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Research Paper

Improvement of adventitious root formation in flax using hydrogen peroxide

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Flax (*Linum usitatissimum* L.) is an important crop for the production of oil and fiber. *In vitro* manipulations of flax are used for genetic improvement and breeding while improvements in adventitious root formation are important for biotechnological programs focused on regeneration and vegetative propagation of genetically valuable plant material. Additionally, flax hypocotyl segments possess outstanding morphogenetic capacity, thus providing a useful model for the investigation of flax developmental processes.

Here, we investigated the crosstalk between hydrogen peroxide and auxin with respect to reprogramming flax hypocotyl cells for root morphogenetic development. Exogenous auxin induced the robust formation of adventitious roots from flax hypocotyl segments while the addition of hydrogen peroxide further enhanced this process. The levels of endogenous auxin (indole-3-acetic acid; IAA) were positively correlated with increased root formation in response to exogenous auxin (1-Naphthaleneacetic acid; NAA). Histochemical staining of the hypocotyl segments revealed that hydrogen peroxide and peroxidase, but not superoxide, were positively correlated with root formation. Measurements of antioxidant enzyme activities showed that endogenous levels of hydrogen peroxide were controlled by peroxidases during root formation from hypocotyl segments.

In conclusion, hydrogen peroxide positively affected flax adventitious root formation by regulating the endogenous auxin levels. Consequently, this agent can be applied to increase flax regeneration capacity for biotechnological purposes such as improved plant rooting.

Introduction

Reactive oxygen species (ROS) are produced by aerobic metabolic processes in living organisms and their generation is elevated under unfavorable conditions. At a physiological level, they play important roles in signal transduction and multiple developmental processes [1–3]. ROS are implicated in the regulation of the cell cycle [4], cell elongation [5,6], root hair formation [7], lateral and adventitious root formation [8,9], root elongation [10], stomatal closure [11] gravitropism [12] and embryogenesis [13,14].

Crosstalk between ROS signalling and auxin or abscisic acid is involved in plant developmental processes [15–17]. ROS that control developmental processes are generated by plasma membrane-localized NADPH oxidase [3,6]. The phytohormones auxin and abscisic acid are capable of promoting the production of ROS through this mechanism [12,18–21]. Auxin-induced mitotic activity of *Arabidopsis* protoplasts occurred in conjunction with accelerated H_2O_2 generation [22]. ROS stimulated cell division only in

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the presence of auxin; in the absence of auxin, they exerted a rather damaging effect on protoplast culture [8]. Auxin controls gravitropic curvature through the generation of ROS [12] and this is dependent on phosphatidylinositol 3-kinase [23]. In addition, a hydrogen peroxide-inducing chemical compound called alloxan stimulates auxin-dependent lateral root formation [8].

The intracellular level of ROS under unfavorable conditions is controlled via enzymatic and non-enzymatic compounds working in co-operation [1,24,25]. Their role in stress response is well documented; however, little is known about the regulation of ROS during developmental processes. Until now, only a limited number of studies have been devoted to the role of positive redox homeostasis and active antioxidant defense during developmental processes such as mitotic activity [22,26], androgenesis [27], somatic embryogenesis [28] and root growth [29]. Hydrogen peroxide decomposing catalase 2 plays an essential role in maintaining root meristem activity in the presence of oxidative stress [26]. The elevated activity of another enzyme involved in hydrogen peroxide decomposition, namely ascorbate peroxidase, corresponded with the mitotic activity accelerated by external auxin in *Arabidopsis* protoplasts [8].

Flax (*Linum usitatissimum* L.) is an important crop for the production of oil and fiber and is therefore an important target for biotechnological use [30–32]. *In vitro* manipulation is an essential tool of flax genetic improvement and breeding. It is generally accepted that flax hypocotyl segments possess outstanding morphogenetic capacity, thus providing a useful model for the investigation of flax developmental processes [13,33].

In this study, we used flax hypocotyl segments to study the processes connected to the reprogramming of cells for root morphogenetic development. We investigated the effect of exogenous hydrogen peroxide and provided new information on hydrogen peroxide-auxin crosstalk in this process. The manipulation of root morphogenesis by hydrogen peroxide and auxin may serve as a new and efficient tool for plant rooting in flax biotechnology.

