ORIGINAL ARTICLE

Nitric oxide regulation of leaf phosphoenolpyruvate carboxylase-kinase activity: implication in sorghum responses to salinity

José A. Monreal · Cirenia Arias-Baldrich · Vanesa Tossi ·
Ana B. Feria · Alfredo Rubio-Casal · Carlos García-Mata ·
Lorenzo Lamattina · Sofía García-Mauriño

Abstract Nitric oxide (NO) is a signaling molecule that mediates many plant responses to biotic and abiotic stresses, including salt stress. Interestingly, salinity increases NO production selectively in mesophyll cells of sorghum leaves, where photosynthetic C_4 phosphoenolpyruvate carboxylase (C_4 PEPCase) is located. PEPCase is regulated by a phosphoenolpyruvate carboxylase-kinase (PEPCase-k), which levels are greatly enhanced by salinity in sorghum. This work investigated whether NO is involved in this effect. NO donors (SNP, SNAP), the inhibitor of NO synthesis NNA, and the NO scavenger cPTIO were used for long- and short-term treatments. Long-term treatments had multifaceted consequences on both *PPCK* gene expression and PEPCase-k activity, and they also decreased photosynthetic gas-exchange parameters and plant growth. Nonetheless, it could be observed that SNP increased PEPCase-k activity, resembling salinity effect. Short-term treatments with NO donors, which did not change photosynthetic gas-exchange parameters and *PPCK* gene expression, increased PEPCase-k activity both in illuminated leaves and in leaves kept at dark. At least in part, these effects were independent on protein synthesis. PEPCase-k activity was not decreased by short-term treatment with cycloheximide in NaCl-treated plants; on the contrary, it was decreased by cPTIO. In summary, NO donors mimicked salt effect on PEPCase-k activity, and scavenging of NO abolished it. Collectively, these results indicate that NO is involved in the complex control of PEPCase-k activity, and it may mediate some of the plant responses to salinity.

Keywords Abscisic acid · Calcium-dependent protein kinase · Nitric oxide · Phosphoenolpyruvate carboxylase · Phosphoenolpyruvate carboxylase-kinase · Protein turnover · Salinity · Sorghum

Abbreviations

CDPK	Ca^{2+}-dependent protein kinase
cPTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
NNA	N-nitro-L-arginine
PA	Phosphatidic acid
PEPCase	Phosphoenolpyruvate carboxylase
PEPCase-k	Phosphoenolpyruvate carboxylase-kinase
PLD	Phospholipase D
qPCR	Quantitative PCR
SNAP	S-nitroso-N-acetyl-1,1-penicillamine
SNP	Sodium nitroprusside

J. A. Monreal · C. Arias-Baldrich · A. B. Feria · A. Rubio-Casal ·
S. García-Mauriño (✉)
Departamento de Biología Vegetal y Ecología, Facultad de Biología, Universidad de Sevilla, Avenida Reina Mercedes n° 6, 41012 Seville, Spain
e-mail: sgarma@us.es

V. Tossi · C. García-Mata · L. Lamattina
Instituto de Investigaciones Biológicas, CONICET-UNMdP, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, 7600 Mar del Plata, Argentina

Introduction

The relevance of nitric oxide (NO) as a signaling molecule in plants is fully acknowledged at present (Fewson and

Nicholas 1960; Leshem 1996; Delledonne et al. 1998; Lamattina et al. 2003). NO has a dual function in both regulating growth and development and mediating plant responses to diverse biotic and abiotic environmental signals. As a consequence, this molecule has been implied in processes as diverse as seed germination, root growth, respiration, stomatal closure, flowering, cell death, and responses to biotic and abiotic stresses (Durner et al. 1998; Lamattina et al. 2003; Wendehenne et al. 2004; Besson-Bard et al. 2008).

The origin of NO in plants is still controversial and it appears that NO does not come from a single source (del Río et al. 2004). Reduction of nitrite produces NO via enzymatic and nonenzymatic routes. The first pathway involves cytosolic nitrate reductase (Yamasaki and Saki-hama 2000), a root-specific plasma-membrane nitrite reductase (Stohr et al. 2001), and reduction of nitrite by electrons from the mitochondrial electron transport chain (Planchet et al. 2005). NO can be produced nonenzymatically by chemical reduction of nitrite at acidic pH in the apoplasm (Bethke et al. 2004). In addition, NO is synthesized from L-Arg by a nitric oxide synthase-like activity (Barroso et al. 1999; Corpas et al. 2006; Tian et al. 2007). Other oxidative pathways use polyamine and hydroxyl-amine as substrates for NO production (Gupta et al. 2011).

There are numerous reports showing that NO increases plant tolerance to salt stress. The beneficial effects of NO have been attributed to different processes. By activating H^+-translocating ATPases of the plasma membrane and tonoplast, NO has been reported to regulate K^+ to Na^+ ratio, contributing to maintain ion homeostasis (Uchida et al. 2002; Shi et al. 2007; Chen et al. 2010). Other valuable effects of NO under salinity depend on the induction of enzymes involved in detoxification (such as antioxidant enzymes) or in the synthesis of protective metabolites (such as proline) (Zhao et al. 2004, 2007; Zhang et al. 2006; Tanou et al. 2009; Bai et al. 2011; Wu et al. 2011; Fan et al. 2012; Keyster et al. 2012).

In addition, NO can enhance salt tolerance by inducing stomatal closure (García-Mata and Lamattina 2001; Li et al. 2006). NO is a signaling component in abscisic acid (ABA)-induced stomatal closure (García-Mata and Lamattina 2002; Neill et al. 2002; Garcia-Mata et al. 2003). ABA promotes NO formation through the reaction of nitrate reductase in guard cells (Desikan et al. 2002). Downstream of NO during stomatal closure are NO-stimulated mitogen activated protein kinase (MAPK) activity and cGMP production (Soko-lovski et al. 2005; Neill et al. 2008), and phospholipase C (PLC)- and phospholipase D (PLD)-derived phosphatidic acid (PA) (Distéfano et al. 2008).

It is known that C_4 plants are able to maintain relatively high rates of CO_2 fixation even when their stomata are in part closed, and C_4 plants perform better than C_3 plants under salt stress. Phosphoenolpyruvate carboxylase (PEP-Case: EC 4.1.1.31), the enzyme responsible for primary carboxylation in C_4 photosynthesis, is phosphorylated by a light-dependent phosphoenolpyruvate carboxylase-kinase (PEPCase-k). Salinity greatly increased PEPCase-k activity in salt-treated sorghum plants (Echevarría et al. 2001; García-Mauriño et al. 2003). Several experimental evidences indicated that this effect could be related to a decreased rate of protein degradation, and that ABA, which increases in response to salinity, was responsible for it (Monreal et al. 2007a). Further investigation on salinity effects on PEPCase-k activity showed that 172 mM salt treatment decreased the rate of PEPCase-k degradation; meanwhile at higher concentration (258 mM) it increased *PPCK* gene expression and/or mRNA stability (Monreal et al. 2013). It has been proposed that phosphorylation of the protein by a calcium-dependent protein kinase (CDPK) could be modulating the turnover of PEPCase-k under salt stress. Both by ABA-dependent and independent mechanisms, NO could be involved in the effects of salinity on PEPCase-k activity and *PPCK* gene expression.

This work was aimed to investigate the effect of NO on PEPCase-k activity and *PPCK* gene expression, and to evaluate the involvement of NO as a mediator of some of the mechanisms, triggered by salt, that impact on PEPCase-k activity of sorghum leaves.

Materials and methods

Plant material and growth conditions

Sorghum plants (*Sorghum bicolor* L., Rhône-Poulenc, Seville, Spain) were grown hydroponically in 12 h light to dark cycles (25 °C, 60 % relative humidity and 20 °C, 70 % relative humidity for each photoperiod, respectively). Light intensity was 350 µmol m^{-2} s^{-1} PAR. Seeds were placed in moist vermiculite and seedlings were transferred to a hydroponic culture system 7 days after sowing. Plants were grown in 1 l polyethylene pots filled with a continuously aerated nitrate-type nutrient solution (Hewitt 1966). Plant material used in the experiments was obtained from plants 3–4 weeks after sowing.

