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Rapid increase of NO release in plant cell cultures induced by cytokinin

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Abstract 4,5-Diaminofluorescein, a fluorescence indicator for NO, was applied to detect the release of NO from plant cells. NO production was increased within 3 min when plant cell cultures (*Arabidopsis*, parsley, and tobacco) were treated by cytokinin and was dose-dependent and signal-specific in that other plant hormones and inactive cytokinin analog were not effective in stimulating of NO release. The response was quenched by addition of 2-(aminoethyl)-2-thiopseudourea, an inhibitor of the animal NO synthase, and by addition of an NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-1-oxy-3-oxide. These results imply that NO may act in cytokinin signal transduction.

Key words: Nitric oxide; Cytokinin; Signal transduction; *Nicotiana tabacum*

1. Introduction

Cytokinins are major plant hormones and consist of a group of compounds which are modified adenines. They regulate a number of important processes in plant life like cell division, branching, senescence, and deetiolation. Although found about 50 years ago [1], very little is known about their mode of action. The recent discovery of the cytokinin receptor gene family, which are two-component receptors in the plasma membrane, and the discovery of rapidly cytokinin-regulated genes, which encode response-relay proteins, were milestones in cytokinin research [2–5]. Obviously, a mechanism for information transfer, i.e. signal transduction, from the plasma membrane to the nucleus involving these proteins must exist [6]. The only other fragment of knowledge about cytokinin signal transduction that we know is the observation of a stimulation of calcium influx across the plasma membrane in the moss *Physcomitrella patens* within minutes [7]. Guided by the observation of a partially similar and overlapping action of phytochrome and cytokinin in N-metabolism and deetiolation and by the observation that cGMP is involved in phytochrome action [8,9] and that cGMP is linked to NO by an NO-activated guanylate cyclase in animals [10] we had launched a first study on the involvement of NO in cytokinin action [11]. We could show that in the classical *Amaranthus* system NO donors and NO-mimicked cytokinin action on betalain accumulation and NO synthase (NOS) inhibitors inhibited betalain accumulation. We expand on this here by showing that cytokinins lead to a rapid stimulation of NO release in cell cultures which has the properties necessary for a potential role in cytokinin signal transduction.

2. Materials and methods

2.1. Cell cultures

Tobacco strain BY-2 (gift from Dr. A.M. Jones, Chapel Hill, NC, USA) was grown in MS medium in a 7 day growth cycle and tobacco strain VB1-0 (gift from Dr. E. Zazímalová, Inst. Experimental Biology, Prague, Czech Republic) in a 21 day growth cycle as described [12]. The *Arabidopsis* cell culture was grown in MS medium in a 7 day growth cycle and a gift from Dr. U. Schmitz (University of Hannover, Germany). Cultured parsley (*Petrosilenum crispum* L.) cells were obtained from Dr. D. Scheel (Institut für Pflanzenbiochemie, Halle, Germany) and grown in HA medium in a 7 day growth cycle with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) [13]. All cell cultures were grown in the dark at 26°C. For use in experiments, cultures were grown for 10 days (VBI-0 tobacco line) or 5 days (all others). The source for 4,5-diaminofluorescein (DAF-2) and inhibitors was Calbiochem (Schwalbach, Germany) and for hormones and media Sigma (Taufkirchen, Germany).

2.2. NO quantification

NO was determined by binding to DAF-2 in a fluorometric assay [14,15]. Cells were weighed into 100 mg portions and transferred into either 0.95 ml 100 mM KH_2PO_4 pH 7.5 (Fig. 1b,c only, according to [14,15]) containing 10 μM DAF-2 or into 0.85 ml hormone-free MS medium and 0.1 ml 100 mM KH_2PO_4 pH 7.5 (all other experiments) containing 5 μM DAF-2 and hormones when indicated. Both media gave similar results but buffered medium stabilized the cells better. Routinely, after 105 min incubation in the dark at 25°C on a rotatory shaker (150 rpm) the supernatant (20 μl or 60 μl) was diluted 1:50 or 1:100 (v/v) into water and emission read at 515 nm by excitation at 495 nm (Figs. 1a, 2–4). For parsley and *Arabidopsis* experiments (Fig. 1b,c) the medium was 100 mM KH_2PO_4 pH 7.5 and cells were incubated for 1 h with hormone and then 10 μM DAF-2 was added and another 30 min incubation followed. For the time course (Fig. 4) reading was started immediately after mixing cells, medium, hormone and DAF-2. Cellular pellets contained very little DAF-2 fluorescence (not shown). Hormones were dissolved in 10% ethanol (1 mM BAP), 0.5% acetic acid (1 mM zeatin), DMSO (auxins), or water as stock solutions and care was taken to add the same amounts of solvent to every assay, not exceeding 0.05% for ethanol or 0.5% for DMSO. When auxin was the agonist to be tested, the cultures were pregrown in auxin-free media. All experiments were done at least twice (Fig. 3) or three or more times (all others) and when inactive compounds were tested one assay with an active compound was conducted for control of activity. Bars indicate the experimental error of the duplicate samples (except for Fig. 4).

