Acta Physiol Plant (2011) 33:1591–1595
DOI 10.1007/s11738-010-0692-6

ORIGINAL PAPER

Effects of low nitrogen and drought stresses on proline synthesis of *Jatropha curcas* seedling

Wen-Guo Wang · Rui Li · Bin Liu · Liang Li · Sheng-Hua Wang · Fang Chen

Received: 28 January 2010 / Revised: 7 December 2010 / Accepted: 29 December 2010 / Published online: 20 January 2011

Abstract Proline is one of the most important osmoregulatory solutes subjected to osmotic stresses. In this study, low nitrogen supply suppressed the dry biomass, leaf area, and proline biosynthesis of the seedlings of the energy plant *Jatropha curcas*, which could grow in poor, dry soil. Low-nitrogen stress induced *JcP5CS* mRNA expression and decreased the activity of P5CS enzyme and the content of free proline in leaves of *J. curcas* seedlings. When the seedlings grown in low-nitrogen conditions were suddenly exposed to PEG-6000 (−1.6 MPa) stress, the expression of *JcP5CS* gene was highly induced, and both the activity of P5CS and the content of free proline increased and maintained at high levels to mitigate the impact of drought stresses. This may be one of the reasons why *J. curcas* could adapt to poor and drought conditions.

Keywords Low nitrogen supply · Proline · Drought stress · P5CS · *Jatropha curcas*

Communicated by G. Klobus.

W.-G. Wang · R. Li · B. Liu · L. Li · S.-H. Wang · F. Chen (✉)
Key Lab of Bio-resources and Eco-environment, Ministry of Education, College of Life Science, Sichuan University, Chengdu 610064, People's Republic of China
e-mail: chenfang@scu.edu.cn

W.-G. Wang
Research Centre of Biomass Energy Technology, Biogas Institute of Ministry of Agriculture, Chengdu 610041, People's Republic of China

Introduction

In plants, free proline accumulation as one of the potential indicators of osmotic tolerance has been correlated with tolerance to drought stresses (Hare and Cress 1997; Ashraf and Foolad 2007), and it also has been thought to play a cardinal role as an osmoregulatory solute subjected to hyperosmotic stresses (Delauney and Vermad 1993). It has been known that there are two alternative routes in proline biosynthesis in higher plants: the L-ornithine and the L-glutamate pathways. And the glutamate pathway is dominant under osmotic stress conditions (Delauney et al. 1993; Kishor et al. 2005). In this pathway, the dual function enzyme Δ^1-pyrroline-5-carboxylate synthetase (P5CS) is a key enzyme, which catalyzes the conversion of glutamate into Δ^1-pyrroline-5-carboxylate, which is finally reduced to proline. Nitrogen is an extremely important element of plant nutrition; lack of nitrogen commonly limits the growth and development of plant. The proline biosynthesis could also be regulated by the flux of nitrogen by controlling the level of P5CS under stress conditions (Delauney et al. 1993; Kishor et al. 1995).

For the production of plant oils that can be converted into biodiesel, *Jatropha curcas* is one species that has received much attention recently (Gubitz et al. 1999; Achten et al. 2007; Fairless 2007; King et al. 2009): not only as an energy plant, but also as a drought-tolerant plant used to describe land of poor quality (King et al. 2009). In China, *J. curcas* has been used to control soil erosion in the Jinsha River dry-hot valley (Li et al. 2007). In this study, we studied the effects of low nitrogen supply and drought stresses on proline synthesis to discuss the tolerance mechanism of *J. curcas* growing in arid land.

