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Original Contribution

The nitric oxide donor sodium nitroprusside regulates polyamine and proline metabolism in leaves of *Medicago truncatula plants*

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ABSTRACT

Nitric oxide (NO), polyamines, and proline have all been suggested to play key roles in a wide spectrum of physiological processes and abiotic stress responses. Although exogenous application of polyamines has been shown to induce NO production, the effect of NO on polyamine biosynthesis has not yet been elucidated. Several reports exist that demonstrate the protective action of sodium nitroprusside (SNP), a widely used NO donor, which acts as a signal molecule in plants responsible for the regulation of the expression of many defense-related enzymes. This study attempted to provide a novel insight into the effects of application of low (100 μM) and high (2.5 mM) concentrations of SNP on the biosynthesis of two major abiotic stress response-related metabolites, polyamines and proline, in mature (40 day) and senescing (65 day) *Medicago truncatula* plants. Physiological data showed that long-term (24 h), higher SNP concentration resulted in decreased photosynthetic rate and stomatal conductance followed by intracellular putrescine and proline accumulation, as a result of an increase in biosynthetic arginine decarboxylase (ADC) and Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) enzymatic activity, respectively. Further analysis of polyamine oxidase (PAO)/diamine oxidase (DAO) polyamine catabolic enzymes indicated that DAO enzymatic activity increased significantly in correlation with putrescine accumulation, whereas PAO activity, involved in spermidine/spermine degradation, increased slightly. Moreover, transcriptional analysis of polyamine and proline metabolism genes (*P5CS*, *P5CR*, *ADC*, *SPMS*, *SPDS*, *SAMDC*, *PAO*, *DAO*) further supported the obtained data and revealed a complex SNP concentration-, time-, and developmental stage-dependent mechanism controlling endogenous proline and polyamine metabolite production. This is the first report to provide a global analysis leading to a better understanding of the role of the widely used NO donor SNP in the regulation of key stress-related metabolic pathways.

Nitric oxide (NO) is a key signaling molecule that mediates a variety of physiological functions including growth and development [1], biochemical interactions, and defense responses against plant abiotic and biotic stresses [2]. Previous reports have suggested NO as a stress-inducing agent [3], whereas others have assigned it as a protective molecule [4,5], functioning as an antioxidant by scavenging reactive oxygen species (ROS)[1] [6].

The cytoprotective or cytotoxic actions of NO in plant metabolism [7] depend to a large extent on its local tissue concentration (C. Antoniou et al., unpublished data) and are affected by the rate of synthesis, rate of displacement, and efficiency of removal of reactive nitrogen species (RNS) and ROS [8]. Interestingly, the existence of a cross talk between ROS and RNS has been recently documented [9]. In regard to cytotoxic action, NO treatment results in the generation of harmful ROS such as superoxide anion($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) (C. Antoniou et al., unpublished data; [10]). These ROS react with lipids, proteins, and nucleic acids, causing lipid peroxidation, membrane damage, and enzyme inactivation (C. Antoniou et al., unpublished data). The NO toxic effect resulting from the oxidative state may be alleviated by several antioxidative defense systems, including induction of polyamines (PAs) and proline [11–13].

To fully understand the diverse bioregulatory functions of nitric oxide, we studied important polyamine and proline metabolic responses following exogenously applied NO in *Medicago truncatula*. For this reason, sodium nitroprusside (SNP) was chosen as the most commonly used NO^+ donor [14,15] because of its relatively low cost and well-documented application [16], as well as the continuous, long-lasting NO production compared with other NO donors [14].

PAs are ubiquitous low-molecular-weight, polycationic, aliphatic amines involved in the regulation of plant growth and development. Putrescine (Put), spermidine (Spd), and spermine

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[1] *Abbreviations used:* ROS, reactive oxygen species; RNS, reactive nitrogen species; SNP, sodium nitroprusside; PA, polyamine; ODC, ornithine decarboxylase; ADC, arginine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase; SAMDC, *S*-adenosylmethionine decarboxylase; DAO, diamine oxidase; PAO, polyamine oxidase; Put, putrescine; Spd, spermidine; Spm, spermine; P5CS, Δ^1-pyrroline-5-carboxylate synthetase; P5CR, pyrroline-5-carboxylate reductase.

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(Spm) are the most abundant PAs in plant cells [17]. Because of their roles in osmotic adjustment, maintenance of membrane stability, and free radical scavenging, they are also important in protecting plants against abiotic stress [11].

Polyamine metabolism (biosynthesis and catabolism) is crucial in regulating polyamine levels in cells. In plants, the simplest polyamine, putrescine, is derived either directly from ornithine by ornithine decarboxylase (ODC) or indirectly from arginine, by arginine decarboxylase (ADC) activity [18]. Spd and Spm are formed by the subsequent addition of an aminopropyl moiety [13] in reactions catalyzed by Spd synthase (SPDS) and Spm synthase (SPMS), respectively. The donor of the aminopropyl group, decarboxylated *S*-adenosylmethionine (SAM), is formed by decarboxylation of SAM in a reaction catalyzed by SAM decarboxylase (SAMDC) [19]. Diamine oxidase (DAO) and polyamine oxidase (PAO) are thought to play a major role in the production of H_2O_2 via catabolism of PAs in plant tissues [20]. In particular, DAO produces Δ^1-pyrroline and H_2O_2 as a result of Put oxidation, whereas PAO uses Spd or Spm to produce Δ^1-pyrroline and 1,3-diaminopropane or 1,3-diaminopropane and 1,5-diazabicyclononane, respectively [21].

