Research Publications
Nitric oxide modulates specific steps of auxin-induced adventitious rooting in sunflower

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Abbreviations: AR, adventitious roots; NPA, 1-napthylphthalamic acid; NO, nitric oxide; NOS, nitric oxide synthase; IAA, indole-3-acetic acid; CsA, cyclosporin A; SNP, sodium nitroprusside; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide; Lat B, latrunculin B

Present work on indole-3-acetic acid (IAA)-induced adventitious rooting in sunflower hypocotyl highlights a clear demarcation of nitric oxide (NO)-dependent and NO-independent roles of auxin in this developmental process. Of the three phases of adventitious rooting, induction is strictly auxin-dependent though initiation and extension are regulated by an interaction of IAA with NO. A vital role of auxin-efflux transporters (PIN) is also evident from 1-napthylphthalamic acid (NPA)-triggered suppression of adventitious roots (AR). Use of actin depolymerizing agent, latrunculin B (Lat B), has demonstrated the necessity of functional actin filaments in auxin-induced AR response, possibly through its effect on actin-mediated recycling of auxin transporter proteins. Thus, evidence for a linkage between IAA, NO and actin during AR formation has been established.

Adventitious roots (AR) are post-embryonic roots known to originate from stem, leaf petiole and non-pericycle tissue of old roots. In young stem, AR commonly arise from the interfascicular parenchyma while they appear from vascular rays near the cambium in older stem. Formation of AR begins with redifferentiation of predetermined cells which switch from their dermal and subtending cortical cells. Polar transport of auxin from the shoot apical meristem to the rooting region is primarily facilitated by auxin-influx (AUX1) and -efflux (PIN) transporters. Asymmetric localization of these transporter proteins in the vascular cambium cells is responsible for differential distribution of auxin in a particular zone of cells. Polar auxin transport is known to be inhibited by 1-napthylphthalamic acid (NPA, a phytotropin). This inhibition is mediated through a binding of NPA molecule to putative NPA-binding protein (NBP), which is functionally associated with PIN proteins. Efflux transporters exhibit rapid turnover in plasma membrane. High affinity of NBP for actin filaments, suggests its involvement in the cycling and polar distribution of PIN proteins. Organization of actin filaments is known to be rapidly, reversibly and specifically disrupted by Latrunculin B (Lat B), a macrolide toxin isolated from Latrunculia magnifica, a red sea sponge. Lat B associates with actin monomers in 1:1 ratio, thereby preventing their repolymerization into filaments, resulting in a complete shift from F-actin to G-actin. Owing to its well-understood and simple mode of action and low effective dosage, Lat B has supplanted the classic actin-depolymerizing drug cytochalasin D in pharmacological...
investigations. In the past few years, significant work has been done on NO as a signaling molecule in a variety of plant developmental processes. Nitric oxide is known to play a crucial role in root development.

Using sunflower as a model system, present work has been undertaken to investigate the possible role of NO during IAA-induced adventitious rooting in hypocotyl explants. Since auxin action is principally based on PIN-regulated polar transport of IAA molecules, and PIN proteins are known to exhibit actin-assisted rapid recycling in the target cells, attempts have been made in the present work to find a correlation between auxin transport, actin and NO, using specific pharmacological agents. Additionally, effect of Cyclosporin A (CsA), an inhibitor of nitric oxide synthase (NOS) in animal systems, has also been investigated. These specific agents have been used to monitor root initiation at the target sites in our attempts to decipher a signaling cascade for AR.

