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ORIGINAL ARTICLE

Fungal elicitor Pep-25 increases cytosolic calcium ions, H_2O_2 production and activates the octadecanoid pathway in *Arabidopsis thaliana*

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Abstract Transgenic *Arabidopsis thaliana* plants expressing the protein aequorin were used to investigate the transient change in cytosolic calcium ions caused by stimulation through the fungal elicitor Pep-25. Our results show that the elicitor Pep-25 derived from *Phytophthora sojae* can induce H_2O_2 production and an increase in cytosolic calcium ions. The transcription of *LOX, OPR3, PED, AOS, AOC* genes and the protein accumulation of AOS were induced by Pep-25 treatment. Pep-25 also induced an accumulation of jasmonic acid (JA). Blocking the production of H_2O_2 and the increase of cytosolic calcium ions both suppressed the transcription of *LOX, OPR3, PED, AOS, AOC* genes, the accumulation of AOS, and the accumulation of JA. These results indicated that the production of H_2O_2 derived from the plasma-membrane NADPH oxidase and the subsequently increase of cytosolic calcium ions are both required for the activation of the octadecanoid pathway by Pep-25 treatment in *A. thaliana*.

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Keywords *Arabidopsis* · Aequorin · Cytosolic calcium ions · Hydrogen peroxide · Octadecanoid pathway

Abbreviation

$[Ca^{2+}]_{cyto}$	Concentration of cytosolic calcium ions
DMTU	Dimethylthiourea
DHC	2,5-Dihydroxycinnamic acid
DPI	Diphenylene iodonium
$LaCl_3$	Lanthanum chloride
RR	Ruthenium red

Introduction

Induction of early defense responses in plants by extracts of pathogens or plant cell walls, called elicitation, involves complex signaling mechanisms of which a number of components have been identified (Ebel and Cosio 1994; Amborabe et al. 2008). After perception of the elicitor stimulation (recognition process), the plant cell at the site of the infection transmits the information inside the cell across the plasma membrane as well as to neighboring cells. As a result, a number of defense responses are induced, including a rapid and localized cell death (hypersensitive responses, HR), a rapid oxidative burst, cross-linking and strengthening of the plant cell wall, the induction of the phenylpropanoid pathway and the synthesis of lignin, the accumulation of antimicrobial compounds and the synthesis of phytoalexins, and finally, the production of ethylene and jasmonic acid (JA) (Lamb and Dixon 1997; Alvarez et al. 1998). Pep-25 is the elicitor derived from the 42-kDa glycoprotein secreted by *Phytophthora sojae*. Treatment of parsley cells with Pep-25 caused the transcriptional accumulation of defense-associated genes

and the production of furanocoumarin phytoalexins (Blume et al. 2000; Fellbrich et al. 2000). Elicitor treatment also stimulated Ca^{2+} and H^+ influx, the efflux of K^+ and Cl^-, an oxidative burst, and the accumulation of jasmonate in parsley cells (Desikan et al. 1996; Cessna and Low 2001).

Ion fluxes across the plasma membrane are involved in the earliest measurable signaling events of plant responses to elicitor stimulation, such as the alkalization of the extracellular medium, the efflux of K^+, and an increased level of intracellular Ca^{2+}. Extracellular Ca^{2+} has been shown the important role for elicitor-induced gene expression and caused an increment of cytosolic calcium ions concentration ($[Ca^{2+}]_{cyto}$) (Jabs et al. 1997; Trewavas and Malho 1998; Cessna and Low 2001; Ogasawara et al. 2008). The oxidative burst, characterized by the transient release of H_2O_2 and superoxide ions, is also an early event in the plant response to elicitor stimuli. H_2O_2 is the product of the oxidative burst, but it also acts as a second messenger and induces defense-related gene expression and defense-related responses (Alvarez et al. 1998; White and Broadley 2003; Davies et al. 2006).

The octadecanoid (ODA) pathway, which is responsible for the accumulation of JA, has been implicated in wounding-induced biosynthesis of proteinase inhibitors (Ellis et al. 2002; He et al. 2002; Wasternack et al. 2006). It has been reported that H_2O_2 is involved in the induction of the ODA pathway that is induced by wounding and pathogen infection (Orozco-Cárdenas et al. 2000; Turner et al. 2002). The $[Ca^{2+}]_{cyto}$ increase also acts upstream to the ODA pathway activated by the extracted elicitor from yeast in parsley suspension cells (Navazio et al. 2002) (more information on the relationships among $[Ca^{2+}]_{cyto}$, H_2O_2 and the octadecanoid pathway will be presented in the discussion). Transformed plants that express the Ca^{2+} sensitive aequorin protein have been successfully used to quantify the intracellular fluxes associated with a range of diverse physical and pharmacological stimuli (Johnson et al. 1995; Blume et al. 2000; Cessna and Low 2001). Such plants permit the measurement of the transient change of intracellular Ca^{2+} concentration and avoid the potential damage to the plant caused by the classical loading method. In the present study, we constructed transgenic *Arabidopsis* plants expressing recombinant aequorin. When we treated such transgenic *A. thaliana* with Pep-25, sharp increases of cytosolic calcium ions and H_2O_2 were measured. In addition, the genes involved in octadecanoid pathway, *LOX, OPR*3, *PED*, *AOS*, *AOC* genes and AOS protein, were all induced, the accumulation of JA was also observed. The role of Ca^{2+} and H_2O_2 in the activation of ODA related gene expressions are discussed.

