

Overexpression of a novel feedback-desensitized Δ^1 -pyrroline-5-carboxylate synthetase increases proline accumulation and confers salt tolerance in transgenic *Nicotiana plumbaginifolia*

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Abstract Δ^1 -Pyrroline-5-carboxylate synthetase (P5CS) is a key enzyme of proline biosynthesis in plants. The *Nicotiana plumbaginifolia* mutant RNA was previously reported as salt tolerant, overproducing proline, and having reduced feedback inhibition of proline biosynthesis. A mutation in an RNA P5CS gene changing a conserved glutamate codon at position 155 into a glycine codon (E155G) is a possible explanation for the phenotypes in the RNA mutant. In this work, the E155G mutation was introduced in the *Arabidopsis thaliana* P5CS1 cDNA driven by the CaMV 35S promoter and expressed in wild type *N. plumbaginifolia* (P2) and in the RNA mutant. The E155G substitution decreased feedback inhibition of P5CS by proline in transgenic lines. Under normal conditions, proline content and growth were comparable in the transgenic lines and the P2 control. In contrast, when plants were exposed to salt stress, proline levels in all transgenic lines increased compared to P2. Growth rate was also less affected by salt treatment in the transgenic lines than in the controls. Semi-quantitative RT-PCR showed higher p35S-P5CS transgene derived mRNA levels in plants submitted to salt stress than in non-stressed plants. To evaluate the role of the CaMV 35S promoter

during salt stress, *N. plumbaginifolia* lines harbouring a p35S-GUS cassette were investigated. Increased GUS activities were observed when submitting leaf discs of transformants to salt stress compared to normal conditions. This result can partly explain increased proline content in the p35S-P5CS transgenic plants when exposed to salt stress.

Keywords Δ^1 -Pyrroline-5-carboxylate synthetase · Proline metabolism · Salt tolerance · CaMV 35S promoter

Introduction

Osmotic stress caused by drought or salt is a major limiting factor for plant productivity in agriculture. Salinity represents the major threat when permanent irrigation with salt containing water is used in agriculture, particularly in arid and semiarid regions of the world (Jury and Vaux 2005). In many organisms including plants, osmotic stress induces rapid accumulation of proline. Proline was shown to play a pivotal role in osmotic stress tolerance (Kavi Kishor et al. 1995; Székely et al. 2008; Szabados and Saviouré 2010; Kavi Kishor and Sreenivasulu 2014). Studies on proline synthesis and catabolism have indicated a variety of functions for this amino acid such as a ROS scavenger, a regulator of protein synthesis and the cell cycle or a source of energy, nitrogen or carbon, but its main role is to act as an osmolyte in response to dehydration, salt stress or freezing (Hare and Cress 1997; Kavi Kishor et al. 2005; Verbruggen and Hermans 2008; Trovato et al. 2008; Szabados and Saviouré 2010; Wang et al. 2014). Biosynthesis and catabolism of proline in plants are highly regulated processes (Verbruggen and Hermans 2008; Szabados and Saviouré 2010). Proline in plants is synthesized via two

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pathways from either glutamate or ornithine (Delauney and Verma 1993). It has been demonstrated that the glutamate pathway is predominant under conditions of osmotic stress (Delauney et al. 1993; Székely et al. 2008). On the other hand, the ornithine pathway can play an important role, for example in young *A. thaliana* plantlets (Roosens et al. 1998) and may contribute to increased proline content and enhanced plant stress tolerance (Roosens et al. 2002). However, *A. thaliana* deletion mutants affecting the function of ornithine- δ -aminotransferase (OAT), a key enzyme of the ornithine pathway, still accumulate proline under stress which indicates that this biosynthetic pathway is not predominant in such conditions (Funck et al. 2008).

In the glutamate pathway, the two first steps of the proline biosynthesis are catalysed by Δ^1 -pyrroline-5-carboxylate synthetase (P5CS), a bifunctional enzyme exhibiting activities for γ -glutamyl kinase (γ -GK) and glutamate-5-semialdehyde (GSA) dehydrogenase. GSA spontaneously cyclizes to Δ^1 -pyrroline-5-carboxylate (P5C), which is then converted to proline by Δ^1 -pyrroline-5-carboxylate reductase (P5CR) (Hu et al. 1992; Delauney and Verma 1993). In bacteria, the pathway of proline biosynthesis has been shown to be regulated by its end product, proline. γ -GK is sensitive to feedback inhibition by proline (Csonka 1989). Similarly, in plants, P5CS is feedback inhibited by proline (Hu et al. 1992) and consequently this step is limiting for proline biosynthesis. Hong et al. (2000) suggested that under stress conditions, proline accumulation in plants might involve the partial loss of feedback regulation of P5CS due to conformational changes around the proline feedback interaction site.

Transgenic tobacco plants overexpressing *Vigna aconitifolia* wild type P5CS were shown to overproduce proline which led to enhanced salt stress tolerance and increases in biomass and seed production of these transgenic tobacco plants when subjected to salt treatment (Kavi Kishor et al. 1995). Zhang et al. (1995), constructed mutated forms of the *V. aconitifolia* P5CS gene encoding enzymes with reduced feedback inhibition; substitution of the Phe-129, situated in the allosteric, site by Ala (P5CSF129A) resulted in a feedback insensitive P5CS (Zhang et al. 1995). Proline levels were subsequently measured in transgenic tobacco plants expressing either a wild-type form of the *V. aconitifolia* P5CS or the P5CSF129A mutated form under the control of the CaMV 35S promoter (Hong et al. 2000). In the absence of stress, transgenic plants expressing P5CSF129A accumulated about twofold more proline than the plants expressing *V. aconitifolia* wild-type P5CS. This difference was further increased in plants treated with 200 mM NaCl. These results demonstrated that the feedback regulation of P5CS plays a role in controlling the level of proline in plants under both normal and stress conditions. In addition salt stress tolerance was higher in

the plants overexpressing the P5CSF129A mutated form than in plants overexpressing the wild type *V. aconitifolia* enzyme.

