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BRIEF COMMUNICATION

Two enzymatic sources of nitric oxide in different organs of apple plant

H.J. GAO[1]*, H.Q. YANG[2,3], J.Y. WANG[1] and J.X. WANG[1]*

Shandong Institute of Pomology, Longtan RD 64, Taian 271000, P.R. China[1]
Shandong Agricultural University, Daizong RD 61, Taian 271018, P.R. China[2]
State Key Laboratory of Crop Biology, Daizong RD 61, Taian 271018, P.R. China[3]

Abstract

Nitric oxide production, nitric oxide synthase (NOS) and mitochondrial nitrite-reducing activities in roots, leaves and stems of different developmental stages were investigated, using potted 3-year-old apple (*Malus domestica* Borkh.) trees. The arginine-dependent NOS activity is sensitive to NOS inhibitor L-NAME and aminoguanidine (AG), with L-NAME being more effective than AG. Endogenous NO production, NOS and mitochondrial nitrite-reducing activities are predominately presented in young leaves and especially in young white roots and young stems. Root and stem mitochondria can reduce nitrite to nitric oxide at the expense of NADH, however, this mitochondrial nitrite-reducing activity is absent in leaves.

Additional key words: chemiluminescence, L-arginine, mitochondria, nitric oxide synthase, nitrite.

Nitric oxide is a multifunctional molecule implicated in numerous physiological and biological processes during plant development and stress response (Neill *et al*. 2003, Grün *et al*. 2006, Besson-Bard *et al*. 2008, Xu *et al*. 2009). In plants, NO can be produced either by reducing inorganic nitrogen by nitrite-dependent pathway or by oxidation of organic nitrogen by arginine-dependent pathway (Crawford and Guo 2005, Stöhr 2007). Nitric oxide synthase (NOS) activities have been detected in several plant species and NOS inhibitors remarkably inhibit NO production (Wendehenne *et al*. 2001, Del Río *et al*. 2004). Although the protein AtNOS1 is no longer considered to be a plant NOS, it is still possible that a unique plant NOS responsible for arginine-dependent NO formation might exist (Yamasaki and Cohen 2006, Wilson *et al*. 2008). Potential enzymatic mechanisms for nitrite-dependent NO production in plants include cytosolic nitrate reductase (NR), a root-specific plasma membrane (PM) nitrite:NO reductase (NI-NOR), nitrite reductase (NiR), and xanthine oxidase (XO) (Salgado *et al*. 2007, Kaiser *et al*. 2007). Additionally, Gupta *et al*. (2005) reported that in higher plants, only root mitochondria but not leaf mitochondria, reduce nitrite to NO, *in vitro* and *in situ*, with both terminal oxidases participating in NO formation. Until now, this mitochondrial nitrite-reducing activity has been observed in *Arabidopsis*, barley, pea and tobacco. It was also proved that this mitochondrial nitrite-dependent NO formation is also lacking in non-green tissues like potato tubers and cauliflower inflorescences. However, mitochondria from non-green tobacco cell suspensions can reduce nitrite to NO, although with a lower capacity than root mitochondria (Gupta *et al*. 2005).

In recent years, most studies focused on the effects of NO on plant physiological and biochemical processes by application of exogenous NO donors (Correa-Aragunde *et al*. 2008). There is a lack of information on the production of endogenous NO (especially the organ- or tissue-specific formation) and its enzymatic sources during normal development of plants. Recently, constitutive NOS activities were detected in roots, stems and leaves of pea seedlings which appeared to be

