Plant Physiology and Biochemistry 115 (2017) 484–496

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Salt-tolerance mechanisms induced in *Stevia rebaudiana* Bertoni: Effects on mineral nutrition, antioxidative metabolism and steviol glycoside content

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ARTICLE INFO

Article history:
Received 3 February 2017
Received in revised form
24 April 2017
Accepted 25 April 2017
Available online 26 April 2017

Keywords:
Chlorophyll fluorescence
Mineral nutrition
Physiological response
Stevia plants
NaCl stress
Oxidative stress
Steviol glycosides

ABSTRACT

In order to cope with challenges linked to climate change such as salinity, plants must develop a wide spectrum of physiological and molecular mechanisms to rapidly adapt. *Stevia rebaudiana* Bertoni plants are a case in point. According to our findings, salt stress has no significant effect on plant growth in these plants, which accumulate sodium (Na^+) in their roots, thus avoiding excessive Na^+ accumulation in leaves. Furthermore, salt stress (NaCl stress) increases the potassium (K^+), calcium (Ca^{2+}), chloride ion (Cl^-) and proline concentrations in Stevia leaves, which could contribute to osmotic adjustment. We also found that long-term NaCl stress does not produce changes in chlorophyll concentrations in Stevia leaves, reflecting a mechanism to protect the photosynthesis process. Interestingly, an increase in chlorophyll *b* (Chlb) content occured in the oldest plants studied. In addition, we found that NaCl induced reactive oxygen species (ROS) accumulation in Stevia leaves and that this accumulation was more evident in the presence of 5 g/L NaCl, the highest concentration used in the study. Nevertheless, Stevia plants are able to induce (16 d) or maintain (25 d) antioxidant enzymes to cope with NaCl-induced oxidative stress. Low salt levels did not affect steviolbioside and rebaudioside A contents. Our results suggest that Stevia plants induce tolerance mechanisms in order to minimize the deleterious effects of salt stress. We can thus conclude that saline waters can be used to grow Stevia plants and for Steviol glycosides (SGs) production.

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1. Introduction

Environmental stress factors, including salt stress, are an important threat to agriculture in the context of climate change (Ahuja et al., 2010). Salinity is considered to be the major environmental factor limiting plant growth, particularly in arid and semi-arid regions such as Mediterranean areas, where water availability is very restricted. The presence of NaCl in soils induces osmotic stress as well as ion toxicity in all plant organs due to excessive accumulation of the phytotoxic ions sodium (Na^+) and chloride (Cl^-). This in turn leads to nutritional imbalance due to a decrease in other important nutrients, such as calcium (Ca^{+2}), magnesium (Mg^{+2}) and potassium (K^+) (Munns and Tester, 2008; Parida and Das, 2005). The accumulation of toxic ions can be a positive mechanism for dealing with osmotic stress, but only if plants have the ability to compartmentalize these ions inside the vacuole (Acosta-Motos et al., 2015b). In addition, plants can induce the synthesis of osmolytes such as amino acids or nitrogen-containing molecules, including proline or glycine-betaine, which also contributes to osmotic adjustment, maintaining plant turgor (Ashraf and Foolad, 2007).

The inhibition of plant growth induced by salinity is associated with a decrease in the photosynthesis rate. It is known that salt

Abbreviations: APX, ascorbate peroxidase; ASC, ascorbate reduced form; CAT, catalase; DHA, ascorbate oxidized form; DHAR, dehydreascorbate reductase; DW, dry weight; FW, fresh weight; GR, glutathione reductase; GSH, glutathione reduced form; GSSG, glutathione oxidized form; gs, stomatal conductance; H_2O_2, hydrogen peroxide; LWC, leaf water content; LP, lipid peroxidation; MDHAR, monodehydroascorbate reductase; MEP, 2-C-methyl-D-erythritol-4-phospate; NADH, nicotinamide adenine dinucleotide reduced form; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; O_2^-, superoxide anion; $^{\cdot}OH$, hydroxyl radicals; PAR, photosynthetically active radiation; POX, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; SG, steviol glycosides; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive-substances; UGTs, UDP-glycosyltransferases.

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http://dx.doi.org/10.1016/j.plaphy.2017.04.023
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stress (NaCl stress) affects the photosynthetic process due to stomatal closure that limits CO_2 fixation (Arbona et al., 2013) and reduces chlorophyll (Chl) content. Certain plants, however, have developed mechanisms to counteract the deleterious effects of salinity, acquiring tolerance to such stress. Furthermore, salt stress also causes oxidative stress mediated by the enhanced generation of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) (Demidchik, 2015; Hernández et al., 1993). The overproduction of these radicals could be explained as an imbalance of the electron transport chain caused by a stressful condition. The role of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and the ascorbate-gluthatione (ASC-GSH) cycle enzymes [ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and gluthatione reductase (GR)] as ROS scavengers during salt stress is another important tolerance mechanism against oxidative stress, and, therefore, against salt stress (Acosta-Motos et al., 2015a, 2015b; Gill and Tuteja, 2010; Hernández et al., 2000, 2001). In fact, numerous authors have reported that NaCl-tolerant plant species either induce or are able to show a much higher antioxidant capacity than NaCl-sensitive species (Hernández et al., 2000, 2003; López-Gómez et al., 2007; Mittova et al., 2003).

