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Expression of the ipomoelin gene from sweet potato is regulated by dephosphorylated proteins, calcium ion and ethylene

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ABSTRACT

A wound-inducible cDNA, ipomoelin (IPO) was isolated from the subtraction library of sweet potato (*Ipomoea batatas* cv. Tainung 57) and used as a molecular probe to investigate the transduction pathway of wounding signal within plant cells. Following mechanical wounding of the leaves of sweet potato, IPO mRNA accumulation peaked at 6 h and then continuously declined. However, IPO gene expression in the apical unwounded leaves began at 6 h after wounding and continued for a further 10 h. Besides mechanical wounding, methyl jasmonate (MeJA) was identified as a signal transducer leading to the accumulation of IPO mRNA. Treatment with salicylic acid reduced the production of IPO mRNA, further supporting the involvement of the octadecanoid pathway in the signal transduction of wounding in sweet potato. In addition, ethylene was involved in the signal pathway and induced the expression of the IPO gene. Furthermore, the application of okadaic acid, a protein phosphatase inhibitor, blocked the accumulation of IPO mRNA induced by MeJA or ethylene, indicating that activation of the IPO gene by both MeJA and ethylene was via dephosphorylated proteins. The presence of a calcium ion chelator or channel blockers also inhibited the expression of the IPO gene after wounding. However, investigation by confocal scanning microscopy further pointed out that mechanical wounding rather than the application of MeJA induced the accumulation of the calcium ion. These results may indicate that the calcium ion is also involved in the activation of IPO mRNA. In addition, wounding signals the accumulation of calcium ion first and then stimulates the biosynthesis of MeJA in sweet potato. Hence, the reaction sequence of signal transducers, including the calcium ion, MeJA and protein kinase/phosphatase, in the wounding signalling pathway of sweet potato is suggested in this report.

Key-words: calcium ion; ethylene; protein phosphorylation; sweet potato; wound induction.

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Abbreviations: ABA, abscisic acid; CEPA, 2-chloroethyl phosphonic acid; DIECA, diethyldithiocarbamic acid; EGTA, ethylene glycol tetraacetic acid; IPO, ipomoelin; JA, jasmonic acid; MeJA, methyl jasmonate; OGA, oligogalacturonic acid; OKA, okadaic acid; PGA, polygalacturonic acid; SA, salicylic acid; STA, staurosporine.

INTRODUCTION

Plants have developed defensive systems as survival mechanisms. External stimuli recognized as attack signals by plants may start the cascades of signal transduction within cells directly or through the receptors in the plasma membrane (Meindl, Boller & Felix 1998). In most cases, certain genes are related to defence systems and wound healing can be activated to protect plants against attack by herbivores and pathogens (Bowles 1990).

Several external signals have been reported to be capable of activating defensive responses in plants. Mechanical wounding and herbivorous attack can simulate the production of systemin, an 18-amino acid polypeptide, which activates approximately 20 genes involved in signal transduction pathways and defensive systems in tomatoes (Bergey, Orozco-Cardenas & Ryan 1999). Oligogalacturonic acid (OGA) produced by mechanical or enzymatic disruption of cell wall components polygalacturonic acid (PGA) induces wound-responsive gene expression (Benhamou, Chamberland & Pauze 1990; Bergey & Ryan 1999) and enhances the phosphorylation of tomato plasma membrane proteins (Farmer, Pearce & Ryan 1989). Furthermore, chitosan, a fungal-derived oligosaccharide, can increase the expression of proteinase inhibitor genes in tomatoes (Doares *et al*. 1995b; Howe *et al*. 1996) and wound-responsive genes in *Arabidopsis thaliana* (Rojo, León & Sánchez-Serrano 1999). These external signals also enable plants to induce the expression of the defensive gene in tissues distant from the primary wound sites (Doares *et al*. 1995b; Rojo *et al*. 1999).

Following the primary external signals, several cell components are involved in the responses and transduction of wounding signals. Systemin was believed to trigger the release of linolenic acid by stimulating the activity of a

lipase in the plasma membrane,and thus increasing the production of methyl jasmonate (MeJA) via the octadecanoid pathway using linolenic acid as a substrate (Farmer & Ryan 1992). The rise in endogenous levels of MeJA following wounding activates wound-inducible genes (Creelman, Tierney, & Mullet 1992; Peña-Cortés *et al*. 1993) and the application of MeJA exogenously can induce wound-responsive genes in undamaged plants (León *et al*. 1998; Rojo *et al*. 1998, 1999).

Additionally, protein kinases and phosphatases were also involved in the transduction of wounding signal and regulated jasmonic acid (JA)-dependent and -independent gene expression (Lee *et al*. 1998; Rojo *et al*. 1998). Membrane proteins of tomato were first found to be phosphorylated by protein kinases during the addition of PGA (Farmer *et al*. 1989). Wounding, systemin and OGA increased the activity of myelin basic protein kinase in tomato leaves (Startmann & Ryan 1997). Wounding also rapidly induced the activities of MMK4, a mitogen-activated protein (MAP) kinase from alfalfa, WIPK, a 46 kDa MAP kinase from tobacco and WAPK, a ser/thr protein kinase from tobacco (Usami *et al*. 1995; Bögre *et al*. 1997; Lee *et al*. 1998). These results indicate that protein phosphorylation cascades are important in wound signal transduction.

Upon different stimuli, such as cold-shock (Knight *et al*. 1991), light (McAinsh *et al*. 1995), elicitors (Knight *et al*. 1991) and hormones (Irving, Gehring & Parish 1992; Moyen *et al*. 1998), the concentration of cytosolic Ca^{2+} within plant cells was increased. This phenomenon resulted from the uptake of Ca^{2+} from the extracellular space via channels in the plasma membrane and endoplasmic reticulum or vacuoles (Gilroy *et al*. 1991). The increase in the level of cytosolic Ca^{2+} with calmodulin may regulate the activities of numerous proteins, including kinases, phosphatases and ion transporters (Enslen *et al*. 1996; Sheen 1996; Qui *et al*. 1998; Bergey & Ryan 1999). Ca^{2+}/calmodulin also differentially controlled JA-dependent and -independent wound signal pathways in *Arabidopsis thaliana* (León *et al*. 1998). Additionally, Ca^{2+} also signalled the expression of genes coding for sporamin and β-amylase of sweet potato (Ohto *et al*. 1995).

