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Effects of high proline accumulation on chloroplast and mitochondrial ultrastructure and on osmotic adjustment in tobacco plants

Lucélia Borgo[1], Celso Jamil Marur[2] and Luiz Gonzaga Esteves Vieira[2*]

*[1]Centro de Ciências Biológicas, Departamento de Biologia Geral, Universidade Estadual de Londrina, Londrina, Paraná, Brazil. [2]Laboratório de Biotecnologia Vegetal, Instituto Agronômico do Paraná, Universidade Estadual de Londrina, Rod. Celso Garcia Cid, km 375, Cx. Postal 481, 86001-970, Londrina, Paraná, Brazil. *Author for correspondence. E-mail: luizvie@gmail.com*

ABSTRACT. Proline accumulates in many organisms in response to abiotic stresses, such as drought and salinity. Reports have discussed the role of proline in relation to osmotic adjustment, even though no clear-cut evidence has yet been provided demonstrating this association as a defense mechanism against drought stress in plants. However, it has been indicated that the application of exogenous proline can damage the ultrastructure of chloroplasts and mitochondria. In this study, using transgenic tobacco plants carrying a mutant *p5cs* gene from *Vigna aconitifolia* under control of the CaMV 35S promoter, we show that the high levels of endogenous proline accumulated in leaves does not contribute to the osmotic adjustment in plants under water deficit conditions. Additionally, electron microscopic observations of mitochondria and chloroplasts show that the ultrastructure of these organelles was not damaged even in the transgenic plants, with the leaves presenting the highest endogenous proline content in (100 μmol g^{-1} dry mass). This investigation demonstrates that in tobacco, high levels of endogenous free proline are not associated with osmotic adjustment and that elevated proline content in leaves, even at levels 10 times higher than normal, do not disturb the chloroplast and mitochondria ultrastructure under both irrigated and water deficit conditions.

Keywords: *p5cs*, transgenic, water deficit.

Efeitos do alto acúmulo de prolina na ultraestrutura de cloroplastos e mitocôndrias e no ajustamento osmótico de plantas de tabaco

RESUMO. Prolina é acumulada por diversos organismos em resposta a estresses abióticos, como déficit hídrico e salinidade. Estudos vêm discutindo o papel deste aminoácido no processo de ajustamento osmótico, embora nenhuma evidência clara tenha sido fornecida demonstrando esta associação como mecanismo de defesa contra seca em plantas. Entretanto, tem sido proposto que a aplicação exógena de prolina pode danificar a ultraestrutura de cloroplastos e mitocôndrias. Neste estudo, usando plantas transgênicas de tabaco contendo o gene mutante *p5cs* de *Vigna aconitifolia* sob controle do promotor CaMV 35S, evidenciou-se que o alto conteúdo de prolina endógena acumulado em folhas não contribuiu para o ajustamento osmótico em plantas sob estresse hídrico. Além disso, análises de microscopia eletrônica em mitocôndrias e cloroplastos mostraram que a ultraestrutura destas organelas não foi afetada, mesmo em plantas transgênicas que apresentaram os níveis mais elevados de prolina endógena em folhas (100 mmol g^{-1} de massa seca). Estes resultados demonstram que em plantas de tabaco, altas concentrações de prolina endógena não estão associadas ao processo de ajustamento osmótico e que o conteúdo de prolina em folhas, mesmo 10 vezes maior do que o normal, não causou danos à mitocôndrias e cloroplastos em condições de déficit hídrico e de irrigação normal.

Palavras-chave: *p5cs*, transgênico, déficit hídrico.

Introduction

Drought is considered one of the most limiting conditions for crop productivity, and water resources for agriculture are increasingly becoming scarce. One of the most well-documented physiological responses to water deficit in plants is the ability of some species to osmotically adjust their cells (CHAVES et al., 2009). This adjustment can be achieved by the accumulation of osmolytes, as organic compounds, such as amino acids (e.g., proline), amines (e.g., glycinebetaine and polyamines) and a variety of sugars and sugar alcohols (e.g., mannitol, trehalose and galactinol), decrease the osmotic potential and therefore maintain the cell water potential and turgidity close to the optimum level (VALLIYODAN; NGUYEN, 2006).

Proline accumulates in a diverse taxonomic group of plants in response to biotic and abiotic

stresses (SZABADOS; SAVOURÉ, 2010). The levels of proline vary from species to species and can be 100 times greater under water deficit compared to well-watered conditions (VERBRUGGEN; HERMANS, 2008). It is suggested that proline acts in membrane and protein protection against the effects of the high concentration of inorganic ions and temperature extremes, in the stabilization of cell structures and detoxification of free radicals (VERBRUGGEN; HERMANS, 2008) and as a way to store carbon, nitrogen and energy (HARE; CRESS, 1997). Studies also have shown that proline can also alter the activities of antioxidant enzymes (CAMPOS et al., 2011).

In plants under osmotic stress, proline is predominantly synthesized from glutamate via D1-pyrroline-5-carboxylate (P5C) by two successive reductions that are catalyzed by P5C synthetase (P5CS), the rate-limiting enzyme for the biosynthetic pathway in higher plants, and P5C reductase (P5CR) (VERBRUGGEN; HERMANS, 2008). Proline accumulation in the leaves of plants exposed to drought stress is not only associated with an increased expression of the *p5cs* gene but also with the decreased expression of proline dehydrogenase genes (*PDH*) coding for the enzymes of proline degradation (MILLER et al., 2005).

