5-2007

Expression of *Bacillus subtilis proBA* Genes and Reduction of Feedback Inhibition of Proline Synthesis Increases Proline Production and Confers Osmotolerance in Transgenic *Arabidopsis*

Mingqing Chen
Wuhan University

Hongbo Wei
Wuhan University

JunWei Cao
Wuhan University

Ruijie Liu
Grand Valley State University, liuruiji@gvsu.edu

Youliang Wang
Wuhan University

See next page for additional authors

Journal of Biochemistry and Molecular Biology, Vol. 40, No. 3, May 2007, pp. 396-403

JOURNAL OF Biochemistry and Molecular Biology

Expression of *Bacillus subtilis proBA* Genes and Reduction of Feedback Inhibition of Proline Synthesis Increases Proline Production and Confers Osmotolerance in Transgenic *Arabidopsis*

Mingqing Chen, Hongbo Wei, JunWei Cao*, Ruijie Liu, Youliang Wang and Congyi Zheng
State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan 430072, P. R. China

Received 13 November 2006, Accepted 2 January 2007

Proline accumulation has been shown to correlate with tolerance to drought and salt stresses in plants. We attempt to introduce the wild-type, mutant, and fusion *proBA* genes derived from *Bacillus subtilis* into *Arabidopsis thaliana* under the control of a strong promoter cauliflower mosaic virus 35S (CaMV35S). The transgenic plants produced higher level of free proline than control and the overproduction of proline resulted in the increased tolerance to osmotic stress in transgenic plants. Besides, the mutation in *proBA* genes, which were proved to lead γ-glutamyl kinase (γ-GK) reduces sensitivity to the end-product inhibition and the fusion of *proB* and *proA* also result in increasing proline production and confer osmotolerance in transgenic lines.

Keywords: Proline, *proBA* gene, Transgenic *Arabidopsis*, Salt stress, Feedback inhibition

Introduction

The accumulation of osmolyte compounds has long been proposed as an adaptative mechanism for drought and salt tolerance, it has received increasing interest during the last 20 years. In response to drought and/or high salinity stresses, which disturb the intracellular water balance, many plants and bacteria synthesize and accumulate several kinds of compatible osmolytes, such as proline (Kavi Kishor *et al*., 1995), mannitol (Tarczynski *et al*., 1993; Shen *et al*., 1997), glycinebetaine (Nomura *et al*., 1995; Huang *et al*., 2000), trehalose (Garg *et al*., 2002), fructan (Pilon-Smits *et al*., 1995) that function as osmotica. Among the compatible solutes, proline appears to be the most widely distributed osmolyte accumulated under stress conditions not only in plants but also in eubacteria.

The pathway of proline synthesis from glutamate, the most common mechanism of proline biosynthesis, comprises three enzymatic steps (Fig. 1). The corresponding genes of *Bacillus subtilis*, *proB*, *proA*, and *proC*, encode γ-glutamyl kinase (γ-GK), γ-glutamyl-phosphate reductase (γ-GPR) and Δ-pyrroline-5-carboxylate (P5C) reductase. The proline pathway in plants has been proposed to be analogous to that in bacteria, although proline can also be made from ornithine (Adams and Frank, 1980). In plants, the Δ1-pyrroline-5-carboxilate synthetase (P5CS) is a bifunctional enzyme with both γ-GK and glutamate semialdehyde (GSA) dehydrogenase activities and it is a rate-limiting enzyme for biosynthetic pathway in higher plants, being feedback inhibited by proline (Hu *et al*., 1992; Zhang *et al*., 1995). In bacteria, proline biosynthesis has been shown to be regulated by the end-product inhibition of γ-glutamyl kinase (γ-GK) activity (Smith *et al*., 1984). A similar situation was also observed in plants. The γ-GK activity of *Vigna* P5CS was inhibitive by proline, but its GSA dehydrogenase activity was not affected, suggesting that the γ-GK is the rate-limiting step in proline biosynthesis in plants (Zhang *et al*., 1995).

In recent years, several studies have demonstrated that genetic manipulation of the accumulation of compatible solutes resulted in increased tolerance to drought or salt stress in transgenic plants (Tarczynski *et al*., 1993; Kavi Kishor *et al*., 1995; Nomura *et al*., 1995; Pilon-Smits *et al*., 1995; Shen *et al*., 1997; Huang *et al*., 2000; Garg *et al*., 2002). Since proline biosynthesis during stress has been demonstrated to be controlled by P5CS activity, many attempts to enhanced osmotolerance by increasing proline accumulation centered on this enzyme. The Δ1-pyrroline-5-carboxylate synthetase (P5CS) cDNA from mothbean (*Vigna aconitifolia* L.) was introduced into rice (*Oryza sati6a* L.) (Shen *et al*., 1998) and tobacco (Kavi Kishor *et al*., 1995).

Abbreviations: γ-GK, γ-glutamyl kinase; P5CS, Δ1-pyrroline-5-carboxilate synthetase; CaMV35S ,cauliflower mosaic virus 35S.

*To whom correspondence should be addressed.
Tel: 86-027-62772461; Fax: 86-027-68752560
E-mail: caojw6131@126.com

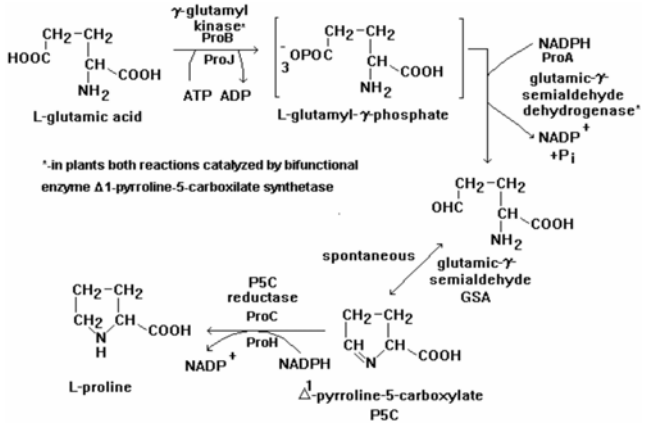

Fig. 1. Pathways of proline biosynthesis from glutamate in *B. subtilis* and plants. Steps in pathway are the same in both bacteria and plants, but the initial two reactions are performed by the bifunctional P5CS enzyme in plants.