In *Nicotiana plumbaginifolia* a mutant named RNA has been isolated that is salt tolerant and overproduces proline (Sumaryati et al. 1992). This RNA mutant accumulates proline at higher levels in stressed as well as in normal conditions compared to the wild type *N. plumbaginifolia* plant (P2). By using NMR spectroscopy and ^{13}C -labeled glutamate it was shown that proline exerts reduced feedback inhibition on its own biosynthesis in this mutant (Roosens et al. 1999). Comparison of the P5CS gene sequences from P2 and the RNA mutant, revealed a single mutation resulting in the replacement of the GAG glutamate codon at position 155 of the coding sequence by a GGG glycine codon (E155G mutation) (Roosens 1999).

Two P5CS genes with different expression patterns and functions have been identified in *Arabidopsis thaliana*. The P5CS1 gene is expressed in most plant organs and is induced by salt and drought stress (Strizhov et al. 1997). In addition, a knockout mutation in the P5CS1 gene increases sensitivity to salt stress (Székely et al. 2008). P5CS2 transcripts, on the other hand, are mainly detected in dividing cells and as a result of incompatible plant/pathogen interactions (Strizhov et al. 1997; Fabro et al. 2004). P5CS2 mutants display developmental abnormalities and are affected in fertility (Székely et al. 2008; Funck et al. 2012; Mattioli et al. 2012).

In this work, a mutated form of the *A. thaliana* P5CS1 gene in which the Glu₁₅₅ codon was replaced with a Gly codon (*mP5CS*), was placed under control of the CaMV p35S promoter and was introduced in *N. plumbaginifolia* with the goal to increase proline content and salt tolerance. To optimize the proline overproduction, the wild type *N. plumbaginifolia* but also the RNA mutant, which was already characterized as expressing a P5CS gene coding for a feedback insensitive enzyme (Sumaryati et al. 1992; Roosens et al. 1999) were used as recipient genotypes. Transformation of the RNA mutant was initiated in order to estimate physiological limitations in the range of proline overproduction. In addition, we assessed the contribution of the CaMV 35S promoter in the enhanced proline concentration observed under salt stress conditions.

Materials and methods

Construction of the *mP5CS* expression cassette

The E155G mutation was introduced in the *Arabidopsis thaliana* P5CS1 cDNA via PCR modification. An Eco72I-BglII fragment of this modified gene, containing the mutation, was introduced in Eco72I-BglII cut pCAM-P5CS

plasmid. pCAM-P5CS is a pCAMBIA 1300 (www.cambia.org) derivative containing the wild type *A. thaliana P5CS1* cDNA under control of the CaMV enhanced 35S promoter and a nopaline synthase 3' polyadenylation signal, as well as the hygromycin phosphotransferase gene driven by a 35S promoter as selectable marker gene. The change at the mutated site (GAG to GGG) in the final clone was confirmed by sequencing. The resulting plasmid was named pCAM-mP5CS (Supplementary Fig. 1), and then transferred to *A. tumefaciens* strain LBA4404 for subsequent transformation of *N. plumbaginifolia*.

pCaMV 35S:gus vector

pATAG3 (Goossens et al. 1999) is a T-DNA vector harbouring the β -glucuronidase gene (*gus*) under control of the CaMV 35S promoter and nopaline synthase 3' polyadenylation signal. The vector also contains an *nptII* gene driven by the nopaline synthase promoter as selectable marker gene.

Plant transformation and analysis of transgenic plants

Wild type *N. plumbaginifolia* (Viviani), referred to as P2, and the proline overproducing *N. plumbaginifolia* mutant, referred to as RN_a (Sumaryati et al. 1992; Roosens et al. 1999) were both used as plant material for *Agrobacterium*-mediated transformation. *N. plumbaginifolia* leaf discs were infected by submersion for 10 min in an overnight *Agrobacterium* culture diluted tenfold with physiological saline solution (0.9 % of NaCl). Further, the leaf pieces were immersed in MS liquid medium containing 2 mg/l benzyladenine (BA) and 1 mg/l naphthalene acetic acid (NAA) for 3 days at 24 ± 2 °C, under 12 h light and 12 h dark cycles. Leaf discs were then transferred to MS solid medium supplemented with the appropriate antibiotic (25 mg/l hygromycin or 100 mg/l kanamycin), 1 mg/l NAA and 2 mg/l BA for 1 month to select transgenic calli. Resistant calli were transferred several times at the interval of 3 weeks to new MS medium containing 0.2 mg/l NAA and 25 mg/l hygromycin or 100 mg/l kanamycin until shoots developed. Individual shoots were excised and transferred to plastic boxes with MS containing 0.5 mg/l NAA for 4–8 weeks. Rooted shoots were transplanted in pots containing soil and transferred to the greenhouse. Progeny seeds from these transgenic plants were obtained by selfing.

The presence of the mutated *mP5CS* gene was confirmed by PCR on genomic DNA from the T0 transgenic plant lines using forward (5' ATGGAGGAGCTAGATCGTTCACGT 3') and reverse (5' GGGTGCTAATAGCATCATTC 3') primers specific for the mutated variant of *AtP5CS1*. The

reaction consisted of 35 cycles of 95 °C (30 s), 58 °C (1 min), 72 °C (1 min) followed by a final extension at 72 °C for 10 min. PCR products were run on a 1 % agarose gel and stained with ethidium bromide.

To evaluate the inheritance of the transgenes in the progeny, T1 seeds produced by the transformants were sown on MS medium containing 25 mg/l hygromycin or 100 mg/l kanamycin.