Materials and methods

Plant material, growth conditions, and drought treatment

The seeds of *J. curcas* were collected from Panxi area in China. After soaking in distilled water for 12 h, the seeds were planted in plastic pot filled with quartz sand and watered with nutrient solution (4 mM N as $Ca(NO_3)_2$ and KNO_3; 0.5 mM K as KH_2PO_4; 1.2 mM Mg as $MgSO_4$; 1.0 mM Ca as $Ca(NO_3)_2$; 10 μM B as H_3BO_3; 15 μM Fe as Fe-EDTA; 0.5 μM Mn as $MnSO_4$; 0.5 μM Zn as $ZnSO_4$; 0.5 μM Cu as $CuSO_4$; 0.1 μM Mo as $(NH_4)_6Mo_7O_{24}$) (Brueck and Senbayram 2009). For low-nitrogen treatments, the N concentrations (KNO_3 instead of $Ca(NO_3)_2$) were 0.05, 0.20, 0.50, and 1.00 mM (Table 1); potassium and calcium supply of low-nitrogen treatments were balanced by addition of the appropriate amount of KCl and $CaCl_2$. The cultures were in greenhouse at 28 ± 1°C and under 16/8 h (light/dark) photoperiod conditions adjusted to an intensity of 1,000 μmol m^{-2} s^{-1}.

Osmotic treatments were carried out under the same culture conditions. After a growing period of 30 days, the seedlings grown in each nitrogen treatment were exposed to PEG-6000 (−1.6 MPa) stress for 48 h.

Biomass determination and leaf area analysis

The plants were harvested after a growing period of 30 days. The roots were rinsed five times with redistilled water and dried on filter paper. The leaf blades were measured using a portable area meter (CI-202, CID). In a given experimental series, the different plant organs were collected and dried at 105°C for 48 h, then weighed for a dry matter (DW) assessment.

Proline content

Proline content was determined according to the method described by Bates et al. (1973). Fresh leaf material (0.50 g) was homogenized in 10 cm^3 of 3% aqueous sulfosalicylic acid and filtered through filter paper. Half milliliter of the filtrate was mixed with 1 ml of acid-ninhydrin and 1 cm^3 of glacial acetic acid in a test tube. The mixture was placed in a water bath at 100°C for 1 h. Then, the mixture was extracted with 4 cm^3 of toluene and the chromophore containing toluene was aspirated, cooled to room temperature, and the absorbance was measured at 520 nm with a UV/visible spectrophotometer. Appropriate proline standards were included for the calculation of proline in the samples.

RNA isolation and real-time RT–PCR analysis of *JcP5CS* gene

Total RNA was isolated from *J. curcas* leaves using RNA prep pure Plant Kit (Tiagen, China) according to the manufacturer's protocol. Reverse transcription of the pooled RNA was carried out with oligo(dT) primers using ReverTra Ace-α-™ (TOYOBO). The expression of *JcP5CS* (GeneBank accession No. GU358610) was analyzed quantitatively using SYBR Green II real-time PCR kit (TaKaRa) with Bio-Rad IQ5 detection system. The reaction mixture contained 2 × SYBR Green PCR Master Mix, 18 pM each of the forward and reverse primers of *JcP5CS* (PR-F: 5′-GGGCAAGCAAGGAGATGAAC-3′, PR-R: 5′-TCCAACCTCAGCACCTAAGC-3′) or 18s rRNA (GeneBank accession No. AY823528) (18sF: 5′-TCGTATC TTGCTGTGACTGG-3′; 18s: 5′-TTGGCAAGCCTTA GCATC-3′), and 1 μl cDNA in a total volume of 25 μl. The thermocycling program was 40 cycles of 95°C for 20 s and 60°C for 20 s with an initial cycle of 95°C for 10 s. After the PCR, a dissociation curve (melting curve) was constructed from the range of 55–95°C to ensure that primer–dimers and other nonspecific products had been eliminated. Data were collected and processed, including baselines subtraction and threshold definition, with iQ5-Cycler software (BioRad).

P5CS activity assay

The extraction of P5CS was processed according to Kishor et al. (1995). The leaves were homogenized in an extraction buffer (100 mM Tris–Cl, pH 7.5, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, and 1 mM PMSF) in pre-chilled eppendorf tubes on ices. The extracts were centrifuged at 4°C for 20 min at 10,000*g*. The supernatants were further clarified by centrifugation at 10,000*g* for 20 min at 4°C.

