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Transformation of tomato with a bacterial *codA* gene enhances tolerance to salt and water stresses

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ABSTRACT

Genetically engineered tomato (*Lycopersicon esculentum*) with the ability to synthesize glycinebetaine was generated by introducing the *codA* gene encoding choline oxidase from *Arthrobacter globiformis*. Integration of the *codA* gene in transgenic tomato plants was verified by PCR analysis and DNA blot hybridization. Transgenic expression of gene was verified by RT-PCR analysis and RNA blot hybridization. The *codA*-transgenic plants showed higher tolerance to salt stress during seed germination, and subsequent growth of young seedlings than wild-type plants. The *codA* transgene enhanced the salt tolerance of whole plants and leaves. Mature leaves of *codA*-transgenic plants revealed higher levels of relative water content, chlorophyll content, and proline content than those of wild-type plants under salt and water stresses. Results from the current study suggest that the expression of the *codA* gene in transgenic tomato plants induces the synthesis of glycinebetaine and improves the tolerance of plants to salt and water stresses.

Introduction

Increasing environmental stresses, such as salinity, drought, and low temperature, pose serious threats to global agricultural productivity (Boyer, 1982). Abiotic stresses adversely affect the physiological status of an organism by altering its metabolism, growth, and development. Thus, the genetic manipulation of plants for enhancement of tolerance to various kinds of abiotic stresses has become utmost important in recent years. Plants accumulate sugars and other compatible solutes in response to such unfavorable environments (Hare et al., 1998). These compounds in some cases stabilize highly ordered biomolecules under stress conditions (Hare et al., 1998). One of such compatible solutes is glycinebetaine, which imparts tolerance in plants to abiotic stresses (Hayashi et al., 1997, 1998; Sakamoto and Murata, 2001, 2002; Chen and Murata, 2002, 2008, 2011).

Glycinebetaine is a highly efficient compatible solute (Le Rudulier et al., 1984) and its accumulation is strongly associated with the growth of plants in dry and saline environments (Rhodes and Hanson, 1993). The level of glycinebetaine is correlated with the degree of enhanced tolerance to stress (Saneoka et al., 1995). At high concentrations, glycinebetaine does not interfere with cytoplasmic functions and it efficiently stabilizes the structure and functions of many macromolecules. Glycinebetaine also stabilizes the oxygen-evolving photosystem II protein–pigment complex at high concentrations of NaCl (Murata et al., 1992; Papageorgiou and Murata, 1995). Glycinebetaine is much more effective than the other compatible solutes in stabilization of the quaternary structure of enzymes and complex proteins, as well as the highly ordered state of membrane at high concentrations of salts (Gorham, 2010; Papageorgiou and Murata, 1995).

Tomato (*Lycopersicon esculentum*) is one of the most important crop plants worldwide, because it propagates easily and has a short lifecycle. Tomato is considered as a model plant for physiological, biochemical and molecular-genetic investigations (Kalloo, 1991; Bordas et al., 1997). Salt and water stresses affect seed germination, root development, shoot development, fruit quality and yield of tomato plants (Foolad, 2004). Conventionally, deleterious effects

Abbreviations: BAP, 6-benzylaminopurine; CTAB, cetyl trimethyl ammonium bromide; DW, dry weight; 1/2-MS, half-strength Murashige & Skoog medium without plant growth regulator; IAA, indole-3-acetic acid; LE, leaf expansion; RWC, relative water content.

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of salt and water stresses on tomato plants can be ameliorated by seed priming, plant breeding, and treatment with mycorrhizae. As compared to these conventional methods, metabolic engineering has proved to be a better alternative for combating deleterious effects of salt and water stresses. Metabolic engineering of tomato could be very useful for increasing crop productivity in salt and drought-affected areas. Since tomato does not have two enzymes required for biosynthesis of glycinebetaine, it cannot produce this compatible solute. Introduction of the glycinebetaine-biosynthetic pathway into tomato plants would enhance the tolerance to a variety of abiotic stresses.

A considerable attention has been given to the genetic engineering of plants for the biosynthesis of glycinebetaine for enhancement of tolerance to salt stress (Hayashi et al., 1998; Sakamoto and Murata, 2000; Chen and Murata, 2002, 2008). The most successful genetic engineering of glycinebetaine biosynthesis has been achieved by introducing choline oxidase, which is encoded by a bacterial *codA* gene, to various plants, such as *Arabidopsis* (Hayashi et al., 1997, 1998; Alia et al., 1998; Sulpice et al., 2003), *Brassica* (Huang et al., 2000; Prasad et al., 2000), persimmon (Gao et al., 2000), rice (Sakamoto et al., 1998; Mohanty et al., 2002; Su et al., 2006; Kathuria et al., 2009), maize (Quan et al., 2004), and potato (Ahmad et al., 2008). Such transformation for the synthesis of glycinebetaine enhanced the tolerance of relevant plants towards various kinds of environmental stress at all stages of lifecycle of plants (Chen and Murata, 2008, 2011).

Park et al. (2004) reported that glycinebetaine synthesis in *codA*-transgenic tomato protected seeds, plants, and flowers from chilling damage. However, the effect of the *codA* transgene on the tolerance of tomato plants to salt and water stress has not been systematically examined. Development of *codA*-transgenic tomato can be employed to minimize the deleterious effects of salt and water stresses in agriculture. To maintain the current level of productivity or to increase the total production in marginal saline lands or in rain-fed areas, tomato cultivars with enhanced levels of glycinebetaine are needed.

Enhanced tolerance to abiotic stress has been achieved when choline oxidase encoded by the *codA* gene is directed to the chloroplast (Hayashi et al., 1997; Sakamoto and Murata, 2000; Park et al., 2007a). Transgenic plants genetically engineered with the *codA* gene whose product is targeted to chloroplasts accumulate glycinebetaine primarily in these organelles, and they exhibit enhanced tolerance to chilling stress at various developmental stages (Park et al., 2004). The concentration and the localization of glycinebetaine in the cell influence the tolerance of plants to abiotic stress. The accumulation of glycinebetaine in chloroplasts of transgenic plants effectively protects the photosynthetic machinery under salt stress conditions. This suggests that the subcellular localization of the synthesis of glycinebetaine might be critical for the enhancement of stress tolerance in tomato plants (Park et al., 2007a).

