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

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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 re-differentiation of predetermined cells which switch from their morphogenetic path to act as mother cells for initiation of root primordia.[1] The process of AR formation consists of three physiologically interdependent phases: induction, initiation and extension.[1] Induction phase comprises of various molecular and biochemical events but no morphologically visible changes appear during this phase. Formation of multilayered cells and conception of root primordia occurs during initiation phase. During expression phase, root primordia exhibit intra-stem growth and their emergence through epidermis. Various environmental and endogenous factors, such as temperature, light, hormones (particularly auxin), sugars and mineral salts, act as cues for promoting redifferentiation of predetermined cells resulting in root induction.

The three phases of AR formation are known to be regulated by alterations in the endogenous level of auxin.[2] A transient increase in auxin concentration has been reported during induction phase, which is followed by a decrease and again an increase during expression phase.[3] Auxin transport to and from the responding region is essential for root organogenesis. Acropetal transport of auxin occurs through the vascular cylinder and the basipetal transport takes place through the epidermal and subtending cortical cells.[4] 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.[5] 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.[6] Efflux transporters exhibit rapid turnover in plasma membrane.[7] High affinity of NBP for actin filaments,[8,9] suggests its involvement in the cycling and polar distribution of PIN proteins.[10] 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.[11] 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.[12] 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[13] in pharmacological
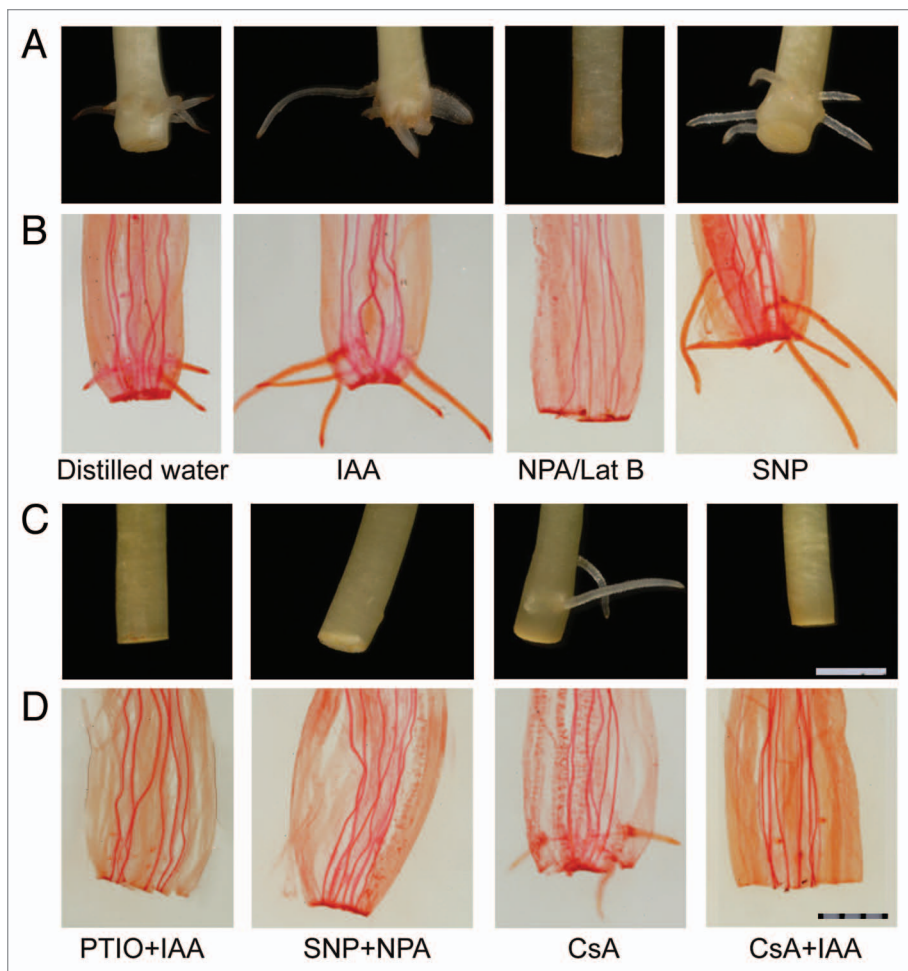
Figure 1. Effect of IAA and various other pharmacological agents on adventitious rooting in hypocotyl explants. Morphological observations of rooting response (A and C). Evaluation of endogenous root initiation and elongation observed in cleared explants stained with safranin (B and D). Scale bar represents 3 mm.