Table 1 Effects of nitrogen supply on the total biomass and leaf area of *Jatropha curcas*, 30 days after emergence

Treatments no.	Nitrogen concentration (mM)	Total biomass (g $plant^{-1}$)	Leaf area (cm^2 $plant^{-1}$)
1	4.00	0.501 ± 0.021 a	125.75 ± 5.23 a
2	1.00	0.367 ± 0.018 b	113.96 ± 7.53 a
3	0.50	0.294 ± 0.014 c	96.50 ± 5.39 b
4	0.20	0.152 ± 0.017 d	82.40 ± 2.65 c
5	0.05	0.106 ± 0.014 e	66.14 ± 7.25 d

Means followed by same letters are not significantly different at 0.05 level of significance

The activity of P5CS was processed according to García-Ríos et al. (1997). The P5CS assay was carried out in 100 mM Tris–Cl (pH 7.2), 25 mM $MgCl_2$, 75 mM Na glutamate, 5 mM ATP, 0.4 mM NADPH, and 50 mg crude extract protein at 25°C. The reaction velocity was measured as the rate of consumption of NADPH, monitored as decrease in absorption at 340 nm as a function of time. The unit of P5CS activity was 0.001 ΔOD_{A340} min^{-1}. Total protein content was determined according to Bradford method (Bradford 1976).

Statistical data analysis

The experiment was conducted in a completely randomized design with 10–15 seedlings per replication. Each treatment was replicated three times. Statistical data analysis was carried out using one-way ANOVAs. Least significant differences (LSD, $P \leq 0.05$) were carried out to evaluate the differences in total biomass and leaf area between treatments. All the data were analyzed using Origin software (version 6.00; Microcal Software, Inc.-Northampton, MA 01060 USA).

Results

Effects of low nitrogen supply on growth of *J. curcas* seedlings

As described in Table 1, the nitrogen supply significantly influenced the growth of *J. curcas* seedlings. After a growing period of 30 days, the dry biomass and leaf area in the control group could reach about 0.5 g $plant^{-1}$ and 125 cm^2 $plant^{-1}$, respectively. The lower nitrogen concentrations caused a steady decline in the dry biomass and leaf area. Under the condition of extra-low nitrogen concentration (0.05 mM), the dry biomass decreased to 0.1 g $plant^{-1}$, which was only one-fifth of the ones in the control group. On the other hand, the leaf area of the seedlings grown in the nutrient solution with 0.05 mM nitrogen was half of the control group.

Effects of low nitrogen and drought stresses on free proline accumulation

Free proline was detected in leaves of *J. curcas* seedlings grown in nutrient solution with different concentrations of nitrogen with or without PEG stress (Fig. 1). For the treatments without PEG stress, the proline content maintained 25 μg/kg FW under the control and higher nitrogen concentrations (1.00 and 0.50 mM), but when the nitrogen concentrations were at 0.20 and 0.05 mM, the proline content decreased to about 20 μg/kg FW. The proline content in leaves of *J. curcas* seedlings rose significantly when the PEG-6000 was added; the highest amount measured was found in 0.20 mM nitrogen concentration, in which the proline content was over 50 μg/kg FW. Under the condition of extra-low nitrogen concentration (0.05 mM), the proline content was 45 μg/kg FW, which was still higher than the ones in the control group.

