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Signaling Effects of Nitric Oxide, Salicylic Acid, and Reactive Oxygen Species on Isoeuphpekinensin Accumulation in *Euphorbia pekinensis* Suspension Cells Induced by an Endophytic Fungal Elicitor

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Abstract Nitric oxide (NO), salicylic acid (SA), and reactive oxygen species (ROS) are important signal molecules that mediate plant resistance reactions and play important roles in secondary metabolism. To research the signal transduction pathway of the endophytic fungal elicitor from *Fusarium* sp. E5 promoting secondary metabolism in *Euphorbia pekinensis* suspension cells, the changes in NO, SA, ROS, and isoeuphpekinensin contents in the cells were investigated after elicitor addition to the cell suspension culture. The elicitor did not change H_2O_2 or O_2^- contents notably, whereas NO and SA contents were enhanced. Both the NO donator sodium nitroprusside (SNP) and SA enhanced isoeuphpekinensin content in the absence of the fungal elicitor, whereas the NO scavenger cPTIO and SA biosynthesis inhibitor cinnamic acid (CA) inhibited isoeuphpekinensin accumulation in the presence of the elicitor. In addition, cPTIO inhibited SA production induced by the fungal elicitor. CA did not inhibit NO production, but it significantly inhibited isoeuphpekinensin accumulation. The results demonstrated that in *Euphorbia pekinensis* suspension cells the endophytic fungal elicitor induced increased NO content and SA production, which promoted isoeuphpekinensin accumulation. ROS are clearly not involved in the endophytic fungus–host interaction signaling pathway.

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Introduction

Synthesis pathways of important medicinal secondary metabolites in plants have been studied extensively in recent years because they often have some efficacy in treating human diseases, such as paclitaxel for cancer (Wang and others 2001) and shikonin for the human immunodeficiency virus (Li and others 2009; Wu and others 2009). Traditional cultivation methods struggle to meet the demand for medicinal herbs because of increasingly serious environmental pollution. Consequently, many studies have focused on methods to improve the yield of plant secondary metabolites in cell suspensions, because the environmental conditions can be precisely and easily controlled, and they show high yield potential and trait stability. Existing methods are changing medium conditions, precursor feeding, and two-phase cultivation, but addition of a pathogenic fungal elicitor is the most rapidly effective and induces the steepest increase in production. A downside of this method is that the elicitor leads to premature aging of the plant cells and a consequent decline in biomass (Petrini 1991; Yuan and others 2002), and consequently overall secondary metabolite production clearly is not increased.

Endophytic fungi are an intriguing group of organisms that live within tissues and organs of higher plants for part of their life cycle without causing obvious symptoms of infection (Dai and others 2008). These fungi can stimulate a variety of secondary metabolic processes, promote plant growth (Lewis 2004), and indirectly increase plant resistance to protect plants against environmental stress

(Tanaka and others 2005; Vega and others 2008; Hao and others 2010). Our previous study showed the endophytic fungus *Fusarium* sp. E5, isolated from endothelial stem cells of *Euphorbia pekinensis*, which is a Chinese medicinal herb used to treat dropsy, hepatocirrhosis, and ascite infections, improved the survival rate of *E. pekinensis* tissue cultures and increased diterpene and triterpene contents in 1-year-old roots of *E. pekinensis* transplanted to a greenhouse (Yong and others 2009). In addition, an elicitor isolated from E5 mycelium and added to *E. pekinensis* suspension cell cultures significantly increased diterpene and triterpene contents, and the cells did not age prematurely but remained stable for a longer period of time (unpublished data). Similar reports are rare; therefore, the internal mechanisms by which endophytic fungi promote the synthesis of secondary metabolites in *E. pekinensis* are worthy of further study.

Nitric oxide (NO) is a small water-soluble and fat-soluble molecule whose role in human and animal nervous, cardiovascular, and immune systems has been studied extensively. In recent years there has been much progress in research into NO in plants, with reports that NO is a signaling molecule regulating plant growth, development, and defense responses (Delledonne and others 1998; Neill and others 2002; Zhang and others 2008). Many reports indicate that NO plays a key regulatory role in promotion by fungal elicitors of the accumulation of plant secondary metabolites. Fang and others (2009) proved that NO is a signaling molecule of the endophytic fungal *Cunninghamella* sp. AL4 elicitor which induces volatile oil synthesis in *Atractylodes lancea* suspension cells.