Using site-directed mutagenesis, a replacement of the amino acid Phe at position 129 by Ala was made in *Vigna aconitifolia* P5CS to eliminate the proline feedback inhibition control of the pathway (ZHANG et al., 1995). Transgenic tobacco plants over-expressing the mutated *V. aconitifolia* gene (*VaP5CS129A*) accumulated high proline levels, leading to enhanced salt, drought and heat tolerance (HONG et al., 2000; GUBIS et al., 2007; CVIKROVÁ et al., 2012). Transgenic Carrizo citrange rootstocks expressing the same mutated gene driven by the 35S promoter exhibited an osmotic adjustment and supported longer periods of severe drought stress (MOLINARI et al., 2004).

Many studies have discussed the role of proline in plant osmoprotection and its application in breeding for water stress tolerance (KISHOR et al., 2005; GUBIS et al., 2007; POUSTINI et al., 2007) using genetic transformation techniques, by identifying genetic differences in proline accumulation under water deficit conditions or by exogenous proline application (ASHRAF; FOOLAD, 2007). Although several studies have demonstrated the importance of proline accumulation for the tolerance of certain adverse environmental conditions, these studies have not provided unequivocal evidence that proline accumulation, in general, improves crop yield/biomass under abiotic stress (SERRAJ; SINCLAIR, 2002; ŠPOLJAREVIĆ et al., 2011). Indeed, it has been reported that exogenous proline application negatively affects chloroplast and mitochondrial ultrastructures (HARE et al., 2001).

To verify if a high endogenous accumulation of proline allows for an osmotic adjustment during drought stress and disrupts organelle structure in a model plant species, physiological and electron microscopy analyses were carried out in tobacco transgenic plants over-accumulating proline, due to the constitutive expression of a heterologous Δ1-pyrroline-5-carboxylate synthetase gene (*VaP5CSF129A*), under water deficit conditions.

Material and methods

Tissue culture and plant transformation

Leaf segments (1 cm^2) of 9-week-old *Nicotiana tabacum* cv. Petit Havana (SR1) plants were placed in Petri dishes with TMS medium - MS medium (MURASHIGE; SKOOG, 1962) plus 0.1 mg L^{-1} naphthaleneacetic acid (NAA), 1 mg L^{-1} 6-benzylaminopurine (BAP) and 2 g L^{-1} Phytagel™, two days before *Agrobacterium tumefaciens* co-cultivation at 27 ± 1ºC with a 16/8h (light/dark) photoperiod (30 μmol m^{-2} s^{-1}).

The disarmed *Agrobacterium tumefaciens* strain EHA105 carrying the binary plasmid pBI-P5CSF129A was used for transformation. This plasmid contains in its T-DNA region the β-glucuronidase (*uidA*) reporter gene under control of theCaMV 35S constitutive promoter, the selectable marker gene *nptII* under control of the NOS promoter and the Δ1-pyrroline-5-carboxylate synthetase mutant gene from *V. aconitifolia* (*VaP5CS129A*), also driven by the CaMV 35S promoter.

The bacterial growth and tobacco transformation were performed according to the protocols described by Brasileiro (1998).After two days of co-culture, the explants were transferred to TSM medium containing kanamycin (100 mg L^{-1}), cefotaxime (250 1 mg L^{-1}) and timentin 250 (mg L^{-1}) and were subcultured weekly. After 3-5 weeks, the shoots were isolated and transferred to Magenta™ boxes with TRM medium (MS medium plus 0.1 mg L^{-1} naphthaleneacetic acid –NAA) containing 50 mg L^{-1} kanamycin.

Micropropagation and greenhouse planting

Plantlets of approximately 2 cm and with one axillary bud were placed individually in

polypropylene-capped test tubes (150 x 25 mm) containing 15 mL of 1/2 MS salt solution without plant growth regulator and solidified with 8 g L^{-1} Bacto Agar. The microcuttings were maintained at 27 ± 1ºC with a 16h photoperiod (30 µmol $m^{-2}s^{-1}$) for 6 weeks. The rooted plantlets were transferred to planting trays containing a commercial propagation substrate (Plantmax™) with organic matter and vermiculite. After acclimatization, the T0 plantlets were transferred to 4 L plastic pots with substrate (3 parts soil:1 sand:1 manure) and grown under greenhouse conditions. All plants were kept in the greenhouse under the same growing conditions and were irrigated regularly to avoid water deficit until seeding.

Molecular analyses and selection of events

Standard PCR techniques were used to detect the presence of the *VaP5CS129A* transgene in the leaf samples from regenerated putative transgenic tobacco plantlets. The primers 5'-AGC AAC TCA ACT CTC TCG GA-3' and 5'-CCA CTC TAG ACT TGT CGC CA-3'were used to amplify a 598 bp gene fragment according to Molinari et al. (2004).

Southern blot analyses were performed to confirm the integration of the *VaP5CS129A* gene in transgenic plants. The DNA samples (20 µg) were extracted according to Dellaporta et al. (1983), digested overnight with *Hin*dIII, electrophoresed on 0.8% (w/v) agarose gel, transferred onto nylon membranes (Hybond-N1, GE Healthcare, Piscataway, NJ) and fixed through a 2h incubation at 80ºC. An *Xba*I 1.6 kb internal gene fragment was labeled using ^{32}P d-CTP by random priming and was used as probe. The hybridization and washings were performed at 65ºC and the membranes were exposed to X-ray film for 24h at -70ºC before developing.

Two different transformation events (E1 and E2) were selected for water deficit studies based on their capacity in accumulating free proline in the leaves under normal water supply conditions and on their Southern blot profiles. The T0 plants of the two events and the control non-transformed plants were kept in the greenhouse while T1 seeds were germinated on MS medium containing kanamycin (100 mg L^{-1}) for selection of antibiotic resistant plantlets.