Experimental treatments

Plants were acclimated to salt by increasing weekly NaCl concentration (43, 86, 172 mM final concentration) in the culture medium. Plants were grown with 172 mM NaCl for 10 days.

Low Fe treatment consisted in reducing Fe in the culture medium from 100 µM (control) to 10 µM NaFe-EDTA (low Fe). The NO levels were increased with the NO

donors SNAP (S-nitroso-N-acetyl-1,1-penicillamine) and SNP (sodium nitroprusside). Decreased NO levels were accomplished with the NO scavenger cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) and the inhibitor of NO synthesis NNA (N-nitro-L-arginine). Long-term experiments were performed by addition of SNP and NNA to the culture medium 1 week after the plants had been transferred to polyethylene pots, and the treatment was prolonged for 10 days. Short-term treatments with SNAP, SNP, cPTIO and NNA consisted of overnight exposure of excised leaves to the pharmaceuticals.

Experiments were carried out with excised, fully expanded youngest leaves, which were transferred to a 3 ml cuvette containing 0.01 M Tris–HCl buffer, pH 8, and the indicated pharmaceuticals. When the leaves had been subjected to overnight treatment, they were provided with fresh chemicals next morning. Leaves were illuminated (750 μmol m^{-2} s^{-1} PAR) or kept in the dark for 2–3 h, prior to the preparation of enzyme extracts.

NO measurement

Fully expanded youngest leaves of control and 172 mM NaCl-treated plants (10 days) were cut and vacuum loaded with 20 mM Hepes (pH 7.5) containing 15 μM of the NO detection probe 4-5-diaminofluorescein-FM diacetate (DAF-FM DA). The leaves were then thoroughly washed to remove the excess of probe. Transversal sections were hand cut with a razorblade and mounted for microscopy. Fluorescent images were obtained by confocal laser microscopy using a Nikon C1 Plus with Eclipse Ti Inverted Microscope. The green fluorescent was quantified as a pixel intensity of a fixed area from mesophyll cells using ImageJ analysis software (NIH, Bethesda, MD, USA). The fluorescence values are represented as relative units (RU) with respect to the control treatments and are expressed as mean ± SE. 80–120 mesophyll cells from at least three different plants were quantified.

Enzyme extraction and analysis

Protein extracts were obtained by grinding 0.2 g fresh weight of leaf tissue in 1 ml of extraction buffer containing: 0.1 M Tris–HCl pH 7.5, 20 % (by vol.) glycerol, 1 mM EDTA, 10 mM MgCl$_2$ and 14 mM β-mercaptoethanol. The homogenate was centrifuged at 15,000g for 2 min and the supernatant was filtered through Sephadex G-25.

Determination of PEPCase activity, malate test, in vitro phosphorylation assay, and SDS-PAGE has been described previously (Echevarría et al. 1994). PEPCase activity was measured spectrophotometrically at optimal pH 8.0 using the NAD-MDH-coupled assay at 2.5 mM PEP. A single enzyme unit is defined as the amount of PEPCase that catalyzes the carboxylation of 1 μmol of phosphoenolpyruvate per minute at pH 8 and 30 °C.

The in vitro PEPCase-k activity of sorghum leaves and leaf disks was measured in aliquots of desalted protein extracts (10 μg) that were incubated in a reaction medium containing 100 mM Tris–HCl, pH 7.5, 20 % (by vol.) glycerol, 5 mM MgCl$_2$, 0.25 mM P1P5-di(adenosine-5′)-pentaphosphate (adenylate kinase inhibitor), 1 mM EGTA and 0.2 units of nonphosphorylated sorghum PEPCase. The phosphorylation reaction was initiated by the addition of 37 kBq of [γ-^{32}P]ATP (15 TBq mmol^{-1}) and incubated at 30 °C for 1 h. The reaction was stopped by boiling the samples for 3 min at 90 °C in the presence of dissociation buffer (100 mM Tris–HCl, pH 8, 25 % glycerol, 1 % SDS, 10 % β-mercaptoethanol, and 0.05 % bromophenol blue (all by vol.). The denatured proteins were separated by SDS-PAGE in a Miniprotean electrophoresis cell (Bio-Rad) and stained with Coomassie Brilliant Blue R-250. The gel was analyzed with a phosphor imager (Fuji FLA-5100; Fuji, Tokyo).

Protein quantification

Protein concentrations were determined using the method of Bradford (1976) with BSA as the standard.

RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of frozen, powdered leaves using the RNeasy Plant Mini kit (Qiagen). Extracted nucleic acids were DNase treated to wipe out genomic DNA. RNA concentrations were determined using a Qubit^{TM} Fluorometer (Invitrogen). Reverse transcription reactions were performed using 1 μg of purified total RNA, 1 μL ImProm-II^{TM} Reverse Transcriptase (Promega) and a reaction buffer containing 0.5 mM dNTP, 6 mM MgCl$_2$, 20 U recombinant RNasin$^{®}$ ribonuclease inhibitor and 0.5 μg oligo(dt)15.

qPCR experiments

Quantitative PCR reactions (qPCR) were performed in a final volume of 20 μL consisting of 1 μL of the cDNA, 15 μM of the specific primers (see Supplementary Table S1), and 10 μL of SensiFAST SYBR No-ROX kit (Roche). PCR was conducted on the MiniOpticon^{TM} Real-Time PCR Detection System (Biorad), and the threshold cycles (Ct) were determined using Opticon Monitor^{TM} analysis software for all treatments. To normalize the obtained values, 18S RNA was used as internal control in each sample.

Gas exchange measurements

Photosynthetic light response curves (from 0 to 1,200 µmol m^{-2} s^{-1} PAR) were determined on adult leaves following the procedure of Rubio-Casal et al. (2010). Measurements were made using an LCpro + portable photosynthesis analyzer (open system) connected to a broad leaf chamber (ADC BioScientific Ltd., Hertfordshire, UK) with an integrated LED light source. Each curve typically involved 25–30 min. Net photosynthetic rate (A) and stomatal conductance (g_s) were determined at a CO_2 concentration of 460 ppm, ambient relative humidity and using standard formulas (Von Caemmerer and Farquhar 1981). Data were fitted to the rectangular hyperbolic model (Chartier and Prioul 1976) using the program SigmaPlot 10.0 (Systat Software Inc., San José, CA, USA). In this model, the upper asymptote is the light-saturated photosynthetic rate (A).

Statistical analysis

The statistical analysis of the results was performed using statistical software (SigmaStat, Systat Software Inc). Statistical differences between groups were tested by t test. The means are considered to be significantly different at $P < 0.05$.

Results

Salinity increases NO production in sorghum leaves

Sorghum plants display Kranz anatomy, with two photosynthetic tissue types (mesophyll and bundle sheath) arranged in concentric circles around veins. Photosynthesis is initiated by HCO_3^- fixation by C_4 PEPCase in mesophyll cells. Salinity (172 mM) increased NO production in leaves of sorghum plants (Fig. 1). Interestingly, NO was preferentially accumulated in mesophyll cells (see Fig. 1, inset).