Salicylic acid (SA) is an inducer of plant systemic acquired resistance (SAR) in plant–pathogen interactions and rapidly accumulates at the site of pathogen invasion and spreads to other parts of the plant, causing a general defensive reaction. Fungal elicitors can also stimulate SA accumulation in plant cells, but many studies indicate that the accumulation of many secondary metabolites is not dependent on SA, which might suggest the accumulation of plant secondary metabolites is a local but systemic reaction (Zhao and others 2005). However, in some plants, SA can indeed induce synthesis of secondary metabolites related to gene expression. SA can stimulate tropane alkaloid synthesis in *Scopolia parviflora* and induce expression of related genes (Kanga and others 2004). *Penicillium citrinum* Thom elicitor stimulates puerarin synthesis in lobed kudzuvine suspension cells, and the elicitor-induced biosynthesis of puerarin increases are dependent on the intermediate signaling molecule SA (Xu and Dong 2005). In addition, an "oxidative burst" always occurred in plant cells under pathogen and elicitor treatment (Baker and Orlandi 1995), and reactive oxygen species (ROS) act as signaling molecules in fungal elicitor-induced synthesis of secondary metabolites (Srivastava and others 2009). To study the function and relationship of NO, SA, and H_2O_2 signaling molecules, we added an elicitor prepared from the fungal endophyte *Fusarium* sp. E5 to *E. pekinensis* cell suspension cultures to investigate the signaling pathway of isoeuphpekinensin synthesis in *E. pekinensis*.

Materials and Methods

Cell Suspension Culture and Treatments

Euphorbia pekinensis plants were collected from Langya Mountain, Anhui, China. The suspension cell line was obtained from the procedures described in our previous report (Dai and others 2005a). The culture medium was MS medium (Murashige and Skoog 1962) supplemented with 0.4 mg l^{-1} naphthalene acetic acid (NAA), 2.0 mg l^{-1} 6-benzyladenine (6-BA), and 30 g l^{-1} sucrose. The medium's pH was adjusted to 5.8 before autoclaving. Cultures were shaken at 120 rpm in darkness at 25°C in 100-ml Erlenmeyer flasks and subcultured every 2 weeks.

All exogenous signaling molecules and inhibitors were filtered using a 0.22-μm-diameter microporous membrane. Unless stated otherwise, inhibitors were applied 30 min before application of signaling molecules.

Endophytic Fungal Elicitor Preparation and Treatment

The endophytic fungus *Fusarium* sp. E5 was isolated from *E. pekinensis* (Dai and others 2005b), cultured on potato dextrose agar, and incubated at 28°C. From 7-day-old cultures, 1 cm^2 of mycelia was transferred to a 250-ml Erlenmeyer flask containing 80 ml potato dextrose medium, and the mycelia were maintained in the medium at 150 rpm at 28°C until harvest. When harvesting, the mycelia were filtered and ground with a mortar and a pestle. The homogenate was diluted in water (10 g l^{-1}) and autoclaved for 20 min at 121°C. The autoclaved fungal suspension was used as the elicitor (Yu and others 2001). The amount of fungal extract was determined by the phenol-sulfuric acid method using glucose as a standard (Dubois and others 1956).

Elicitor treatments of the 14-day-old *E. pekinensis* cultures were at the rate of 7.85 mg l^{-1} carbohydrate equivalents. In the meantime, a control was inoculated with an equal volume of sterile double-distilled water.

Measurement of H_2O_2

Active oxygen species in the medium of suspension-cultured *E. pekinensis* cells were measured by chemiluminescence in a ferricyanide-catalyzed oxidation of luminol. A 100-μl aliquot of the medium (cells had been removed

by filtration through a nylon net or a column), 50 µl luminol (5-amino-2,3-dihydro-l,4-phthalazinedione) (1.1 mM in KPi buffer, 50 mM, pH 7.9), and 800 µl KPi buffer (50 mM, pH 7.9) were mixed in a cuvette. The reaction was initiated with 100 µl $K_3[Fe(CN)_6]$ (14 mM in H_2O, freshly prepared). The assay method was according to Schwacke and Hager (1992). To compare independent experiments we used an internal standard of H_2O_2. Fifty µl of H_2O_2 (1 µM, freshly prepared) was added to the assay mixture containing 750 µl KPi buffer. One U of H_2O_2 concentration was defined as the chemiluminescence caused by the internal standard of 1 µM H_2O_2.

