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Research article

Polyamine-induced nitric oxide generation and its potential requirement for peroxide in suspension cells of soybean cotyledon node callus

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ABSTRACT

Polyamines (PAs) induce nitric oxide (NO) generation in plant tissues; however, their mechanism is still unclear. In the present study, suspension cells of soybean cotyledon-node callus were employed. Using a NO-specific fluorescent dye, DAF-FM-DA (3-amino, 4-aminomethyl-2′, 7′-difluorescein, diacetate), and laser confocal scanning microscopy, changes in NO generation induced by exogenous PAs were examined. The results of this study showed that NO fluorescence was significantly induced above endogenous levels when callus cells were treated with 0.05 mM PAs. However, putrescine (Put) was the most active PA. The observed NO release by PAs was rapid and without an apparent lag phase. The response was quenched when the suspension cells were treated with the NO-specific scavenger cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-1-oxy-3-oxide). When 0.01 mM L-aminoguanidine (L-AG) was applied prior to the PA treatments, the NO fluorescence was diminished, and the inhibition of NO fluorescence was correlated with a decrease in diamine oxidase (DAO) activity. When callus cells were incubated with 0.1 mM catalase (CAT) and 1.0 mM N′N-dimethylthiourea (DMTU) prior to PA application, NO release was significantly reduced. In sum, our data provided evidence for PA-induced NO generation in suspension cells of soybean cotyledon node callus and demonstrated that peroxide, potentially derived from PA oxidative degradation, was involved in NO release induced by PAs.

1. Introduction

Polyamines (PAs) are small, aliphatic polycations that are ubiquitously present in all living organisms. Putrescine (Put), spermidine (Spd), and spermine (Spm) are the major PAs in plants, and they are involved not only in various processes, such as cell proliferation (Cona and Cenci, 2003), growth (Neves et al., 2002), morphogenesis (Hummel et al., 2002), differentiation (Cona et al., 2006), and programmed cell death (Yoda et al., 2003; Bouchereau et al., 1999), but also in adaptive responses to various environmental stresses (Cvikrová et al., 2013; An et al., 2008). To date, the genes involved in PA biosynthesis and degradation have already been identified and cloned, which enable PA gain-of-function and loss-of-function studies (Kusano et al., 2007). However, PA-dependent signal transduction processes have not been revealed as well as their important physiological roles.

NO is a labile gaseous free radical that plays a pivotal role as an intra- and intercellular messenger to induce various physiological processes in plants, such as stomata closure (Bright et al., 2006), root development (Pagnussat et al., 2002; Correa-Aragunde et al., 2004), seed germination (Bethke et al., 2006), expression of defence-related genes and programmed cell death (Beligni et al., 2002). In addition, NO participates in plant responses to biotic and abiotic stresses, including interactions with pathogenic microorganisms (Delledonne et al., 1998), wounding (Grün et al., 2006), salinity (Kopyra and Gwóźdź, 2003), drought (Neill et al., 2002), and hypoxia (Wimalasekera et al., 2011a). In plants, NO can be synthesised by nitrate reductase (NR) and can also be formed by the oxidation of arginine via enzyme(s) similar to animal nitric oxide synthase (NOS) (delRío et al., 2004). NO can also be generated via non-enzymatic pathways. Nitrification/denitrification cycles generate NO as a by-product of N_2O oxidation into the

Abbreviations: Arg, arginine; L-AG, L-amino guanidine; B5 medium, Gamborg medium; CAT, catalase; cPTIO, 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-1-oxy-3-oxide; 2,4-D, 2,4-dichlorophenoxyacetic acid; DAF-FM-DA, 3-amino,4-aminomethyl-2′,7′-difluorescein-diacetate; DAO, diamine oxides; DMTU, N′N-dimethylthiourea; MS, Murashige and Skoog medium; NO, nitric oxide; PAO, polyamine oxidases; PAs, polyamines; Put, putrescine; RFU, relative fluorescence units; SNP, sodium nitroprusside; Spd, spermidine; Spm, spermine.

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atmosphere (Wojtaszek, 2000). Nitrite can also be chemically reduced by ascorbic acid from pH 3–6 to yield NO and dehydroascorbic acid (Henry et al., 1997). Although NO is a key signalling molecule, the mechanisms by which plants or tissues regulate its levels remain unclear and controversial.

Tun et al. (2006) presented pharmacological evidence that polyamines induced the production of nitric oxide in various tissues in seedlings of *Arabidopsis thaliana*. Silveira et al. (2006) also found that Put instead of Spd and Spm induced NO release in embryogenic suspension cultures of *Araucaria angustifolia*. Recently, Wimalasekera et al., (2011b) observed that a mutant lacking a copper amine oxidase gene, CuAO1, showed impaired PA-induced NO release due to a T-DNA insertion. Moreover, compared to PA-treated WT, CuAO1 knockouts showed significantly reduced NO-fluorescence in the primary root tips. Thus, they speculated that as yet unknown enzyme(s) or diamine oxidases (CuAO)/polyamine oxidases (PAO) are responsible for NO synthesis (Tun et al., 2006; Wimalasekera et al., 2011b). To date, evidence for PA-induced NO production in cytological levels is limited.

In the present study, we selected suspension cells of soybean cotyledon-node callus, which have abundant soluble proteins with higher CuAO activity. Suspension cells of callus are of good uniformity and without intracellular interference. We next examined the regulation of NO biosynthesis by exogenous polyamines (Put, Spd and Spm), catalase (CAT, a H_2O_2 scavenger), N'N-dimethylthiourea (DMTU, a H_2O_2 quenching agent), L-aminoguanidine (L-AG, an inhibitor of DAO), and cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimid- azoline-1-1-oxy-3-oxide, a specific scavenger of NO) in suspension cells of soybean cotyledon node callus. The primary aim was to obtain further evidence for NO biosynthesis induced by PAs in soybean cells and to generate a better biochemical understanding of PA-induced NO generation.