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Abbreviations: ACC, 1-amino-carboxy-cyclopropane; AET, 2-(aminoethyl)-2-thiopseudourea; BAP, benzyl aminopurine; PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-1-oxy-3-oxide; DAF-2, 4,5-diaminofluorescein

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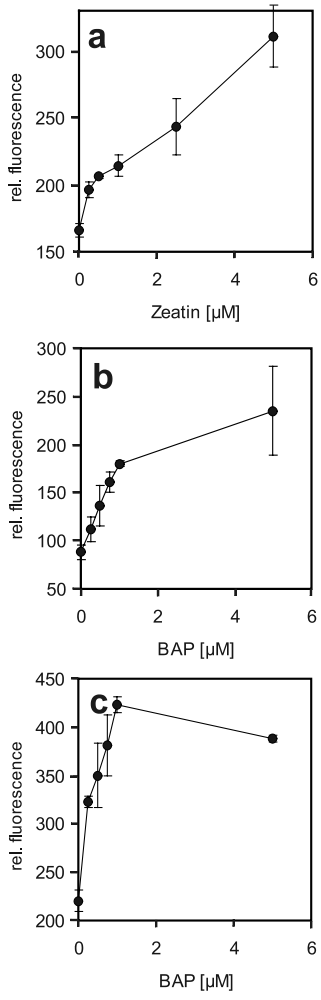

Fig. 1. Dose dependence of NO biosynthesis on cytokinins in several plant cell cultures. a: Tobacco BY-2 cells. b: Parsley cells. c: *Arabidopsis* cells.

3. Results and discussion

When cultured plant cells (tobacco, parsley, *Arabidopsis*) were treated by low concentrations of cytokinins a hormone-dependent increase of NO release was observed above an apparent endogenous level observable without hormone addition (Fig. 1). At 5 μM benzyl aminopurine (BAP) or zeatin the amount of NO released by the cells was doubled as compared to no cytokinin. As cultured plant cells contain cytokinins the control cultures probably were not cytokinin-free [12]. Moreover, a general capacity for NO exudation by plants is well documented [16–19]. Plants synthesize NO by a side reaction of nitrate reductase catalysis [20] and by at least one more enzyme which is localized in chloroplasts and endomembranes [21,22] but too little is known about plant NO biosynthesis at this point so that both source(s) and regulation of possible constitutive and/or cytokinin-induced NO release remained unknown.

To test the signal specificity, cytokinin analogs and other plant hormones were also tested (Fig. 2). Adenosine and the plant hormones abscissic acid (ABA), gibberellic acid (GA_3), and the ethylene precursor 1-amino-carboxy-cyclopropane (ACC) showed no effect, whereas the active auxins indolyl-3-acetic acid (IAA) and indolyl-3-propionic acid (IPA) decreased NO release somewhat. Both zeatin and BAP were effective (Fig. 1a; see below). Taken together, this shows that the increase in NO release was signal-specific.

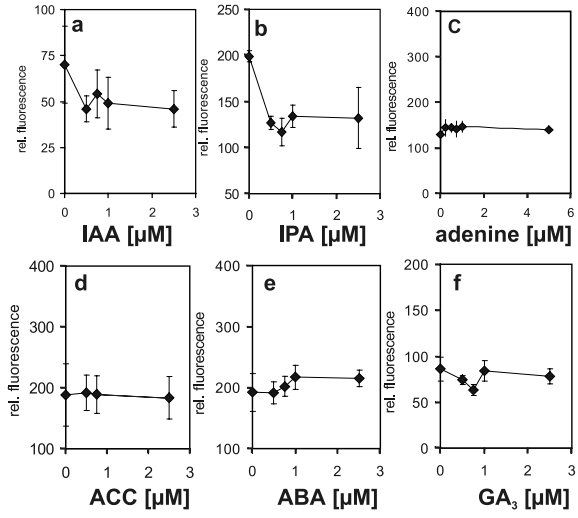

Fig. 2. Effect of various plant hormones on NO biosynthesis in tobacco BY-2 cell cultures. a: IAA. b: IPA. c: Adenine, an inactive cytokinin analog. d: ACC. e: ABA. f: GA_3.

