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Research article

Biochemical composition of symplastic sap from sugarcane genetically modified to overproduce proline

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ABSTRACT

Global interest in sugarcane has increased significantly in recent years because of its economic impact on sustainable energy production. The purpose of the present study was to evaluate changes in the concentrations of total sugars, amino acids, free proline, and total proteins by colorimetric analyses and nuclear magnetic resonance (NMR) to perform a metabolic profiling of a water-soluble fraction of symplastic sap in response to the constitutive expression of a mutant Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*) gene from *Vigna aconitifolia*. However, there was not a significant increase in the free proline content in the sap of transgenic plants compared to the non-transformed control plants. The most noticeable difference between the two genotypes was an almost two-fold increase in the accumulation of sucrose in the stem internodes of P5CS transgenic sugarcane plants. The results presented in this work showed that transgenic sugarcane plants with increased levels of free proline accumulates high soluble sugar content and, therefore, may represent a novel genotype for improving sugarcane cultivars.

1. Introduction

Sugarcane (Saccharum spp.) is the main crop for the production of sucrose and byproducts worldwide (Costa et al., 2013; Kelbert et al., 2015; Matsuoka et al., 2009). The stem internodes of sugarcane store high concentrations of reserve carbohydrates that are deposited in both the vacuoles of parenchyma cells (symplasts) and intercellular spaces (apoplasts) that surround these cells (Welbaum and Meinzer, 1990). In sugarcane stem sap, sucrose not only represents the carbohydrate storage form but is also the principal photosynthesis product and transport carbohydrate.

Although current research programs seek to improve technological solutions for sugarcane production, higher levels of sustainability and production efficiency are needed, which in turn will depend largely on new knowledge. The development and utilization of genetic modification to incorporate new genes into sugarcane are presently promising tools for the sugarcane industry (Cheavegatti-Gianotto et al., 2011; Lakshmanan et al., 2005). Genetically modified (GM) sugarcane that possess genes that mitigate damages inflicted by abiotic stress is a promising approach to reduce crop losses to economically acceptable levels (Cheavegatti-Gianotto et al., 2011; Kishor et al., 2005; Molinari et al., 2007).

One of the strategies that have been used to obtain transgenic sugarcane with higher tolerance to abiotic stress relied on the increased accumulation of the amino acid proline. In plants, proline is formed mostly from glutamate via Δ^1-pyrroline-5-carboxilate (P5C) by two successive reductions that are catalyzed by P5C synthetase (P5CS) and P5C reductase (P5CR) (Hu et al., 2007), with the former considered the key enzyme for its synthesis (Zhang et al., 1995). Transgenic sugarcane plants with high proline production were shown to be more drought-tolerant under severe water-

Abbreviations: GM, genetically modified; SAC, apoplastic sap extracted from control sugarcane; SAT, apoplastic sap extracted from transgenic sugarcane.

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deficit conditions (Molinari et al., 2007). These plants provide valuable material not only for studies on the mechanisms of tolerance to such abiotic stress but also for potential use in production systems.

In addition, it has been reported that exogenous foliar application of proline increased the accumulation of soluble sugars in the model plant *Arabidopsis thaliana* (Moustakas et al., 2011). These authors suggested that higher free proline content triggers soluble sugars accumulation by the interaction of both proline and soluble sugars signaling pathways. Also, Rasheed et al. (2011) observed that soaking of sugarcane buds in proline increases the concentrations not only of this amino acid but also of soluble sugars, suggesting a relationship between the accumulation of these metabolites.

Thus, this study aimed to evaluate the biochemical composition of sugarcane sap, mainly free proline and soluble sugars, using colorimetric and NMR analysis of a water-soluble fraction of the symplastic sap in sugarcane plants constitutively expressing a mutant Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*) gene from *Vigna aconitifolia* in comparison to non-transformed control plants.

2. Material and methods

2.1. Plant materials

Transgenic and control plants (*Saccharum* spp.) were cultivated in a greenhouse at the Agronomic Institute of Paraná (IAPAR) under identical growth conditions. The transgenic event was produced with the sugarcane cultivar RB855536, which was transformed with a mutant Δ^1-pyrroline-5-carboxylate synthetase (*P5CSF129A*) gene of *Vigna aconitifolia* under control of the constitutive ubiquitin promoter from maize (*Ubi-1*) according to a previously reported procedure (Molinari et al., 2007), including the tissue culture protocol, target tissue preparation, micro-projectile bombardment, and subsequent regeneration. Embryogenic calli from immature leaves were bombarded with the pIBI31 plasmid that contained the pUbi-1:: *VaP5CSF129A*::Tnos construct. The integration of the transgene into the plant genome was assessed by polymerase chain reaction and Southern blot, while the transgene expression was directly confirmed by the increased free proline content in the leaves compared with control plants (data not shown).