NO impacts on PEPCase-k activity

The contribution of nitric oxide to the effects caused by salinity in sorghum leaves was evaluated with pharmacological tools. Long-term treatments (10 days) with either the NO donor sodium nitroprusside (SNP) or the inhibitor of NO synthesis N-nitro-L-arginine (NNA) decreased plant growth (Fig. 2a; Table 1) and photosynthetic gas-exchange parameters (Fig. 2b). Besides of these wide detrimental effects, long-term treatments with either SNP or NNA increased PEPCase-k activity in the light (Fig. 3a). On the other hand, the more noteworthy effect was the marked enhancement of PEPCase-k activity caused by SNP in the dark; meanwhile, this activity was totally suppressed by
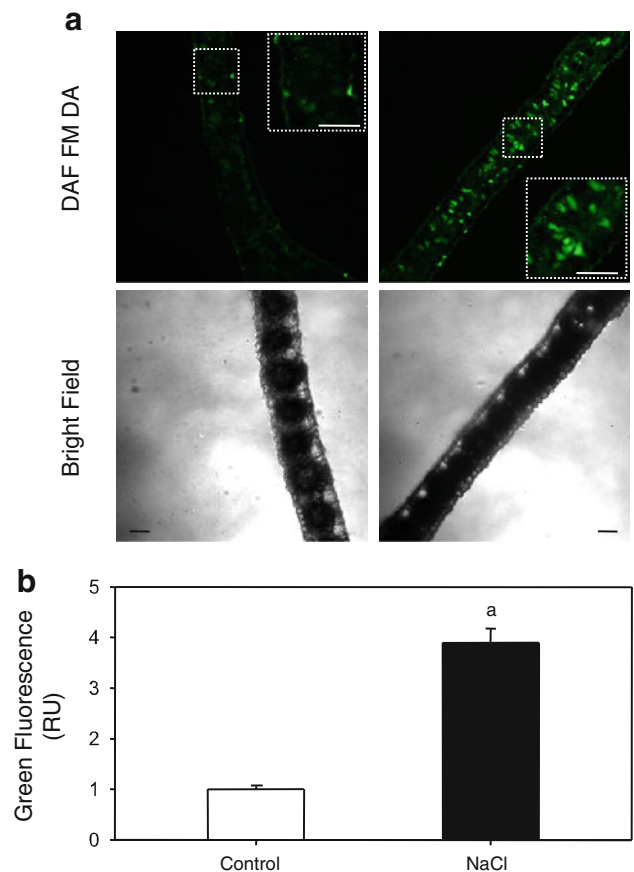

Fig. 1 Nitric oxide production in sorghum leaves. Transversal leaf cuts from control or salt-treated sorghum leaf were vacuum loaded with 20 mM Hepes (pH 7.5) containing 15 µM DAF-FM DA. After washing the excess of dye, the cuts were mounted and visualized by confocal laser microscopy. **a** Images depict one representative picture of the leaf cuts of each treatment. Inset shows a close up (2×) of leaf tissues. *Bar* 50 µM. **b** Quantification of the green fluorescence pixel intensity expressed as relative units with respect to the control treatment, from 80 to 120 mesophyll cells taken from at least three different plants. Values are expressed as mean ± SE. *a* $P < 0.05$ versus control (t test)

NNA. Although dark PEPCase-k activity was very low in control plants, and, for this reason, the depressing effect of NNA was of little extent, it was consistently shown in following experiments. Neither SNP nor NNA increased PEPCase activity, but moderately decreased it (Fig. 3b). PEPCase activity, measured at pH 8, is the same with independence the leaves are illuminated or not.

Three putative *PPCK* genes have been reported in sorghum (Shenton et al. 2006; Paterson et al. 2009). *PPCK1* gene product is the PEPCase-k isoenzyme responsible for the phosphorylation of the C_4 photosynthetic PEPCase, and its expression is triggered by light, unlike *PPCK2* or *PPCK3* (Monreal et al. 2013). The expression of *PPCK* genes in sorghum leaves was analyzed by quantitative RT-PCR (qPCR). Both SNP and NNA treatments increased *PPCK1* expression in the light; however, SNP and not

Fig. 2 Effect of the NO synthesis inhibitor NNA and the NO donor SNP on growth, photosynthesis, and stomatal conductance of sorghum in long-term experiments. Plants were supplied with 200 μM of N-nitro-L-arginine (NNA) or 200 μM of sodium nitroprusside (SNP) for 10 days. Subsets of plants were cultured under Fe restriction (10 μM instead of 100 μM NaFe-EDTA) for the same time. NNA and SNP treatment was performed by addition of the compound to the culture medium. **a** Effect of NNA, SNP, or low Fe on sorghum growth. **b** Gas-exchange analysis of sorghum leaves. A, net photosynthetic rate; g_s, stomatal conductance
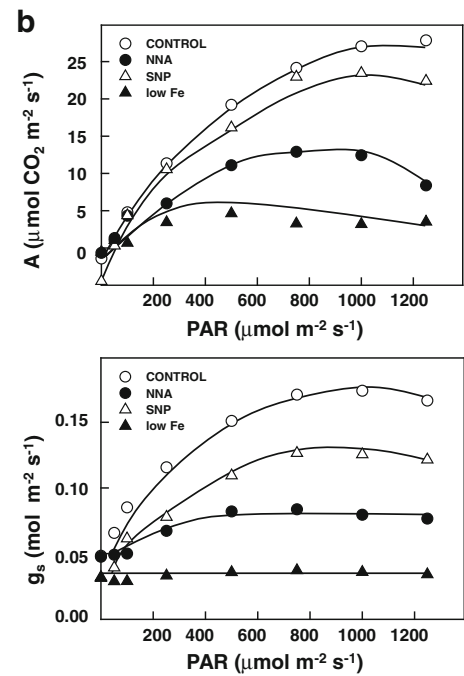

Table 1 Effect of long-term treatment with SNP, NNA and low Fe on sorghum growth

Treatment	Shoots (g)		Roots (g)	
	FW	DW	FW	DW
Control	6.74 ± 0.84	0.82 ± 0.12	5.27 ± 0.65	0.31 ± 0.04
SNP	2.98 ± 1.50	0.42 ± 0.21	3.45 ± 1.74	0.23 ± 0.12
NNA	1.55 ± 0.94	0.23 ± 0.14	1.41 ± 0.97	0.14 ± 0.1
Low Fe	1.29 ± 0.61	0.14 ± 0.07	1.36 ± 0.85	0.09 ± 0.06

Plants used for the experiments in Fig. 2 were collected and fresh weight (FW) and dry weight (DW) of shoots and roots were weighed. Data are mean ± SE of three plants

NNA markedly enhanced *PPCK3* transcript levels in the dark (Fig. 3c).

Short-term experiments were carried out with the NO donor SNAP (S-nitroso-N-acetyl-1,1-penicillamine), which has a faster NO releasing rate (Lander et al. 1993), and with the NO scavenger cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) (Fig. 4). These compounds showed less pleiotropic effects than SNP and NNA. Indeed, overnight treatment of excised leaves with SNAP or cPTIO did not change photosynthetic gas-exchange parameters (Fig. 4a), PEPCase activity (Fig. 4c) or *PPCK* gene expression (Fig. 4d). Both SNAP and cPTIO treatments moderately increased PEPCase-k activity in the light (Fig. 4b), and SNAP treatment greatly enhanced PEPCase-k activity in the dark (Fig. 4b). Comparable effects on PEPCase-k and PEPCase activity were produced by short-term treatments with SNP and NNA instead of SNAP and cPTIO (Table 2).

The ability of the NO donor SNP to change NO levels in sorghum plants was assessed by measuring nitrite concentration by Griess assay (Supplementary Fig. S1). Nitrite is one of the two primary stable and non-volatile breakdown products of NO and its level was increased by SNP treatment, both in long-term (Supplementary Fig. S1a) and in short-term (Supplementary Fig. S1b) experiments. Similar results were obtained with NO quantification by fluorescent microscopy imaging (Supplementary Fig. S2).

Collectively, the data show that increased NO levels caused enhanced PEPCase-k activity in the dark, the same effect previously observed in salt-treated plants (Echevarría et al. 2001; García-Mauriño et al. 2003). As in the case of salinity (Monreal et al. 2013), this effect was not dependent solely on changes of *PPCK* mRNA levels.

