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Research article

Exogenous hydrogen peroxide inhibits primary root gravitropism by regulating auxin distribution during Arabidopsis seed germination

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ABSTRACT

Hydrogen peroxide (H_2O_2) is the key factor in many physiological and metabolic processes in plants. During seed germination, exogenous H_2O_2 application influences gravitropism and induces curvature of the primary root in grass pea and pea seedlings. However, it remains unclear whether and how this happens in the model plant *Arabidopsis thaliana*. In the present study, the effect of exogenous H_2O_2 on the gravitropic response of primary roots during Arabidopsis seed germination was studied using histology and molecular biology approaches. Appropriate H_2O_2 treatment not only restrained primary root growth, but also disrupted gravitropism and induced root curvature. Histological staining and molecular analysis demonstrated that exogenous H_2O_2 correlated with lack of starch-dense amyloplasts in root tip columella cells, which ultimately results in the lack of gravisensing. Detection of calcium ion (Ca^{2+}) by a fluorescent probe showed that Ca^{2+} distribution changed and intracellular Ca^{2+} concentration increased in H_2O_2-treated primary root, which was consistent with alterations in auxin distribution and concentration triggered by H_2O_2 treatment. Furthermore, the normally polar localization of Pin-formed 1 (PIN1) and PIN2 became uniformly distributed on root tip cell membranes after treatment with H_2O_2. This leads to speculation that the IAA signaling pathway was affected by exogenous H_2O_2, causing asymmetrical distribution of IAA on both sides of the primary root, which would influence the gravitropic response.

1. Introduction

Gravitropism is one of the most important tropic movements during plant growth and development. Plants sense gravity and guide growth of new tissues with respect to the gravity vector (Baldwin et al., 2013). The root tip columella cells are essential for sensing gravity, but there are only a couple dozen in Arabidopsis root tips (Digby and Firn, 2002). In the columella cells, there are some specialized, dense starch-filled amyloplasts, the sedimentation of which are responsible for gravity sensing (Baldwin et al., 2013). In vascular plants, the starch-statolith hypothesis states that the amyloplasts in columella cells act as statoliths and fall to the bottom of cells, thereby signaling the direction of gravity (Leitz et al., 2009). However, there is still a secondary mechanism that can direct the growth of plant root downward. According to the Cholodny-Went theory, the asymmetrical accumulation of auxin on opposite sides of the elongation zone leads to the gravi-bending, resulting in the differential growth and root tip curvature (Baldwin et al., 2013; Baluška et al., 2007). Multiple lines of evidence have suggested that the secondary messengers calcium ion (Ca^{2+}) and inositol 1,4,5-triphosphate (IP_3) are also involved in root gravitropism (Geldner et al., 2001; Fasano et al., 2002; Kordyum, 2003). Earlier research showed that application of the calcium chelator EGTA to maize root tips causes the loss of gravitropic sensitivity, but that asymmetric application of calcium chloride near the root tips induces root curvature toward higher calcium sources (Lee et al., 1983). Thus, the temporal and spatial redistribution of Ca^{2+} in the root tip can influence the development of gravitropic bending.

The phytohormone auxin (primarily in the form of IAA) is involved in virtually every aspect of plant growth and development (Band and Bennett, 2012; Palme, 2006). After cellular synthesis of IAA, it is transported into specific tissues by PIN-FORMED (PIN) proteins, where it induces a signaling cascade and causes correspondent plant growing

Abbreviations: ABP1, auxin binding protein1; APX1, ascorbate peroxidase 1; AMY3, α-amylase 1; AUX, auxin resistant 1; Ca^{2+}, calcium ion; CAT3, catalases 3; GPX1, glutathione peroxidase 1; H_2O_2, hydrogen peroxide; IAA, indole-3-acetic acid; IP_3, inositol 1,4,5-triphosphate; MCA, mid1-complementing activity; PGM, phosphoglycerate/bisphosphoglycerate mutase; PIN1, pin-formed 1; PIN2, pin-formed 2; ROS, reactive oxygen species; SOD, superoxide dismutase; SS3, starch synthase 3; TIR1, transport inhibitor response 1