Seeds of Sunflower (Helianthus annuus L. cv. Morden) were germinated on moist germination sheets at 25 ± 2°C under continuous illumination of 4.3 Wm⁻². Hypocotyls from 4 d old seedlings and with similar growth rate, were excised up to 6 cm below the cotyledonary node. Hypocotyl explants with apical meristem intact but cotyledons excised were selected for the present work with a view to provide a continuity of the endogenous auxin source. Similar explants were also recently used by Huang et al. to investigate indole-3-butyric acid (IBA)-induced AR formation in mung bean (Phaseolus radiatus L.). Using IAA instead of IBA for such investigations was preferred for the present work keeping in view that the two auxins seem to employ different transport proteins for their polar transport. Freshly harvested explants were put upright in glass vials with their proximal cut ends dipped in 1 ml of different concentrations (1, 5, 10 and 15 µM) of IAA, thus bathing the hypocotyls up to 5 mm of their lower ends. Explants were maintained in dark during the course of experiments. The number of AR visible on hypocotyl surface was recorded daily up to 4 days of incubation. Concentration of IAA thus observed optimal for rooting (10 µM) was used for all further experiments. Similarly, various concentrations of other test solutions, namely NPA (auxin efflux blocker; 1 and 10 µM), Lat B (an inducer of actin depolymerization; 25, 50 and 100 nM), CsA (an inhibitor of cyclophilins; 1, 5 and 10 µM), sodium nitroprusside (SNP; NO donor; 1, 5, 10 and 100 µM) and 2-phenyl-4,4,5,5-tetramethyllimidazoline-1-oxyl-3-oxide (PTIO; NO scavenger; 1 and 1.5 mM), were initially used to select their respective optimal concentrations. Based on these preliminary experiments NPA, Lat B, CsA, SNP and PTIO were used at 10 µM, 100 nM, 10 µM, 100 µM and 1.5 mM, respectively, for all subsequent experiments. Some other treatment combinations, namely NPA (10 µM) + IAA (10 µM); LatB (100 nM) + IAA (10 µM); CsA (10 µM) + IAA (10 µM); SNP (100 µM) + NPA (10 µM) and PTIO (1.5 mM) + IAA (10 µM) were also used to investigate their effects on adventitious rooting. Hypocotyl explants incubated in distilled water served as control. Morphological observations of rooting response were imaged after 7 days of incubation, using Nikon digital camera fitted on a stereomicroscope (Stemi 2000, Zeiss, Germany). Detailed evaluation of root initiation was observed after clearing by immersing the explants in a 3:1 solution of ethanol; acetic acid overnight. They were then transferred to 2 N NaOH solution, left overnight, washed once with distilled water and stained with safranin solution for 2–3 min. Excess stain was
removed by repeated washing in distilled water. The lower 2 cm region of hypocotyl explants was then cut and mounted on a glass slide to examine endogenous root initials, using a stereomicroscope (Stemi 2000, Zeiss, Germany) fitted with a Nikon camera.

Root initiation and extension in the basal region of hypocotyl explants maintained in distilled water indicates the expected basipetal transport of the inducing factor (endogenous IAA) from the intact meristem, as also reported earlier. Treatment with IAA (10 µM) elicited two effects on hypocotyl explants in comparison to those subjected to distilled water treatment: (1) Formation of greater number of root initials, (2) Greater extension of the initiated roots (Figs. 1 and 2). A response similar to that evoked by IAA is also evident in hypocotyl explants treated with 100 µM of SNP (Figs. 1 and 2). Recently SNP (NO donor) has been reported to evoke dose-dependent response on AR formation in marigold. In the present work, treatment with variable concentrations of SNP, ranging from 1–100 µM, lead to a gradual increase in the number and extension growth of AR till 100 µM. Pagnussat et al. and Liao et al. have used 10 µM and 50 µM as effective SNP concentration in cucumber and marigold, respectively. Thus, optimal concentration of SNP for AR formation is species-dependent. In presence of PTIO (1.5 mM; a specific NO scavenger), complete suppression of AR was evident in sunflower, as also reported earlier in mung bean. Combination of PTIO with IAA lead to root initiation only (no extension growth). NPA (10 µM) blocked AR initiation by endogenous (distilled water treatment) and exogenous IAA (Fig. 1). Application of NPA inhibits polar auxin transport, thus reducing the optimal concentration of IAA required for AR formation at the hypocotyl base (zone of AR formation). Thus, no evidence of root initials was evident in presence of NPA, which is also reported in cucumber and loblolly pine, respectively. Though NO is expected to act downstream of IAA but a treatment of SNP in combination with NPA (present work) lead to complete suppression of AR formation. Our unpublished observations have indicated the expression of NO in the interfascicular cells after induction phase (i.e., during AR initiation and extension). Thus, it can be proposed that IAA is involved in induction phase of adventitious rooting independent of NO, while initiation and extension phases appear to involve IAA-NO interaction. CsA-cyclophilin complex is known to inhibit calcineurin (a protein phosphatase) and NOS activity in animal systems. Treatment of hypocotyl explants with CsA (10 µM) lead to formation of fewer number of roots which exhibited extension growth. Oh et al. reported a significant reduction in the number of roots in the presence of CsA in hypocotyl explants from tomato. Subjecting hypocotyl explants with a combination of CsA and IAA lead to formation of fewer number of root initials, reaffirming the involvement of NO in auxin action in the developmental process under investigation (AR). However, further investigations on the role of cyclophilins and NOS in auxin-modulated AR formation are required to pinpoint their specific
sites of action in this developmental process. Treatment with Lat B (+ and - IAA) lead to complete AR suppression in sunflower hypocotyl explants. Actin-mediated polar localization of PIN proteins is responsible for polar auxin transport and disruption of microfilaments by Lat-B would thus, directly affect IAA transport leading to the observed AR suppression. These observations indicate a convergence of the effect of IAA with that of NO and a role of a well organized actin in the responding cells.