Few authors have focused their attention, however, on the response to salt stress in the *Asteraceae* family, and studies concerning the effect of such stress on Stevia are scarce. *Stevia rebaudiana* Bertoni is a perennial shrub belonging to the *Asteraceae* family. The leaves of *S. rebaudiana* contain a high concentration of steviol glycosides (SGs). Stevioside and Rebaudioside A are the major SGs (Zeng et al., 2013). These molecules are glucosylated derivatives of diterpenoid steviol, and they are used as non-caloric sweeteners in many countries due to the fact that steviol glycosides are sweeter than sucrose (Lorenzo et al., 2014). Moreover, SGs have also shown multiple therapeutic properties, most notably their antihyperglycemic and antihypertensive effects (Abudula et al., 2004; Mishra et al., 2010).

SGs are biosynthetised by the plastidial 2-C-methyl-D-erythritol-4-phospate pathway, also known as the MEP pathway, which is a common pathway with gibberelins. The final step of SGs biosynthesis occurs in cytosol catalysed by 4 UDP-glycosyltransferases (UGTs). UGT85C2 attaches one molecule of D-glucose to C-13 of steviol to produce steviolmonoside. The next reaction of glycosylation leads to the formation of steviolbioside, catalysed by a UGT that has not yet been characterised (Modi et al., 2012). UGT74G1 is involved in the formation of stevioside by the glycosylation of C-4 carboxilic acid of steviolbioside. Rebaudioside A is formed by the addition of a D-glucose molecule of the C-3' of the C-13 glucose of stevioside, catalysed by UGT76G1 (Geuns and Ceunen, 2013). It has previously been suggested that salinity might affect the transcription of some genes involved in the biosynthetic pathway of SGs (Zeng et al., 2013). These authors used adult plants grown in hydroponic cultures to study the effect of NaCl (0–120 mM) on chlorophyll and Pro levels; the activity of some antioxidant enzymes (SOD, CAT and POX); the K, Na, Mg and Ca concentration in different plant organs; and the glycoside content (Zeng et al., 2013).

In this study, we used *S. rebaudiana* Bertoni plants acclimatised to *ex*-vitro conditions to analyse the effects of different NaCl treatments on several more parameters, including plant growth; chlorophyll content and chlorophyll fluorescence parameters; mineral nutrition; proline content; antioxidant metabolism (the ASC-GSH cycle enzymes, SOD, CAT, POX); oxidative stress parameters (lipid peroxidation (LP) and ROS accumulation); and steviol glycoside content (steviolbioside, stevioside and rebaudioside A). We also analysed the distribution of Na^+ and Cl^- in leaves and roots.

2. Material and methods

2.1. Plant material and experimental design

The plants were obtained from micropropagated Stevia shoot cultures (MS medium supplemented with 0.8 mg/L of metatopolin, 6 mg/L of adenenine sulphate and 0.040 mg/L of indole butyric acid, 3% sucrose and 7% agar). For elongation and rooting, shoots with three internodes were transferred to 1/2 MS medium without growth regulators. Under these conditions, the shoots elongated and rooted in 6 weeks. All cultures were maintained at 25 ± 2 °C in a growth chamber with a 16 h photoperiod (80 μmol m^{-2} s^{-1} PAR). The rooted shoots were taken out of the medium and washed with distilled water to remove medium attached to the roots. The shoots were then acclimatised in pots containing a mixture of perlite and peat (1:2) in a controlled growth chamber. The temperature in the chamber was set at 24/18 °C (light/darkness), and there was 80% relative humidity and 350 μmol m^{-2} s^{-1} PAR, supplied by cold white fluorescent lamps with a 16 h photoperiod.

After 8 weeks of acclimatisation to *ex-vitro* conditions, the *S. rebaudiana* plants were transplanted to pots (2 L) containing the same proportion of substrate used for acclimatisation. Seven days later, the plant tips were cut in order to standarise the size of all plants. The plants were then exposed to one of three different irrigation treatments for 16 and 25 d. The control plants (8 plants) were watered with 250 mL of distilled water, while the other plants were irrigated with the same volume of distilled water containing either 2 or 5 g/L NaCl (8 and 9 plants, respectively), corresponding to 34 and 90 mM, respectively. All plants (control and NaCl-treated plants) were watered twice a week with these solutions at the beginning of and throughout the experimental period.

2.2. Growth parameters and nutrient content analysis

At the end of the salinity treatment, the substrate was gently washed from the roots of four plants per treatment, and each plant was divided into leaves, stems and roots. The leaf water content (LWC) was determined in leaves from control and NaCl-treated plants. We first measured the fresh weight (FW) of the leaves, which were then oven-dried at 80 °C until they reached a constant weight in order to measure their dry weight (DW). LWC was determined using the following formula: LWC = [(FW−DW)/FW] ×100.

The measurement of different macronutrients [Ca, K, nitrogen (N), carbon (C), phosphorus (P), sulphur (S) and Mg)], micronutrients [cupper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), thallium (Tl), zinc (Zn) and borum (B)], and the Na and Cl levels was carried out in an ICAP 6000SERIES spectrometer (Ionomic Services of CEBAS-CSIC; Thermo Scientific, Madrid, Spain). To measure Cl content, an aqueous extraction (0.1g/10 mL miliQ water) was shaken for 24 h, and the results were analysed by ion chromatography (Ionomic Services of CEBAS-CSIC; Metrohm Ltd., Herisau, Switzerland). Total C and N contents were determined by combustion at 950 °C. Total C was analysed by infrared detection and N by termic conductivity in the Ionomic Services of CEBAS-CSIC (Murcia, Spain).

2.3. Proline determination

The proline in leaf samples was analysed at the end of the saline treatment (after 25 d of NaCl exposure). Briefly, 0.1 g of frozen (in liquid nitrogen) plant tissue (leaves) was homogenised in a mortar with 5 mL of 3% sulfosalicylic acid. After extraction, homogenates

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