Ethylene is a simple and readily diffusible plant hormone that regulates plant growth and development, including germination, senescence, abscission and fruit ripening. The response of plants to ethylene was mediated by specific ethylene receptors (for review see Ciardei & Klee 2001), which activated a cascade pathway involving Ca^{2+} (Raz & Fluhr 1992) and reversible protein phosphorylstion (Raz & Fluhr 1993). Subsequently, a wide range of genes involved in pathogen defence, wound signalling and fruit ripening was regulated by ethylene (O'Donnel *et al*. 1996, 2001; Watanabe, Fujita & Sakai 2001).

Little is known about the signal transduction in sweet potato and furthermore, the reaction sequence of signal transducers in the wounding signalling pathway of plants has not been fully studied. Therefore, this investigation uses the expression of a wound-inducible gene, ipomoelin (IPO), isolated from the subtraction library of sweet potato, as a molecular probe to analyse the effects of wounding signals, signal transducers, Ca^{2+} and plant hormones on the signal transduction of sweet potato. In particular, the response sequence of these signal transducers after wounding is deduced.

MATERIALS AND METHODS

Plant materials and assay conditions

Sweet potato (*Ipomoea batatas* cv. Tainung 57) plants were vegetatively propagated from cuttings and grown in a controlled environment (16 h/25 °C day, 8 h/22 °C night, humidity 70%, light 100 μmol photons m^{-2} s^{-1}). Plants with six to eight fully developed leaves were used in this study. During the time-course study, local injury was defined as the mechanical wounding at the third fully expanded leaf counted from the terminal bud and the second unwounded leaf from the terminal bud in the same plant was treated as the systemic leaf. Plants were wounded with forceps by pressing around the edges of leaves. For the rest of this study, the second and third fully expanded leaves counted from the terminal bud were excised and their petiole cuts were immersed in the chemicals as indicated. Reagents were added to reach the final concentration of 50 μM MeJA, 0.5 μM okadaic acid (OKA), 1 μM staurosporine (STA), 10 μM salicylic acid (SA), 100 μM ABA, 1 mM 2-chloroethyl phosphonic acid (CEPA), 50 mM diethyldithiocarbamic acid (DIECA) and Ca^{2+} signal blockers, which include 1 mM ethylene glycol tetraacetic acid (EGTA), 10 mM LiCl and 100 μM neomycin in each treatment, respectively. For the ethylene assays, 1 × MS (Murashige & Skoog 1962; pH 5.8) was used as a solution base instead of water to provide a suitable pH for CEPA to release ethylene. All chemicals were purchased from Sigma (St Louis, MO, USA), except for MeJA, which was obtained from Bedoukian Research Inc., Danbury, CT, USA.

RNA isolation and analysis

Total RNA was isolated from leaves ground in liquid N_2 by using a phenol-based extraction procedure, as described by Chomczynski & Sacchi (1987) except that guanidium-HCl was used instead of guanidium thiocyanate. The quantity of RNA was estimated by a spectophotometer and its quality was analysed by agarose gel with formaldehyde. Ten micrograms of total RNA was separated on formaldehyde-agarose gels, transferred to nylon membranes and hybridized with radiolabelled probes following standard laboratory procedures (Sambrook, Fritsch & Maniatis 1989). The radiolabelled probe for IPO mRNA was produced by polymerase chain reaction using the template of IPO cDNA isolated from the subtraction library in this study. The radiolabelled probe for ribosomal RNA was also produced from polymerase chain reaction using the 25S rDNA template from tobacco. Both IPO mRNA and rRNA were saturated with the excess radiolabelled probes (data not shown). Prehybridization was performed in 5 × SSPE

(0.05 M NaH_2PO_4 (pH 6.8), 0.9 M NaCl and 5 mM EDTA), 0.5% SDS, 5 × Denhard's solution (0.1% Ficoll, 0.1% bovine serum albumin, 0.1% polyvinyl pyrrolidine) at 65 °C for 1 h, and upon the addition of the radiolabelled probe, hybridization was performed under the same conditions for another 16 h. Blots were washed twice in 0.1 × SSPE and 0.1% SDS at 65 °C for 15 min. Hybridization was performed with the IPO-radiolabelled probe first and then with the rRNA radiolabelled probe in the same membrane after stripping its previous labelling. Radioactive blots were displayed on a Molecular Dynamics phosphoimager (Sunnyvale, CA, USA) and their autoradiographs were printed using a Kodak XLS 8600 PS printer (Rochester, NY, USA). The relative radioactivity of both the IPO mRNA and rRNA bands was quantified using the Molecular Dynamics IMAGEQUANT software.

Construction of subtraction cDNA library

The procedure for isolating wound-inducible mRNA using the paramagnetic beads with oligo(dT) from Dynabeads (Dynal. Inc., Lake Success, NY, USA) is described (Lambert & Williamson 1993). Following the isolation of the wound-inducible mRNA, Dynabead with oligo(dT) was added again to bind the wound-inducible mRNA. The synthesis of the first strand cDNA(W+) on beads, the digestion of the single-strand poly(dT) on beads and the removal of mRNA from the bead-bound cDNA(W+) were similarly performed. After the bead-bound cDNA(W+) were washed twice with 5 × TdT buffer (500 mM cacodylate buffer pH 6.8, 5 mM $CoCl_2$, 0.5 mM DTT), the poly(dC) tail at the 3′ end of cDNA on beads was formed by using 50 units of the terminal deoxynucleotidyl transferase (Promega, Madison, WI, USA) with 1 × TdT buffer and 1 mM dCTP at 37 °C for 30 min. The beads were again washed twice with 10 × polymerase chain reaction (PCR) buffer (100 mM Tris-HCl pH 8.8, 15 mM $MgCl_2$, 500 mM KCl, 0.1% TritonX-100) and cDNA(W+) was amplified and released from the beads by the PCR, including 1 × PCR buffer, 4 mM $MgCl_2$, 0.4 mM dNTP, 15 pmoles of primers CCTCTAGATTTTTTTTTTTTTTTTTTTTT and TCAAGCTTGGGGGGGGGG and 2 units of Deep vent DNA polymerase (New England Biolabs, Beverly, MA, USA). PCR products digested by *Hin*dIII and *Xba*I were ligated to the corresponding sites of vector pTZ18u (Jeng, Gardner & Gumport 1992) and the subtraction cDNA library was constructed.