Isolation of total RNA and RT-PCR analysis

Total RNA was extracted from 100 mg of plant leaves exposed or not to 200 mM NaCl for 12 h. RNA from transgenic *N. plumbaginifolia* lines, RN_a and P2 (as controls) was extracted with TRI-reagent (Thermo Fischer Scientific, Germany) or Qiagen RNeasy minikit (Stanford, California, USA) following the manufacturer's instructions. To remove DNA contamination, a DNaseI (Thermo Fischer Scientific, Germany) treatment was carried out during 30 min at 37 °C. The reverse transcription (RT) was carried out via the RevertAid H minus first strand synthesis cDNA kit according to manufacturer's recommendations (Fermentas Life Sciences, Germany). 1 μ l of the first strand synthesized cDNA was used directly for PCR amplification using the *mP5CS* forward (5' ATGGAGGAGCTAGATCGTTCACGT 3') and reverse (5' GGGTGCTAATAGCATCATTC 3') primers specific for the mutated variant of *AtP5CS1*. The reaction consisted of 35 cycles of 95 °C (30 s), 55–60 °C (1 min), 72 °C (1 min) followed by a final extension at 72 °C for 5 min. PCR products were run on a 1 % agarose gel. Amplification of translation elongation factor *EF-1 α* cDNA was used as internal control for relative quantification of messenger RNA, using *Nicotiana benthamiana* EF-1 α forward (5' CTCCAAGGC-TAGGTATGATG 3') and EF-1 α reverse (5' CTTCGTGG-TTGCATCTCAAC 3') primers (Dean et al. 2002). RT-PCR analyses were done in three replicates.

P5CS extraction and activity assay

P5CS activity in plant extracts was determined according to the method described by Parre et al. (2010), which measures the phosphate released in the second enzymatic step catalysed by P5CS, i.e. the reduction of gamma-glutamyl phosphate to glutamate semi-aldehyde. One gram of leaf discs obtained from 1-month-old plants grown in vitro was immersed in liquid nitrogen and ground in a mortar at 4 °C in 2 ml of extraction buffer (1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM MgCl₂, 10 mM dithiothreitol (DTT), 1x protease inhibitor tablet (Roche), 10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.5. After centrifugation (20,000 \times g) for 15 min at 4 °C, the supernatant was centrifuged again at

(154,000 \times g) for 30 min at 4 °C and then desalted by passing through a PD10 column (GE Healthcare) equilibrated with activity buffer (pH 7.5, 1 mM EDTA, 50 mM KCl, 3 mM MgSO₄, 10 mM MOPS). Active fractions were collected and used as enzyme source for measuring P5CS activity. Protein content was determined following the Bradford method (1976). The P5CS activity assay was done as described by Parre et al. (2010) with 50 μ g protein and released Pi was measured spectrophotometrically at 623 nm after addition of malachite green. To investigate feedback inhibition properties of P5CS, the activity assay was performed in the presence of 0, 5 or 10 mM proline.

Salt stress treatments and growth conditions

The seeds were surface sterilized by immersion in a sodium hypochlorite solution for 15 min and then thoroughly washed several times with sterile distilled water. MS agar based medium (Murashige and Skoog 1962) containing hygromycin (25 mg/l) was used for seed germination and selection. Seeds were germinated and grown in the culture room at 24 \pm 2 °C, with a 16 h light/8 h dark cycle. For the short term salt stress experiments, 3-week-old plantlets were transferred to Petri dishes with liquid MS medium for a 48-h adaptation period. Afterwards the seedlings were transferred to a MS liquid medium supplemented with or without 200 mM NaCl for 72 h; both in the 48-h adaptation period and in the 72-h treatment the seedlings were supported with filter paper, thus ensuring submersion of the roots, but not the leaves, in the liquid medium. For the long term stress experiments, 3-week-old seedlings were transferred to plastic boxes with MS agar medium supplemented or not with 200 mM NaCl. Evaluation of the growth was assessed after 5 weeks in the culture room. At the end of these periods, plants were harvested, washed several times with distilled sterile water and fresh weight was measured in order to evaluate the growth rate. Relative growth rate was expressed as (Final Fresh Weight – Initial Fresh Weight)/(Initial Fresh Weight) according to Smith and McComb (1981). The harvested plants were immediately frozen in liquid nitrogen for further analysis.

Determination of proline content

The method of Bates et al. (1973) was applied for measuring proline content in plantlets or leaves using 0.1–1.0 g fresh weight material. The aqueous phase of the supernatant was used to measure the optical density in a spectrophotometer at 520 nm. Toluene was used as blank. The proline concentration was determined based on a proline standard curve. Proline content was expressed as μ mol proline per gram fresh weight.

Fluorometric assay of GUS activity

A fluorometric assay method was used for a quantitative determination of GUS enzymatic activity (Jefferson 1987). Leaf discs of about 1 cm² cut from the first and second basal leaf were placed in liquid MS medium containing 10 g/l sucrose and with or without 200 mM NaCl. 100 mg of transgenic leaf discs were homogenized in 200 μ l GUS extraction buffer (50 mM sodium phosphate buffer pH 7.0, 10 mM mercaptoethanol, 10 mM EDTA pH 8.0, 0.1 % Sarcosyl and 0.1 % Triton-X100). Fifty μ l of supernatant were added in 500 μ l of assay buffer (1 mM 4-methylumbelliferyl β -D-glucuronide (4-MUG) in extraction buffer). After incubation for 2 min, the enzyme reaction was stopped by the addition of 0.2 M Na₂CO₃ and measured by spectrofluorometry at 455 nm. The fluorometry was calibrated using 4-methylumbelliferone (4-MU) as a standard. The amount of proteins was determined according to Bradford (1976) using BSA as a standard.

Statistical analysis

Significant differences in enzymatic assays were calculated using the *t* test. The differences are mentioned as significant for $P \leq 0.01$. Comparison of multiple means was done by one-way ANOVA and a Tukey post hoc test at $P \leq 0.01$.

Results

Introduction of the E155G mutation in *AtP5CS1* and RT-PCR analysis

Proline overproduction and osmotic stress tolerance in the *N. plumbaginifolia* RNA mutant was suggested to be due to the replacement of the Glu₁₅₅ codon by a Gly codon in a *P5CS* gene (Roosens 1999; Roosens et al. 1999). Multiple sequence alignment demonstrates that the glutamate at position 155 is absolutely conserved in plant *P5CS* sequences (see Supplementary Fig. 2). The E155G mutation was subsequently introduced in the Arabidopsis *P5CS1* cDNA sequence of expression vector pCAM-P5CS (see “Materials and Methods” section), yielding expression vector pCAM-mP5CS in which the mutated *P5CS* gene is under control of the CaMV 35S promoter.