However, it has not been reported that introduced *proBA* genes into plants. It was reported that the monthbean P5CS cDNA clones complemented *Escherichia coli proB*, *proA* and *proBA* mutants deficient in γ-GK, GSA dehydrogenase and both enzymes, respectively (Hu *et al*., 1992); however, it is not known whether γ-GK and GSA dehydrogenase exhibited enzymatic activity as P5CS in plants.

Since proline biosynthesis of γ-GK or P5CS is subject to feedback inhibition by the elevated level of proline, some work attempt to reduce the sensitivity of these enzymes to end-product inhibition. It was extensively studied that mutations of *proB* gene led γ-GK reduce sensitivity to proline -mediated feedback inhibition, resulting that proline overproduced and osmotolerance enhanced in bacteria (Csonka, 1981; Dandekar and Uratsu, 1988; Sleator *et al*., 2001). A similar situation was also observed in *Vigna* P5CS, the substitution of an alanine for a phenylalanine at amino acid residue 129 of the P5CS resulted in a significant reduction of proline feedback inhibition (Zhang *et al*., 1995). The mutation responsible for the resistance of *proB*74 encoded mutant γ-GK to proline inhibition involves a substitution of an A to a G nucleotide with a consequent alteration of aspartate at position 107 in the polypeptide to asparagines (Csonka *et al*., 1988; Dandekar and Uratsu, 1988). This aspartate residue (at position 128) conserved in the *Vigna aconitifolia* P5CS is not involved in the feedback inhibition (Zhang *et al*., 1995), while a replacement of Phe at position 129 by Ala resulted in removal of feedback inhibition of P5CS (Zhang *et al*., 1995; Hong *et al*., 2000).

In our previous work, *B. subtilis* 93151 was treated with N-methy-N'-nitro-N-nitrosoguanidine (NTG). Subsequently, we obtained a mutant (*B. subtilis* 93151-14) which could resist high NaCl (14%) stress by screening in minimal medium (MM) containing 3, 4-didehydroproline. The *proBA* genes were cloned from the wild strain and mutant, respectively. We found mutation sites in *proB*, which lead in an amino acid substitution (Ser216Thr), also in *proA* genes, which resulted three residue changes (Thr150 → Ala, Arg408 → Lys, Gln412 → Arg) (Miao *et al*., 2002; Liu *et al*., 2004). Besides, the *proB* and *proA* genes from *B.subtilis* overlap by 4 nt and this leads to the formation of a tight genic domain (GenBank accession number AY601668) (Miao *et al*., 2002; Liu *et al*., 2004). A fusion *proBA* gene was constructed by removing the stop codon of *proB* gene and replacing it with a restriction enzyme site to serve as a linker between the mutated *proB* and *proA* genes, which expressed an artificial bifunctional enzyme, γ-glutamyl kinase/γ-glutamyl phosphate reductase (γ-GK/γ-GPR) (Liu *et al*., 2005).

In this work, we attempt to introduce the wild-type, mutant, and fusion *proBA* genes derived from *B. subtilis* into *Arabidopsis thaliana* Another goal for this research was to establish the expression of *proBA* in *Arabidopsis*.

Materials and Methods

Plant material and growth conditions. Sterilized seeds of *Arabidopsis* ecotype Columbia (Col-0) were used for all transformation experiments and subsequent tissue analysis. Plants were either grown in vermiculite in an environmentally controlled green house at 24°C with 16/8 h of light/dark cycle and 80% humidity or aseptically on germination in medium (half strength Murashige-Skoog salts pH 5.7 with KOH, 0.8% agar) under continuous light at 24°C.

Isolation of *B. subtilis proBA* genes and plasmid construction. The wild-type and mutant *proBA* genes were cloned from the wild-type strain *B. subtilis* 93151 and mutant strain *B. subtilis* 93151-14, respectively (Miao *et al*., 2002). The fusion *proBA* gene was constructed by removing the stop codon of *proB* gene and replacing it with a restriction enzyme site to serve as a linker between the mutant *proB* and *proA* genes (Liu *et al*., 2005). These sequences were subcloned into pBE2 and the positive constructs were named as pBE2/*proBA*/W, pBE2/*proBA*/M and pBE2/*proBA*/MF respectively. The wild-type and mutant *proBA* genes were amplified by PCR from pBE2/*proBA*/W, pBE2/*proBA*/M with primers P1 (5'-ggctctagaacggagg agaaactat gaaa-3', the underlining indicates the positions of *Xba*I) and P2 (5'-ggcgagctcatcgtcaatctccccgcaca-3', the underlining indicates the positions of *Sac*I). The *proBA* fusion gene were amplified by PCR from pBE2/*proBA*/MF with primers P3 (5'-ggcggatccgaaactatgaaaaag-3', the underlining indicates the positions of *BamH*I) and P2. The wild-type and mutant *proBA* genes were ligated into the *Xba*I/*Sac*I sites and the fusion *proBA* gene were ligated into the *BamH*I/*Sac*I sites of the binary vector pMD1 which was promoted by the cauliflower mosaic virus 35S (CaMV35S) promoter and terminated by nopaline synthase terminator. The constructs were transformed into *E. coli* DH5α and the positive colonies were further characterized by the restriction enzyme analysis and PCR analysis. The resulting recombinant plasmids named pMD1-*proBA*/W, pMD1-*proBA*/M and pMD1-*proBA*/MF, respectively. These recombinant plasmids and the control plasmid

pMD1 were transformed into *Agrobacterium tumefaciens* strain LBA4404. The positive colonies were characterized by PCR analysis.

