*Plant, Cell and Environment* (2008) **31**, 62–72 doi: 10.1111/j.1365-3040.2007.01742.x

# Calcium influxes and mitogen-activated protein kinase kinase activation mediate ethylene inducing ipomoelin gene expression in sweet potato

YU-CHI CHEN[1,2]*, HSIN-HUNG LIN[3]* & SHIH-TONG JENG[3]

[1]*Department of Horticulture and Biotechnology, Chinese Culture University, Taipei, Taiwan,* [2]*Institute of Biological Chemistry, Washington State University, Pullman, WA 99164 USA; and* [3]*Institute of Plant Biology and Department of Life Science, National Taiwan University, Taipei 106, Taiwan*

## ABSTRACT

**The ipomoelin gene (*IPO*) was identified to be a wound-inducible gene from *Ipomoea batatas*, and its expression was stimulated by methyl jasmonate (MeJA) and hydrogen peroxide. IPO protein was also characterized as a defence-related protein, and it is also a carbohydrate-binding protein. In this study, the expression of *IPO* was used as a molecular probe to study the effects of $Ca^{2+}$ on the signal transduction of ethylene. A confocal microscope monitored the $Ca^{2+}$ within cells, and Northern blotting examined *IPO* expression. The presence of $Ca^{2+}$ channel blocker, including diltiazem, neomycin or ruthenium red, abolished the increase of cytosolic $Ca^{2+}$, and reduced the *IPO* expression in the cells induced by ethylene. Furthermore, both $Ca^{2+}$ influxes and *IPO* expression stimulated by ethylene were prohibited in the presence of 10 mM ethylene glycol-*bis*(2-aminoethyl ether)-*N*, *N*, *N′*, *N′*-tetraacetic acid (EGTA). These results indicated that $Ca^{2+}$ influxes into the cytosol induced by ethylene are from both apoplast and organelles, and are required for activating *IPO* expression. However, in the presence of 1 mM EGTA, ethylene can still stimulate *IPO* expression, but mechanical wounding failed to do it. Therefore, $Ca^{2+}$ channels in the plasma membrane induced by ethylene have higher affinity to $Ca^{2+}$ than that stimulated by wounding. Moreover, the addition of A23187, an ionophore, raised cytosolic $Ca^{2+}$, but was unable to stimulate *IPO* expression. These findings showed that *IPO* induction did not solely depend on $Ca^{2+}$, and $Ca^{2+}$ elevation in cytosol is necessary but not sufficient for *IPO* expression. The application of PD98059, a mitogen-activated protein kinase kinase (MAPKK) inhibitor, did not prevent $Ca^{2+}$ from increasing in the cytosol induced by ethylene, but inhibited the *IPO* expression stimulated by staurosporine (STA), a protein kinase inhibitor. Conclusively, elevation of cytosolic $Ca^{2+}$ by ethylene may stimulate protein phosphatase and MAPKK, which finally activates *IPO* expression.**

*Correspondence: S.-T. Jeng. Fax: 886-2-23918940; e-mail: stjeng@ntu.edu.tw*

*Contributed equally to this work.

*Key-words*: calcium ion; ethylene signalling; membrane channel.

*Abbreviations*: CEPA, 2-chloroethyl phosphonic acid; EGTA, ethylene glycol-*bis*(2-aminoethyl ether)-*N*, *N*, *N′*, *N′*-tetraacetic acid; IPO, ipomoelin; MAPKK, mitogen-activated protein kinase kinase; MeJA, methyl jasmonate; OKA, okadaic acid; STA, staurosporine.

## INTRODUCTION

Ethylene is produced by most plants in response to developmental stages, such as seed germination, tissue differentiation, root formation and elongation, lateral bud development, flower initiation and opening, fruit ripening and leaf abscission (Jackson & Osborne 1970; Yeang & Hillman 1981; Whalen & Feldman 1988; Abeles, Morgan & Saltveit 1992; Payton *et al.* 1996; Deikman 1997; Lelièvre *et al.* 1997; Zhu *et al.* 2006). It also serves as signal mediator responding to environmental stresses, including flooding, wounding and pathogen infection (O'Donnell *et al.* 1996; Grichko & Glick 2001; Oñate-Sánchez & Singh 2002). Ethylene also triggered programmed cell death via calcium ion ($Ca^{2+}$), reactive oxygen species and mitogen-activated protein kinase (MAPK) in tomato suspension cells (de Jong *et al.* 2002). Furthermore, $Ca^{2+}$ was involved in the ethylene-mediated pathogenesis response, and the ethylene-dependent induction of chitinase accumulation was inhibited by blocking $Ca^{2+}$ influxes (Raz & Fluhr 1992).

In eukaryotic cells, $Ca^{2+}$ is one of the most common secondary messengers responding to extracellular stimuli (Pozzan *et al.* 1994; Miedema *et al.* 2001). In plant cells, the $Ca^{2+}$ concentration increased in cytosol either as a result of uptake from apoplastic space or of release from internal stores including vacuoles and endoplasmic reticulum (ER) (Gilroy, Read & Trewavas 1990). External signals may lead to the opening of $Ca^{2+}$ channels in the plasma membrane and in the membrane of organelles (Allen & Sanders 1994; Ward & Schroeder 1994). In animal system, the $Ca^{2+}$-permeable channels of the plasma membrane were grouped into three different families: voltage-operated $Ca^{2+}$ channels, which include L, N, T, P/Q and R subtypes;

receptor-operated $Ca^{2+}$ channels; and the second messenger-operated channels (Rizzuto & Pozzan 2003). Moreover, two different intracellular $Ca^{2+}$ channels located in the organelles within the cells were reported. They are the inositol 1,4,5-trisphosphate ($IP_3$) receptors and ryanodine receptors (Nucifora *et al.* 1996). On the other hand, calcium-permeable channels have been classified on the basis of their voltage dependence into depolarization-activated, hyperpolarization-activated and voltage-independent cation channels in the plasma membrane of plants (White *et al.* 2000; Miedema *et al.* 2001; Sanders *et al.* 2002). Plants also have $Ca^{2+}$ channels similar to the L-type channels characterized in animal systems by patch clamp techniques (Thuleau *et al.* 1994; White *et al.* 2000). Several highly selective $Ca^{2+}$ channels activated by cytosolic second messengers, including $IP_3$ and cyclic adenosine diphosphoribose (cADPR), are also present in tonoplast (Allen & Sanders 1997; White *et al.* 2000; Sanders *et al.* 2002). Nevertheless, the interaction between ethylene and $Ca^{2+}$ influxes within plant cells is still not clear.