To sum up, present investigations provide evidence for a link-age between auxin-induced AR response in seedling hypocotyls and NO (Fig. 3). Both endogenous and exogenous IAA-mediated AR induction seem to depend on actin. Significance of actin in this developmental response has become evident via its role in the cycling of auxin efflux proteins (PIN). The three phases of AR formation can be differentiated from each other in terms of their sensitivities to IAA and NO. AR induction phase seems to be governed by auxin alone, independent of NO. NO seems to become operative in this auxin-modulated response (AR) during initiation and extension phase only. Investigations are being undertaken in the author’s laboratory to visualize and quantitate the NO signal in the IAA-responding hypocotyl explants so that the phasing of the role of NO during AR formation can be precisely predicted.

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**References**

Nitric oxide accumulation and actin distribution during auxin-induced adventitious root development in sunflower

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Auxin-mediated adventitious root (AR) formation from the basal ends of hypocotyl segments in sunflower (Helianthus annuus L., cv. Morden) is associated with gradual subcellular and anatomical changes. Recent work in the author's laboratory has demonstrated that different phases of auxin-induced AR in sunflower hypocotyls are either NO-independent (induction phase) or NO-dependent (initiation and extension phases). These observations indicated a possible interaction between auxin efflux transporters (PIN) and actin during adventitious rooting (Yadav et al., 2010). Based on our present understanding of the signaling events associated with adventitious rooting, the temporal accumulation of endogenous NO in the adventitious root differentiating zone at various stages of development, has been monitored by fluorescence microscopy, using DAF-2DA for detection of NO, in presence of various pharmacological agents. These observations highlight the effects of various promoters and inhibitors of AR on the spatial distribu-

1. Introduction

Adventitious root (AR) formation begins with redifferentiation of predetermined interfascicular parenchymatous cells in the basal region of the stem after the removal of primary root system. This response is known to be evoked by endogenous auxins, primarily indole-3-acetic acid (Taiz and Zeiger, 2011). Although genetic approaches have clarified some discrete aspects of auxin transport and related signal transduction events, not much is known about the molecular mechanisms associated with AR formation. Endogenous auxin concentrations are known to alter during the three phases of AR i.e., induction, initiation and extension (Nag et al., 2001). Using various pharmacological agents, a probable signaling cascade (linking NO, cyclic GMP and mitogen-activated protein kinases) for auxin-induced adventitious root formation has been proposed in cucumber seedlings (Pagnussat et al., 2002, 2003, 2004). These observations are, however, devoid of any extensive analysis of the modulation of endogenous NO distribution. The mode of auxin interaction with NO, resulting in adventitious root formation, still remains unknown.