In this study, we report the development of transgenic tomato plants with the ability to synthesize glycinebetaine by genetic transformation with the *codA* gene from *Arthrobacter globiformis*. The expression of the *codA* transgene resulted in glycinebetaine synthesis in transgenic tomato plants, which subsequently enhanced the tolerance to salt and water stress.

Materials and methods

Plant materials and culture conditions

Seeds of tomato (*Lycopersicon esculentum* Mill.) cv. Pusa Ruby were procured from Division of Vegetable Science, Indian Agricultural Research Institute, New Delhi, India. Seeds were treated with 4% sodium hypochlorite for 8 min and washed several times with sterile distilled water. After surface sterilization, the seeds were cultured on half-strength Murashige and Skoog (1962) medium (1/2-MS). The pH of the medium was adjusted to 5.8 ± 0.02 using 0.1 N NaOH and 0.1 N HCl prior to addition of 0.8% (w/v) agar (Bacteriological grade, Hi-media, Mumbai, India). The cultures were maintained at $24 \pm 2\,^{\circ}C$, at relative humidity 60–65% and under a 16/8-h light/dark photoperiod at a light intensity of 50 $\mu mol\ m^{-2}\ s^{-1}$ from cool white fluorescent tubes (Phillips, Mumbai, India).

Vector plasmid

The pGAH/*codA* binary vector (Hayashi et al., 1997), which had been introduced into *Agrobacterium tumefaciens* (strain GV 2260), was used for transformation of tomato. The vector contained the *codA* gene ligated at the N-terminal end with cDNA that encoded the transit peptide of the small subunit of Rubisco of tobacco, the 35S promoter of cauliflower mosaic virus, and the terminator of the gene for nopaline synthase, as described previously (Hayashi et al., 1997).

Transformation procedure

Cotyledons of tomato cv. Pusa Ruby were pre-cultured for 2 d on the solid MS medium (Murashige and Skoog, 1962) supplemented with 2.5 mg/L BAP and 0.5 mg/L IAA. The cotyledon explants were immersed for 15 min in a cell suspension of *A. tumefaciens* that harbored the pGAH/*codA* binary vector. After transfer on the MS medium, which contained 2.5 mg/L BAP, 0.5 mg/L IAA, 50 mg/L kanamycin, 20 mg/L hygromycin, and 250 mg/L cefotaxime, the explants were kept in darkness for 2 d. Then, after growth for 3–4 weeks at 16/8 h light/dark photoperiod, the explants with emerging shoot buds were isolated and cultured on MS medium supplemented with 0.5 mg/L BAP, 50 mg/L kanamycin, 20 mg/L hygromycin, and 250 mg/L cefotaxime. For rooting, shoots (15–20 mm in length) were transferred onto 1/2-MS medium that contained 25 mg/L kanamycin and 250 mg/L cefotaxime. Resultant plantlets with shoots and roots were transferred to liquid 1/2-MS medium and were allowed to grow for 10 d. Finally, the plantlets were transferred to pots that contained autoclaved soilrite moistened with 1/4-MS salts and were covered with transparent polythene bags to maintain high humidity. After 15 d, plants were transferred to pots that contained a mixture of soilrite, sand, and vermiculite in the ratio of 2:1:1 (by weight) and were kept in a phytotron.

Isolation of intact chloroplasts

T1 transgenic plants of 46-1, 46-2 and 46-3 lines (see, Results and discussion section) were kept in darkness for 3 d. Intact chloroplasts were isolated from leaves of these plants with the Chloroplast Isolation Kit (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol.

Analysis and quantification of glycinebetaine

Glycinebetaine was extracted and analysed by HPLC, as described by Naidu (1998). The quantification of glycinebetaine was done as demonstrated earlier by Park et al. (2004).

Analysis of genomic DNA by polymerase chain reaction and by blot hybridization

Genomic DNA was isolated from leaves of T1 transgenic plants by CTAB (cetyl trimethyl ammonium bromide) method (Murray and Thompson, 1980). Isolated DNA was subjected to

polymerase chain reaction (PCR) to amplify the *nptII* and *codA* genes. The oligonucleotides used for PCR analysis of the *nptII* gene were 5′-AAGATGGATTGCACGCAGGTTC-3′ (forward primer), and 5′-GAAGAACTCGTCAAGAAGGCA-3′ (reverse primer). Primers used to amplify the *codA* gene were 5′-CGACTACATCGTCGTCGGCG-3′ (forward primer) and 5′-CGGTGTTGTGCGTCTTGCGG-3′ (reverse primer). The amplified products were electrophoresed on a 1.0% agarose gel.

For DNA blot analysis, 10 μg genomic DNA, isolated as described above, was digested with *Bam*H1 for 14 h and separated on a 0.8% agarose gel. DNA was then transferred to a nylon membrane (Hybond N$^+$, Amersham, Piscataway, NJ, USA). The coding sequence of the *cod*A gene was used as a probe for DNA blot hybridization. The probe was labeled with (α-^{32}P)-dCTP labeled probe using Random Primer Labeling Kit (Stratagene). Hybridization was carried out for 16 h at 65 °C in the presence of the labeled probe. After hybridization, the membrane was washed with 2 × solution of sodium citrate (SSC) + 0.1% SDS, 1 × SSC + 0.1% SDS, and 0.1 × SSC + 0.1% SDS at 60 °C. An autoradiogram was obtained after 2 d exposure of the membrane at −80 °C.

Analysis of RNA

Total RNA was isolated from young leaves of T1 transgenic plants using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). RT-PCR was performed using one-step RT-PCR kit (Qiagen) to amplify the *nptII* and *codA* genes. The amplified products were analysed by electrophoresis on a 1.0% agarose gel.