Superoxide Anion Assay

The amount of superoxide anion in leaves was detected using the nitro blue tetrazolium (NBT) colorimetric method. The basic principle was to use NBT transformed in the presence of O_2^- into NBT formazan, which shows a maximum absorption peak at 530 nm. *Euphorbia pekinensis* cells were washed with 10 mM PBS (pH 7.8) three times, then resuspended in 10 mM PBS containing 1% (w/v) sucrose and 0.5 mM $CaCl_2$ (0.1 g FW cells ml^{-1}) for 3 h, after which 20 µM NBT and elicitor were added. Five ml of suspension-cultured cell filtrate after different treatment times were used to measure absorbance at 530 nm. The untreated filtrate was used as the control.

Measurement of NO

The amount of NO in the suspension cells of the different treatments was measured spectrophotometrically. Suspension-cultured cells were filtered with a microporous membrane at 4°C. A mixture of 1 ml filtrate and 1 ml Greiss reagent was incubated at room temperature for 30 min. Absorbance was determined at 550 nm. The NO content was calculated by comparison to a standard curve for $NaNO_2$. Measurements were recorded for five individual plants as biological replicates.

Measurement of SA

Extraction and analysis of SA followed the method of Verberne and others (2002) with some modifications. One g of cells was ground in liquid nitrogen and extracted with 2 ml methanol using sonication. After centrifugation at 14,000×*g* for 5 min, the supernatant was collected for rotary evaporation, and the residue was resuspended with 250 µl of 5% trichloroacetic acid. The mixture was re-extracted with 800 µl acetic acid ester:cyclohexane (1:1 v/v) and mixed well; then the organic phase was rotary evaporated until dry, dissolved with 600 µl organic phase, and filtered with a 0.2-µm microporous membrane.

SA was quantified by high-performance liquid chromatography (HPLC) using a reverse-phase column (Hedera Packing Material Lichrospher 5-C18, 4.6 × 200 mm^2, 5 µm). The mobile phase was methanol:H_2O (80:20 v/v) at 1 ml min^{-1} and detected at 217 nm at 25°C.

Phenylalanine Ammonia-Lyase Activity

Phenylalanine ammonia-lyase (PAL) activity was analyzed following the method of Modafar and others (2001) with some modifications. Cells (500 mg) were homogenized for 2 min in 5 ml of 0.1 M borate buffer (pH 8.8) containing 600 mg polyvinylpyrrolidone, 5 mM β-mercaptoethanol, and 2 mM EDTA. The homogenate was centrifuged for 15 min at 14,000×*g* and the supernatant was collected for enzyme activity determination. The PAL activity was measured by incubating 0.5 ml supernatant with 2 ml of 0.1 M borate buffer (pH 8.0) containing 3 mM L-phenylalanine for 1 h at 30°C. The increase in absorbance at 290 nm because of the formation of *trans*-cinnamate was measured spectrophotometrically. The PAL activity was expressed as the change in OD_{290} per hour per gram of fresh weight. One U is equivalent to a 0.01 increase in absorbance in OD_{290}.

The cells in the suspension cultures were filtered under vacuum. The dry weight (DW) was obtained by drying the fresh cell mass at 50°C in an oven until constant weight, and both the DW and the fresh weight (FW) were recorded with a physical balance. All the experiments were repeated three times.

Extraction and Analysis of Isoeuphpekinensin

Dried cells (500 mg) were ground to a powder, then sonicated for 30 min in 30 ml methanol. The extract solution was filtered and evaporated, and the residue was dissolved in 1 ml methanol. The solution samples were transferred to an Eppendorf tube and centrifuged at 12,000×*g* for 5 min. The supernatant was filtered through a 0.45-µm membrane and transferred to clean glass vials for high-performance liquid chromatography (HPLC) analysis. The isoeuphpekinensin content was determined by HPLC using a reverse-phase column (Hedera Packing Material Lichrospher 5-C18, 4.6 × 200 mm^2, 5 µm). The mobile phase was methanol:H_2O (80:20 v/v) at 1 ml min^{-1} for isoeuphpekinensin. Isoeuphpekinensin was detected at 268 nm at 30°C. The isoeuphpekinensin and euphol standards were obtained from Dr. Qiao-Li Liang (Liang and others 2008).