Ca^{2+} detection by confocal scanning microscope

Leaf pieces of sweet potato were cut into small pieces (0.5 cm × 0.5 cm) and immersed in 1 × MS solution for 12 h. Leaf pieces were then transferred to 0.55 M mannitol with fluorescent dye fluo-3 AM (Sigma F-6142) at a final concentration of 1 μM. Fluo-3 AM, which binds Ca^{2+} specifically, is highly lipophilic and easily crosses the plasma membrane (Tsien 1981; Kao, Harootunian & Tsien 1989). The extra fluo-3 AM was removed by washing leaf pieces with 0.55 M mannitol for 1 h. Leaf pieces were wounded by needle, or 50 μM MeJA was added. A Leica TCS-SP2 confocal laser scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) with excitation wavelength at 488 nm and emission wavelength between 500 and 535 nm was then used to investigate the leaf pieces with fluo-3 AM. Images were taken by microscope at 0, 60, 120, 180 and 240 s after the images of leaf pieces were focused. It took 90 s for the sample pretreatment, including mechanical wounding or MeJA application, before leaf pieces were analysed by microscope.

RESULTS

Construction of subtraction cDNA library

The subtraction cDNA library with the wound-inducible genes was constructed using the paramagnetic beads with oligo(dT). A clone from this cDNA library was identified as a MeJA-inducible gene, IPO (Imanishi *et al*. 1997), after comparison with the database in GenBank. To further understand, the transduction process of the wound signals during activation of the IPO gene, this study analysed several factors involved in signal transduction, namely: (1) external stimulus, such as mechanical damage; (2) signal transducers including MeJA, protein phosphatases and protein kinases; (3) plant hormone including ABA, SA and ethylene; and (4) secondary messenger, Ca^{2+}.

After the plants were treated with these chemicals, their total RNAs were isolated and analysed by Northern blotting to detect the amount of IPO mRNA and rRNA sequentially. The radiolabelled probes for IPO mRNA and rRNA were produced by PCR using the templates of isolated IPO cDNA and tobacco rDNA, respectively. Both IPO mRNA and rRNA were saturated with the excess radiolabelled probes. In particular, the amount of rDNA probe used in this study can saturate the rRNA up to 40 μg of the total RNA, although 10 μg of total RNA was always used in this study (data not shown).

Mechanical damage induces IPO gene expression

The time-course of IPO gene expression upon wounding was analysed. Mechanical wounding by forceps at the third fully developed leaf from the terminal bud was studied as a local injury, and the induction of the IPO gene at the second fully expanded and unwounded leaf in the same plant was considered a systemic effect. Total RNAs of both the local and systemic leaves were isolated after wounding and analysed by Northern blotting to determine the amount of IPO mRNA and rRNA (Fig. 1). The value of IPO mRNA was adjusted by its corresponding amount of rRNA to achieve loading equality. Following the adjustment by rRNA, the amount of IPO mRNA from the unwounded leaves was treated as the normalized reference, with a value of one, for determining the relative amount of IPO mRNA in other treatments. In locally injured leaf, the

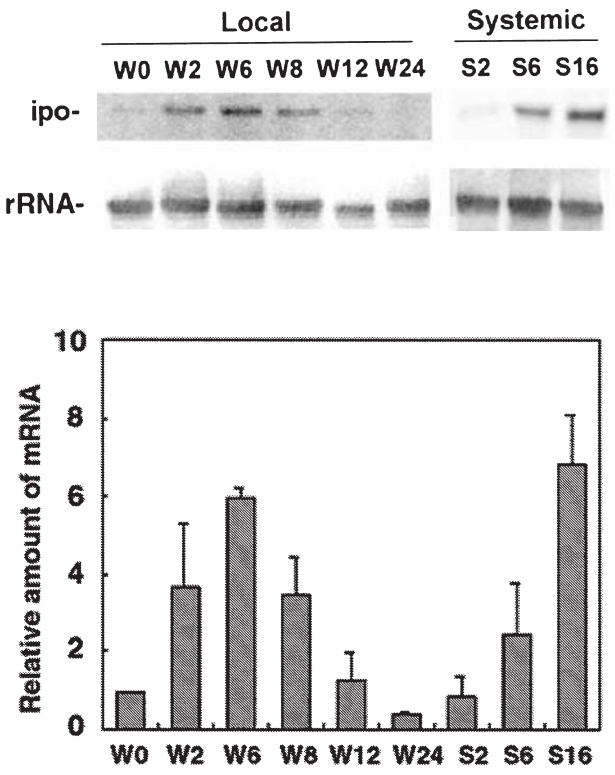
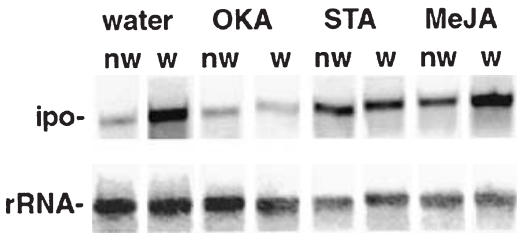


Figure 1. Time-course expression of IPO gene upon wounding. Forceps wounding of the third fully expanded leaf counted from the terminal bud was treated as a local injury and the unwounded second leaf on the same plant was treated as the systemic leaf. The total RNAs of the local leaves were isolated at 0, 2, 6, 8, 12 and 24 h after wounding (W0, W2, W6, W8, W12 and W24) and those of the systemic leaves were isolated at 2, 6 and 16 h after wounding (S2, S6 and S16). Their total RNAs were analysed by Northern blotting to detect the level of IPO mRNA (ipo) and rRNA sequentially. The value of IPO mRNA was adjusted by its corresponding amount of rRNA for equality of loading. After the adjustment by rRNA, the amount of IPO mRNA from the unwounded leaves (W0) was treated as the normalized reference, with a value of one, for determining the relative amount of IPO mRNA. Data are presented as the mean ± standard deviation from three independent experiments.

expression of the IPO gene increased 2 h after wounding, rose to six times that of the unwounded leaf at 6 h after wounding and reduced to its background level 12 h later (Fig. 1). Meanwhile, the accumulation of IPO mRNA in the apical unwounded leaf (systemic leaf) gradually increased to seven times more than that in the unwounded leaf by 16 h after wounding (Fig. 1). The above phenomenon may imply that the IPO gene was expressed not just locally but also systemically upon wounding and that this gene was induced faster in the local leaf than in the systemic leaf.