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Abbreviations: AG - aminoguanidine; BH_4 - tetrahydrobiopterin; CL - chemiluminescence; EDTA - ethylenediamine tetraacetic acid; FAD - flavin adenine dinucleotide; FMN - flavin mononucleotide; HEPES - hydroxyethyl piperazinyl ethanesulfonic acid; L-NAME - N^G-nitro-L-arginine methyl ester; NiR - nitrite reductase; NOS - nitric oxide synthase; NR - nitrate reductase; PM - plasma membrane; PMSF - phenylmethylsulphonyl fluoride; XO - xanthine oxidase.
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* Corresponding authors; fax.: (+86) 538 8225267, e-mails: hjgaosdau@hotmail.com and jxwang66@163.com

developmentally regulated (Corpas *et al*. 2006). During spring, apple plants undergo very rapid growth and development of shoots and roots. In this work, NO production and two enzymatic sources for NO generation (NOS activities and mitochondrial nitrite-reducing activities) were investigated in white roots, brown roots, young leaves, mature leaves and young stems of apple plants growing under normal conditions. These data will contribute to a better understanding of the physiological functions of NO as well as its generating mechanisms in plants.

Three-year-old apple (*Malus domestica* Borkh. cv. Fuji) trees on *Malus hupehensis* Rehd. rootstock were planted in 0.02 m^3 pots containing a 1:1 (v/v) mixture of peat moss and sandy loam soil and well watered at 1 - 3 d intervals. On April 21, fine roots, young leaves, mature leaves and young stems were collected from three individual trees and put into an ice box. The fine roots were then divided into white roots and brown roots by floating on icy water. White roots refer to the new fine roots as they are white in color when first produced, and most of them become brown with age (brown roots) (Wells and Eissenstat 2003). Young leaves refer to the not-fully-expanded and yellowish green leaves (1^{st} and 2^{nd} leaf from the top of the shoots) and mature leaves refer to the fully-expanded and dark green leaves (in the middle part of the shoots). Young stems refer to stems that are not lignified. All the samples were frozen in liquid nitrogen and stored at -70 °C until use.

NO production and NOS activity were estimated by the luminol-H_2O_2 chemiluminescence (CL) method according to Gao *et al*. (2009). For NO determination, samples were ground in an ice bath at a ratio of 200 mg fresh mass per cm^3 of cold deoxygenated water (freshly prepared by boiling distilled deionized water for 1 h). The extracts were centrifuged at 12 000 *g* for 20 min and the supernatant was immediately used for CL detection using a BPCL ultra-weak CL analyzer (Institute of Biophysics, Academia Sinica, China). Relative NO production was expressed as CL counts per microgram of protein. For the assay of NOS activity, plant tissues together with 5 % (m/m) polyvinylpolypyrrolidone (PVPP) were ground with liquid nitrogen and then resuspended in cold extraction buffer containing 50 mM Tris-HCl in distilled deionized water, pH 7.4, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM PMSF, 320 mM sucrose, 1 μM leupeptin. The homogenate was centrifuged at 12 000 *g* for 20 min and the supernatant fraction was immediately used for enzyme assay. Assay of NOS activity was performed in a reaction medium containing 50 mM HEPES buffer, pH 7.4, 1 mM dithiothreitol, 10 μg cm^{-3} calmodulin, 0.5 mM $CaCl_2$, 1 mM β-NADPH, 10 μM FAD, 10 μM FMN, 10 μM BH_4 and 2 mM L-arginine. The reaction mixture was incubated at 30 °C for 30 min. To check the specificity of this luminol-H_2O_2 CL method used to assay the NOS activity of plant samples, NOS activities in different organs of apple plant were also assayed in the presence of 5 mM L-NAME or aminoguanidine (AG), by directly adding each inhibitor to the reaction medium. The production of NO in samples was calculated by subtracting the blank value (zero time), which represent the non-enzymatic NO production. NOS activity was expressed as CL counts mg^{-1}(protein) s^{-1}.