For RNA blot hybridization, total RNA was fractioned on a 1% (w/v) agarose gel that contained 2% (w/v) formaldehyde. Subsequently, RNA was transferred on to a zeta probe membrane and then hybridized with a [^{32}P]-dCTP-labeled probe (a *codA* gene-specific probe) for 24 h. After hybridization, the membrane was washed as described above for DNA blot hybridization. An autoradiogram was obtained by exposing the membrane to X-ray film at −80 °C for 2 d.

Segregation pattern in T1 plants

To evaluate the segregation pattern, seeds that had been harvested from T0 transgenic plants were surface-sterilized and were cultured on 1/2-MS medium that contained 50 mg/L kanamycin and 20 mg/L hygromycin. The T1 transgenic lines 46-1, 46-2 and 46-3 (see, Results and discussion section) were self-crossed to obtain T2 seeds. In order to evaluate further segregation, seeds obtained from T1 transgenic plants were germinated on antibiotic-containing 1/2-MS medium as described above. Cultures were maintained under conditions that were similar to those as described above. After 10–12 d of incubation, kanamycin-resistant and kanamycin-sensitive seedlings were scored. Kanamycin-resistant seedlings were grown further and then transferred to phytotron to perform various physiological experiments.

Assessment of salt tolerance during germination of seeds

Transgenic plants were assessed for changes in tolerance to salt (NaCl) stress by frequency of seed germination in NaCl-supplemented medium. T1 seeds obtained from T0 progeny were surface sterilized as described above and germinated on 1/2-MS medium that contained 0, 100 and 150 mM NaCl. Germination and growth of seedlings were analysed after growth for 10 d under salt stress conditions.

Assessment of salt tolerance in whole plants and leaves

To assess the tolerance of whole plants to salt stress, plants were irrigated with 150 mM NaCl for 10 d. To assess the tolerance of whole plants to water (deficient) stress, the irrigation was withheld for 7 d.

The tolerance of leaves to salt stress was examined by leaf-disc assay. The youngest fully expanded leaves collected from wild-type and transgenic (T1) plants were washed with distilled water. Leaf discs (10 mm) were obtained from the leaves using cork borers. The discs were placed in Petri dishes that contained a solution of 200 mM NaCl and incubated at 25 ± 2 °C for 7 d under continuous light of 50 μmol m^{-2} s^{-1} that was provided by cool white fluorescent tubes.

For physiological assessment, T1 transgenic plants of 46-1 and 46-2 lines and wild-type plants were kept in a greenhouse under controlled temperature and humidity conditions. For measurement of relative water content (RWC), leaf discs were obtained as described above. Fresh weight (FW) of leaf discs was determined immediately. The leaf discs were placed in distilled water for 4 h at room temperature to regain turgidity, and then were placed on tissue paper to remove excess water to determine the turgid weight (TW). Subsequently, leaf discs were oven-dried for 12 h at 80 °C to determine the dry weight (DW). Relative water content was calculated according to the formula RWC (%) = [(FW − DW)/(TW − DW)] × 100

Leaf expansion and plant height of transgenic and wild-type plants were measured every day. Chlorophyll content was quantified by the dimethylsulphoxide (DMSO) method (Prieto et al., 2004); a 250 mg leaf was immersed in 5 mL of DMSO at 65 °C in darkness for 12 h. The absorbance of the extract was recorded at 649.1 and 665.1 nm using a spectrophotometer. Proline content was measured in leaves by the method of Bates et al. (1973).

Results and discussion

Generation of transgenic plants

The *codA* gene driven by the CaMV 35S promoter was introduced in tomato plants by *Agrobacterium*-mediated transformation. The construct used for transformation included the transit peptide that facilitated the targeting of choline oxidase to chloroplasts. About 80% pre-cultured cotyledonary explants, which had been co-cultivated with *Agrobacterium*, formed calli on MS medium that contained 2.5 mg/L BAP, 0.5 mg/L IAA, 50 mg/L kanamycin, and 20 mg/L hygromycin. During subsequent culture on the same medium for three weeks, shoots regenerated from the calli. These shoots were elongated and subsequently multiplied as independent putative transgenic lines on the MS medium that had been supplemented with 0.5 mg/L BAP. We obtained 10 putative transformed T0 plants that were resistant to both kanamycin and hygromycin.

An initial screening of the putative transgenic plants was carried out by polymerase chain reaction (PCR) analysis with primers for *nptII* and *codA* genes as described in Materials and methods section. The result showed that six among the 10 putative T0 transgenic plants revealed two bands on the agarose gel at 0.7 and 1.3 kb, which corresponded to *nptII* and *codA* genes, respectively (data not shown). These six T0 transgenic plants were then subjected to the RT-PCR analysis to determine the level of expression of *nptII* and *codA* genes. Among the six T0 plants, we selected three plants of independent lines, which showed the highest levels of expression of the *codA* gene at 1.3 kb (data not shown), and termed them 46-1, 46-2 and 46-3. They were self-crossed to generate T1 seeds. The T1 seeds were, then, germinated on kanamycin and hygromycin-enriched MS basal medium. The resultant plants, which were resistant to both antibiotics, were transplanted in pots in a greenhouse and self-crossed to generate T2 seeds.