Plant transformation. *Arabidopsis* plants were transformed using a modified vacuum infiltration method (Bechtold *et al*., 1993). *Arabidopsis* seeds produced by the first generation of transformed plants were harvested and selected on half strength Murashige-Skoog salts medium containing 50 mg/l kanamycin. The 10-day-old seedlings can be different on the 1/2 MS medium containing 50 mg/l kanamycin. The resulting kanamycin-resistant plants (T1) were transferred to soil in pots and grown in chambers at 24 with 16/8 h of light/dark cycle and 80% humidity for the production of seeds (T2).

PCR analysis of transgenic plants. Transgenic *Arabidopsis* seeds (T2) were germinated on MS medium containing Kanamycium for ten days then transferred to soil in pots. The total DNA was extracted from the leaves of transgenic *Arabidopsis* seedlings of T2 generation. Leaf extracts were analyzed by PCR for the presence of the *proBA* genes or *proBA* fusion gene using the primer P1 and P2. The positive transgenic lines were grown for the production of seeds (T3).

Northern-blot analyses. Total RNA isolated from the transgenic and control *Arabidopsis* seedlings was electrophoresed, blotted, and hybridized with the DNA fragment corresponding to the *proB* gene as a radioactive probe. Hybridization and washing of the filters were preformed by the method of Sambrook's (Sambrook *et al*., 1989).

Germination under salt stress. 200 µl sterilized seeds of wild type and T3 transgenic *Arabidopsis* were cultured in MS medium with 0 and 300 mM NaCl on a glass plate for 10 days after 48 h at 4°C.

Salt treatment on *Arabidopsis*. The seeds of wild type and T3 transgenic *Arabidopsis* were germinated in vermiculite in small pots with holes in the bottom (4 plants per pot). The pots were kept in flat-bottomed trays. The seedlings were grown for 4-5 weeks. For salt stress, 4-week-old plants were watered with equal volume Hoagland solution containing 200 mM NaCl for 20 days, and the NaCl solution was added every 2 days to maintain the constant concentration of NaCl in the vermiculite. The stressed plants were re-supplied with water to allow them recover and grow without stress.

Measurement of proline contents. Leaves of wild type and T3 transgenic *Arabidopsis* were used for determination of proline content as described previously (Bates *et al*, 1973). Precaution was taken to select leaves of similar age and size. About 0.5 g of leaf tissue collected from normal and stressed leaves was extracted overnight in 1 ml 3% (w/v) aqueous 5-sulphosalicylic acid. Precipitated protein and other debris were removed by centrifugation at 8,000 g for 10 min. Acid ninhydrin was prepared by dissolving 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6M H_3PO_4. The cellular extract was mixed with 0.5 ml acid ninhydrin and 0.5 ml glacial acetic acid. The mixture reacted in 100°C for 1 h. Samples were cooled and mixed vigorously with 2 ml toluene. The absorbance of samples was read at 520 nm against a toluene blank. Values of proline contents were taken from measurements of three independent samples.

Extract preparation and enzyme assay. 0.5 L bacterial cultures of LB broth containing 80 µg ml^{-1} of ampicillin were grown at 37°C with shaking until mid-log phase. Cultures were harvested by centrifugation, washed in TD buffer (50 mM Tris, 1 mM dithiothreitol, pH 7.2). Cell-free extracts were prepared by resuspending cell pellets in TD buffer followed by sonication. Cellular debris was removed by centrifugation at 14,000 g for 20 min at 4°C. The activities of GK and GPR in crude bacterial homogenates were determined using the coupled reaction (Smith *et al*., 1984). The buffer contained 100 mM Tris-HCl pH 7.2, 50 mM glutamate, 0.15 mM NAPDH, 1 mM $MgCl_2$, 5 mM ATP and 1 mM DTT. The oxidation of NAPDH was monitored at 340 nm. One unit of GK/GPR corresponds to the oxidation of 1 nmole NAPDH per minute at room temperature. Protein concentrations were determined according to Bradford using bovine serum albumin as standard (Bradford, 1976).

Results

Production of transgenic plants. The genes derived from *B. subtilis*: the wild-type *proBA* genes, the mutant *proBA* genes, and the fusion *proBA* gene respectively were transformed into *Arabidopsis* using a modified vacuum infiltration method. Selected on half strength MS salts with 50 mg/l kanamycin, about 0.2% seedlings of the total were proved antibiotic-resistant lines. To confirm their transgenic nature, PCR amplifications were performed. Among the antibiotic-resistant lines, 23 lines carrying the wild-type *proBA* genes, 27 lines carrying the mutant *proBA* genes and 49 lines carrying the fusion *proBA* gene were PCR+ (Fig. 2). The transgenic lines carrying the wild-type *proBA* genes, the mutant *proBA* genes and the fusion *proBA* gene respectively named as At*proBA*/w, At*proBA*/M and At*proBA*/MF, the control transgenic lines carrying the empty vector pMD1 named AtpMD1.

Northern blot analyses of transgenic *Arabidopsis*. The *proBA* genes transcriptional levels of transgenic *Arabidopsis*

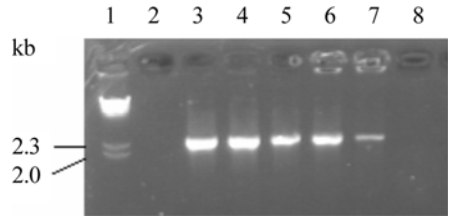

Fig. 2. PCR analysis confirming the presence of gene into *Arabidopsis* genome Lane 1: λDNA/*HindIII* marker; Lane 2, wild-type *Arabidopsis* plant; Lanes3, 4: transformants carry the wild-type *proBA* genes of *B. subtilis*; Lanes 5, 6: transformants carry the mutant *proBA* genes of *B. subtilis*; Lanes 7: transformants carry the fusion *proBA* gene of *B. subtilis*; Lane 8: pMD1 plant.