investigations. In the past few years, significant work has been done on NO as a signaling molecule in a variety of plant developmental processes.[14] Nitric oxide is known to play a crucial role in root development.[15]

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-asssisted 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,[16] 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.[17] 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.[18] 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
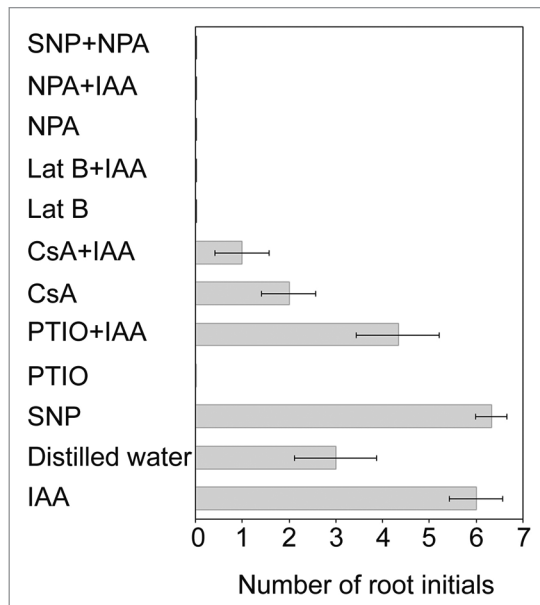
Figure 2. Quantitative analysis of AR initiation in presence of distilled water, IAA and various pharmacological agents in hypocotyl explants of sunflower. Each datum presents a mean and standard error from at least three observations.

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.[19] 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.[20] 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.[21] and Liao et al.[20] 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.[17] 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[22] and loblolly pine,[23] respectively. Though NO is expected to act downstream of IAA[24] 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 iniation 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.[25] Treatment of hypocotyl explants with CsA (10 µM) lead to formation of fewer number of roots which exhibited extension growth. Oh et al.[26] 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
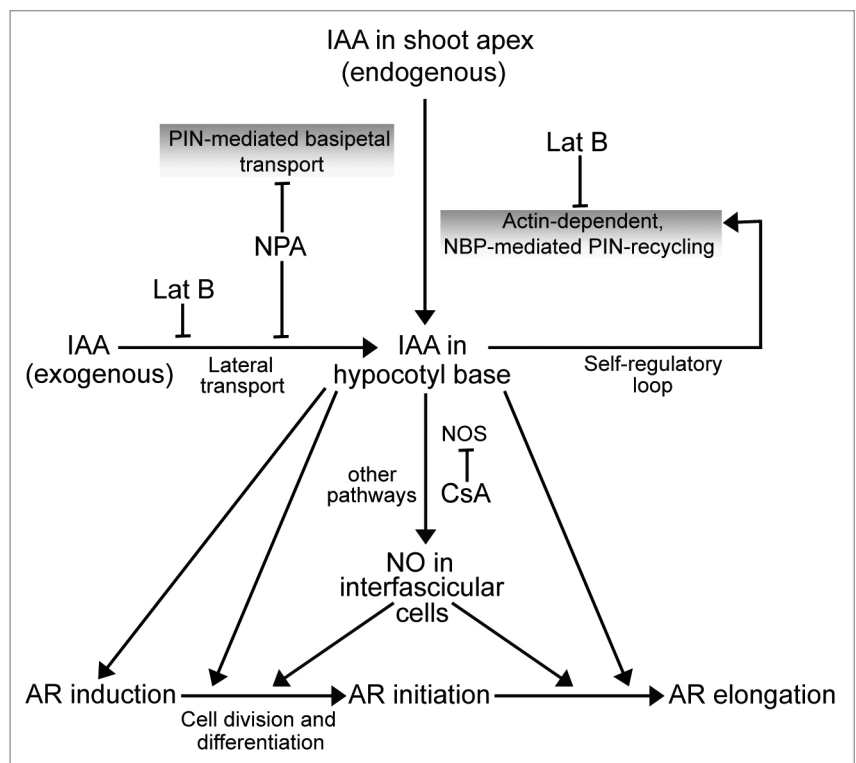
Figure 3. Schematic presentation of the probable events associated with NO-mediated adventitious rooting.

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 linkage 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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