Materials and methods

Arabidopsis transformation

Arabidopsis thaliana (L.) Heynh. (Landsberg *erecta*, from ABRC, Columbus, OH, USA) was grown in a greenhouse under 16-h light and 8-h dark cycle at 25°C. pMAQ 2.4 was kindly provided by Heather Knight (Oxford University, UK). The plasmid includes the cauliflower mosaic virus 35S promoter and the octopine synthase terminator. A DNA segment encoding *aequorin* (*aeq*) was inserted between the 35S-promoter and the NOS termination site (Knight et al. 1991). The plasmid was mobilized to *Agrobacterium tumefaciens* strains LBA4404 by liquid nitrogen fast freezing and used for plant flower transformation. *A. thaliana* adult plants (5 weeks old) were subjected to *Agrobacterium tumefaciens* mediated transformation, infected by vacuum infiltration and grown in the greenhouse to collect seeds. These seeds were screened in MS medium supplemented with 50 mg/l kanamycin. The seedlings that grow normally on kanamycin for 3 weeks were selected as the experimental materials. The induction of seedlings for *A. thaliana* was accomplished as previously reported (Knight et al. 1997).

Measurement of $[Ca^{2+}]_{cyto}$

Transgenic *Arabidopsis* expressing aequorin was used to measure the change of $[Ca^{2+}]_{cyto.}$ Seedlings were grown for 2 weeks on 0.8% agar in MS medium supplemented with 50 mg/l kanamycin at 21°C under a 16-h photoperiod. Reconstitution of aequorin was performed in vivo essentially as described previously by floating seedlings on water containing 2.5 μM coelenterazine in the dark overnight at 21°C (Knight et al. 1997). The measurement of $[Ca^{2+}]_{cyto}$ was performed by placing individual seedlings in a transparent plastic cuvette containing 0.2 ml water. After approximately 5 s of background counting, different volumes of the Pep-25 (sequence DVTAGAEVWNQPVR GFKVYYEQTEMT) and its derivatives were diluted to appropriate concentration from the original stock solution (1 mg/ml, about 320 μM) and injected into the cuvette via a light-tight 1 ml syringe inserted into a light-tight port in the luminometer sample housing, to give different final concentrations of Pep-25. The injection of Pep-25 stock solution was performed so as to induce a negligible touch response in the cytosolic Ca^{2+} levels within the seedling. For inhibitor or scavenger experiments, the seedlings were preincubated for 10 min with different inhibitors or scavengers, or water as the control. After 10 min, the cells were placed into the luminometer cuvette, the intensity of luminescence was recorded immediately after stimulation with Pep-25. Luminescence was calibrated as Ca^{2+} concentration as

described previously (Knight et al. 1997; Sai and Johnson 1999, 2002).

Measurement of H_2O_2

The collected seedlings (0.2 g) were ground to a powder under liquid nitrogen and homogenized with 1 ml of 0.2 mol/l $HClO_4$ at 4°C. The extract was held on ice for 5 min and centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was collected and either processed immediately or quick-frozen at −70°C until further analysis could be performed. The concentration of H_2O_2 was measured by the method reported elsewhere (Chen et al. 1993). For inhibitor or scavenger experiments, the seedlings of *A. thaliana* were preincubated for 10 min with different inhibitors or scavengers, or water as the control. After 10 min, Pep-25 was added and the concentration of H_2O_2 was measured at the indicated times.

Quantification of JA

Treated seedlings at indicated times were collected and immediately frozen in liquid nitrogen to minimize wound-induced JA accumulation. Samples were finely ground in liquid nitrogen followed by stirring in 80% methanol at 4°C overnight. The extract was methylated as described in Royo et al. (1999). JA was quantified by GC–MS (GCD plus, electron ionization mode, 30 m × 0.25 mm HP-5 column, Hewlett-Packard, Palo Alto, CA, USA). For inhibitor or scavenger experiments, the seedlings were preincubated for 10 min with different inhibitors or scavengers, or water as the control. After 10 min, Pep-25 was added, and the concentration of JA was measured at the indicated times.

RT-PCR and Northern-blot hybridization

Total RNA was extracted from seedlings of *A. thaliana* by different treatments as described in Sambrook et al. (1989). RT-PCR was manipulated as described method using the primers (Forward primer atgacaagcaaacaatact and Reverse primer caaaatcctaaatgttgtct) special for aequorin gene. For Northern blotting, RNA was separated by electrophoresis (30 μg per lane) on denaturing 1.2% agarose gels containing 2.2 mM formaldehyde and blotted onto membranes (Hybond N, Amersham Pharmacia Biotech, Bucks, UK) by standard methods (Sambrook et al. 1989). After cross-linking for 2 h at 80°C in a vacuum oven, blots were prehybridized for 1 h at 50°C in 20 mM Na_2HPO_4, pH 6.8, containing 6× SSC, 0.4% SDS, 5× Denhardt's solution, and 0.5 mg/ml denatured salmon sperm DNA. Overnight hybridization was carried out in this medium (without Denhardt's solution) with Dig-labeled, gene-specific oligonucleotide probes generated from *A. thaliana* cDNAs. Blots were washed twice for 15 min in 6× SSC and 0.1% SDS at room temperature and twice for 15 min at 55°C before autoradiography. Equal loading of RNA samples was assessed by hybridization with 18S rRNA labeled by random priming. The Dig-labeled probes of *aequorin* (*aeq)*, *LOX, OPR3, PED, AOS, AOC* genes were generated according to the manufacturer's recommendation (Dig DNA labeling and Detection kit, Cat No.1 093 657, Roche Diagnostics Corp, Indianapolis, IN, USA). The hybridization and detection was performed by the manufacturer's instructions. The probes for *LOX, OPR3, PED, AOS, AOC* genes were obtained by RT-PCR using the procedure and the primers for RT-PCR as reported previously (He et al. 2002).