MeJA induces IPO gene expression via dephosphorylated proteins

MeJA is known to be able to induce the expression of the IPO gene (Imanishi *et al*. 1997), but its relation to protein phosphorylation and dephosphorylation is unclear. The petiole cuts of the excised leaves were placed in OKA as a protein phosphatase inhibitor, STA as a protein kinase inhibitor, MeJA, or water as a control for 12 h. The wounding effect owing to the separation of leaf-petiole cuttings from plants should be diminished during this long incubation period (Fig. 1). Some of the leaves, which had been immersed in each solution for 12 h, were then wounded by forceps, while the others remained unwounded. After another 6 h, the total RNAs of the wounded and unwounded leaves were analysed by Northern blotting. The presence of STA or MeJA enhanced the expression of the IPO gene, even in the unwounded leaves (Fig. 2). Meanwhile, even in wounded leaves, the appearance of OKA reduced the expression of the IPO gene (Fig. 2). These results indicated that MeJA and dephosphorylated protein(s) were involved in the transduction process of induc-



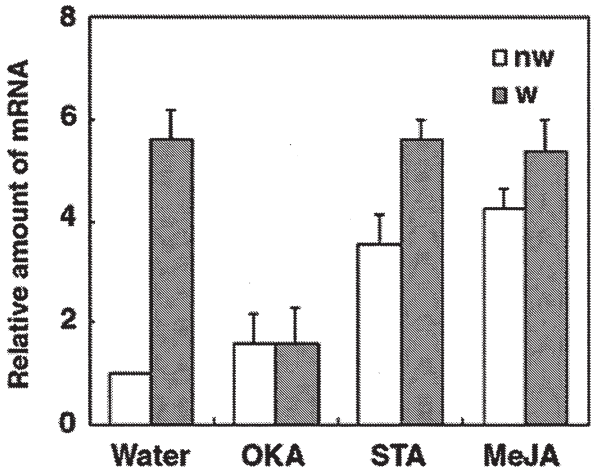


Figure 2. Influence of OKA, STA and MeJA on the expression of IPO gene. The petiole cuts of the second and third fully expanded leaves from the terminal bud were immersed in water, 0.5 μM OKA, 1 μM STA and 50 μM MeJA, respectively, for 12 h. OKA is a protein phosphatase inhibitor and STA is a protein kinase inhibitor. Some of the leaves in each solution were wounded using forceps, whereas the others remained unwounded. Six hours after wounding, the total RNAs of both the wounded (w) and unwounded (nw) leaves were analysed by Northern blotting to detect IPO mRNA (ipo) and rRNA sequentially. The value of IPO mRNA was adjusted by its corresponding amount of rRNA for equality of loading. After the adjustment by rRNA, the amount of IPO mRNA from the unwounded leaves in water was treated as the normalized reference, with a value of one, for determining the relative amount of IPO mRNA. Data are presented as the mean ± standard deviation from three independent experiments.

ing the IPO gene, whereas phosphorylated protein(s) interfered in IPO gene expression.

Since MeJA and protein dephosphorylation are important for stimulating the IPO gene, this work studied the order in which these factors participate in the wound signal transduction. The leaf-petiole cuttings were first placed in OKA solution for 3 h, then MeJA was added to the OKA solution for another 12 h before IPO mRNA was measured (OKA + MeJA in Fig. 3). The induction of IPO mRNA by MeJA in the presence of OKA was inhibited. Without OKA the expression of IPO mRNA stimulated by MeJA was expected (MeJA in Fig. 3). Furthermore, OKA alone inhibited the expression of IPO mRNA (OKA in Fig. 3). Therefore, the presence of OKA interfered with the function of MeJA in stimulating the expression of the IPO gene. These results indicate that the wounding signals were transferred to MeJA and then passed to dephosphorylated protein(s) causing the activation of IPO gene.

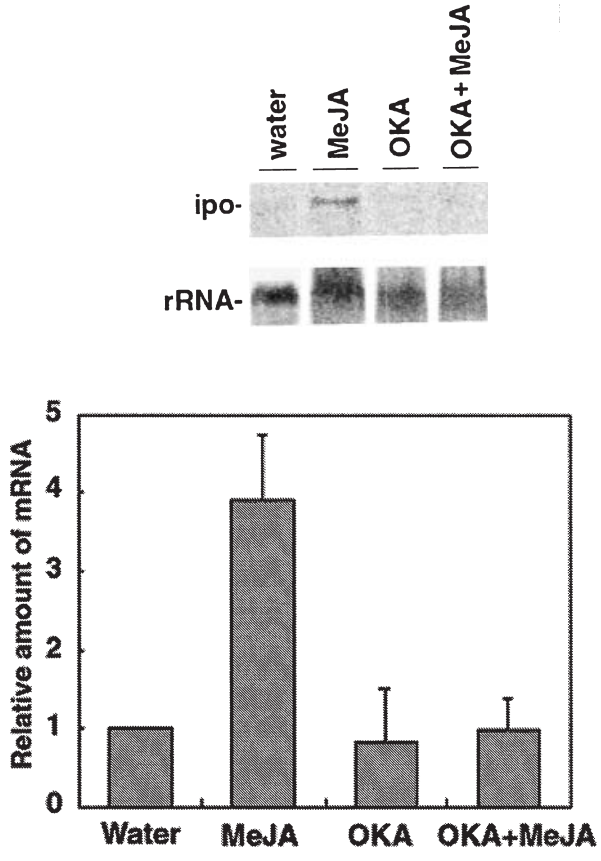


Figure 3. Determination of the signal transduction order of MeJA and dephosphorylated proteins. The petiole cuts of the second and third fully expanded leaves from the terminal bud were immersed in 0.5 μM OKA for 3 h. MeJA was then added to a final concentration of 50 μM and incubated for another 12 h (OKA + MeJA). The control leaves were incubated separately in water, MeJA and OKA for 15 h. Total RNAs were analysed by Northern blotting to detect IPO mRNA (ipo) and rRNA sequentially. The value of IPO mRNA was adjusted by its corresponding amount of rRNA for equality of loading. After the adjustment by rRNA, the reaction with water alone was treated as the normalized reference, with a value of one, for determining the relative amount of IPO mRNA. Data are presented as the mean ± standard deviation from three independent experiments.

