Free Radical Biology and Medicine 71 (2014) 36–48

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Original Contribution

Sodic alkaline stress mitigation by interaction of nitric oxide and polyamines involves antioxidants and physiological strategies in *Solanum lycopersicum*

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ARTICLE INFO

Article history:
Received 20 September 2013
Received in revised form
13 February 2014
Accepted 17 February 2014
Available online 28 February 2014

Keywords:
Tomato
Alkaline stress
Oxidative stress
Nitric oxide
Polyamines
Alleviating effects
Free radicals

ABSTRACT

Nitric oxide (NO) and polyamines (PAs) are two kinds of important signal in mediating plant tolerance to abiotic stress. In this study, we observed that both NO and PAs decreased alkaline stress in tomato plants, which may be a result of their role in regulating nutrient balance and reactive oxygen species (ROS), thereby protecting the photosynthetic system from damage. Further investigation indicated that NO and PAs induced accumulation of each other. Furthermore, the function of PAs could be removed by a NO scavenger, cPTIO. On the other hand, application of MGBG, a PA synthesis inhibitor, did little to abolish the function of NO. To further elucidate the mechanism by which NO and PAs alleviate alkaline stress, the expression of several genes associated with abiotic stress was analyzed by qRT-PCR. NO and PAs significantly upregulated ion transporters such as the plasma membrane Na^+/H^+ antiporter (*SlSOS1*), vacuolar Na^+/H^+ exchanger (*SlNHX1* and *SlNHX2*), and Na^+ transporter and signal components including ROS, MAPK, and Ca^{2+} signal pathways, as well as several transcription factors. All of these play important roles in plant adaptation to stress conditions.

Plants are frequently challenged by abiotic stresses including drought, salinity, alkalinity, and extreme temperatures, which negatively affect their growth and productivity. Among these, salinity and alkalinity most chronically limit economic crop yields. Worldwide, salt stress affects 831 million hectares, 434 million of which also suffer from sodic alkaline conditions [1]. Sodic alkaline stress manifests as three major conditions detrimental to plant growth and development: high soil pH, water deficit, and toxicity associated with excess Na^+ uptake. Excess Na^+ has been shown to cause K^+ deficiency as well as other nutrient imbalances [2]. At the subcellular level, alkaline stress can induce oxidative stress mediated by reactive oxygen species (ROS), enhancing the deleterious effects of these three factors. Recently, increasing attention has been paid to the effect of alkaline stress on plants, including redox homeostasis and signal transduction factors [3].

Nitric oxide (NO) is a hydrophobic, highly diffusible gaseous molecule with a broad spectrum of regulatory functions involved in various plant growth and developmental processes including germination, metabolism, signal transport, flowering, and senescence [4]. In addition, increasing evidence indicates that NO is a ubiquitous and important endogenous signal molecule that plays a critical role in plant disease resistance, ion homeostasis, cell death, hormone responses, and stress tolerance [4,5]. Previous reports suggested that NO, as either a stress-inducing agent or a protective molecule, plays an important role in ROS scavenging [6]. Exogenous NO can elevate antioxidant levels under various stresses by activating antioxidant defense systems, which can play key roles in plants' tolerance to stress [7]. NO also acts as a secondary messenger implicated in many plant cell signaling events, including a ROS-based signaling pathway induced by stress [8].

Polyamines (PAs), including putrescine (Put), spermidine (Spd), and spermine (Spm), are low-molecular-weight aliphatic polycations that are quite common in living organisms. Being positively charged, they can interact with negatively charged molecules such as proteins, nucleic acids, membrane phospholipids, and cell wall constituents, thereby activating or stabilizing these molecules to alleviate cell injury under stress conditions [9]. The strong antioxidant nature of PAs results in the neutralization or scavenging of ROS under normal and oxidative stress conditions [10]. In addition,

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http://dx.doi.org/10.1016/j.freeradbiomed.2014.02.018
0891-5849/

there is also evidence that interplay occurs among PAs, ROS generation, and NO signaling in abscisic acid (ABA)-mediated responses to osmotic stresses including drought, salt, and alkaline stress [11]. ABA and PAs can, along with amine oxidase activity, increase H_2O_2 and Ca^{2+} concentrations in guard cells to induce stomatal closure [12]. PAs can also play an important role in secondary messenger signaling cross-talk [9]. In accordance with the broad physiological functions of PAs, exogenous PAs have also been reported to improve stress tolerance in various plants [13–16].

PAs and cytokinins share some overlapping physiological functions in processes involving NO [17]. It has been observed that cytokinins rapidly induce NO biosynthesis in plant cell cultures of *Arabidopsis,* parsley, and tobacco [18]. Similarly, PA metabolism also has an impact on NO formation [17]. Spd and Spm were able to induce rapid NO biosynthesis, whereas Put had little effect. Such induced NO activity may act as a link between PA-mediated stress response and other stress mediators [19]. In turn, the effect of NO on PA biosynthesis was demonstrated in *Medicago truncatula* [20], suggesting that NO and PAs are interlinked, forming part of a signaling cross-talk mechanism [16].

Because of our interest in the effects of both PAs and NO on stress response, as well as our preliminary observation of an enhanced alkaline stress tolerance in tomato seedlings treated with either NO or Spm in culture, we were inclined to investigate whether PAs and NO might have an interactive effect on tomato tolerance to alkaline stress. We hypothesized that NO and PAs may stimulate and induce accumulation of each other. Interestingly, it was possible to inhibit Spm's mitigative effect on alkaline stress by using the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). In contrast, the mitigative effect of NO could not be removed by the PA synthesis inhibitor methylglyoxalbisguanylhydrazone (MGBG). These results indicate that NO may act as a downstream signal of PAs to enhance plants' stress tolerance. In this study, the roles of Spm and NO in regulating $NaHCO_3$-induced Na^+ toxicity and oxidative stress were assessed in tomato seedlings. Pharmacologic and biochemical analyses were used to determine whether there was an interaction between PAs and NO in tomato seedlings under alkaline stress. The results could deepen our understanding of the overlapping functions of PAs and NO in plant response to sodic alkaline stress.