Except $Ca^{2+}$ mediating the signal transduction, MAPKs were also known as intracellular signal mediators. MAPKs form a family of protein kinases that are activated in response to extracellular stimuli in eukaryotic organisms. In plants, MAPKs have been demonstrated to be an important enzyme in different types of stresses, including cold, droughts and salt stresses (Seo *et al.* 1995; Jonak *et al.* 1996; Bogre *et al.* 1997). MAPKs were also involved in the defence-related signal transduction (Ichimura *et al.* 2000; Meskiene & Hirt 2000; Tena *et al.* 2001; Zhang & Klessig 2001), and the wound-inducible protein kinase (WIPK) was found to respond to wounding in tobacco (Seo, Sano & Ohashi 1999). Moreover, *OsMSRMK2* gene, which was cloned and demonstrated to be included in a MAPK family, was modulated by MeJA, salicylic acid, ethylene, temperature, ozone and sulfur dioxide in rice (Agrawal, Rakwal & Iwahashi 2002). In *Arabidopsis*, MAPKs were activated by the ethylene precursor, aminocyclopropane-1-carboxylic acid, to demonstrate that the MAPK pathway mediated part of the ethylene signalling in plants (Ouaked *et al.* 2003). These results suggested that MAPK cascade mediates stress signal transductions and plays an important role in regulating the physical reactions.

In the previous study, IPO protein was characterized as a defence-related protein by the insect-feeding experiment, and it reduced both silkworm survival rate and weight gain. Based on its sequence, IPO protein is a jacalin-like lectin, and its C-terminus is highly homologous with those of mannose-binding lectin, jacalin and agglutinin (Chen *et al.* 2005). Lectin was known as a carbohydrate-binding protein and is widely existed in certain species. In the previous carbohydrate-binding assay, IPO protein recognized monosaccharides, such as glucose, galactose and mannose, rather than polygalacturonic acid and sucrose. Additionally, IPO protein strongly bound methyl glucoside including methyl $\alpha$-D-mannopyranoside and methyl $\alpha$-D-glucopyranoside. However, IPO protein cannot bind sugar alcohols like mannitol and sorbitol (Chen *et al.* 2003). *IPO* in sweet potato was also reported to be a wound-inducible gene. The *IPO* mRNA was also accumulated after the stimulation of sweet potato by ethylene, MeJA and $H_2O_2$ (Imanishi *et al.* 1997; Chen *et al.* 2003; Jih, Chen & Jeng 2003). The signal transduction model for *IPO* expression in sweet potato was suggested by Chen *et al.* (2003). Following the stimulation by wounding, the cytosolic $Ca^{2+}$ quickly increased, and further enhanced the release of $Ca^{2+}$ from organelles. The elevation of cytosolic $Ca^{2+}$ stimulated the biosynthesis of MeJA through the octadecanoid pathway. Then, MeJA may induce the expression of *IPO* via dephosphorylated proteins. On the other hand, ethylene caused the opening of $Ca^{2+}$ channels in the vacuolar membrane, and thus resulted in the activation of protein phosphatases (Chen *et al.* 2003). In addition, hydrogen peroxide and nitric oxide were indicated to be involved in the signal transduction of *IPO* in sweet potato (Jih *et al.* 2003). However, the signal transduction of the *IPO* expression induced by ethylene is not known in detail. We report here that the influxes of cytosolic $Ca^{2+}$ induced by ethylene was from the apoplastic space via the L-type $Ca^{2+}$ channel in plasma membrane, and then more $Ca^{2+}$ released from intracellular organelles was stimulated. Consequently, the accumulation of $Ca^{2+}$ in cytosol induced the expression of *IPO* via dephosphorylated protein(s) and MAPKK cascade.

## MATERIALS AND METHODS

### Plant materials and assay conditions

Sweet potato (*Ipomoea batatas* cv. Tainung 57) plants were grown in a controlled environment (16 h/25 °C day; 8 h/22 °C night; humidity 70%; light 100 $\mu$mol photons $m^{-2}$ $s^{-1}$). Plants with six to eight fully developed leaves were used in this study. The second and third fully expanded leaves counted from the terminal bud were excised, and their petiole cuts were immersed in water or 1× Murashige–Skoog (MS) (3 mM $CaCl_2$, 1.25 mM $KH_2PO4$, 0.1 mM $Na_2EDTA$, 0.1 mM $FeSO_4$, 0.1 mM $MnSO_4$, 0.037 mM $ZnSO_4$, 0.1 $\mu$M $CuSO_4$, 18.8 mM $KNO_3$, 0.1 $\mu$M $CoCl_2$, 30 mM $NH_4Cl$, 1.5 mM $MgSO_4$ and 5 $\mu$M KI) (Murashige & Skoog 1962) (pH 5.8) with the final concentration of 1, 5 or 10 mM EGTA; 0.1 mM neomycin; 0.1 mM diltiazem; 50 $\mu$M ruthenium red; 0.1 $\mu$M PD98059; or 1 $\mu$M A23187 under 100 $\mu$mol photons $m^{-2}$ $s^{-1}$ for 16 h at 25 °C. Leaves with their petiole in water or 1× MS without any chemical treatment were used as controls. The effect of wounding caused by the separation of leaves from the mother plants should be reduced during this long incubation period in water or MS solution. Then, the leaves were stimulated by 1 mM CEPA or 1 $\mu$M STA for another 2 h before Northern assay. The CEPA releases ethylene (Min & Bartholomew 1996), while STA inhibits protein kinase (Barwe, Sathiyabama & Jayabaskaran 2001). Leaves were otherwise wounded by tweezers, and Northern assays were subsequently applied 2 h later. All chemicals were purchased from Sigma (St Louis, MO, USA).

## RNA isolation and analysis

Total RNA was isolated from liquid $N_2$-ground leaves with Trizol reagent (Gibco BRL, Carlsbad, CA, USA) according to the procedure suggested by the manufacturer. The quality of RNA was analysed by agarose gel with formaldehyde, while its quantity was estimated by a spectrophotometer. Ten micrograms of total RNA was separated on formaldehyde–agarose gels, and the digital images of RNA gels were taken by the CCD camera with Scion Visicapture software (Scion Corporation, Frederick, MD, USA) after gels were stained with 2 $\mu$g mL$^{-1}$ ethidium bromide for 10 min. Then, the RNA gels were 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 the PCR using the template of the full-length *IPO* cDNA isolated from the cDNA library in a previous study (Chen *et al.* 2003). Prehybridization was performed in 5× SSPE [0.05 M $NaH_2PO_4$ (pH 6.8), 0.9 M NaCl and 5 mM ethylenediaminetetraacetic acid (EDTA)], 0.5% sodium dodecyl sulphate (SDS), 5× Denhardt's solution (0.1% Ficoll, 0.1% bovine serum albumin, 0.1% polyvinyl pyrrolidine) at 60 °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 60 °C for 15 min. Radioactive blots were displayed on the Molecular Dynamics Phosphoimage (Molecular Dynamics, Sunnyvale, CA, USA). Images of Northern assays were produced by Phosphage as TIFF-formatted files, while those of ethidium-bromide-stained gels were produced by CCD camera as TIFF-formatted files as mentioned earlier. The amounts of IPO mRNA fragments and 28S rRNA in Northern and ethidium-bromide-stained gels, respectively, were then quantified by software Zero-Dscan 1.1 (BD Biosciences, Rockville, MD, USA), and their ratios of IPO mRNA to 28S rRNA, the relatively normalized mRNA values, are indicated in Figs 3–5.