NO effects on PEPCase-k activity are not dependent on protein synthesis

In the leaves of C_4 plants, light upregulates PEPCase-k activity in a process dependent on protein synthesis, and cycloheximide (CHX) has been shown to suppress PEP-Case-k activity and PEPCase phosphorylation (Jiao et al. 1991; Giglioli-Guivarc'h et al. 1996). Light-triggered PEPCase-k activity in excised leaves (Fig. 5a) either supplied overnight with SNP or not. SNP increased PEPCase-k activity to some extent, both in the light and in the dark, as has been shown in previous experiments. In a subsequent experiment, leaves were illuminated to induce PEPCase-k synthesis, and then CHX was added (Fig. 5b). This caused
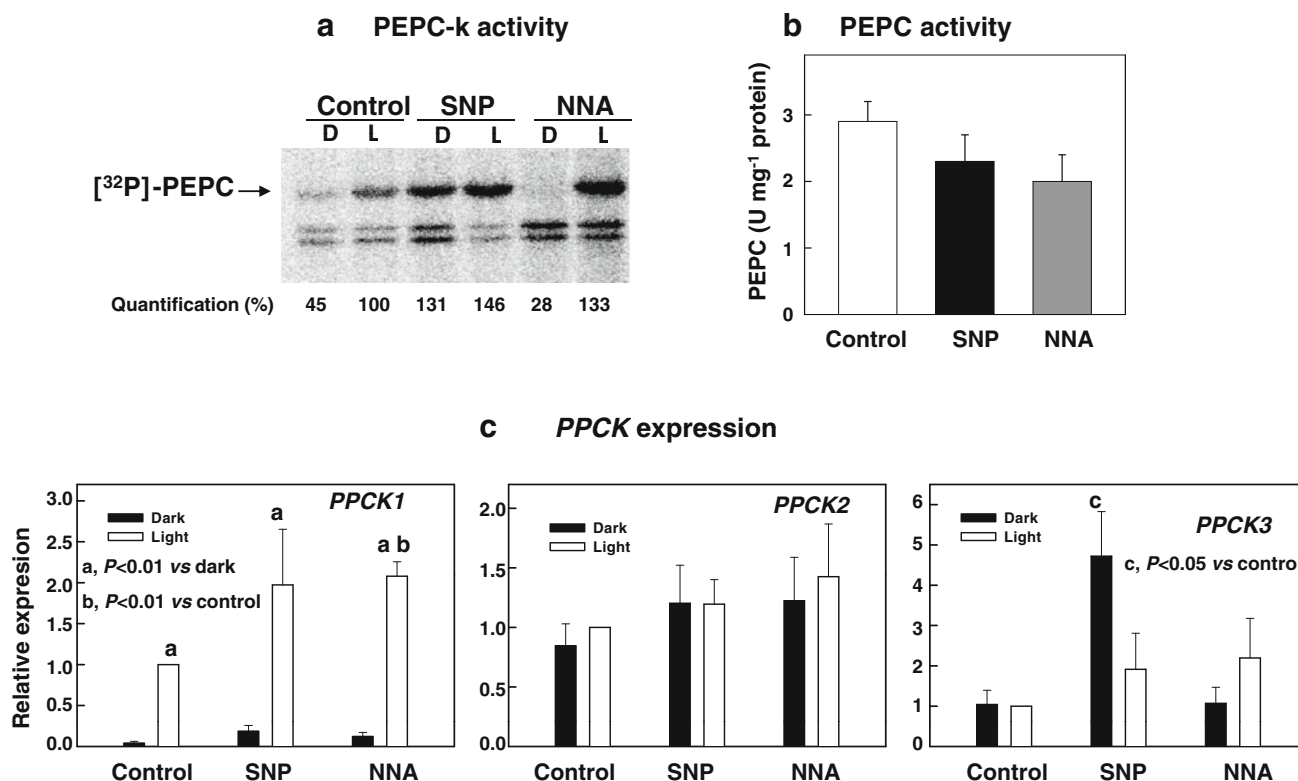
Fig. 3 Effect of SNP and NNA on PEPCase activity, PEPCase-k activity, and *PPCK* gene expression in long-term experiments. Plants were treated with 200 μM SNP or 200 μM NNA for 10 days. Excised leaves were placed in a 3 ml cuvette containing 0.01 mM Tris–HCl buffer, pH 8, in the presence or absence of 200 μM SNP or 200 μM NNA, and illuminated for 2 h (L) or kept at dark (D). **a** The in vitro PEPCase-k activity was assayed using 15 μg of desalted protein extracts, in the presence of 37 kBq of [γ-^{32}P]ATP and 0.2 U exogenous purified C$_4$ PEPCase, as described in "Materials and methods". Phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. *Arrow* shows the phosphorylated PEPCase. The PEPCase bands were quantified by phosphor imager (Fuji FLA-5100). **b** In parallel, PEPCase activity was measured in leaf extracts. Values of PEPCase activity the same in illuminated leaves as in leaves kept at dark. **c** Quantitative PCR (qPCR) analysis of the relative *PPCK1*, *PPCK2* and *PPCK3* transcript levels. Data are means ± SE from three independent experiments

PEPCase-k activity disappearance at a higher rate in control than in SNP-treated leaves. In the latter case, PEPCase-k activity kept at 70 % of leaves with SNP and without CHX, and nearly as high as in control leaves. This effect was not due to a decreased rate of absorption of CHX. This compound increased the level of *PPCK3* mRNA (Fig. 5c), and the same increase was produced in the presence or in the absence of SNP.

Some effects of salt on PEPCase-k activity are dependent on NO

Salinity increases PEPCase-k activity in sorghum leaves by several mechanisms (Monreal et al. 2013). Nonetheless, at 172 mM, the main effect of NaCl was to decrease the rate of PEPCase-k protein turnover. As expected, CHX did not decrease PEPCase-k activity in the light in 172 mM NaCl-treated plants (Fig. 6a), given that CHX was added after the light induction of the kinase. On the contrary, the effect of salt on PEPCase-k was abolished by the NO scavenger

cPTIO (Fig. 6b). This result demonstrates the involvement of NO in the effects of salt on PEPCase-k activity.

Salinity greatly decreased the rate of photosynthesis and the stomatal conductance in sorghum leaves (see Supplementary Fig. S3). Even so, the effect of cPTIO on leaves from salt-treated plants was not related to alteration of photosynthetic gas exchange, as it was not changed by cPTIO. On the contrary, cPTIO was able to decrease NO level in salt-treated plants (Supplementary Fig. S4).