Leaf discs of wild type *N. plumbaginifolia* (P2) and of the RNA mutant were transformed with pCAM-mP5CS, yielding TP2P5CS and TRNaP5CS transgenic lines, respectively. PCR amplification was carried out to confirm the presence of the *mP5CS* gene in TP2P5CS and TRNaP5CS transgenic lines. The *mP5CS* gene was found

to be integrated in the genome of all selected transgenic plants analysed. T1 plants segregated according to a 3:1 ratio suggesting a single transgene locus, with the exception of TRNaP5CS/05 line (ratio 15:1). Three TP2 and three TRNa transgenic lines were chosen and their homozygous T2 progenies, selected on hygromycin containing medium, were used for further analysis.

The expression of the *mP5CS* gene in T2 transgenic plants was evaluated by RT-PCR. A clear expression of the *mP5CS* transgene was observed in all transgenic lines tested (TP2P5CS 06, 07, 08 and TRNaP5CS 03, 05 and 08) while no detectable expression was obtained in P2 wild type (Fig. 1a). The *EF-1 α* gene used as a control was amplified at a similar intensity in the corresponding transgenic and control lines as shown in Fig. 1b.

Evaluation of the feedback sensitivity of the *mP5CS* encoded enzyme

To confirm that the E155G substitution confers reduced feedback sensitivity to P5CS, enzymatic assays on extracts of P2, the RNA mutant and two TP2P5CS plants were performed (Fig. 2). When the P5CS activity assay was performed in the presence of 5 and 10 mM proline, the enzyme activity in P2 was significantly inhibited by 47.2 and 62.3 % respectively. Proline feedback inhibition was clearly reduced in the tested transgenic and mutant lines. In the presence of 5 mM proline, the inhibition reached only 5.7 % for TP2P5CS/07, 16.6 % for TP2P5CS/08 and 14.4 % for RNA. When 10 mM proline was used in this assay, these values were respectively 15.8, 30.4 and 22.0 %. These results indicate that the mutated *P5CS* gene leads effectively to the synthesis of an enzyme that is less sensitive to feedback inhibition.

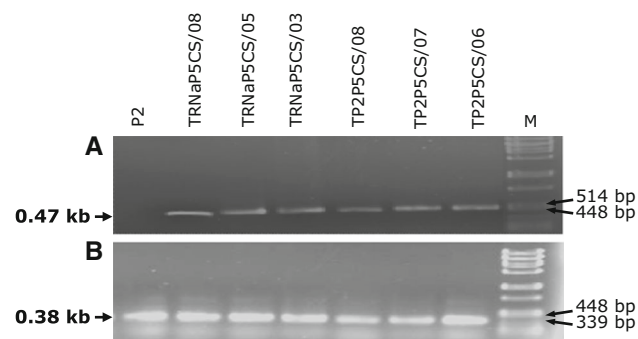


Fig. 1 RT-PCR analysis of leaves of 8-week-old T2 transgenic plant lines grown on MS medium. **a** Amplification of *mP5CS* mRNA in TP2P5CS/06, 07, 08 and TRNaP5CS/03, 05, 08 transgenic plants and the P2 wild type plant (expected size 0.47 kb). **b** Amplification of *EF-1 α* mRNA in the same lines (expected size 0.38 kb). M, λ *Pst*I molecular mass marker

Growth of TP2P5CS and TRNaP5CS transgenic lines submitted to a 5-week stress period

The relative growth rate was measured after 5 weeks in vitro culture on MS with or without 200 mM NaCl (Fig. 3a). The transgenic TP2P5CS and TRNaP5CS lines did not differ statistically in relative growth rate compared to their controls when grown on a non-stress MS medium. However, a significant difference was observed between the controls and *mP5CS* transgenic lines when the plants were grown on MS supplemented with 200 mM NaCl. Compared to P2 plants, the growth rates of TP2P5CS lines 06, 07 and 08 were increased by 2.5-, 1.9- and 1.5-fold, respectively. In the case of the TRNaP5CS transgenic lines, the growth rates were increased by 2.0-, 2.8- and 2.1-fold respectively in the lines 03, 05 and 08 compared to P2 (Fig. 3a). However, the performance of the TRNaP5CS lines was not significantly different compared to RNA when stress was applied except for the TRNaP5CS/05 line.