Recent work in the author's laboratory has demonstrated that different phases of auxin-induced AR in sunflower hypocotyls are either NO-independent (induction phase) or NO-dependent (initiation and extension phases). These observations indicated a possible interaction between auxin efflux transporters (PIN) and actin during adventitious rooting (Yadav et al., 2010). Based on our present understanding of the signaling events associated with adventitious rooting, the temporal accumulation of endogenous NO in the adventitious root differentiating zone at various stages of development, has been monitored by fluorescence microscopy, using DAF-2DA for detection of NO, in presence of various pharmacological agents. These observations highlight the effects of various promoters and inhibitors of AR on the spatial distribu-

Abbreviations: AR, adventitious roots; AtNOA1, Arabidopsis thaliana nitric oxide associated 1; CLSM, confocal laser scanning microscope; CS, cyclosporin A; DAF-2DA, 4,5-diaminofluorescein diacetate; GDC, glycine decarboxylase; GEF, guanine nucleotide exchange factor; GMP, guanine mononucleotide phosphate; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; iNOS, inducible nitric oxide synthase; Lat B, latrunculin B; NBP, NPA binding receptor protein; Ni–NOR, nitrite–NO oxidoreductase; NPA, 1-napthylphthlamic acid; NO, nitric oxide; NOS, nitric oxide synthase; PMSF, phenylmethylsulfonyl fluoride.

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tion of NO in the responding cells. Estimation of total NO content and NO content due to putative NOS activity has been undertaken in the hypocotyl explants subjected to pharmacological treatments (known to stimulate or inhibit AR). Lastly, a comparative confocal microscopic image analysis of the bundling of actin in presence of auxin efflux blocker (NPA) and nitric oxide scavenger [2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO)] has highlighted the common features of their molecular action during AR formation.

2. Materials and methods

2.1. Plant material and explant treatments

Raising of hypocotyl explants from sunflower seedlings (Helianthus annuus L. cv. Morden) and their treatment with various pharmacological agents was undertaken according to Yadav et al. (2010). Briefly, the hypocotyls from light-grown, 4 d old seedlings with similar growth rate, were excised up to 6 cm below the cotyledonary node. Cotyledons were carefully excised, retaining the shoot meristem. The resulting explants were used for adventitious rooting experiments. Freshly harvested explants were put upright in glass vials with their cut ends dipped in 1 ml of various test solutions in 5 ml corn inglass vials. Explants were maintained in dark during the course of experiments (i.e., till 4 d). The test solutions of IAA (10 μM), NPA (10 μM), Lat B (100 nM), cyclosporin A (CsA, 10 μM) and PTIO (1.5 mM) were used either alone or in combinations, as described in Section 3. Hypocotyl explants incubated in distilled water for 4 d served as control.

2.2. Anatomical changes and epifluorescence microscopic visualization of fluorescence due to endogenous NO distribution using DAF-2DA

Hypocotyl explants subjected to various pharmacological agents (for a period of 4 d) were used for cutting (using razor blade) fine sections from the basal 2–4 mm regions and they were observed for various stages of AR formation, using a confocal microscope. Digital images were taken using AxioCam digital camera (Zeiss, Germany). Cross sections obtained from the basal 2–4 mm regions of various explants were incubated for 40 min in 10 μM of DAF-2DA prepared in 10 mM Tris–HCl buffer set at pH 7.4 (according to Corpas et al., 2006). Sections were then washed twice for 5 min each with the same buffer (without DAF-2DA) and observed for fluorescence due to DAF-2DA binding with NO (exc 485 nm; em 515 nm), using epifluorescence microscope (Axioskop from Zeiss, Germany). In order to check the specificity of fluorescence due to NO (control), sections were preincubated for 1 h with 1 mM PTIO in 10 mM Tris–HCl buffer (pH 7.4), subsequently incubated for 40 min with 10 μM DAF-2DA in presence of 1 mM PTIO and then examined for fluorescence quenching microscopically.