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responses (Davies, 1987; Adamowski and Friml, 2015). Polarity of subcellular PINs localization are very important for directional auxin flow and root gravitropic growth (Wisniewska et al., 2006). PINs-directed translocation of IAA is first transported from stem apex to root apex, and then it is diverted up along the root cortex, which is normally called the "*fountain stream of auxin*" model (Bennett, 2003). According to this model, PIN1 is mainly located in the lower cell membrane of vascular cylinder cells, from where it can send IAA downward toward the columella cells of the root cap. PIN2 is responsible for bringing IAA up from the root tip to the elongation zone along the outer layer cells belonging to root cortex (Blakeslee et al., 2005). Therefore, PIN1 and PIN2 are vital for gravi-bending. IAA is perceived by auxin receptors in the TRANSPORT INHIBITOR RESPONSE 1 (TIR1) family (Kepinski and Leyser, 2005; Benjamins and Scheres, 2008), which results in the proteolysis of AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins, after which they can release their inhibitory effect on AUXIN RESPONSE FACTORS (ARFs) and regulate auxin-responsive gene expression (Benjamins and Scheres, 2008; Sauer et al., 2006).

Hydrogen peroxide (H_2O_2) is the most stable of the Reactive Oxygen Species (ROS). H_2O_2 is produced via many different enzyme-catalyzed processes (Forman and Torres, 2002). H_2O_2 can be regarded as a growth factor that regulates different plant growth and development processes, such as cell division, differentiation and migration (Forman and Torres, 2002; Veal and Day, 2011). Therefore, it can be treated as a kind of signaling molecule that controls diverse biological processes (Neill et al., 2002). On the other hand, H_2O_2 causes oxidative damage (even cell apoptosis) when the plant is subjected to different biotic and abiotic stresses, which, although it seems damaging, is an important biological role (Veal and Day, 2011; Henle and Linn, 1997; Mittler, 2002). Additionally, H_2O_2 functions as a signaling molecule in auxin-regulated root gravitropism (Neill et al., 2002). We and others have reported that exogenous H_2O_2 treatment can influence primary root gravitropism and induce root bending in grass pea and pea during initial development stage (Jiang et al., 2012; Li et al., 2015a). However, the more detailed mechanism remains unknown.

To further unravel the detailed mechanism concerning the loss of gravitropic sensitivity and root curvature after H_2O_2 treatment, the effect of exogenous H_2O_2 application on primary root growth was explored during seed germination in Arabidopsis. We present that appropriate exogenous H_2O_2 treatment can induce primary root bending via adjusting the concentration and distribution of intracellular Ca^{2+} and IAA in Arabidopsis root tips during the early seed germination stage. Studies in Arabidopsis will be greatly beneficial to understanding the role of H_2O_2 in gravitropic regulation.

2. Materials and methods

2.1. Plant material and growth conditions

Four *Arabidopsis thaliana* lines, namely ecotype Col-0, *PIN1::PIN1-GFP, PIN2::PIN2-GFP* and *DR5::DR5-GFP*, were used in our experiments. Seeds were vernalized at 4 °C for 48 h in darkness, sterilized with 75% (v/v) ethanol (30 s) and 0.1% Mercuric Chloride (6 min), and rinsed with sterile water (six times). Seeds were then germinated on 1/2 MS solid medium (1% agar) at 21 °C day/18 °C night with a photoperiod of 16-h light/8-h dark. For different H_2O_2 concentration treatments, seeds were germinated on 1/2 MS medium for 60 h supplemented with 1 mM, 2 mM, 3 mM, 4 mM, or 5 mM H_2O_2. Seedlings grown on 1/2 MS medium without H_2O_2 were used as controls. Seedlings after treated with or without H_2O_2 were then photographed with a camera (CANON G15). Seeds of *PIN1::PIN1-GFP, PIN2::PIN2-GFP* and *DR5::DR5-GFP* were germinated to observe cell membrane localization and concentrations of IAA in root tip cells.