Statistical Analysis

The mean and standard error were calculated for each biochemical measurement. All data were analyzed by

repeated analysis of variance (ANOVA) to compare the differences between treatments using SPSS v13.0 (IBM Corporation, Somers, NY, USA).

Results

Effect of Elicitor on NO Production, PAL Activity, and SA and Isoeuphpekinensin Accumulation

The NO content of *E. pekinensis* cells increased following elicitor addition and peaked after 7.5 h, the value of which was 2.9-fold that of the control, then decreased gradually to the control level after 20 h (Fig. 1a). The NO content of the same culture without elicitor remained at a low level throughout the experimental period (Fig. 1a).

Catalysis of PAL is the first step in the phenylpropanoid metabolic pathway and is the first rate-limiting enzyme of SA biosynthesis. PAL activity is considered to be the major source of SA in plant cells (Mauch-Mani and Slusarenko 1996). Activity of PAL increased in response to elicitor addition and peaked after 10 h, the value of which was 3.2-fold that of the control, then decreased gradually but remained slightly higher than that of the control (Fig. 1b).

The SA level first increased after 12.5 h, then decreased and returned to the control level after 20 h (Fig. 1c). Thus, NO production was first stimulated by the endophytic fungal elicitor, then activation of PAL, followed by SA accumulation.

Isoeuphpekinensin content peaked on the fourth day of elicitor treatment and was 2.4-fold that of the control (Fig. 1d). Subsequently, the isoeuphpekinensin content decreased gradually to be more similar to that of the control after 7 days. The isoeuphpekinensin content of the control peaked at 5.19 g g^{-1} DW on the sixth day (Fig. 1d). In sum, endophytic fungal elicitor improved the isoeuphpekinensin accumulation of *E. pekinensis* suspension culture significantly, and it also made the peak concentration emerge 2 days ahead of control; this is important for industrial production.

Effect of ROS on Isoeuphpekinensin Accumulation

To understand the role of ROS in the host reaction to the endophytic fungal elicitor, we first measured the H_2O_2 and O_2^- contents of *E. pekinensis* suspension cultures with elicitor applied on day 14. The H_2O_2 and O_2^- levels did not change significantly within 25 h (Fig. 2a, b). Then we determined the isoeuphpekinensin content of *E. pekinensis*

