

Extracellular ATP and nitric oxide signaling pathways regulate redox-dependent responses associated to root hair growth in etiolated *Arabidopsis* seedlings

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Abbreviations: cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; eATP, extracellular adenosine triphosphate; GSH, reduced glutathione; NO, nitric oxide; PPADS, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid

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Extracellular ATP (eATP) and nitric oxide (NO) have emerged as crucial players in plant development, stress responses and cell viability. Glutathione (GSH) is an abundant reducing agent with proposed roles in plant growth, development and stress physiology. In a recent publication, we demonstrated that eATP and NO restore hypocotyl elongation of etiolated *Arabidopsis* seedlings treated with GSH. Here it is reported that exogenous ATP also restores root hair growth suggesting a role for ATP and NO in the regulation of redox balance associated to specific processes of plant morphogenesis. A tentative model integrating redox-, eATP- and NO-signaling pathways during root hair growth in *Arabidopsis* seedlings is presented.

Linking Signaling Pathways to Gain Understanding on the Regulation of Morphological Processes in Plants

The first living organisms evolved under reducing conditions a long time before the environment became oxidizing due to oxygen produced by photosynthesis. As a consequence, the most fundamental signaling pathways were initially adapted to reducing conditions. Plants, as sessile organisms have taken advantage of the regulatory mechanisms evolved on a changing redox-status and nowadays they are strongly dependent of environmental redox conditions. An efficient redox control together with other signaling

mechanisms cooperates to maintain the steady-state redox homeostasis in plant cell. In this scenario, two small signaling molecules such as ATP and NO have a very ancient origin and are involved in cell redox physiology.^{1,2} ATP and NO play diverse biological functions in phylogenetically distant species. Intracellular ATP is the major source of energy for cellular reactions. However, ATP also acts as a signaling molecule at the extracellular face of the plasma membrane in animals as well as in plants.³ NO is an important regulatory molecule in eukaryotes. Several roles have been attributed to extracellular ATP (eATP) and NO in plants, ranging from cell viability to pathogen defence and cell death.^{2,4} NO production stimulated by eATP was shown in tomato suspension cells,⁵ hairy roots of *Salvia miltiorrhiza*⁶ as well as in algae.⁷ Reichler et al.⁸ showed that the intersection of eATP signaling and NO via the cellular messenger cGMP regulates pollen germination and pollen tube growth in *Arabidopsis*. Thus, the biochemical and molecular mechanisms underlying eATP and NO signaling pathways in plants have just begun to emerge. Lately, we have described an interconnection between eATP, NO and redox system during hypocotyl elongation in etiolated *Arabidopsis* seedlings.⁹ It was demonstrated that a fine-tuning of redox balance and endogenous NO level is associated to eATP action. Therefore, these findings open a new window in the poorly explored cross-talk between eATP, NO and redox balance in the plant kingdom.

Extracellular ATP Signaling Pathway in Plants

Despite the emerging role of eATP as a signal molecule, little is known about how eATP is transmitted to the apoplast. Apparently, ATP is released from plant cells by wounding¹⁰ or by plasma membrane ABC transporters.¹¹ In addition, a G-protein complex participates in the fine-tuning of touch-induced ATP release in *Arabidopsis* roots.¹² As summarized by Roux and Steinebrunner,³ cellular responses to eATP include perception, increases of cytoplasmic calcium concentration $[Ca^{2+}]_{\text{cyt}}$ and superoxide production. Plants perceive eATP throughout receptors that are structurally distinct from those found in animals.¹³ Demidchik et al.¹⁴ proposed that in root cell protoplasts eATP is sensed at the plasma membrane by an unknown receptor and that reactive oxygen species (ROS) are produced by a co-localized NADPH oxidase. Subsequently, ROS production stimulates the activation of Ca^{2+} channels, which contributes to the increase of $[Ca^{2+}]_{\text{cyt}}$. NADPH oxidases are haem-containing flavoproteins with multiple transmembrane-spanning domains. These enzymes reduce oxygen to form superoxide anion. Previously, Song et al.¹⁵ showed that after eATP perception, $[Ca^{2+}]_{\text{cyt}}$ level is transiently elevated and modulates eATP-induced superoxide accumulation via NADPH oxidase in *Arabidopsis* leaves. The spatial variation of Ca^{2+} channel activation by superoxide was also described in *Arabidopsis* root cells.¹⁶ More recently, the link between eATP, NO and phospholipid signaling has been revealed in tomato suspension cells.¹⁷

eATP, NO and Redox Balance Interplay during Root Hair Formation in *Arabidopsis* Seedlings