SDS-PAGE and Western blotting

Total protein separation was conducted using a discontinuous SDS-PAGE (12% separation gel) system (Sambrook et al. 1989). After electrophoresis, the proteins were tranferred to nitrocellulose using a semidry blotting instrument. Protein immunoblotting was performed by using antibody against tomato AOS (1:1,000). The antibody against *A. thaliana* Sumo3 (1:5,000, ab5317, Abcam Inc, Cambridge, UK) was used as the loading control. Protein concentration was measured by the Bradford (1976) method with BSA as a standard.

Results

Transformation of Arabidopsis with the aequorin construct

The plasmid containing recombinant aequorin was mobilized into LBA4404, and was then transformed into 5-week-old *A. thaliana* seedlings by floral-dipping infiltration. The F1 seeds were obtained and cultivated on resistance plates containing 50 μg/ml kanamycin. F2 seedlings that grew normally on resistance plates were assayed by RT-PCR. As shown in Fig. 1a, RT-PCR results demonstrated that the aequorin transgene was strongly expressed (Fig. 1a), but the phenotypic appearance and growth behavior of transgenic Araibidopsis were indistinguishable from those of non-transformed *Arabidopsis*. We directly added two volumes of 37.5 mM $CaCl_2$ into the transformed and non-transformed seedlings. A sharp, transient and strong increase of luminescence, which indicates the increase of $[Ca^{2+}]_{cyto}$, was observed for transformed *Arabidopsis* seedlings, but no strong increase was seen in the non-transformed *Arabidopsis* seedlings. The maximum luminescence is shown in Fig. 1b. Such results further confirmed that aequorin was functional in the transformed seedlings.

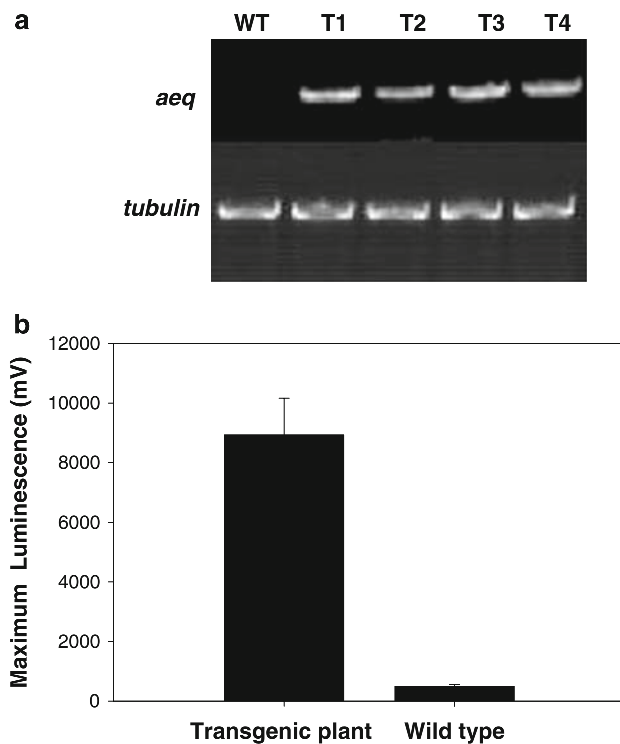

Fig. 1 Transformation of *Arabidopsis* with the *aequorin* gene. **a** Test of the *aequorin* gene transcriptional level by RT-PCR. Four individual transgenic line (T1, T2, T3, T4) and wild type line (*WT*) were used to extract total RNA for RT-PCR using the described primers. **b** Maximum luminescence reflected by calcium signals in transgenic and wild-type plants. The transgenic line expressing *aequorin* and its control wild type were treated with extracelluar calcium ions and the maximum luminescence was recorded using a luminometer. Data represent the mean ± SE from six experiments. *aeq*, aequorin transcript

Pep-25 increases $[Ca^{2+}]_{cyto}$

Treating the *Arabidopsis* seedlings expressing recombinant aequorin with 200 nM Pep-25 produced a characteristic increase of $[Ca^{2+}]_{cyto}$. In contrast, only a small peak was immediately detected after addition of water. The extent of the $[Ca^{2+}]_{cyto}$ response depended on the concentration of Pep-25 added (Fig. 2a). The $[Ca^{2+}]_{cyto}$ response was saturated at a concentration of 160 nM Pep-25. Chelation of extracellular Ca^{2+} by EGTA strongly reduced the change in $[Ca^{2+}]_{cyto}$ induced by Pep-25 in *A. thaliana*. Lanthanide is frequently used to inhibit Ca^{2+} transport across the plant plasma membrane. Pre-incubating *Arabidopsis* seedlings with $LaCl_3$ for 10 min prior to Pep-25 abrogated the $[Ca^{2+}]_{cyto}$ peak by 62% as compared with the Pep-25-only treatment. Ruthenium red (RR), a membrane-permeable Ca^{2+} channel inhibitor, can block the release of Ca^{2+} from intracellular compartments. RR pretreatment for 10 min prior to Pep-25 also blocks the $[Ca^{2+}]_{cyto}$ increase. Inhibitors of plasma membrane NADPH oxidase (DPI and pyridine) were not capable of blocking the Pep-25 induced increment of $[Ca^{2+}]_{cyto}$ after a 10 min preincubation with the inhibitors. Because *A. thaliana* required a little bit higher concentration of Pep-25 to induce the increase of $[Ca^{2+}]_{cyto}$ compared with the Pep-13 induced defense response in other plant such as parsley and potato (usually, parsley needs only about 10 nM; Nürnberger et al. 1994; Blume et al. 2000), it is possible that the effect induced by the high concentration of Pep-25 is not specific. To rule out this possibility, we used two Pep-25 derivatives; one is Pep-25/A19, which replaced the Y19 (Tyr at position 19) with alanine (Pep-25/A19). The other is Pep-25/A9, which replaced the W9 (Trp at position 9) with alanine (Pep-25/A9). We also synthesized Pep-13 (VWNQPVRGFKVYE, deletion some C-terminal and N-terminal amino acid residues from Pep-25). As shown in Fig. 2b, Pep-25/A19 still remained the ability to induce an increase in $[Ca^{2+}]_{cyto}$, while Pep-25/A9 lost the capability to trigger the Ca^{2+} uptake. Pep-13 cannot significantly improve the increase of $[Ca^{2+}]_{cyto}$, which is different from its effect on parsley or potato.