Proline content in TP2P5CS and TRNaP5CS transgenic lines

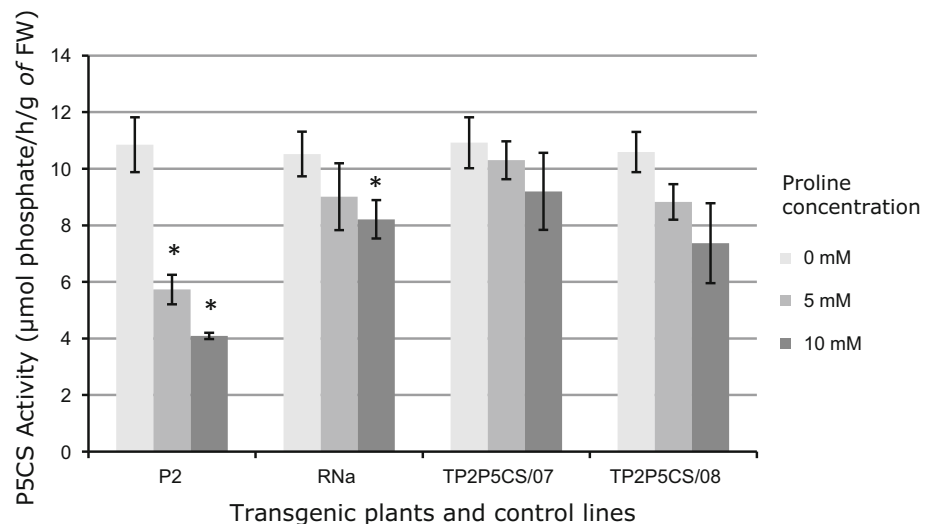
Short stress period

Three-week-old T2 plantlets were first transferred in liquid MS medium for 48 h and then subjected during 72 h to a treatment in liquid MS medium with or without 200 mM NaCl. The proline levels were measured for TP2P5CS, TRNaP5CS lines and their respective controls P2 and RNA (Fig. 3b). In TP2P5CS lines, the plantlets presented a low level of proline on MS medium, while marked differences appeared when they were grown on MS supplemented with 200 mM NaCl. The proline content increased around threefold in the tested lines TP2P5CS 06, 07, and 08 when compared to stressed P2 plants. In T2 TRNaP5CS lines, the proline content also remained at a low level similar to the one obtained in P2 and RNA when grown on a MS basal medium. In contrast, on MS medium supplemented with 200 mM NaCl, proline levels increased by a factor 2.3, 2.6 and 2.3 in TRNaP5CS lines 03, 05 and 08 respectively when compared to the wild type but not significantly when compared to the RNA control line (Fig. 3b).

Long stress period

To evaluate the proline level in the transgenic P5CS lines after a longer period of stress, the plants were grown in plastic boxes for 5 weeks on semi-solid MS medium with or without 200 mM NaCl and then analysed for their proline content (Fig. 3c). In TP2P5CS transgenic lines 06, 07 and 08 the proline levels were increased 2.5-, 1.8- and 1.4-fold respectively compared to the wild type level when

Fig. 2 Inhibition of P5CS activity by proline in transgenic lines TP2P5CS/07 and TP2P5CS/08 and P2 and RNA as control plants. Assays were carried out in the presence of 0, 5 and 10 mM of proline. P5CS activity was expressed in μmol of phosphate (Pi) per hour per gram of fresh weight of plant samples. Average values and standard deviations are based on three replicates. *Asterisk* significantly different from the value at 0 mM within the same line according to Student's *t* test ($P \leq 0.01$)



grown on MS supplemented with 200 mM NaCl. However, in non-stress conditions the level of proline for these transgenic lines was comparable to (TP2P5CS/07) or even significantly less (TP2P5CS/06 and 08) than the values observed for P2. In TRNaP5CS lines, proline content remained at levels comparable or lower than in the RNA control line when MS was used as growth medium. When salt was supplemented in the culture medium, the proline contents were increased by 3.2-, 3.5- and 2.8-fold respectively in the TRNaP5CS/03, 05 and 08 lines compared to wild type plants. However the proline content in the transgenic lines did not differ significantly from the one observed for the stressed RNA control (Fig. 3c).

Relationship between growth inhibition and proline content in plants submitted to salt stress

Overall, the data presented indicate that plant growth on a stress medium is more affected in P2 than in the RNA mutant and the TP2P5CS and TRNaP5CS transgenic lines.

Here, we assessed the relative performance of these transgenic lines by comparing the values of relative growth inhibition, obtained by expressing the fresh weight of plants grown under salt stress conditions as a percentage of the weight of the corresponding lines in non-stress conditions (Fig. 4). In the case of the P2 control, the growth inhibition is quite strong, reaching up to 81 %, while in the transgenic TP2P5CS lines it varies from 50 to 69 %. The RNA mutant confirms also its relative salt tolerance (55 % inhibition). When we consider the transgenic lines obtained in the RNA background, the range of growth inhibition varies from 47 to 57 %. Thus, a clear correlation ($R^2 = 0.81$) was noticeable between the salt tolerance measured as relative growth inhibition rate with the level of proline accumulation under stress (Fig. 4).

Effect of salt stress on the expression of the 35S promoter

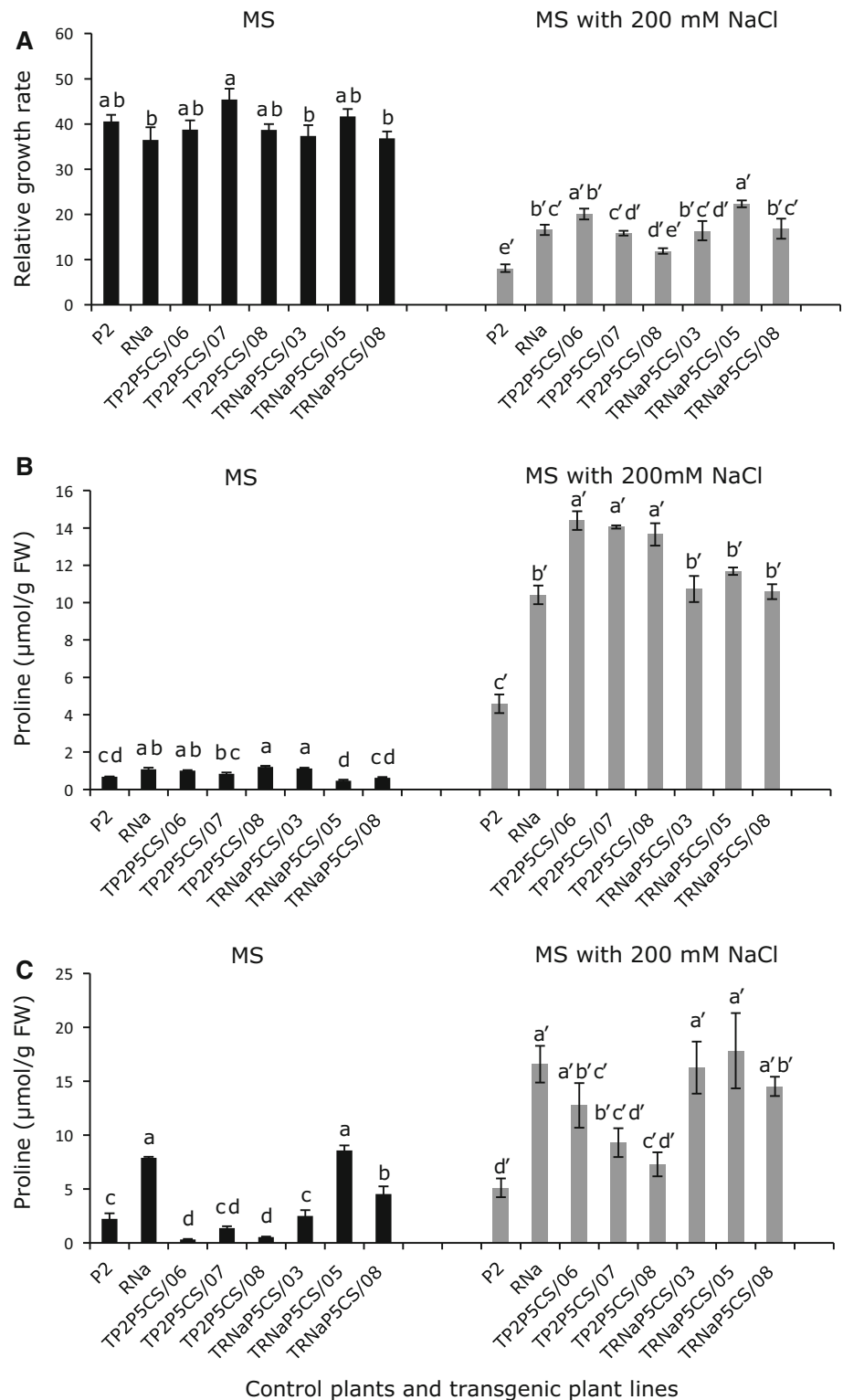
Proline content in the transgenic lines overexpressing the *mP5CS* gene did not appear to increase when the plants were grown in non-stress culture conditions, while proline accumulation was observed on salt containing-medium. This consideration led us to analyse the impact of salt treatment on the expression level conferred by the 35S promoter which was used in the *mP5CS* construct.