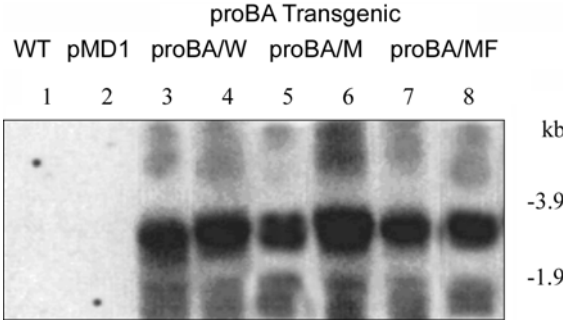

Fig. 3. Expression of *proBA* genes in transgenic *Arabidopsis* plants. Northern blot of total RNA from wild-type (WT) and pMD1 plants (lanes 1 and 2) and the *proBA* transgenic lines: At*proBA*/w (Lanes 3-4), At*proBA*/M (Lanes 5-6) and At*proBA*/MF (Lanes 7-8).

lines were analyzed by the Northern blot. The results showed that the *proBA* genes constitutively expressed as expected in transgenic *Arabidopsis* (Fig. 3).

Germination under salt stress. We examined the effects of salt stress on the germination of seeds from the wild type and the *proBA*-transgenic plants (Fig. 4). After five days, 100% germination was observed in the seeds from wild-type as well as transgenic plants in the presence of 50 mM NaCl or in its absence (Fig. 4A). At 200 mM NaCl, more than 50% of the transformed plants seeds germinated, whereas only 30% of the seeds of the wild-type plant did so. At 250 mM NaCl, seeds of the wild-type plant did not germinate at all, whereas 10% of seeds of At*proBA*/w, 30% of At*proBA*/M and 40% of At*proBA*/MF transgenic plants germinated. These findings indicate that the transformed plants had enhanced ability to germinate under salt-stress conditions.

In the *proBA*-transgenic plants, the transgenic lines carrying the mutant *proBA* genes germinated faster than the wild-type *proBA*-transgenic lines in 100-300 mmol NaCl (Fig. 4B). At 300 mM NaCl, some of At*proBA*/M and At*proBA*/MF transgenic plants germinated whereas no At*proBA*/w transgenic lines did so. It seems that the mutation in the *proBA* genes confer transgenic plants tolerance to salt-stress in some extent.

The fusion *proBA*-transgenic lines showed the best ability to germinate under salt-stress: its percent of germination was the highest in all kinds of NaCl contents (Fig. 4).

Growth under salt stress. Fig. 5 shows the effects of salt stress on the growth of seedlings after germination. Seeds of the wild-type plant and the T3 seeds of the fusion *proBA*-transgenic plants were allowed to germinate on solidified Murashige and Skoog's medium supplemented with 100 mM NaCl, and roots and cotyledons were allowed to develop for a further 20 days. The growth of the wild-type plants was weak and the leaves turned white after 10 days. By contrast, the transformed plants remained good growth. One hundred lines of At*proBA*/MF transgenic plants and the wild-type plants were tested to assess

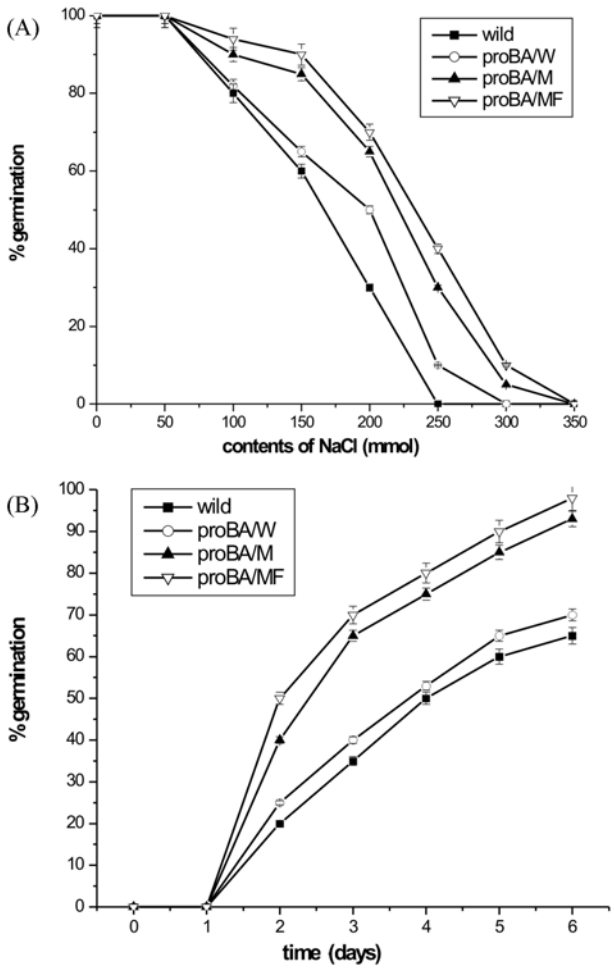

Fig. 4. The effects of salt stress on the germination of seedlings wild-type and transformed plant. Seeds from wild-type plants and T3 seeds from *proBA*-transformed plants were germinated on plates of 1/2 MS medium that had been supplemented with NaCl (from 0 to 350 mM) after incubation at 4°C for 2 days. Results are means ± S.E. (A) The percent of germination of wild-type and transformed plants after five days under several of NaCl content. (B) The effects of germination of seedlings wild-type and transformed plant under 150 mM NaCl-stress.

the development of the roots. The development of the roots of the transformed plants was markedly more efficient than that of the wild-type plants. The root length of At*proBA*/MF transgenic plants was 2 to 3-fold longer than that of the wild-type plants (Fig. 5B). Uniform growth rates for all of wild type and transformed plants were observed in the absence of NaCl. The rates of growth in the presence of NaCl were lower than those in the absence of NaCl. At 200 mM NaCl, the transformed plants grew slowly, whereas the wild-type plants did not grow at all. At 250 mM NaCl, no growth was observed in both transformed and wild-type plants.