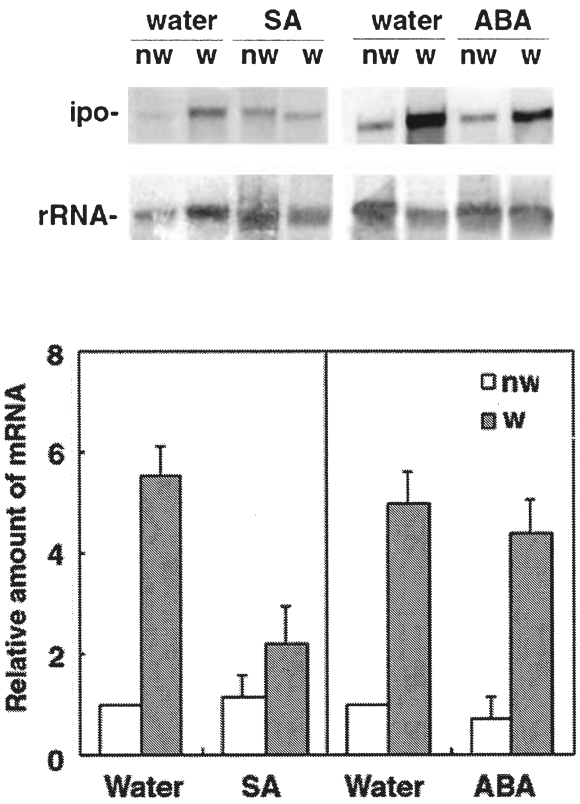


Figure 4. Effects of SA and ABA on IPO gene expression. The petiole cuts of the second and third fully expanded leaves from the terminal bud were immersed in water, 10 μM SA and 100 μM ABA, respectively, for 12 h. Some of the leaves in each solution were then wounded with forceps and the remainder were left unwounded. Six hours after wounding, the total RNAs of both wounded (w) and unwounded (nw) leaves were analysed by Northern blotting to detect IPO mRNA (ipo) and rRNA sequentially. The value of IPO mRNA was adjusted by its corresponding amount of rRNA for equality of loading. After the adjustment by rRNA, the reaction with the unwounded leaves in water was treated as the normalized reference, with a value of one, for determining the relative amount of IPO mRNA. Data are presented as the mean ± standard deviation from three independent experiments.

Effects of ABA and SA on IPO gene expression

The petiole cuts of the excised leaves were placed in 100 μM ABA or 10 μM SA for 12 h and then some of these leaves immersed in each solution were mechanically wounded using forceps. After another 6 h incubation, the total RNAs of the wounded and unwounded leaves were analysed by Northern blotting (Fig. 4). In the unwounded leaves, the amount of IPO mRNA following the treatment with either hormone was similar to that with water only. However, in the wounded leaves, leaves treated with SA displayed less IPO mRNA than those treated with water, whereas ABA did not alter the accumulation of IPO mRNA (Fig. 4). These

results implied that SA interfered with the expression of IPO gene in sweet potato, whereas ABA had no influence.

Ca^{2+} is involved in the activation of IPO gene

Ca^{2+} was suggested as a secondary messenger during signal transduction within plants. External signals may stimulate the opening of Ca^{2+} channels in the plasma membrane first, followed by the opening of Ca^{2+} channels in the vacuolar membrane and finally activate the Ca^{2+}-dependent proteins (Allen & Sanders 1994; Ward & Schroeder 1994). Therefore, Ca^{2+} chelator EGTA, vacuolar Ca^{2+} channel blockers LiCl and neomycin (Frankling-Tong *et al.* 1996) were used in this study. The petiole cuts of the excised leaves were immersed in these solutions individually for 12 h and then the edges of some leaves were wounded using forceps. At 6 h after wounding, the total RNAs of the wounded and unwounded leaves from each solution were isolated and analysed by Northern blotting. Leaves treated with the Ca^{2+} blockers and chelator all displayed less IPO mRNA than those treated with water alone in both the wounded and unwounded leaves (Fig. 5). This phenomenon may imply that the induction of IPO gene by mechanical wounding includes Ca^{2+}.

MeJA does not induce the accumulation of Ca^{2+}

The presence of Ca^{2+} in the cells stained with fluo-3 AM was investigated by confocal scanning microscopy. The induction of Ca^{2+} was clearly visualized several minutes after mechanical wounding (Fig. 6A). However, the amount of fluorescent signals did not increase but was gradually quenched by the scanning of laser beam when MeJA by itself was added (Fig. 6B). These results indicate that the accumulation of Ca^{2+} was induced by mechanical wounding but not the application of MeJA. Furthermore, the wounding signal induced the accumulation of Ca^{2+} first and then stimulated the biosynthesis of MeJA in sweet potato.

Ethylene is involved in the induction pathway of IPO gene

Ethylene has been reported to be an inducer of wound-responsive genes in plants (León, Rojo & Sánchez-Serrano 2001) and the effect of ethylene on the expression of IPO gene was examined. The petiole cuts of the excised leaves were immersed in 1 × MS with OKA, STA, neomycin, EGTA and DIECA as a jasmonate biosynthesis inhibitor (Menke *et al.* 1999), respectively, for 16 h. Subsequently, 1 mM CEPA was added to each solution to produce ethylene (Min & Bartholomew 1996) and incubated for another 6 h before their RNAs were harvested. The pH 5.8 of the 1 × MS solution allows CEPA to release ethylene. Northern blotting results reveal that ethylene alone enhanced IPO gene expression (Fig. 7). OKA, a protein phosphatase inhibitor, reduced the expression of the IPO gene even in the presence of ethylene, and STA, a protein kinase inhibitor, did not influence the IPO mRNA expression induced by ethylene. This phenomenon may indicate that the dephosphorylated protein(s) was involved in the ethylene signal transduction in activating the IPO gene. Furthermore, in the presence of neomycin, a vacuolar Ca^{2+} channel blocker, ethylene was unable to induce IPO gene expression, but ethylene did induce the expression of the IPO gene in the presence of EGTA, a Ca^{2+} chelator (Fig. 7). This result indicates that the action of ethylene may require the Ca^{2+} from vacuoles but not from the extracellular space. To examine the relationship between ethylene and MeJA, the application of DIECA, a MeJA biosynthesis inhibitor, did not interfere with the expression of the IPO gene induced by ethylene and this may indicate that the stimulation of ethylene in IPO gene expression is MeJA-independent.