Semiquantitative RT-PCR was performed to determine the expression level of the *p35S-mP5CS* gene under salt stress in the TP2P5CS plants. Leaf discs were incubated on liquid MS medium with or without 200 mM NaCl during 12 h and RNA was extracted as mentioned in “[Materials and methods](#)” section. In three replicates, the TP2P5CS/06, 07 and 08 transgenic lines showed a marked increase of the *mP5CS* RNA expression when submitted to salt stress compared to the level observed under normal conditions (Fig. 5). For the internal control (*EF-1 α* gene), differences between stress and non-stress conditions were barely noticeable.

To specifically assess the role of salt on the expression conferred by the 35S promoter, a p35S-GUS construct was introduced in both P2 and the RNA mutant via *Agrobacterium*-mediated transformation, generating TP2GUS and TRNaGUS lines respectively. Leaf discs (area $\pm 1 \text{ cm}^2$) of the transgenic lines (T2 generation) were incubated on liquid MS medium with or without 200 mM NaCl during 12 and 24 h.

Results of fluorometric GUS analyses showed that leaf discs incubated on MS without NaCl for 12 or 24 h showed higher GUS activity compared to the values observed for the same lines at time zero (Fig. 6). The increased values may be the consequence of stress due to wounding and

Fig. 3 Influence of salt stress on relative growth rate and proline content. **a** Effect of salt stress on growth rate of T2 transgenic lines grown on MS supplemented with or without 200 mM NaCl during 5 weeks in the culture room. Relative growth rate values of T2 transgenic lines TP2P5CS/06, 07, 08 and TRNaP5CS/03, 05, 08, with P2 and RNA as controls. Average values and standard deviations are based on three replicates. **b** Effect of salt stress on proline content of T2 transgenic lines TP2P5CS/06, 07, 08 and TRNaP5CS/03, 05, 08 with P2 and RNA mutant as controls. Plants were grown in vitro on MS medium with or without 200 mM NaCl for 72 h. Average values and standard deviations are based on three replicates. **c** Effect of salt stress on proline content of T2 transgenic lines TP2P5CS/06, 07, 08 and TRNaP5CS/03, 05, 08 with P2 and RNA as controls. Plants were grown in vitro on MS medium with or without 200 mM NaCl for 5 weeks. Average values and standard deviations are based on three replicates. Within one treatment, bars with the same letter are not significantly different at $P \leq 0.01$ after one-way ANOVA and post hoc Tukey test



culture in liquid medium, as it is known that 35S promoter activity is influenced by a variety of hormonal, environmental and stress factors, e.g. by methyl jasmonate, salicylic acid, auxins and sucrose (Xiang et al. 1996; Cooke and Webb 1997). Importantly, however, for both time

points and in all transgenic lines GUS activity was markedly higher in leaf discs submitted to salt stress than in non-stressed discs (Fig. 6). When the leaf discs of TP2GUS plants were incubated on MS with NaCl for 12 h, the GUS enzyme activity increased in lines TP2GUS/01, 02 and 03

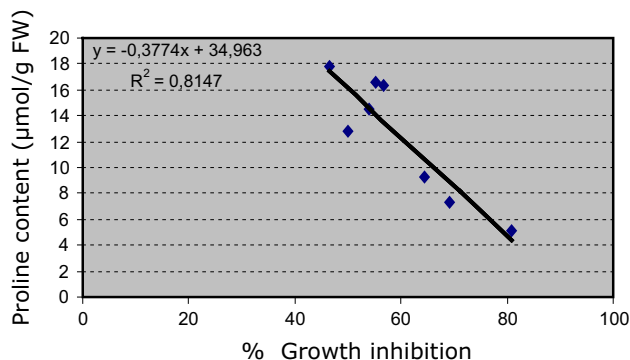


Fig. 4 Correlation between relative growth inhibition and proline content of T2 transgenic TP2P5CS lines 06, 07, 08 and TRNaP5CS lines 03, 05, 08 as well as P2 and RNA growing on MS medium supplemented with 200 mM NaCl during 5 weeks. Growth inhibition values are derived from the data reported in Fig. 3a