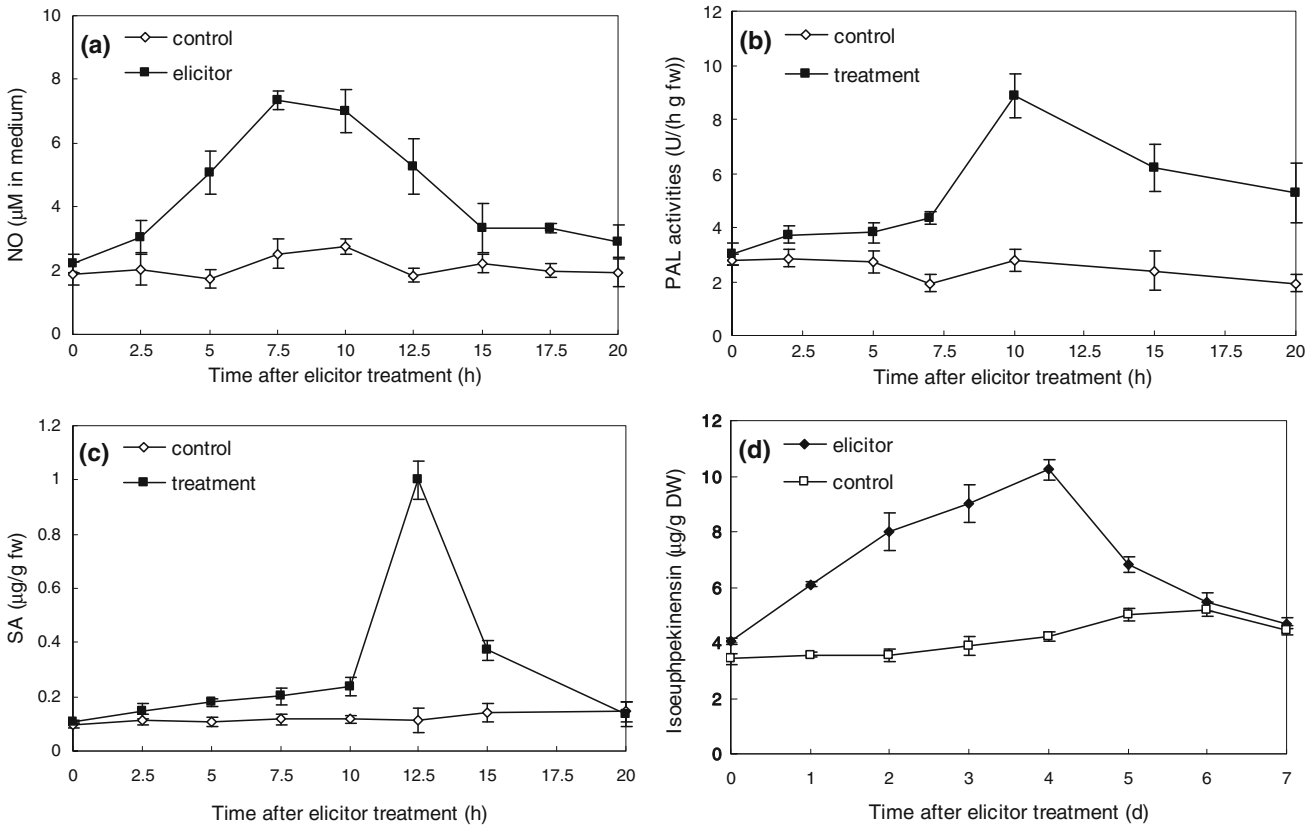

Fig. 1 The effects of elicitor on NO accumulation, PAL activation, SA accumulation, and isoeuphpekinensin accumulation of *E. pekinensis* cultures. **a** Effects on NO accumulation. **b** Effects on PAL activation. **c** Effects on the accumulation of SA. **d** Effects on isoeuphpekinensin accumulation. Values are the mean of three replicates ± standard error

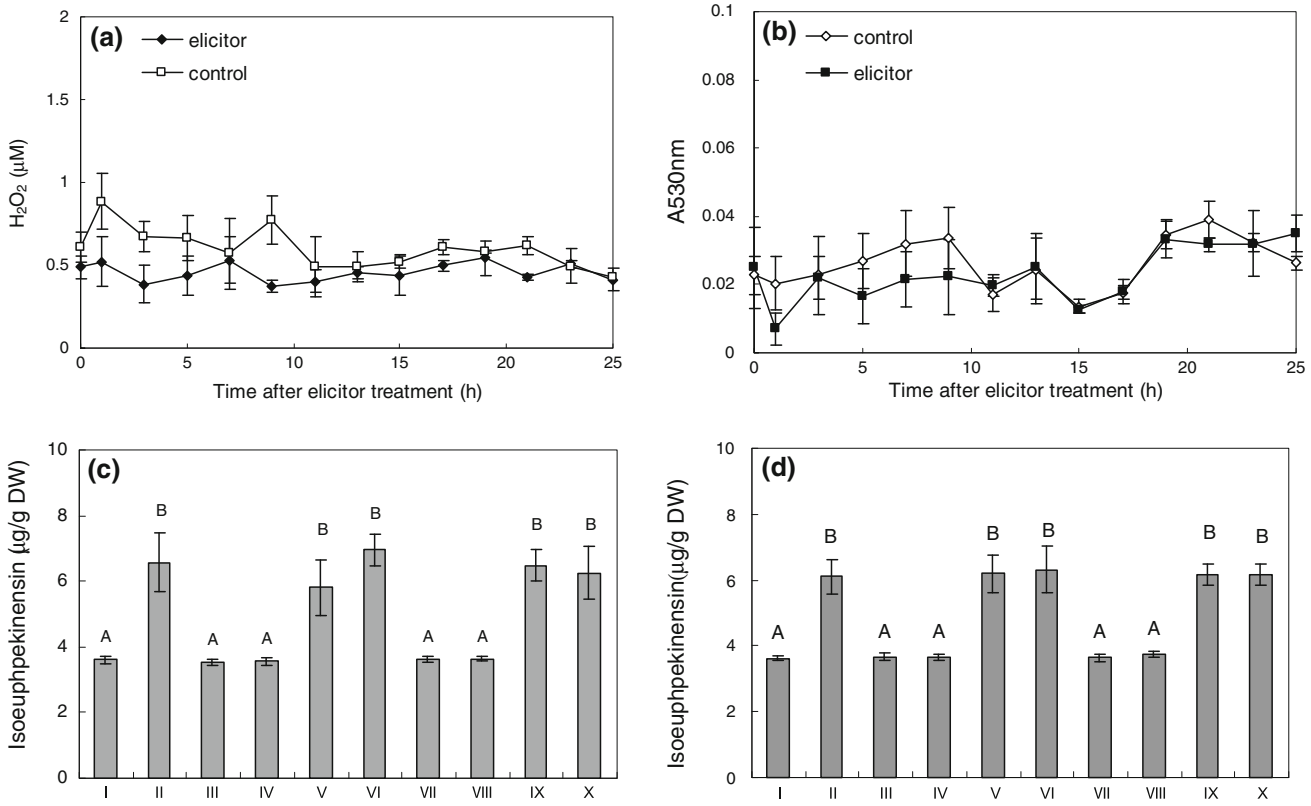

Fig. 2 The effects of ROS on isoeuphpekinensin accumulation. **a** Effects of elicitor on H_2O_2 accumulation. **b** Effects of elicitor on O_2^- accumulation. **c** Effects of O_2^- and H_2O_2 on isoeuphpekinensin accumulation. *I* Control, *II* elicitor, *III* 0.5 mM H_2O_2, *IV* 1.0 mM H_2O_2, *V* elicitor + 0.5 mM H_2O_2, *VI* elicitor + 0.5 mM H_2O_2, *VII* 0.5 mM flavin + flavin oxidase, *VIII* 1 mM flavin + flavin oxidase, *IX* elicitor + 0.5 mM flavin + flavin oxidase, *X* elicitor + 1 mM flavin + flavin oxidase. **d** Effects of DPI and CAT on isoeuphpekinensin accumulation; inhibitors were applied 30 min before ROS. *I* control, *II* elicitor, *III* 2 mkat/L CAT, *IV* 4 mkat/L CAT, *V* elicitor + 2 mkat/L CAT, *VI* elicitor + 4 mkat/L CAT, *VII* 0.5 mM DPI, *VIII* 1 mM DPI, *IX* elicitor + 0.5 mM DPI, *X* elicitor + 1 mM DPI. Cells were harvested 4 h after treatment. Values are the mean of three replicates ± standard error. *Bars* with *different capital letters* indicate significant differences at $p < 0.01$

suspension cultures with ROS applied on day 14. The H_2O_2 and O_2^- (flavin oxidase and flavin) contents did not affect isoeuphpekinensin content compared to the control (Fig. 2c).

Isoeuphpekinensin accumulation was unaffected by addition of diphenyleneiodonium (DPI), a NADPH oxidase inhibitor (Rosenwasser and others 2010), and the H_2O_2 scavenger catalase (CAT) to *E. pekinensis* suspension cultures. In addition, DPI and CAT plus elicitor individually did not change isoeuphpekinensin content (Fig. 2d). Thus, ROS were not involved in isoeuphpekinensin biosynthesis in *E. pekinensis*.

Effects of cPITO and CA on NO Production, PAL Activation, and SA Accumulation

To determine the relationship between the signal molecules NO and SA, we investigated NO and SA contents of *E. pekinensis* suspension cultures after addition of 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) (a NO scavenger) and CA (a SA inhibitor). CPTIO significantly reduced the elicitor-induced stimulation of NO, but CA did not have the same effect (Fig. 3a). This result implied that SA does not mediate NO production. However, cPTIO reduced elicitor-induced PAL activation and SA accumulation (Fig. 3b), which indicates that NO production is located upstream of SA signaling, PAL activation, and induction of SA accumulation.

Effects of NO and SA on Isoeuphpekinensin Biosynthesis

To confirm the role of NO and SA in isoeuphpekinensin biosynthesis induced by the endophytic fungal elicitor, we investigated the impact of these signaling molecules and inhibitors of their synthesis on isoeuphpekinensin biosynthesis. The elicitor, SNP, and SA significantly increased isoeuphpekinensin biosynthesis, whereas cPTIO and CA inhibited isoeuphpekinensin biosynthesis (Fig. 4), which indicated that NO and SA play a key role in isoeuphpekinensin biosynthesis induced by the endophytic fungal elicitor. Furthermore, CA suppressed stimulation of

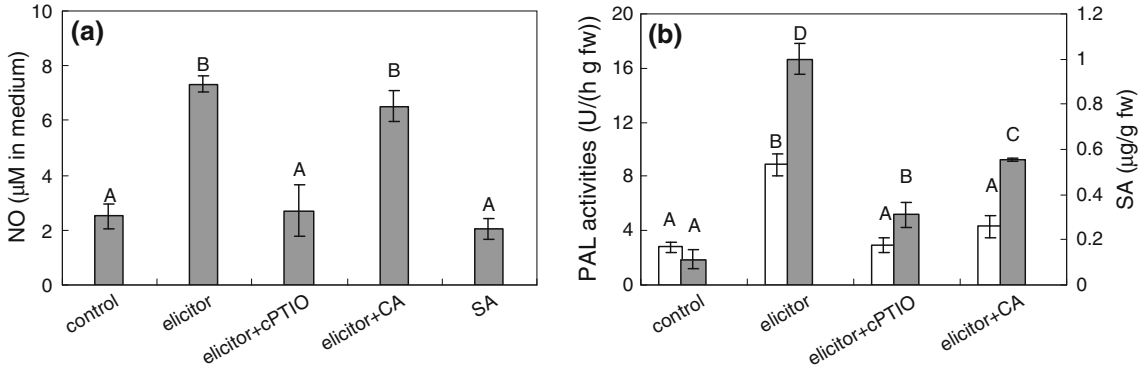

Fig. 3 The mediate relationship between NO and SA in *E. pekinensis* cells. **a** Effects of inhibitor on NO level. **b** Effects of inhibitor on PAL activity (*white bars*) and SA level (*gray bars*). Concentration of elicitor applied was 7.85 mg/L, cPTIO was 30 μM, CA was 1 mM, SA was 1 mM. Cells were harvested 4 h after treatment. Values are the mean of three replicates ± standard error. *Bars* with *different capital letters* indicate significant differences at $p < 0.01$

isoeuphpekinensin biosynthesis induced by SNP, exogenous SA reversed the inhibition of isoeuphpekinensin biosynthesis by cPTIO and CA, and cPTIO did not inhibit the promotion of isoeuphpekinensin biosynthesis induced by SA (Fig. 4). These results proved that SA is located downstream of the signaling pathway of isoeuphpekinensin biosynthesis. Collectively, our results implied that the signaling pathway of isoeuphpekinensin biosynthesis in *E. pekinensis* suspension cells induced by the endophytic fungal elicitor was as follows: the endophytic fungal elicitor induced NO production, which mediated isoeuphpekinensin biosynthesis dependent on SA (Fig. 5).

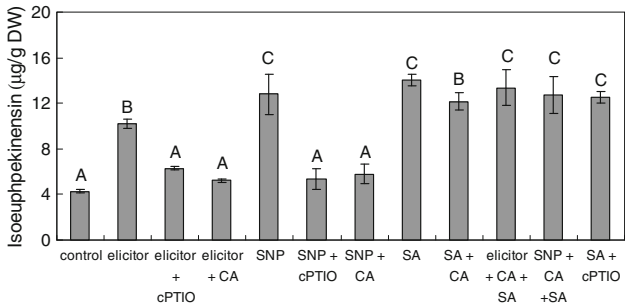

Fig. 4 The effects of NO and SA on isoeuphpekinensin biosynthesis in suspension cells of *E. pekinensis*. The concentrations of reagents applied were: elicitor, 7.85 mg/L; cPTIO, 30 μM; CA, 1 mM; SNP, 0.1 mM; SA, 1 mM. Cells were harvested 4 h after treatment. Values are the mean of three replicates ± standard error. *Bars* with *different capital letters* indicate significant differences at $p < 0.01$

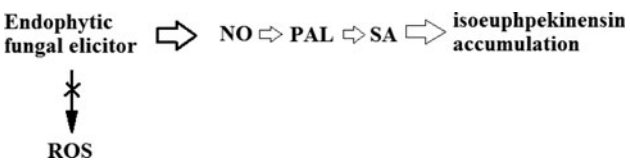

Fig. 5 Signaling model of isoeuphpekinensin accumulation induced by endophytic fungal elicitor