## $Ca^{2+}$ detection by confocal scanning microscope

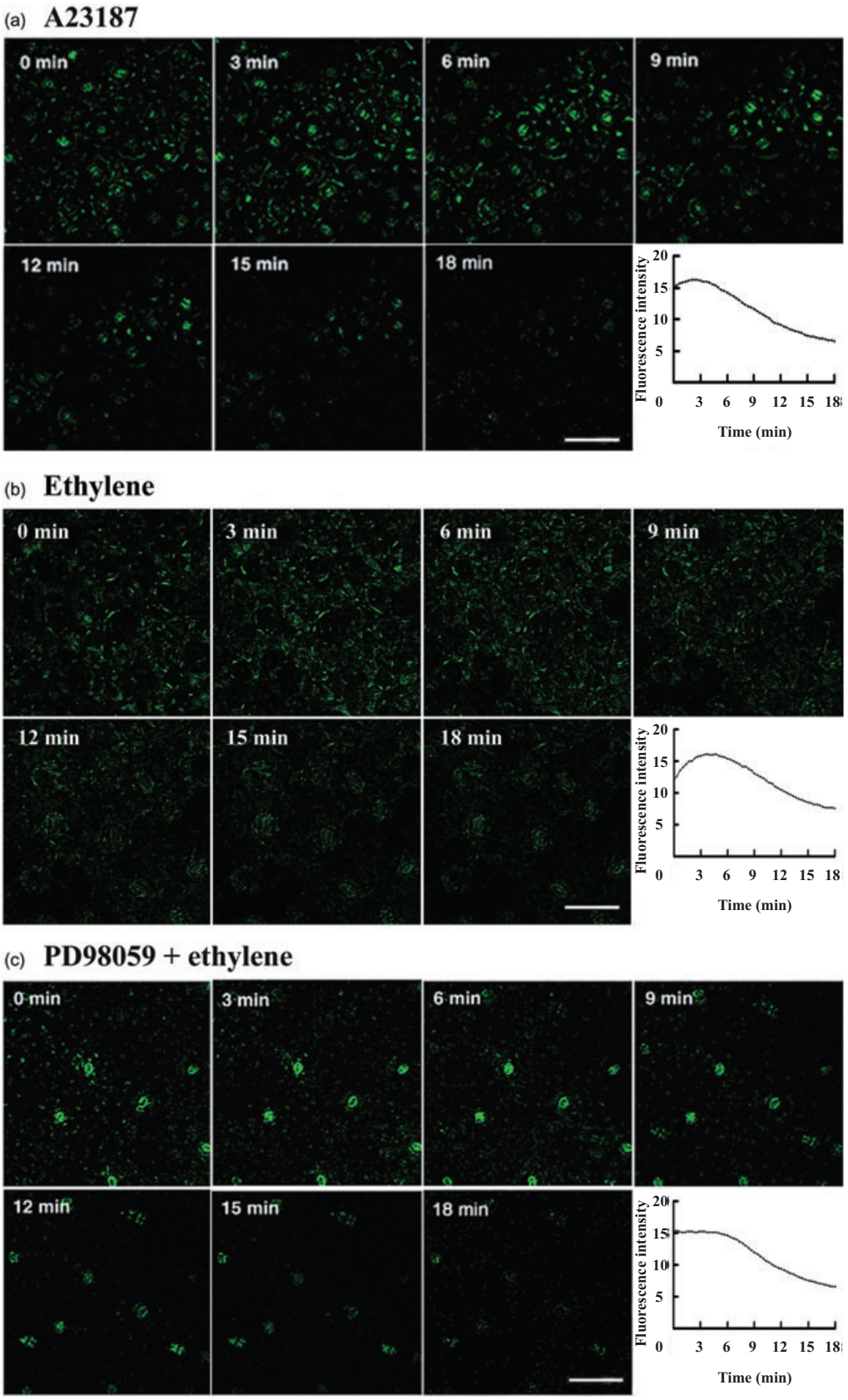
Leaves of sweet potato were cut into 0.5 × 0.5 cm pieces, and floated abaxial upwardly in 1× MS solution with the final concentration of 1 or 10 mM EGTA, 0.1 mM neomycin, 0.1 mM diltiazem, 50 $\mu$M ruthenium red or 0.1 $\mu$M PD98059 for 16 h. The leaf pieces were then transferred to 0.55 M mannitol, which maintained the osmotic pressure of plant cells, with fluorescent dye fluo-3 AM (Sigma F-6142) at a final concentration of 1 $\mu$M for 1 h. 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 the leaf pieces with 0.55 M mannitol for 15 min (three times). Then, the addition of 1 mM CEPA or 1 $\mu$M A23187 was performed, or the mechanical wounding was applied to initiate $Ca^{2+}$ influxes into cytosol. During this treatment period, inhibition reagents always existed at a final concentration as described earlier. Leaf pieces without inhibition reagents were also added with 1 mM CEPA or 1 $\mu$M A23187 as control reactions. Leica TCS-SP2 confocal laser scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) with an excitation wavelength at 488 nm and emission wavelength between 500 and 535 nm was used to investigate the leaf pieces with fluo-3 AM. The focal plane of the confocal microscope in this study was always set at the epidermis that includes guard cells and epidermal cells. The images were taken every 20 s or 1 min after the images of leaf pieces were focused, and then their fluorescence intensities were recorded. Data were presented as fluorescence intensities, whose values were directly from microscope (Fig. 1), or were indicated as relative fluorescences, whose values were normalized to their initial fluorescence at time zero (Fig. 2). It always took 90 s for the application of CEPA, A23187 or mechanical wounding before the leaf pieces were analysed by the confocal microscope.