Mitochondria isolation was conducted according to Gupta *et al*. (2005) with some modifications. Plant tissues, together with 5 % PVPP, were ground with extraction buffer (pH 7.4) containing 50 mM Tris-HCl, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM PMSF, 320 mM mannitol, 0.1 % (m/v) bovine serum albumin (BSA), at a ratio of 300 mg(f.m.) cm^{-3}. The homogenate was centrifuged at 1 000 *g* for 15 min, and the supernatant was centrifuged again at 10 000 *g* for 20 min. The resulting pellet was suspended in suspension medium containing 25 mM HEPES-KOH, pH 7.4, 0.1 % BSA, 250 mM mannitol, 1 mM dithiothreitol, 2 mM $MgCl_2$, 0.5 mM KH_2PO_4. All the procedures were conducted at 4 °C. Estimation of NO emission from mitochondrial suspensions followed the same procedure as in the assay of NOS activity (Gao *et al*. 2009), and the reaction mixture was 50 mM sodium phosphate buffer (pH 7.5), containing 0.5 mM $NaNO_2$, 1 mM NADH (Gupta *et al*. 2005). Relative mitochondrial nitrite-reducing activity was expressed as CL counts mg^{-1}(protein) s^{-1}. Protein content was determined according to Bradford (1976), using BSA as standard. Each experiment was conducted with three replicates and the statistical analysis was done by Duncan's test ($P < 0.05$) using *SPSS* software (*SPSS 16.0 for Windows*).

In apple, endogenous NO production and NOS activity are mainly presented in young tissues/organs, namely white roots, young stems and young leaves (Table 1). The highest NO production and NOS activity was found in white roots, followed by young stems and young leaves, while in mature leaves and brown roots, both NO production and NOS activity were rather low (Table 1). Considering the different developmental stages of the different plant organs investigated, it could be concluded that roots and stems exhibit higher NO production and NOS activity than leaves, in agreement with findings in pea seedlings (Corpas *et al*. 2006). NOS inhibitor L-NAME or AG, each significantly reduced NOS activity in all the plant organs investigated, especially in young tissues/organs (Table 2). L-NAME produced an inhibition

Table 1. NO production [CL counts μg^{-1}(protein)], NOS activity and mitochondrial nitrite-reducing activity [CL counts mg^{-1}(protein) s^{-1}] in different organs of apple plant. Means ± SE, $n = 3$. Different letters within each column indicate significant differences at $P < 0.05$.

Plant organs	NO production	NOS activity	Nitrite-reducing activity
White roots	759 ± 89a	219.0 ± 24.0a	16.9 ± 3.3b
Brown roots	289 ± 21c	31.9 ± 8.6d	5.0 ± 0.6c
Young leaves	301 ± 38c	68.6 ± 13.1c	0
Mature leaves	77 ± 6d	24.7 ± 3.6d	0
Young stems	575 ± 48b	112.0 ± 9.2b	49.7 ± 6.4a

Table 2. Effects of L-NAME or aminoguanidine (AG) on NOS activities [CL counts mg^{-1}(protein) s^{-1}] in different organs of apple plant. Means ± SE, $n = 3$. Different letters within each column indicate significant differences at $P < 0.05$.

Treatment	White roots	Brown roots	Young leaves	Mature leaves	Young stems
Control	219.0 ± 24.0a	31.9 ± 8.6a	68.6 ± 13.1a	24.7 ± 3.6a	112.0 ± 9.2a
L-NAME	24.2 ± 3.1b	20.8 ± 1.7b	10.6 ± 1.9b	11.1 ± 1.7b	39.4 ± 4.2c
AG	43.3 ± 8.6b	22.2 ± 2.8b	11.4 ± 1.7b	19.2 ± 2.2a	57.8 ± 4.7b

of 89, 85 and 65 % of the NOS activity in white roots, young leaves and young stems, respectively. As for AG, the inhibition rate was 80, 83 and 48 % in white roots, young leaves and young stems, respectively, suggesting that L-NAME was more effective than AG. Beligni and Lamattina (2001) pointed out that NO is mainly formed in actively growing tissues, functioning as a non-traditional regulator of plant growth. Extending white roots undergo elongation, formation of secondary and hairy roots to facilitate water and nutrient uptake, which may require higher concentrations of NO (Correa-Aragunde *et al*. 2008, Neill *et al*. 2003). As for young stems, in addition to the involvement in xylem cell wall lignification and differentiation (Gabaldón *et al*. 2005), higher NO levels in young stems might participate in long-distance translocation of root-sourced signals to shoots, acting as a systemic signaling molecule (Capone *et al*. 2004, Corpas *et al*. 2004, 2006, Gaupels *et al*. 2008). Higher content of NO in young leaves than in mature leaves may be related to the involvement of NO in leaf expansion (Han *et al*. 2009).