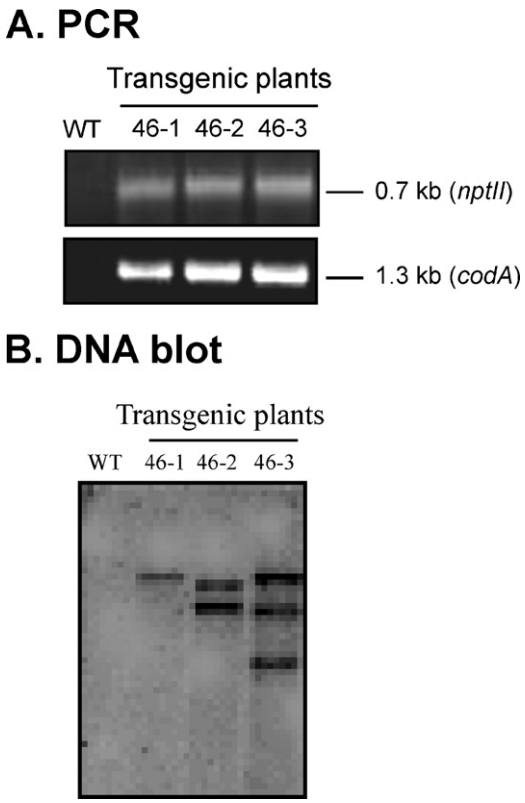

Fig. 1. Integration of the *codA* transgene in the genome of T1 transgenic tomato plants of three independent lines 46-1, 46-2 and 46-3. (A) PCR analysis of the genomic DNA from leaves of transgenic plants, using *nptII* and *codA* gene-specific primers. Bands at 0.7 and 1.3 kb corresponded to PCR products of the *nptII* and *codA* genes, respectively. (B) DNA blot analysis of the genomic DNA from leaves of transgenic plants, using the *codA*-specific probe. The genomic DNA was digested with *Bam*HI.

Polymerase chain reaction and DNA blot analysis of transgenic plants

We examined, by PCR and DNA blot hybridization, the mode of integration of the recombinant *codA* gene into the genome of transformed T1 plants of the three independent lines. We extracted the genomic DNA from leaves of T1 plants of each of the three independent lines 46-1, 46-2 and 46-3. Analysis by PCR of genomic DNA with primers, described in Materials and methods section, showed two bands at 0.7 and a 1.3 kb that corresponded to the *nptII* and *codA* genes, respectively, in each of the three lines of transgenic plants (Fig. 1A). These results suggested that the recombinant *codA* gene had been integrated into the genome of transgenic plants in each of the three independent lines.

Transgenic plants were also subjected to DNA blot analysis using the *codA*-coding region as a probe. The result revealed that T1 plants of independent 46-1, 46-2, and 46-3 lines, showed one, two, and three bands, respectively, whereas no hybridization signal was observed in wild-type plants (Fig. 1B). Positions of the hybridization bands on the blot analysis varied among the three lines, suggesting that the sites of insertion of the recombinant *codA* gene were different among the three lines. These results of PCR and DNA blot hybridization analyses might suggest that the number of integrated copies of the recombinant *codA* gene was one, two, and three in the T1 plants of 46-1, 46-2, and 46-3 lines, respectively.

Both the location in the genome and the copy number of the transgene is known to affect the level of transgene expression. Therefore, these findings may account for the difference in phenotype among the transgenic plants, which are described below.

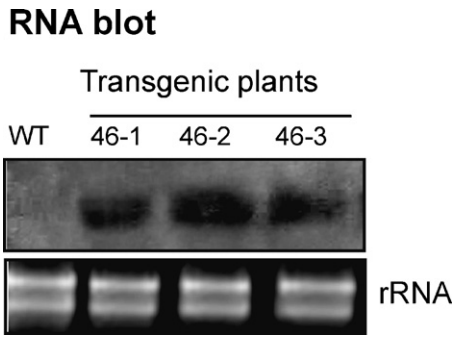

Fig. 2. Expression of the *codA* transgene in T1 transgenic tomato plants of three independent lines 46-1, 46-2 and 46-3. RNA blot analysis of RNA from leaves of transgenic plants, using the *codA*-specific probe. Equal loading of total RNA was assessed by ethidium bromide staining of rRNA bands.

Expression of the codA gene in transgenic plants

The expression of the *codA* gene in leaves of T1 plants was analysed by RNA blot hybridization using the *codA*-coding region as a probe. The result revealed that levels of the *codA* transcripts were different among the three lines of plants. The level of *codA* transcripts in line 46-1 and 46-2 plants was higher than that in 46-3 plants (Fig. 2). By contrast, the *codA* transcripts were not detected in wild-type plants. The expression of the *codA* gene in leaves of T1 transgenic and wild-type plants were examined also by RT-PCR using *nptII* and *codA* gene-specific primers. The result indicated that both *codA* and *nptII* genes were expressed in the transgenic plants of lines 46-1, 46-2, and 46-3. However, transcripts for *codA* and *nptII* genes were not detected in wild-type plants (data not shown).

Segregation analysis

To analyse the stable integration, the copy number, and heritability of the *codA* transgene in the nuclear genome, T1 seeds from T0 transgenic plants were germinated on 1/2-MS medium supplemented with kanamycin and hygromycin. Wild-type plants were sensitive to the antibiotics and none of the seeds germinated. Antibiotic-resistant and PCR-positive T1 transgenic plants were grown and self-crossed in order to obtain T2 seeds. The resultant T2 seeds thus obtained from individual T1 transgenic lines 46-1, 46-2 and 46-3 were again subjected to segregation analysis. T2 seeds of 46-1 and 46-2 lines showed 100% germination in the presence of both kanamycin and hygromycin, suggesting that these two lines were homozygous for the *codA* transgene. By contrast, T2 seeds of transgenic lines 46-3 showed segregation of 3:1 for antibiotic-resistant germination, suggesting that this line was hemizygous for the *codA* transgene. Therefore, T1 transgenic plants of 46-1 and 46-2 lines were used for the following experiments.

Accumulation of glycinebetaine in codA-transgenic plants

Table 1 shows that T1 transgenic plants of 46-1, 46-2 and 46-3 lines accumulated glycinebetaine in leaves at levels of 297, 256, and 214 nmol g^{-1} fresh weight, respectively. These plants accumulated glycinebetaine in intact chloroplasts from leaves at levels of 8.6, 7.2 and, 5.5 nmol mg^{-1} chlorophyll, respectively (Table 1). By contrast, wild-type plants did not accumulate glycinebetaine either in leaves or in chloroplasts (Table 1). These values were consistent with the result of Park et al. (2007a) in *codA*-transgenic tomato of another variety, which revealed the glycinebetaine content at levels of 9 nmol mg^{-1} chlorophyll.