# RESULTS

## Induction of A23187 and ethylene on the accumulation of cytosolic $Ca^{2+}$

*IPO* in sweet potato was stimulated by mechanical wounding (Chen *et al.* 2003), and its expression induced by ethylene was further studied here. The induction of ethylene on the accumulation of cytosolic $Ca^{2+}$ within the cells stained with fluo-3 AM was investigated by a confocal scanning microscope. To test the working system, $Ca^{2+}$ influxes of leaves, which were abaxial upwardly floated on the medium, was artificially increased by the application of ionophore A23187, which is widely used in biological systems to study the regulatory roles of $Ca^{2+}$ (Rasmussen & Goodman 1997). The fluorescence of fluo-3AM increased right after the addition of A23187 (Fig. 1a), indicating the occurrence of $Ca^{2+}$ influxes within cytosol. The same working condition was performed to study the effects of ethylene on the accumulation of cellular $Ca^{2+}$ (Fig. 1b), and leaves treated with

**Figure 1.** The induction of cytolic $Ca^{2+}$ by A23187, ethylene and PD98059. The 0.5 × 0.5 cm leaf pieces were abaxial upwardly floated in 1× Murashige–Skoog (MS) solution without or with PD98059 (C) for 16 h, and then the final concentration of 1 $\mu$M fluo-3 AM in 0.55 M mannitol was added for 1 h. Leaf pieces were then added with 1 $\mu$M A23187 (a) or 1 mM CEPA (b & c), and investigated by a confocal scanning microscope to analyse the appearance of cytosolic $Ca^{2+}$. The focal plane of the confocal microscope was always set at the epidermis that includes guard cells and epidermal cells. The fluorescence intensity was recorded every 20 s, and the serial pictures shown here were taken at 0, 3, 6, 9, 12, 15 and 18 min after the images of cells were focused. It took 90 s for reagent application and image focusing. The fluorescence intensities read directly from microscope of this observation view versus time were showed in the last panel. Bar = 80 $\mu$m.



CEPA releasing ethylene showed a similar fluorescence profile as that of Fig. 1a. Therefore, the elevation of $Ca^{2+}$ in cytosol was rapidly induced by ethylene, and that the $Ca^{2+}$ increment disappeared soon.

## Effects of diltiazem, neomycin and ruthenium red on the accumulation of cytosolic $Ca^{2+}$ induced by ethylene

Diltiazem, neomycin and ruthenium red are different types of $Ca^{2+}$ channel blockers, and they inhibit L-type $Ca^{2+}$ channel in plasma membrane, $IP_3$-mediated $Ca^{2+}$ channels in organelles and cADPR-mediated $Ca^{2+}$ channels in organelles, respectively (Tsien *et al.* 1987; Hess 1988; Frankling-Tong *et al.* 1996; Durner, Wendehemme & Klessig 1998). The small pieces of sweet potato leaves pretreated with diltiazem, neomycin or ruthenium red in the 1× MS buffer were probed by fluo-3 AM. The leaves were then added with 1 mM CEPA to release ethylene, and the images of these leaves were taken by a confocal scanning microscope. The change of fluorescence intensity with each $Ca^{2+}$ channel blocker was not significant during the investigation, demonstrating that diltiazem, neomycin and ruthenium red inhibited ethylene inducing $Ca^{2+}$ accumulation (Fig. 2a). Therefore, $Ca^{2+}$ from both apoplast and organelles is involved in the signal transduction of ethylene. Furthermore, fluorescence variations in this study were simplified into two patterns: an increase of fluorescence intensity indicated the accumulation of $Ca^{2+}$ in cytosol, while the fluorescence intensity remained unchanged pointed out that $Ca^{2+}$ did not enter into cytosol.

## Effects of EGTA on the accumulation of cytosolic $Ca^{2+}$

The influxes of intercellular $Ca^{2+}$ induced by ethylene were blocked by the presence of diltiazem (Fig. 2a), indicating the involvement of apoplastic $Ca^{2+}$ in ethylene stimulation. The roles of apoplastic $Ca^{2+}$ during the stimulation of ethylene and wounding were further studied separately in the presence of EGTA, a cation chelator. The leaf pieces of sweet potato were floated in 1× MS buffer with the final concentration of 1 mM or 10 mM EGTA for 16 h. After being probed by fluo-3 AM, the leaf pieces were treated with CEPA to release ethylene (Fig. 2b) or were wounded by tweezers (Fig. 2c). The emission of fluorescences caused by the influxes of $Ca^{2+}$ was recorded by the confocal microscope. In the absence of EGTA, the treatment of ethylene (Fig. 2b) or mechanical wounding (Fig. 2c) resulted in the increment of $Ca^{2+}$ in cytosol. However, in the presence of 1 mM or 10 mM EGTA in which sweet potato looked healthy, the increase of $Ca^{2+}$ in cytosol was not observed in either ethylene or wounding treatments (Fig. 2b,c), demonstrating that EGTA prevented apoplastic $Ca^{2+}$ from entering into the cells that were treated with ethylene or wounding. Furthermore, apoplastic $Ca^{2+}$ is involved in the signal tansduction of ethylene and wounding.

## Involvement of $Ca^{2+}$ from organelles and apoplast in *IPO* expression

Ethylene was able to induce the expression of *IPO*, and the action of ethylene may require $Ca^{2+}$. The operation of $Ca^{2+}$ channels in both plasma membrane and organelles was required for ethylene to increase $Ca^{2+}$ within cytosol (Fig. 2a,b). However, whether the expression of *IPO* induced by ethylene was influenced by $Ca^{2+}$ from $Ca^{2+}$ channels was not clear. The petiole cuts of the excised leaves were placed in 1× MS with $Ca^{2+}$ channel blockers including neomycin, diltiazem or ruthenium red for 16 h before the CEPA was added to release ethylene. After another 2 h, the total RNAs of leaves were analysed by Northern blotting. The presence of ethylene alone enhanced the *IPO* expression (Fig. 3), but the presence of any $Ca^{2+}$ signal blocker including neomycin, diltiazem or ruthenium red significantly abolished the expression of *IPO* based on the relative amount of IPO mRNA (Neo, Dilt and RR in Fig. 3). These results showed that ethylene inducing *IPO* expression required $Ca^{2+}$ released from $Ca^{2+}$ channels, which were located in both organelles and plasma membrane.

Additionally, the ionophore A23187 was used to increase the intracellular $Ca^{2+}$ concentration artificially and to study the regulatory role of $Ca^{2+}$ in cells. The cytosolic $Ca^{2+}$ concentration was induced by A23187 within several minutes (Fig. 1a), but the application of A23187 did not induce the expression of *IPO* (A23 in Fig. 3). These results point out that the induction of *IPO* did not solely depend on the $Ca^{2+}$ increase in cytosol, and moreover the increase of $Ca^{2+}$ in cytosol is necessary but not sufficient for inducing *IPO* expression.