Tolerance of mature plants to salt stress. To determine the tolerance of mature plants to salt stress, we measured the dry

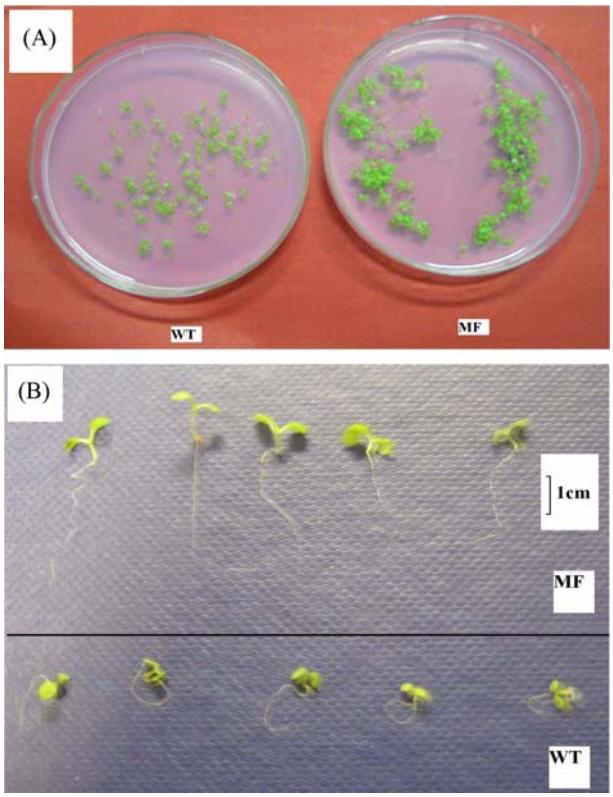

Fig. 5. Effects of salt stress on the growth after germination of wild-type and transformed plant. Seeds from wild-type plants and T3 seeds from At*proBA*/MF transformed plants were germinated on plates of MS medium that had been supplemented with 100 mM NaCl. After incubation at 4°C for 2 days, the plates were kept in the vertical position at 24°C for 6 days under continuous light. (A) Effects of the growth on the plates (B) Effects of the development of the roots.

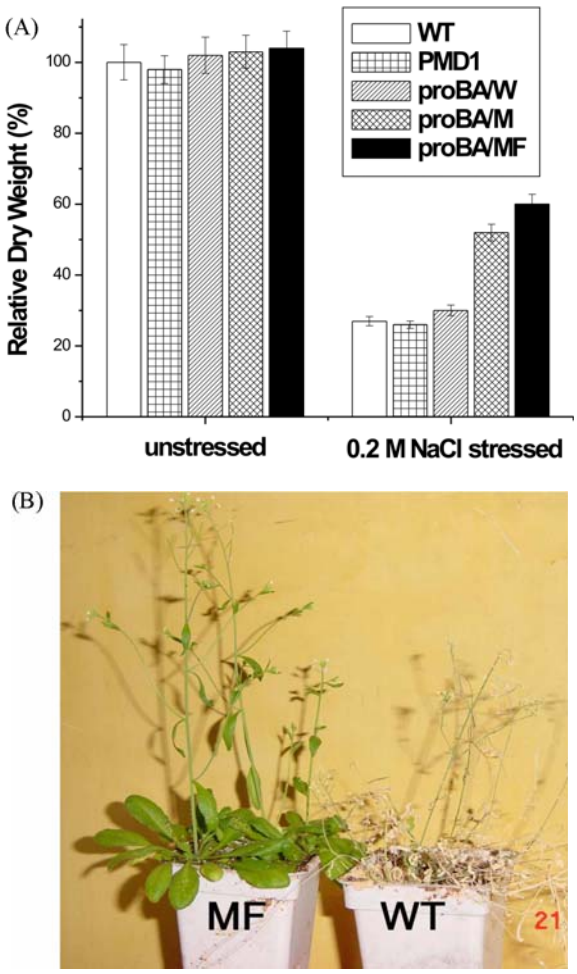

Fig. 6. Effects of salt stress on mature plants of wild-type and the fusion *proBA*-transgenic plants. 4-week-old plants were watered with equal volume Hoagland solution containing 200 mM NaCl for 20 days, and the NaCl solution was added every 2 days to maintain the constant concentration of NaCl in the vermiculite. (A) Comparison of the wild-type, pMD1, and *proBA*-transgenic plants at normal and 0.2 M NaCl salt-stressed conditions. 30 seedlings were dried and weighed. Data are means ± S.D. (B) Growth performance of wild-type and the fusion *proBA*-transgenic plants at normal and 0.2 M NaCl salt-stressed conditions.

weight of control and transgenic plants under normal and 200 mM NaCl stressed conditions. Control and transgenic plants did not differ when grown under normal conditions (Fig. 6A). However, after 10 days streess, the control plants (the wild-type *Arabidopsis* plants and pMD1 transgenic plants) and the wild-type *proBA*-transgenic plants started to wilt and the leaves began to turn yellow, whereas the mutant *proBA*-transgenic plants and the fusion *proBA*-transgenic plants still showed healthy growth. After 20 days of NaCl stress, the control plants died away, whereas the mutant *proBA*-transgenic plants and the fusion *proBA*-transgenic plants started to seed (Fig. 6B). At last, 80% of 50 lines At*proBA*/M and 90% of 70 lines At*proBA*/MF could adapt and complete their life cycle under 200 mM NaCl stress. Compared to control plants, At*proBA*/M plants had 1.9-fold greater biomass and At*proBA*/MF plants had 2.2-fold greater biomass (Fig. 6A). Significant difference between control and *proBA*-transgenic plants (the mutant and the fusion) in growth under salt-stressed conditions demonstrated introduction the mutant *proBA* genes in plants may lead to increased tolerance to drought and salinity stress.

