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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

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Chen, Mingqing; Wei, Hongbo; Cao, JunWei; Liu, Ruijie; Wang, Youliang; and Zheng, Congyi, "Expression of Bacillus subtilis proBA Genes and Reduction of Feedback Inhibition of Proline Synthesis Increases Proline Production and Confers Osmotolerance in Transgenic Arabidopsis" (2007). Peer Reviewed Articles. 33. [https://scholarworks.gvsu.edu/bms_articles/33](https://scholarworks.gvsu.edu/bms_articles/33?utm_source=scholarworks.gvsu.edu%2Fbms_articles%2F33&utm_medium=PDF&utm_campaign=PDFCoverPages)

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

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Received 13 November 2006, Accepted 2 January 2007

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 Journal of Biology, the state of Context Case of the Context Case of the Context Case of the Context Case of the State Context Context Case Proline accumulation has been shown to correlate with tolerance to drought and salt stresses in plants. We attempt to
therivate the while-type, mutant, and fusion $probBA$ genes
derived from *Bacillus subilis* into *Arabidopsis thatiana* under
the control of a strong promoter ca tolerance to drought and salt stresses in plants. We attempt to introduce the wild-type, mutant, and fusion *proBA* genes
derived from *Bacillus whithis* into *Archidapsis fudiana* under
the control of a strong promoter cauliflower mosaic virus 35S
(CaMV35S). The transgenic plants pro 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

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resulted in the increased tolerance to osmotic stress in
transgenic plants. Besides, the mutation in $proBA$ thee proline than control and the overproduction of proline
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transgenic plants. Besides, the mutation in *proBA* genees,
which were proved to lead γ -glutamyl kinase (γ -GK)
reduces sensitivity to the end-product inhibiti transgenic plants. Besides, the mutation in *proBA* genes, which were proved to lead γ -glutamyl kinase (γ -GK) ether dues sensitivity to the end-product inhibition and the mediators function and confer osmotolerance where were proved to read r_{reg} ratany in the endouce sensitivity to the end-product inhibition and the fusion of *proB* and *proA* also result in increasing proline production and confer osmotolerance in transgenic l **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
Intro Example 18. Proline, *proBA* gene, Transgenic Arabidops.
Salt stress, Feedback inhibition
Introduction
The accumulation of osmolyte compounds has long be
proposed as an adaptative mechanism for drought and stolerance, i **Keywords:** Proline, *proBA* gene, Transgenic *Arabidopsis*, Salt stress, Feedback inhibition

The accumulation of osmolyte compounds has long been

proposed as an adaptative mechanism for drought and salt

olerance, it h 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)

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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.

JOURNAL

Biochemistry

Molecular Biology

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Sine Key Laboratoy of Visiong, College of Life Sciences, Wuhan University, Wuhan 430022, PR China
 Received 13 Newstehs PAM, *Accepted 2 January 2007*
 and the local only and fluorism of the relation are such as the se 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 ∆1-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 $\Delta 1$ -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, $\Delta 1$ -pyrroline-5-carboxilate synthetase; CaMV35S , cauliflower mosaic virus 35S.

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 proB74 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). **EVALUAT SET THE SET THANGES AND MONUMBER CONDUST THANGES AND MONUMBER (MONUMBER THANGES PROPRET) THOW THE INTERFER (FOR PROPRET) THOW THE INTERFER (FOR PROPRET) THOW THOW THOW THOW THOW THOW THANGES CONDEQ AND A CONDEQUE** bacteria and plants, but the initial two reactions are performed by
the bifunctional PSCS enzyme in plants.

However, it has not been reported that introduced $proB\beta$
genes into plants. It was reported that the monthbean P the bifunctional PSCS enzyme in plants.