2.3. Estimation of NO production due to putative NOS activity

Putative NOS activity was estimated spectrofluorometrically, according to David et al. (2010). Briefly, 1 g tissue from the basal 4 mm hypocotyl segments (pooled from several explants subjected to specific treatments till 4 d) was homogenized in the extraction buffer (50 mM Tris–HCl, 250 mM sucrose and 1 mM EDTA, pH 7.4) containing 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 2 μg ml−1 aprotinin, 25 μg ml−1 soybean trypsin inhibitor and 10 μg ml−1 protein inhibitor cocktails procured from Sigma (USA). The homogenate was frozen and thawed with liquid nitrogen thrice and centrifuged at 10,000 g for 20 min at 4 °C, according to Butt et al. (2003). The supernatant was collected and stored at −20 °C. Protein concentration was determined by Bradford assay (Bradford, 1976). Total NO content was estimated in triplicate for each sample in a reaction buffer containing 1 mM L-arginine and 1 μM β-NADPH. After addition of 10 μM DAF-2DA, the reaction mixture was incubated at 25 °C for 30 min, according to Corpas et al. (2004). In order to determine putative NOS activity, samples were preincubated with 10 mM aminoguanidine (iNOS inhibitor) in the reaction buffer for 30 min, followed by the addition of 1 mM L-arginine, 1 μM β-NADPH and 10 μM DAF-2DA (Valderrama et al., 2007). The reaction mixture was incubated at 25 °C for 30 min. Reaction mixture without protein served as control for the respective sample. Fluorescence was measured using a spectrofluorometer (Model: FS920; Edinburgh Instruments, UK) at the excitation and emission wavelengths of 485 and 515 nm, respectively, according to Kojima et al. (1998). Data was presented as fluorescence units (counts per second).

2.4. Localization of actin

Actin was visualized according to Blancalflor (2000), using Alexa Fluor phalloidin (Molecular Probes Inc., USA) as the fluorescent probe. Fine sections obtained by razor blade from the basal 4 mm regions of control explants and those treated with Lat B, IAA, NPA and PTIO till 4 d, were immersed for 30 min in 50 mM PIPES buffer (pH 6.9) containing 4 mM MgSO4 and 10 mM EGTA. Sections were then incubated for 10 min in the same buffer containing 0.1 μM Alexa Fluor phalloidin, 0.3 M mannitol and 2% glycerol. Sections, subsequently washed once with PIPES buffer, were mounted on a glass slide and immediately observed for fluorescence under CLSM using argon laser.

All experiments were performed at least thrice and statistically analyzed wherever necessary.

3. Results and discussion

3.1. Pre-mitotic events (induction phase) leading to AR formation are evident in the interfascicular parenchyma within 1 d (24 h)

Auxin-mediated AR formation in the hypocotyl explants is associated with gradual anatomical changes in the interfascicular parenchyma. During the induction phase (1 d), predetermined cells became cytoplasmically dense. Initiation of cell division became evident after 2 d (Fig. 1). After 3 d of IAA treatment (Fig. 1M), tiers of cells became prominent in this region. Few cells of the inner tier exhibited characteristic cell wall thickenings, which proliferated to a greater extent by day 4. These presumably differentiate into vasculature in the developing root primordium. Root primordium, thus initiated after 4 d of IAA treatment, showed a well developed meristem (m) and procambial tissues (pc) (Fig. 1Q). AR formation is stimulated as a result of root excision, which possibly reduces the sink size for basipetally translocating substances, leading to their build up in the hypocotyl base. The view gains support from our preliminary experiments, where explants with intact apical meristem exhibited AR formation but those with decapitated apical meristem did not form AR. Root excision could also probably remove the inhibitors of AR being synthesized in roots.