Free proline level in transgenic plants. Proline content in the wild-type *Arabidopsis* and the transgenic lines including AtpMD1, At*proBA*/w, At*proBA*/M and At*proBA*/MF respectively was determined at normal and salt-stressed conditions. We measured the free proline contents of new leaf cells of 5-week-old plants. Proline levels were increased in both control and transgenic plants when it was imposed 200 mM NaCl stress (Fig. 7). This values increased from 185 μg g^{-1} fresh weight (before stress) to about 3000 μg g^{-1} fresh weight (after stress) in control (wild-type and pMD1) plants, from 315 μg g^{-1} to about 3423 μg g^{-1} in the wild-type *proBA*-transgenic lines, from 315 μg g^{-1} to about 5876 μg g^{-1} in the mutant *proBA*-transgenic lines, and from about 300 μg g^{-1} to an

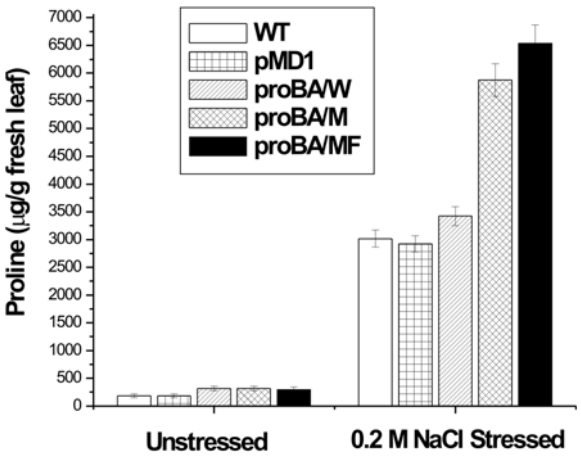

Fig. 7. Proline content in leaves of the wild-type and pMD1, At*proBA*/w, At*proBA*/M and At*proBA*/MF transgenic plants at normal and 0.2 M NaCl salt-stressed conditions. Data are mean and S.E.

average of 6543 μg g^{-1} in the fusion *proBA*-transgenic lines.

Without stress, proline content was approximately 2- fold greater in transgenic lines than in control plants. After stress, no significant differences in proline content were observed in the wild-type *proBA*-transgenic lines when compared with the control (wild-type and pMD1) plants. However, the level of free proline was 2 to 2.5-fold greater in the mutant *proBA*-transgenic lines and the fusion *proBA*-transgenic lines than in control plants after stress. Proline content in the fusion *proBA*-transgenic lines was greater than in the mutant *proBA*-transgenic lines after 200 mM NaCl stress.

Effect of *proBA* mutation and fusion on the activity of GK/GPR. To assess the effect of *proBA* mutation and fusion on the activity of GK/GPR, the wild-type, mutant, and fusion *proBA* gene respectively were ligated into vector pBE2 and transformed into *E.coli* JM83 (Δ*proBA*) (Fiedler and Skerra 2001). Cell homogenates of above transformants were analyzed for the activities and the response to feedback inhibition of GK/GPR. The wild-type GK/GPR produced by the plasmid pBE2/*proBA*-W is very sensitive to proline-mediated feedback inhibition (Fig. 8). However, the mutant GK/GPR was about 30-fold less sensitive to proline inhibition than the wild-type enzyme. Fifty percent inhibition of the wild-type enzyme was observed in the presence of 7.5×10^{-6} M proline, whereas 2.5×10^{-4} M proline was required to inhibit 50% of the mutant enzyme activity. Furthermore, the fused GK/GPR was about 2-fold more active than the separate GK and GPR.

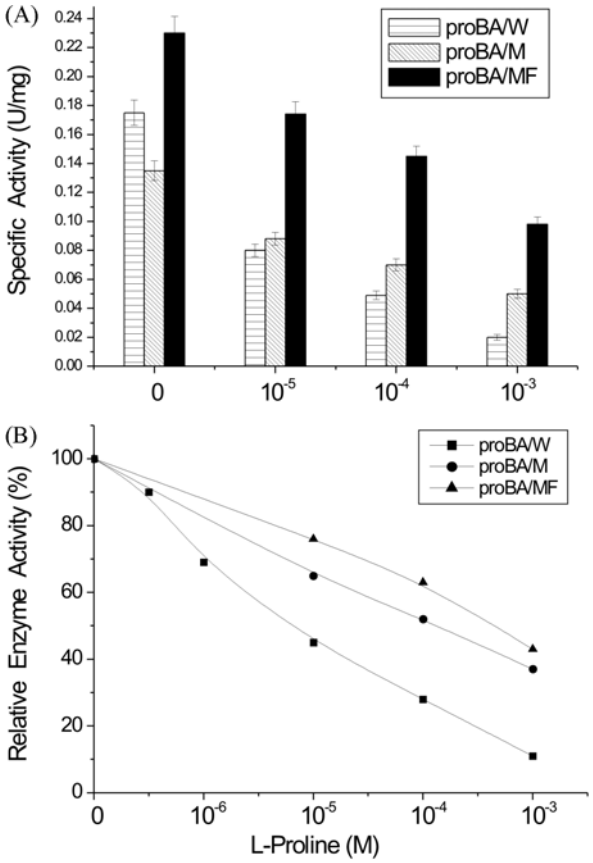

Fig. 8. Effect of *proBA* mutation and fusion on the specific activity of GK/GPR in the presence or absence of L-proline. GK/GPR activities of JM83/ *proBA*-W, JM83/ *proBA*-M, JM83/ *proBA*-MF were measured by the stand coupled assay. Samples assayed in the absence and presence of L-proline as indicated, respectively. Data are means of three independent determinations. (A) Effect of the *proBA* mutation and fusion on the specific activity of GK/GPR in the presence or absence of L-proline. Results are means ± S.E. (B) Effect of L-proline on the relative activity of GK/GPR.