Root hairs increase the root surface area allowing a most efficient anchorage in the soil and water uptake. Root hair formation is controlled by several endogenous and exogenous factors. In *Arabidopsis*, root hair formation occurs in light- and dark-grown seedlings. It has been demonstrated

that cell elongation during post-embryonic hypocotyl and root hair growth are under the control of light-regulated developmental switch.¹⁸ However, it seems that light requirements for phytochrome action may be different for hypocotyl and root hair growth. Hypocotyl elongation and root hair growth are regulated by eATP.^{3,19,20} It was reported that root hair growth is under mechanistically different forms of redox control in *Arabidopsis*.²¹ Current knowledge of redox control in the apoplast and cytoplasm predicts that the plasma membrane is an important site for perception and transduction of environmental changes through redox-based signaling.²² GSH-mediated inhibition of etiolated hypocotyl growth and rooting was described by Cano et al.²³ in lupin. More recently, we demonstrated that in the presence of GSH, etiolated *Arabidopsis* seedlings showed inhibition of hypocotyl elongation and altered root system architecture including changes in primary root length, root hair number and agravitropic growth. All these morphological traits were restored by eATP and NO.⁹ In this addendum, we show that growth and differentiation of root hairs in *Arabidopsis* provide a simple and suitable model to study mechanisms underlying the interconnection between eATP, NO and redox status. Here, we examined root hair growth of 5-day-old *Arabidopsis* seedlings grown in the presence of GSH, ATP or combinatorial treatments in darkness. Harvested seedlings were recorded as digital images and root hair number was quantified. A similar amount of root hairs was detected in control and 1 mM ATP-treated seedlings (Fig. 1). However, in presence of 1.5 mM GSH, root hairs were almost undetected. Figure 1A and B shows that root hair development was almost completely recovered in GSH-treated seedlings upon the addition of exogenous 1 mM ATP. The purinoreceptor antagonist, PPADS and the specific NO scavenger, carboxy-PTIO (cPTIO) reduced the development of root hairs in ATP plus GSH-treated seedlings (Fig. 1B). In contrast, the addition of 100 μM H_2O_2 to GSH-treated seedlings did not restore the development of root hairs in GSH-treated seedlings

(data not shown) suggesting that the recovery of root hair growth mediated by exogenous nucleotides is specific. Moreover, the NO donor *s*-nitroso-*N*-acetyl-D-penicillamine (SNAP) added to GSH-treated seedlings did not restore the root hair number (data not shown). However, we can not discard that a combination of external factors such as GSH and darkness may affect NO release from SNAP.²⁴ Our findings suggest that eATP is sensed by purinoreceptor-like proteins to exert its effect on root epidermal cells and that endogenous NO is implicated in this process (Fig. 1B). This is in agreement with the finding that NO is involved in auxin-induced root hair formation.²⁵ Thus, eATP-NO action on GSH-treated seedlings grown in darkness is restricted not only to hypocotyl elongation but also to root hair development.

Monshausen et al.²⁶ demonstrated that ATRBOH C, a plasma membrane NADPH oxidase seems to be a key element in modulating root growth-related ROS production in *Arabidopsis*. In this way, our evidence is in agreement with the current model which assumes that root hair growth is dependent on eATP and ROS production.^{20,27} According to Foreman et al.²⁷ ROS production by NADPH oxidase induces opening of Ca^{2+} channels and elongation of the root hair. Furthermore, ROS control the activity of Ca^{2+} channels required for polar growth.²⁸ Wu and Wu⁶ demonstrated that eATP induces a rapid increase in the $[Ca^{2+}]_{\text{cyt}}$ level, which is dependent on NO in *Salvia miltiorrhiza* hairy root culture. It was also demonstrated that a NADPH oxidase similar to the mammalian gp91^{phox} is necessary to elongate root hairs in *Arabidopsis*.²⁷ However, the balance between eATP and NO is tightly regulated³ possibly by impairing auxin transport.²⁹ Other results also indicate that NO and cGMP operate downstream of auxin promoting root morphological changes.³⁰ Furthermore, the involvement of NO in actin cytoskeleton and vesicle trafficking in root apices has been demonstrated.³¹ Thus, the currently available data allowed us to speculate that NO can directly influence the activity of target proteins through S-nitrosylation or acting