Pep-25 induces H_2O_2 accumulation

Addition of 160 nM Pep-25 to *A. thaliana* seedlings caused a transient and rapid release of H_2O_2. Increased extracellular H_2O_2 was detected as early as 5 min after addition of Pep-25, which peaked at about 60 min and subsequently declined to lower levels. The maximum concentration of extracellular H_2O_2 was 542 μM. A Pep-25 dependent dosage effect was observed in the seedlings of *A. thaliana*. The H_2O_2 increase was saturated by Pep-25 at the concentration of 200 nM. Pretreatment with scavengers of H_2O_2 (DMTU or DHC) almost abolished the Pep-25-induced increase of H_2O_2. This result confirms the production of H_2O_2 induced by Pep-25. The plasma membrane NADPH oxidase inhibitors DPI and pyridine pretreatment for 10 min prior to Pep-25 blocked the increase of cytosolic H_2O_2 by 62 and 54%, respectively. The extracellular Ca^{2+} chelator EGTA, the plasma membrane Ca^{2+} channel blocker $LaCl_3$, and RR pretreatment for 10 min also inhibited the Pep-25 induced increase of $[H_2O_2]_{cyto}$ (Fig. 3a). As shown in Fig. 3b, we also found that Pep-25/A19 likely Pep-25 can induce the increase of cytosolic H_2O_2, but Pep-25/A9 and Pep-13 cannot induce such effect, which is similar their effects to $[Ca^{2+}]_{cyto.}$

Induction of JA accumulation by Pep-25

The accumulation of JA could be measured in seedlings of *A. thaliana* treated with Pep-25. After 18 h treatment with 160 nM Pep-25, the accumulation of JA reached the maximal level of 28 ng/g DW (Fig. 4a). This level could be sustained at least for 24 h. Inhibitors of plasma membrane NADPH oxidase (DPI and pyridine), H_2O_2 scavengers

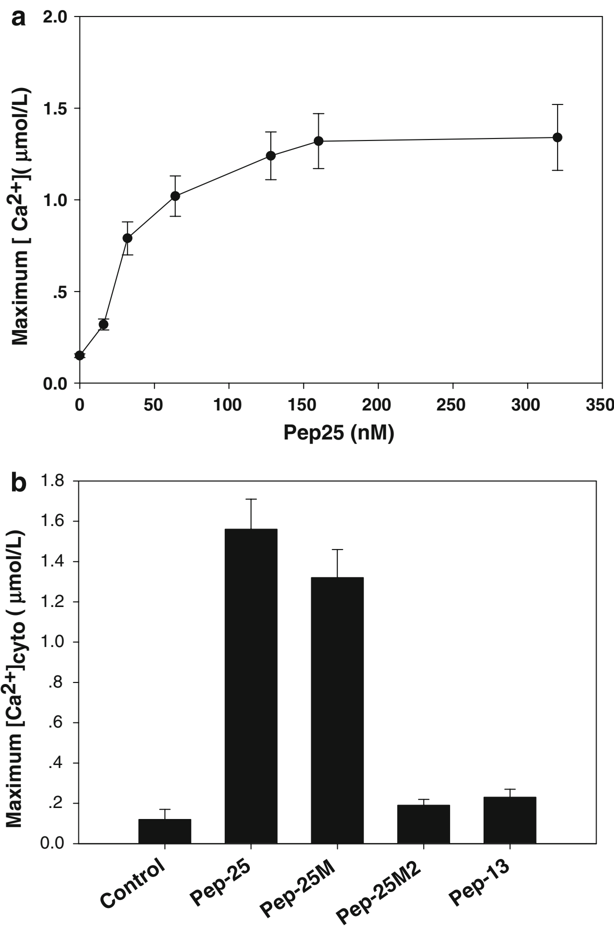

Fig. 2 Effects of Pep-25 and its derivatives on inducing the transient increase in $[Ca^{2+}]_{cyto}$ in *A. thaliana* seedlings. **a** Dose–response for maximum increase in $[Ca^{2+}]_{cyto}$ against Pep-25 concentration in the seedlings of *Arabidopsis thaliana*. **b** The different effects of 160 nM Pep-25 and its derivatives on the induction of the increase of $[Ca^{2+}]_{cyto}$ in *A. thalinana* seedlings. Data represent the mean ± SE from six experiments