Discussion

There are conflicting evidence about proline role and the relevance of its accumulation in the maintenance of yield. In many report, osmolyte accumulation has been proposed as an effective tolerance mechanism for water deficits and salt tolerance (Tarczynski *et al*., 1993; Nomura *et al*., 1995; Pilon-Smits *et al*., 1995; Shen *et al*., 1997; Huang *et al*., 2000; Garg *et al*., 2002), which could be enhanced in crops (Kavi Kishor *et al*., 1995). Others have suggested that osmolyte accumulation and crop yield have no consistent benefit, with probably no link with osmotic adjustment (Blum *et al*., 1996; Hare *et al*., 1998; Serraj and Sinclair, 2002). In this work, the development of the roots of the transformed plants was markedly more efficient than that of the wild-type plants in the presence of 100 mM NaCl (Fig. 5B). The mutant *proBA*-transgenic plants and the fusion *proBA*-transgenic plants could adapt and complete their life cycle under 200 mM NaCl stress. These findings suggested that proline accumulation have positive effect on osmotolerance and root length under salt stress. The

transgenic plants produced higher level of free proline than control suggested that overexpression of *proBA* genes leads to proline accumulation in the transgenic lines and *proBA* genes derived from *B. subtilis* could perform function as P5CS in *Arabidopsis*.

In *B. subtilis* 93151, the *proB* and *proA* constitute an operon and are suggested to be overlapped by 4 nt. When the wild-type and mutant *proBA* genes were introduced into *Arabidopsis thaliana*, the transcripts of *proBA* genes might be readthrough and express a fusion protein as P5CS in transgenic plants At*proBA*/w and A*tproBA*/M, because the expression system of plants could not recognize the promoter and SD sequence ahead of *proA* gene.

Proline biosynthesis in many bacteria is frequently regulated through feedback inhibition of the first biosynthetic enzyme (γ-GK). The intracellular concentration of proline is raised to over 1 M under truly hypertonic growth conditions (Kempf and Bremer, 1998). Scince the γ-GK of Gram-negative organisms is inhibited by relatively low proline concentrations, it was thought that the regulation of salt tolerant Gram-positive bacteria should be somewhat different in proline-producing (Whatmore *et al.*, 1990). In this work, proline was found to inhibition both the mutant and wild-type γ-GK/γ-GPR, but a 30-fold greater concentration of proline was required to inhibition the mutant enzyme than was required to inhibit the wild-type enzyme. Thus we presumed that γ-GK enzyme in *B. subtilis* also is subject to feedback inhibition by proline. In addition to *proBA* pathway, recent studies revealed an additional proline biosynthetic pathway: *proHJ*, which is responsible for the high-level accumulation of proline under hyper-osmotic growth conditions (Wood *et al*., 2001).

For *proBA*-transgenic plants, reduction of feedback inhibition in the mutant *proBA*-transgenic lines resulted in a 2-fold increase in proline content compared with that in the wild-type *proBA*-transgenic lines. This difference also show that feedback regulation of the wild-type γ-GK in *proBA*-transgenic lines is not completely eliminated under stress, because the wild-type *proBA*-transgenic lines and the mutant *proBA*-transgenic lines should accumulate uniform proline content if the wild-type γ-GK completely lose its feedback regulation property.

To obtain an expected γ-GK/γ-GPR enzyme complex that may function as bifunctional enzyme (P5CS), the fusion *proBA* gene was constructed by fusing the mutated *proB* and *proA* genes. The fused GK/GPR was about 2-fold more active than the separate GK and GPR.

In this test, expression of the fused *proBA* rendered transgenic plant accumulation more proline at 200 mM NaCl stress and more salt-tolerance than expression of the separate mutant *proBA*. Thus, in addition to the mutation of *proB* confers the transformants less sensitivity to the end -product inhibition, the fusion of *proB* and *proA* also contributed to the overproduction of proline. One plausible explanation for the increased proline content is that the fusion *proBA* gene should be more propitious to express a fusion protein than the wild-type *proBA* in transgenic plants. The artificial bifunctional enzyme was propitious to ensure closeness between the enzyme moieties and increase substrate transfer taken place between GK and GRP. Such proximity reduces the breakdown of the labile γ-glutamyl-phosphate which thereby increases the overall proline production.

Acknowledgments This work was supported by the Innovation Fund in Wuhan University of P. R. China.

References

Adams, E. and Frank, L. (1980) Metabolism of proline and the hydroxyprolines. *Annu. Rev. Biochem*. **49**, 1005-1061.

Bates, L. S., Waldren, R. P. and Teeare, I. D. (1973) Rapid determination of free proline for water-stress studies. *Plant Soil* **39**, 205-207.

Bechtold, N., Ellis, J. and Lelletier, G. (1993) In planta Agrobacterium mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C. R Acad. Sci. Paris Life Sci.* **316**, 1194-1199.

Blum, A. (1996) Crop responses to drought and the interpretation of adaptation. *Plant Growth Regulation* **20**, 135-148.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **72**, 248-254.

Csonka, L. N. (1981) Proline over-production results in enhanced osmotolerance in *Salmonella typhimurium*. *Mol. Gen. Genet.* **182**, 82-86.

