

ARTICLE ADDENDUM



Role of mitochondrial cyanide detoxification in Arabidopsis root hair development

Lucía Arenas-Alfonseca, Cecilia Gotor , Luis C. Romero , and Irene García 

Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas and Universidad de Sevilla, Sevilla, Spain

ABSTRACT

In non-cyanogenic plants, cyanide is produced during ethylene biosynthesis and is mainly detoxified by the β -cyanoalanine synthase CAS-C1. Arabidopsis plants lacking CAS-C1 show abnormal root hairs, which stop growing at early stages. Root hair elongates by polarized cell expansion at the tip, and we have observed that CAS-C1-driven GFP fluorescence locates in mitochondria and accumulates in root hair tips during root hair elongation. Genetic crosses have been performed between *cas-c1* plants and *scn1-1* mutants, defective in the *SCN1* protein that regulates the NADPH oxidase RHD2/AtrbohC, and between *cas-c1* and *rhd2-1*, defective in the NADPH oxidase necessary for the generation of ROS and the Ca^{2+} gradient necessary for root hair elongation. The phenotypic and molecular analysis of these crosses indicates that *cas-c1* is hypostatic to *scn1-1* and epistatic to *rhd2-1*. Furthermore, the action of cyanide in root hair development is independent of ROS and of direct NADPH oxidase inhibition by cyanide.

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Mitochondria are double-membrane-bound organelle found in most eukaryotic organisms including plants. Mitochondria oxidize carbon fuels to form cellular energy through the oxidative phosphorylation, which requires electron transfer through several large protein complexes, some of which pump protons, forming a proton gradient that powers the synthesis of ATP. The generation of ATP through mitochondrial respiration also produces reactive oxygen species (ROS). In excess, ROS can damage the cell, but they are also an important signal produced in response to varied stresses.¹ In addition to the classical electron transport chain (ETC), plant mitochondria possess an alternative ETC that modulates mitochondrial ROS production.² Cyanide is a disruptor of the ETC because it binds to the heme iron of cytochrome *c* oxidase, thereby impairing vital functions.³

Despite its toxicity, cyanide has been proposed to act as a regulator of several biological processes such as seed dormancy and germination;^{4–8} plant immunity^{9–12} and root hair development.¹³ Our previous work has shown that Arabidopsis null mutants in the mitochondrial β -cyanoalanine synthase, CAS-C1, accumulate cyanide to non-toxic level because the plant is completely viable but shows a root hairless phenotype suggesting a signaling role of CAS-C1 and cyanide in root development.^{7,13} *cas-c1* root hair bulges start growing correctly but they do not elongate and form malformed protuberances. The root hairless phenotype is phenocopied when wild type plants grow in the presence of cyanide and is reverted in *cas-c1* in the presence of the antidote hydroxocobalamin.¹³

Plant root hairs are tubular protrusions of root epidermal cells that increase the root surface to absorb nutrients and water, establish relationships with soil microbiota and contribute to

anchoring the plant to the soil. An epidermal cell is differentiated to a root hair following a well-studied process involving position-dependent signaling and molecular feedback loops.¹⁴ Once differentiated, the root hair elongates unidirectionally through polarized cell expansion, similarly to other polarized cell growth such as pollen tube or filamentous fungi growth. Root hair can reach growth rates of more than $1 \mu\text{m min}^{-1}$,¹⁵ which implies, that an extremely efficient mechanism for the delivery and modification of cell wall at the tip of the root hair and not along the shanks is taking place. The polar growth has been extensively studied and it is well known that polysaccharides, proteins and other molecules necessary for the cell wall formation are enclosed in vesicles that are directed to the cell tip by cytoplasmic streamings.^{14,16–20} pH, ROS and calcium gradient signaling are involved in the establishment and organization of this traffic.^{14,18,20–22} For this, the endoplasmic reticulum and the Golgi apparatus are important for the formation of vesicles and mitochondria provide the high amounts of energy required for the active transport and are calcium stores that move along the cytoskeleton to provide Ca^{2+} to the cell tip.^{23,24} Therefore, the subapical zone of the root hair is rich in these organelles including mitochondria.^{17,18,25}

To deepen in the function of CAS-C1 in the root hair growth and to delimit the position of the action of this protein in this process, several cellular and genetic strategies have been used. First of all, total ATP levels of wild type and *cas-c1* plants have been measured, and no differences have been found, suggesting that *cas-c1* mutants, despite they accumulate high levels of cyanide, have an appropriate energy homeostasis. Plants expressing a fusion protein CAS-C1-GFP under the control of the *cas-c1*

CONTACT Irene García  irene.garcia@ibvf.csic.es  Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas and Universidad de Sevilla, Avenida Américo Vespucio 49, Sevilla E-41092, Spain

