Growth phase-dependent subcellular localization of nitric oxide synthase in maize cells

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Abstract A protein band of approximately 166 kDa was detected in the soluble fraction of root tips and young leaves of maize seedlings, based on Western blot analysis using antibodies raised against mouse macrophage nitric oxide synthase (NOS) and rabbit brain NOS. NOS activity was present in these soluble fractions, as determined by L-[U-^{14}C]citrulline synthesis from L-[U-^{14}C]arginine. Immunofluorescence showed that the maize NOS protein is present in the cytosol of cells in the division zone and is translocated into the nucleus in cells in the elongation zone of maize root tips. These results indicate the existence of a NOS enzyme in maize tissues, with the localization of this protein depending on the phase of cell growth.

Key words: Plant nitric oxide synthase; Root growth; Immunolocalization; *Zea mays*

1. Introduction

Nitric oxide synthase (NOS) is responsible for the synthesis of nitric oxide, from the guanidine-nitrogen of L-arginine during the five-electron oxidation to L-citrulline [1,2]. Different isoforms of NOS, originally identified in the nervous, immune and cardiovascular systems of mammalian tissues [3–5], have also been detected in non-mammalian vertebrate groups, as well as in a variety of invertebrates [6], including crab [7]. The widespread presence of NOS in the animal kingdom suggests an old evolutionary pathway for this enzyme.

Plant metabolism is influenced by exogenously applied NO. The exposure of spruce needles to gaseous NO leads to a marked, rapid increase in the intracellular concentration of cGMP [8], probably through the activation of guanylyl cyclase, an important target for the physiological effects of NO in animal cells [9]. Guanylyl cyclase activation could also account for the stimulation of germination and the modified light requirement of the phytochrome-broken dormancy of *Paulownia tomentosa* seeds induced by NO donors [10] since cGMP, the product of this enzyme, participates in the signaling pathway of phytochrome phototransduction [11]. Low concentrations of NO donors promote growth in pea foliage [12] and maize root tips [13] while higher concentrations are necessary to induce the accumulation of phytoalexin in potato tuber tissues [14].

The ability of plants to respond to NO is indicative of the presence of many molecular targets for NO or its derivatives [15,16]. Evidence for the existence of an endogenous pathway for NO synthesis in the plant kingdom has increased in recent years. NOS activity in plant cells, based on the formation of L-citrulline from L-arginine, was initially observed in extracts of the legume *Mucuna hassjoo* [17] and in lupine roots, where a NADPH-diaphorase activity was also detected [18]. NO production by a NOS-like enzyme in cultured soybean cells was recently reported, and shown to induce plant defense responses to pathogens [19]. Inducible NOS activity in resistant, but not susceptible, tobacco leaves infected with tobacco mosaic virus has also been shown to activate plant defense responses [20]. Inhibitors of NO synthesis potentiated *Pseudomonas syringae* growth in *Arabidopsis thaliana* leaves [19]. These observations, and the known multifunctional actions of NO in mammalian cells [15,16], indicate that the participation of NOS is likely to be demonstrated for many cellular processes in plants. In this study, we present evidence for the existence of a NOS-like enzyme in maize cells and suggest that this molecule may be involved in the signal transduction pathway that controls root growth.

2. Materials and methods

2.1. Plant material

Maize seeds (*Zea mays* L., cv. Maia Normal) were germinated aseptically at 28°C in the dark, in water supplemented with 1 mM EGTA. Young leaves and root segments (5 mm) excised from 3-day-old seedlings were used in all experiments.

2.2. Preparation of soluble fractions

Maize tissue (0.5 g/ml) was homogenized in 100 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 250 mM sucrose, 20 mM KCl, supplemented with 10 mM dithiothreitol, 50 μg PMSF/ml, 100 μM benzamidine and 50 μM antipain. The homogenate was frozen in liquid N_2 and ground in a mortar and pestle three times. The pH of the crude extract was adjusted to 7.2 (with NaOH) and was centrifuged at $3000\times g$ for 20 min and then at $12\,000\times g$ for 15 min at 4°C. The resulting supernatant was concentrated using Centricon-10 membranes (Amicon, Beverly, MA, USA). Protein concentrations were determined by a modified Lowry procedure [21], using BSA as the standard.

