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Abscisic acid and nitric oxide signaling in two different portions of detached leaves of *Guzmania monostachia* with CAM up-regulated by drought

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ABSTRACT

Guzmania monostachia is an epiphyte tank bromeliad capable of up-regulating crassulacean acid metabolism (CAM) in response to several environmental stimuli, including drought and light stress. In other plant species, abscisic acid (ABA) and nitric oxide (NO) seem to be involved in CAM induction. Because the leaves of tank bromeliads perform different functions along their length, this study attempted to investigate whether ABA and NO are involved in regulation of CAM expression in this species by quantifying these compounds in apical and basal portions of the leaf, and whether there would be differences in this event for each leaf portion. Detached leaves exposed to a 30% polyethylene glycol solution showed a significant upregulation of CAM on the seventh day of treatment only in the apical portion, as indicated by nocturnal acid accumulation and phosphoenolpyruvate carboxylase (PEPC) activity. On the three days prior to CAM induction, ABA, NO and H_2O_2 were quantified. The amounts of ABA were higher in PEG-exposed leaves, along their entire length. NO, however, was higher only in the apical portion, precisely where CAM was up-regulated. H_2O_2 was higher only in the basal portion of PEG-exposed leaves. Our results suggest that ABA might be a systemic signal to drought, occurring in the entire leaf. NO and H_2O_2, however, may be signals restricted only to the apical or basal portions, respectively.

Introduction

Crassulacean acid metabolism (CAM) is estimated to occur in species belonging to 343 genera, distributed in 35 families (Silvera et al., 2010). Among epiphytes, CAM is even more abundant as a large part of all epiphytic bromeliads and orchids are believed to perform some degree of this photosynthetic adaptation (Holtum et al., 2007). Basically, CAM consists of a nocturnal accumulation of organic acids originating from the incorporation of atmospheric and/or respiratory CO_2 on a phosphoenolpyruvate (PEP) molecule, forming oxaloacetate, and its subsequent reduction to malate, which is stored in the vacuole as malic acid. These two steps are catalyzed by the enzymes phosphoenolpyruvate carboxylase (PEPC – EC 4.1.1.31) and malate dehydrogenase (MDH – EC 1.1.1.82), respectively. During the day, malic acid leaves the vacuole and is decarboxilated in the cytosol, generating CO_2, which powers the Calvin cycle, even when all stomata are closed. As a result, these plants usually keep the stomata closed during most of the daytime, opening them only at periods with higher relative humidity (usually at night, early morning or late afternoon). This process significantly reduces water loss and protects the photosynthetic machinery against high irradiance (Lüttge, 2004).

Even more importantly, CAM photosynthesis might also be considered as an additional source of physiological plasticity for plants living under continuously changing environments. For instance, some species are capable of switching from C_3 to CAM photosynthesis under different environmental conditions or ontogenetic stages (Sleslak et al., 2003). These species are known as C_3-CAM facultatives and represent a fascinating theme, as the switch from C_3 to CAM is a complex metabolic change, requiring a fine-tuned regulation among cell, tissue and the whole organism (Freschi and Mercier, 2012). Although the signaling events controlling the C_3-CAM transition are widely unknown, the amount of information on this topic for some plant species is increasing. Based on the current literature, the main hormones that seem to regulate this process are abscisic acid (ABA), as a positive regulator (Dai et al., 1994; Taybi et al., 1995; Taybi and Cushman, 1999; Cushman and Borland, 2002; Freschi et al., 2010a), and cytokinins, as a negative signal (Peters et al., 1997; Freschi et al., 2010a). In addition, at least in *Mesembryanthemum crystallinum*, Ca^{2+}-dependent protein kinases and Ca^{2+} itself have also been demonstrated to regulate CAM expression (Taybi and Cushman, 1999). Recently, we have shown that in *in vitro*-grown pineapple (*Ananas comosus*) plants, in addition to ABA, cytokinins and Ca^{2+}, nitric oxide (NO) also appear to modulate CAM expression (Freschi et al., 2010a). Interestingly,

Abbreviations: ABA, abscisic acid; MDH, malate dehydrogenase; NO, nitric oxide; PEG, polyethylene glycol; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase.

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although having being identified as plant signaling molecule only recently, NO was found to be involved in several other plant stress responses, such as stomatal control (Desikan et al., 2002; García-Mata and Lamattina, 2003, 2007), a key requirement for the correct functioning of CAM photosynthesis.

Although nitric oxide has already been linked to CAM modulation in young plants (Freschi et al., 2010a), whether this gas can induce CAM in a species capable of regulating CAM expression even when adult, as is the case of *G. monostachia*, has yet to be investigated. In this tank bromeliad, environmental stresses such as intense light (Maxwell et al., 1994) and drought (Maxwell et al., 1994; Freschi et al., 2010b) have been demonstrated to induce and/or up-regulate CAM photosynthesis. Interestingly, as other tank bromeliads, *G. monostachia* seems to have some functional division along its leaf length, with the apical portion mainly performing photosynthesis and the final steps of nutrient assimilation, whereas the basal portion is primarily involved in nutrient and water uptake (Sakai and Sandford, 1980; Popp et al., 2003; Takahashi et al., 2007; Freschi et al., 2010b; Takahashi and Mercier, 2011). Some division along the leaf blade in other rosette monocots, like agaves, has also been reported (Olivares and Medina, 1990). In bromeliads and agaves, the basal portion of the leaf is younger than the apical, and because some aspects (like CAM) are likely to increase with age, it is possible that the age of each leaf portion may influence the degree of CAM (Olivares and Medina, 1990; Popp et al., 2003).