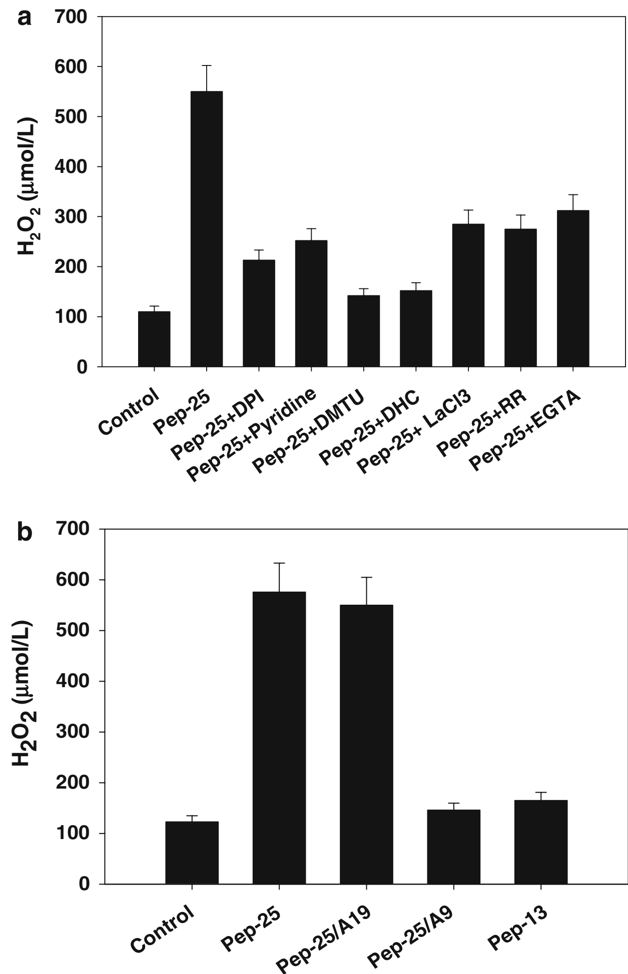

Fig. 3 Effects of Pep-25 and its derivatives on inducing the production of H_2O_2 in the seedlings of *A. thaliana*. **a** Effects of different inhibitors or scavengers on 160 nM Pep-25 induced H_2O_2 production. For inhibitors or scavengers treatment, *A. thaliana* was pretreated with inhibitors or scavengers (10 µM $LaCl_3$, 100 µM ruthenium red, 1 mM EGTA,10 µM DPI, 10 mM pyridine;,1 mM DMTU and 5 µM DHC) for 10 min and the production of H_2O_2 was measured after 60 min. Data represent the mean ± SE from six experiments. **b** Effect of 60-min treatment of 160 nM Pep-25, Pep-25/A19, Pep-25/A9 and Pep-13, respectively, on the induction of H_2O_2 production in *A. thaliana* seedlings. Data represent the mean ± SE from six experiments

(DMTU and DHC), calcium channel inhibitors ($LaCl_3$ and RR), and the extracellular Ca^{2+} chelator EGTA could all block the increase of JA accumulation to different degrees in Pep-25-treated seedlings of Arabidopsis (Fig. 4b). H_2O_2 treatment also directly induced the accumulation of JA in the seedlings (Fig. 4b)

Activation of the octadecanoid pathway by Pep-25

The *LOX, OPR3, PED*, *AOS* and *AOC* genes and allen oxidase (AOS) are the key genes/enzyme of the octadecanoid pathway involved in the synthesis of JA. The increase of transcriptional rate of the *LOX, OPR3, PED, AOS* and *AOC* genes indicated the activation of the octadecanoid pathway. Transcriptions of these genes could be detected within the first 3 h after Pep-25 treatment and reached a maximum after 12 h (data not shown). To investigate the roles of H_2O_2 and $[Ca^{2+}]_{cyto}$ in the defense responses by Pep-25, the effects of DPI, pyridine, DMTU, EGTA, $LaCl_3$, and RR on Pep-25 induction of ODA-related genes were investigated. Pretreatment with all of these reagents could suppress Pep-25-induced transcription of *LOX, OPR3, PED, AOS* and *AOC* genes in *A. thaliana* (Fig. 5). Pep-25 at 160 nM induced the gradual accumulation of AOS protein. This AOS accumulation reached the maximum level after 36 h (Fig. 6a). DMTU, DPI, pyridine, $LaCl_3$, RR, and EGTA all suppressed the Pep-25-induced increase of AOS protein (Fig. 6b). H_2O_2 treatment after 36 h also directly induced the accumulation of AOS protein (Fig. 6a, the right lane labeled with star).