Stem internodes from all portions of both GM and control sugarcane plants were grown in 17 L plastic pots filled with equal volumes of substrate (3:1, soil:sand) under the same conditions until maturity. After this, the sugarcane stalks were cut at their base and left to grow again in the same pots until sampling at the beginning of the sucrose accumulation stage (180–190 days). The internodes were divided and then immediately frozen in liquid nitrogen and stored at −80 °C until sap extraction. The pots were fertilized twice with a 10:10:10 ratio of nitrogen:phosphorus:potassium at 0.5 g/L soil at the initial growth and after the first cutting, and the plants were equally irrigated and placed in similar positions relative to incident solar radiation.

2.2. Sap extraction

Apoplastic sap extraction from the plant stems was performed according to the methodology described by Dong et al. (1994). Stem internodes that were pooled from all parts of the cane were transversely cut into pieces (3–4 cm length). The stems pieces were immersed in ethanol in a glass container for 10 min and then exposed to a flame within a laminar flow hood. After this treatment, sap was collected by centrifugation at 3000 × *g* for 20 min at 5 °C (Tejera et al., 2006). Apoplastic sap that was extracted from transgenic sugarcane (SAT) and control sugarcane (SAC) was centrifuged to remove impurities and then stored at −20 °C.

After centrifugation, the remaining fragments were crushed in a blender to release the symplastic sap (i.e., vacuolar fluid from the cell) and filtered through a cheese cloth. Lignified material was removed by centrifugation at 5000 × *g* for 10 min at 5 °C and then frozen.

Symplastic sap that resulted from the extraction was lyophilized and stored in a freezer at −20 °C until the chemical analysis.

2.3. Total amino acid and proline determination

Free amino acid determination was performed by colorimetric analysis using ninhydrin reagent (Magné and Larher, 1992). Solutions were prepared from dried symplastic sap at a concentration of 1.0 mg/mL. These solutions were transferred to 0.5 mL test tubes that contained 0.2 mol/L citrate buffer, pH 4.6 (0.21 g citric acid in 40.0 mL of distilled water and 40.0 mL of 1 mol/L NaOH in 100.0 mL of distilled water). Subsequently, 1.0 mL of sap solution or L-leucine standard solution and 1 mL of ninhydrin solution were added to the reaction mixture. The tubes were then cooled to room temperature in a tap water bath, and 3 mL of 60% ethyl alcohol in water was added as a diluent. The absorbance of the chromophore was taken within 1 h at 570 nm using a Varian Cary Modelo 1E UV-VIS spectrophotometer, and the linear regression curve was constructed using L-leucine standard at concentrations of 10–100 μg/mL.

Free proline determination was performed using 1% ninhydrin solution that was prepared in glacial acetic acid:water (60:40, v/v). An aliquot of 0.5 mL of the sample (50 mg/mL) was pipetted into a test tube with 2 mL of the ninhydrin solution (Bates et al., 1973). After shaking, the tube was heated in a boiling water bath for 1 h. The tubes were then cooled to room temperature in a tap water bath, and 5 mL of toluene was added. After 15 s of vigorous shaking, was observed the phases separation and the absorbance of the upper phase was taken at 520 nm. The linear regression curve was constructed using L-proline standard at concentrations of 10–60 μg/mL.

2.4. Sugar determination

The determination of total sugars was performed after preparing a solution of symplastic sap at a concentration of 50 μg/mL and analyzed using the phenol-sulfuric acid method (Dubois et al., 1956). An aliquot of 1 mL of the sap solution were poured into test tubes with 0.5 mL of reagent solution A (5.0 g phenol in water to 100.0 mL) and stirred for 10 s. Then was added 2.5 mL of reagent solution B (concentrated sulfuric acid) with vigorous stirring. The solutions were heated in a water bath for 10 min and then cooled for 15 min. Absorbance was taken at 490 nm, and a standard curve was constructed using glucose at concentrations of 10–60 μg/mL.

The determination of reducing sugars was performed at a concentration of 40 μg/mL and analyzed using the *p*-hydroxybenzoic

Table 1
Proximate composition of the symplastic sap from *VaP5CSF129A* genetically modified (GM) and control sugarcane plants (mean ± SD; $n = 3$).

Component	GM (mg/g)	Control (mg/g)
Total amino acids	2.80 ± 0.01[a]	2.00 ± 0.02[b]
Proline	0.068 ± 0.001	0.050 ± 0.002
Total sugars	133.58 ± 1.84[a]	67.73 ± 1.13[b]
Reducing sugars	20.00 ± 0.61[a]	7.22 ± 0.39[b]
Glucose	6.26 ± 0.08[a]	1.77 ± 0.03[b]
Fructose	13.72 ± 0.77[a]	5.45 ± 0.41[b]
Sucrose	113.58 ± 2.45[a]	60.51 ± 1.52[b]

Within each row, different lowercase letters indicate a significant difference ($p < 0.05$). The absence of lowercase letter indicates no significant difference.