1)[c]
18	5	68	65	49	16	0.005 (3:1)[a]
21	3	43	33	20	13	1.4 (1:1)[c]
22	1	68	66	37	29	0.96 (1:1)[c]
23	1	52	52	35	17	1.61 (3:1)[c]

[a] $0.95>p>0.80$; [b] $0.80>p>0.50$; [c] $0.50>p>0.20$

band was detected in wild-type plants. In addition, we also detected a faint band at the approximately 70-kDa region on long exposure. This could be a precursor of choline oxidase, as has been shown earlier by Hayashi et al. (1997), revealing thereby that the precursor protein containing a transit peptide was targeted to the chloroplast and processed correctly. A good correlation could be established between the transcript levels and protein levels in the respective plants. For example, plant lines 4, 14, 18 and 21, which show high levels of transcript, also show higher protein levels.

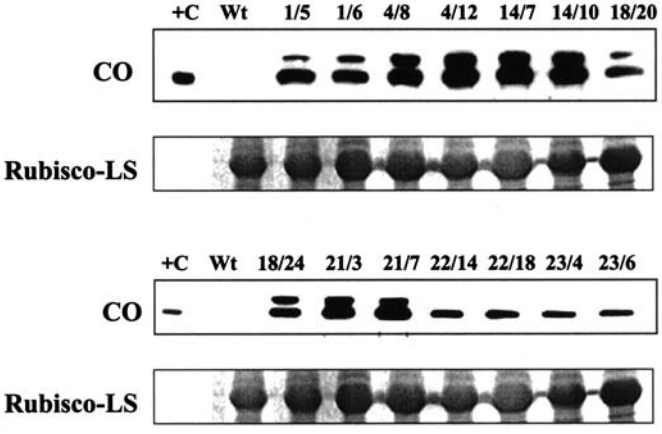

Fig. 4 Western analysis of R1 generation PB1/*codA* transgenic rice plants. +C is choline oxidase from *Arthrobacter globiformis* loaded as a positive control. *Wt* represents wild-type plants as a negative control. Numbers on the top depict the identity of the R1 generation transgenic plants from transgenic lines 1, 4, 14, 18, 21, 22 and 23. CO represents choline oxidase and Rubisco-LS represents a large subunit of Rubisco as a loading control

Glycine betaine accumulation

Glycine betaine levels were detected by employing proton-NMR studies in six lines (1, 14, 17, 18, 22 and 23). All of these showed accumulation of glycine betaine and also significant levels of choline. Similar data collected for R1 progeny plants are subsequently provided in this paper.

Genetic analysis of hygromycin resistance in the progeny of R0 plants

Seeds obtained from seven R0 plants were analyzed for segregation of the hygromycin resistance trait, as shown in Table 1. Mendelian as well as non-Mendelian segregation ratios were obtained. Interestingly, two single-copy plants segregated in a 1:1 ratio for hygromycin resistance. On the other hand, two transgenic lines with multiple copies of the transgene show a 3:1 segregation ratio for hygromycin resistance.

Analysis of R1 plants and their progeny

Expression of the codA gene in R1 transgenic plants

For Western analysis of progeny plants, we selected two R1 plants that were hygromycin-resistant from each of the seven independent R0 lines. As in R0 lines, the analysis of R1 plants revealed the presence of a 64-kDa protein which corresponds to choline oxidase (Fig. 4). No such band was detected in wild-type plants. In addition, we detected a band at about 70 kDa representing a precursor of choline oxidase, in lines showing higher levels of choline oxidase. Variable levels of protein were detected among various transgenic lines. Overall, the expression levels of R1 lines were similar to that of their parents.

Glycine betaine accumulation

NMR analysis was also performed with leaves of R1 transgenic plants for quantitation of accumulated glycine betaine. A total of seven R1 plants from seven independent lines were selected for NMR analysis. R1 transgenic line 21 accumulated the highest amount of glycine betaine (about 2.12 μmol/g of dry weight). Except for transgenic line 22 and 23, all other lines accumulated about 1 μmol/g dry weight of glycine betaine (Table 2). Interestingly, all lines also accumulated highly significant levels of choline, almost to the level of the wild-type.