Material and methods

Plant materials, growth conditions, and stress treatments

Tomato seeds (*Solanum lycopersicum* L.) were sterilized in 2.5% NaClO and germinated in vermiculite. After emergence, batches of five seedlings were grown hydroponically in a plastic container filled with 5 L of Hoagland nutrient solution [21]. Plants were cultivated in a glasshouse maintained at 25–30:15–20 °C (day: night) with a photoperiod of 12–14 h. Sodium nitroprusside (SNP; a NO donor, 0.1 mM), 0.4 mM *S*-nitrosoglutathione (GSNO; another NO donor), 0.25 mM Spm, 1 mM cPTIO (a specific scavenger of NO), and 1 mM MGBG (a specific inhibitor for polyamine biosynthesis) were used in various treatments 24 h before stress. $NaHCO_3$ (0 and 75 mM) was applied separately for the control and alkaline stress. Nutrient solution including the above reagents in each treatment was renewed every 2 days. The experiment was arranged in a randomized complete block design with five replicates. Each replicate included two containers of 10 plants. Gene expression in tomato roots was analyzed by qRT-PCR 15 h after treatment, and other physiological indices were assessed 10 days after treatment.

Photosynthetic apparatus analysis

The third fully expanded leaf of each plant was used to determine photosynthetic parameters by a photosynthesis system (LI-6400, Lincoln, USA). Chlorophyll was extracted in 80% acetone and measured according to previous descriptions [1]. Chlorophyll fluorescence parameters were measured on the third fully expanded leaf after 30 min in the dark using chlorophyll fluorescence imaging (FluorCam7, Photon Systems Instruments, USA) [22]. The maximum PSII quantum yield [$F_v/F_m = (F_m - F_o)/F_m$], photochemical efficiency of open PSII centers [$F_v'/F_m' = (F_m' - F_o')/F_m'$], actual photochemical efficiency of PSII [$\Phi_{PSII} = (F_m' - F_s)/F_m'$], photochemical quenching coefficient [$q_P = (F_m' - F_s)/(F_m'-F_o')$], nonphotochemical quenching [NPQ = $(F_m - F_m')/F_m'$], imbalance of excitation energy distribution between PSII and PSI [$\beta/\alpha - 1 = (F_m' - F_o')/(F_m' - F_s) - 1$], fraction of light absorbed in PSII antennae that was utilized in photosynthetic electron transport ($P = F_v'/F_m' \times q_P$), fraction of light absorbed in PSII antennae that was dissipated via thermal energy dissipation in the antennae ($D = 1 - F_v'/F_m'$), and fraction of light absorbed in PSII antennae that was in excess [$E_X = F_v'/F_m' \times (1 - q_P)$] were determined. For immunoelectron microscopic analysis of chloroplast ultrastructure, the samples were fixed as described by Liu and Guo [23].

Determination of Na^+ and K^+ contents

An atomic absorption spectrophotometer (TAS-990, Purkinje General, China) was used to determine concentrations of Na^+ and K^+ [24].

Analysis of ROS generation

The histochemical staining of $O_2^{\bullet-}$ and H_2O_2 was performed as follows. In the case of $O_2^{\bullet-}$, leaves were vacuum infiltrated directly with 0.1 mg ml^{-1} nitroblue tetrazolium (NBT) in 25 mM K-Hepes buffer (pH 7.8) and incubated at 25 °C in the dark for 2 h. In the case of H_2O_2, leaves were vacuum infiltrated with 1 mg ml^{-1} 3,3'-diaminobenzidine (DAB) in 50 mM Tris–acetate (pH 3.8) and incubated at 25 °C in the dark for 24 h. In both cases, leaves were rinsed in 80% ethanol five times at 70 °C and then photographed [25].

$O_2^{\bullet-}$ was quantified using the method of hydroxylamine oxidation. Extract (0.5 ml) was mixed with 1 ml hydroxylamine and incubated at 25 °C for 1 h. Then 1 ml of 17 mM *p*-aminobenzene sulfonic acid and 7 mM α-naphthylamine solution was added and the solution was incubated for an additional 20 min. The reaction was measured spectrophotometrically at 530 nm and the $O_2^{\bullet-}$ concentration was obtained using a linear calibration curve of $NaNO_2$ [1]. The H_2O_2 concentration was determined according to Xia et al. [25]. The assay was based on the absorbance change in the titanium peroxide complex at 415 nm. Absorbance values were quantified using a standard curve generated from known concentrations of H_2O_2.

In situ localization of H_2O_2 was performed using the highly sensitive, cell-permeative probe 2',7'-dichlorofluorescin diacetate (DCFH-DA) according to Capone et al. [26]. Roots were incubated in 1 ml buffer (20 mM K-phosphate, pH 6.0, containing 50 mM DCFH-DA and 3 mg ml^{-1} horseradish peroxidase) for 20 min at 25 °C in the dark. Roots were removed, washed in the same buffer, and visualized immediately. Images were captured with a confocal laser scanning microscope system (Leica TCS SL, Leica Microsystems, Germany), using standard filters and collection modalities for DCFH-DA green fluorescence (excitation 488 nm; emission 525 nm). The pixel intensities of fluorescence images, acquired using a confocal microscope, were determined by using ImageJ software (National Institutes of Health, USA).