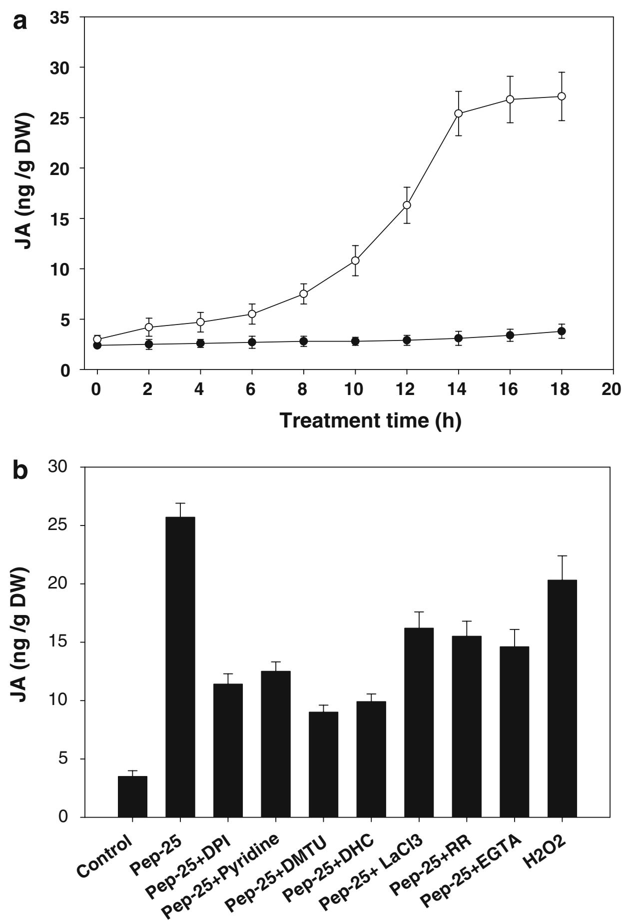

Fig. 4 Pep-25 at 160 nM induced the accumulation of JA in the seedlings of *A. thaliana*. **a** Time course of JA induced by Pep-25 at 160 nM in the seedlings of *A. thaliana*. **b** Effects of different inhibitors and scavengers on Pep-25 induced JA accumulation. For inhibitors or scavengers treatment, the seedlings of *A. thaliana* were pretreated with inhibitors or scavengers for 10 min and the product of JA was measured after 60 min. The concentrations of different inhibitors or scavengers used were as in Fig. 3. The concentration of H_2O_2 is 1 mM; Data represent the mean ± SE from six experiments

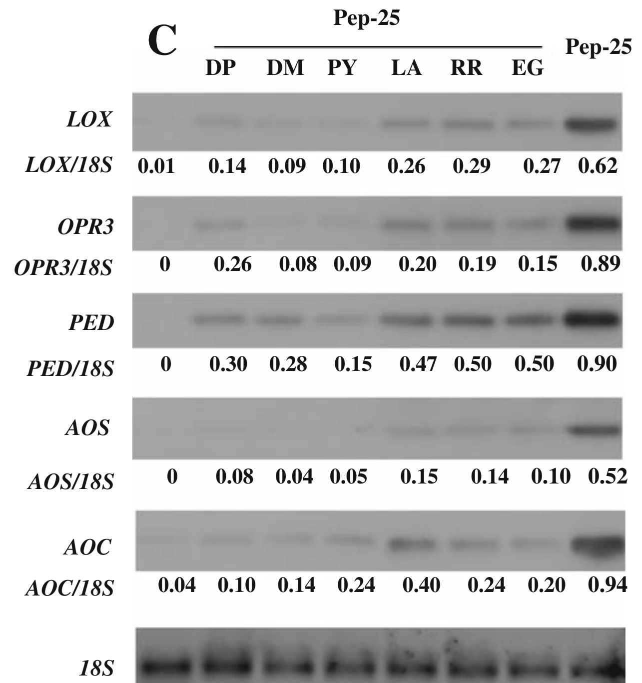

Fig. 5 Pep-25 at 160 nM induced the transcription of genes involved in the octadecanoid pathway. The seedlings of *A. thaliana* was pretreated with different inhibitors (DPI), H_2O_2 scavengers (DMTU and DHC) or Ca^{2+} channel inhibitors (EGTA, $LaCl_3$ and pyridine) for 10 min and then treated with Pep-25 for 12 h. The transcription of the *LOX, OPR3, AOS, AOC* and *PED* genes were assayed by Northern blotting, the *18S* was used as the control, the ratios among these genes were assayed by Imagine J software (http://rsbweb.nih.gov/ij/download.html). The concentrations of different inhibitors or scavengers used were as in Fig. 3

Discussion

To investigate the role of calcium ions during plant response to different stimulus, we used transgenic *A. thaliana* expressing the protein aequorin. The transgenic seedlings were confirmed by RT-PCR (Fig. 1a; Hu et al. 2004). For further confirmation that the aequorin protein is functional in transgenic *Arabidopsis* seedlings, we applied 37.5 mM $CaCl_2$ to *A. thaliana* expressing or not expressing aequorin. A strong luminescence was detected in the transgenic seedlings expressing aequorin. This phenomenon correlates with previous reports. Thus the transgenic seedlings provide a convenient way to measure transient changes in $[Ca^{2+}]_{cyto}$ induced by environmental stress.

Calcium ion fluxes have been generally accepted as a second messenger system to induce a sequence of physiological reactions and defense-related gene expression in plant and animal cells (Trewavas and Malho 1998; Blume et al. 2000). An increase of $[Ca^{2+}]_{cyto}$ is usually regarded as the result of the influx of extracellular Ca^{2+} across the plasma membrane and/or the efflux Ca^{2+} from an intracellular Ca^{2+} store (Cessna and Low 2001). In our system, the extracellular Ca^{2+} and intracellular Ca^{2+} pools provide the sources of Ca^{2+}-involved changes in $[Ca^{2+}]_{cyto}$. This can be concluded from the facts that a plasma membrane Ca^{2+} channel inhibitor ($LaCl_3$), and an intracellular membrane Ca^{2+}channel inhibitor (RR) both suppressed the Pep-25-induced increase of of $[Ca^{2+}]_{cyto}$ in the seedlings of *A. thaliana*. Therefore, these results indicated that extracellular and endogenous calcium pools were both required for the increase of cytosolic Ca^{2+}. It has been reported that elicitors can induce a biphasic Ca^{2+} peak, the first peak arising from the extracellular Ca^{2+} pool, the second peak arising from intracellular Ca^{2+} pool. In Pep-25-treated cells of *A. thaliana* we also found a biphasic Ca^{2+} peak.