2.3. Western blotting

The polypeptides in solubilized samples were separated by SDS-

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Abbreviations: BH_4, 6(*R*)-5,6,7,8-tetrahydrobiopterin; BSA, bovine serum albumin; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; FITC, fluorescein isothiocyanate; FMN, flavin mononucleotide; LPS, lipopolysaccharide; NOS, nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate

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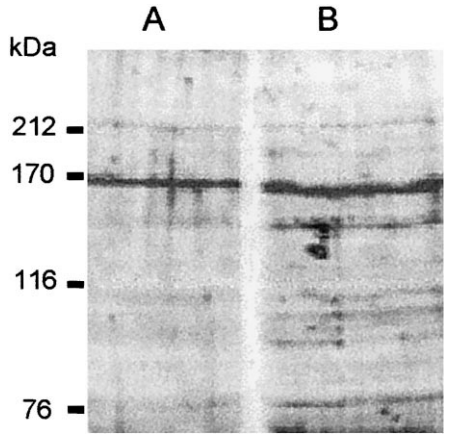

Fig. 1. Western blot analyses of extracts from maize tissues using antibodies raised against macrophage NOS. Concentrated solubilized proteins extracted from 3-day-old maize seedlings were fractionated by SDS-PAGE, blotted to nitrocellulose membranes and probed with mouse anti-macNOS antibodies. Solubilized proteins (150 μg) from young leaves (lane A) and root tips (lane B). Molecular weight standards as follows: myosin (212 kDa), α-macroglobulin (170 kDa), β-galactosidase (116 kDa) and transferrin (76 kDa).

polyacrylamide gel electrophoresis (SDS-PAGE) according to [22], using a 7% acrylamide slab gel with a 3% acrylamide stacking gel, in a Mini-Protean II Dual Slab Cell (Bio-Rad, Hercules, CA, USA). The current was set at 25 mA and the gels were run for 2 h. After electrophoresis, the gels were blotted onto 0.45 μm nitrocellulose membranes (Schleicher and Schüll, Keene, NH, USA) for 2 h, at 180 mA [23]. The membranes were then blocked by incubation for 1 h with 5% non-fat dry milk in Tris-buffered saline (TBS: 20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.05% Tween 20 (TBS-Tween), and then rinsed three times with TBS-Tween. The protein blots were probed with IgG_{2a} mouse anti-macNOS at 0.5 μg/ml or polyclonal rabbit anti-brainNOS at 2 μg/ml (Transduction Laboratories, Lexington, KY, USA). After overnight incubation at 4°C, the blots were washed six times with TBS-Tween for 10 min at room temperature followed by treatment with horseradish peroxidase-conjugated rabbit IgG anti-mouse Ig (for anti-macNOS) or peroxidase-conjugated sheep IgG anti-rabbit Ig (for anti-brainNOS) at 2 μg/ml in TBS-Tween, overnight at 4°C. The blots were then washed six times with TBS for 10 min at room temperature and the color reaction developed using 0.6 mg 3,3′-diaminobenzidine/ml (Sigma), in 50 mM Tris-HCl, pH 7.4, and 3% H_2O_2.

2.4. Immunofluorescence

Longitudinal root tip sections from maize seedlings were immersed in TMK buffer (20 mM Tris-HCl, pH 7.2, 50 mM mannitol, 50 mM KCl) and fixed with formol in TMK (1:6, v/v). After 30 min, the sections were washed at least four times with TMK and permeabilized with 0.2% Triton X-100 in TMK for 30 min. After washing in TMK, the sections were treated for 1 h in TMK containing 3% BSA and incubated overnight with fluorescein isothiocyanate (FITC)-conjugated mouse IgG_{2a} anti-macNOS (10 μg/ml; Transduction Laboratories) or with irrelevant antibody (FITC-conjugated monoclonal IgG_{2a} anti-surface protein of *Trypanosoma cruzi* prepared in our laboratory) diluted to 250 μg/ml in TMK containing 3% BSA. Excess antibody was removed by four washes with TMK for 30 min each. The sections were then mounted in TMK containing 50% glycerol on slides covered with a coverslip and examined under a dual-channel (Bio-Rad) laser confocal system (MRC 1024UV) coupled to an Axiovert 100 inverted microscope (Zeiss) and equipped with Ar-Kr and UV lasers. The 488 nm line of the Ar-Kr laser was used to excite the FITC-labeled root tip. Manufacturer-supplied software (OS/2) running on a Pentium 150 MHz computer (Compaq) was used to control image acquisition and processing.