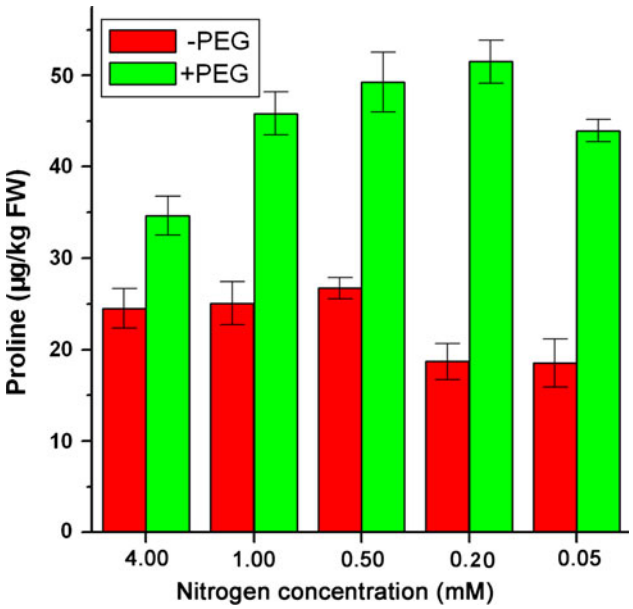

Fig. 1 Effects of low nitrogen supply on free proline accumulation of *Jatropha curcas* with or without PEG stresses

Effects of low nitrogen and drought stresses on P5CS gene expression and enzyme activity

To determine the effects of low nitrogen supply on the biosynthesis of proline, the mRNA level of *JcP5CS* gene and P5CS activity were further detected. Low-nitrogen stresses induced *JcP5CS* gene expression. Regardless of whether the PEG was added, the lowest amounts of the mRNA level of *JcP5CS* were all observed in the control group, while the highest values were found in the lowest nitrogen concentration (0.05 mM) (Fig. 2). On the other hand, when the nutrient solutions were without PEG-6000, the P5CS activity significantly decreased with the nitrogen concentrations (Fig. 3). However, similar to the free proline accumulation and *JcP5CS* gene expression, P5CS activity also rapidly increased along with the decline in nitrogen concentration under the PEG stress.

Discussion

Nitrogen supply effects on the biomass have frequently been reported (Wu et al. 2008; Brueck and Senbayram 2009).

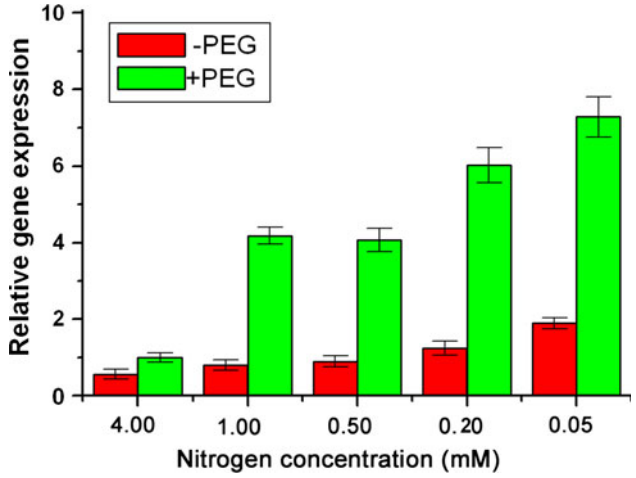

Fig. 2 Effects of low nitrogen on *JcP5CS* gene expression *Jatropha curcas* with or without PEG stresses

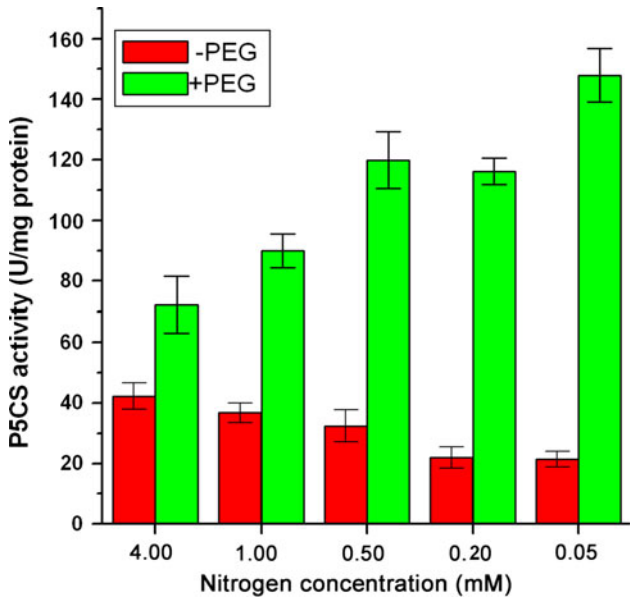

Fig. 3 Effects of low nitrogen supply on P5CS activity *Jatropha curcas* with or without PEG stresses