Water deficit experiment

For the water deficit experiment, 6-week-old homogeneously sized T1 transgenic lines (E1 and E2) and control plants were cultivated under greenhouse conditions in 4 L plastic pots with 3.5 kg substrate (3 parts soil: 1 sand: 1 manure). The plastic pots were fully irrigated and drained, after which, the water was withheld for 12 days. Plants were grown under natural light in the greenhouse (approximately 800–1000 μmol m^{-2} s^{-1} PAR – photosynthetically active radiation) with a 12h photoperiod and temperature between 25 and 28ºC.

The leaf water status was monitored through thermocouple psychrometer chambers (model C-30, Wescor, Inc.) assembled with a datalogger (model CR-7, Campbell Scientific, Inc.). Two leaf discs of approximately 13 mm diameter were collected from the fourth fully expanded leaf and placed in the chambers. After obtaining the total leaf water potential (Ψw), the sensors were immersed in liquid nitrogen for the determination of the osmotic potential (Ψs). The pressure potential was obtained by the difference between the Ψw and Ψs. All components were determined in well-watered conditions and during the period of water withholding.

The photosynthetic rates (µmol CO_2 m^{-2} s^{-1}) were determined using a portable photosynthesis system (LI-COR, model LI-6200), with a 0.25 L chamber. The water status measurements were made in the same leaves under both water stressed and irrigated conditions.

Proline content

Leaf and root samples (100 mg) were ground in liquid nitrogen and free proline was extracted with 3% (w/v) sulfosalicylic acid. After centrifugation, the supernatant was used for the determination of the proline content at 520 nm as described by Bates (1973). The values are expressed in μmol proline g^{-1} leaf FW. The assays were performed in triplicate for each biological replicate.

Electron microscopy

The leaf samples of event 1 and non-transgenic plants were collected at day 0 and 12 days after withholding water. The samples were fixed in 2.5% glutaraldehyde diluted in phosphate buffer (0.1 M, pH 7.3) and the washed in phosphate buffer and post-fixed in 1% osmium tetroxide also diluted in phosphate buffer. The samples were washed in distilled water and placed on uranyl acetate 0.5% for 2h, after which, the material was dehydrated in an increscent series of acetone (50-100%), transferred to a mixture of acetone and 100% resin Araldite™ (1:1) and left overnight.. After incubation (37ºC for 1h), the samples were embedded in the appropriate Araldite™ resin molds and incubated at 60ºC for 48h. The cuttings were made with an ultrafine diamond knife microtome Ultracut (Leica™) and sections were examined and photographed in a Philips CM-100™ electron microscope.

Statistical analysis

The data were analyzed using a completely randomized design with four replicates. The differences among treatments were analyzed by one-way ANOVA, taking $p < 0.05$ as significant according to Tukey's multiple range test.

Results and discussion

Analysis of the transformation events

To determine the number of T-DNA copies that were inserted into the tobacco genome, a Southern blotting analysis was performed on 4 randomly selected plants that were GUS (data not shown) and PCR positive (Figure 1B).

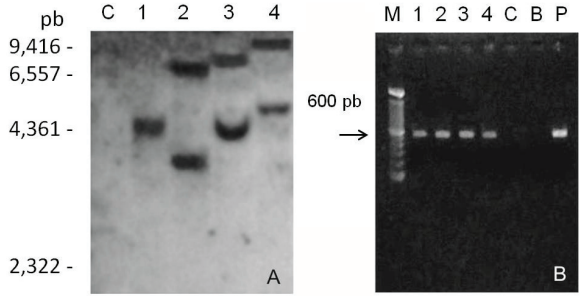

Figure 1. (A) Southern blot analysis. lane c: non-transformed control; lanes 1–4: P5CS129A transgenic events. (B) PCR amplifications of DNA from leaves of transgenic events using P5CS129A gene-specific primers to amplify a 598 bp product. Lane m: DNA marker; lane b: no DNA; lane c: negative control; lanes 1–4: transgenic events; lane p: pBI-P5CSF129A plasmid.

The Southern blotting analysis of all plants showed the integration of the transgene into the tobacco genome (Figure 1A). The pattern of the hybridization signal and the size of bands indicated that all events were independent. As expected, no hybridization signal was detected in the non-transformed control tobacco plants.

The number of hybridizing bands indicates the number of integration sites in the nuclear genome of transgenic tobacco plants. The E1 event presented only one insertion of the transgene (Figure 1A, lane 1), whereas E2, E3 and E4 showed two insertions of the transgene in their genomes. A segregation analysis by seed germination in 100 mg L^{-1} kanamycin confirmed the presence of more than one copy of the transgene in E2, E3 and E4 and a single-copy insertion in E1 (data not shown).

The choice of events for the subsequent analysis was based on preliminary examination of differences in the free proline content in the leaves of transgenic plants under normal water supply conditions.

Proline accumulation in leaves and roots

Confirming data obtained by some authors (KONSTANTINOVA et al., 2002; POSPISILOVA et al., 2011; ZHU et al., 1998), the insertion of the heterologous *p5cs* gene into tobacco plants resulted in an increased proline accumulation in leaf tissues. Under irrigated conditions (day 0), E1 and E2 presented similar leaf proline content, with approximately 10-fold higher proline levels than control plants (Figure 2).

A distinct pattern in leaf proline accumulation in the transgenic plants was observed from the 9th day of water stress until the end of the experiment, with E1 showing increased levels of proline, whereas E2 presented only a slight increase after 12 days of water deprivation (Figure 2).