However, it has not been reported that introduced *proBA*

ended by and plants. It was respected that the monthbean PSCS

eDNA clones complemented *Excherichia coli proB*, pro*Z* a

In our previous work, B. subtilis 93151 was treated with Nmethy-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

Expression of Determins sionins product substitution (Ser216Thr), also in *proB*, which lead in an amino acid

substitution (Ser216Thr), also in *proB* genes, which resulted

the resisting the consistence of the proB (Thr found mutation sites in *proB*, which lead in an amino acid substitution (Ser216Thr), also in *proA* genes, which resulted three residue changes (Thr150 \rightarrow Ala, Arg408 \rightarrow Lys, Gln412 \rightarrow 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

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.

Arabidopsis ecotype Columbia (Col-0) were used for all
transformation experiments and subsequent tissue analysis. Plants
were either grown in vermiculatie in an environmentally controlled
green house at 24°C with 168 h transformation experiments and subsequent tissue analysis. Plants
transformation experiments and subsequent tissue analysis. Plants
were either grown in vermiculate in an environmentally controlled
blumidity or asspitcial were either grown in vermiculite in an environmentally controlled
green house at 24°C with 168 h of light/dark cycle and 80%
humidity or aseptically on germination in medium (half strength
Murashige-Skoog salts pH 5.7 wit green house at 24°C with 16/8 h of light/dark cycle and 80%
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Isolation of *B. subili*.
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were subcloned into
as pBE2/ ally on germination in medium (half strength
alts pH 5.7 with KOH, 0.8% agar) under
4°C.

dits proBA genes and plasmid construction.
uttant $prob$ B agenes were cloned from the wild-
s 93151 and mutant strain *B. subtilis* 93 Murashige-Skoog salls 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 continuous light at 24°C.
 Isolation of *B. subilils proBA* **genes and plasmid construction.**

The wild-type and mutant *proBA* genes were cloned from the wild-
type strain *B. subililis* 93151 and mutant strain *B. subil* continuous light at 24²
 Isolation of *B. subtili*