In a previous study, we demonstrated that suspending the water supply to *G. monostachia* for seven days lead to a significant CAM upregulation, which took place exclusively at the apical leaf portion, whereas the leaf base continued to perform C_3 in spite of the fact that loss of water was mainly detectable in this basal region. These findings encouraged us to investigate whether the plant hormone ABA and/or the free radical NO participated in the signaling cascades controlling CAM expression along the leaves of *G. monostachia*. Moreover, because leaves of *G. monostachia* seem to have a separation of functions along their length, it is possible that each part of the leaf perceives and responds differently to a water shortage. Therefore, we questioned whether there would be specific signals for each leaf portion.

Materials and methods

Plant growth, treatments and sampling

Plants of *Guzmania monostachia* (L.) Rusby ex Mez var. *monostachia* were cultivated in a shaded greenhouse at the Department of Botany at the University of São Paulo (São Paulo, Brazil) until adulthood. Light intensity never exceeded 300 μmol photons m^{-1} s^{-1}, and temperature varied with the environment, ranging from 10.6 °C to 27.2 °C. Photoperiod was also determined by the environment, ranging from 11 to 13 h. A mix of pinus bark and Tropstrato® was used as substrate. These plants were subsequently transferred to a controlled environment chamber with the following conditions: photosynthetic flux density (PFD) of 200 μmol photons m^{-1} s^{-1}, 12 h photoperiod, constant temperature of 25 ± 1 °C, and relative humidity of 60/70%.

After at least two weeks of acclimation under the above-mentioned controlled environment, leaves from the 8th to the 12th node, representing the youngest fully expanded leaves, were detached and used in the experiments. The detached leaves were kept up to seven days in water (control condition) or in 30% (w/w) polyethylene glycol 6000 (PEG 6000) solution (water deficit treatment). In the experiment using whole plants, the control group consisted of plants watered in the tank daily, while in the treatment group, the tank was dried and water was withheld for seven days. After this period, a minimum of five leaves (belonging to at least five individuals) were collected and divided into three portions. The basal portion comprised the leaf sheath, and the remaining part of the leaf was equally divided, forming the so-called middle and apical leaf portions. The leaf portions were separately fragmented, forming a pool of at least five leaves from five different individuals. This pool was then sampled to perform each analysis in triplicate. The samples were then frozen in liquid nitrogen and remained at −80 °C until the moment of analysis. For each harvest point, samples were collected 1 h after the onset of the light period (dawn), and for titratable acidity analysis, new samples were collected 1 h before the end of the light period (dusk).

Measurements of water percentage

The water percentage of each leaf portion was assessed by determining the fresh weight (FW), and after drying at 65 °C to a constant weight, the dry weight (DW) was determined. The water percentage was then calculated from the formula [(FW − DW)/FW × 100]. Measurements were made in triplicate.

Measurements of water potential

A detached leaf kept in PEG 30% solution was attached to a L-51-A leaf psychrometer (Wescor) and allowed to equilibrate for at least 30 min. Water potential (expressed as MPa) was measured by the wet bulb depression method. Measurements were made in the apical portion of the leaf for seven days. The experiment was repeated to measure water potential in the basal region.

Titratable acidity and PEPC activity assays

The titratable acidity was determined following Freschi et al. (2010b). The acids were extracted in distilled boiled water and then titrated with NaOH. The variation was assessed by subtracting the acid content obtained at dusk from that obtained at dawn. The measurement was made in triplicate. The method for measuring PEPC activity was performed exactly as outlined in Freschi et al. (2010b). The values of titratable acidity and PEPC activity were normalized by mg of total chlorophyll, measured according to Lichtenthaler (1987).

ABA quantification by GC–MS

ABA content was measured by GC–MS according to Ludwig-Müller et al. (2008), with modifications. Frozen samples were ground in 95% isopropanol, 5% acetic acid (v/v) in a proportion of 1 mL extraction buffer per 200 mg of fresh weight, and kept at 4 °C under shaking for 2 h in complete darkness. About 100 μg of [$^2H_6$](+)-*cis*, *trans*-abscisic acid (D-ABA) was added to each sample as internal standard. The samples were then centrifuged (4 °C; 15,000 × *g*) and the supernatant collected and dried under N_2 flux. Then, 500 μL of ethyl acetate was added to the dried samples, followed by centrifugation at 15,000 × *g* (20 °C). The upper phase was collected, and the phase separation process was repeated. The upper phase was then dried under N_2 flux, methylated with trimethylsilyl-diazomethane (Sigma Cat No. 362832), dried again, resuspended in ethyl acetate and, finally, injected in a Hewlett-Packard 6890 gas chromatograph coupled with a 5973 mass spectrometer. The column used was an HP-1701 (30 m, D.I. 0.25 mm, I.T. 0.5 μm), with helium as a carrier gas in a 1 mL min^{-1} flux. The column remained at 150 °C for 3 min, followed by a temperature ramp from 150 °C to 200 °C at a rate of 4 °C min^{-1}, and finally, from 200 °C to 300 °C at a rate of 10 °C min^{-1}. The quantification of ABA was performed by gas chromatography associated with mass spectrometry, with selective ion monitoring (GC–MS–SIM),