Higher plant mitochondria are a major source for NO production (Kaiser *et al*. 2007). In recent years, it was proved that root mitochondria of tobacco, pea, barley and *Arabidopsis* can reduce nitrite to NO in the presence of NADH, with terminal oxidases (cytochrome oxidase and alternative oxidase) participating in NO formation. Surprisingly, this capability is absent in leaf mitochondria of all the above-mentioned plant species (Gupta *et al*. 2005, Planchet *et al*. 2005, Kaiser *et al*. 2007). This mitochondrial-dependent nitrite reduction may be a general mechanism for NO generation in eukaryotic organisms, originating independently of NOS activity (Chen *et al*. 2005, Salgado *et al*. 2007). In this research, we further investigated this mitochondrial nitrite-reducing activity in roots, leaves, and stems of different developmental stages of apple plants, using a luminol-H_2O_2 CL method for the detection of NO emission from mitochondrial suspensions. The results showed that, in addition to root mitochondria, stem mitochondria are also able to reduce nitrite to NO in the presence of NADH (Table 1). The highest level of NO emission was observed from mitochondria of young stems, followed by white roots, then brown roots. However, no NO production was detected from mitochondrial suspensions of either young leaves or mature leaves, in agreement with findings in *Arabidopsis*, barley, pea and tobacco (Gupta *et al*. 2005).

From these results, we concluded that *1*) higher levels of NO and NOS exist in roots and stems than in leaves; *2*) root and stem mitochondria but not leaf mitochondria reduce nitrite to NO at the expense of NADH; and *3*) L-arginine-dependent NOS activity and nitrite-dependent mitochondrial reducing activity are two important mechanisms responsible for NO formation in apple, although the capacity of the latter is much lower than that of the former (Table 1). Serving as interfaces between plants and the soil, roots, in contrast to leaves, are much more exposed to many biotic and abiotic stresses, such as pathogens, flooding, drought, and salt (Wells and Eissenstat 2003). Higher contents of NO in roots than in leaves might be related to the responses of plant roots to various biotic/abiotic stresses. As for stems, higher contents of NO might be required to trigger cell death in differentiating xylem elements, especially under hypoxic/anoxic conditions (Gabaldón *et al*. 2005, Gupta *et al*. 2005). Higher NO production in roots might also be attributed to substrates availability, as roots are regularly exposed to NO_3^-/NO_2^- in the bulk soil. Furthermore, plant nitrogen metabolism provides substrates (both nitrite and L-arginine) for NO synthesis (Stöhr 2007, Salgado *et al*. 2007). In apple, nitrate content in roots are much higher than in leaves (Klepper and Hageman 1969), and L-arginine was also found to be mainly located in roots and in cotyledons of germinating seeds, serving as an important nitrogen reserve (Gao *et al*. 2008, 2009). In addition to the mitochondrial nitrite-reducing activity, a PM-bound nitrite:NO reductase (NI-NOR) associated with the PM-bound NR was reported to be root-specific that generate NO from nitrite (Stöhr *et al*. 2001, Stöhr and Ullrich 2002, Stöhr and Stremlau 2006). Bethke *et al*. (2004) also reported a nonenzymatic reduction of apoplastic nitrite to NO in roots and germinating seeds but unlikely in leaves. Further investigation of organ- or tissue-specific formation of NO will contribute to a better understanding of NO producing mechanisms and NO signaling.

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