Discussion

Fungal components can be used as an elicitor, which can evoke multiple responses in plant cells, including production of a variety of secondary metabolites (Modafar and others 2001). However, the mechanisms of signal transduction in the fungal elicitor-evoked synthesis of secondary metabolites in plant cells are unclear. Generally, it is believed that a fungal elicitor applied as an extracellular stimulus first recognizes and binds to a specific receptor on the plant cell membrane, thus stimulating the cells to produce a specific intracellular messenger and regulate the expression of nuclear genes through the corresponding signal transduction pathways (Nürnberger and others 1994), and ultimately activate defensive secondary metabolic systems for synthesis of secondary metabolites. Under biotic stress such as fungal infection, plant cells sense and transmit stress signals by a variety of signaling molecules and signaling pathways (Ligterink and others 1997). Crosstalk between signaling molecules has been reported. Xu and others (2005) found that hypericin biosynthesis from hypericum cells mediated by the NO pathway depended on JA. In the present study, we showed that an endophytic fungal elicitor induces NO production, and NO-mediated isoeuphpekinensin biosynthesis in *E. pekinensis* suspension cells is dependent on SA. ROS is not involved in the mediation process, which is notably different from the host response to pathogenic fungal infection associated with the ROS production phenomenon (Fig. 5). In addition, NO and SA levels peaked at 7.5 and 12.5 h, respectively, after addition of the fungal elicitor, while isoeuphpekinensin accumulation peaked on day 4. These results implied that NO and SA act as signals to initiate continuous biosynthesis of secondary metabolites in the host plant.

Many studies have focused on pathogen elicitor-induced plant secondary metabolite accumulation and the signaling

transduction pathway (for example, Schwacke and Hager 1992). Wu and others (2009) demonstrated that NO induced expression of the shikonin biosynthesis genes *PAL*, *HMGR*, and *PGT*, and shikonin synthesis. SA enhanced accumulation of secondary metabolites and expression of defense genes in *Scopolia parviflora* (Kang and others 2004; Wang and others 2004; Chen and others 2006).

A pathogen elicitor initially induces the synthesis of target compounds, of which the accumulation quickly peaks, but also leads to accelerated aging of plant cells, which prematurely enter the decline phase. Consequently, the synthesis of target compounds is not significantly increased (Petrini 1991; Yuan and others 2002). However, endophytes, as microorganisms that colonize plants in the long term, formed a mutually beneficial symbiotic relationship with host plants in the long-term evolution of the ecosystem, so it is different from the pathogen–host interaction. Our previous studies showed that the endophytic fungus *Fusarium* sp. E5, isolated from endothelial stem cells of *E. pekinensis*, increases isoeuphpekinensin production and promotes proliferation of *E. pekinensis* suspension cells, but the mechanisms underlying both the increasing phenomenon of isoeuphpekinensin production and cell proliferation are not yet clear. This research indicates that the endogenous fungal elicitor does not cause the oxidative burst in *E. pekinensis* cells and ROS do not play a role in the signaling pathway of elicitor-induced isoeuphpekinensin synthesis; this differs markedly from other host plant responses to pathogen elicitors. ROS are an important signal that mediates some plant defense responses and phytoalexin accumulation, promotes generation of other signaling molecules (Mehdy 1994), and induces the hypersensitive response and other defense responses in plants. However, the mechanism by which ROS regulate fungal elicitor-induced production of plant secondary metabolites is unclear. ROS are believed to induce expression of defense genes and secondary metabolite biosynthesis genes such as sesquiterpene synthase and PAL (Baker and Orlandi 1995). In wheat inoculated with pathogenic and nonpathogenic strains of *Puccinia striiformis* f. sp. *tritici*, H_2O_2 and O_2^- levels were higher in response to the pathogenic strain than the nonpathogenic strain (Neill and others 2002). This finding implied that accumulation of H_2O_2 related to programmed cell death was closely associated with pathogen infection. Thus, it is hypothesized that ROS levels showed little change when *E. pekinensis* cells were incubated with the endophytic fungus *Fusarium* sp. E5 because the fungus is symbiotic with *E. pekinensis* and does not cause any disease. On the other hand, Kawano and Muto (1999) found that H_2O_2 content was reduced by SA via peroxidase catalysis in a tobacco cell suspension culture.

In the present study, we investigated the signaling pathway of isoeuphpekinensin biosynthesis in *E. pekinensis*. Unraveling the precise relationship between NO and SA and excluding the function of ROS in the process will certainly improve our understanding of endophyte symbiosis with the host plant to enhance production of plant secondary metabolites. Moreover, the difference in signaling between pathogen–host and endophyte–host interactions is an intriguing topic for future exploration.

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