

RESEARCH PAPER

Carbon monoxide regulates the expression of the wound-inducible gene *ipomoelin* through antioxidation and MAPK phosphorylation in sweet potato

Jeng-Shane Lin^{1,*}, Hsin-Hung Lin^{1,2,*}, Yu-Chi Li¹, Yu-Chi King¹, Ruei-Jin Sung¹, Yun-Wei Kuo¹, Chih-Ching Lin^{1,3}, Yu-Hsing Shen¹ and Shih-Tong Jeng^{1,†}

¹ Institute of Plant Biology and Department of Life Science, National Taiwan University, Taipei 10617, Taiwan

² Biodiversity Research Center, Academia Sinica, Taipei 11529, Taiwan

³ Institute of Plant and Microbial Biology, Academia Sinica, Taipei 11529, Taiwan

* These authors contributed equally to this work.

† To whom correspondence should be addressed. E-mail: stjeng@ntu.edu.tw

Received 1 April 2014; Revised 4 June 2014; Accepted 6 June 2014

Abstract

Carbon monoxide (CO), one of the haem oxygenase (HO) products, plays important roles in plant development and stress adaptation. However, the function of CO involved in wounding responses is seldom studied. A wound-inducible gene, *ipomoelin* (*IPO*), of sweet potato (*Ipomoea batatas* cv. Tainung 57) was used as a target to study the regulation of CO in wounding responses. After wounding for 1 h, the endogenous CO content and *IbHO* expression level were significantly reduced in leaves. *IPO* expression upon wounding was prohibited by the HO activator hemin, whereas the HO inhibitor zinc protoporphyrin IX elevated *IPO* expression. The *IPO* expression induced by wounding, H₂O₂, or methyl jasmonate was inhibited by CO. CO also affected the activities of ascorbate peroxidase, catalase, and peroxidase, and largely decreased H₂O₂ content in leaves. CO inhibited the extracellular signal-regulated kinase (ERK) phosphorylation induced by wounding. *IbMAPK*, the ERK of sweet potato, was identified by immunoblotting, and the interaction with its upstream activator, *IbMEK1*, was further confirmed by bimolecular fluorescence complementation and co-immunoprecipitation. Conclusively, wounding in leaves repressed *IbHO* expression and CO production, induced H₂O₂ generation and ERK phosphorylation, and then stimulated *IPO* expression.

Key words: Carbon monoxide, ERK phosphorylation, H₂O₂, ipomoelin, sweet potato, wounding.

Introduction

Carbon monoxide (CO) is an odourless, tasteless, and colourless diatomic gaseous molecule that has been widely considered to be a poisonous gas since 17th century. It can be produced by haem oxygenase (HO; E.C. 1:14:99:3) in biological systems in plants (Sjostrand, 1952; Siegel *et al.*, 1962), and acts as a physiological messenger (Shekhawat and Verma, 2010). HO is a ubiquitous enzyme that has been identified

in different organisms including bacteria, algae, fungi, animals, and plants (Terry *et al.*, 2002). HO is an evolutionarily conserved enzyme that catalyses haem degradation leading to the production of equimolar amounts of CO, biliverdin, and free iron (Muramoto *et al.*, 2002; Terry *et al.*, 2002). Recent studies indicate that HO plays multiple roles in growth, development, and the stress response. It participates in the

Abbreviations: APX, ascorbate peroxidase; BiFC, bimolecular fluorescence complementation; CAT, catalase; CO, carbon monoxide; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; Hb, haemoglobin; Hm, hemin; HO, haem oxygenase; IPO, ipomoelin; MAPK, mitogen-activated protein kinase; MJ, methyl jasmonate; NO, nitric oxide; POX, peroxidase; qRT-PCR, quantitative reverse transcription-PCR; RACE, rapid amplification of cDNA ends; SA, salicylic acid; SD, standard deviation; SOD, superoxide dismutase; STA, staurosporine; YFP, yellow fluorescent protein; ZnPP, zinc protoporphyrin IX.