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\text$ Isolation of B. subtilis proBA genes and plasmid construction. **Isolation of B. subtilis proBA genes and plasmid construction.**
The wild-type ard mutant *rendA* genes were cloned from the wild-
Type strain B. subtilis 93151 and mutant strain B. subtilis 93151-14,
respectively (Miao The wild-type and mutant *proBA* genes were cloned from the wildtype 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 $proBA$ gene and replacing it with a restriction enzyme site to serve as a linker between the mutant $proBA$ genes OSE and 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 mutatar $probB$ and $probA$ genes (Liu et al , 2005). These sequences
were subcloned into pB 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/*proBAN*, pBE it with a restriction enzyme site to serve as a linker between the mutant *proB* and *prod* 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 wid-type and mutant *p* 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 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'-ggetclagaacggagg agaaactat gaaa-3', as pBE2/proBA/W, pBE2/proBA/M and pBE2/proBA/MF respectively. The wild-type and mutant *proBA* genes were amplified by PCR
The wild-type and mutant *proBA* genes were amplified by PCR
from pBE2/*proBA/W*, pBE2/*proBA/M* with primers P1 (5'-
ggctctagaacegaage agaaactat gaaa-3', the u 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 *Xbal*) and P2 (5'-ggcgagctcategtcaatectccccgcaca-3', the underlining indicates the po from pBE2/proBA/W, pBE2/proBA/M with primers P1 (5'from pBE2/proBA/W, pBE2/proBA/M with primers P1 (5'-ggetdagaacggagg agaaactat gaaa-3', the underlining indicates the positions of Xba1) and P2 (5'-ggegaggetdacegtecacteccegcaca-3', the underlining indicates the positions ggetctagaacggagg agaaactat gaaa-3', the underlining indicates the ggctctagaacggagg agaaactat gaaa-3', the underlining indicates the positions of *Xba*l) and P2 (5'-ggcgaggtcategtcaatctccccgcaca-3', the underlining indicates the positions of *Sach*). The *proBA* fusion general priids by positions of XbaI) and P2 (5'-ggcgageteategteaateteceegeaea-3', the positions of *XbaI*) and P2 (5'-ggcgagctcategtcaatctccccgcaca-3', the underlining indicates the positions of *SacI*). The *proBA* fusion gene were amplified by PCR from pBE2/*proBA/MF* with primers P3 (5'-ggcggatccgaaacta underlining indicates the positions of SacI). The proBA fusion gene underlining indicates the
were amplified by PCR f
ggcggatccgaaactatgaaaaa
of *BamHI*) and P2. The
ligated into the *Xbal/Sa*
ligated into the *BamHI/S*
was promoted by the ca
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constructs were further
 prom pBE2/*proBA*/MF with primers P3 (5'-
g-3', the underlining indicates the positions
wild-type and mutant *proBA* genes were
cl sites and the fusion *proBA* gene were
acl sites of the binary vector pMD1 which
auliflowe were amplified by PCR from pBE2/proBA/MF with primers P3 (5'were amplified by PCR from pBE2/*proBA/MF* with primers P3 (5'-ggcggatccgaacatagaacag-3', the underlining indicates the positions of *BamHI*) and P2. The wild-type and mutant *proBA* genes were ligated into the *XbaUSacl* ggcggatccgaaactatgaaaaag-3', the underlining indicates the positions ggcggatccgaaactatgaaaaag-3', the underlining indicates the of *BamH*) and P2. The wild-type and mutant *proBA* geligated into the *Xbal/SacI* sites and the fusion *proBA* geligated into the *Rbal/SacI* sites of the binary positions were
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*oBA/*MF, plasmid of BamHI) and P2. The wild-type and mutant proBA genes were ligated into the *Xbal/SacI* sites and the fusion $probA$ gene were ligated into the *BamHI/SacI* sites of the binary vector pMD1 which was promoted by the cauliflower mosaic virus 35S (CaMV35S) promoter and terminated by no ligated into the Xbal/SacI sites and the fusion proBA gene were ligated into the *BamHL/SacI* 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 ligated into the BamHI/SacI 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 DH5 α and the positive
colonies were further characterized was promoted by the cauliflower mosaic virus 35S (CaMV35S) promoter and terminated by nopaline synthase terminator. The
constructs were transformed into E. coli DH5 α and the positive
colonies were further characterized by the restriction enzyme
analysis and PCR analysis. The r promoter and terminated by nopaline synthase terminator. The constructs were transformed into *E. coli* DH5 α and the positive colonies were further characterized by the restriction enzyme analysis and PCR analysis. The resulting recombinant plasmids named pMD1*-proBA*/W, pMD1*-p* constructs were transformed into E. coli DH5 α 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 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
discriming that the control plasmid analysis and PCR analysis. The resulting recombinant plasmids analysis and PMD1-proBA/W, pMD1-proBA/M and pMD1-proBA/MF, respectively. These recombinant plasmids and the control plasmid named pMD1-proBA/W, pMD1-proBA/M and pMD1-proBA/MF, named parameters, produced probability, probability, probability and properties and the control plasmid respectively. These recombinant plasmids and the control plasmid respectively. These recombinant plasmids and the control plasmid 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.

pMI) were transformed into Approximation Americans consider the SUM (EIDA-140). The positive columns verte characterized by FCR independent

EThat transformation. Anothelaysis plane were transformed using a LTB both

FTan **Plant transformation.** *Arabidopsis* plants were transformation in single and the second modified secure materials of the first generation of transformation and transformation by the first generation of transformation. T **PCR analysis of transgenic plants. Transgenic christial consists seeds and the systemation of the constraining Kanamycium for the dyst then days then transferred to soil in post. The total DNA was extracted from the leav Norther-blot analyses.** Total RNA isolated from the transgenical
and control *Arabidopsis* seedlings was electrophoresed, botted, and
hybridized with the DNA fragment corresponding to the *proB* gene
as a radioactive pro **Germination under salt stress**. 200 µl sterilized seeds of wild type
and 73 transpenic *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 *A* **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 Measurement of proline contents. Leaves of wild type and T3 **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 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 H3PO4. 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.