## Effects of EGTA on *IPO* expression

To further understand the effects of apoplastic $Ca^{2+}$ on *IPO* expression, the petiole cuts of the excised sweet potato leaves were placed in 1× MS or water with 1, 5 or 10 mM EGTA for 16 h, and then CEPA was added to release ethylene or mechanical wounding was applied with tweezers. After another 2 h, their total RNAs were analysed by Northern blotting. Figure 4a shows that in the presence of 10 mM EGTA, the expression of *IPO* induced by ethylene was similar to the background reaction without EGTA. Surprisingly, the activation of *IPO* was not inhibited in the condition with 1 or 5 mM EGTA, although the ethylene-stimulated $Ca^{2+}$ influxes did not occur in the presence of 1 mM EGTA (Fig. 2b). Therefore, these results demonstrated that only high concentration of EGTA is able to chelate the $Ca^{2+}$ that is participated in ethylene activating the expression of *IPO*.

Solution 1× MS contains $Ca^{2+}$, which might interfere EGTA in chelating apoplastic $Ca^{2+}$ during the 16 h incubation. MS solution with 0, 1, 5 and 10 mM EGTA contains 92.68, 67.50, 7.86 and 2.65% free calcium ion, respectively, calculated by GEOCHEM PC software. Therefore, water

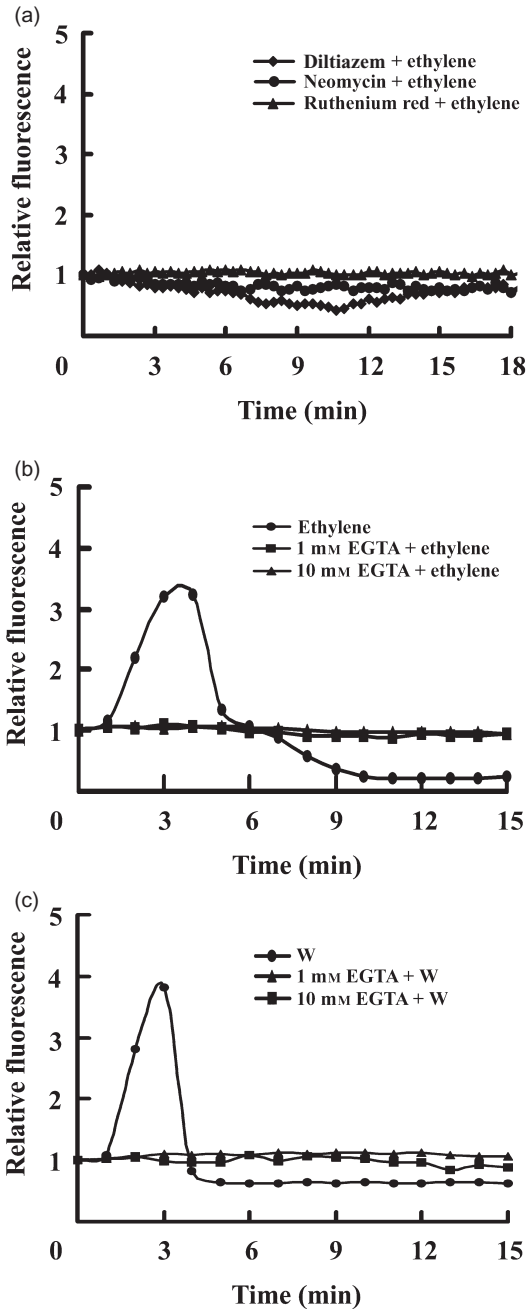



**Figure 2.** Effects of $Ca^{2+}$ channel blockers and EGTA on cytosolic $Ca^{2+}$ induced by ethylene and mechanical wounding. The 0.5 × 0.5 cm leaf pieces were abaxial upwardly floated in 1× Murashige–Skoog (MS) solution without or with a channel blocker (a), including 0.1 mM diltiazem, 0.1 mM neomycin or 50 μM ruthenium red, or with 1 mM (b & c) or 10 mM (b & c) EGTA for 16 h, and then transferred into 0.55 M mannitol with 1 μM fluo-3 AM for 1 h. Leaf pieces were then added with 1 mM CEPA (a & b) to release ethylene or wounded by tweezers (c) before they were investigated by the confocal microscope. The focal plane of the confocal microscope in this study was always set at the epidermis. The intensities of fluorescence were scanned by confocal microscope every 20 s (a) or 1 min (b & c). It took 90 s for reagent application and image focusing. The relative fluorescences, whose values were normalized to their initial fluorescence at time zero, versus time were shown.

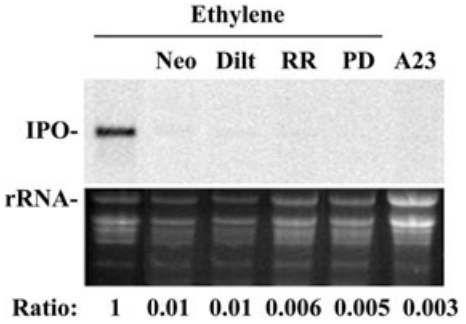



**Figure 3.** Effects of $Ca^{2+}$ channel blockers and A23187 on the expression of *IPO*. The petiole cuts of the second and third fully expanded leaves from the terminal bud were immersed in 1× Murashige–Skoog (MS) with an inhibitor, including 0.1 mM neomycin (Neo), 0.1 mM diltiazem (Dilt), 50 μM ruthenium red (RR) or 0.1 μM PD98059 (PD). CEPA (1 mM) was added to release the ethylene 16 h later. In addition, leaves were put in 1× MS without any inhibitor for 16 h, and A23187 (A23), an ionophore, was added. Neomycin, diltiazem and ruthenium red are $Ca^{2+}$ channel blockers, while PD98059 is a MAPKK inhibitor. For another 2 h, the total RNAs of these leaves were analysed by Northern blotting to detect *IPO* mRNA. Ethidium-bromide-stained agarose gel presents ribosome RNA (rRNA) as a loading control. The ratios of IPO mRNA to rRNA in each reaction were calculated, and the ratio of the reaction treated with ethylene alone was treated as a value of one for determining the relative ratios of other reactions.

instead of 1× MS solution was used to perform the ethylene induction and mechanical wounding assays. Again, the expression of *IPO* induced by ethylene was still observed in the presence of 1 or 5 mM EGTA, and the addition of 10 mM EGTA was high enough to inhibit *IPO* expression (Fig. 4b). Hence, high concentration of EGTA even in water is still needed to chelate $Ca^{2+}$ involved in the expression of *IPO* induced by ethylene. However, the presence of 1 mM EGTA was already able to abolish the expression of *IPO* induced by wounding (Fig. 4b), and also prohibited the increase of $Ca^{2+}$ within the cytosol of the wounded cells (Fig. 2c). These results may point out that the mechanisms of apoplastic $Ca^{2+}$ entering into cytosol under the induction of ethylene and wounding are different, and also that $Ca^{2+}$ affinity of the $Ca^{2+}$ channels induced by ethylene is higher than that of $Ca^{2+}$ channels stimulated by mechanical wounding.