3.2. Significant NO accumulation occurs in the interfascicular cells within 2 d (48 h)

Within the plant cells, NO is not as mobile as expected because of its trapping in the hydrophobic core of the membranes, as well as due to its chemical reactivity with lipids and/or proteins (Stöhr, 2007). The diffusion coefficient of NO is highly dependent on membrane composition. Cell membranes exhibit varying lipid composition in different regions. Thus, they are not homogenous
Fig. 1. Fluorescence microscopic visualization of NO signal in hypocotyl sections. Gradual changes in endogenous NO distribution in parenchyma cells between the neighbouring vascular bundles in hypocotyl explants accompanying adventitious rooting, upon treatment with 10 μM IAA. Sections were observed for fluorescence in the absence of DAF-2DA, in presence of DAF-2DA and in presence of DAF-2DA (10 μM) + PTIO (1 mM). Arrowhead in 'S' indicates intense fluorescence in the epidermal cells of a young primordium. m represents meristem and pc procambial tissue. Magnification: 200×.
lipid layers. Fluid phase membranes have been observed to act as strong barriers to NO transport. Based on these facts, it is technically possible to visualize NO accumulation in viable cells by fluorescence microscopy. DAF-2DA, a widely used fluorescent probe for NO, is employed in plant and animal systems for endogenous NO visualization (Kojima et al., 1998). As also reported earlier in mung bean (Huang et al., 2007), coinciding with AR differentiation in sunflower hypocotyl explants (present work), a gradual increase in fluorescence due to endogenously localized NO was observed in the interfascicular parenchyma with increasing period of IAA treatment. Distribution of NO during lateral root formation has also been investigated earlier in tomato, supporting the involvement of NO in the auxin signaling pathway (Correa-Aragunde et al., 2004). NO signal did not exhibit any significant difference up to 1 d after IAA treatment (Fig. 1C and G). After 2 d of treatment, intense green symplastic and apoplastic fluorescence was observed in the interfascicular region (Fig. 1K). Our previous report (Yadav et al., 2010) on the demarcation of auxin-mediated induction phase and IAA–NO interaction–mediated initiation phase, is strengthened by the present observations. These results implicate the possible involvement of IAA alone during the induction phase (1 d). A lag phase (when IAA probably leads to NO synthesis) is, however, essential for the transition from induction to initiation phase (2 d stage) and thereafter. NO generation in cells exhibiting AR initiation appears to accompany mitotic activity during the initiation phase. Further increase in fluorescence intensity was observed after 3 d of IAA treatment (Fig. 1O). Interestingly, appreciably intense fluorescence due to NO was visualized in the root primordium after 4 d, it being maximum in the apical meristematic cells (Fig. 15). This may be attributed to the possible involvement of NO during cell division and differentiation. In the root primordium, NO signal was uniform and symplastic in the epidermal layer of the primordium. Treatment with NO specific scavenger (PTIO) leads to a significant reduction in fluorescence at all stages of AR development, indicating that the green fluorescence is, in fact, due to NO. The persistent fluorescence contained in the lignified tissue (xylem) in control and PTIO-treated sections can be attributed to autofluorescence of lignin at the excitation wavelength (ex. 485 nm) being employed for detecting fluorescence due to DAF-2DA and NO interaction. At this point, it will be worthwhile to mention that while evaluating NO signal in plant cells by DAF treatment, care must be taken to take note of such autofluorescence from lignified cells and it may be pointed out that this lignin autofluorescence is not quenchable by PTIO treatment. Thus, it can be distinguished from fluorescence due to NO. Evidence for NO generation in the interfascicular cells 2 d after IAA treatment highlights the involvement of NO in the adventitious rooting process accompanying root initiation (after the auxin-mediated induction phase).

3.3. A correlation between IAA, NO and actin distribution is evident during AR formation

Auxin (principally IAA) synthesized in the apical tissues moves downward through its characteristic polar transport mechanism. The central feature of this transport is that IAA-efflux is directed by polar localization of some efflux carriers (mainly PIN proteins) which determine polar intracellular transport of IAA. Polarity of PIN distribution on the plasma membrane of a cell involves movement of newly synthesized PIN proteins through the endomembrane secretory system. PIN is rapidly cycled between the plasma membrane at the base of the cell and an unidentified endosomal compartment, by an actin-dependent mechanism (Morris, 2000). NPA, an inhibitor of polar auxin transport, has been widely used as a tool to investigate the mechanism and regulation of polar auxin transport (Muday and Murphy, 2002). NPA-binding receptor protein (NBP) is functionally associated with PIN proteins. The link between NBP and the actin cytoskeleton suggests its involvement in directing the polar distribution of PIN, which has been shown to cycle along actin filaments (Geldner et al., 2001; Taiz and Zeiger, 2011).

Treatment of hypocotyl explants with NPA (10 μM) leads to complete abolition of NO-associated fluorescence detected by DAF-2DA (Fig. 2C), establishing that NO acts downstream of auxin during AR formation. In the non-inductive conditions of NPA + IAA, some fluorescence was observed (Fig. 2G) which was quenchable by PTIO (Fig. 2H).