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Walues of proline contents were taken from measurements of these

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in the bacterial culture of the model of the sympetrol of the sympetric phase of the sympetrol of the phase control of the phase contro 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, nU 72). Call free where of ampicillin were grown at 37°C with shaking until mid-log phase. Cultures were harvested by 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, $1 \text{ mM } MgCl_2$, $5 \text{ mM } ATP$ and $1 \text{ mM }DTT$. 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).

Production of transgenic plants. The genes derived from B . subtilis: the wild-type *proBA* genes, the mutant *proBA* genes, *Arabidopsis* using a modified vacuum infiltration method. Selected on half strength MS salts with 50 mg/l kanamycin, *Arabidopsis* using a modified vacuum and the fusion *proBA* gene respectively were transformed into
 Arebidopsis using a modified vacuum infiltration method.
 Selected on half strength MS salts with 50 mg/l kanamycin,

about 0.2% seedlings of the total w *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 amibiotic-
resistant lines. To confirm their transgenic n Selected on half strength MS salts with 50 mg/l kanamycin, about 0.2% seedlings of the total were proved antibioticresistant 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 $probBA$ genes and 49 lines carrying the
fusion $probA$ gene were PCR+ (Fig. 2). The transgenic lines
carrying the wild-type $probA$ genes, the mutant carrying the empty vector pMD1 named AtpMD1.

carrying the mutant *proBA* genes and 49 lines carrying the
fusion *proBA* genes were PCR+ (Fig. 2). The transperic lines
carrying the wild-type *proBA* genes, the mutant *proBA* genes
and the fusion *proBA* gene respectiv fusion *proBA* gene were PCR+ (Fig. 2). The transgenic lines
carrying the wild-type *proBA* genes, the mutant *proBA* genes
and the fusion *proBA* genes the mutant *proBA* genes
AtyroBA/M and AtyroBA/MF, the control trans carrying the wild-type *proBA* genes, the mutant *proBA* genes
and the fusion *proBA* gene respectively named as AtproBA/w,
AtproBA/M and AtproBA/MF, the control transgenic lines
carrying the empty vector pMD1 named AtpMD and the fusion *proBA* gene respectively named as Atpro*BA*/w,
Atpro*BA*/M and Atpro*BA*/MF, the control transgenic lines
carrying the empty vector pMD1 named AtpMD1.
Northern blot analyses of transgenic *Arabidopsis*. Th AtproBA/M and AtproBA/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
is $\frac{$ proBA genes transcriptional levels of transgenic Arabidopsis
 $\frac{1}{2}$ 3 4 5 6 7 8
 $\frac{1}{2}$
 $\frac{2}{2}$
 $\frac{2}{2}$
 $\frac{2}{2}$
 $\frac{1}{2}$
 $\frac{$ Fig. 2. PCR analysis confirming the presence of gene into *Arabidopsis* genome Lane 1: λ DNA/*HindIII* marker; Lane 2, wild-type *Arabidopsis* plant; Lanes3, 4: transformants carry the wild-type *proBA* genes of *B. subt* 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.

plants. Northern blot of total RNA from wild-type (WT) and 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 transgenic *Arabidopsis* (Fig. 3).
 Germination under salt stress

salt stress on the germination of

the *proBA*-transgenic plants (F

germination was observed in the

as transgenic plants in the press

absence (Fig. 4 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
destroce (Fig. 4A). At 200 mM NaCl, more than 50% of the seeds from wild-type as well assests g 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 indicate that the transformed plants had enhanced ability to germinate under salt-stress conditions. Fig. 3. Expression of proBA genes in transgenic *Arabidopsis*
plans. Northem blot of total RNA from wild-type (*WT*) and
plans. Northem blot of total RNA from wild-type (*WT*) and
 Δ *proBA* (lanes 3-4), Δ *proBAiM* pMD1 plants (lanes 1 and 2) and the *proBA* transgenic lines:
AtproBA/w (Lanes 3-4), AtproBA/M (Lanes 5-6) and AtproBA/
MF (Lanes 7-8).
MF (Lanes 7-8).
Ilnes were analyzed by the Northern blot. The results showed
that the AtproBA/w (Lanes 3-4), AtproBA/M (Lanes 5-6) and AtproBA/M

MF (Lanes 7-8).