Discussion

Despite the great advance in the knowledge of NO functions and mechanisms of action in plants, a relatively small amount of protein targets for NO have been identified. This work demonstrates that PEPCase-k activity is modulated by NO. In addition, results in this paper show that salinity increases endogenous NO specifically in mesophyll cells of sorghum leaves. The photosynthetic PEPCase isoenzyme
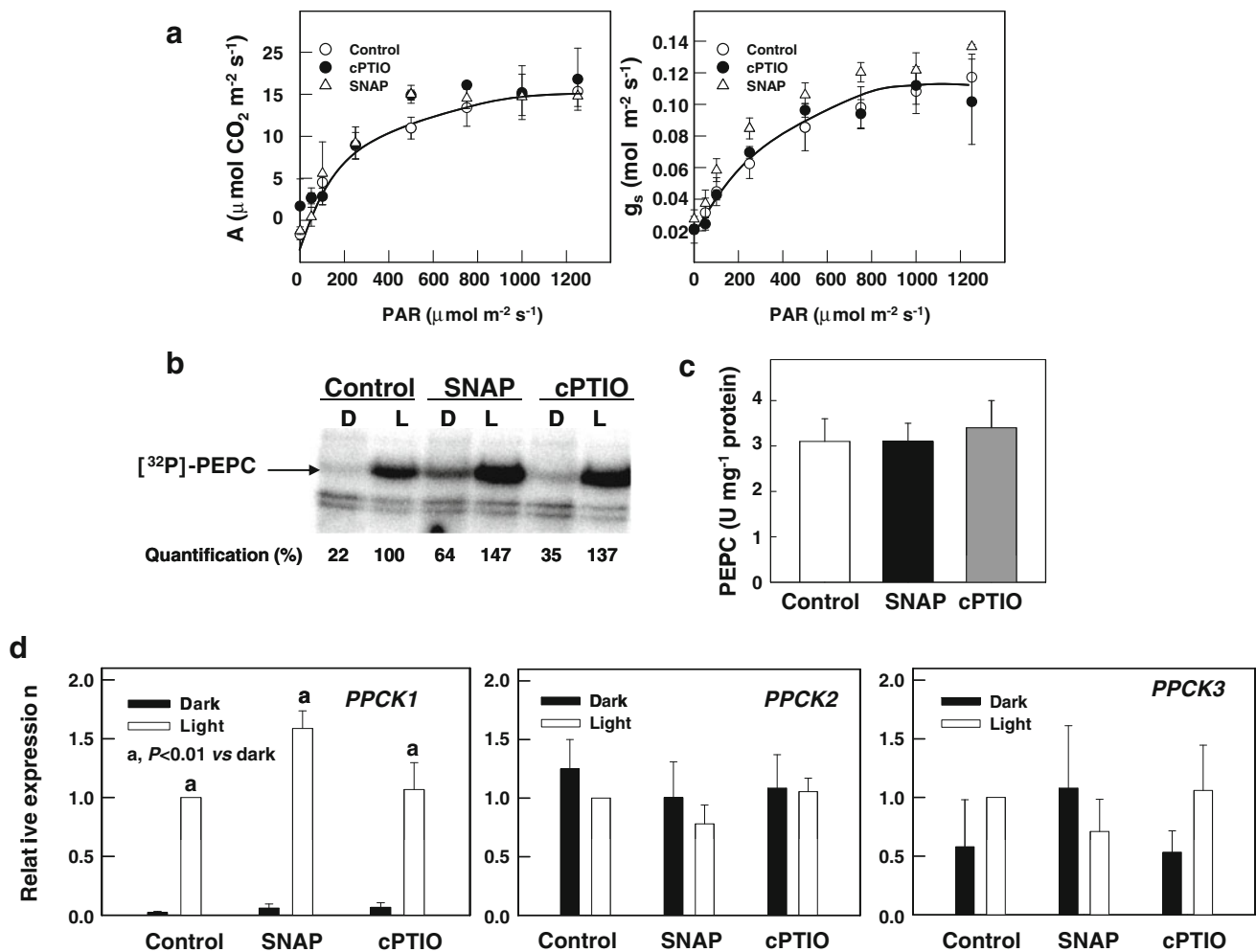
Fig. 4 Effect of SNAP and cPTIO on net photosynthetic rate, stomatal conductance, PEPCase activity, PEPCase-k activity, and *PPCK* gene expression in short-term experiments. Excised leaves were supplied overnight with 400 μM SNAP or 400 μM cPTIO. **a** Gas-exchange analysis of sorghum leaves. **b** The leaves were illuminated for 2 h (L) or kept in dark (D), and the in vitro PEPCase-k activity (**b**), PEPCase activity (**c**), and quantitative PCR (qPCR) analysis of the relative *PPCK1*, *PPCK2* and *PPCK3* transcript levels (**d**) were measured as in Fig. 3. PEPCase activity was the same in illuminated leaves as in leaves kept at dark Data are means ± SE from three independent experiments

Table 2 Effect of short-term treatment with SNP and NNA on PEPCase-k and PEPCase activity

Treatment	PEPCase-k activity (%)		PEPCase activity
	Dark	Light	(U mg^{-1} protein)
Control	34 ± 10	100	3.5 ± 0.1
SNP	83 ± 33	145 ± 35	3.8 ± 0.5
NNA	39 ± 4	108 ± 17	3.7 ± 0.1

Excised sorghum leaves were supplied overnight with 200 μM of the NO donor SNP or 200 μM of the NO synthesis inhibitor NNA. Other experimental conditions as in Fig. 3. Data are mean ± SE of two independent experiments

(C$_4$ PEPCase), which is located in mesophyll cells, is the most abundant PEPCase isoenzyme in sorghum leaves. Based on sequence and expression data, it was suggested that *PPCK1* encodes the kinase in mesophyll cells being responsible for the phosphorylation of the C$_4$ PEPCase (Shenton et al. 2006). *PPCK1* expression is triggered by light, and (according to Ct values; Monreal et al. 2013) the expression level of this gene is higher than those of *PPCK2* and *PPCK3*. Salinity changes the phosphorylation status of PEPCase in the light and in the dark, and these changes are followed by increased malate synthesis in the dark period that is subsequently used in photosynthesis in the light (Echevarría et al. 2001; García-Mauriño et al. 2003). This is in good accordance to the hypothesis that NO mediates some of the effects of salinity on PEPCase-k.

Long-term treatments with SNP (NO donor) and with NNA (inhibitor of NO synthesis) had a negative impact on sorghum plants. These broad deleterious effects could be due to NO imbalance and/or to specific effects of the
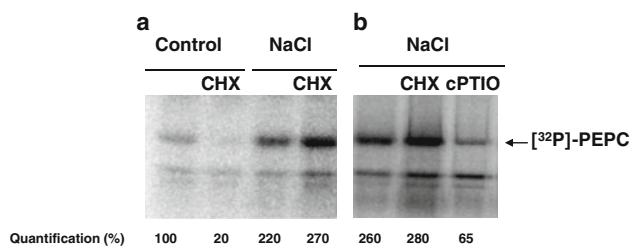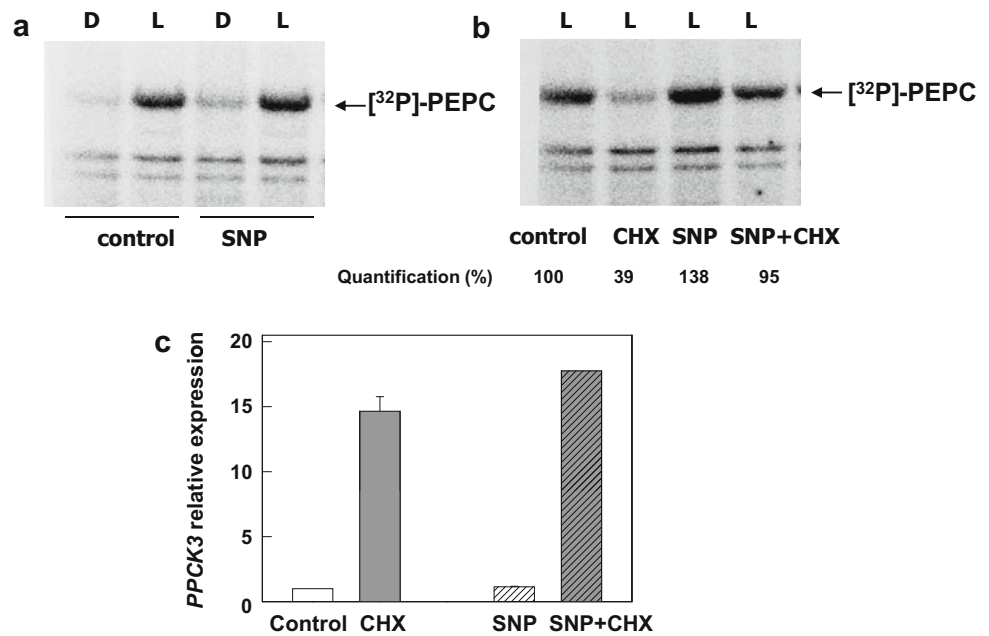
Fig. 6 The NO scavenger cPTIO decreases PEPCase-k activity in illuminated leaves from salt-stressed plants. **a** Leaves from control and 172 mM NaCl-treated plants (10 days) were illuminated for 30 min and then supplied with 20 μM cycloheximide (CHX) when indicated, and illuminated subsequently for 2.5 h. **b** Leaves from 172 mM NaCl-treated plants (10 days) were supplied overnight with 400 μM cPTIO when indicated. The leaves were illuminated for 30 min and then supplied with 20 μM cycloheximide (CHX) when indicated, and illuminated subsequently for 2.5 h. The in vitro PEPCase-k activity was measured as in Fig. 3