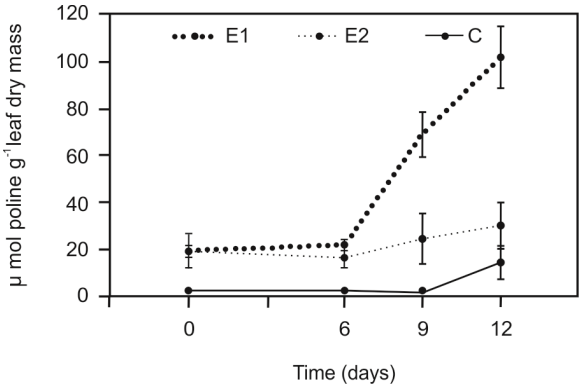

Figure 2. Proline content in leaves of non-transformed plants (c) and *P5CS129A* transgenic events E1 and E2 during 12 days of water withholding. Results are expressed in micromoles of proline per gram of leaf dry mass. Vertical bars represent standard error (n = 4).

Some factors may explain differences in the transgene expression among independent events, including the number and location of transgene insertion, effects of silencing, or even the physiological stage of the tissue (DALAKOURAS et al., 2011).

The proline content was slightly higher in leaves than in roots in the non-transformed control plants under severe water deficit stress at the end of the stress period (Figure 3).

Although the proline accumulation in roots differed considerably between the transformation events (165 and 98 μmoles g^{-1} dry weight for E1 and E2, respectively), both events presented higher values of this aminoacid in roots than in leaves (Figure 3). These data are in agreement with published reports that indicate a high proline accumulation in the growing region of maize primary roots at low water potentials as a result of an increased transportation of proline to the root tip (VERSLUES; SHARP, 1999) and that the delivery of proline to the root growing region is critical for the continued growth at low water potentials (SHARMA et al., 2011). However, Dobrá et al. (2011) showed that the proline content in *N. tabacum* cv. Wisconsin 38 under strong stress was the

highest in the upper leaves and the lowest in the roots. High proline levels in the upper leaves coincided with the highly elevated transcriptional activity of a more active isoform (*P5CSA*) in these leaves. The*P5CSA* transcript level in roots was approximately 5 times lower than the average values in leaves under strong stress.

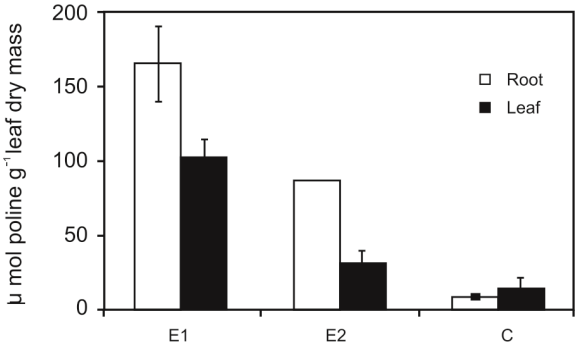

Figure 3. Proline accumulation in roots (□) and leaves (■) of *VaP5CS129A* transgenic events (E1 and E2) and control plants at the end of the experiment (12 days of water with holding). Results are expressed in micromoles of proline per gram dry mass. Vertical bars represent standard error (n = 4).

The inverse proline content observed in the roots and leaves of the CaMV 35S::*VaP5CS129A* transgenic events compared to the non-transformed control plants may be due to the differential patterns of gene expression. The CaMV 35S promoter is presumed to be a constitutive promoter, but it contains several domains and subdomains that can confer different developmental and tissue-specific expression patterns in different plants. Benfey et al. (1989) showed that 35S promoter-driven GUS activity was much higher in the root cap. Another possible explanation might be that the proline dehydrogenase genes (*PDH*), which code for the enzymes responsible for proline degradation, are dissimilarly expressed in different plant organs. Dobrá et al. (2011) reported that the expression of *PDH* genes drops rapidly both in leaves and roots in early stages of dehydration and is maintained at low levels in severe drought stress in wild-type tobacco plants; however, the relative extent of the down-regulation of *PDH* transcription was more pronounced in the leaves than in the roots. Additionally, the genotypic effects (RAMPINO et al., 2006), length and severity of the water deficit (SEČENJI et al., 2010) and pleiotropic effects of the insertion of the orthologous mutant *VaP5CS129A* on the expression of the proline metabolism-related genes cannot be ruled out in explaining the higher proline content in the roots of the transgenic plants.

Plant water status

The photosynthetic and transpiration rates of the transgenic events were superior to those of the non-transformed plants until the sixth day of water withholding, but they did not show higher rates than the control plants after 12 days without irrigation. The stomatal resistance values remained constant in both the transgenic and control plants (approximately 0.7 s.cm-1) until day 9, but increased to approximately 8 s.cm-1 at the end of the water deficit period. These data indicate that water stress limited the diffusion of gases in the transgenic plants as well confirming that neither event E1 nor E2 had a greater tolerance to water stress compared to the control plants (Figure 4A-C). In well-watered conditions, the leaves of the transgenic plants showed total water potential very close to that of the control plants. After water withholding, the total and osmotic potentials in the transgenic plants increased slightly until the sixth day without irrigation, however, from the ninth day until the end of the water deficit stress, these two parameters showed a strong decrease.

The high levels of proline accumulated in the transgenic plants (Figure 2) did not lead to osmotic potentials lower than the total water potentials (Figure 5A and B).

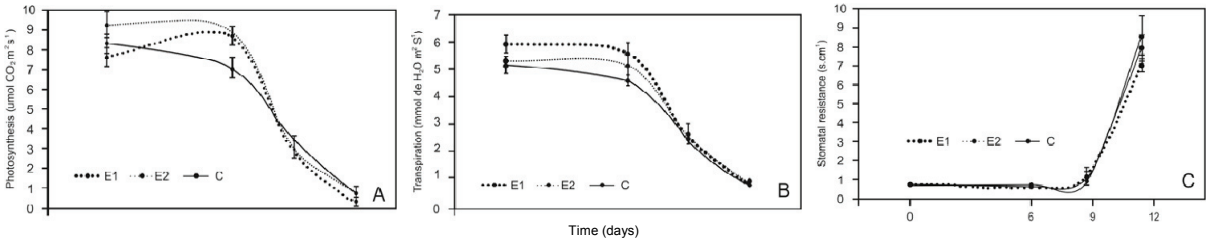

Figure 4. Changes in the (A) photosynthetic rate (μmol CO_2 m^{-2} s^{-1}), (B) transpiration (mmol H_2O m^{-2} s^{-1}) and (C) stomatal resistance ($s.cm^{-1}$) in *VaP5CS129A* transgenic tobacco plants (events E1 and E2) and control plants (c) during 12 days without irrigation. Vertical bars represent standard error (n = 4).