MF (Lanes 7-8).

lines were analyzed by the Northern blot. The results showed

that the *proBA* genese constitutively expressed as expected in

transgenic plants tolerance to salt-stress in some extent.

to germinate under salt-stress: its percent of germination was the highest in all kinds of NaCl contents (Fig. 4).

10% of seeds of AtproBA/w, 30% of AtproBA/M and 40% of seeds of AtproBA/W transperic plants germinated. These findings of proBA-transperic plants are mained at biling to germinate under salt-stress conditions. In the *pro* AtproBA/MF transgenic plants germinated. These findings
indicate that the transformed plants had enhanced ability to
indicate that the transformed plants, the transgenic lines carrying
the mutant *proBA* transgenic plants In the *proBA*-transgenic plants, the transgenic lines carrying
 m mutant *proBA* genes germinated faster than the wild-type
 bBA -transgenic lines in 100-300 mmol NaCl (Fig. 4B). At
 b 0 mM NaCl, some of AtproBA/M an 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 A*tproBAI*M and A*proBBA*M ransgenic lines diplants germinated whereas no A*tp* proBA-transgenic lines in 100-300 mmol NaCl (Fig. 4B). At 300 mM NaCl, some of AtproBA/M and AtproBA/MF transgenic plants germinated whereas no AtproBA/w transgenic lines dioso. It seems that the mutation in the proBA gen 300 mM NaCl, some of At*proBA/*M and A*tproBA/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-str plants germinated whereas no AtproBA/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 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 The fusion *proBA*-transgenic lines showed the best ability
germinate under salt-stress: its percent of germination was
highest in all kinds of NaCl contents (Fig. 4).
owth under salt stress. Fig. 5 shows the effects of Growth under salt stress. Fig. 5 shows the effects of salt stress on the growth of seedlings after germination. Seeds of the wildtype 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 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 AtproBA/MF
transgenic plants and the wild-type plants were tested to assess 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 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 \pm 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 wildtype 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 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. **Fig. 4.** The effects of salt stress on the germination of seedlings wild-type and transformed plant. Seeds from *wold*-transformed plants were germinated on plates of 1/2 MS medium that had been supplemented with NaCl (f and T3 seeds from *proBA*-transformed plants were germinated
on plats of 1/2 MS medium that had been supplemented with
NaCl (from 0 to 350 mM) after incubation at 4⁻⁰C for 2 days.
Results are means \pm S.E. (A) The perc

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 AtproBA/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.

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 plan 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 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 *proB* 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 plant 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 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 plan 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* 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 fold greater biomass and AtproBA/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 d 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 salinit in growth under salt-stressed conditions demonstrated introduction the mutant *proBA* genes in plants may lead to increased tolerance to drought and salinity stress. increased tolerance to drought and salinity stress. Fig. 5. Effects of salt stress on the growth after germination of
via divpore and transformed plant. Seeds from wild-yep a pants
and T3 seeds from AlproBA/MF transformed plants were
exermined on plates of MS medium that h

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 \pm S.D. (B) Growth performance of wild-type and the fusion proBA-transgenic plants at normal and 0.2 M NaCl salt-stressed conditions.