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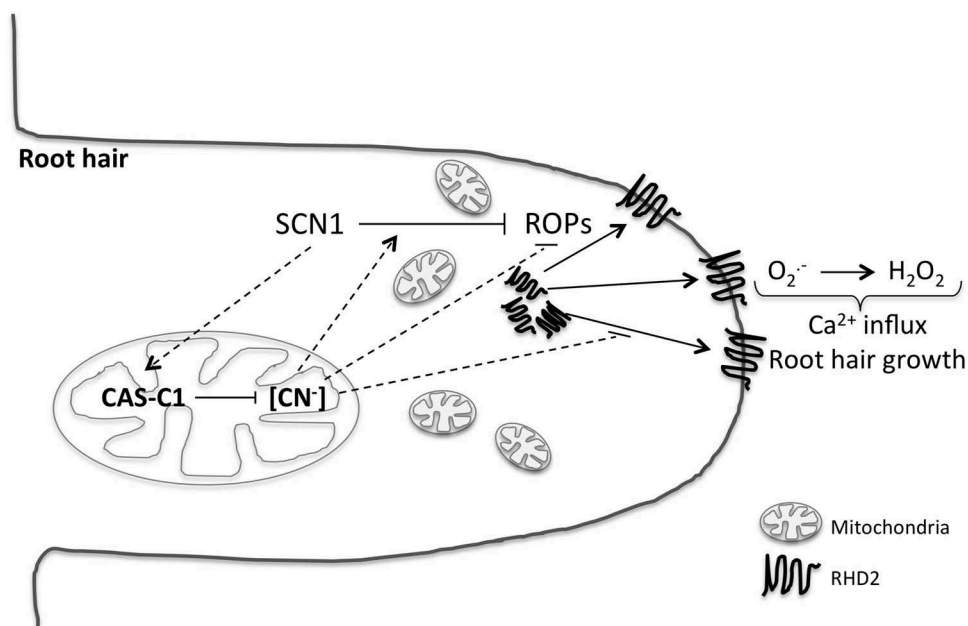


Figure 1. Mitochondria, which are located at the subapical zone of the elongating root hair, possess a mechanism for cyanide detoxification based in the CAS-C1 protein. Cyanide action in the root hair elongation process is exerted in a step between the SCN1 action, which inhibits ROP proteins, and the RHD2 NADPH oxidase action, which is regulated by ROPs and is essential for ROS production, Ca²⁺ influx and, ultimately, polar root hair growth. Full lines: established relationships; dashed lines: proposed relationships.

promoter have been constructed, showing that CAS-C1 locates in mitochondria and accumulates in the subapical region of the root hair tip, suggesting that it could have a function in maintaining the appropriate levels of cyanide for root tip elongation.

Mutants affected in essential early steps of the root hair elongation have been crossed with *cas-c1* in order to establish the epistatic relationships between them. SUPERCENTPEDE (SCN1) is a Rho GTPase GDP dissociator inhibitor that acts as a negative regulator of Rho-related GTPases (ROPs). ROPs are essential for the regulation of the activity of the NADPH oxidase RHD2/AtbrohC, which produces ROS specifically at the root hair tip and therefore stimulates local Ca²⁺ influx. The appropriate localization of the RHD2 activity at the root hair tip guarantees the correct elongation of the root hair, thus mutants in *SCN1* show supernumerary bulges that do not elongate,²⁶ whereas the phenotype of *rhd2* mutants is similar to *cas-c1*, i.e., it has abnormal short root hairs.²⁷ Double mutants *scn1-1 cas-c1* and *rhd2-1 cas-c1* have been generated and their phenotypic analysis has established that *cas-c1* mutation is hypostatic to *scn1-1* and epistatic to *rhd2-1*, since the phenotype of the double *scn1-1 cas-c1* mutant is identical to the single *scn1-1* mutant and the phenotype of the double *rhd2-1 cas-c1* is identical to the single *cas-c1* mutant (Figure 1). Hydroxocobalamin treatment reverts the *rhd2-1 cas-c1* phenotype to *rhd2-1*, whereas it has no effect in the double *scn1-1 cas-c1* mutant or the *scn1-1* single mutant. Molecular data support this epistatic relationship because several genes involved in the root hair development are not significantly affected by the *cas-c1* mutation in a *scn1-1* background while they show a further decrease compared to the single *rhd2-1* mutation when combining the *rhd2-1* and *cas-c1* mutations.

Finally, the effect of the *cas-c1* mutation is independent of ROS production, since the double *rhd2-1 cas-c1* mutant has a *cas-c1* phenotype but it shows neither O₂⁻ nor H₂O₂ staining. Moreover, it has been confirmed that the NADPH oxidase activity is not inhibited by cyanide: root protein extracts of *cas-c1*, wild type and wild type extracts treated with 0,1 mM KCN show very similar NADPH oxidase activities.

In conclusion, we suggest that cyanide itself could have a signaling role. Indeed, cyanide has been proposed to act as a regulator of several biological processes⁴ and besides its possible role as a ROS producer for signaling purpose, a direct effect of cyanide as signaling molecule cannot be discarded.

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ORCID

Cecilia Gotor  <http://orcid.org/0000-0003-4272-7446>
Luis C. Romero  <http://orcid.org/0000-0002-2414-4813>
Irene García  <http://orcid.org/0000-0001-7586-5664>

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