Table 1
Levels of glycinebetaine in the leaves and intact chloroplasts isolated from leaves of three antibiotic-resistant T1 transgenic plants. Plants were incubated in darkness for 3 days, and intact chloroplasts were isolated from leaves using Chloroplast Isolation Kit (Sigma). Values are the average of three replicates ± SD.

Parameter	Wild-type	Transgenic lines		
		46-1	46-2	46-3
Glycinebetaine in the leaves (nmol g^{-1} fresh weight)	0	297 ± 18	256 ± 15	214 ± 12
Glycine betaine in intact chloroplasts isolated from leaves (nmol mg^{-1} chlorophyll)	0	8.6 ± 1.2	7.2 ± 0.9	5.5 ± 0.7

Enhanced tolerance of codA-transgenic seeds to salt stress

To examine the tolerance of *codA*-transgenic plants to salt stress during germination of seeds and growth of young seedlings, seeds of transgenic plants and wild-type plants were allowed to germinate on MS medium that contained 0, 100 and 150 mM NaCl. While seeds of wild-type plants germinated at a high frequency in the absence of NaCl, none of such seeds germinated in the presence of 100 and 150 mM NaCl (Fig. 3A). By contrast, transgenic T1 seeds showed a high frequency of germination in the presence of 100 and 150 mM NaCl (Fig. 3B). However, although the germination frequency was unaffected by salt stress, growth of young seedlings of *codA*-transgenic plants was repressed at 100 mM NaCl and was severely repressed at 150 mM NaCl (Fig. 3B).

Germination of seeds and growth of young seedlings are most important developmental stages in the plant lifecycle and are highly sensitive to salt stress (Bewley and Black, 1982; Mayer and Polijakoff-Mayber, 1982). The results in the present investigation suggest that the transformation of tomato plants with the *codA* gene is a powerful strategy to increase the productivity of tomato in the saline field.

Enhanced tolerance of codA-transgenic plants to salt stress

To investigate the effect of the *codA* transgene on the tolerance to salt stress in whole plants, T1 transgenic plants were compared with wild-type plants under stress conditions by irrigation with 150 mM NaCl for 10 d. Old transpiring leaves of wild-type plants turned yellow, and finally the whole plant died after exposure to the salt stress for 10 d. By contrast, the *codA*-transgenic plants grew well (Fig. 4A). These results suggest that the biosynthesis of glycinebetaine is critical for attaining the enhancement of salt tolerance of plants.

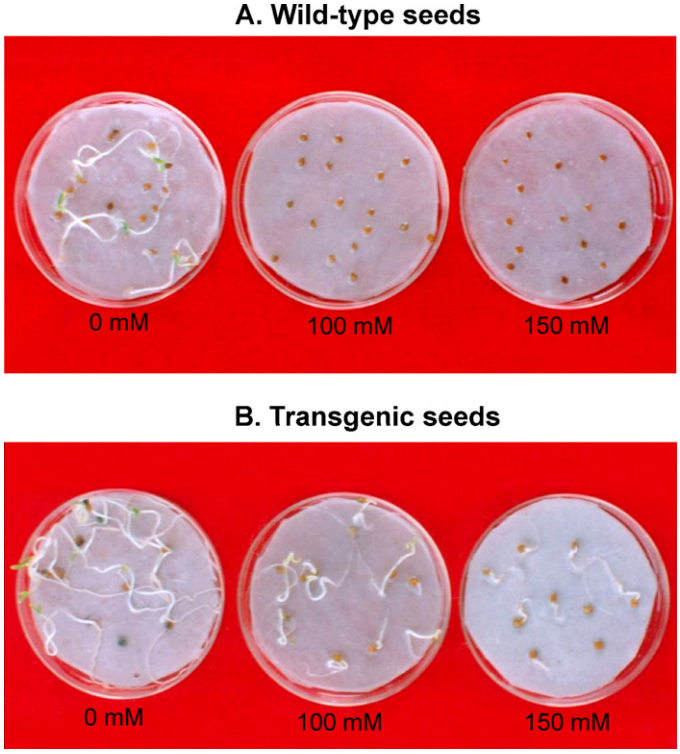

Fig. 3. Difference in the tolerance to salt stress during germination between seeds from wild-type plants and seeds from T1 transgenic plants of line 46-2. Seeds were allowed to germinate for 10 d on MS medium supplemented with 0, 100 and 150 mM NaCl.

Similarly, *codA*-transgenic plants survived and performed better under water stress conditions. By contrast, non-transgenic wild-type plants failed to withstand water stress conditions (Fig. 4B).

When salt and water stresses were removed, *codA*-transgenic plants recovered fully while wild-type plants did not recover at all (data not shown).

Improved tolerance of *codA*-transgenic plants to salinity was reported in *Arabidopsis* (Hayashi et al., 1997, 1998; Sulpice et al., 2003), Japonica rice (Sakamoto et al., 1998), Indica rice (Mohanty et al., 2002), Indian mustard (Prasad et al., 2000), and tobacco (Konstantinova et al., 2002). Improved tolerance of glycinebetaine-biosynthetic transgenic plants to water stress has been observed in cotton (Lv et al., 2007), maize (Quan et al., 2004), and wheat (Wang et al., 2010). The results in present study are consistent with previous investigations.

Enhanced tolerance of leaves from codA-transgenic plants to salt stress

The tolerance of T1 transgenic plants to NaCl stress was analysed by leaf disc assay. When leaf discs from *codA*-transgenic and wild-type plants were treated with 200 mM NaCl for 7 d, leaf discs from the transgenic plants remained green, whereas leaf discs from wild-type plants showed bleaching symptoms (Fig. 5). These results suggest that *codA*-transgenic plants conferred the tolerance to salt stress at the leaf level.