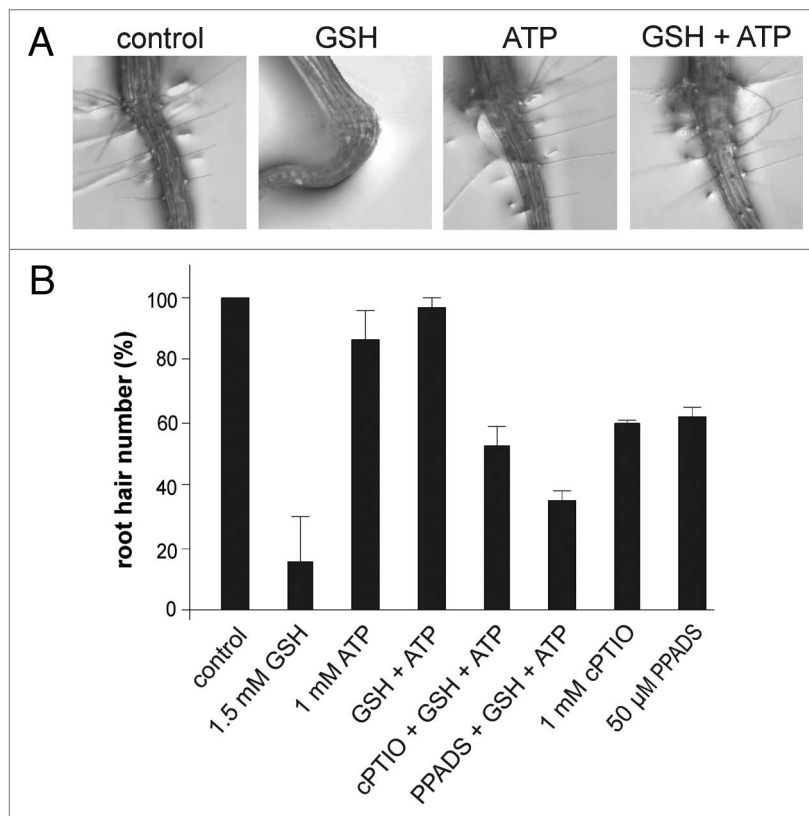


Figure 1. eATP restores root hair growth of GSH-treated *Arabidopsis* seedlings in darkness. (A) Representative 5-d-old seedlings (*Arabidopsis thaliana* ecotype Col 0) grown vertically in the absence or presence of 1 mM ATP, 1.5 mM GSH, GSH + ATP in darkness. (B) Measurement of root hair number of etiolated seedlings grown in the absence or presence of 1 mM ATP, 1.5 mM GSH, GSH + ATP, 1 mM cPTIO, 50 μ M PPADS. Combinatorial treatments are indicated. Data are means (\pm SD) of at least three independent experiments.

as a Ca^{2+} -mobilizing intracellular messenger. All these findings support an interesting convergence of eATP, NO and redox system operating in plants.

Furthermore, a complex signaling network including eATP, NO, auxin, ROS, $[\text{Ca}^{2+}]_{\text{cyt}}$ and cGMP may take place during root hair growth in *Arabidopsis* seedlings. Finally, eATP and NO appear as multipurpose signaling messengers that regulate specific target molecules impacting in a cell-type specific manner in *Arabidopsis* seedlings. For such reasons, we should recognize that we are only beginning to understand the intricate puzzle that interplays in plant morphogenesis. To summarize previous evidence and the potential interactions between eATP, NO and their cross-talks with other signalings, a schematic model is shown in Figure 2. This model is based on those proposed by Wu and Wu,⁶ Demidchik et al.¹⁴ and Lombardo et al.²⁵ It integrates

both external and internal cues into the root hair cell. Nevertheless, crucial questions remain to be answered i.e., plant cell matrix, as well as plasma membrane contains a suite of proteins capable of being activated through exogenous ATP but which are their specific molecular targets? Overall, ROS, Ca^{2+} and NO seem to be critical messengers acting on multiple targets in the plant cell.

Future studies will allow us to elucidate the expanding role of eATP, NO and the finely regulated cross-talk between auxin and redox system. Indeed, redox-dependent modulation by eATP and NO signaling may be critical to optimize root morphology as an adaptive strategy under environmental stress conditions.