The radical scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-1-oxy-3-oxide (PTIO) is known to directly react with NO [23]. When PTIO was added to the cytokinin-containing test medium, a strong decrease of the fluorescence signal was observed (Fig. 3b). We assume that PTIO competed with the DAF-2 in the test medium to bind NO so that a reduction of the measurable amount of NO resulted. When 2-(aminoethyl)-2-thiopseudourea (AET), an inhibitor of animal NOS, was used to inhibit NO release 100 μM inhibitor strongly decreased NO release (Fig. 3a). Since thiourea inhibited the betalaine accumulation response to cytokinin in *Amaranthus* [11] we assume that rather a NOS-like enzyme in the cells was inhibited and not that NO was quenched after release by AET. Whether PTIO and AET are cytokinin antagonist remains to be seen but preliminary data showed that both inhibited cytokinin-induced deetiolation in *Arabidopsis* seedlings (not shown). Involvement of NO in deetiolation was suggested [24].

The increase above control levels by cytokinin of NO release in BY-2 cells was very rapid and apparent after 3 min (Fig. 4). Quite similar results were obtained with the VBI-0

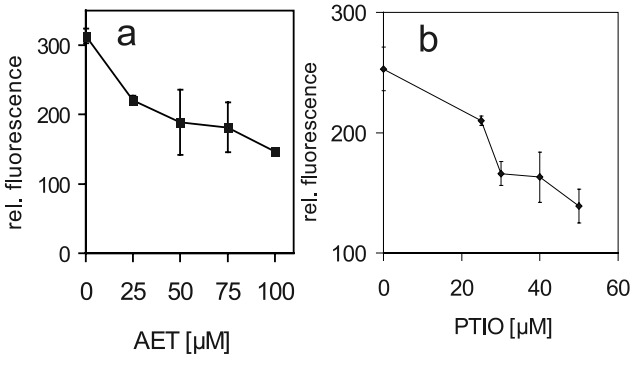

Fig. 3. Effect of inhibitors on NO biosynthesis in tobacco cell cultures. a: AET in the presence of 1 μM zeatin in cell line BY-2. b: PTIO in the presence of 1 μM zeatin in cell line BY-2.

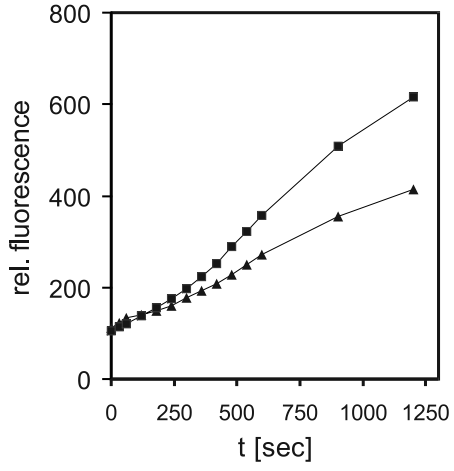

Fig. 4. Time course of NO biosynthesis induced by 3 μM BAP in tobacco BY-2 cells. Control: ▲; 3 μM BAP: ■.

tobacco cell line (not shown). This is comparable to observations on the time course of stimulation by elicitor of NO biosynthesis which was observed after 3 min to occur in chloroplasts of tobacco leaf cells by an imaging method using DAF-2 [22]. Thereafter, NO appeared to also be synthesized in these cells in endomembranes. Other authors found an NOS-like plant enzyme in peroxisomes [21] and another possible source for NO in plants is nitrite and nitrate converted by nitrate reductase [20]. It is tempting to speculate that cytokinin may increase NO biosynthesis in plastids, which could also provide an explanation for the known positive effects of cytokinin on plastid development [25–28], however, it is not yet clear how many sources or enzymes for NO biosynthesis in plants exist and whether etioplasts – which would be the plastids in the dark-grown cell cultures used here – could have a capacity to generate NO.

The interval of 3 min observed here and previously after which a clear rise of NO release above the control was found is still close to the range of very rapid gene activation of ARR genes by cytokinin after 10 min [3] so that gene activation as a mechanism to increase NO cannot be completely discarded for the observations which are reported here since the time course of cytokinin-induced ARR homologs in BY-2 cells is unknown. Hwang and Sheen [6] suggested a direct pathway from cytokinin receptors via phosphorylated AHP relay proteins to gene regulation of ARR genes. Clearly, a phosphorelay mechanism not only could lead to gene activation but also to unknown regulatory steps for NO release, possibly by phosphorylation of the relevant enzyme(s) but other models cannot be excluded yet. However, we regard gene activation as an unlikely mechanism for such rapid activation of NO release by cytokinin. Upstream and downstream elements known to interact with NO as a second messenger in animals are present in plants: (1) cGMP is implied in phytochrome, gibberellin, and elicitor action in plants [3,8,9,29] and cGMP is elevated by NO in plants [30] and animals by guanylate cyclase activation [10]. (2) cADPR-activated calcium channels were found in plants in the tonoplast [31] and are present in the ER where in animals cADPR synthesis is found to be activated by NO [32,33]. Our findings may pave the way to further elucidate the mechanism of action of cytokinin signalling.

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