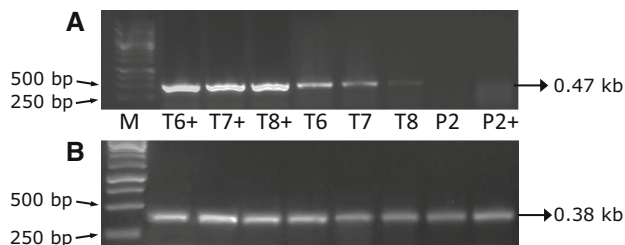
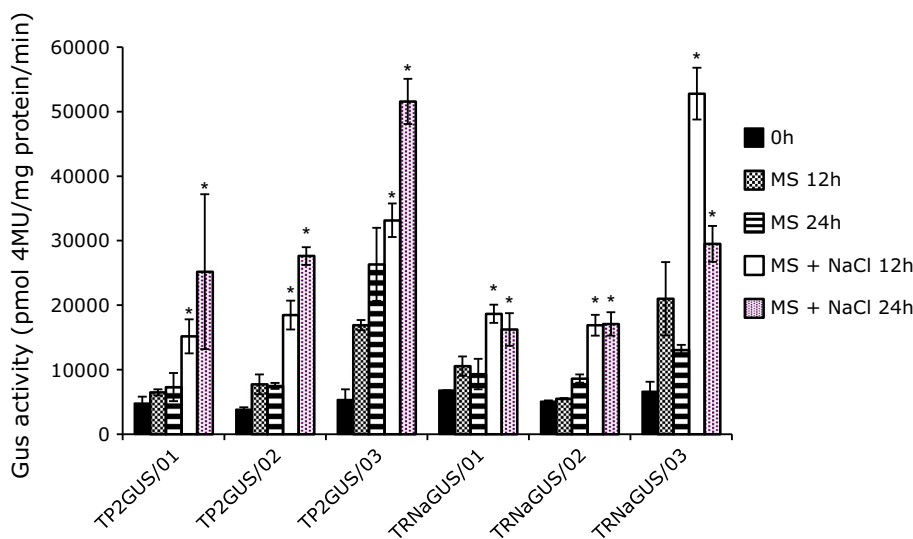


Fig. 5 Gene expression of *mP5CS* (a) and *EF-1α* as internal control (b) measured by semiquantitative RT-PCR. RNA was extracted from leaf discs of 1-month-old T2 plants exposed to MS medium with or without 200 mM of NaCl for 12 h. T6: TP2P5CS/06, T7: TP2P5CS/07, T8: TP2P5CS/08; *plus* designates the same lines but exposed to 200 mM salt stress. M, GeneRuler 1-kb molecular mass marker (Fermentas). Expected sizes of the amplification products are 0.47 kb for *mP5CS* and 0.38 kb for *EF-1α*

Fig. 6 GUS activity in leaf discs of T2-generation TP2GUS and TRNaGUS transgenic lines, incubated on MS medium with or without 200 mM NaCl for 0, 12 and 24 h in the culture room. Average values and standard deviations are based on six replicates. Asterisk indicates that GUS activity in a line grown under salt stress is significantly different according to Student's *t* test ($P \leq 0.01$) from GUS activity in the same line grown for the same time under non-stress conditions



by a factor 2.3, 2.4 and 2.0 respectively compared to the values observed in the same transgenic lines after 12 h on MS medium without NaCl. After 24 h salt stress, GUS activity values further increased and were higher by a factor 3.4, 3.7 and 2.0 respectively in transgenic lines TP2GUS/01, 02 and 03 as compared to non-stress conditions (Fig. 6). In the case of transgenic TRNaGUS leaf discs incubated on MS with NaCl, a somewhat different result was obtained. After 12 h on NaCl containing MS medium, the GUS activity increased by a factor 1.8, 3.1 and 2.5 in transgenic lines TRNaGUS/01, 02, and 03 compared to the values for the same transgenic lines incubated 12 h on MS without NaCl (Fig. 6). Interestingly and in contrast to the TP2GUS lines, this increase was not further expressed after 24 h, the induction value for each line being even lower than the ones obtained after 12 h of stress, i.e. 1.7, 2.0 and 2.3 respectively for lines TRNaGUS/01, 02, and 03.

Discussion

Several mutations in plant and bacterial P5CS or γ -GK encoding genes that reduce feedback inhibition by proline have previously been identified (Rushlow et al. 1985; Zhang et al. 1995; Sleator et al. 2001; Fujita et al. 2003; Sekine et al. 2007). Here we describe a new mutation at the position of the Glu₁₅₅ residue that relieves feedback inhibition by proline. The glutamate at this position is absolutely conserved in plant P5CS enzymes and is adjacent to sites where mutations may give rise to feedback-

desensitized P5CS or γ -GK isoforms (Supplementary Fig. 3). Moreover Glu₁₅₅ is located in a region corresponding to a loop in *E. coli* and *Campylobacter jejuni* γ -GK that is implicated in binding inhibitory proline (Pérez-Arellano et al. 2010).

A positive correlation between the capacity for proline accumulation and osmotolerance has been established in various plants, for instance tobacco (Kavi Kishor et al. 1995; Hong et al. 2000), *Medicago truncatula* (Armengaud et al. 2004; Verdoy et al. 2006), *Arabidopsis* (Yoshiba et al. 1995; Székely et al. 2008), rice (Su and Wu 2004; Karthikeyan et al. 2011), wheat (Sawahel and Hassan 2002), potato (Hmida-Sayari et al. 2005), chickpea (Bhatnagar-Mathur et al. 2009) and pigeonpea (Surekha et al. 2014). To evaluate if an increase in proline content confers enhanced salt stress tolerance in transgenic *N. plumbaginifolia* we inserted the *A. thaliana* P5CS1 gene with the E155G mutation in the genome of *N. plumbaginifolia* wild type (P2) plants and of the RNA mutant, already characterized by a feedback insensitive P5CS gene. In both cases the mutant P5CS1 gene was driven by the CaMV 35S promoter. Under normal conditions, the growth rate of the P2 and RNA controls and the transgenic TP2P5CS and TRNaP5CS lines did not differ in 5-week-old plants (Fig. 3a), and also the proline content was comparable to the values obtained for the P2 and RNA controls (Fig. 3b, c). On the other hand, significant differences between the P2 control and transgenic TP2P5CS plants submitted to salt stress were observed in terms of growth rate as well as proline content (see Fig. 3a–c). However, when the comparison was made between the RNA control line and the derived TRNaP5CS lines, growth rate and proline content values were not significantly different. We also observed a clear correlation between salt tolerance measured as relative growth inhibition in the presence of 200 mM NaCl in the culture medium and proline content. The highest proline content values are clearly associated with the less pronounced growth inhibition (Fig. 4). Full tolerance to 200 mM salt could not be obtained in any of the lines we generated. Nevertheless, the less affected the plants were, the easier they recovered in growth when stress was released. These recovered transgenic plants were able to set some seeds, while in similar conditions, wild type plants could not recover from salt stress and died (data not shown).