Physiological assessment of transgenic plants under salt and water stresses

Relative water content (*RWC*) *in leaves*: under non-stressed, salt and water stress conditions, transgenic T1 plants of lines 46-1 and 46-2 showed a high level of RWC than that of wild-type plants (Fig. 6A). When wild-type plants were subjected to salt stress at 150 mM of NaCl for 10 d, the RWC decreased from 77 ± 5% to 57 ± 4%. By contrast, when T1 plants were exposed to 150 mM NaCl for 10 d, the RWC decreased from 87 ± 6% to 82 ± 5%. During exposure to water stress due to withdrawal of irrigation for 7 d, the RWC decreased from 77 ± 5% to 55 ± 5% in wild-type plants but from 87 ± 6% to 78 ± 5% in *codA*-transgenic plants (Fig. 6A). These results suggested that the expression of the *codA* gene and the resultant accumulation of glycinebetaine in transgenic tomato plants had a strong effect in maintaining the RWC in leaves, which could be achieved by preventing the loss of water from leaves or by promoting the incorporation of water in roots.

Leaf expansion: leaf expansion was significantly higher in transgenic plants in comparison to wild-type plants under non-stressed, salt stress and water stress conditions (Fig. 6B). When wild-type plants were exposed to salt stress at 150 mM of NaCl for 10 d, leaf expansion decreased from 1.1 ± 0.07 cm to 0.5 ± 0.07 cm. In con-

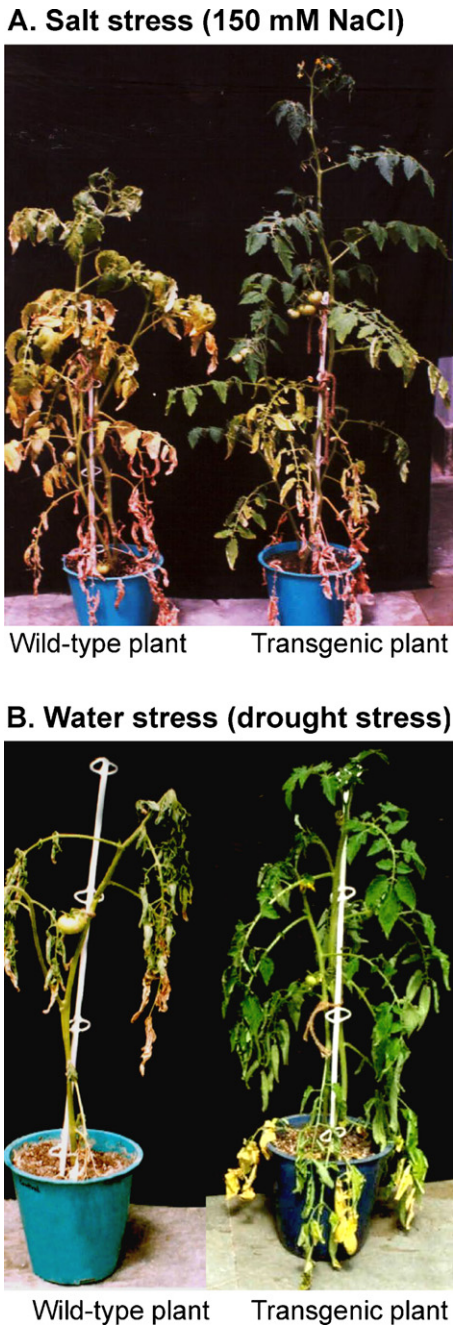

Fig. 4. Difference in the tolerance to salt and water stresses at the whole plant level between wild-type plants and T1 transgenic plants of line 46-2. (A) Salt stress. Plants were irrigated with 150 mM NaCl for 10 d. (B) Water stress. The irrigation was withheld for 7 d.

Fig. 5. Difference in the tolerance to salt stress between leaves from wild-type plants and T1 transgenic plants of line 46-2. Leaf discs were excised from transgenic and wild-type plants and were immersed in 200 mM NaCl for 7 d.

trast, when *codA*-transgenic plants were subjected to the same conditions, the leaf expansion decreased from 1.4 ± 0.09 cm to 1.2 ± 0.09 cm. During exposure to water stress due to withdrawal of irrigation for 7 d, the leaf expansion decreased from 1.1 ± 0.07 cm to 0.6 ± 0.05 cm in wild-type plants but from 1.4 ± 0.09 cm to 1.0 ± 0.07 cm in *codA*-transgenic plants (Fig. 6B). These results showed that the leaf expansion was significantly repressed when wild-type plants were subjected to salt and water stress. In contrast, only slightly reduced expansion of leaves was observed in transgenic plants under salt and water stress conditions.

Plant height: similarly, when plant height was measured, the transgenic plants showed significant increase in plant height compared to wild-type plants under non-stressed, salt stress and water stress conditions (Fig. 6C). When wild-type plants were subjected to salt stress at 150 mM of NaCl for 10 d, the plant height decreased from 15.0 ± 1.1 cm to 9.5 ± 0.1 cm. By contrast, when *codA*-transgenic plants were exposed to 150 mM NaCl for 10 d, the plant height decreased from 20.5 ± 1.2 cm to 18.0 ± 1.5 cm. During exposure to water stress due to withdrawal of irrigation for 7 d, the plant height decreased from 15.0 ± 1.1 cm to 3.5 ± 1.1 cm in wild-type plants but from 21.0 ± 1.2 cm to 8.5 ± 1.5 cm in *codA*-transgenic plants (Fig. 6C). These results suggested that the increase in plant height was significantly affected by salt and water stress conditions in wild-type plants, whereas it was reduced only marginally in *codA*-transgenic plants under salt and water stress conditions.