2. Materials and methods

2.1. Callus and suspended cell culture

Soybean (*Glycine max* (Linn.) Merr.) seeds (provided by the soybean institute of Jilin province Academy of Agricultural Sciences) were rinsed for 1 h and soaked with 75% ethanol for 50 s, and were then sterilised with 0.1% $HgCl_2$ for 8 min. After 7 or 8 washes with distilled water, these seeds were sown into 1/2 MS medium with 6 g/L agar. Petri dishes were placed in a growth chamber under conditions of darkness at 25 ± 2 °C for 5 d. The seeds were germinated and ultimately grew into aseptic seedlings.

Parts of the soybean cotyledon node were excised from aseptic seedlings, and inoculated into the callus growth medium containing 1/2 MS mineral salts +1/2 B5 organics +3 mg/L 2, 4-D+30 g/L sucrose +6 g/L agar, and then cultured at 25 ± 2 °C in darkness. Several days later, calli developed and were used for subculture. After 4 subcultures at the same condition, a colony of yellowish, well-developed and structure-loosening calli of the soybean cotyledon node was available.

Approximately 1.5 g of calli were transplanted into sterile solution medium containing 1/2 MS mineral salts +1/2 B5 organics +3 mg/L 2,4-D +2% sucrose +1% mannitol +500 mg/L pectinase +3 ml/L 2,4-D (10 ml). The suspended cells were obtained by shaking the culture in darkness at 120 r/min and 25 ± 2 °C. Cells at the logarithmic growth phase (density 10^4/ml) were selected for the treatments with *exogenous PAs*, *SNP*, *CAT, DMTU and AG*.

2.2. Treatments with exogenous PAs and SNP

A NO-specific fluorescent probe, DAF-FM DA, which was purchased from Beyotime Institute of Biotechnology (Shanghai, China), was diluted to a final concentration of 15 μM with kit diluents. The suspension cells were centrifuged at 1000 r/min for 2 min at 4 °C. Sediment cells were collected and re-suspended in 15 μM DAF-FM DA at 25 °C for 30 min. To mix the probe and cells sufficiently, eppendorf tubes were turned upside down every three minutes. To remove the superfluous DAF-FM DA that did not enter into the cells, the probe-loaded cells were rinsed 3 times with sodium phosphate buffer (pH7.4). Next, 0.05 mM PAs (Put, Spd and Spm, Sigma), 0.05 mM PAs + 0.1 mM cPTIO (Sigma), 0.01 mM SNP (NO donor, Fluka), 0.01 mM SNP+ 0.1 mM cPTIO were loaded at 25 °C for 35 min.

2.3. CAT, DMTU and AG treatments

After centrifugation at 1000 r/min for 2 min at 4 °C, sediment cells were incubated in darkness with 0.01 mM L-AG (Sigma), 1.0 mM DMTU (Sigma) and 0.1 mM CAT, respectively, for 30 min at 25 °C. Next, 15 μM DAF-FM DA probe was loaded for an additional 30 min and 0.05 mM PAs (Put, Spd and Spm) for 35 min under the same condition. NO was visualised using a laser confocal scanning microscope. In addition, 100 μl of suspended cells were used to test the NO RFU using an automatic microplate reader.

2.4. NO determination

NO was visualised using a laser confocal scanning microscope (TCS-SP2, Leica, Germany). Excitation at 488 nm and emission at 510 nm was used. In addition, 100 μl of suspended cells was used to test the NO RFU using an automatic microplate reader (Greiner Labortechnik, Ficheenhausen, Germany).

2.5. Measurement of DAO activity

The suspension cells of soybean cotyledon-node callus were centrifuged at 3000 r/min for 2 min at 4 °C and were collected and ground using a cell grinder to determine the DAO activity. The DAO activity was detected according to the method previously described by Su et al. (2005). The protein contents in the supernatant were determined according to the Bradford method (1976) using bovine serum albumin as a standard (Bradford, 1976). Moreover, 0.01 changes in the absorbance value at 555 nm were regarded as one activity unit. The DAO activity was expressed as U/min. mg protein.

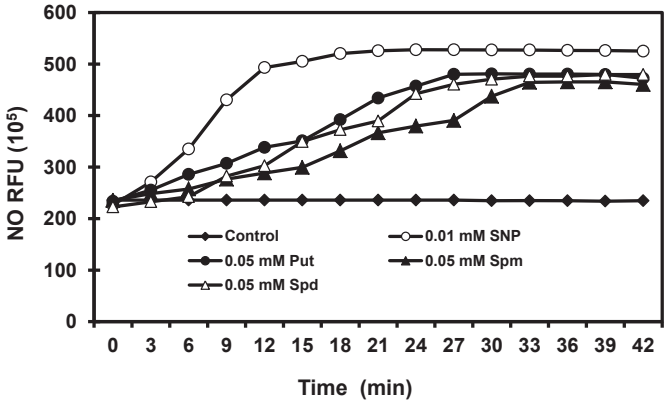

Fig. 1. Time curves of the SNP- and PA-induced NO relative fluorescence units in suspension cells of soybean cotyledon-node callus. The suspended cells (cell density 10^4/ml) were incubated with 15 μM DAF-FM DA at 25 ± 2 °C for 30 min. Next, 0.05 mM PAs (Put, Spd and Spm) and 0.01 mM SNP were loaded for another 35 min at 25 ± 2 °C. Using an automatic microplate reader (Greiner Labortechnik, Ficheenhausen, Germany), 100 μl of the suspended cells were used tested for NO RFU.