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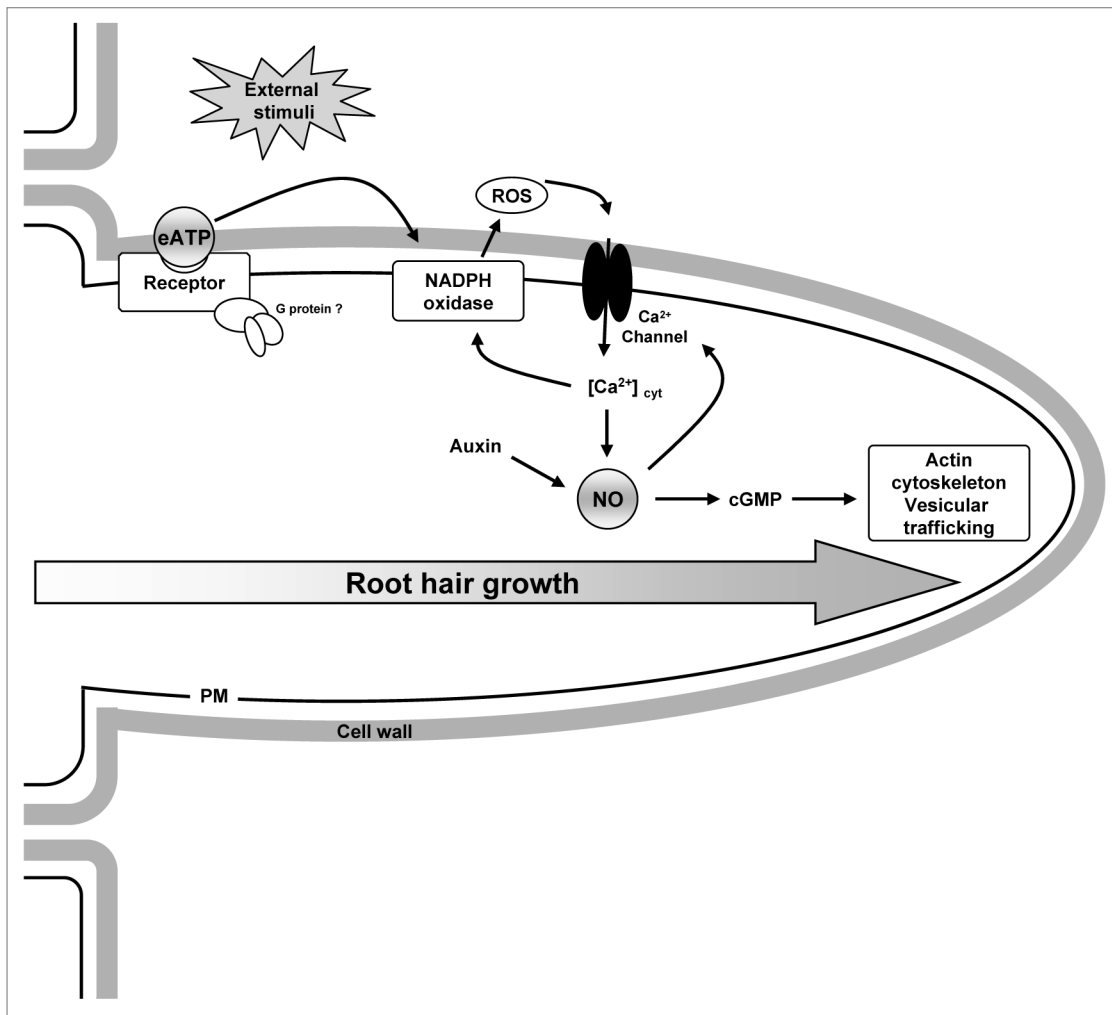


Figure 2. Putative model integrating eATP and NO-regulated signaling pathways for root hair growth in Arabidopsis. eATP putative receptor, NADPH oxidase and Ca²⁺ channel are shown in the plasma membrane (PM). Depending on the cell type and the external stimulus, eATP would induce Ca²⁺ channel activity and increase [Ca²⁺]_{cyt} leading to NO accumulation and ROS production via NADPH oxidase stimulation. In addition, NO may regulate Ca²⁺ channels by feedback activation. NO may also promote the formation of cGMP messenger. In turn, actin cytoskeleton may act as a downstream effector of NO signal transduction pathway. In parallel, auxin through NO could modulate root hair growth.

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