In plants, proline (Pro) is synthesized from either glutamate or ornithine [22,23]. The first two steps of proline biosynthesis from glutamate are catalyzed by the rate-limiting bifunctional enzyme Δ^1-pyrroline-5-carboxylate synthetase (P5CS), which produces glutamic-γ-semialdehyde (GSA). This GSA is spontaneously converted to pyrroline-5-carboxylate (P5C) that is reduced by P5C reductase (P5CR) to proline [22]. Environmental stresses induce the accumulation of Pro in many plant species [22]. Proline provides protection against stress by acting as an N-storage compound; as an osmolite, a hydrophilic protectant for enzymes and cellular structures; and as a free radical scavenger [24].

The polyamine metabolic pathway is interconnected with other metabolic routes involved in the formation of various signaling molecules and metabolites that are relevant in plant stress responses [19]. For instance, interactions between stress-induced Pro [25] and polyamine accumulation in response to various abiotic stresses may reflect the fact that they share ornithine as a common precursor [19]. Furthermore, recent studies indicate that polyamines are related to NO through arginine [26], a common precursor in their biosynthetic routes. A recent publication by Tun et al. [27] presents evidence that PAs induce production of NO in *Arabidopsis thaliana* and reports that NO could be a link between polyamine-mediated stress responses and other stress mediators. Although further confirmation of PA-induced NO production is necessary, the observations previously reported [27] could imply the presence of an unknown enzyme responsible for direct conversion of PAs to NO. A schematic representation of the suggested link between PAs, Pro, and NO is shown in Supplementary Fig. 1.

In this study, the effects of SNP application on polyamine and proline levels and metabolism were determined in *M. truncatula* plants in relation to developmental stage-, time-, and concentration-dependent response. Free PAs and Pro levels were quantified in leaves after 3 and 24 h SNP application, and key polyamine and proline metabolism enzymatic activities were determined along with their respective gene expression levels.

Material and methods

Plant material and growth conditions

This study was conducted using *M. truncatula* genotype Jemalong A17. After scarification, seeds were sown in sterile perlite:sand (3:1) pots and placed at 4 °C for 4 days for stratification. Plants were grown in a growth chamber at 22/16 °C day/night temperatures, at 60–70% relative humidity, with a photosynthetic photon flux density of 100 μmol m^2 s^{-1} and a 16/8-h photoperiod.

Plant materials were treated as follows: (1) control–H_2O treatment and (2) SNP treatment. SNP treatment was imposed by vacuum infiltration of two different SNP concentrations (100 μM and 2.5 mM) at two different developmental stages: mature (40 days) and senescing (65 days) plants. Leaf samples were harvested at specific time points (3 and 24 h after SNP application), frozen in liquid nitrogen, and stored at −80 °C for subsequent analyses.

Physiological measurements

Stomatal conductance was measured using a Δ*T*-Porometer AP4 (Delta-T Devices, Cambridge, UK) according to the manufacturer's instructions.

Chlorophyll fluorescence parameters (F_v/F_m) of leaves representing the maximum photochemical efficiency of photosystem II (PSII) were measured with an OptiSci OS-30p chlorophyll fluorometer (Opti-Sciences, Hudson, NH, USA). Leaves were incubated in the dark for 30 min before measurements.

Polyamine extraction

Plant material (0.1 g) was homogenized in 1.5 ml of 5% (v/v) prechilled perchloric acid, kept at 4 °C for 1 h, and then centrifuged at 15,000 g for 40 min. The resulting supernatant phase, containing the free polyamine fraction, was further analyzed. Standards and plant extracts were then subjected to benzoylation reaction. One milliliter of 2 N NaOH was mixed with 500 μl plant extract. After addition of 10 μl benzoyl chloride, vortexing for 30 s, and incubation for 20 min at room temperature, 2 ml of saturated NaCl was added. Benzoyl polyamines were extracted in 2 ml diethyl ether and 1 ml of the ether phase was collected, evaporated to dryness, and redissolved in 200 μl methanol. Polyamine standards were treated in a similar way with the cell extract in the reaction mixture [28].

HPLC analysis of benzoylated derivatives

The benzoyl derivatives were separated and analyzed by an HPLC system (Waters Series HPLC, Milford, MA, USA), in which 20 μl of each benzoyl polyamine was injected into a 20-μl loop, loaded onto a C18 reverse-phase column (25 cm × 2.1 mm, 5 μm; Supelco Analytical, Bellefonte, PA, USA) at room temperature. Samples were eluted from the column in a gradient program at a flow rate of 0.2 ml/min, according to [29], with some modifications. Polyamine peaks were detected with a UV detector at 254 nm. Three polyamine standards (Sigma Chemical Co.) of Put, Spd, and Spm were prepared at various concentrations for the production of the appropriate standard curves.

Determination of proline content

The levels of free proline in leaf samples were determined using the ninhydrin reaction according to [30]. Proline concentration was determined using a standard curve and calculated on a fresh weight basis (μmol proline/g FW) [31].

Enzymatic activity assays

P5CS enzyme activity assay

Plant cell extraction and P5CS activity measurements were processed according to [32]. Leaves were homogenized in an extraction buffer (100 mM Tris–Cl, pH 7.5, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride)