Auxin-induced reactive oxygen species production requires the activation of phosphatidylinositol 3-kinase

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Abstract  We recently reported that production of reactive oxygen species (ROS) is essential for auxin-induced gravitropic signaling. Here, we investigated the role of phosphatidylinositol 3-kinase and its product, PtdIns(3)P, in auxin-mediated ROS production and the root gravitropic response. Pretreatment with LY294002, an inhibitor of PtdIns 3-kinase activity, blocked auxin-mediated ROS generation, and reduced the sensitivity of root tissue to gravistimulation. The amount of PtdIns(3)P increased in response to auxin, and this effect was abolished by pretreatment with LY294002. In addition, sequestration of PtdIns(3)P by transient expression of the endosome binding domain in protoplasts abrogated IAA-induced ROS accumulation. These results indicate that activation of PtdIns 3-kinase and its product PtdIns(3)P are required for auxin-induced production of ROS and root gravitropism.

Keywords: Phosphatidylinositol 3-kinase; Phosphatidylinositol(3)phosphate; Reactive oxygen species; Gravitropism; Maize; Arabidopsis thaliana

1. Introduction

Auxin is an essential plant hormone that mediates global cellular responses, such as tropism, apical dominance, lateral root formation, and vascular differentiation as well as cell elongation \cite{1,2}. The proteosome-mediated degradation of AUX/IAA transcriptional repressors by a auxin, and cytoplasmic events including activation of the plasma membrane H\textsuperscript{+}-ATPase, members of the mitogen activated protein kinase (MAPK) cascade, phospholipase A2, and phospholipase C have been extensively studied in various experimental systems \cite{3–5}. Recently, we reported that reactive oxygen species (ROS) were generated in response to auxin, and that they were essential for elaboration of the root gravitropic response in maize root and protoplast, suggesting that they are part of the auxin signaling pathway \cite{6}.

ROS such as superoxides and H\textsubscript{2}O\textsubscript{2} are produced in response to many endogenous and/or environmental stimuli in many cell types \cite{7}. The mechanism of cell surface receptor-mediated ROS generation has been studied in animal system, especially in phagocytic neutrophil cells. ROS are usually produced by a multicomponent NADPH oxidase system triggered by the activation of cell surface receptors \cite{8}. Although all of the molecular links between stimulus and ROS production have not been identified, it is known that the binding of phosphatidyl 3-phosphate (PI3P) to one of the components of NADPH oxidase can activate the enzyme complex \cite{9}. Activation of PtdIns 3-kinase is necessary and sufficient for PDGF-induced ROS generation in non-phagocytic human hepatoma cells \cite{10}. PtdIns 3-kinase, the enzyme responsible for D-3 phosphorylation of phosphoinositides, is involved in a wide variety of cellular processes, including mitogenesis, membrane trafficking and ruffling, the oxidative burst, chemotaxis, and apoptosis \cite{11,12}. Eucaryotic PtdIns 3-kinases can be divided into four groups based on sequence homology and preferred inositol lipid substrates \cite{11}. In plants, only one type of PtdIns 3-kinase, which is related to yeast Vps34, has been identified. This enzyme phosphorylates phosphatidyl inositides in soybean and Arabidopsis \cite{13}. Antisense inhibition of Arabidopsis PtdIns 3-kinase (AtVPS34) led to severe defects in growth and tissue development \cite{14}.

In plant cells, it is likely that the effects of ROS vary with their concentration. While high levels of ROS (oxidative burst) result in hypersensitive cell death \cite{15}, low level of ROS have been reported to promote many cellular processes including cell cycle progression \cite{16} and onset of secondary cell wall differentiation \cite{17}. Interest in ROS in plants has been heightened by the recent findings that Arabidopsis respiratory burst oxidase homologues are related to gp91phox, one of the subunits of the mammalian NADPH oxidase complex. These gene products function in Ca\textsuperscript{2+} uptake and subsequent cell expansion in roots \cite{18}, as well as in ROS-dependent ABA signaling in Arabidopsis guard cells \cite{19} and in the leaves of maize seedlings \cite{20}.

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To investigate a possible link between activation of PtdIns 3-kinase leading to auxin-mediated ROS production and the gravitropic responses in plant cells, we used pharmacological inhibitors of PtdIns 3-kinase as well as transient expression of the endosome binding domain (EBD) of human early endosome antigen 1 (EEA1). The latter is a scavenger of PtdIns(3)P, the product of PtdIns 3-kinase. We report here that auxin-mediated ROS production and the root gravitropic response require PtdIns 3-kinase activation in both maize and Arabidopsis.

2. Materials and methods

2.1. Chemicals

Dihydororhodamine-123 and 2',7'-dichlorofluorescin diacetate (DCF-DA) were purchased from Molecular Probe (USA). LY294002 and wortmannin were obtained from BIOMOL (PA, USA), and macerozyme for protoplast isolation was bought from Yakult Pharmaceutical Company (Tokyo). Protease inhibitor cocktail was bought from Roche (Germany). Other chemicals were purchased from Sigma (USA).

2.2. Plant materials and growth conditions

Maize grains (Zea mays L., Golden Cross Bantam) were germinated on wet paper towels as described in a previous report [6]. Arabidopsis thaliana (Columbia) seedlings were grown at 23 °C with a 16 h light/8 h dark cycle.

2.3. Assay of ROS

Intracellular ROS production in root tissues was measured using the oxidation sensitive fluorescence of dihydororhodamine-123. Two day-grown maize roots (n = 30 for each treatment) were bisected longitudinally, stained with 0.003% dihydororhodamine-123 and observed with a fluorescence microscope (excitation = 485 nm, emission = 535 nm, Carl Zeiss). Photographs were taken with a PIXERA visual communication suite, version 1.1.0 for the Macintosh operating system (PIXERA Corp., CA, USA). The pictures shown are areas of the microscope field, and are representative of the entire field.

For the flow cytometric analysis, protoplasts treated with auxin for the indicated times were incubated with 5 μM DCF-DA for 5 min. This compound is converted to 2',7'-dichlorofluorescin, which is then oxidized by H2O2 to the highly fluorescent DCF. The fluorescence intensity was measured using a FACScan (Beckton Dickinson, Bedford, USA) with excitation and emission settings of 488 and 530 nm, respectively.

H2O2 was quantified with a Biosystech H2O2-560 assay kit (OxIS International, Inc., USA), which is based on the oxidation of ferrous ion (Fe2+) to ferric ion (Fe3+) by hydrogen peroxide under acidic condition.

2.4. Determination of the activity of PtdIns 3-kinase

A previously described method [14] was used with little modification. Arabidopsis root samples were homogenized with extraction buffer (50 mM HEPES-KOH, pH 7.4, 5 mM MgCl₂, 1 mM EDTA, 10 mM DTT, 1 mM 2-mercaptoethanol and protease inhibitor cocktail). After spinning down the homogenate, the membrane fraction was pelleted by centrifugation of the resulting supernatant at 100,000 x g for 1 h at 4 °C. The assay mixtures contained 50 mM HEPES-KOH, pH 7.4, 5 mM MgCl₂, 10 mM DTT, 10 mg of membrane proteins, and 100 μM of [γ-32P-ATP] (3000 Ci/mmol, 10 μCi/μl, Amersham) in a total volume of 50 μl. The reaction was started by adding 10 μg of phosphatidyl inositol and incubated for 10 min at 30 °C. Lipids were extracted after adding 100 μl of 1 M HCl, 200 μl of chloroform-methanol (1:1, v/v) was added to the sample and it was vigorously vortexed.

The lower chloroform phase was spotted onto a TLC plate (trans-1,2-diaminocyclohexane-N,N',N'-tetraacetic acid-treated silica gel 60, Merck), and the radiolabeled PI phosphate was separated in a borate buffer system followed by autoradiography.

2.5. Transient expression of EBD in Arabidopsis protoplasts

Arabidopsis protoplasts were isolated with an enzyme mixture containing 0.25% macerozyme R-10, 400 mM mannitol, 1 mM CaCl₂ and 5 mM MES-KOH (pH 5.6) and transfected by a polyethylene glycol-based method as previously described [21]. The viability of protoplasts was determined by staining with 1% Evans Blue. Typically, 0.3 ml of protoplast suspension (10⁶ cells/ml) was incubated with 30 μg of DNA constructs containing GFP-EBD or GFP only. The transfected protoplasts were collected after 12 h at 23 °C, and treated with 5 μM of indole acetic acid (IAA) for 10 min. Intracellular ROS production in the protoplasts was observed by fluorescence microscopy after staining with dihydroorhodamine-123. All transient expression experiments were performed at least five times with similar results.

2.6. Determination of the curvature of the maize root

Several pretreated or untreated maize seedlings were grown vertically in duplicate sets for 2 days in a clear plastic Petri-dish. One set was then placed in a horizontal position to impose a gravistimulus and the other set was left undisturbed to measure root length at the indicated times. To measure root length and root curvature, ten-times magnified root image were recorded and displayed automatically every 30 min for 3 h on a computer monitor and a custom software program (Image Pro-plus, Yongma, KOREA) was used.

3. Results

3.1. Activation of phosphatidylinositol 3-kinase is essential for auxin-induced production of ROS

In order to identify cytoplasmic effector molecules involved in auxin-induced ROS generation, we first examined PtdIns 3-kinase because several lines of evidences implicate this en-
zyme in agonist-induced ROS generation in mammalian cells [7,10]. We examined the effects of two compounds, LY294002 and wortmannin, specific PtdIns 3-kinase inhibitors, which are widely used in animal cell biology [22,23]. Pretreatment with LY294002 or wortmannin for 1 h totally abolished auxin-induced ROS production in intact maize root tissues (Fig. 1A) and also in root protoplast (Fig. 1B and C), as observed by fluorescence microscopy and flow cytometry, respectively. Over that time period, we did not detect microscopically any damage to the cells due to these inhibitors.

We also measured PtdIns 3-kinase activity by determining the amount of its product, PtdIns(3)P. The production of PtdIns(3)P increased within 10 min in response to auxin treatment of Arabidopsis roots, reached a maximum at 30 min and then gradually declined (Fig. 2A). Moreover, the increase in PtdIns(3)P was dependent on the dose of auxin, and was abolished by pretreatment with LY294002 for 30 min (Fig. 3B). These results show that activation of PtdIns 3-kinase is necessary for the production of ROS, which is likely to act downstream of reception of the auxin signal.

3.2. Sequestration of PtdIns(3)P leads to failure of ROS production in response to auxin

To confirm that PtdIns(3)P is involved in ROS-mediated auxin signaling, we used the endosome-binding domain (EBD; amino acids 1257–1411). The EBD, also known as FYVE finger, of human EEA1 (NM_003566) can sequester PtdIns(3)P by binding it [24]. We transiently overexpressed the EBD tagged with GFP in Arabidopsis protoplasts. While ROS were easily detected in the non-transfected protoplasts in response to auxin, no ROS were detected in the protoplasts expressing EBD (Fig. 3E and F). As a control, in protoplasts expressing only GFP produced ROS upon auxin treatment (Fig. 3H and I). These observations confirm the idea that the activation of PtdIns 3-kinase, and its enzymatic product PtdIns(3)P play essential roles in ROS-mediated auxin signaling.

Fig. 2. In vitro PtdIns 3-kinase assays. (A) Membrane fractions of Arabidopsis root tissues treated with IAA were assayed as described in Section 2. (B) The amount of PtdIns(3)P produced in Arabidopsis root tissues was analyzed in response to increasing concentrations of IAA with or without 10 µM LY294002 for 30 min.

Fig. 3. Effect of the EBD on auxin-mediated ROS generation. Arabidopsis protoplasts were transfected with GFP:EBD (A–F) or GFP alone (G–I) and treated with IAA for 10 min. Protoplasts were visualized with a bright field microscope (A, D and G). Transfection was confirmed by GFP fluorescence (B, E and H) and intracellular ROS was detected with dihydrorhodamine-123 (C, F and I).
3.3. Activation of PtdIns 3-kinase is required for the gravitropic response

We next asked whether inactivation of PtdIns 3-kinase impairs root gravitropism. The increase of ROS in the gravitistemulated-root was blocked by pretreatment of 50 μM of LY294002 for 1 h (Fig. 4A), and a similar result was obtained with whole root tissue. The ROS normally present in the lower cortex of the root after gravistimulation was absent in roots pretreated with LY294002 (Fig. 4B). We measured gravitropic curvature over the gravistimulation time in maize roots pretreated for 2 h with LY294002. The length of the root of the LY294002-treated plants was also measured without gravistimulation to check for impairment of growth by the inhibitor. As shown in Fig. 5A, the root length of the inhibitor-treated plants was more or less the same as that of the control plants ($P$ value is less than 0.0001 at each time point in t test except at 180 min ($P < 0.0111$)). However, the degree of curvature of the roots in response to gravistimulation was reduced by 50% in the LY294002-treated roots ($P < 0.0001$) (Fig. 5B). It appears therefore, that preventing ROS production in response to auxin signaling by inhibiting PtdIns 3-kinase, results in diminished sensitivity to gravity in primary root tissues.

4. Discussion

The rapid turnover of PtdIns(3)P and PtdIns(3,4)P2 in plants could reflect a role in cellular signaling [25,26]. Although type III PtdIns 3-kinase has also been identified in plant cells, its role has yet to be determined. It has been suggested that PtdIns(3)P is involved in intracellular vesicle trafficking by binding to the FYVE domain [11,21]. Bunney et al. demonstrated that PtdIns 3-kinase is associated with active nuclear or nucleolar transcription sites, suggesting a role in the regulating transcription [27]. It was also reported that ABA-induced ROS production in guard cells, involved in stomatal closure, is inhibited by the PtdIns 3-kinase inhibitors wortmannin and LY294002 [28]. Here, we have provided evidence that PtdIns(3)P is a signaling molecule in gravitropic responses by showing that inhibition of PtdIns 3-kinase abrogated auxin-induced ROS production and
resulted in reduced root curvature when *Arabidopsis* and maize were gravistimulated.

ROS are produced by the NADPH oxidase complexes in various eucaryotic cells. In plant, it has been reported that the *Atrboh* (Arabidopsis thaliana respiratory burst oxidase homologs), *AtrbohD* and *AtrbohF* are Arabidopsis gp91phox homologs required for ROS-dependent ABA signaling and plant defense responses [19,29]. Moreover, the facts of *rdh2*, previously defined as *AtrbohC*, also encodes a homolog of gp91phox, and that the *rdh2* mutant has short root hair and stunted roots, indicate that ROS can function as a signaling molecule not only in activation of the defense system but also in major aspects of plant development [18]. It is likely that plant and mammalian NADPH oxidases are similar both structurally and functionally. Diphenylene iodonium (DPI), an inhibitor of mammalian NADPH oxidase, inhibits ROS generation in response to pathogen infection in soybean cells [4]. However, no p40phox has yet been reported in plants.

PtdIns(3)P production by PtdIns 3-kinase and ROS formation via NADPH oxidase complexes seem to be coupled by activation of Rac, a subfamily of Rho-GTPases. In mammalian cells, Rho-GTPases are activated by GDP/GTP exchange factors (GEFs) that contain Dbl homology (DH) domains as catalytic sites and plextrin homology (PH) domains as binding sites for PtdIns 3-kinase products [31]. The lipid phosphorylated by PtdIns 3-kinase recruit Dbl family GEFs that activate Rac and induce membrane ruffling. Even though no Dbl family GEF has been reported in plant cells, a constitutively active OsRac1 is reported to induce ROS production. In addition, a dominant negative form of OsRac1 suppressed elicitor-induced ROS generation and HR-like cell death in rice [32]. These results indicate that Rac also plays an important role in the oxidative burst resulting from activation of the NADPH oxidase complex in plants cells. The additional mechanisms that connect the activity of PtdIns 3-kinase with the activation of Rac remain to be investigated.

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