2.5. NOS activity

The total NOS activity in the root tips and young leaves of 3-day-old maize seedlings was determined by the citrulline assay method [24]. Briefly, 500 mg of tissue was homogenized in 0.2 ml of cooled homogenizing buffer (Tris-HCl 50 mM, pH 7.4, 3.2 mM sucrose, 5 mM dithiothreitol, 10 μg leupeptin/ml, 10 μg soybean trypsin inhibitor/ml, and 2 μg aprotinin/ml). The homogenate was centrifuged at 10 000×*g* for 20 min at 4°C. 40 μl of the resulting supernatant was added to 100 μl of assay buffer (KH_2PO_4 50 mM, pH 7.2, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 mM valine, 20 μM L-citrulline, 60 μM L-arginine, 1 mM dithiothreitol, 100 μM NADPH, 50 μM BH_4, 4 μM FMN, 3.5 μg calmodulin/ml and 1 μM (0.05 μCi) L-[U-^{14}C]arginine. Following a 30 min incubation at 25°C, the reaction was terminated by adding 1:1 (v/v) suspension of Milli-Q water:Dowex-Ag50W (200–400, 8% cross-linked, Na^+ form). The resin was removed by centrifugation (5000×*g*, 10 min) and the supernatant collected. NOS activity in the supernatant was assayed by the conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline in samples with and without the NOS inhibitors L-NAME (3 mM) and L-aminoguanidine (3 mM). The protein content of the supernatants was determined by the Coomassie blue binding method using Bio-Rad protein reagent with BSA as the standard.

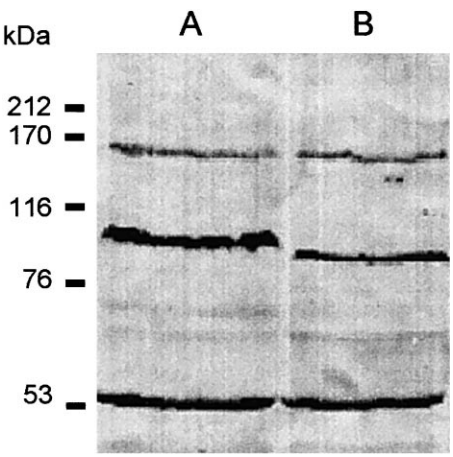

Fig. 2. Immunoreactivity of maize proteins with brain NOS antibodies. Concentrated solubilized proteins extracted from 3-day-old maize seedlings were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with rabbit anti-brainNOS antibodies. Solubilized proteins (150 μg) from young leaves (lane A) and root tips (lane B). Molecular weight standards as in Fig. 1 including glutamic dehydrogenase (53 kDa).

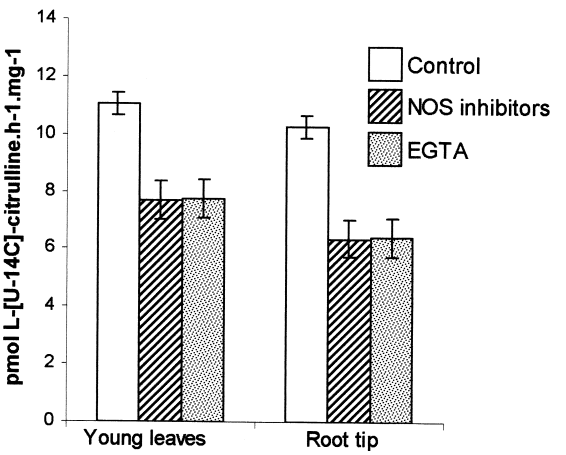

Fig. 3. NOS activity assayed by L-[U-^{14}C]citrulline formation in maize tissues. Extracts derived from young leaves and root tips were incubated in the presence of 60 μM L-[U-^{14}C]arginine as described in Section 2. EGTA (2 mM) or NOS inhibitors (3 mM L-NAME and 3 mM L-aminoguanidine) were present in the reaction mixture where indicated. Data are mean values ± S.E.M. of five independent experiments.

