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Research article

Chloroplast functionality has a positive effect on nitric oxide level in soybean cotyledons

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ABSTRACT

The subcellular localization of NO generation in soybean cotyledons, and the relationship between NO synthesis and *in vivo* chloroplast performance were studied. Employing the NO probe 4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-FM DA) and fluorescence microscopy, a strongly punctuated fluorescence was detected in mesophyll cells. The co-localization of DAF-FM and chlorophyll fluorescence, in confocal laser microscopy images, indicated the presence of NO in the chloroplasts. NO visualization was dependent on light, seedling age, and chloroplast function throughout cotyledons lifespan. The addition of herbicides with action in chloroplasts (DCMU and paraquat) dramatically reduced the quantum yield of photosystem II (ϕ_{PSII}), and lead to images with absence of punctuated green fluorescence. Moreover, electron paramagnetic resonance signals corresponding to NO-spin trap adduct observed in cotyledon homogenates decreased significantly by the treatment with herbicides, as compared to controls. Neither chloroplast function nor NO content were significantly different in cotyledons from plants growing in the presence of ammonium or nitrate as the nitrogen source.

These findings suggest that chloroplasts are organelles that contribute to NO synthesis *in vivo*, and that their proper functionality is essential for maintaining NO levels in soybean cotyledons.

1. Introduction

Nitric oxide (NO) plays important roles in plants, where it is involved in physiological processes like stomatal closure [1], seed germination [2], root development [3], senescence [4], expression of defense-related genes and programmed cell death [5]. In addition, NO participates in plant responses to biotic and abiotic stresses, such as interactions with pathogenic microorganisms [6,7], wounding [8], salinity, drought, and hypoxia [9]. Although it is a key signaling molecule acting throughout the lifespan of plants, how plants or tissues regulate NO levels remains unclear, controversial and is currently a subject of study [10,11].

At least seven different pathways to produce NO in plants appear to exist, and they can be classified as either oxidative or reductive [11]. Briefly, nitrate reductase (NR), and mitochondrial or plasma membrane-associated NO production (NR:NiNOR system) are all reductive pathways and depend on nitrite as a primary substrate, whereas NO production from arginine (Arg), polyamine or hydroxylamine are among the oxidative pathways [11].

Nitric oxide synthases (NOS) are present in almost all known organisms except plants, where no NOS genes or enzymes have been identified yet. Even though NO plays a crucial role in plant physiology, higher plants seem to have lost the specific NOS in the course of evolution [10]. Recently, Foresi et al. [12] characterized the sequence, protein structure and biochemistry of an NOS from the green alga *Ostreococcus tauri*, that contains the main characteristics of animal NOS [12]. NO generation in this alga is dependent on irradiance and growth phase. This single-cell alga is of particular interest because it shares a common ancestor with higher plants, providing compelling evidence that an active NOS functions in a photosynthetic organism belonging to the plant kingdom [12].

Although Arabidopsis Nitric Oxide Associated 1 (*NOA*1), or *RIF1* [13] was reported to encode a protein with NOS activity [14], the biological role of AtNOA1/RIF1 is currently believed to be primarily associated with chloroplasts ribosome functions [15–17]. In *rif1* seedlings, not only chloroplast ultrastructure, but also the level of proteins encoded by the chloroplastic genome were affected [13],

Abbreviations: cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA, 4-aminomethyl-2′,7′-difluorofluorescein diacetate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; PQ, 1,1′-dimethyl-4,4′-bipyridinium dichloride.

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suggesting that *NOA1/RIF1* might bind plastidial ribosomes and is required for the normal function and proper protein synthesis in plastids [16]. Recently, it has been reported that NO accumulation in Arabidopsis is independent of NOA1 in the presence of sucrose [18]. It is possible that the primary requirement for NOA1 activity is efficient chloroplast function to generate photosynthates. Provision of sucrose enables *noa1* to accumulate NO, and this fact raised the question why fixed carbon may be necessary for NO accumulation in Arabidopsis [18].

The first reports describing chloroplasts as an NO source dealt with tobacco leaf cells subjected to a fungal elicitor from *Phytophthora cryptogea* and a range of abiotic stressors [19,20], and these were followed by studies in Arabidopsis cell cultures exposed to iron overload [21]. Furthermore, Jasid et al. [22], employing isolated chloroplasts, evidenced NO generation *in vitro* that was inhibited by the Arg analogs L-NAME or LNNA, and also a nitrite-dependent NO generation that was reduced in the presence of DCMU, showing that soybean chloroplasts are able to produce NO *in vitro*, with the supplementation of adequate substrates.

It is clear that NO content in plants varies among tissues, depends on physiological status and also occurs as a generalized stress response. Previous studies have indicated a high NO production in cotyledons [23]. In addition, in soybean cotyledons NO content strongly depends on seedling age, showing a maximum value at around day 7 of seedling development [4,24]. Here, we explore the hypothesis that the content of NO in soybean cotyledons is related to chloroplast functionality *in planta*. Employing fluorescence microscopy and electron paramagnetic resonance (EPR) we show here that chloroplasts contribute to NO synthesis *in vivo*. Moreover, the level of NO in the whole tissue is related to chloroplasts functionality. Finally, as nitrate reduction could be involved in NO synthesis, we explore NO generation in plants grown with ammonium as the unique nitrogen source.