## Effects of PD98059 on *IPO* expression

PD98059 is an MAPKK inhibitor, which was widely used in biological analysis (Burnett *et al.* 2000), and was used to study whether the MAPK pathway was involved in the activation of *IPO* induced by ethylene. It was added into the incubation medium with leaf pieces for 16 h before CEPA application to release ethylene, and the confocal scanning microscope was then used to investigate the leaf pieces with fluo-3 AM. The fluctuation of cytosolic $Ca^{2+}$ was observed after the addition of CEPA in the presence of PD98059 (Fig. 1c). The relative concentration pattern of $Ca^{2+}$ with time course of the PD98059-treated leaf pieces was similar

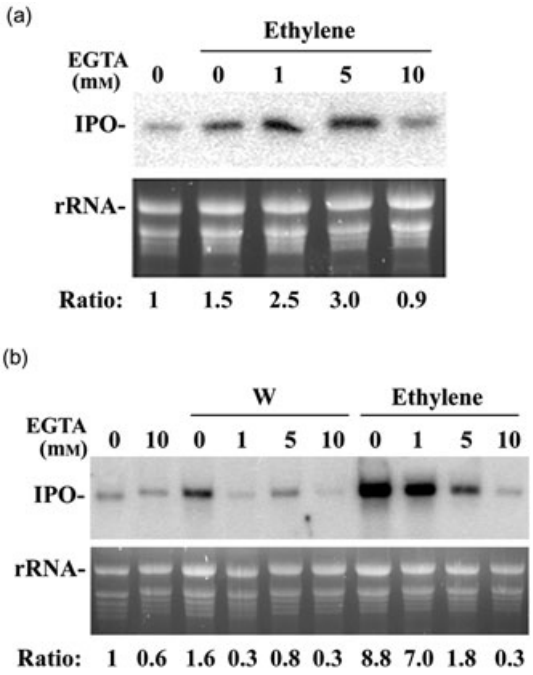



**Figure 4.** Effects of EGTA on the expression of *IPO* stimulated by ethylene and wounding. The petiole cuts of the second and third fully expanded leaves from the terminal bud were immersed in 1× Murashige–Skoog (MS) (a) or water (b) without or with 1, 5 or 10 mM EGTA for 16 h. Leaves were added with 1 mM CEPA to release ethylene (Ethylene) or wounded by tweezers (W). The control leaves without or with 10 mM EGTA were not wounded or not added with CEPA. The total RNAs of these leaves were analysed by Northern blotting to detect *IPO* mRNA. Ethidium-bromide-stained agarose gel presents ribosome RNA (rRNA) as a loading control. The ratios of IPO mRNA to rRNA in each reaction were calculated, and the ratio of the reaction without any treatment was treated as a value of one for determining the relative ratios of other reactions.

to that treated with CEPA alone (Fig. 1b). This result indicated that the MAPKK inhibitor, PD98059, cannot affect the cytosolic $Ca^{2+}$ increment induced by ethylene. Interestingly, the comparison among Fig. 1a–c also shows that the concentration of $Ca^{2+}$ in the guard cells treated with PD98059 increased significantly once the addition of ethylene (Fig. 1c).

However, the *IPO* expression induced by ethylene was inhibited in the presence of PD98059 (PD in Fig. 3). This result implied that PD98059 interfered with the expression of *IPO* in sweet potato, while it did not prevent $Ca^{2+}$ from releasing in the presence of ethylene. Therefore, MAPKK is involved in the *IPO* expression pathway, but the regulatory role of MAPKK in ethylene transduction pathway is in the downstream of $Ca^{2+}$ accumulation in cytosol.

### Signal transduction of MAPKK and dephosphorylated proteins in the *IPO* expression induced by ethylene

The application of OKA, a protein phosphatase inhibitor, and STA, a protein kinase inhibitor (Barwe *et al.* 2001), had the opposite effects on the accumulation of *IPO* mRNA induced by wounding and ethylene (Chen *et al.* 2003). OKA inhibited the *IPO* expression, while STA induced it. These results indicated that *IPO* gene induced by wounding and ethylene was activated by dephosphorylated proteins, but inhibited via protein phosphorylation (Chen *et al.* 2003). Because both dephosphorylated proteins and MAPKK are important for stimulating *IPO*, the order of these factors participating in the signal transduction of ethylene was studied here. The leaf petiole cuttings were first placed in PD98059 solution for 16 h, then STA was added to the PD98059 solution for another 2 h before *IPO* mRNA was measured. The induction of *IPO* mRNA by STA in the presence of PD98059 was inhibited (Fig. 5). Therefore, the presence of PD98059 interfered the function of STA in stimulating *IPO* expression. These results also indicated that the position of MAPKK in the signal transduction of ethylene is downstream of the dephosphorylated protein(s). In addition, the signal induced by ethylene is transferred to dephosphorylated protein(s) first, and then passes to MAPKK to cause the activation of *IPO* (Fig. 6).

## DISCUSSION

In this study, the signal transduction of ethylene inducing the expression of *IPO* was investigated. Right after the stimulation of ethylene, $Ca^{2+}$ from both apoplast and organelles entered into cytosol in minutes. The involvement of apoplastic $Ca^{2+}$ was identified by the application of channel blocker diltiazem, which inhibited the influxes of $Ca^{2+}$ from apoplast (Fig. 2a) and decreased *IPO* expression (Fig. 3). The application of cation chelator EGTA in cells (Fig. 4a,b) also indicated the involvement of apoplastic $Ca^{2+}$

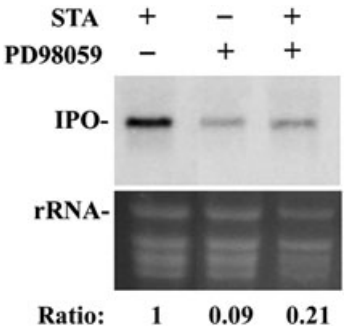



**Figure 5.** Effects of PD98059 on the expression of *IPO* stimulated by STA. The petiole cuts of the second and third fully expanded leaves from the terminal bud were immersed in 0.1 μM PD98059, an MAPKK inhibitor, for 16 h. The final concentration of 1 μM STA, a protein kinase inhibitor, was then added for another 2 h. The control leaves were incubated separately in 1 μM STA or 0.1 μM PD98059 alone. Their total RNAs were analysed by Northern blotting to detect *IPO* mRNA (IPO). Ethidium-bromide-stained agarose gel presents ribosome RNA (rRNA) as a loading control. The ratios of IPO mRNA to rRNA in each reaction were calculated, and the ratio of the reaction treated with STA alone was treated as a value of one for determining the relative ratios of other reactions.