Materials and methods

Plant material

Flax (*Linum usitatissimum* L.; cv. Super, AGRITEC, Research, Breeding & Services, Ltd., Czech Republic) seedlings were grown on vertically oriented Phytagel square plates containing ½ Murashige and Skoog (MS) medium (pH 5.7) (16-h light/8-h dark; 22°C) for 4 days. Then, the hypocotyls were collected and segmented into 2 mm long segments and incubated in liquid ½ MS media containing 0.5 or 1 mg l^{-1} NAA with or without the addition of 100 μM H_2O_2 for two days. One part of the segments was transferred to hormone- and H_2O_2-free solid ½ MS media, while the remainder was used for biochemical and histochemical assays.

Protein extraction for enzymatic analyses

Flax hypocotyl segments were ground in the presence of liquid nitrogen and extraction buffer (100 mM sodium-phosphate buffer, pH 7.8, 2 mM EDTA, 2 mM ascorbate) using a mortar and pestle. The homogenate was centrifuged at 15 000 × *g* for 20 min at 4°C. The supernatant was collected and desalted on Amicon Ultra YM-10 centrifugation columns (Merck Millipore, Germany). The protein content was determined according to Bradford using BSA as standard [34].

Enzyme activity measurements

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by monitoring the inhibition of the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) at 560 nm [35]. One unit of SOD is defined as the extract volume required for 50% inhibition of MTT reduction. Catalase (EC 1.11.1.6) activity was measured by following the decrease in absorbance of H_2O_2 at 240 nm [36], and guaiacol peroxidase (GPX) activity was determined by following the absorbance decrease in guaiacol concentration at 470 nm [37]. The values presented are the means from at least three independent experiments.

Histochemical and fluorescent staining of flax hypocotyls for the visualization of viability, hydrogen peroxide, superoxide, and peroxidase activity

For the evaluation of viability, the hypocotyl segments were incubated in 0.001% fluorescein diacetate (FDA) (w/v) for 1 h in the dark at room temperature. Then, the segments were washed in distilled water and processed as described below for ROS localization.

For visualization of superoxide, the hypocotyl segments were incubated in 1% (w/v) nitroblue tetrazolium chloride in NBT solution in 10 mM potassium phosphate buffer (pH 7.8) under vacuum for 10 min [38].

ROS localization was monitored using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes, Invitrogen, Carlsbad, CA) according to Fryer [39]. The segments were incubated in 25 μM H_2DCFDA on a variable speed rocker at room temperature in the dark for 2 h. Next, the segments were incubated in distilled water in the dark for 1 h. The fluorescent signal was documented using a stereomicroscope (LEICA M165FC, Wetzlar, Germany) equipped with a GFP3 filter set (excitation 450–490 nm, emission 500–550 nm).

To localize peroxidase activity, the hypocotyl segments were incubated in 0.2 M potassium buffer containing 0.33 M o-dianisidine and 3 mM H_2O_2 at 25°C for 1 h.

Endogenous free indole-3-acetic acid measurement

The quantification of endogenous IAA was performed as described previously [40]. Briefly, approximately 10 mg of ground plant material (in triplicate) was incubated for 10 minutes in 1 ml of cold phosphate buffer (50 mM; pH 7.0) containing 0.02% sodium diethyldithiocarbamate and supplied with [$^2H_5$]IAA internal standard. After centrifugation at 36 000 × *g*, the samples were acidified with 1 M HCl to pH 2.7, subjected to C8-based solid-phase extraction and subsequently evaporated to dryness under vacuum. Final analyses were conducted by means of ultra-high performance liquid chromatography (Acquity UPLC system, Waters) coupled with mass spectrometry (micro API tandem quadrupole mass spectrometer, Waters).

Results

Hydrogen peroxide stimulated the NAA-induced morphogenetic reprogramming towards adventitious root formation

We incubated the flax hypocotyl segments in liquid ½ MS media containing two different auxin (NAA) concentrations for 2 days. Afterwards, they were transferred to solid MS hormone-free media. As expected, these growing conditions caused the intensive