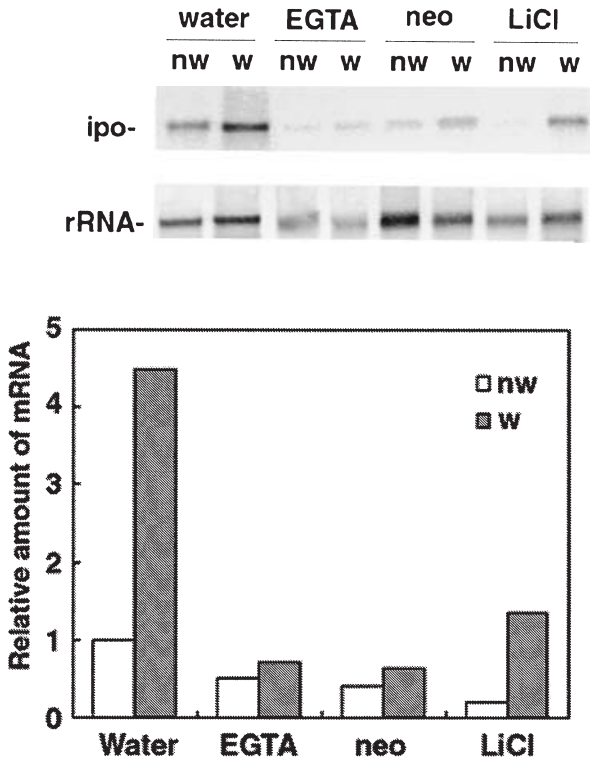


Figure 5. Effects of calcium chelator and channel blockers on the IPO gene expression. The petiole cuts of the second and third fully expanded leaves from the terminal bud were immersed in water, 1 mM ethylene glycol tetraacetic acid (EGTA), 100 μM neomycin (neo) and 10 mM LiCl, respectively, for 12 h before wounding. Six hours after wounding, the total RNAs of both the wounded (w) and unwounded (nw) leaves were analysed by Northern blotting to detect IPO mRNA (ipo) and rRNA sequentially. The value of IPO mRNA was adjusted by its corresponding amount of rRNA for equality of loading. After the adjustment by rRNA, the amount of IPO mRNA from the unwounded leaves in water was treated as the normalized reference, with a value of one, for determining the relative amount of IPO mRNA. Data are presented as the mean values from two independent experiments.

DISCUSSION

Sweet potato is an important crop and a major source of starch world-wide. Sweet potato is also relatively resistant

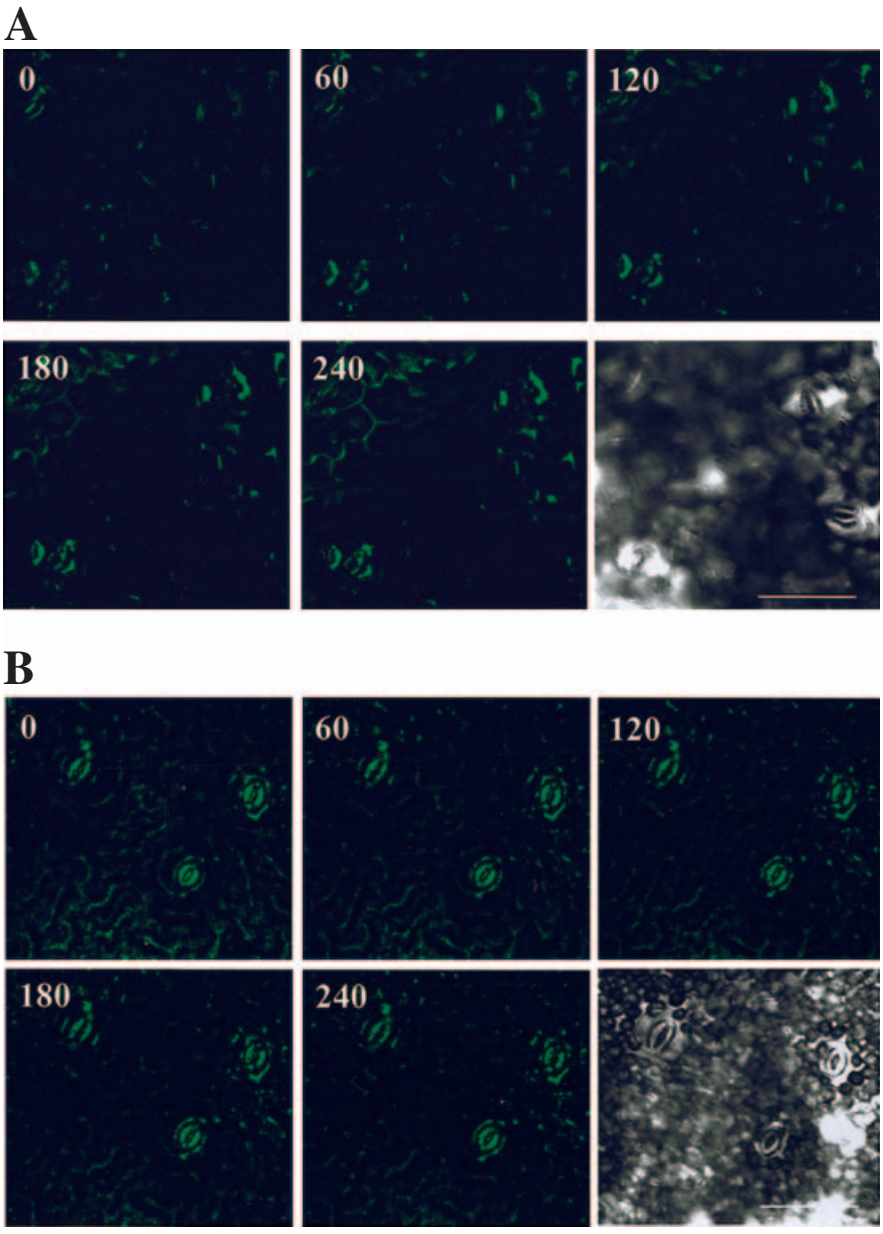


Figure 6. Visualization of Ca^{2+} by confocal microscope. The leaf pieces as 0.5 cm × 0.5 cm were immersed in 1 × MS solution for 12 h and then transferred into 0.55 M mannitol with fluo-3 AM at a final concentration of 1 μM. Leaf pieces were washed by 0.55 M mannitol to remove the extra fluorescent dye, before were wounded by needle (A) or 50 μM MeJA was added (B). The confocal laser scanning microscope was set with excitation wavelength at 488 nm and emission wavelength between 500 and 535 nm and used to investigate the appearance of Ca^{2+}. The serial pictures were taken at 0, 60, 120, 180 and 240 s after the images of leaves were focused. It spent 90 s in the wounding or MeJA application to the leaf pieces and the image focusing. Bright field images are also included for comparison. Bar = 40 μm.

to herbivore biting. The roles of specific genes in the defensive system of sweet potato are interesting topics. A mechanical wound-inducible gene, IPO, was isolated from the subtraction library and occurred locally and systemically following wounding (Fig. 1). In particular, several chemical inhibitors were used to determine the reaction sequence of the signal transducers inducing the expression of the IPO gene (Fig. 8). Inhibitors are frequently used in research on signal transduction because they provide a means for investigating possible transduction routes. However, it should be remembered that inhibitors might have more than one effect and thus there is always uncertainty in the deduction to be made about their effects.

