Plant Physiology and Biochemistry 115 (2017) 354–359

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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

Research article

Evidence towards the involvement of nitric oxide in drought tolerance of sugarcane

Neidiquele M. Silveira [a], John T. Hancock [b], Lucas Frungillo [d], Eleni Siasou [b], Fernanda C.C. Marcos [c], Ione Salgado [c], Eduardo C. Machado [a], Rafael V. Ribeiro [c, *]

[a] Laboratory of Plant Physiology "Coaracy M. Franco", Center R&D in Ecophysiology and Biophysics, Agronomic Institute (IAC), Campinas, SP, Brazil
[b] Centre for Research in Biosciences, University of the West of England (UWE), Bristol, UK
[c] Department of Plant Biology, Institute of Biology, University of Campinas (UNICAMP), Campinas, SP, Brazil
[d] School of Biological Sciences, Institute of Molecular Plant Sciences, University of Edinburgh, Edinburgh, UK

ARTICLE INFO

Article history:
Received 8 November 2016
Received in revised form
26 March 2017
Accepted 7 April 2017
Available online 8 April 2017

Keywords:
Nitrate reductase
S-nitrosoglutathione reductase
NO metabolism
Genotype dependent

ABSTRACT

Exogenous supply of nitric oxide (NO) increases drought tolerance in sugarcane plants. However, little is known about the role of NO produced by plants under water deficit. The aim of this study was to test the hypothesis that drought-tolerance in sugarcane is associated with NO production and metabolism, with the more drought-tolerant genotype presenting higher NO accumulation in plant tissues. The sugarcane genotypes IACSP95-5000 (drought-tolerant) and IACSP97-7065 (drought-sensitive) were submitted to water deficit by adding polyethylene glycol (PEG-8000) in nutrient solution to reduce the osmotic potential to −0.4 MPa. To evaluate short-time responses to water deficit, leaf and root samples were taken after 24 h under water deficit. The drought-tolerant genotype presented higher root extracellular NO content, which was accompanied by higher root nitrate reductase (NR) activity as compared to the drought-sensitive genotype under water deficit. In addition, the drought-tolerant genotype had higher leaf intracellular NO content than the drought-sensitive one. IACSP95-5000 exhibited decreases in root *S*-nitrosoglutathione reductase (GSNOR) activity under water deficit, suggesting that *S*-nitrosoglutathione (GSNO) is less degraded and that the drought-tolerant genotype has a higher natural reservoir of NO than the drought-sensitive one. Those differences in intracellular and extracellular NO contents and enzymatic activities were associated with higher leaf hydration in the drought-tolerant genotype as compared to the sensitive one under water deficit.

1. Introduction

Despite evidence regarding the importance of nitric oxide (NO) in plant signaling, the mechanism responsible for NO synthesis is still controversial. It is now widely accepted that NO plays a key role in signaling among plant cells, however, it has been a challenge to determine the sources of NO in plants and there is considerable discussion of how exactly NO is formed in plant cells (Hancock, 2012; Salgado et al., 2013). In biological systems, NO can be formed both enzymatically and non-enzymatically. In mammals, the enzyme responsible for NO generation is NO synthase (NOS), with L-arginine being converted to citrulline, using NADPH as electron donor and O_2 as co-substrate and producing NO and water (Alderton et al., 2001). The existence of NOS remains questionable in plants. Although NO production is dependent on L-arginine and its production is sensitive to inhibitors of NOS (Moreau et al., 2010), a homologous gene for this protein has not been found in plants. While a recent extensive survey of higher plant genomes failed to uncover the presence of a NOS encoding region in any species (Jeandroz et al., 2016), Foresi et al. (2010) characterized the sequence, protein structure, phylogeny, biochemistry and NOS expression in green algae of the *Ostreococcus* genus, in which the amino acid sequence was 45% similar to human NOS.