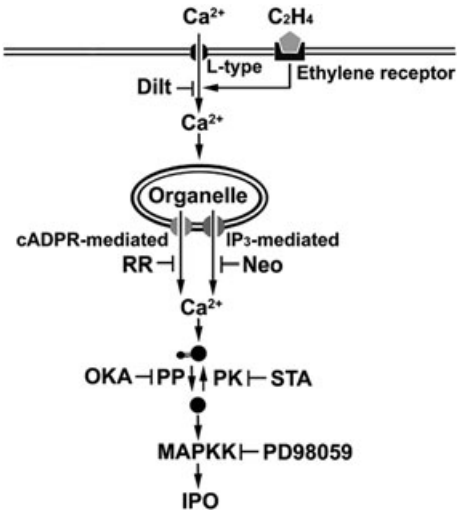



**Figure 6.** Model for the signal transduction of ethylene activating *IPO* in sweet potato. Ethylene binding to its receptor allows the influxes of apoplastic $Ca^{2+}$ into cytosol through the L-type voltage $Ca^{2+}$ channel in the plasma membrane, and then activates the release of $Ca^{2+}$ via inositol 1,4,5-trisphosphate ($IP_3$)-mediated and cyclic adenosine diphosphoribose (cADPR)-mediated $Ca^{2+}$ channels in organelle membrane. The elevation of cytosolic $Ca^{2+}$ may stimulate the protein dephosphorylation, and then the signal was passed through MAPKK to induce the *IPO* expression. Diltiazem (Dilt) blocks the L-type voltage-operated $Ca^{2+}$ channel, neomycin (Neo) blocks the $IP_3$-mediated $Ca^{2+}$ channel, whereas ruthenium red (RR) blocks the cADPR-mediated $Ca^{2+}$ channel. STA inhibits protein kinase (PK), OKA inhibits protein phosphatase (PP) and PD98059 inhibits MAPKK. The arrows represent induction, while the blunted lines indicate repression.

during ethylene activation. Neomycin and ruthenium red blocking $IP_3$-mediated and cADPR-mediated $Ca^{2+}$ channels in organelles, separately, also inhibited the entry of $Ca^{2+}$ from organells (Fig. 2a) and decreased the expression of *IPO* (Fig. 3), indicating the involvement of organelles' $Ca^{2+}$ in the ethylene signal transduction for inducing *IPO*. The accumulation of $Ca^{2+}$ in the cytosol induced by ethylene may stimulate protein dephosphorylation, which further activated MAPKK (Fig. 5), and then induced the expression of *IPO* (Fig. 6).

IPO protein is a defence-related protein by the insect-feeding experiment, and it decreased silkworm survival rate. IPO protein is a lectin based on its sequence, and its C-terminus is highly homologous with those of mannose-binding lectin, jacalin and agglutinin (Chen *et al.* 2003). In the previous study, IPO protein is a carbohydrate-binding protein. However, IPO protein cannot bind sugar alcohols like mannitol and sorbitol (Chen *et al.* 2003). Regarding the gene expression, accumulation of *IPO* mRNA appeared 2 h, peaked at 6 h and then continuously declined up to 16 h after wounding the sweet potato leaves (Chen *et al.* 2003). The expression of *IPO* was also induced by the stimulus of MeJA (Imanishi *et al.* 1997; Chen *et al.* 2003). The influences of $Ca^{2+}$, dephosphorylated proteins, $H_2O_2$ and nitric oxide on *IPO* expression were also studied (Chen *et al.* 2003; Jih *et al.* 2003). This study further investigates the signal transduction of ethylene in inducing the expression of *IPO* in sweet potato.

During the signal transduction induced by external stimuli, $Ca^{2+}$ is always considered to be the first response messenger. Monitoring the quick accumulation of cytosolic $Ca^{2+}$ by confocal microscope is a convenient method to investigate the activation of cells. However, the increase of $Ca^{2+}$ in cytosol is very fast, and it did take 90 s for sample treatment and microscope focusing in order to record the change of $Ca^{2+}$ by confocal microscope. Consequently, the increase of $Ca^{2+}$ in cells may not be recorded thoroughly, and only the second half of the increasing peak was documented by the confocal microscope (Fig. 1a–c). The $Ca^{2+}$ rising pattern of the cells treated with A23187, which is an ionophore allowing $Ca^{2+}$ to influxes into cytosol, was a half-peak shape monitored by confocal microscope (Fig. 1a). Therefore, the $Ca^{2+}$ rising pattern of the cells induced by ethylene showed the same half-peak shape, and was considered to be the consequence of $Ca^{2+}$ influxes into cells (Fig. 1b). However, the full $Ca^{2+}$ induction peak was observed in Fig. 2b, indicating $Ca^{2+}$ accumulation in the cells stimulated by ethylene initiated at the different time points. The variations of plant tissues used in each assay were used to explain this phenomenon. Conclusively, fluorescence variations in this study were simplified into two patterns. The increase in fluorescence indicates an increment of $Ca^{2+}$ in cytosol, while the unchanged fluorescence intensity with time demonstrates that the concentration of $Ca^{2+}$ in cytosol is unaltered.

Antagonists to $Ca^{2+}$ channels have been frequently used to characterize transport processes. To understand the effects of $Ca^{2+}$ in the signal transduction of ethylene, $Ca^{2+}$ channel blockers and chelators were used in this study. Ruthenium red and neomycin were used to block the $Ca^{2+}$ release from internal ER and vacuoles. Ruthenium red was treated as a cADPR-mediated channel blocker in this study. The synthesis of the second messenger cADPR was inducible by nitric oxide in animal cells, and cADPR activated $Ca^{2+}$ release from the specific subset of membrane vesicles (Wendehenne *et al.* 2001). In addition, cADPR in plant cells was able to induce $Ca^{2+}$ release from ER and vacuoles (Allen, Muir & Sanders 1995; Leckie *et al.* 1998). In tobacco cells, cADPR induced *PR-1* and *PAL* gene expression, and the induction of cADPR was inhibited by ruthenium red (Durner *et al.* 1998). On the other hand, neomycin was able to inhibit phospholipase C (Frankling-Tong *et al.* 1996), which broke down phosphatidyl inositol 4,5-bisphosphate into $IP_3$ that was responsible for the release of $Ca^{2+}$ from intracellular stores (Berridge 1984). Neomycin was used previously as a phospholipase C inhibitor to study $Ca^{2+}$ metabolism in plant cells (Frankling-Tong *et al.* 1996; Knight, Trewavas & Knight 1997). Hence, neomycin in this study was used as a channel blocker, which inhibited $IP_3$-mediated channel. In addition, diltiazem was used as an inhibitor to block L-type channel, which is one of the voltage-operated $Ca^{2+}$ channels in plasma membrane (Tsien *et al.* 1987; Hess 1988).