2.2. Scanning electron microscopy observation for root cell length

To detect the cell length of primary root elongation zone, seedlings treated with or without H_2O_2 for 60 h were immediately frozen in liquid nitrogen and photographed with scanning electron microscopy (SEM) (TM-3000; Hitachi, http://www.hitachi.com/) under 220 V conditions.

2.3. Observation of amyloplasts in columella cells in primary roots

To observe the amyloplasts of columella cells in Arabidopsis primary root tips, Lugol's solution was used to stain starch according to the procedure described by Jiang et al. (Jiang et al., 2012; Fujihira et al., 2000). Seedlings germinated and grown for 60 h with or without H_2O_2 were stained with Lugol's solution for 1 min (including 2.0 g potassium iodide, 1.0 g iodine and 300 ml distilled water) and then washed with distilled water three times. Amyloplasts located in root tip columella cells were then observed using light microscope (DMLB, Leica, Germany).

2.4. Detection of intracellular free calcium ion in root tips

Cytosolic-free calcium ions were visualized using the Ca^{2+}-specific fluorescent probe Fluo-8® AM (AAT Bioquest, Inc., USA) according to a previously described procedure with some modifications (Zhou et al., 2016). In brief, seedlings grown for 60 h on 1/2 MS medium supplemented with or without H_2O_2 were washed with 20 mM HEPES-NaOH (pH 7.5) buffer three times and then incubated in 200 μL of 20 mM HEPES-NaOH (pH 7.5) solution containing 10 μM of Fluo-8 AM, Pluronic F-127 (1 mL incubation solution including 5 μL of 25% Pluronic F-127) in darkness at 37 °C for 90 min. The seedlings were then rinsed with distilled water three times. Fluorescent images of root tips were captured immediately with confocal microscopy (excitation at 488 nm and emission at 525 nm, Olympus FV 1000 MPE).

2.5. RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from seedlings treated with or without 4 mM H_2O_2 for 60 h using the MiniBEST plant RNA extraction kit (TaKaRa, Japan) according to the manufacturer's instructions. Synthesis of cDNA was performed with PrimeScript™ RT Master Mix using the following cycle: 37 °C 15 min, 85 °C 5 s, and 16 °C storage. Real-time PCR amplification using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) was carried out with the following program: 95 °C 30 s, 95 °C 5 s and 60 °C 30 s (40 cycles), and then 95 °C 15 s, 60 °C 15 s. The primers for this study are listed in Supplementary Table. The *Actin2* gene was used as the standard reference for quantification.

2.6. Immunofluorescent tissue localization of auxin

The distribution of IAA in the primary root tip elongation zone was detected using immunofluorescent staining. A paraformaldehyde stationary liquid was prepared by adding 500 μl of 1 N of NaOH to 900 ml ddH_2O and then stirring in 40 g of paraformaldehyde using a magnetic stirrer at 65 °C. After cooling down the above solution at room temperature, 100 ml of 10× TBS buffer was mixed into it and the pH was adjusted to 7.4 with HCl (Ditengou et al., 2008; Schlicht et al., 2006). The Immunol Fluorescence Staining Kit (KeyGEN BioTECH, Nanjing, China) was used according to manufacturer's detailed instructions. Generally, seedlings treated with or without H_2O_2 for 60 h were fixed with 4% paraformaldehyde stationary liquid for 1 h at 4 °C, and then the samples were washed three time with ice-cold PBS buffer solution. Next, the samples were submerged in diluted monoclonal antibody anti-IAA (diluted 1:20 in PBST supplemented with 1% BSA) and incubated in a humidity box overnight at 4 °C. After washing the seedlings three times in PBS (5 min each wash), they were incubated in secondary antibody FITC-labeled goat anti-mouse IgG in 1% BSA (diluted 1:1000),