chemicals. The excess NO, as a consequence of extended SNP supply, could promote nitrosative stress (Corpas et al. 2011), and NNA is known to cause iron deficiency chlorosis in plants (Graziano and Lamattina 2005). A primary symptom resulting from iron deficiency is leaf yellowing, and this effect could be appreciated in NNA-treated sorghum plants. Iron deficiency induces a decline of rates of net photosynthesis and severely Fe chlorotic leaves result in lower stomatal opening (Larbi et al. 2006). Even so, the effects of NNA on photosynthetic gas-exchange parameters were smaller than the drop caused by reducing Fe supply from 100 to 10 μM NaFe-EDTA, and photosynthetic activity was noticeable, although reduced, in NNA-treated plants. This is a fundamental fact, because the light-mediated up-regulation of PEPCase-k is dependent on

photosynthetic activity (Jiao and Chollet 1992; Monreal et al. 2010). Despite of the diverse effects of long-term treatment, it could be observed that SNP increased PEPCase-k activity, resembling salinity effect. Short-term treatments with NO donors confirmed that they increased PEPCase-k activity both in illuminated leaves and in leaves kept at dark. At least in part, these effects were independent on protein synthesis, the same as previously demonstrated for salt stress (Monreal et al. 2013). In addition, not only NO donors mimicked salt effect on PEPCase-k activity, but also scavenging of NO abolished it. This strongly suggests that some effects of salinity on sorghum plants are mediated by NO.

NO does not seem to be indispensable for light-triggered up-regulation of PEPCase-k activity and *PPCK1* expression, which are not impaired by NNA or cPTIO. This suggests that the control of PEPCase-k activity by NO is related to stress responses more than being involved in the basic functioning of C_4 photosynthesis. In this line, increased PEPCase-k activity has been reported to be associated to nitrogen stress (Ueno et al. 2000), phosphate starvation (Gregory et al. 2009), oxidative stress (Izui et al. 2004) and salinity (Echevarría et al. 2001; García-Mauriño et al. 2003). Further studies will clarify whether NO regulates PEPCase-k activity in every stressful condition or if its action is restricted to salinity. Particularly appealing is the effect of long-term treatment with SNP on *PPCK3* expression. There is no information about the localization or the biological role for this kinase, and it may function in stress responses.

The turnover of PEPCase-k is thought to be regulated by the ubiquitin/proteasome pathway (Agetsuma et al. 2005),

and salinity (at least in part by increasing ABA; Monreal et al. 2007a), augmented PEPCase-k activity because of decreased rate of protein degradation (Monreal et al. 2013). Reducing the level of NO by cPTIO abolished this effect of salinity, and this result establishes a link between salinity and NO. Lithium decreased the rate of PEPCase-k activity disappearance caused by CHX (Monreal et al. 2007b). In this case, changes of PEPCase-k activity were directly related to the amount of protein, establishing that ionic stress, due to lithium treatment, was decreasing the rate of protein turnover. In the same line, ABA effects are also related to slower PEPCase-k degradation. Thus, it is possible that NO is also impacting on the turnover of the protein, although direct effects on PEPCase-k activity cannot be discarded.

NO and derived nitrogen species, such as peroxynitrite ($ONOO^-$), exert part of their biological activities by chemical modification of target proteins. These modifications include S-nitrosylation, nitration and oxidation of proteins (Besson-Bard et al. 2008; Astier et al. 2011; Vandelle and Delledonne 2011). Therefore, it could be also possible that NO controls PEPCase-k turnover and/or protein activity by direct modification of the protein. Furthermore, Ca^{2+} and protein kinases are increasingly recognized as prevalent mediators of NO effects. The interplay between NO and Ca^{2+} is highly complex and has important functional implications (Courtois et al. 2008). Calcium-dependent protein kinases (CDPKs) that are regulated through NO-dependent mechanisms have been implied in processes such as root growth (Lanteri et al. 2006) and stress responses and tolerance (Das and Pandey, 2010). Recombinant PEPCase-k protein was phosphorylated in vitro by the catalytic subunit of mammalian cyclic AMP-dependent protein kinase (PKA) in a conserved phosphorylation motif, which can be recognized by PKA and by plant CDPKs (Monreal et al. 2013). Thus, it is possible that the turnover of PEPCase-k is regulated by phosphorylation by a CDPK, as it has been reported for ACC synthase (Kamiyoshihara et al. 2010) and pyruvate kinase (Tang et al. 2003). Further work is necessary to clarify if nitrosylation/nitration and phosphorylation occur in parallel or if only one of these modifications is involved in the control of PEPCase-k turnover and/or protein activity.

On the other hand, phospholipase D (PLD) and phosphatidic acid (PA) have been reported to be involved in NO signaling (Laxalt et al. 2007; Lanteri et al. 2008; Distéfano et al. 2012) and in the signaling pathway that triggers PEPCase-k synthesis in response to light in sorghum leaves (Monreal et al. 2010). Interestingly, high salinity (258 mM NaCl) also increased *PPCK* mRNA levels (Monreal et al.
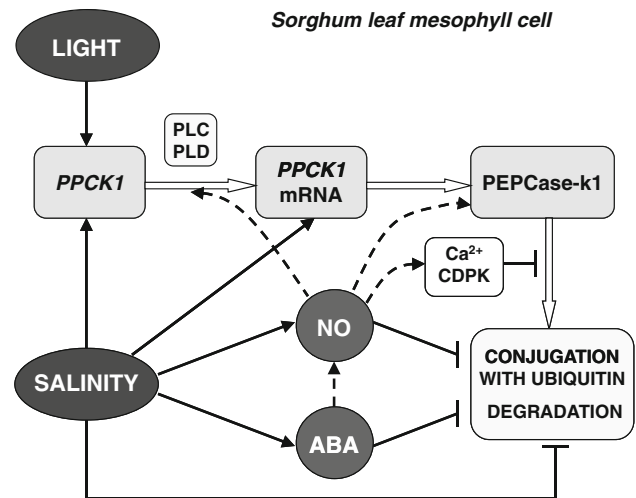

Fig. 7 Mechanism of the regulation of PEPCase-k1 activity under salt stress. Light triggers *PPCK*1 gene expression and PEPCase-k1 synthesis via activation of PLC (Giglioli-Guivarc'h et al. 1996) and PLD (Monreal et al. 2010). Salinity greatly enhances PEPCase-k activity (Echevarría et al. 2001; García-Mauriño et al. 2003); 172 mM salt treatment decreases the rate of PEPCase-k degradation, meanwhile 258 mM salt treatment increases *PPCK* gene expression and/or mRNA stability (Monreal et al. 2013). Salinity increases ABA level, which decreases the rate of PEPCase-k turnover (Monreal et al. 2007a). This mechanism proposes that NO mediates some of the effects of salinity on PEPCase-k1 activity, increasing *PPCK1* mRNA level, decreasing protein degradation and/or activating PEPCase-k1. A CDPK, which could be up-regulated by NO, has been proposed to regulate PEPCase-k1 degradation (Monreal et al. 2013). This complex system impacts on photosynthetic C_4 PEPCase and contributes to a better carbon balance under salt stress (García-Mauriño et al. 2003) Fig. 1

2013). This could be related to long-term treatment with NO donors on *PPCK* gene expression.

Finally, further work will be necessary to clarify the interplay between ABA and NO regulating PEPCase-k levels. In addition to guard cells and salinity, there are other metabolic backgrounds where NO and ABA work together (Hancock et al. 2011). The proposed mechanisms of regulation of PEPCase-k1 activity under salt stress are summarized in Fig. 7.