Although previous reports indicated a role of NO in mediating auxin response during AR formation in cucumber (Pagnussat et al., 2002, 2003), a cross talk between IAA and NO still remains to be elucidated. In order to further explore IAA–NO interaction during AR formation in sunflower, Latrunculin B (Lat B), an actin depolymerizing agent was employed. Lat B has been widely used to investigate the function of actin in gymnosperms, monocotyledonous and dicotyledonous plants. It induces profound changes in the organization of microfilaments while leaving the organization of microtubules unaltered. Lat B associates with actin monomers in 1:1 ratio, thereby preventing their repolymerization into actin filaments (Morton et al., 2000). In the present work, Lat B leads to a negative impact on NO accumulation in the responding cells (Fig. 2K). Actin depolymerization using Lat B would supposedly lead to substantial inhibition of IAA transport, thus resulting in reduced accumulation of IAA in the basal responding region of the hypocotyl segments. This might explain the observed significant reduction in NO accumulation. While NO fluorescence was evident in Lat B + IAA treatment, it was relatively less as compared to treatment with IAA only (Fig. 2O). The interfascicular NO fluorescence, quenchable by PTIO, authenticates NO-associated DAF-2DA fluorescence.

Cyclophilins constitute a family of ubiquitous proteins known to bind immunosuppressant drugs. These proteins belong to the cluster of immunophilin proteins and catalyze cis–trans isomerization of nascent proteins, thereby assisting protein folding. Cyclophilins (which are known to bind with cyclosporin) have recently been reported to be a component in auxin regulation of plant growth and development (Oh et al., 2006). They interact with the N-terminal region of the GNOM protein, a guanine nucleotide exchange factor (GEF) required for the proper localization of the auxin efflux carrier PIN1 (Geldner et al., 2003). Cyclosporin A (CsA) inhibits NOS activity in animal systems (Diaz-Ruiz et al., 2005). The cyclophilin inhibitor, CsA, inhibits auxin-induced adventitious root initiation in tomato hypocotyl sections and also reduces the expression of auxin-induced early auxin response genes (Oh et al., 2006). Compared to the IAA-induced NO fluorescence, intensity of NO fluorescence in root initials of CsA-treated explants was faint. Moreover, the intense fluorescence due to NO from epidermis in the root initials of CsA-treated explants was not detectable (Fig. 2S). Interestingly, CsA + IAA treatment markedly reduced the intensity of NO-associated symplastic fluorescence in the dividing cells of interfascicular region and the developing root initials. Thus, a potential involvement of cyclophilins in AR formation is apparent from the present work. Further investigations in this direction would provide evidence for cyclophilins as potential signaling intermediates during AR response.

3.4. Differences in total NO content and NO due to putative NOS activity are evident in hypocotyl explants subjected to AR inducing and inhibitory treatments

In higher plants, both enzymatic and non-enzymatic (Bethke et al., 2004) sources of NO have been documented. Enzymatic sources mainly involve nitrate reductase (NR) (Neill et al., 2008), as described in auxin-induced formation of lateral root initials (Kolbert et al., 2008); plasma membrane-associated nitrite–NO
Fig. 2. NO visualization in 4 d hypocotyl explants in presence of various pharmacological agents. Distribution of endogenous NO content in the parenchyma cells between the neighbouring vascular bundles of 4 d old hypocotyl explants treated with NPA (10 µM), Lat B (100 µM) and CsA (10 µM), alone or in combination with IAA (10 µM). Sections were observed for fluorescence in the absence of DAF-2DA (10 µM), in presence of DAF-2DA (10 µM) and in presence of DAF-2DA (10 µM) + PTIO (1 mM). Magnification: 200×.
Spectrofluorometric estimation of NO content. Effect of various treatments on total NO content and NO content due to putative NOS activity in the basal part of 4 d hypocotyl explants, accompanying expression/suppression of AR. Data represents mean and standard errors of fluorescence intensity (counts per second) from three replicates.