Table 2 Determination of the concentration (μmol/g dry weight) of choline and glycine betaine in R1 generation PB1/*codA* transgenic rice plants

Plant identity	Choline in the R1 generation			Glycine betaine in the R1 generation		
	Experiment 1	Experiment 2	Average	Experiment 1	Experiment 2	Average
C	5.73	4.65	5.19 ± 0.54	0.00	0.00	0.00
1	4.06	3.04	3.55 ± 0.51	1.19	1.394	1.29 ± 0.1
4	5.35	7.54	6.45 ± 1.1	1.148	1.007	1.07 ± 0.07
14	5.16	3.04	4.10 ± 1.06	0.96	1.025	0.99 ± 0.03
18	4.33	3.14	3.74 ± 0.59	0.93	1.025	0.97 ± 0.04
21	5.65	4.12	4.89 ± 0.76	1.92	2.333	2.12 ± 0.21
22	5.45	3.93	4.69 ± 0.76	0.66	0.769	0.71 ± 0.05
23	5.06	3.49	4.27 ± 0.79	0.23	0.216	0.22 ± 0.007

Table 3 Agronomic traits of PB1/*codA* R1 generation transgenic plants recovered from salt stress and wild-type (WT) rice. Wild-type plants failed to recover after salt stress

Plant identity	NaCl 150 mM	No. of seedlings evaluated	No. of surviving plantlets	Number of surviving plants in two experiments grown to maturity	Average height (cm)	Average no. of panicles	Average no. of seeds
WT	–	17	17	17	88.3	10	388
WT	+	32	0	0	–	–	–
1	+	22	15	5	63.8	5	172
4	+	15	10	3	80.2	6	200
14	+	33	8	8	69.3	5	102
18	+	27	20	17	70.4	5	174
21[a]	+	10	5	3	55.0	5	100
22	+	21	11	8	71.8	6	166
23	+	17	5	5	72.8	6	172

[a] Result of one experiment only

Agronomic performance of transgenic lines under salt stress

When grown in liquid medium, 150 mM of NaCl is harmful to rice seedlings. However, several *codA* transgenic rice seedlings survived a 150-mM NaCl stress for a week before being transferred to normal rice growth medium. From two independent experiments, approximately 51% (74 out of 145) transgenic plants recovered well after exposure to salt stress. Out of the seven independent transgenic lines analyzed for their performance under salinity stress, four lines (1, 4, 18 and 22) showed high levels of stress tolerance (about 66% survival, 56 out of 85) against salt stress, whereas the wild-type plants failed to recover after exposure to salt stress and died. Some of the surviving seedlings were transferred to soil and have set seed (Table 3). A total of 49 plants were grown to maturity. In comparison to unstressed wild-type plants, transgenic plants exposed to salt stress gave rise to marginally shorter plants with a lesser number of panicles. The number of seeds obtained varied from approximately 100–200 seeds among various lines, whereas the unstressed wild-type plants yielded on average 388 seeds per plant. Obviously there is some degree of penalty on exposure to salt. However, the yield is significant keeping in view of the fact that all wild-type plants failed to recover on exposure to salt stress.

Genetic analysis of hygromycin resistance in progeny of R1 plants

Seeds from 3–5 R1 plants, representing the R2 generation, of six different R0 lines having 1–5 copies of the transgene were grown on hygromycin-containing medium. Among the various transgenic lines analyzed, seed germination was found to be 77.7 to 93.3%. As in the R1 progeny, Mendelian as well as non-Mendelian segregation patterns were obtained (data not shown). These plants are being grown for further analysis.

Discussion

Agrobacterium-mediated transformation of an elite *indica* variety Pusa Basmati 1 has been accomplished. Although the transformation efficiency obtained with binary vectors was less than that achieved with superbinary vectors (Mohanty et al. 1999, 2000), we could still obtain several transgenic plants for analysis of abiotic stress tolerance. This work, together with earlier reports of Hayashi et al. (1997) for *Arabidopsis* and Sakamoto et al. (1998) for *japonica* rice, conclusively proves that engineering for glycine betaine, as accomplished by a gene (*codA*) encoding for the enzyme choline oxidase, is an effective way for imparting stress

tolerance to non-accumulators such as rice. In addition, this work has been carried out utilizing a popular and economically important *indica* variety Pusa Basmati 1.