The result in Fig. 6 indicates that the *codA*-transgenic tomato plants performed better than wild-type plants, in terms of RWC, leaf expansion, and plant height, under non-stress conditions. These findings might be related to previous observations; namely, Sulpice et al. (2003) observed that *codA*-transgenic *Arabidopsis* plants produced more numbers of flowers and seeds than wild-type plants when grown under non-stress conditions; Park et al. (2007b) observed that the *codA* transgene increased the sizes of flower buds and flowers of tomato plants under non-stress conditions. Microarray analysis of the expression of genes in flower buds of wild-type and *codA*-transgenic tomato revealed that the *codA* transgene enhanced the expression of a number of genes for cell division (Park et al., 2007b). The *codA*-transgenic rice plants enhanced the expression of many genes that are involved in the response of plants to abiotic stress (Kathuria et al., 2009). These studies provided evidence that the enhanced expression of stress-responsive and cell cycle-related genes in glycinebetaine accumulating transgenic plants might be a plausible explanation for the enhanced performance of *codA*-transgenic plants under non-stress conditions.

Chlorophyll content in leaves: transgenic plants showed high chlorophyll content as compared to wild-type plants under non-stressed, salt stress and water stress conditions (Fig. 6D). When wild-type plants were exposed to salt stress at 150 mM of NaCl for 10 d, chlorophyll content decreased from 2.50 ± 0.30 mg g^{-1} FW to 1.29 ± 0.25 mg g^{-1} FW. In contrast, when T1 transgenic plants were subjected to 150 mM NaCl for 10 d, the chlorophyll content decreased from 3.40 ± 0.30 mg g^{-1} FW to 3.26 ± 0.25 mg g^{-1} FW. During exposure to water stress due to withdrawal of irrigation for 7 d, the chlorophyll content decreased from 2.50 ± 0.30 mg g^{-1}

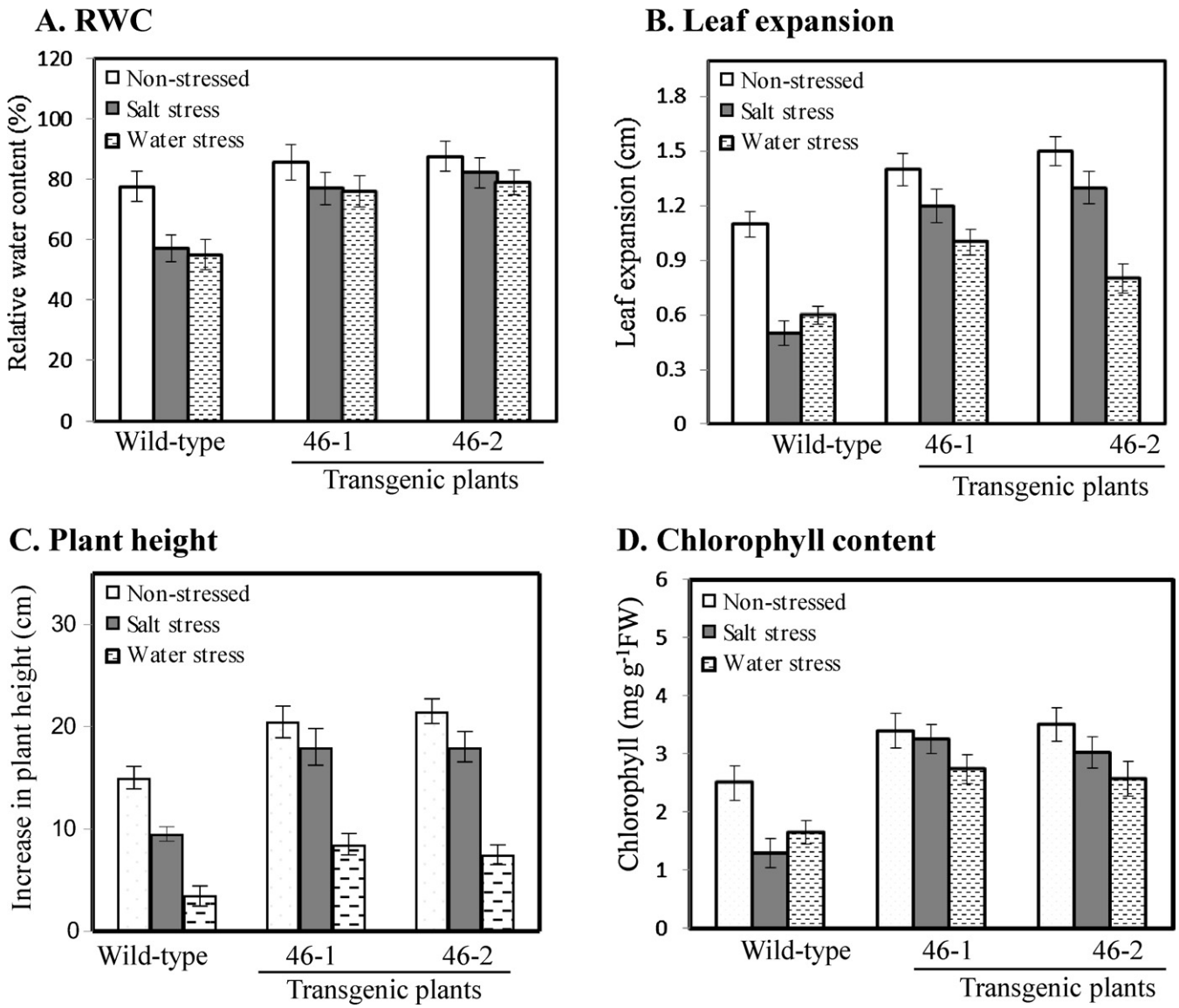

Fig. 6. Physiological assessment of wild-type plants and T1 transgenic plants of lines 46-1 and 46-2 under salt and water stresses. Physiological activities were measured in terms of (A) relative water content, (B) leaf expansion, (C) increase in plant height, and (D) chlorophyll content. Plants were grown in a greenhouse under controlled environmental conditions as described in the Materials and method section. Then, plants were irrigated with 150 mM NaCl for 10 d for salt stress or the irrigation was withheld for 7 d for water stress. Each value represents the average of three independent experiments ± standard deviation (SD).