A low nitrogen supply usually resulted in very low dry matter production (Sheehy Skeffington and Jeffrey 1985). Under the conditions of low nitrogen concentrations, the growth of *J. curcas* was significantly inhibited. Low nitrogen supply suppressed the biosynthesis of protein, nucleic acid, and other metabolites, eventually resulting in low biomass and leaf area. Several studies have demonstrated that the nitrogen starvation-stress and low-nitrogen stress could induce genes expression in plants (Chen et al. 2003; Fang et al. 2010). The level of P5CS could also be regulated by the nitrogen supply (Delauney et al. 1993; Kishor et al. 1995). In this study, under the conditions of low-nitrogen concentrations, the mRNA levels of *JcP5CS* gene were higher; however, the P5CS activity and the proline content were significantly less than that of the control group. This indicated that low-nitrogen stress could induce the *JcP5CS* gene expression, but it also suppressed the P5CS activity and some other metabolites in proline biosynthesis pathway, and eventually led the decline in the content of free proline.

Accumulation of free proline in plant leaves is a commonly observed response to drought stress (Bandurska and Stroiński 2003; Schafleitner et al. 2007). It could act as an osmoregulatory solute to alleviate the damages caused by environmental stresses (Ashraf and Foolad 2007). Although the content of proline in seedlings under the extra-low nitrogen condition was lower than the control group, however, when the *J. curcas* seedlings grown in the nutrient solution with different nitrogen concentrations were exposed to PEG stresses, the free proline accumulated rapidly, and the content of proline of *J. curcas* seedlings grown under low-nitrogen conditions were all higher than the control group. The high level of proline contributes to stabilizing sub-cellular structures, scavenging free radicals, and buffering cellular redox potential. Furthermore, it may also function as a protein-compatible hydrotrope (Srinivas and Balasubramanian 1995), alleviating cytoplasmic acidosis, and maintaining appropriate NADP+/NADPH ratios compatible with metabolism (Hare and Cress 1997). That may be one of the reasons why *J. curcas* could suffer drought stress in arid land.

In plants, the process of proline biosynthesis is orderly and complex, and many factors are involved (Kishor et al. 2005). In this study, the three important factors—content of free proline, mRNA level of *JcP5CS* gene, and P5CS activity—in proline biosynthesis were detected in *J. curcas* seedlings under nitrogen and drought stresses. We can clearly find that with the reduction in nitrogen concentration, the counts of all the three factors increased, and among them, the mRNA level of *JcP5CS* had the highest growth rate, followed by activity of P5CS and the lowest one was the content of free proline. Low-nitrogen stress suppressed some factors in proline biosynthesis pathway, which could result in free proline content decrease. However, when the seedlings stressed by low-nitrogen conditions were exposed to drought stress, the upstream factors of the biosynthesis pathway should be largely synthesized to get sufficient products for the next step. And the ultimate goal of this series of acts was to get enough end-product (proline) to mitigate the impact of drought stress.

In conclusion, our results have shown that low-nitrogen supply suppressed the growth and development of *J. curcas* seedlings, including the proline biosynthesis. But when the seedlings grown in low-nitrogen conditions were exposed to drought stresses, the *JcP5CS* gene could be highly induced that led to high P5CS enzyme activity, eventually resulting in proline content's increase to protect organism

from harm. This should be one of the reasons why *J. curcas* could adapt to poor and drought conditions.

Acknowledgments This work was funded by the National Natural Science Foundation of China (No. 31070301).