3. Results and discussion

To identify a putative NOS enzyme in *Zea mays*, we used antibodies raised against two different mammalian NOS isoforms to perform Western blots of the soluble fractions of maize seedling homogenates. A monoclonal antibody raised against mouse macrophage NOS recognized a ~166 kDa protein in homogenates from young leaves and root tips (Fig. 1A,B). As expected, this antibody recognized the 130 kDa protein of macrophage cell extracts (not shown). Polyclonal antibodies raised against rabbit brain NOS also recognized a protein of approximately 166 kDa in soluble fractions from young maize leaves and root tips (Fig. 2A,B). This compared favorably with the expected 155 kDa NOS present in brain homogenates (not shown). Reactive polypeptides of lower molecular weight (103–109 kDa and 51 kDa) were present in plant extracts probed with anti-brainNOS (Fig. 2A,B). Since the level of these low molecular weight bands increased as the higher bands decreased, and since such bands were also seen in brain extracts allowed to age (not shown), they probably represent degradation products. Such a phenomenon has been reported previously for NOS [25].

The observation that antibodies raised against two different isoforms of mammalian NOS show cross-reactivity with the same 166 kDa protein band in maize tissues suggests the existence of a homologous NOS enzyme in this plant species with antigenic epitopes common to both mammalian isoforms. The antibodies used in the present study were raised against epitopes from the NADPH binding region of mammalian NOS. Although this region is structurally homologous to the NADPH-cytochrome P450 reductase and other plant NADPH oxidoreductases [26,27], the molecular weight of these proteins is in the range of 36–78 kDa, instead of the 166 kDa observed with maize tissues.

The 155 kDa NOS is a Ca^{2+}-dependent isoform constitutively expressed in neuronal cells while the 130 kDa NOS of macrophages is Ca^{2+}-independent, and is induced in inflammatory responses [28]. Maize tissues possessed Ca^{2+}-dependent NOS activity. As shown in Fig. 3, in the presence of 60 μM L-[U-^{14}C]arginine, extracts derived from young maize leaves and root tips produced 11.05 pmol and 10.26 pmol L-[U-^{14}C]citrulline/h/mg, respectively. When the NOS inhibitors L-NAME and L-aminoguanidine were added to the reaction mixture, both at 3 mM, these values decreased to 7.69 pmol and 6.38 pmol L-[U-^{14}C]citrulline/h/mg, respectively. Based on the above data the actual NOS activities estimated for young leaves and root tips were 3.36 pmol and 3.88 pmol L-[U-^{14}C]citrulline/h/mg, respectively. These NOS activities were completely blocked by EGTA, suggesting that a constitutive enzyme is expressed in these tissues, even though it was rec-