Once plants receive the wound signal, increasing Ca^{2+} concentration within cells is the first step in the signal transduction pathway that promotes defensive reactions. The application of systemin significantly raised the concentration of cytosolic Ca^{2+} in tomato cells within 10 min (Moyen *et al*. 1998) and mechanical swirling also immediately and transiently increased cytosolic Ca^{2+} of tobacco protoplast (Haley *et al*. 1995). Furthermore, calmodulin with Ca^{2+} modulated cellular functions and its RNA appeared within 30 min after mechanical wounding in young tomato plants (Bergey & Ryan 1999). The effect of Ca^{2+} blockers and chelator on the IPO gene expression after wounding was also observed (Fig. 5), and in addition, mechanical wounding increased the accumulation of cellular Ca^{2+} within minutes (Fig. 6). These results strongly suggest that increase in cytosolic Ca^{2+} is the first response after wounding. Furthermore, Ca^{2+} release from vacuoles is slowly activated by the presence of Ca^{2+} (Allen & Sanders 1994; Ward & Schroeder 1994). Consequently, the influx of

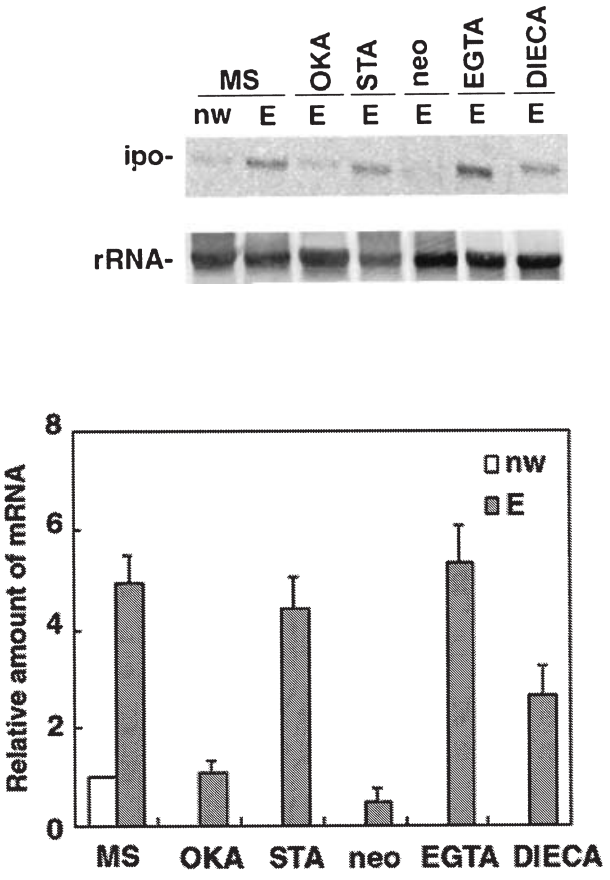


Figure 7. Effect of ethylene on IPO gene expression. The petiole cuts of the second and third fully expanded leaves from the terminal bud were immersed in 1 × MS or 1 × MS containing 0.5 μM OKA, 1 μM STA, 100 μM neomycin, 1 mM EGTA and 50 mM DIECA, respectively. After 16 h, 1 mM CEPA was added to release ethylene (E). For another 6 h, the total RNAs from the treated and untreated leaves (MS) were analysed by Northern blotting to detect IPO mRNA (ipo) and rRNA sequentially. The value of IPO mRNA was adjusted by its corresponding amount of rRNA for equality of loading. After the adjustment by rRNA, the amount of IPO mRNA from the unwounded leaves in 1 × MS was treated as the normalized reference, with a value of one, for determining the relative amount of IPO mRNA. Data are presented as the mean ± standard deviation from three independent experiments.

extracellular Ca^{2+} may cause the release of Ca^{2+} from intracellular stores.

MeJA has been considered a key signal molecule during the activation of defence-related proteins and its biosynthesis and function are related to Ca^{2+}. MeJA biosynthesis in periwinkle cells required the increase of Ca^{2+} within the cytosol (Memelink, Verpoorte & Kijne 2001) and the influx of Ca^{2+} into cells was required for MeJA response (Kenton, Mur & Draper 1999). However, mechanical wounding rather than the application of MeJA induced the accumulation of Ca^{2+} (Fig. 6). Therefore, the wounding signal in sweet potato first stimulates the influx of Ca^{2+}, which may further induce the biosynthesis of MeJA. Furthermore, SA decreased the IPO gene expression in the wounded sweet potato (Fig. 4), suggesting that MeJA biosynthesis is through the octadecanoid pathway. This may also imply that the increase of Ca^{2+} within the cytosol induces the activity of the octadecanoid pathway.

MeJA-responsive genes were strongly induced by wounding and MeJA, but only wound-responsive genes were activated by wounding in *Arabidopsis thaliana* (León *et al*. 1998). Both mechanical wounding and MeJA activated the expression of IPO gene (Figs 1 & 2) and thus the IPO gene is MeJA responsive according to this definition. Furthermore, MeJA activated the accumulation of proteinase inhibitor II and other mRNAs in the systemic leaves, but the expression of these genes was not detected in the treated roots (Dammann, Rojo & Sánchez-Serrano 1997). IPO mRNA was present in sweet potato leaves and little or none was detectable in sweet potato roots even after wounding (data not shown). Therefore, the occurrence of IPO mRNA is also organ-specific.

