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SHORT COMMUNICATION

Apocynin-induced nitric oxide production confers antioxidant protection in maize leaves

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Summary

The effect of apocynin on nitric oxide (NO) synthesis and oxidative stress was studied in corn (*Zea mays*) seedlings. After treatment with 100 μM apocynin, strongly increased amounts of NO were detected in the leaves. This NO production was reduced by more than 70% by N^G-nitro-l-arginine methyl ester (L-NAME), a NO synthase (NOS) inhibitor, but there was no reduction in NO production when apocynin was applied in combination with diphenylene iodonium (a plant NOX inhibitor). When maize seedlings were UV-B-irradiated, cellular damage occurred and reactive oxygen species (ROS) were found widely distributed in chloroplasts and mesophyll cells. Pre-treatment with apocynin and coinciding NO accumulation prevented this damage. However, the protective effect was averted by L-NAME application. Leaf discs placed in 1 M H_2O_2 for 24 h showed a reduction in chlorophyll content that could also be avoided by apocynin treatment.

Our results show that apocynin induces the accumulation of NO in leaves of maize seedlings through a NOS-like activity, a mechanism alternative to NOX inhibition, and confers an augmented tolerance to different types of abiotic oxidative stress. Indeed, we propose the use of apocynin as an alternative approach to study NO functionality in plants.

Introduction

Apocynin (4-hydroxy-3-methoxyacetophenone, acetovanillone, CAS 498-02-2) is a methoxy-substituted catechol originally extracted from the roots of *Picrorrhiza kurroa*, a small perennial herb that grows in the Himalayas. Extracts of *P. kurroa* are used in traditional medicine for the treatment

Abbreviations: DAB, diamino benzidine; DAF-FM, 4,5-diamino-fluorescein diacetate; DPI, diphenylene iodonium; NO, nitric oxide; NOS, NO synthase; NOX, NADPH oxidase; L-NAME, N^G-nitro-l-arginine methyl ester; c-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide.

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of diseases associated with chronic inflammation (Meyer and Schmitt, 2000 and references therein). Metabolized apocynin reduces oxidative stress in phagocytes, inhibiting the formation of superoxide by NADPH oxidase (NOX) (Vejrazka et al., 2005). Furthermore, it was recently demonstrated that apocynin increases the steady-state level of nitric oxide (NO) in endothelial cells (Steffen et al., 2007; Riganti et al., 2008). NO is a small, highly diffusible atmospheric gas and an ubiquitous bioactive molecule, proposed as a broad spectrum anti-stress factor both in plants and animals (Furchgott, 1995; Lamattina et al., 2003). Information regarding the effect of apocynin on plants is scarce. The aim of this work was to test the hypothesis that apocynin can increase NO concentration in plants and protect them from oxidative stress generated by UV-B irradiation and H_2O_2.

Material and methods

Plant material

Maize (*Zea mays* N107B, W23.) seeds were supplied by the Maize Genetics Cooperation Stock Center (University of Illinois, Urbana). After surface sterilization with 0.5% hypochlorite for 20 min, seeds were washed and germinated on water-saturated filter paper at 25 °C in the dark. Germinated seedlings were grown in soil:vermiculite (3:1, v/v) in a controlled growth chamber under 14 h of light of 120 μmol photons $m^{-2} s^{-1}$ at 25 °C. Fourteen-day-old vigorous seedlings were used in the experiments.

NO fluorescence

NO content was analyzed in the second leaves using 100 μM 4,5-diaminofluorescein diacetate (DAF-FM), which was loaded to the leaves for 1 h. After thorough washing, green fluorescence (515–555 nm), due to NO production, was visualized in a Nikon Eclipse E200 microscope and images of at least four individual experiments analyzed using IMAGEJ 1.3 software (NIH) for quantification of fluorescence.

UV-B treatment and reactive oxygen species (ROS) detection

Seedlings treated with H_2O, 100 μM apocynin or 100 μM apocynin+100 μM N^G-nitro-l-arginine methyl ester (L-NAME) were irradiated for 3 h with white light supplemented with UV-B produced by Philips tubes (TL100W/12), filtered with 0.13 mm thick cellulose diacetate. The spectral irradiance was 3.3 W m^{-2}. After UV-B irradiation, plants were supplied through the cut stems with a 1 mg mL^{-1} solution of diamino benzidine (DAB), pH 3.8, overnight under light at 25 °C. ROS were visualized in transversal sections of the second leaf of these plants as dark-polymerization products due to the reaction of DAB with H_2O_2.

Cell damage

Damage caused to cells by stress treatments was determined as relative ion leakage. Leaf discs of 25 mm were washed in deionised water and placed in Petri dishes with 15 mL of deionised water at 25 °C for 3 h. Conductivity in the bathing solution was determined ($C1$). After that, samples were heated at 80 °C for 2 h and conductivity measured again ($C2$). Relative ion leakage was expressed as percentage of the total conductivity after heating (relative ion leakage % = $(C1/C2)100$).

H_2O_2 stress and chlorophyll content

Seedlings were treated for 12 h with 0, 10, 50, 100 or 200 μM apocynin. After that, 70-mm leaf discs were placed for 24 h in multi-well plates containing 1 M H_2O_2 or H_2O. For chlorophyll (Chl) measurement, 100 mg leaves were grounded in liquid N_2, and extracted with 1 mL of 80% (v:v) acetone at 4 °C. After centrifugation at 12,000*g* for 10 min, chlorophyll was quantified in the supernatant according to the formula: $A_{652}/34.2$ = mg Chl mL^{-1}.

Results and discussion

Apocynin induces NO production in maize leaves

We performed a time-course experiment of endogenous NO production in maize seedlings treated with 100 μM apocynin. This is the concentration of apocynin reported to induce NO in endothelial cells (Steffen et al., 2007). Endogenous NO generation was visualized in leaves as green fluorescence with the permeable fluorophore DAF-FM (Garcia-Mata and Lamattina, 2002; Corpas et al., 2004, 2006). After 2 h of apocynin treatment, fluorescence was increased (Figure 1A) and clearly visible in vascular tissue and epidermal cells after 4 h of treatment (Figure 1B). Fluorescence continued to increase after 8, 12 and 24 h of apocynin