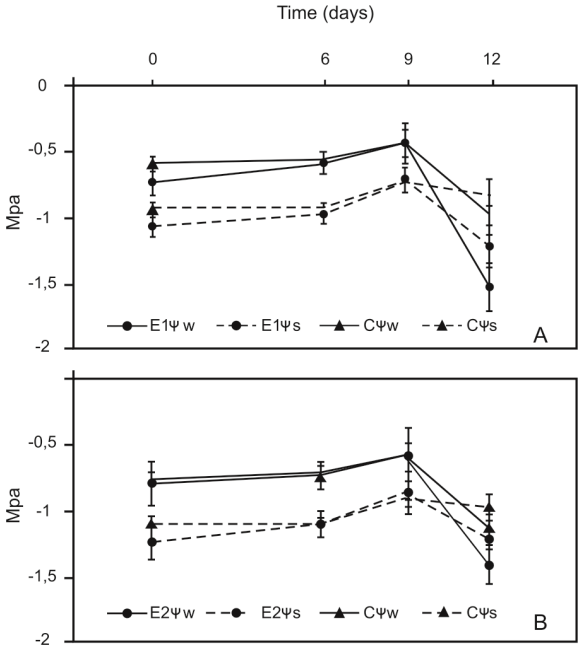

Figure 5. Estimated total water (Ψw) and osmotic (Ψs) potentials in the leaves of the non-transformed control (c) and *P5CSF129A* transgenic events E1 (a) and E2 (b) submitted to 12 days of water deficit. Potential values expressed in mega Pascal (MPa). Vertical bars represent standard error (n = 4).

The decline of the pressure potentials after 9 days of water deficit also indicates the lack of osmotic adjustment in the transgenic events (Figure 6).

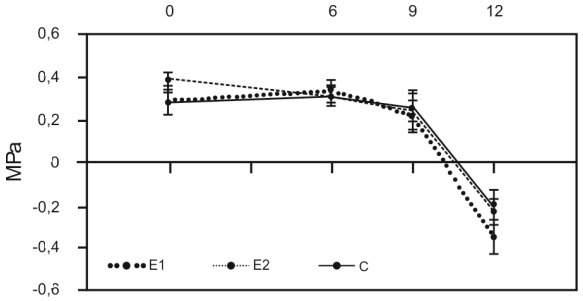

Figure 6. Pressure potential of the *VaP5CS129A* transgenic (E1 and E2) and control tobacco plants (c) submitted to 12 days of water withholding. Potential values expressed in mega Pascal (MPa). Vertical bars represent standard error (n = 4).

It has been reported that high levels of proline can be accompanied by an osmotic adjustment (ASHRAF; FOOLAD, 2007; DELAUNEY; VERMA, 1993; KISHOR et al., 1995; MOLINARI et al., 2004). Compared to the work of Molinari et al. (2004), which demonstrated that Carrizo citrange transgenic plants accumulating approximately 250 μmol g^{-1} proline were able to adjust osmotically and maintain turgor under osmotic stress, the tobacco transgenic events in this study accumulated much less proline in their leaves and roots (approximately 100 and 160 μmol g^{-1}, respectively). In our study, the levels of proline accumulated by the transgenic plants were not sufficient to allow for an osmotic adjustment.

Other authors have reported that proline accumulation does not lead to drought tolerance (LUTTS et al., 1996; RHODES; HANSON, 1993; RODRIGUEZ et al., 1997), suggesting that the significance of proline accumulation in osmotic adjustment is much less than expected and can vary according to the species. Gomes et al. (2010) demonstrated that two contrasting ecotypes of coconut palm with equivalent responses to drought presented a low osmotic adjustment despite an expressive accumulation of proline in their leaves under water deficit. Similar findings were reported for calli of *Triticum durum* genotypes after water stress (LUTTS et al., 2004) and for drought-stressed *Phragmites australis* (PAGTER et al., 2005). Additionally, the accumulation of free proline in the leaves of drought-susceptible genotypes of cassava and common beans is considered only as a single indicator of the stress condition (SUNDARESAN; SUDHAKARAN, 2006).

Mitochondria and chloroplast ultrastructure

The molar concentrations of exogenous proline were shown to have toxic effects on subcellular structures such as mitochondria and chloroplasts (HARE et al., 2001). According to these authors, the damage observed in the chloroplasts of plants subjected to the exogenous application of high concentrations of proline is consistent with the hypothesis that the synthesis of proline may contribute to the continued activity of the photosystems, avoiding an over-reduction of the photosynthetic electron acceptor pools. To determine whether higher endogenous levels of proline have some toxic effect on the mitochondria and chloroplasts, we carried out a transmission electron microscopy analysis of the leaf tissue of event 1 and non-transformed control plants grown under normal water supply and severe water deficit conditions (12 days of water withholding).

No changes were observed in either mitochondria or chloroplast ultrastructure, indicating that the constitutive levels of endogenous proline in these plants had no toxic effect on these organelles (Figure 7).