Our results with *N. plumbaginifolia* overexpressing P5CS and submitted to salt stress conditions are in line with those mentioned for other species. For instance, transgenic tobacco lines overexpressing a wild type *V. aconitifolia* P5CS produced 10- to 18-fold more proline and exhibited better growth performance under salt stress than control plants (Kavi Kishor et al. 1995, 2005). Furthermore, total removal of feedback inhibition of the same *V. aconitifolia* P5CS results in a higher level of proline accumulation (Hong et al. 2000). In petunia plants transformed with an *Arabidopsis*

wild type *AtP5CS1* gene or *Oryza sativa* wild type *OsP5CS1*, proline content increased 1.5–2.6 times compared to wild type plants when they were exposed to drought stress. They showed also better growth values under drought stress than the control lines (Yamada et al. 2005). However, under non-stress conditions, our results differ from those reported in the literature in the sense that, in many studies, the proline level was already markedly increased in transgenic lines not submitted to salt stress. In the case where a P5CS gene was under the control of the 35S promoter, an increase in proline from a moderate 1.5-fold up to about 15-fold in defined transgenic lines has been reported depending on the plant species (Kavi Kishor et al. 1995; Hong et al. 2000; Sawahel and Hassan 2002; Yamada et al. 2005; Verdoy et al. 2006; Karthikeyan et al. 2011; Surekha et al. 2014). These examples show that effects of increasing proline biosynthesis can vary widely among species. In our *N. plumbaginifolia* transformants there was no clear increase of proline under non-stress conditions; similarly in the RNA mutant there is at most a slight increase in proline under non-stress conditions (see also Roosens et al. 1999). When grown on salt containing medium, the TRNaP5CS lines show proline contents in the range of those observed for the RNA mutant. It thus appears that the presence of one mutated P5CS gene as in the RNA mutant, already allows to obtain a proline accumulation which cannot be much amplified by an additional mutated P5CS gene in the transgenic plants; the lack of further increase in proline may be attributed to many causes including post-transcriptional regulation mechanisms and proline degradation. Proline accumulation is the result of proline synthesis and catabolism. Under salt stress conditions proline degradation is repressed (Delauney et al. 1993; Peng et al. 1996) and this certainly plays a role in the difference in proline levels in transgenic plants grown under stress or non-stress conditions.

The observed increase in proline in the transgenic lines when they were grown on salt containing medium, led us to assess whether expression of the *p35S-mP5CS* transgene is induced by salt stress. Therefore, the influence of a short salt treatment on the expression level of the *p35S-mP5CS* gene was evaluated by semi-quantitative RT-PCR in TP2P5CS transgenic lines. We noticed that the transgenic lines, submitted to salt stress, showed a marked increase of the *mP5CS* RNA level compared to the same lines under normal conditions. Interestingly, similar conclusions can be drawn from experiments with potato lines transformed with an *A. thaliana* P5CS1 cDNA under the control of the 35S promoter (Hmida-Sayari et al. 2005) and *A. thaliana* lines expressing *Phaseolus vulgaris* P5CS1 or P5CS2 genes placed under the control of a 35S promoter (Chen et al. 2013). In both studies, P5CS mRNA levels were clearly higher in plants subjected to NaCl treatment compared to non-stressed plants. Such higher P5CS mRNA levels may

be due to increased RNA stability or to inducibility of the 35S promoter by salt. To more specifically assess the impact of salt on p35S, we introduced a p35S-GUS construct in the P2 wild type and the RNA mutant background. GUS activity was found to increase 2- to 2.5-fold in leaf discs of TP2GUS transgenic lines exposed to salt stress during 12 h and to increase further after 24 h of stress. This shows that activity of the CaMV 35S promoter is indeed enhanced by salt stress. Interestingly, in the case of TRNaGUS leaf discs, the increase in activity was also present after 12 h but no further increase was observed after 24 h of stress exposure. This observation may be due to the fact that RNA leaf discs are less severely stressed on a salt medium than P2 leaf discs. The CaMV 35S promoter is considered as a constitutive promoter. Studies have shown that the upstream sequences of the CaMV 35S promoter contain several elements mediating developmental and organ-specific regulation (Benfey and Chua 1990; Benfey et al. 1990). Also a defined element in the CaMV 35S promoter was detected as responsive to salicylate (Qin et al. 1994). Schnurr and Guerra (2000) showed the CaMV 35S promoter is up-regulated by shortened photoperiod and that the light conditions, including photoperiod, play an important role in the regulation of the CaMV 35S promoter. Our data concerning the expression of the p35S-GUS construct suggest that the CaMV 35S promoter also contains elements responsive to salt stress.

In conclusion, this work points out that the overexpression of a mutated form of P5CS encoding a feedback-insensitive enzyme leads to a significant increase of proline content conferring enhanced tolerance to salt stress as exemplified by evaluating the growth rate of transgenic plants submitted to such conditions. Although developing tolerance to salt and drought stress in crops is a complex issue involving various categories of approaches (Deinlein et al. 2014), manipulation of proline biosynthesis appears as a valid way to minimize the adverse effects of salt stress and to obtain adapted plants.

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Conflict of interest The authors declare that they have no conflict of interest.

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