In summary, this paper shows that the effects of salinity on PEPCase-k activity in sorghum leaves can be mimicked with NO donors, and blocked by NO scavengers. In addition, salinity increases endogenous NO production specifically in mesophyll cells, in which photosynthetic C_4 PEPCase and PEPCase-k1 are located, and were changes of activity of these proteins would be most important in carbon economy under prolonged salt stress. It can be concluded that NO is involved in the complex control of PEPCase-k activity in sorghum leaves, and it may mediate some of plants responses to salinity that are related to this enzyme.

References

Agetsuma M, Furumoto T, Yanagisawa S, Izui K (2005) The ubiquitin-proteasome pathway is involved in rapid degradation of phosphoenolpyruvate carboxylase kinase for C_4 photosynthesis. Plant Cell Physiol 46:389–398

Astier J, Rasul S, Koen E, Manzoor H, Besson-Bard A, Lamotte O, Jeandroz S, Durner J, Lindermayr C, Wendehenne D (2011) S-nitrosylation: an emerging post-translational protein modification in plants. Plant Sci 181:527–533

Bai X, Yang L, Yang Y, Ahmad P, Yang Y, Hu X (2011) Deciphering the protective role of nitric oxide against salt stress at the physiological and proteomic levels in maize. J Proteome Res 10:4349–4364

Barroso JB, Corpas FJ, Carreras A, Sandalio LM, Valderrama R, Palma JM, Lupiáñez JA, del Río LA (1999) Localization of nitric-oxide synthase in plant peroxisomes. J Biol Chem 274:36729–36733

Besson-Bard A, Alain Pugin A, Wendehenne D (2008) New insights into nitric oxide signaling in plants. Annu Rev Plant Biol 59:21–39

Bethke PC, Badger MR, Jones RL (2004) Apoplastic synthesis of nitric oxide by plant tissues. Plant Cell 16:332–341

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chartier P, Prioul JL (1976) The effects of irradiance, carbon dioxide and oxygen on the net photosynthetic rate of the leaf: a mechanistic model. Photosynthetica 10:20–24

Chen J, Xiao Q, Wu FH, Dong XJ, He JX, Pei ZM, Zhang HL (2010) Nitric oxide enhances salt secretion and Na^+ sequestration in a mangrove plant, *Avicennia marina*, through increasing the expression of H^+-ATPase and Na^+/H^+ antiporter under high salinity. Tree Physiol 30:1570–1585

Corpas FJ, Barroso JB, Carreras A, Valderrama R, Palma JM, Leon AM, Sandalio LM, del Rio LA (2006) Constitutive arginine-dependent nitric oxide synthase activity in different organs of pea seedlings during plant development. Planta 224:246–254

Corpas FJ, Leterrier M, Valderrama R, Airaki M, Chaki M, Palma JM, Barroso JB (2011) Nitric oxide imbalance provokes a nitrosative response in plants under abiotic stress. Plant Sci 181:604–611

Courtois C, Besson A, Dahan J, Bourque S, Dobrowolska G, Pugin A, Wendehenne D (2008) Nitric oxide signalling in plants: interplays with Ca^{2+} and protein kinases. J Exp Bot 59:155–163

Das R, Pandey GK (2010) Expressional analysis and role of calcium regulated kinases in abiotic stress signalling. Curr Genomics 11:2–13

del Río LA, Corpas FJ, Barroso JB (2004) Nitric oxide and nitric oxide synthase activity in plants. Phytochemistry 65:783–792

Delledonne M, Xia YJ, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature 394:585–588

Desikan R, Griffiths R, Hancock J, Neill S (2002) A new role for an old enzyme: Nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 99:16314–16318

Distéfano AM, García-Mata C, Lamattina L, Laxalt AM (2008) Nitric oxide-induced phosphatidic acid accumulation: a role for phospholipases C and D in stomatal closure. Plant Cell Environ 31:187–194

Distéfano AM, Scuffi D, García-Mata C, Lamattina L, Laxalt AM (2012) Phospholipase Dδ is involved in nitric oxide-induced stomatal closure. Planta 236:1899–18907

Durner J, Wendehenne D, Klessig DF (1998) Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proc Natl Acad Sci USA 95:10328–10333

Echevarría C, Pacquit V, Bakrim N, Osuna L, Delgado B, Arrio-Dupont M, Vidal J (1994) The effect of pH on the covalent and metabolic control of C_4 phosphoenolpyruvate carboxylase from *Sorghum* leaf. Arch Biochem Biophys 315:425–430

Echevarría C, García-Mauriño S, Alvarez R, Soler A, Vidal J (2001) Salt stress increases the Ca^{2+}-independent phosphoenolpyruvate carboxylase kinase activity in *Sorghum* plants. Planta 214:283–287

Fan F, Du CX, Guo SR (2012) Effect of nitric oxide on proline metabolism in cucumber seedlings under salinity stress. J Amer Soc Hort Sci 137:127–133

Fewson CA, Nicholas DJD (1960) Utilization of nitric oxide by micro-organisms and higher plants. Nature 188:794–796

García-Mata C, Lamattina L (2001) Nitric Oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiol 126:1196–1204

García-Mata C, Lamattina L (2002) Nitric oxide and abscisic acid cross talk in guard cells. Plant Physiol 128:790–792

Garcia-Mata C, Gay R, Sokolovski S, Hills A, Lamattina L, Blatt MR (2003) Nitric oxide regulates K^+ and Cl^- channels in guard cells through a subset of abscisic acid-evoked signaling pathways. Proc Natl Acad Sci USA 100:11116–11121

García-Mauriño S, Monreal JA, Alvarez R, Vidal J, Echevarría C (2003) Characterization of salt stress-enhanced phosphoenolpyruvate carboxylase kinase activity in leaves of *Sorghum vulgare*: independence from osmotic stress, involvement of ion toxicity and significance of dark phosphorylation. Planta 216:648–655

Giglioli-Guivarc'h N, Pierre JN, Brown S, Chollet R, Vidal J, Gadal P (1996) The light-dependent transduction pathway controlling the regulatory phosphorylation of C_4-phospho*enol*pyruvate carboxylase in protoplasts from *Digitaria sanguinalis*. Plant Cell 8:573–586

Graziano M, Lamattina L (2005) Nitric oxide and iron in plants: an emerging and converging story. Trends Plant Sci 10:4–8

Gregory AL, Hurley BA, Tran HT, Valentine AJ, She YM, Knowles VL, Plaxton WC (2009) In vivo regulatory phosphorylation of the phosphoenolpyruvate carboxylase AtPPC1 in phosphate-starved Arabidopsis thaliana. Biochem J 420:57–65

Gupta KJ, Fernie AR, Kaiser WM, van Dongen JT (2011) On the origins of nitric oxide. Trend Plant Sci 16:160–168

Hancock JT, Neill SJ, Wilson ID (2011) Nitric oxide and ABA in the control of plant function. Plant Sci 181:555–559

Hewitt EJ (1966) Sand and water culture methods used in the study of plant nutrition. Commonwealth Bureau of Horticultural and Plantation Crops, East Malling Tech Commun No 22

Izui K, Matsumura H, Furumoto T, Kai Y (2004) Phosphoenolpyruvate carboxylase: a new era of structural biology. Annu Rev Plant Biol 55:69–84

Jiao J, Chollet R (1992) Light activation of maize phosphoenolpyruvate carboxylase protein-serine kinase activity is inhibited by mesophyll and bundle sheat-directed photosynthesis inhibitors. Plant Physiol 98:152–156

Jiao JA, Echevarría C, Vidal J, Chollet R (1991) Protein turnover as a component in the light/dark regulation of phosphoenolpyruvate carboxylase protein-serine kinase activity in C_4 plants. Proc Natl Acad Sci USA 88:2712–2715

Kamiyoshihara Y, Iwata M, Fukaya T, Tatsuki M, Mori H (2010) Turnover of LeACS2, a wound-inducible 1-aminocyclopropane-

1-carboxylic acid synthase in tomato, is regulated by phosphorylation/dephosphorylation. Plant J 64:140–150

Keyster M, Klein A, Ludidi N (2012) Caspase-like enzymatic activity and the ascorbate-glutathione cycle participate in salt stress tolerance of maize conferred by exogenously applied nitric oxide. Plant Signal Behav 7:349–360