2. Results

2.1. NO localizes in chloroplasts of soybean cotyledons

To investigate NO production, 4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-FM DA) was employed as a fluorescent probe [25]. DAF-FM DA is a membrane-permeant substance that reacts with a product of NO oxidation (N_2O_3) [26]. Cotyledons from 7-day old seedlings were excised, cut in thin sections, and loaded with DAF-FM DA. Microscopic observations showed a strongly punctuated fluorescence in mesophyll cells (Fig. 1Aa). When cotyledons were simultaneously incubated in the presence of cPTIO as NO scavenger, the images showed a much lower fluorescence signal, evidencing the specificity of DAF-FM DA for NO (Fig. 1Ab). Non-significant autofluorescence background was observed in tissues incubated without DAF-FM DA (Fig. 1Ac).

In order to gain insight into the subcellular localization of endogenous NO, 7-day old cotyledons were observed employing confocal laser microscopy after incubation in the presence of the same fluorescent probe, DAF-FM DA. Cotyledons exhibited fluorescence corresponding to NO detection (pseudocolored green, Fig. 1Ba), and the chlorophyll autofluorescence (in red, Fig. 1Bb and Supp. Fig. 1). The co-localization of DAF-FM and chlorophyll fluorescence is shown pseudocolored in yellow-orange (Fig. 1Bc)

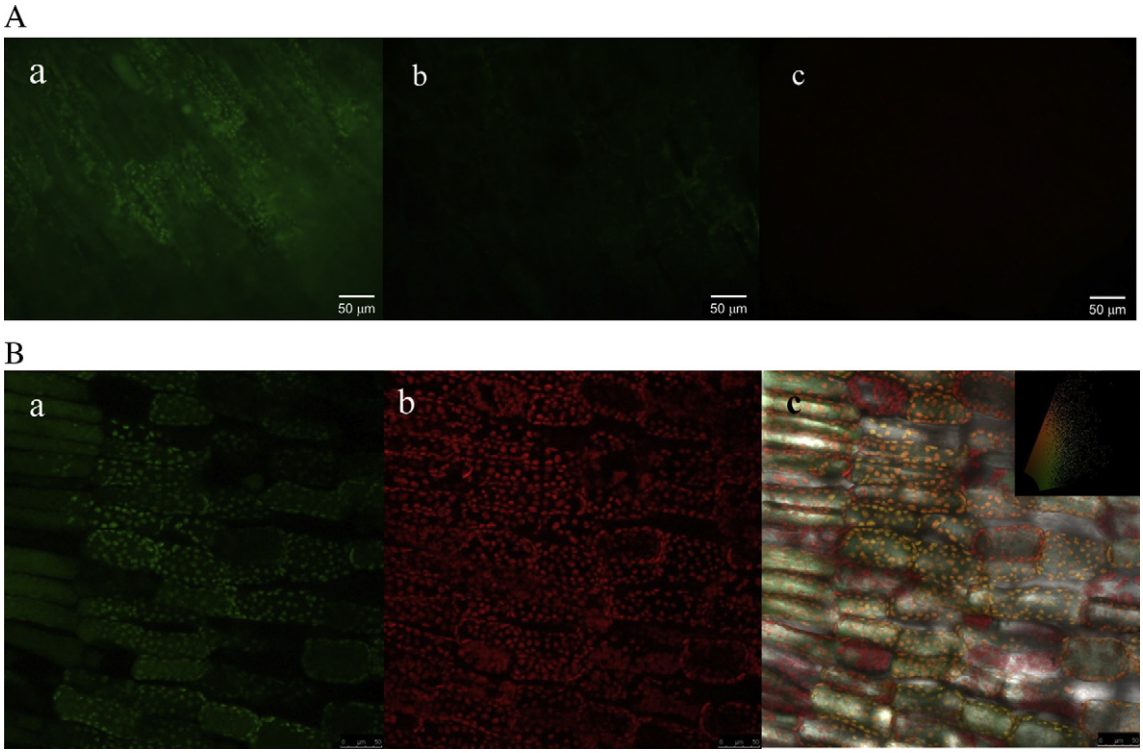

Fig. 1. NO detection in soybean cotyledons sections employing DAF-FM DA fluorescence. A. Fluorescence microscopy. Soybean plants were grown in vermiculite, daily irrigated with Steinberg solution and cotyledons were excised on day 7 after planting. a. Cotyledon sections loaded with 17 μM DAF-FM DA for 30 min and washed for 10 min. b. Cotyledon sections loaded with DAF-FM DA for 30 min in the presence of the specific NO scavenger cPTIO (400 μM), washed and observed. c. Cotyledon sections incubated for 30 min without DAF-FM DA (autofluorescence) (green color; $\lambda_{excitation} = 450–490$ nm and $\lambda_{emission} = 500–550$ nm). Images are representative of at least three independent experiments. B. Confocal laser microscopy. Cotyledons from 7-day old plants were incubated in the presence of 17 μM DAF-FM DA for 30 min ($\lambda_{excitation} = 488$ nm and $\lambda_{emission} = 500–525$ nm). a. Fluorescence corresponding to NO detection with DAF-FM (pseudocolored green). b. Chlorophyll autofluorescence ($\lambda_{excitation} = 488$ nm, $\lambda_{emission} = 670–730$ nm, pseudocolored red). c. Merged image showing co-localization of DAF-FM fluorescence and chlorophyll signals (shown in orange-yellow). Embedded scattergram in the upper right side of picture Bc was produced by the co-localization analysis software Leica SP-5. Images are representative of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)