FW to 1.64 ± 0.20 mg g^{-1} FW in wild-type plants but from 3.40 ± 0.30 mg g^{-1} FW to 2.73 ± 0.25 mg g^{-1} FW in *codA*-transgenic plants (Fig. 6D). The enhanced tolerance of the transgenic plants to salt and water stresses was possibly related to the high content of chlorophyll in leaves as compared to non-transgenic wild-type plants under salt and water stress conditions. Blunden et al. (1997) and Whapham et al. (1993) reported that treatment of tomato leaves with seaweed extracts that contained glycinebetaine increased the content of chlorophyll in leaves. Matoh et al. (1987) suggested that betaines play a role, as cytokinins, in increasing the content of chlorophyll in leaves.

In separate experiments, we observed that, during exposure to 150 mM NaCl, young seedlings of *codA*-transgenic plants grew significantly faster than that of wild-type plants in terms of length, fresh weight, and dry weight of leaves (data no shown). Significant improvement in growth of seedlings in transgenic plants over wild-type plants under salt stress could be due to enhancement of their capacity to photosynthesize even in the presence of NaCl at levels that were inhibitory to wild-type seedlings.

Increase in proline levels by the codA transformation

Previous studies have demonstrated that plants accumulate proline in the response to various kinds of environmental stress and, in particular, salt and drought stresses (Trovato et al., 2008). Therefore, we examined the level of proline in wild-type and *codA*-transgenic plants under non-stressed and salt-stressed conditions. Interestingly, the level of proline accumulated in *codA*-transgenic plants was much higher than that in wild-type plants under both non-stressed and salt-stressed conditions (Fig. 7). The result also showed that the level of proline accumulation increased with an increase in concentration of NaCl. Under non-stressed conditions, the proline level was 20.0 ± 2.20 μg g^{-1} FW in wild-type plants, while the proline level was 37.0 ± 2.5 μg g^{-1} FW in *codA*-transgenic plants. When wild-type plants were subjected to salt stress at 150 and 200 mM NaCl for 10 d the proline levels were 35.0 ± 2.5 and 48.0 ± 3.5 μg g^{-1} FW, respectively. In contrast, when *codA*-transgenic plants were exposed to salt stress at 150 and 200 mM NaCl for 10 d, the proline levels were 64.0 ± 3.0 and

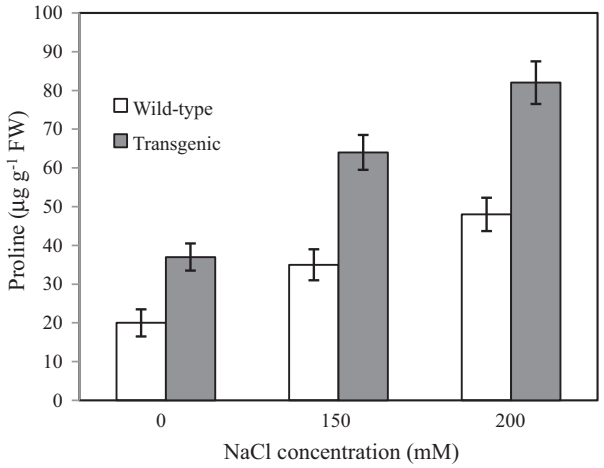

Fig. 7. Proline content in leaves of wild-type plants and T1 transgenic plants of line 46-2. Plants were grown in a greenhouse under controlled environmental conditions as described in the Materials and method section and then exposed to salt stress at 0, 150 and 200 mM NaCl for 10 d. Each value represents the average of three independent experiments ± standard deviation (SD).

$82.0 \pm 4.5\ \mu g\ g^{-1}$ FW, respectively. An increase in levels of proline in response to salt stress was observed also in *codA*-transgenic rice plants (Sakamoto et al., 1998).

Among compatible solutes, proline is considered to be of major importance. A large number of plant species accumulate proline in response to stresses, such as salinity and drought (Trovato et al., 2008). However, the mechanism for the enhanced accumulation of proline in *codA*-transgenic plants remains to be clarified.

Conclusions

In the present investigation, we transformed tomato plants with the bacterial *codA* gene that encodes choline oxidase and isolated three independent transgenic lines. Analyses by PCR and DNA blotting clearly indicated that the *codA* gene had been successfully integrated in transgenic tomato plants but at different positions of the genome among the three transgenic lines. Analyses by RT-PCR and RNA blotting indicated that the integrated *codA* gene was expressed in the three lines of transgenic plants. Direct measurement of glycinebetaine suggested that transgenic plants synthesized this compatible solute by the action of choline oxidase.

The *codA*-transgenic tomato plants revealed a much higher frequency of seed germination and faster growth of young seedlings under salt stress conditions than non-transformed tomato plants. Enhanced tolerance of *codA*-transgenic tomato plants to salt stress was observed also at the whole plant level. The transgenic plants revealed enhanced leaf expansion and increase in plant height as compared to wild-type plants under salt stress, as well as non-stressed conditions.

In other plants, such as Arabidopsis (Hayashi et al., 1997) and rice (Sakamoto et al., 1998; Mohanty et al., 2002; Su et al., 2006), the *codA* transgene enabled the accumulation of glycinebetaine and enhanced the tolerance against salt stress. The present study also demonstrated that the *codA*-transgenic tomato enhanced the tolerance to salt stress. Park et al. (2007a) observed that the *codA* transgene enhanced the salt tolerance of the photosynthetic machinery of tomato plants; the results are consistent with those in the present study.

The present investigation demonstrated that transformation of tomato plants with the *codA* gene also enhanced the tolerance to water stress at the whole plant level. Protection by the *codA* transgene in tomato plants against water stress was observed in the investigation of relative water content, leaf expansion, and plant growth measured by plant height, and chlorophyll content. An increase in the levels of glycinebetaine accumulation enhanced drought tolerance also in other transgenic plants, such as cotton (Lv et al., 2007), maize (Quan et al., 2004), and wheat (Wang et al., 2010). The enhanced tolerance of the transgenic plants was possibly due to the higher water retention capacity and the higher chlorophyll content in leaves than wild-type plants under salt and water stress conditions.

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