Lamattina L, García-Mata C, Graziano M, Pagnussat G (2003) Nitric oxide: the versatility of an extensive signal molecule. Annu Rev Plant Biol 54:109–136

Lander HM, Sehajpal P, Levine DM, Novogrodsky A (1993) Activation of human peripheral blood mononuclear cells by nitric oxide-generating compounds. J Immunol 150:1509–1516

Lanteri ML, Pagnussat GC, Lamattina L (2006) Calcium and calcium-dependent protein kinases are involved in nitric oxide- and auxin-induced adventitious root formation in cucumber. J Exp Bot 57:1341–1351

Lanteri ML, Laxalt AM, Lamattina L (2008) Nitric oxide triggers phosphatidic acid accumulation via phospholipase D during auxin-induced adventitious root formation in cucumber. Plant Physiol 147:188–198

Larbi A, Abadía A, Abadía J, Morales F (2006) Down co-regulation of light absorption, photochemistry, and carboxylation in Fe deficient plants growing in different environments. Photosyn Res 89:113–126

Laxalt AM, Raho N, Ten Have A, Lamattina L (2007) Nitric oxide is critical for inducing phosphatidic acid accumulation in xylanase-elicited tomato cells. J Biol Chem 282:21160–21168

Leshem YY (1996) Nitric oxide in biological systems. Plant Growth Regul 18:155–159

Li S, Assmann SM, Albert R (2006) Predicting essential components of signal transduction networks: a dynamic model of guard cell abscisic acid signalling. PLoS Biol 4:1732–1748

Monreal JA, Feria AB, Vinardell JM, Vidal J, Echevarría C, García-Mauriño S (2007a) ABA modulates the degradation of phosphoenolpyruvate carboxylase kinase in sorghum leaves. FEBS Lett 581:3468–3472

Monreal JA, López-Baena FJ, Vidal J, Echevarría C, García-Mauriño S (2007b) Effect of LiCl on phosphoenolpyruvate carboxylase kinase and the phosphorylation of phosphoenolpyruvate carboxylase in leaf disks and leaves of Sorghum vulgare. Planta 225:801–812

Monreal JA, López-Baena FJ, Vidal J, Echevarría C, García-Mauriño S (2010) Involvement of phospholipase D and phosphatidic acid in the light-dependent up-regulation of sorghum leaf phosphoenolpyruvate carboxylase-kinase. J Exp Bot 61:2819–2827

Monreal JA, Arias-Baldrich C, Pérez-Montaño F, Gandullo J, Echevarría C, García-Mauriño S (2013) Factors involved in the rise of phosphoenolpyruvate carboxylase-kinase activity caused by salinity in sorghum leaves. Planta 237:1401–1413

Neill SJ, Desikan D, Clarke A, Hancock JT (2002) Nitric oxide is a novel component of abscisic acid signaling in stomatal guard cells. Plant Physiol 128:13–16

Neill S, Barros R, Bright J, Desikan R, Hancock J, Harrison J, Morris P, Ribeiro D, Wilson I (2008) Nitric oxide, stomatal closure, and abiotic stress. J Exp Bot 59:165–176

Paterson AH, Bowers JE, Bruggmann R, Dubchak I, Grimwood J, Gundlach H, Haberer G, Hellsten U, Mitros T, Poliakov A, Schmutz J, Spannagl M, Tang H, Wang X, Wicker T, Bharti AK, Chapman J, Feltus FA, Gowik U, Grigoriev IV, Lyons E, Maher CA, Martis M, Narechania A, Otillar RP, Penning BW, Salamov AA, Wang Y, Zhang L, Carpita NC, Freeling M, Gingle AR, Hash CT, Keller B, Klein P, Kresovich S, McCann MC, Ming R, Peterson DG, Mehboobur R, Ware D, Westhoff P, Mayer KF, Messing J, Rokhsar DS (2009) The Sorghum bicolor genome and the diversification of grasses. Nature 457:551–556

Planchet E, Jagadis Gupta K, Sonoda M, Kaiser WM (2005) Nitric oxide emission from tobacco leaves and cell suspensions: rate limiting factors and evidence for the involvement of mitochondrial electron transport. Plant J 41:732–743

Rubio-Casal AE, Leira-Doce P, Figueroa ME, Castillo JM (2010) Contrasted tolerance to low and high temperatures of three tree taxa co-occurring on coastal dune forests under Mediterranean climate. J Arid Environ 74:429–439

Shenton M, Fontaine V, Hartwell J, Marsh JT, Jenkins GI, Nimmo HG (2006) Distinct patterns of control and expression amongst members of the PEP carboxylase kinase gene family in C_4 plants. Plant J 48:45–53

Shi Q, Ding F, Wang X, Wei M (2007) Exogenous nitric oxide protect cucumber roots against oxidative stress induced by salt stress. Plant Physiol Biochem 45:542–550

Sokolovski S, Hills A, Gay R, Garcia-Mata C, Lamattina L, Blatt MR (2005) Protein phosphorylation is a prerequisite for intracellular Ca^{2+} release and ion channel control by nitric oxide and abscisic acid in guard cells. Plant J 43:520–529

Stohr C, Strube F, Marx G, Ullrich WR, Rockel P (2001) A plasma membrane-bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite. Planta 212:835–841

Tang GQ, Hardin SC, Dewey R, Huber SC (2003) A novel C-terminal proteolytic processing of cytosolic pyruvate kinase, its phosphorylation and degradation by the proteasome in developing soybean seeds. Plant J 34:77–99

Tanou G, Molassiotis A, Diamantidis G (2009) Hydrogen peroxide- and nitric oxide-induced systemic antioxidant prime-like activity under NaCl-stress and stress-free conditions in citrus plants. J Plant Physiol 166:1904–1913

Tian QY, Sun DH, Zhao MG, Zhang WH (2007) Inhibition of nitric oxide synthase (NOS) underlies aluminium-induced inhibition of root elongation in Hibiscus moscheutos. New Phytol 174:322–331

Uchida A, Jagendorf AT, Hibino T, Takabe T, Takabe T (2002) Effects of hydrogen peroxide and nitric oxide on both salt and heat stress tolerance in rice. Plant Sci 163:515–523

Ueno Y, Imanari E, Emura J, Yoshizawa-Kumagaye K, Nakajima K, Inami K, Shiba T, Sakakibara H, Sugiyama T, Izui K (2000) Immunological analysis of the phosphorylation state of maize C_4-form phosphoenolpyruvate carboxylase with specific antibodies raised against a synthetic phosphorylated peptide. Plant J 21:17–26

Vandelle E, Delledonne M (2011) Peroxynitrite formation and function in plants. Plant Sci 181:534–539

Von Caemmerer S, Farquhar GD (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta 153:376–387

Wendehenne D, Durner J, Klessig DF (2004) Nitric oxide: a new player in plant signalling and defense responses. Curr Opin Plant Biol 7:449–455

Wu X, Zhu W, Zhang H, Ding H, Zhang HJ (2011) Exogenous nitric oxide protects against salt-induced oxidative stress in the leaves from two genotypes of tomato (Lycopersicom esculentum Mill.). Acta Physiol Plant 33:1199–1209

Yamasaki H, Sakihama Y (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. FEBS Lett 468:89–92

Zhang YY, Wang LL, Liu YL, Zhang Q, Wei QP, Zhang WH (2006) Nitric oxide enhances salt tolerance in maize seedlings through increasing activities of proton-pump and Na^+/H^+ antiport in the tonoplast. Planta 224:545–555

Zhao LQ, Zhang F, Guo JK, Yang YL, Li BB, Zhang LX (2004) Nitric oxide functions as a signal in salt resistance in the calluses from two ecotypes of reed. Plant Physiol 134:849–857

Zhao MG, Tian QT, Zhang WH (2007) Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in Arabidopsis. Plant Physiol 144:206–217