The cation chelator EGTA, which bound apoplastic $Ca^{2+}$, was applied to the reaction in order to further confirm the roles of apoplastic $Ca^{2+}$ during ethylene activation. In the previous study, *IPO* treated by mechanical wounding induced both the influxes of apoplastic $Ca^{2+}$ and the release of organelles' $Ca^{2+}$. Nevertheless, the ethylene-dependent *IPO* expression was reported to be regulated by the release of $Ca^{2+}$ from intracellular stores only because neomycin but not EGTA (1 mM) inhibited the expression of *IPO* induced by ethylene (Chen *et al.* 2003). The same results showed in this study that in the presence of 1 mM EGTA, ethylene can still induce *IPO* expression (Fig. 4a,b), but the increase of cytosolic $Ca^{2+}$ was not detected by confocal microscope (Fig. 2b). However, in the same concentration of 1 mM EGTA, both $Ca^{2+}$ influxes (Fig. 2c) and IPO expression (Fig. 4b) were inhibited in the cells treated with mechanical wounding. The high concentration of EGTA (10 mM) in both ethylene- and wounding-stimulated cells was needed to prevent $Ca^{2+}$ from entering into cytosol (Fig. 2b,c), and reduced the expression of *IPO* (Fig. 4a,b). Hence, both signal transduction pathways induced by ethylene and wounding did require apoplastic $Ca^{2+}$ to activate *IPO*, but the nature of $Ca^{2+}$ channels induced by ethylene and wounding was different. The possible reason for 1 mM EGTA not inhibiting the ethylene-induced *IPO* but repressing the wound-induced *IPO* is that ethylene and wounding triggered the expression of *IPO* via different plasma membrane $Ca^{2+}$ channels that may have different affinity with $Ca^{2+}$. Because only high concentration of EGTA can retard the activation of ethylene, $Ca^{2+}$ channels induced by ethylene may have higher affinity to $Ca^{2+}$ than those stimulated by wounding. Therefore, after harvesting of plants, high concentration of EGTA is required to delay the action of ethylene. At the same time, the presence of 1 mM EGTA can block the induction of mechanical wounding, but it still allows the stimulation of ethylene.

$Ca^{2+}$ influxes into cytosol are mainly from apoplastic space and internal organelles, but $Ca^{2+}$ from different source for activating genes was reported. $Ca^{2+}$ antagonists, including EGTA (up to 10 mM), lanthanum, verapamil and ruthenium red, were used to test the involvement of the apoplastic and the organelles' $Ca^{2+}$ in the ethylene responses of *Pisum sativum* (Petruzzelli *et al.* 2003). Results indicated that $Ca^{2+}$ release from organelles, but not apoplastic space, was involved in activating *PS-ACO1* gene, and also that both the apoplastic and the organelles' $Ca^{2+}$ were required for negatively regulating *PS-ACS2* gene and positively activating class 1 $\beta$-1,3-glucanase (Petruzzelli *et al.* 2003). However, our study indicated that $Ca^{2+}$ from both apoplast and organelles was involved in the activation of *IPO* induced by ethylene or wounding (Figs 3 & 4).

Additionally, $Ca^{2+}$ is an important secondary messenger and regulates the complicated gene expression in both animals and plants. The concentration and localization of $Ca^{2+}$ were responsible for the specific regulation of $Ca^{2+}$ between stimuli and responses (Sanders *et al.* 2002). The $Ca^{2+}$ channel was also regulated by $Ca^{2+}$ feedback regulation. The cADPR-dependent $Ca^{2+}$ channels opened at physiological *trans*-tonoplast voltages, but repressed when the $Ca^{2+}$ concentration was greater than 600 nM (Leckie *et al.* 1998). Therefore, even though the cytosolic $Ca^{2+}$ was increased largely by the application of A23187, the expression of *IPO* did not occur (Fig. 3). This result further indicated that the unsuitable increase of $Ca^{2+}$ cannot turn on transcription, at least the transcription of *IPO* gene.

MAPK plays an important role in signal transcription in all eukaryotic cells, and it represents the convergence point for many signalling pathways and modulates a variety of cellular events. Mechanical manipulation of *Arabidopsis* leaves induced the gene expressions of MAPKKK and MAPK (Bogre *et al.* 1996, 1997). MAPK and other protein kinases were induced by touch, cold and water stress within 30 min in *Arabidopsis* (Mizoguchi *et al.* 1996). Wounding alfalfa leaves specifically induces the transient activation of the $p44^{MMK4}$ kinase, which belongs to the MAPK family (Bogre *et al.* 1997). These evidences displayed MAPK pathway mediating the plant stress signal, and thus it was worth finding out the regulation of MAPKs within the ethylene transduction pathway inducing the *IPO* expression in sweet potato. PD98059, a MAPKK inhibitor, blocked the expression of *IPO* induced by ethylene (Fig. 3), but it did not inhibit the influxes of $Ca^{2+}$ in the cells treated by ethylene (Fig. 1c). These results showed that MAPKK was involved in the ethylene transduction activating *IPO*, and its action was located after the $Ca^{2+}$ accumulation. STA, a protein kinase inhibitor, was demonstrated to induce *IPO* expression (Fig. 5), while OKA, a protein phosphatase inhibitor, decreased the *IPO* expression activated by ethylene (Chen *et al.* 2003). However, leaves of sweet potato were treated with PD98059 before STA was added, and its *IPO* expression was still reduced as those treated with PD98059 alone (Fig. 5). These results indicated that MAPKK was involved in the ethylene-induced signal pathway, and the reaction order of MAPKK in the ethylene transduction pathway was behind the dephosphorylated proteins (Fig. 6).

Conclusively, the ethylene transduction pathway inducing the expression of *IPO* is shown in Fig. 6. Following ethylene stimulation, $Ca^{2+}$ from the intercellular space quickly enters into cytosol via L-type voltage-operated $Ca^{2+}$ channel in the plasma membrane , and it further activates the $IP_3$-mediated and cADPR-mediated $Ca^{2+}$ channels in organelles to release $Ca^{2+}$ to enter into cytosol. The elevation of cytosolic $Ca^{2+}$ may stimulate the protein dephosphorylation, which further activates MAPKK, and finally, *IPO* gene is induced.

## ACKNOWLEDGMENTS

This work was supported by the National Science Council under grant 95-2313-B-002-011 to S.-T.J. We wish to thank Technology Commons, College of Life Science, National Taiwan University for technical support.

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*Received 17 July 2007; accepted for publication 10 September 2007*

 