In contrast to the wild-type, all transgenic lines containing the *codA* gene showed the presence of glycine betaine, reflecting the fact that all the transgenic lines have acquired the ability to synthesize this compatible solute. Effectiveness of glycine betaine for salt and heat tolerance has also been shown by genetic mutation in maize by Saneoka et al. (1995) and Yang et al. (1996). Stable integration and faithful transmission of the transgene are of paramount importance in transgene technology. We have obtained Mendelian as well as non-Mendelian segregation ratios for the transgene(s). Similar observations have been made earlier (Komari et al. 1996; Mohanty et al. 1999). Interestingly, transgenic line 18 with five copies of the transgene segregated in a 3:1 ratio for hygromycin resistance, indicating single-locus integration of the transgenes.

Although the glycine betaine level obtained (0.22–2.12 μmol per gram of dry weight) for R1 lines was much lower than that of natural accumulators such as spinach and sugar beat, it was more than that reported earlier for transgenic tobacco (Holmström et al. 2000; see Sakamoto and Murata 2000, 2001), and even this much glycine betaine was capable of imparting a high level of salt tolerance as revealed by challenge studies. Holmstörm et al. (2000) reported that even 0.035 μmol/g fresh weight of glycine betaine was capable of imparting salinity and low temperature tolerance to transgenic tobacco plants. Unlike the apprehensions raised (Nuccio et al. 1998, 1999; McNeil et al. 1999), the availability of choline does not seem to be a problem in rice. Interestingly, the level of choline in transgenic plants was found to be only marginally different from wild-type plants even though part of it would have been used for the synthesis of glycine betaine. Probably the reason for this is the fact that the transgenic plants have started to produce glycine betaine, and the drive to reach homeostasis has resulted in more choline synthesis in the transgenic plants to be able to replenish choline utilized for glycine betaine synthesis. The level of expression of glycine betaine in transgenic plants points towards the stabilization of cellular structures and macromolecules rather than osmotic adjustments alone (Holmberg and Bülow 1998; Sakamoto et al. 1998; Sakamoto and Murata 2001). The agronomic performance of the transgenic plants under stress is significant. More than 50% plants of some transgenic lines survived on exposure to salt stress and yielded seeds, which could provide a significant yield gain under stress conditions. This could be of use for crop productivity in saline areas. To the best of our knowledge this is the first report involving transgenic *indica* rice plants with a *codA* gene and providing data on yield under salt stress.

Acknowledgements Our research programme is supported by grants from the Department of Biotechnology, New Delhi, India, and the Rockefeller Foundation, New York, USA. A.M. and H.K. are grateful to the University Grants Commission, New Delhi, India, and the Council of Scientific and Industrial Research, New Delhi, India, for the award of a Senior Research Fellowship and a Junior Research fellowship, respectively.

References

Alia, Hayashi H, Sakamoto A, Murata N (1998) Enhancement of the tolerance of *Arabidopsis* to high temperature by genetic engineering of the synthesis of glycine betaine. Plant J 16:155–161

Alia, Kondo Y, Sakamoto A, Nonaka H, Hayashi H, Pardha Saradhi P, Chen THH, Murata N (1999) Enhanced tolerance to light stress of transgenic *Arabidopsis* plants that express the *codA* gene for a bacterial choline oxidase. Plant Mol Biol 40:279–288

Arakawa T, Chang DKX, Merritt JL, Langridge WHR (1997) Expression of cholera toxin B subunit oligomers in transgenic potato plants. Trans Res 6:403–413

Bajaj S, Targolli J, Liu L-F, David Ho T-H, Wu R (1999) Transgenic approaches to increase dehydration-stress tolerance in plants. Mol Breed 5:493–503

Bradford MM (1976) A rapid and sensitive method for quantification of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72:248–254

Brouquisse R, Weigel P, Rhodes D, Yocum CF, Hanson AD (1989) Evidence for a ferredoxin-dependent choline mono-oxygenase from spinach chloroplast stroma. Plant Physiol 90:322–329

Christou P (1994) Rice biotechnology and genetic engineering. Technomic Publishing Co. Inc. Lancaster, USA

Csonka LN (1989) Physiological and genetic responses of bacteria to osmotic stress. Microbiol Rev 53:121–147

Garcia-Perez A, Burg MB (1991) Renal medullary organic osmolytes. Physiol Rev 71:1081–1073

Gorham J (1995) Betaines in higher plants-biosynthesis and role in stress metabolism. In: Wallsgrove RM (ed) Amino acids and their derivatives in higher plants. Cambridge University Press, Cambridge, pp 171–203