Csonka, L. N., .Gelvin, S. B, Goodner, B. W., Orser, C. S., Siemieniak, D. and Slightom, J. L. (1988) Nucleotide sequence of a mutation in the *proB* gene of Escherichia coli that confers proline overproduction and enhanced tolerance to osmotic stress. *Gene* **64**, 199-205.

Dandekar, A. M. and Uratsu, S. L. (1988) A single base pair change in proline biosynthesis genes causes osmotic stress tolerance. *J. Bacteriol*. **170**, 5943-5945.

Fiedler, M. and Skerra, A. (2001) *proBA* complementation of an auxotrophic *E. coli* strain improves plasmid stability and expression yield during fermented production of a recombinant antibody fragment. *Gene* **274**, 111-118.

Garg, A. K., Kim, J. K., Owens, T. G., Ranwala, A. P., Choi, Y. D., Kochian, L. V. and Wu, R. (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proc. Natl. Acad. Sci. USA* **99**, 15898-15903.

Hare, P. D., Cress, W. A. and Van Staden, J. (1998) Dissecting the roles of osmolyte accumulation during stress. *Plant, Cell and Environment* **21**, 535-553.

Hong, Z., Lakkineni, K., Zhang, H. and Verma D. P. S. (2000) Removal of feedback inhibition of D1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. *Plant Physiol.* **122**, 1129-1136.

Hu, C. A., Delauney, A. J. and Verma, D. P. (1992) A bifunctional enzyme (D1-pyrroline-5-carboxylate synthetase) catalyses the first two steps in proline biosynthesis in plants. *Proc. Natl. Acad. Sci. USA* **89**, 9354-9358.

Huang, J., Hirji, R., Adam, L., Rozwadowski, K. L., Hammerlind, J. K., Keller, W. A. and Selvaraj, G. (2000) Genetic engineering of glycinebetaine production toward enhancing stress tolerance in plants: metabolic limitations. *Plant Physiol.* **122**, 747-756.

Kavi Kishor, P. B., Hong, Z., Miao, G., Hu, C. A. and Verma, D. P. S. (1995) Overexpression of D 1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol.* **108**, 1387-1394.

Kempf, B. and Bremer, E. (1998) Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Arch. Microbiol.* **170**, 319-330.

Liu, R. J., Cao, J. W. and Miao, L. X. (2004) *proA* gene cloning and function analysis of *proBA* gene in osmoregulation from a salt-tolerant mutant of *Bacillus subtilis*. *Acta. Microbiol. Sinica.* **44**, 452-456.

Liu, R. J., Chen, T. and Cao, J. W. (2005) Construction of fused osmoregulation proBA gene from a salt-tolerant mutant of *Bacillus subtilis* and its influence on the osmotolerance of *E. coli*. *Acta Microbiol. Sinica*. **45**, 23-26.

Miao, L. X., Cao, J. W., Liu, R. J., Wang, Y. L. and Zeng, Y. H. (2002) Cloning and sequencing of *proBA* gene from the selected mutant resistant to proline analogue from *Bacillus subtilis*. *Acta. Genetica. Sinica*. **29**, 1111-1117.

Nomura, M., Ishitani, M., Takabe, T., Rai, A. K. and Takabe, T. (1995) Synechococcus sp. PPC 7942 transformed with *E. coli* bet genes produces glycine betaine from choline and acquires resistance to salt stress. *Plant Physiol*. **107,** 703-708.

Pilon-Smits, E. A. H., Ebskamp, M. J. M., Paul, M. J., Jeuken, M. J. W., Weisbeek, P. J. and Smeekens, S. C. M. (1995) Improved performance of transgenic fructan-accumulating tobacco under drought stress. *Plant Physiol.* **107**, 125-130.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York, USA.

Serraj, R. and Sinclair, T. R. (2002) Osmolyte accumulation: can it really help increase crop yield under drought conditions? *Plant, Cell and Environment* **25**, 333-341

Shen, B., Jensen, R. G. and Bohnert, H. J. (1997) Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. *Plant Physiol.* **113**, 1177-1183.

Sleator, R. D., Gahan, C. G. M. and Hill, C. (2001) Mutations in the Listerial *proB* gene leading to proline overproduction: effects on salt tolerance and murine infection. *Appl. Environ. Microbiol.* **67**, 4560-4565.

Smith, C. J., Deuth, A. H. and Rushlow, K. E. (1984) Purification and characteristics of a γ-glutamyl kinase involved in *Escherichia coli* proline biosynthesis. *J. Bacteriol.* **157**, 545-551.

Su, J., Shen, Q., Ho, T. H. D. and Wu, R. (1998) Dehydration-stress regulated transgene expression in stably transformed rice plants. *Plant Physiol.* **117**, 913-922.

Tarczynski, M. C., Jensen, R. G. and Bohnert, J. H. (1993) Stress protection of transgenic tobacco by production of the osmolyte Mannitol. *Science* **259**, 508-510.

Whatmore, A. M., Chudek, J. A. and Reed, R. H. (1990) The effects of osmotic upshock on the intracellular solute pools of *Bacillus subtilis*. *J. Gene. Microbiol.* **136**, 2527-2535.

Wood, J. M., Bremer, E., Csonka, L. N., Karaemer, R., Poolman, B., Van der Heide, T. and Smith, L. T. (2001) Osmosensing and osmoregulatory compatible solute accumulation by bacteria. *Com. Biochem. Physiol. Part A* **130**, 437-460.

Zhang, C. S., Lu, Q. and Verma, D. S. P. (1995) Removal of feedback inhibition of delta-1-pyrroline-5-carboxylate synthetase, a bifunctional enzyme catalyzing the first 2 steps of proline biosynthesis in plants. *J. Biol. Chem.* **270**, 20491-20496.