The nitrate reductase (NR) enzyme is essential for nitrogen assimilation and also involved in NO production both *in vitro* (Rockel et al., 2002) and *in vivo* (Kaiser et al., 2002). In the first case,

Abbreviations: GSH, glutathione; GSNO, *S*-nitrosoglutathione; GSNOR, *S*-nitrosoglutathione reductase; GSSG, oxidized glutathione; NH_4^+, ammonium; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; PEG, polyethylene glycol; PPFD, photosynthetic photon flux density; RSNO, *S*-nitrosothiol; RWC, relative water content; WD, water deficit.

* Corresponding author.
E-mail address: rvr@unicamp.br (R.V. Ribeiro).

http://dx.doi.org/10.1016/j.plaphy.2017.04.011
0981-9428/

NR catalyzes the transfer of two electrons from NADPH to nitrate to produce nitrite. As secondary activity, NR also reduces nitrite to NO using NADPH, being NO synthesis dependent on nitrite content of plant tissues. The efficiency of this reaction for NO production is considered low and requires high concentrations of nitrite (Yamasaki and Sakihama, 2000; Rockel et al., 2002). Modolo et al. (2005) have suggested that the primary role of NR for NO production is as a pathway to provide nitrite. Electrons required for the reduction of nitrite to NO can be provided by the mitochondrial respiratory chain (Planchet et al., 2005) or by the photosynthetic system (Jasid et al., 2006).

The NO bioavailability may be affected by glutathione (GSH), an antioxidant present at high intracellular concentrations. Spontaneous reaction of NO with the thiol grouping of GSH will form *S*-nitrosoglutathione (GSNO). The control of intracellular GSNO is partly regulated by degradation catalyzed by *S*-nitrosoglutathione reductase (GSNOR) (Frungillo et al., 2014). The GSNOR catabolizes GSNO to oxidized glutathione (GSSG) and ammonium (NH_4^+), resulting in depletion of intracellular levels of GSNO and reduction of *S*-nitrosothiol (RSNO) formation by transnitrosation processes. In fact, GSNO has an important role in *S*-nitrosation and also represents a natural intracellular reservoir of NO (Ji et al., 1999; Liu et al., 2001).

Recent studies have shown that NO plays an important role in plants under stressful conditions, such as drought (Santisree et al., 2015; Farnese et al., 2016; Silveira et al., 2016). For instance, Arasimowicz-Jelonek et al. (2009) found that *Cucumis sativus* subjected to mild water deficit enhanced NO synthesis in root cells, with an intense NO production in root elongation zone. Although several reports have shown increased NO production under drought (Filippou et al., 2011; Fan and Liu, 2012; Xiong et al., 2012; Cai et al., 2015), there is no information about how plant species/varieties differ in NO production and how this differential NO production is related to drought tolerance. The aim of this work was to test the hypothesis that drought-tolerance in sugarcane is associated with NO production and metabolism, with the more drought tolerant genotype presenting higher NO accumulation in plant tissues.

2. Material and methods

2.1. Plant material and growth conditions

Two sugarcane genotypes (*Saccharum* spp.) developed by the Sugarcane Breeding Program of the Agronomic Institute (ProCana, IAC, Brazil) with differential biomass production and drought tolerance were studied: IACSP95-5000 is a drought-tolerant genotype (Marchiori, 2014), whereas IACSP97-7065 is sensitive to water deficit (Oliveira, 2012; Sales et al., 2013). Plants of both genotypes were obtained through vegetative propagation. Small stem segments (with one bud each) of mature plants were selected and planted on commercial substrate (Levington M2 Compost, Heerlen UK). After 50 days, plants with five to six leaves were transferred to modified Sarruge (1975) nutrient solution with 15 mmol L^{-1} N (7% as NH_4^+); 4.8 mmol L^{-1} K; 5.0 mmol L^{-1} Ca; 2.0 mmol L^{-1} Mg; 1.0 mmol L^{-1} P; 1.2 mmol L^{-1} S; 28.0 μmol L^{-1} B; 54.0 μmol L^{-1} Fe; 5.5 μmol L^{-1} Mn; 2.1 μmol L^{-1} Zn; 1.1 μmol L^{-1} Cu and 0.01 μmol L^{-1} Mo. The pH of nutrient solution was kept between 5.5 and 6.0 and its electrical conductivity between 1.53 and 1.70 mS cm^{-1} by weekly monitoring and corrected when necessary. Plants were grown in growth chamber, with a 12-h photoperiod, air temperature of 30/20 °C (day/night), air relative humidity of 80% and the photosynthetic photon flux density (PPFD) about 700 μmol m^{-2} s^{-1}.