Fig. 6. Effects of salt stress on mature plants of wild-type and the fusion *proBA*-transgenic plants. 4-week-old plants were watered the with equal volume Hoagland solution containing 200 mM NaCl for 20 days, and the N Free proline level in transgenic plants. Proline content in **Free proline level in transgenic plants.** Proline content in
the wild-type Arabidopsis and the transgenic lines including
AtpMD1, AtproBA/w, AtproBA/M and AtproBA/MF respectively
was determined at normal and salt-stresse the wild-type *Arabidopsis* and the transgenic lines including
AtpMD1, AtproBA/w, AtproBA/M and AtproBA/MF respectively
was determined at normal and salt-stressed conditions. We
measured the free proline contents of new l AtpMD1, AtproBA/w, AtproBA/M and AtproBA/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 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 μ g g⁻¹ fresh stress (Fig. 7). This values increased from 185 µg g^{-1}
weight (before stress) to about 3000 µg g^{-1} fresh weight
stress) in control (wild-type and pMD1) plants, from 3 fresh weight (after stress) in control (wild-type and pMD1) plants, from 315 μ g g^{-1} to about 3423 µg g⁻¹ in the wild-type *proBA*-transgenic g^{-1} to about 3423 µg g^{-1}
lines, from 315 µg g^{-1} to
proBA-transgenic lines, in the wild-type *proBA*-transgenic
b about 5876 μ g g⁻¹ in the mutant
and from about 300 μ g g⁻¹ to an lines, from 315 µg g^{-1} to about 5876 µg g^{-1}
proBA-transgenic lines, and from about 300 in the mutant $proBA$ -transgenic lines, and from about 300 µg g⁻¹ to an

AtproBA/w, AtproBA/M and AtproBA/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 control (wild-type and pMD1) plants. However, the level of transgenic lines after 200 mM NaCl stress.

in the fusion *proBA*-transgenic lines.

me content was approximately 2- fol

mes than in control plants. After stress

es in proline content were observed in

mg/D1) plants. However, the level *O*

2.5-fold greater in th the wild-type *proBA*-transgenic lines when compared with the control (wild-type and pMD1) plants. However, the level of the control ferme profestransgenic lines and the fision *proBA*-transgenic lines than in control pla 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 great 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 was greater th 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 activit** 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 **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 ($\Delta probA$) (Fi 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 ($\Delta proBA$) (Fiedler and Skerra
2001). Cell homogenates of above transformants were
analyzed for the activities and the response to fee transformed into *E.coli* JM83 ($\Delta probA$) (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 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 in 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 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 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. 7. Proline content in leaves of the wild-type and pMD1,
RAproBAIw, AlproBAIM and AlproBAIMF transgenic plans at
normal and 0.2 M NaCl salt-stressed conditions. Data are mean
normal and 0.2 M NaCl salt-stressed condit

Discussion

There are conflicting evidence about proline role and the relevance of its accumulation in the maintenance of yield. In

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 \pm S.E. (B) Effect of L-proline on the relative activity of GK/GPR.

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.*, 2002), whic 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 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., 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) 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-ty 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 ha 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 sa 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 Fig. 8. Effect of *proBA* mutation and fusion on the specificativity of GK/GPR in the presence or alsence of L-proline GK/GPR activities of JM83*i proBA*-W, JM83*i proBA*-M, JM83*i proBA*-M, JM83*i proBA*-M, JM83

transgenic plants produced higher level of free proline than

and are suggested to be overlapped by 4 nt. When the wildand express a fusion protein as P5CS in transgenic plants of plants could not recognize the promoter and SD sequence