References

Achten WMJ, Mathhijs E, Verchot L, Singh VP, Aerts R, Muys B (2007) Jatropha biodiesel fueling sustainability? Biofuels Bioprod Biorefining 1:283–291

Ashraf M, Foolad MR (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 59:206–216

Bandurska H, Stroiński A (2003) ABA and proline accumulation in leaves and roots of wild (*Hordeum spontaneum*) and cultivated (*Hordeum vulgate* 'Maresi') barley genotypes under water deficit conditions. Acta Physiol Plant 25:55–61

Bates LS, Waldren RP, Teare JD (1973) Rapid determination of proline for water stress studies. Plant Soil 39:205–207

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

Brueck H, Senbayram M (2009) Low nitrogen supply decreases water-use efficiency of oriental tobacco. J Plant Nutr Soil Sci 172:216–223

Chen QS, Yi KK, Huang G, Wang XB, Liu FY, Wu YR, Wu P (2003) Cloning and expression pattern analysis of nitrogen-starvation-induced genes in rice. Acta Botanica Sinica 45:974–980

Delauney AJ, Vermad PS (1993) Proline biosynthesis and osmoregulation in plants. Plant J 4:215–223

Delauney AJ, Hu CAA, Kishor PBK, Verma DPS (1993) Cloning of ornithine δ-aminotransferase cDNA from *Vigna aconitifolia* by transcomplementation in *Escherichia coli* and regulation of proline biosynthesis. J Biol Chem 268:18673–18678

Fairless D (2007) Biofuel: the little shrub that could: maybe. Nature 499:652–655

Fang CX, He HB, Wang QS, Qiu L, Wang HB, Zhuang YE, Xiong J, Lin WX (2010) Genomic analysis of allelopathic response to low nitrogen and barnyardgrass competition in rice (*Oryza sativa* L.). Plant Growth Regul 61:277–286

García-Ríos M, Fujita T, Larosa PC, Locy RD II, Clithero JM, Bressan RA, Csonka LN (1997) Cloning of a polycistronic cDNA from tomato encoding γ-glutamyl kinase and γ-glutamyl phosphate reductase. Proc Natl Acad Sci 94:8249–8254

Gubitz GM, Mittelbach M, Trabi M (1999) Exploitation of the tropical oil seed plant *Jatropha curcas* L. Bioresour Technol 67:73–82

Hare PD, Cress WA (1997) Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Regul 21:79–102

King AJ, He W, Cuevas JA, Freudenberger M, Ramiaramanana D, Graham IA (2009) Potential of *Jatropha curcas* as a source of renewable oil and animal feed. J Exp Bot 60:2897–2905

Kishor PBK, Hong ZL, Miao GH, Hu CAA, Verma DPS (1995) Overexpression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol 108:1387–1394

Kishor PBK, Sangam S, Amrutha RN, Laxmi PS, Naidu KR, Rao KRSS, Rao S, Reddy KJ, Theriappan P, Sreenivasulu N (2005) Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. Curr Sci 88:424–438

Li J, Wu PZ, Li MR, Wu GJ (2007) Development of energy plant: progress and suggestions. Chinese J Nat 29:21–25

Schafleitner R, Gaudin A, Rosales ROG, Aliaga CAA, Bonierbale M (2007) Proline accumulation and real time PCR expression analysis of genes encoding enzymes of proline metabolism in relation to drought tolerance in Andean potato. Acta Physiol Plant 29:19–26

Sheehy Skeffington MJ, Jeffrey DW (1985) Growth performance of an inland population of *Plantago maritima* in response to nitrogen and salinity. Plant Ecol 61:265–272

Srinivas V, Balasubramanian D (1995) Proline is a protein-compatible hydrotrope. Langmuir 11:2830–2833

Wu F, Bao W, Li F, Wu N (2008) Effects of drought stress and N supply on the growth, biomass partitioning and water-use efficiency of *Sophora davidii* seedlings. Environ Exp Bot 63:248–255