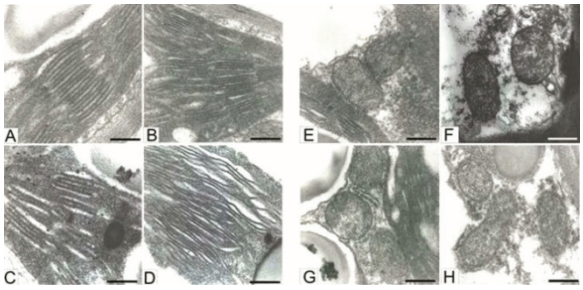

Figure 7. Electron micrographs of tobacco leaves, illustrating chloroplast (A-D) and mitochondria (E-H) ultrastructure of *VaP5CS129A* transgenic plants (Event 1) and non-transformed control plants after 12 days of water with holding and under normal water supply conditions. Transgenic plants under water deficit (A, E) and normal water supply (B, F). Control plants under water deficit (C, G) and normal water supply (D, H). Scale bars = 0,1 μm.

Therefore, the data obtained in this study using *VaP5CS129A* transgenic plants did not substantiate the damage to mitochondrial cristae and chloroplast thylakoid membranes in *Arabidopsis thaliana* plants observed by Hare et al. (2001) while exogenously applying proline at concentrations ranging from 5-20 mM.

Other studies also have shown that the external supply of proline has a toxic effect on plant and animal cells (DEUSCHLE et al., 2004; DONALD et al., 2001; NANJO et al., 2003). In our study, even significantly higher levels of endogenous free proline in the tobacco leaves under water deficit conditions (100 μmol g^{-1} dry mass, approximately equivalent to 100 mM) were not able to cause such toxic effects in the organelle ultrastructure.

It has been suggested that the hypersensitivity to exogenous proline may be mediated by P5C as a degradation intermediate of proline (HELLMANN et al., 2000). The inhibition of P5C catabolism led to the toxicity of proline in a yeast mutant deficient in P5C dehydrogenase (P5CDH) (DEUSCHLE et al., 2001), which was attributed to the P5C-induced production of reactive oxygen. Based on the fact that the external supply of proline induced *P5CDH* expression at a much more level than *PDH* expression, the toxicity effects of exogenously applied proline in *Arabidopsis thaliana* have been credited to the uncoupled induction of the *PDH* and *P5CDH*genes (DEUSCHLE et al., 2001; DEUSCHLE et al., 2004). Miller et al. (2009) showed that P5C generated from proline oxidation in mitochondria can be transported to the cytosol and then converted back to proline by P5C reductase (P5CR), thus helping to maintain the intracellular proline:P5C ratio. A high level of exogenously supplied proline increases the Proline-P5C cycle in the plant cell, causing an overflow of electrons into the generation of ROS (MILLER et al., 2009). In this work, the apparent lack of proline toxicity to chloroplasts and mitochondria observed in the high-producing proline transformation event (Event 1) suggests that the plants were able to maintain cellular homeostasis. Further studies will be required to assess if the high endogenous over-production of this amino acid, different from externally supplied proline, causes a lesser imbalance between proline biosynthesis and catabolism, resulting in minor underlying consequences.

Thus, we have demonstrated that the increased endogenous proline concentrations were not able to provide an osmotic adjustment in transgenic tobacco plants constitutively expressing the mutant gene *VaP5CS129A* subjected to drought stress. We have also shown that very high levels of endogenous proline in tobacco plants under both normal irrigated and severe water deficit conditions did not cause any visible damage on the chloroplast and mitochondria ultrastructure.

Conclusion

The main conclusion of this research comes from the finding that high proline production induced by water deficit in transgenic tobacco plants had no relation with osmotic adjustment, based in physiological parameters as total and osmotic potentials that showed strong decrease at the end of water stress.

We can also conclude that high proline accumulation in leaves induced by water stress did not cause morphological disturbances in chloroplast and mitochondria ultrastructure.

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References

ASHRAF, M.; FOOLAD, M. R. Roles of glycine betaine and proline in improving plant abiotic stress resistance. **Environment and Experimental Botany**, v. 59, n. 2, p. 206-216, 2007.

BATES, L. S. Rapid determination of free proline for water stress studies. **Plant and Soil**, v. 39, n. 1, p. 205-207, 1973.

BENFEY, P. N.; REN, L.; CHUA, N. H. The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. **EMBO Journal**, v. 8, n. 8, p. 2195-2202, 1989.

BRASILEIRO, A. C. M. Interação *Agrobacterium* - Hospedeiro. In: BRASILEIRO, A. C. M.; CARNEIRO,

V. T. C. (Ed.). **Manual de transformação genética de plantas**. Brasília: Embrapa-SPI/Embrapa-Cenargen, 1998. p. 76-89.

CAMPOS, M. K. F.; CARVALHO, K.; SOUZA, F. S.; MARUR, C. J.; PEREIRA, L. F. P.; BESPALHOK FILHO, J. C.; VIEIRA, L. G. E. Drought tolerance and antioxidant enzymatic activity in transgenic 'Swingle' citrumelo plants over-accumulating proline. **Environment and Experimental Botany**, v. 72, n. 2, p. 242-250, 2011.

CHAVES, M. M.; FLEXAS, J.; PINHEIRO, C. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. **Annals of Botany**, v. 103, n. 4, p. 551–560, 2009.

CVIKROVÁ, M.; GEMPERLOVÁ, L.; DOBRÁ, J.; MARTINCOVÁ, O.; PRÁSIL, I. T.; GUBIS, J.; VANKOVÁ, R. Effect of heat stress on polyamine metabolism in proline-over-producing tobacco plants. **Plant Science**, v. 182, p. 49-58, 2012.