Imagenic plants moduced higher level of her proline than be more
control signalsed that overcapters in the measure control signalsed that overcapters in the transport
in the transport lines and growth genes corporations a control suggested that overexpression of *proBA* genes leads to
proline accumulation in the transpearie lines and *proBA* genes
derived from *B. subilits* could perform fuction as PSCS in
Arabidopsis.
In *B. subilits* 9 proline accumulation in the transgenic lines and *proBA* genes
derived from *B. subtilis* could perform function as PSCS in
Arabidopsis.
In *B. subtilis* 93151, the *proB* and *proA* constitute an operon
and are suggested derived from *B*. *subtilis* could perform function as P5CS in $Arabidopsis$
In B. *subtilis* 99151, the *proB* and *proA* constitute an operon
and are suggested to be overlapped by 4 nt. When the wild-
type and mutat *proBA* g *Arabidopsis.*
In *B. subti*
and are suggetype and mutual
and are suggetype and mutual
thaliana, the and express
At*proBA*/w
of plants couse ahead of *pro*
regulated threnzyme (γ -C
raised to overagrative organize org In B. subtilis 93151, the *proB* and *proA* constitute an operon
d are suggested to be overlapped by 4 nt. When the wild-
are and mutant *proBA* genes were introduced into *Arabidopsis*
liana, the transcripts of *proBA* type and mutant *proBA* genes were introduced into *Arabidopsis* thaliana, the transcrips of *proBA* genes might be readthrough
and express a fusion protein as PSCS in transgenic plants $\Delta{tprobAB}$ are math to recognize the *thaliana*, the transcripts of *proBA* genes might be readthrough
and express a fusion protocia as PSCS in transgenic plants
AtproBA/w and AtproBA/M, because the expression system
of plants could not recognize the promote AtproBA/w and AtproBA/M, because the expression system
of plants could not recognize the promoter and SD sequence
of plants could not recognize the promoter and SD sequence
ahead of prod gene.
Proline biosynthesis in many ahead of *proA* gene.

Proline biosynth

regulated through fe

enzyme (γ -GK). Th

raised to over 1 M₁

raised to over 1 M₁

(Kempf and Brem

negative organisms

concentrations, it v

tolerant Gram-positi

in prolin 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 Gramnegative 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 proline. γ -GK/ γ -GRP, but a 30-fold greater concentration of proline was required to inhibit 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 studies revealed an additional proline biosynthetic pathway:

that γ-GK enzyme in B. subtilis also is subject to feedback
inhibition by proline. In addition to *proBA* pathway, recently, inclusion
studies revealed an additional proline biosynthetic pathway;
proHJ, which is responsi inhibition by proline. In addition to *proBA* pathway, recent
studies revealed an additional proline biosynthetic pathway:
fored, profil, which is responsible for the high-level accumulation
of proline under hyper-osmotic proHJ, which is responsible for the high-level accumulation
of proline under hyper-osmotic growth conditions (Wood et
of proline under hyper-osmotic growth conditions (Wood et
al., 2001).
For proBA-transgenic plants, redu of proline under hyper-osmotic growth conditions (Wood *et*
L, 2001).
For pro*BA*-transgenic plants, reduction of feedback inhibition
in the mutant *proBA*-transgenic lines resulted in a 2-fold
increase in proline conte *al.*, 2001).
For *prou*
in the muincrease in
type *proB*
feedback r
lines is not feedback r
lines is not wild-type
transgenic the wild-type *proBA* gene
proBA gene
proBA gene
than the sk In this
transgenic stress and
 For *proBA*-transgenic plants, reduction of feedback inhibition
the mutant *proBA*-transgenic lines resulted in a 2-fold
rease in proline content compared with that in the wild-
tree *proBA*-transgenic lines. This differe in the mutant *proBA*-transgenic lines resulted in a 2-fold
increase in proline content compared with that in the wild-
type $prob$ -transgenic lines. This difference also show that
feedback regulation of the wild-type γ -G increase in proline content compared with that in the wildlines is not completely eliminated under stress, because the 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 than the separate GK and GPR.

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 an 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 thould accumulate u 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 -G 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 accum 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-to In this test, expression of the fused $probA$ rendered nsgenic plant accumulation more proline at 200 mM NaCl ess and more salt-tolerance than expression of the separate thant $probA$. Thus, in addition to the mutation of $prob$ a 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 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 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 wildtype *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 t 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.

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