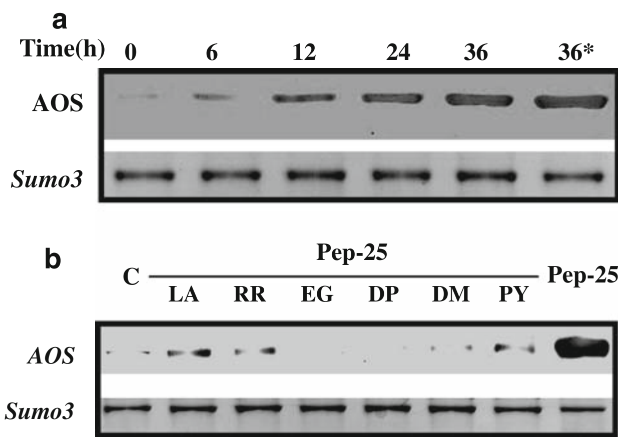

Fig. 6 Effect of Pep-25 at 160 nM and different inhibitors or scavengers on the accumulation of AOS protein. **a** The seedlings of *A. thaliana* were treated with 160 nM Pep-25 and the accumulation of AOS was assayed by Western blotting. **b** Effects of different inhibitors or scavengers on the accumulation of AOS in seedlings of *A. thaliana*. The seedlings were pretreated with different inhibitors and scavengers (*LA* $LaCl_3$, *RR* ruthenium red, *EG* EGTA, *DP* DPI, *DM* DMTU, *PY* pyridine) for 10 min and then with Pep-25 for 3 days. The accumulation of AOS was assayed by Western blotting. *The lane labeled with asterisk* shows the treatment with 1 mM H_2O_2 for 36 h. The concentrations of different inhibitors or scavengers used were as in Fig. 3

Our study also indicated that Ca^{2+} influx may regulate the release of H_2O_2 induced by Pep-25. Since $LaCl_3$ and RR could suppress the release of H_2O_2, the simplest interpretation of the data is that the increase of $[Ca^{2+}]_{cyto}$ is required for the production of H_2O_2. On the other hand, scavengers of H_2O_2 (DMTU and DHC) could not block the increase of cytosolic Ca^{2+} concentration. These results suggest that H_2O_2 is not necessary for the $[Ca^{2+}]_{cyto,}$ increase, and that the increase in $[Ca^{2+}]_{cyto}$ possibly acts upstream of the production of H_2O_2 induced by Pep-25. In soybean or tobacco cells, fungal elicitor induced the observed Ca^{2+} pulses were essential for the transduction of the oxidative burst signals (Chandra et al. 1997; Lecourieux et al. 2002). Blocking the increase of cytosolic calcium also suppressed the transcriptions of the *LOX, OPR3, PED, AOS, AOC* genes, the expression of AOS protein, and the accumulation of JA. These results indicated that the calcium influx from the extracellular calcium pool and the efflux from the intracellular calcium pool were both required for the induction of the octadecanoid pathway by Pep-25 in *A. thaliana*.

Pep-25 could induce the accumulation of H_2O_2, which is released in a biphasic pattern. Similar results were previously reported (Blume et al.2000; Grant and Loake 2000a, b; Grant et al. 2000). Most evidence to date support the conclusion that plasma membrane NADPH oxidase is responsible for the accumulation of H_2O_2. DPI and pyridine could efficiently block the accumulation of H_2O_2, which indicated that the accumulation of H_2O_2 results from the activation of plasma membrane NADPH oxidase in Pep-25-induced *A. thaliana* seedlings. $LaCl_3$ and RR both block Pep-25-induced accumulation of hydrogen peroxide suggesting that the increase of cytosolic calcium is required for the accumulation of hydrogen peroxide in Pep-25-induced *A. thaliana* seedlings. In the present study, Pep-25 also induced the transcription of genes related to the octadecanoid pathway and the synthesis of JA. Orozco-Cárdenas et al. (2000) have developed a model in which wounding induces OGA production which then leads to the accumulation of JA that, in turn, stimulates H_2O_2 accumulation. However, in tomato seedlings, wounding can only induce the synthesis of JA in cells of the vascular bundles only, not in mesophyll cells. Therefore, another possibility is that H_2O_2 is functionally upstream of the JA synthesis in Pep-25 treated *A. thaliana*. On the other hand, it is functionally downstream of JA synthesis in wounded tomato seedlings, since mechanical wounding and pathogen (or its deviated elicitor) attack are two different stimuli for *A. thaliana* (Thomma et al. 1998). Suppressing the cytosolic Ca^{2+} increase and the accumulation of hydrogen peroxide also blocked the activation of the octadecanoid pathway and the accumulation of JA. DMTU and DHC scavenged Pep-25-induced accumulation of H_2O_2 and also suppressed the transcription of defense-related genes and the accumulation of JA. These results show that H_2O_2 is required for the Pep-25-induced accumulation of JA and functions upstream of the octadecanoid pathway during the response of *A. thaliana* to elicitor stimulation.

In summary, we provided evidence to support the conclusion that the production of H_2O_2 and the increase of cytosolic Ca^{2+} are required for Pep-25-induced accumulation of JA and the activation of the octadecanoid pathway, including the transcriptions of *LOX, OPR3, PED, AOS, AOC* genes and the accumulation of AOS. In addition, the increase of cytosolic Ca^{2+} functions upstream of the production of H_2O_2 and JA. Finally, the source of Ca^{2+} that increase cytosolic Ca^{2+} levels came from both, extracellular and intracellular Ca^{2+} pools.