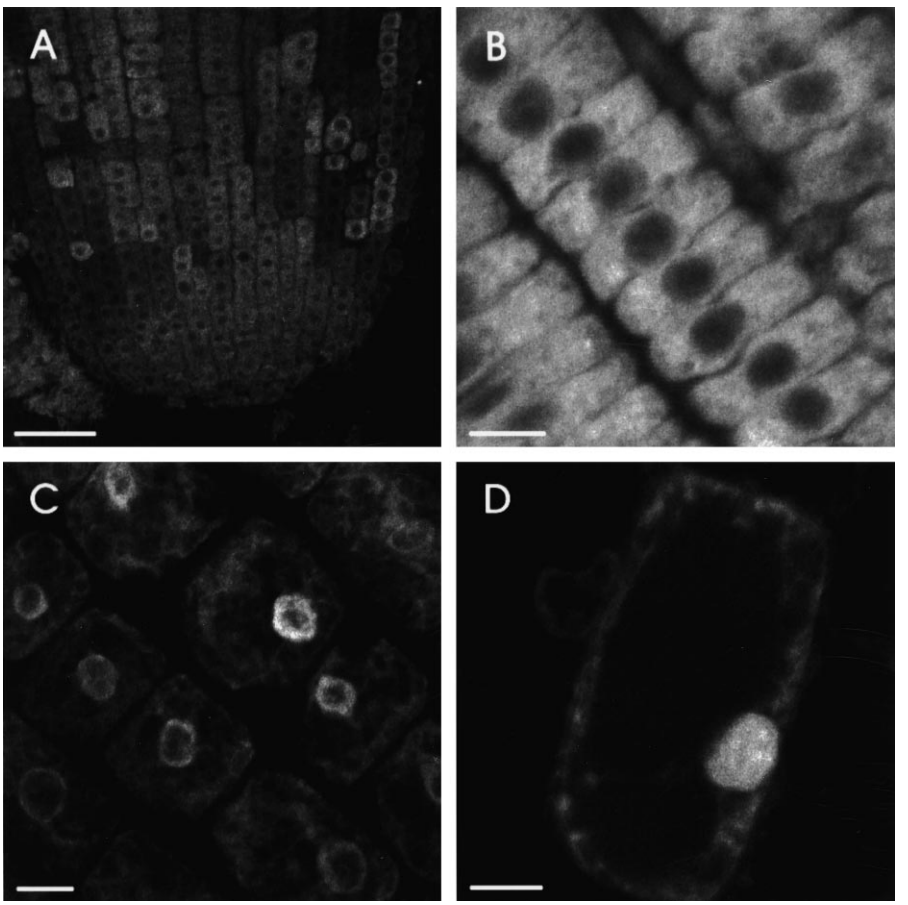

Fig. 4. Immunofluorescence of maize root tips labeled with anti-macNOS conjugated to FITC. Longitudinal sections of root tips from 3-day-old maize seedlings were immunolabeled with the fluorescent antibody and examined using a Bio-Rad 1024 UV laser scanning fluorescence confocal microscope. A and B: Sections showing intense labeling with anti-macNOS antibody in the cytoplasm of the cells in the apical meristem and subjacent meristematic tissues. C: Section of tissue in the transition between the cell division and cell elongation zones, showing labeling with anti-macNOS around the nucleus. D: Cell from the elongation zone with intense NOS immunolabeling in the nucleus. Scale bars: 50 μm for A; 10 μm for B–D.

ognized by antibodies directed against both mammalian isoforms (Figs. 1 and 2).

Plant root apices have a zone of cell division that contains the apical meristem and the subjacent meristematic tissues, followed by a zone of cell elongation. Between these two major zones there is a region characterized by the presence of postmitotic cells where the rate of cell division is decreased and rapid cell elongation has not yet been triggered [29]. We used an antibody raised against macrophage NOS conjugated with fluorescein isothiocyanate (anti-macNOS-FITC) to locate the immunoreactive protein in maize root apices. Fig. 4 shows that the cells of root tips contain considerable amounts of immunoreactive NOS protein differentially distributed in the subcellular compartments according to cell type. In the apical meristem and subjacent meristematic tissues, the immunoreactive protein was widely distributed in the cell cytoplasm and absent from the nucleus (Fig. 4A,B). As cells moved away from the cell division zone, the cytosolic immunolabeling with anti-macNOS decreased. The cells present in the transition between the zones of cell division and cell elongation showed immunolabeling to macNOS around the nucleus (Fig. 4C). The preferential nuclear localization of the immunoreactive protein was evident in cells in the elongation zone (Fig. 4D). These cells were characterized by a prominent vacuolar compartment and increased length, denoting differentiation. In control experiments where buffer alone or antibody raised against surface proteins of *Trypanosoma cruzi* conjugated with FITC replaced anti-macNOS-FITC, no fluorescence was observed in the root tip sections (not shown).

The observation that the immunoreactivity to NOS in the cytosol of meristematic cells in the division zone was displaced towards the nucleus in the elongation zone indicated that the subcellular localization of the immunoreactive NOS protein in maize roots depended on the phase of cell growth. In mammalian cells, NO has been implicated as an important mediator of the differentiation response to growth factors. NOS expression in the mitogenic phase of growth is a prerequisite for growth arrest which is necessary for further differentiation of PC12 neuronal cells induced by neuronal growth factors [30]. NO formation is also required in the early stages of the monocytic differentiation of U937 human monoblasts induced by vitamin E-succinate [31].

The subcellular distribution of NOS immunoreactive protein in the maize root apex might indicate a parallel function for this enzyme in the cellular differentiation of maize root cells. The migration of this cytosolic enzyme towards the nucleus in the elongation zone suggests the participation of this protein in the activation of nuclear transcription factors necessary to support the rapid growth of the elongation zone [32]. The possibility that NO may participate in signal transduction pathways associated with plant cell growth agrees with our previous observation on the effects of NO-releasing substances on maize root elongation [13]. In the latter study, we showed that NO-releasing compounds induced maize root growth at nanomolar concentrations. This growth was prevented by methylene blue, a compound known to inhibit NO actions, suggesting that NO was the mediator of the observed response [13].

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