DALAKOURAS, A.; MOSER, M.; BOONROD, K.; KRCZAL, G.; WASSENEGGER, M. Diverse spontaneous silencing of a transgene among two Nicotiana species. **Planta**, v. 234, n. 4, p. 699-707, 2011.

DELAUNEY, A. J.; VERMA, D. P. S. Proline biosynthesis and osmoregulation in plants. **Plant Journal**, v. 4, n. 2, p. 215-223, 1993.

DELLAPORTA, S. L.; WOOD, J.; HICHS, J. B. A plant minipreparation: version II. **Plant Molecular Biology Reporter**, v. 1, n. 4, p. 19-20, 1983.

DEUSCHLE, K.; FUNCK, D.; FORLANI, G.; STRANSKY, H.; BIEHL, A.; LEISTER, D.; VAN DER GRAAFF, E.; KUNZE, R.; FROMMER, W. B. The role of Δ1-pyrroline-5-carboxylate dehydrogenase in proline degradation. **Plant Cell**, v. 16, n. 12, p. 3413-3425, 2004.

DEUSCHLE, K.; FUNCK, D.; HELLMANN, H.; DÄSCHNER, K.; BINDER, S.; FROMMER, W. B. A nuclear gene encoding mitochondrial Δ1-pyrroline-5-carboxylate dehydrogenase and its potential role in protection from proline toxicity. **Plant Journal**, v. 27, n. 4, p. 345-355, 2001.

DOBRÁ, J.; VANKOVÁ, R.; HAVLOVÁ, M.; BURMAN, A. J.; LIBUS, J.; ŠTORCHOVÁ, L. Tobacco leaves and roots differ in the expression of proline metabolism-related genes in the course of drought stress and subsequent recovery. **Journal of Plant Physiology**, v. 168, n. 13, p. 1588-1597, 2011.

DONALD, S. P.; SUN, X. Y.; HU, C. A.; YU, J.; MEI, J. M.; VALLE, D.; PHANG, J. M. Proline oxidase, encoded by p53-induced gene-6, catalyzes the generation of proline-dependent reactive oxygen species. **Cancer Research**, v. 61, n. 3, p. 1810-1815, 2001.

GOMES, F. P.; OLIVA, M. A.; MIELKE, M. S.; ALMEIDA, A. A. F.; AQUINO, L. A. Osmotic adjustment, proline accumulation and cell membrane stability in leaves of *Cocosnucifera* submitted to drought stress. **Scientia Horticulturae**, v. 126, n. 3, p. 379-384, 2010.

GUBIS, J.; VANKOVÁ, R.; CERVENÁ, V.; DRAGUNOVÁ, M.; HUDCOVICOVÁ, M.; LICHTNEROVIA, H.; DOKUPIL, T.; JUREKOVÁ, Z. Transformed tobacco plants with increased tolerance to drought. **South Africa Journal of Botany**, v. 73, n. 4, p. 505-511, 2007.

HARE, P. D.; CRESS, W. A. Metabolic implications of stress-induced proline accumulation in plants. **Plant Growth Regulation**, v. 21, n. 2, p. 79-102, 1997.

HARE, P. D.; CRESS, W. A.; VANSTADEN, J. Disruptive effects of exogenous proline on chloroplast and mitochondrial ultrastructure in Arabidopsis leaves. **South Africa Journal of Botany**, v. 68, n. 3, p. 393-396, 2001.

HELLMANN, H.; FUNCK, D.; RENTSCH, D.; FROMMER, W. B. Hypersensitivity of an Arabidopsis sugar signaling mutant toward exogenous proline application. **Plant Physiology**, v. 122, n. 2, p. 779-789, 2000.

HONG, Z.; LAKKINENI, K.; ZHANG, Z.; VERMA, D. P. S. Removal of feedback inhibition of Δ1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. **Plant Physiology**, v. 122, n. 4, p. 1129-1136, 2000.

KISHOR, K. P. B.; HONG, Z.; MIAO, G. H.; HU, C. A. A.; VERMA, D. P. S. Overexpression of Δ1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. **Plant Physiology**, v. 108, n. 4, p. 1387-1394, 1995.

KISHOR, K. P. B.; SANGAM, S.; AMRUTHA, R. N.; LAXMI, P. S.; NAIDU, K. R.; RAO, K. R. S. S.; RAO, S.; REDDY, K. J.; THERIAPPAN, P.; SREENIVASULU, N. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. **Current Science**, v. 88, n. 3, p. 424-438, 2005.

KONSTANTINOVA, T.; PARVANOVA, D.; ATANASSOV, A.; DJILIANOV, D. Freezing tolerant tobacco, transformed to accumulate osmoprotectans. **Plant Science**, v. 163, n. 1, p. 157-164, 2002.

LUTTS, S.; ALMANSOURI, M.; KINET, J. M. Salinity and water stress have contrasting effects on the relationship between growth and cell viability during and after stress exposure in durum wheat callus. **Plant Science**, v. 167, n. 1, p. 9-18, 2004.

LUTTS, S.; KINET, J. M.; BOUHARMONT, J. Effects of salt stress on growth, mineral nutrition and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) cultivars differing in salinity tolerance. **Plant Growth Regulation**, v. 19, n. 3, p. 207-218, 1996.

MILLER, G.; STEIN, H.; HONIG, A.; KAPULNIK, Y.; ZILBERSTEIN, A. Responsive modes of *Medicago sativa* proline dehydrogenase genes during salt stress and recovery dictate free proline accumulation. **Planta**, v. 222, n. 1, p. 70-79, 2005.

MILLER, G.; HONIG, A.; STEIN, H.; SUZUKI, N.; MITTLER, R.; ZILBERSTEIN, A. Unraveling Δ1-pyrroline-5-carboxylate-proline cycle in plants by uncoupled expression of proline oxidation enzymes. **Journal of Biological Chemistry**, v. 284, n. 39, p. 26482-26492, 2009.