Grover A, Sahi C, Sanan N, Grover A (1999) Taming abiotic stresses in plants through genetic engineering: current strategies and perspective. Plant Sci 143:101–111

Hayashi H, Alia, Mustardy L, Deshnium P, Ida M, Murata N (1997) Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. Plant J 12:133–142

Holmberg N, Bülow L (1998) Improving stress tolerance in plants by gene transfer. Trends Plant Sci 3:61–66

Holmstörm K-O, Somersalo S, Mandal A, Palva TE, Welin B (2000) Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. J Exp Bot 51:177–185

Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J 10:165–174

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem 163:16–20

McNeil SD, Nuccio ML, Hanson AD (1999) Betaines and related osmoprotectants, targets for metabolic engineering of stress tolerance. Plant Physiol 120:945–949

Mohanty A, Sarma NP, Tyagi AK (1999) *Agrobacterium*-mediated high frequency transformation of an elite indica variety Pusa Basmati 1 and transmission of the transgenes to R2 progeny. Plant Sci 147:127–137

Mohanty A, Grover M, Chaudhury A, Haq QR, Sharma AK, Maheshwari SC, Tyagi AK (2000) Analysis of the activity of promoters from two photosynthesis-related genes *psaF* and *petH* of spinach in a monocot plant, rice. Ind J Biochem Biophys 37:447–452

Nelson DE, Shen B, Bohnert HJ (1998) Salinity tolerance-mechanisms, models and the metabolic engineering of complex traits. In: Setlow JK (ed) Genetic engineering, vol 20. Plenum press, New York, pp 153–176

Nuccio ML, Russell BL, Nolte KD, Rathinasabapathi B, Gage DA, Hanson AD (1998) The endogenous choline supply limits glycine betaine synthesis in transgenic tobacco expressing choline monooxygenase. Plant J 16:487–496

Nuccio ML, Rhodes D, McNeil SD, Hanson AD (1999) Metabolic engineering of plants for osmotic stress resistance. Curr Opinion Plant Biol 2:128–134

Papageorgiou GC, Murata N (1995) The unusually strong stabilizing effects of glycine betaine on the structure and function in the oxygen-evolving photosystem II complex. Photosyn Res 44:243–252

Rhodes D, Hanson AD (1993) Quaternary ammonium and tertiary sulphonium compounds in higher plants. Annu Rev Plant Physiol Plant Mol Biol 44:357–384

Robinson SP, Jones GP (1986) Accumulation of glycine betaine in chloroplast provides osmotic adjustment during stress. Aust J Plant Physiol 13:659–668

Sakamoto A, Murata N (2000) Genetic engineering of glycine betaine synthesis in plants: current status and implications for enhancement of stress tolerance. J Exp Bot 51:81–88

Sakamoto A, Murata N (2001) The use of bacterial choline oxidase, a glycine betaine-synthesizing enzyme, to create stress-resistant transgenic plants. Plant Physiol 125:180–188

Sakamoto A, Alia, Murata N (1998) Metabolic engineering of rice leading to biosynthesis of glycinebetaine and tolerance to salt and cold. Plant Mol Biol 38:1011–1019

Sakamoto A, Valverde R, Alia, Chen THH, Murata N (2000) Transformation of *Arabidopsis* with the *codA* gene for choline oxidase enhances freezing tolerance of plants. Plant J 22:449–453

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Second Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Saneoka H, Nagasaka C, Hahn DT, Yang W-J, Premachandra GS, Joly RJ, Rhodes D (1995) Salt tolerance of glycine betaine-deficient and -containing maize lines. Plant Physiol 107:631–638

Tyagi AK, Mohanty A (2000) Rice transformation for crop improvement and functional genomics. Plant Sci 158:1–18

Tyagi AK, Mohanty A, Bajaj S, Chaudhury A, Maheshwari SC (1999) Transgenic rice: a valuable monocot system for crop improvement and gene research. Crit Rev Biotech 19:41–79

Weigel P, Weretilnyk EA, Hanson AD (1986) Betaine aldehyde oxidation by spinach chloroplasts. Plant Physiol 82:753–759

Yang G, Rhodes D, Joly RJ (1996) Effects of high temperature on membrane stability and chlorophyll fluorescence in glycine betaine-deficient and glycine betaine-containing maize lines. Aus J Plant Physiol 23:437–443

Yeo A (1998) Molecular biology of salt tolerance in the context of whole-plant physiology. J Exp Bot 49:915–929