2.2. Water deficit induced by polyethylene glycol (PEG)

Sugarcane plants growing in nutrient solution were submitted to water deficit (WD) by adding polyethylene glycol (PEG-8000, Fisher Scientific, Leicestershire, UK) to the solution. To prevent osmotic shock, PEG-8000 was added to the nutrient solution to cause a gradual decrease in its osmotic potential until −0.4 MPa. All evaluations were taken 24 h after the solution reached the desired osmotic potential, being the short-term responses to water deficit evaluated. Leaf and root samples were collected, immediately immersed in liquid nitrogen and then stored at −80 °C for further enzymatic analyses.

2.3. Leaf relative water content (RWC)

The relative water content was calculated using the fresh (FW), turgid (TW) and dry (DW) weights of leaf discs according to Jamaux et al. (1997): RWC = 100 × [(FW−DW)/(TW−DW)].

2.4. Extracellular and intracellular NO detection

Diaminofluoresceins (DAFs) are markers used for detecting NO in tissues by fluorescence emission (Yao et al., 2004; Kojima et al., 1998). The chemical transformation of 4,5-diaminofluorescein compound (DAF-2) is based on the reactivity of aromatic diamines with NO in the presence of O_2. *N*-nitrosation of DAFs yields the highly green-fluorescent triazole form (DAF-2T) (Kojima et al., 1998). DAFs do not react directly with NO, but with their oxidized forms such as N_2O_3 (Mur et al., 2011). Among DAFs most used as NO indicators, the plasma membrane-permeable compound DAF-2 diacetate (DAF-2DA) is prominent, being hydrolyzed by esterases to form NO-sensitive DAF-2 (Kojima et al., 1998). As DAF-2DA is able to enter the cell, this compound cannot be used to monitor NO extracellular content, unlike DAF-2 that is impermeable to plasma membrane.

Leaf and root samples (100 mg) were incubated in 10 mM Tris, 50 mM KCl, pH 7.2 buffer in 1 mL microcentrifuge tubes for 40 min, before the addition of 5 μM 4,5-diaminofluorescein diacetate (DAF-2). The sample was placed into a quartz cuvette and fluorescence measured for 30 min (Suppl. Fig. S1) using a fluorescence spectrophotometer (F-2500, Hitachi Science & Technology, Berkshire, UK) with excitation and emission at 488 and 512 nm, respectively (Bright et al., 2009). For the negative control, samples were incubated in the absence of DAF-2. Data are shown as average value (n = 3) for each treatment and they represent the fluorescence signal after 30 min, considering the negative control (data shown = sample − negative control).

Intracellular NO was visualized using the cell permeable NO-specific dye 4,5 diaminofluorescein-2 diacetate (DAF2-DA). Fresh leaf and root segments were incubated in MES-KCl buffer (10 mM MES, 50 mM KCl, 0.1 mM $CaCl_2$, pH 6.15), at room temperature for 15 min. Then, these segments were incubated in solution of 10 μM DAF2-DA, mixing gently per 40 min in dark and at room temperature (Desikan et al., 2002; Bright et al., 2009). The samples were washed with buffer to remove the excess of DAF2-DA and placed onto a glass slide and covered with a glass slip before observing fluorescence using laser-scanning microscopy with excitation at 488 nm and emission at 515 nm (Nikon PCM, 2000, Nikon, Kingston-upon-Thames, UK). Photos were taken with a 10× magnification, 15 s exposure and 1× gain. Images were analyzed using ImageJ software (NIH, Bethesda, MD, USA) and data are presented as mean pixel intensities.