NOS is known to be active only when dephosphorylated. Cyclosporin–cyclophilin A dimer is known to bind calcineurin (a class PP2B phosphatase), inhibiting its phosphatase activity (Romano et al., 2005). The observed inhibition in NO synthesis due to putative NOS activity in CsA-treated explants may be explained as a probable result of CsA-mediated maintenance of phospho-rylated, inactive state of putative NOS. Similar reduction in NOS activity in rat spinal cord cells on treatment with CsA, has also been reported earlier (Diaz-Ruiz et al., 2005). It is further suggested that binding of CsA to cyclophilin D could block mitochondrial transition pore permeability, thus decreasing intracellular calcium concentration, resulting in a reduction in the activity of calcium-dependent NOS. A complete inhibition of putative NOS activity in presence of CsA throws light on the presumption about the presence of a NOS-like enzyme. A reduction in NO content and NO due to putative NOS evident in NPA, Lat B and CsA treatments, indicates the involvement of the three components of AR signaling (NO, IAA and actin) and their interaction. Thus, the inhibitory effect of aminoguanidine suggests that the generation of endogenous NO may be catalyzed by a NOS-like enzyme contributing to NO accumulation in the root initiation region, resulting in the observed morphological response.

Actin organization associated with AR formation exhibits marked differences in AR inducing and inhibitory treatments

Any signal (like IAA) leading to a polarized developmental event, such as AR formation, would very likely affect cytoskeleton organization, in particular actin (Xuan et al., 2008). Auxin promotes the localization of cycling PIN proteins on the plasma membrane (Paciorek et al., 2005). Recently, Nick et al. (2009) have observed auxin-induced transformation of actin bundles into finer strands and have proposed a self-regulating circuit between polar auxin transport and actin organization. Confocal laser scanning microscopic (CLSM) imaging of fluorescence due to actin upon treatment with Alexa Fluor phalloidin (present work) shows contrasting differences between auxin-treated hypocotyl segments and those treated with NPA and Lat B (present work). The basal region of IAA-treated hypocotyl segments responding by exhibiting AR formation, shows enhanced and well distributed actin in the interfascicular cells after 4 d of incubation (Fig. 4A). This is in accordance with the findings of Nick et al. (2009) that auxin availability is a requisite for the maintenance of actin cytoskeleton. Enhanced expression of actin in presence of auxin has also been observed in...
the hypocotyl cuttings from loblolly pine (Diaz-Sala et al., 1996). In the present work on sunflower hypocotyls, Lat B treatment led to bundling of actin and markedly reduced fluorescence due to actin. Similar pattern of actin disintegration was also visualized in NPA-treated hypocotyl explants (Fig. 4B). Actin disintegration by NPA (present work) seems contradictory to the reports of Petrášek et al. (2003) who reported that inhibitory action of NPA on auxin efflux had no observable effect on the arrangement of actin filaments. But, NPA leads to inhibition of polar auxin transport, thereby drastically reducing the cellular auxin concentration and consequently leading to observed actin disintegration. Interestingly, PTIO treatment (Fig. 4C) also lead to a drastic actin cytoskeleton breakdown, appearing as a thick fluorescent layer below the cell membrane and bundling of actin, visible as spots of intense fluorescence. As NO appears downstream of auxin in the signaling pathway (Pagnussat et al., 2003), auxin-mediated actin cytoskeleton maintenance could possibly be modulated by NO. Recently, NO has been reported to modulate actin cytoskeleton in the root apices of maize seedlings (Kasprowicz et al., 2009).

4. Conclusions

To sum up, present work provides evidence for changes in temporal and spatial expression of endogenous NO in the interfascicular cells of hypocotyl segments, accompanying auxin-induced adventitious rooting. NO generation during AR initiation and extension (and not AR induction) has been demonstrated. Endogenous NO accumulation in the AR differentiating zone has been shown to be largely due to the enhanced activity of putative NOS. A possible correlation between NO, IAA and actin has become evident. Evidence has also been provided for a self-regulatory role of IAA in actin-mediated PIN recycling, thus altering auxin transport to the target sites. Involvement of NO in auxin-mediated actin cytoskeleton maintenance is also apparent during the differentiation of adventitious roots. Present findings using cyclosporin indicate the probable involvement of cyclophilins in PIN localization, thereby opening a new front for further investigations with regard to biochemical events associated with AR formation. Specifically, the impact of cyclosporin on the phosphorylation status of putative NOS holds promise for newer and significant findings.

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