MOLINARI, H. B. C.; MARUR, C. J.; BESPALHOK, J. C. F.; KOBAYASHI, A. K.; PILEGGI, M.; LEITE, R. P. J.; PEREIRA, L. F. P.; VIEIRA, L. G. E. Osmotic adjustment in transgenic citrus rootstock Carrizo citrange (*Citrus sinensis* Osb. X *Poncirustrifoliata* L. Raf.) overproducing proline. **Plant Science**, v. 167, n. 6, p. 1375-1381, 2004.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissues culture. **Physiologia Plantarum**, v. 15, n. 3, p. 473-479, 1962.

NANJO, T.; FUJITA, M.; SEKI, M.; KATO, T.; TABATA, S.; SHINOZAKI, K. Toxicity of free proline revealed in an Arabidopsis T-DNA-tagged mutant deficient in proline dehydrogenase plant. **Cell Physiology**, v. 44, n. 5 , p. 541-548, 2003.

PAGTER, M.; BRAGATO, C.; BRIX, H. Tolerance and physiological responses of *Phragmitesaustralis* to water deficit. **Aquatic Botany**, v. 81, n. 4, p. 285-299, 2005.

POSPISILOVA, J.; HAISEL, D.; VANKOVA, D. Responses of transgenic tobacco plants with increased proline content to drought and/or heat stress. **American Jounal of Plant Science**, v. 2, n. 3, p. 318-324, 2011.

POUSTINI, K.; SIOSEMARDEH, A.; RANJBAR, M. Proline accumulation as a response to salt stress in 30 wheat (*Triticumaestivum* L.) cultivars differing in salt tolerance. **Genetic Resources and Crop Evolution**, v. 54, n. 5, p. 925-934, 2007.

RAMPINO, P.; PATALEO, S.; GERARDI, C.; MITA, G.; PERROTTA, C. Drought stress response in wheat: physiological and molecular analysis of resistant and sensitive genotypes. **Plant Cell Environment**, v. 29, n. 12, p. 2143-2152, 2006.

RHODES, D.; HANSON, A. D. Quaternary ammonium and tertiary sulfonium compounds in higher plants. **Annual Review Plant Physiology and Plant Molecular Biology**, v. 44, p. 357-384, 1993.

RODRIGUEZ, H. G.; ROBERTS, J. K. M.; JORDAN, W. R.; DREW, M. C. Growth, water relations, and accumulation of organic and inorganic solutes in roots of maize seedlings during salt stress. **Plant Physiology**, v. 113, n. 3, p. 881-893, 1997.

SEČENJI, M.; LENDVAI, Á.; MISKOLCZI, P.; KOCSY, G.; GALLÉ, Á.; SZŰCS, A.; HOFFMANN, B.; SÁRVÁRI, É.; SCHWEIZER, P.; STEIN, N.; DUDITS, D.; GYÖRGYEY, J. Differences in root functions during long-term drought adaptation: comparison of active gene sets of two wheat genotypes. **Plant Biology**, v. 12, n. 6, p. 871-882, 2010.

SERRAJ, R.; SINCLAIR, T. R. Osmolyte accumulation: can it really help increase crop yield under drought conditions? **Plant Cell Environment**, v. 25, n. 2, p. 333-341, 2002.

SHARMA, S.; VILLAMOR, J. G.; VERSLUES, P. E. Essential role of tissue-specific proline synthesis and catabolism in growth and redox balance at low water potential. **Plant Physiology**, v. 157, n. 1, p. 292-304, 2011.

ŠPOLJAREVIĆ, M.; AGIĆ, D.; LISJAK, M.; GUMZE, A.; WILSON, I. D.; HANCOCK, J. T.; TEKLIĆ, T. The relationship of proline content and metabolism on the productivity of maize plants. **Plant Signaling and Behavior**, v. 6, n. 2, p. 251-257, 2011.

SUNDARESAN, S.; SUDHAKARAN, P. R. Water stress-induced alterations in the proline metabolism of drought-susceptible and drought-tolerant cassava (*Manihot esculenta*) cultivars. **Physiologia Plantarum**, v. 94, n. 4, p. 635-642, 2006.

SZABADOS, L.; SAVOURÉ, A. Proline: a multifunctional amino acid. **Trends in Plant Science**, v. 15, n. 2, p. 89-97, 2010.

VALLIYODAN, B.; NGUYEN, H. T. Understanding regulatory networks and engineering for enhanced drought tolerance in plants. **Current Opinion in Plant Biology**, v. 9, n. 2, p. 189-195, 2006.

VERBRUGGEN, N.; HERMANS, C. Proline accumulation in plants: a review. **Amino Acids**, v. 35, n. 4, p. 753-759, 2008.

VERSLUES, P. E.; SHARP, R. E. Proline accumulation in maize (*Zea mays* L.) primary roots at low potentials. Metabolic source of increased proline deposition in the elongation zone. **Plant Physiology**, v. 119, n. 4, p. 1349-1360, 1999.

ZHANG, C. S.; LU, Q.; VERMA, D. P. S. Removal of feedback inhibition of Δ-1-pyrroline-5-carboxylate synthetase, a bifunctionalenzyme catalyzing the first 2 steps of proline biosynthesis in plants. **Journal of Biological Chemistry**, v. 270, n. 35, p. 20491-20496, 1995.

ZHU, B.; SU, J.; CHANG, M. C.; VERMA, D. P. S.; FAN, Y. L.; WU, R. Overexpression of Δ1-pyrroline-5-carboxylate synthetase gene and analysis of tolerance to water and salt-stressed in transgenic rice. **Plant Science**, v. 139, n. 1, p. 41-48, 1998.

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