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References

Alvarez ME, Pennell RI, Meijer P-J, Ishikawa A, Dixon RA, Lamb C (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell 92:773–784

Amborabe BE, Bonmort J, Fleurat-Lessard P, Roblin G (2008) Early events induced by chitosan on plant cells. J Exp Bot 59:2317–2324

Blume B, Nürnberger T, Nass N, Scheel D (2000) Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. Plant Cell 12:1425–1440

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Cessna SG, Low P (2001) Activation of the oxidative burst in aequorin-transformed *Nicotiana tabacum* cells is mediated by protein kinase- and anion channel-dependent release of Ca^{2+} from internal stores. Planta 214:126–134

Chandra S, Stennis M, Low PS (1997) Measurement of Ca^{2+} fluxes during elicitation of the oxidative burst in aequorin-transformed tobacco cells. J Biol Chem 272:28274–28280

Chen Z, Silva H, Klessig DF (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science 262:1883–1886

Davies DR, Bindschedler LV, Strickland TS, Bolwell GP (2006) Production of reactive oxygen species in *A. thaliana* cell suspension culture in response to an elicitor from *Fusarium oxysporum*: implication for basal resistance. J Exp Bot 57:1817–1827

Desikan R, Hancock JT, Coffey MJ, Neill SJ (1996) Generation of active oxygen in elicited cells of *A. thaliana* is mediated by a NADPH oxidase-like enzyme. FEBS Lett 382:213–217

Ebel J, Cosio EG (1994) Elicitors of plant defense responses. Int Rev Cytol 148:1–36

Ellis C, Karafyllidis I, Turner JG (2002) Constitutive expression of jasmonate signalling in an *Arabidopsis* mutant correlates with enhanced resistance to *Erysiphe cichoracearum*, *Pseudomonas syringae*, and *Myzus persicae*. Mol Plant Microbe Interact 15:1025–1030

Fellbrich G, Blume B, Brunner F, Hirt H, Kroj T, Ligterink W, Romanski A, Nürnberger T (2000) *Phytophthora parasitica* elicitor-induced reactions in cells of *Petroselinum crispum*. Plant Cell Physiol 41:692–701

Grant JJ, Loake GJ (2000a) Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. Plant Physiol 124:21–30

Grant JJ, Loake GJ (2000b) Evidence of reactive oxygen intermediates and cognate redox signaling in disease resistance. Plant Physiol 124:21–29

Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J (2000) The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J 23:441–450

He Y, Fukushige H, Hildebrand DF, Gan S (2002) Evidence supporting a role of jasmonic acid in *Arabidopsis* leaf senescence. Plant Physiol 128:876–884

Hu X, Neill SJ, Cai W, Tang Z (2004) Induction of defense gene expression by oligogalacturonic acid requires increase in both cytosolic calcium and hydrogen peroxide in *A. thaliana*. Cell Res 14:234–240

Jabs T, Tschope M, Colling C, Hahlbrock K, Scheel D (1997) Elicitor-stimulated ion fluxes and O_2^- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. Proc Natl Acad Sci USA 94:4800–4805

Johnson CH, Knight MR, Kondo T, Masson PH, Sedbrook JC, Haley A, Trewavas AJ (1995) Circadian oscillations of cytosolic and chloroplastidic free calcium in plants. Science 269:1863–1865

Knight MR, Campbell AK, Smith SM, Trewavas AJ (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. Nature 352:524–526

Knight H, Trewavas AJ, Knight MR (1997) Calcium signaling in *A. thaliana* responding to drought and salinity. Plant J 12:1067–1078

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 48:251–275

Lecourieux D, Mazars C, Pauly N, Ranjeva R, Pugin A (2002) Analysis and effects of cytosolic free calcium increases in response to elicitors in *Nicotiana plumbaginifolia* cells. Plant Cell 14:2627–2641

Navazio L, Moscatilelllo R, Bellincampi D, Baldan B, Meggio F, Brini M, Bowler C, Mariani P (2002) The role of calcium in oligogalacturonide-activated signalling in soybean cells. Planta 215:596–605

Nürnberger T, Nennstiel D, Jabs T, Sacks WR, Hahlbrock K, Scheel D (1994) High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. Cell 78:449–460

Ogasawara Y, Ogasawara Y, Kaya H, Hiraoka G, Yumoto F, Kimura S, Kadota Y, Hishinuma H, Senzaki E, Yamagoe S, Nagata K, Nara M, Suzuki K, Tanokura M, Kuchitsu K (2008) Synergistic activation of the Arabidopsis NADPH oxidase AtrbohD by Ca^{2+} and phosphorylation. J Biol Chem 283:8885–8892

Orozco-Cárdenas ML, Narváez-Vásquez J, Ryan CA (2000) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. Plant Cell 13:179–191

Royo J, Leon J, Vancanneyt G, Albar JP, Rosahl S, Ortego F, Castanera P, Sanchez-Serrano JJ (1999) Antisense-mediated depletion of a potato lipoxygenase reduces wound induction of proteinase inhibitors and increases weight gain of insect pests. Proc Natl Acad Sci USA 96:1146–1151

Sai J, Johnson CH (1999) Different circadian oscillators control Ca^{++} fluxes and *Lhcb* gene expression. Proc Natl Acad Sci USA 96:11659–11663

Sai J, Johnson CH (2002) Dark-stimulated calcium ion fluxes in the chloroplast stroma and cytosol. Plant Cell 14:1279–1291

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Thomma BPHJ, Eggermont K, Penninckx IAMA, Mauch-Mani B, Vogelsang R, Cammue BPA, Broekaert WF (1998) Seperate jasmonate-dependent and salicylate-dependent defense-response pathway in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc Natl Acad Sci USA 95:15107–15111

Trewavas AL, Malho R (1998) Ca^{2+} signaling in plant cells: the big network. Curr Opin Plant Biol 1:428–433

Turner JG, Ellis C, Devoto A (2002) The jasmonate signal pathway. Plant Cell 14:S153–S164

Wasternack C, Stenzel I, Hause G, Kutter C, Maucher H, Neumerkel J, Feussner I, Miersch O (2006) The wound response in tomato—role of jasmonic acid. J Plant Physiol 163:297–306

White PJ, Broadley MR (2003) Calcium in plants. Ann Bot 92:487–511