Protein kinase and phosphatase have been reported to be involved in signal transduction pathways. When STA and OKA were utilized, a reversible phosphorylation step was identified in a transduction pathway related to MeJA-induced gene transcription in *Arabidopsis thaliana* (Rojo *et al*. 1998). STA inhibiting protein kinase enhanced the expression of JA-responsive genes, whereas OKA inhibit-

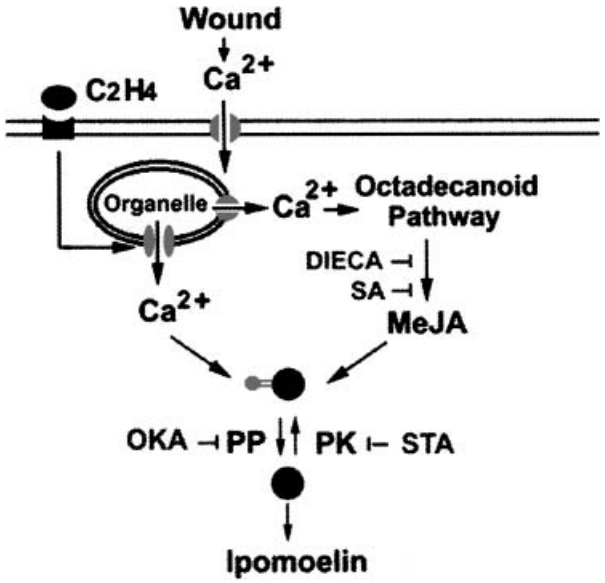


Figure 8. Model for the signal transduction of the IPO gene in sweet potato. Following stimulation by wounding, cytosolic Ca^{2+} quickly rises, which may further enhance the release of Ca^{2+} from vacuoles. The elevation of cytosolic Ca^{2+} may stimulate the biosynthesis of MeJA through the octadecanoid pathway. Then, MeJA may induce the expression of the IPO gene via dephosphorylated protein(s). The binding of ethylene receptors with ethylene causes the opening of Ca^{2+} channels in the vacuolar membrane and thus results in the activation of protein phosphatase(s). STA inhibits protein kinase (PK), whereas OKA inhibits protein phosphatase (PP). SA and DIECA was observed to block the synthesis of MeJA (Peña-Cortés *et al*. 1993; Doares *et al*. 1995a; Menke *et al*. 1999). The arrows represent positive action, while the blunted lines indicate negative regulation.

ing protein phosphatase 1 and 2 A decreased JA-responsive gene transcription (Rojo *et al*. 1998). Furthermore, OKA abolished the response of MeJA and implicates a protein phosphatase in the MeJA signal tansduction of tobacco (Kenton *et al*. 1999). Similar studies here indicate that dephosphorylated protein(s) activated the MeJA-inducible IPO gene in sweet potato (Fig. 2) and also suggest the positive regulation of dephosphorylated protein(s) during the activation of MeJA-responsive genes in sweet potato. However, a JA-independent pathway was positively regulated by protein kinase and negatively controlled by protein phosphatase in *Arabidopsis thaliana* (Rojo *et al*. 1998). Consequently, reversible protein phosphorylation is critical during signal transduction in plants.

SA induced the synthesis of pathogenesis-related (PR) protein and enhanced resistance to a number of pathogens (Chen *et al*. 1995). However, SA was demonstrated to inhibit the accumulation of JA and the subsequent activation of gene expression (Peña-Cortés *et al*. 1993; Doares *et al*. 1995a). Further studies indicated that SA or aspirin blocked the production of allene oxide synthase that mediated the conversion of lipoxygenase-derived fatty acid hydroperoxides to allene epoxides, precursors of the synthesis of the JA (Peña-Cortés *et al*. 1993; Harms, Ramirez & Peña-Cortés 1998). SA also interfered with IPO gene expression in sweet potato (Fig. 4). Since the IPO gene is MeJA inducible, SA may act similarly, blocking the octadecanoid pathway and reducing the production of the MeJA precursor in sweet potato.

The phytohormone ABA has various influences on the regulation of the wounding signal pathway. ABA-deficient mutants of tomato and potato failed to accumulate proteinase inhibitor II mRNA upon wounding, but treating these mutants with ABA could induce the expression of proteinase inhibitor II mRNA in undamaged tissues (Peña-Cortés *et al*. 1989, 1996; Dammann *et al*. 1997). ABA was suggested to be a key component in wound signal transduction, which activates defensive genes (Peña-Cortés *et al*. 1996; Wasternack *et al*. 1996). However, ABA induced lower levels of proteinase inhibitor proteins and mRNAs than systemin and JA, which suggested that ABA was not a primary signal for defensive gene activation, but might accumulate upon wounding because of desiccation (Birkenmeier & Ryan 1998). ABA was further observed to inhibit alkaline lipase and proteinase in germinating apple embryos (Ranjan & Lewak 1995). In this case, the sweet potato IPO gene was wound inducible, but the accumulation of IPO mRNA upon wounding was not influenced by the ABA treatment (Fig. 4). Imanishi *et al*. (1997) also showed that the IPO gene was ABA-independent. Apparently, the role of ABA varies in different plants.

The signal transduction pathways conducted by mechanical wounding and ethylene leading to the expression of IPO gene is suggested here (Fig. 8). The concentration of cytosolic Ca^{2+} increases after cells receive the wound signals. Ca^{2+} within cells may come from the extracellular space and vacuoles. Since each Ca^{2+} chelator EGTA and vacuolar Ca^{2+} channel blockers LiCl and neomycincan abolishes IPO gene expression independently (Fig. 5), the influx of Ca^{2+} into cytosol may follow a sequential order. External signals may first lead to the opening of Ca^{2+} channels in the plasma membrane, followed by the opening of Ca^{2+} channels in the membrane of vacuoles (Allen & Sanders 1994; Ward & Schroeder 1994). Meanwhile, the elevation of cytosolic Ca^{2+} may stimulate the biosynthesis of MeJA (Memelink *et al*. 2001). Furthermore, the decrease in IPO gene expression in the wounded plant following the treatment with SA (Fig. 4) indicates that MeJA synthesis occurs through the octadecanoid pathway. In the ethylene transduction pathway, neomycin, a vacuolar Ca^{2+} channel blocker abolishes the IPO gene expression in the presence of ethylene, whereas EGTA does not (Fig. 7). This phenomenon may indicate that the influx of Ca^{2+} from vacuoles but not from extracellular space is required for ethylene action in IPO gene expression. However, the presence of DIECA, a MeJA synthesis inhibitor, does not inhibit the stimulation of IPO gene expression by ethylene (Fig. 7). This result may indicate that the signal transduction pathways conducted by ethylene and wound signal are separated (Fig. 8). In particular, both signal pathways were inhibited by OKA (Figs 2 & 7), and also the wound signal was already identified as being transferred to MeJA and then to protein phosphatase(s) (Fig. 3). Therefore, both the wound signal and ethylene pathways may stimulate the same protein phosphatase(s), which dephosphorylates the regulatory protein(s), thus inducing IPO gene expression.

In the present study the signal transductions of wounding and ethylene in activating IPO gene of sweet potato were studied in detail. In particular, the reaction sequence and functions of second messenger and signal transducers including Ca2 +, MeJA and protein kinase/phosphatase in the wound-signalling pathway of sweet potato were determined. Furthermore, the interaction of wounding and ethylene signal pathways in activating the IPO gene was suggested.

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