© The Author 2014. Published by Oxford University Press on behalf of the Society for Experimental Biology.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0/>), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

phytochrome chromophore formation to affect photomorphogenesis and light signalling in plants (Davis *et al.*, 2001; Gisk *et al.*, 2010; Shekhawat and Verma, 2010). In addition, HO acts as a downstream mediator in auxin signalling to affect root development (Cao *et al.*, 2007; Xuan *et al.*, 2007, 2008b; Gisk *et al.*, 2012). In soybean (*Glycine max*) and *Arabidopsis*, HO is involved in reactive oxygen species scavenging to against salinity and heavy metal-induced oxidative stresses (Balestrasse *et al.*, 2008a, b; Zilli *et al.*, 2009). The addition of HO1 products, especially CO, can partially rescue the UV-C hypersensitivity in the *hy1-100* mutant (Xie *et al.*, 2012).

CO is a physiological messenger involved in various plant growth and development. It promotes root elongation (Xuan *et al.*, 2007), root hair development (Guo *et al.*, 2009), adventitious root generation (Xuan *et al.*, 2008b), and stomatal closure (Cao *et al.*, 2007; Song *et al.*, 2008; Xuan *et al.*, 2008a). In addition, CO activates antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), to protect plant from the oxidative damage induced by salt (Xie *et al.*, 2008; Bose *et al.*, 2013), cadmium (Han *et al.*, 2008; Cui *et al.*, 2012), paraquat (Sa *et al.*, 2007), and UV irradiation (Xie *et al.*, 2012). It also improves adaptation of iron deficiency in *Arabidopsis* (Kong *et al.*, 2010) and delays gibberellin-mediated programmed cell death in wheat aleurone layers (Wu *et al.*, 2011). In addition, CO induces the production of nitric oxide (NO) in plants (Xuan *et al.*, 2007; Song *et al.*, 2008; Xuan *et al.*, 2008b), and alters the phosphorylation of p38 mitogen-activated protein kinases (MAPK) and extracellular signal-regulated kinases (ERK) in animals (Song *et al.*, 2002; Kim *et al.*, 2005; Basuroy *et al.*, 2011; Schallner *et al.*, 2012). Thus, CO participates in not only physiological regulation but also in defence responses.

In natural environments, plants develop inducible defence systems to survive biotic and abiotic threats. During pathogenic and herbivorous attacks, plants produce a wide variety of defence-related hormones, including ethylene, methyl jasmonate (MJ), salicylic acid (SA) (Pieterse & Van Loon, 2004; Manavella *et al.*, 2008), and peptide-hormones (Ryan *et al.*, 2002), to unlock the defence-related regulatory networks. Second messengers, including NO, cytosolic calcium (Ca²⁺) (Capiati *et al.*, 2006; Chen *et al.*, 2008; Howe and Jander, 2008), and reactive oxygen species (Orozco-Cardenas *et al.*, 2001; Jih *et al.*, 2003; Le Deunff *et al.*, 2004), are generated to induce defence-related genes (El-kereamy *et al.*, 2011). Post-transcriptional regulators such as microRNAs (Lin *et al.*, 2012) and post-translational regulation including MAPK cascades (Chen *et al.*, 2008; Howe and Jander, 2008) are also involved in various defence responses.

MAPK cascades, consisting of a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK, mediate signalling transductions in various stresses in plants. AtMPK3 and AtMPK6 can be activated after treated with flagellin flg22, a pathogen-derived peptide (Galletti *et al.*, 2011). The AtMKK1–AtMPK6 module controls the H₂O₂ signalling in an abscisic acid-dependent pathway to regulate stress responses (Xing *et al.*, 2008). In tobacco, wounding activates wounding-induced protein kinase, the orthologous

of AtMAPK3, and SA-induced protein kinase, the orthologous of AtMAPK6, and further regulates the levels of jasmonic acid and SA (Yang *et al.*, 2001; Menke *et al.*, 2004; Seo *et al.*, 2007). The addition of an MAPKK inhibitor, PD98059, blocks the expression of *Ipomoein* (*IPO*) induced by wounding and ethylene (Chen *et al.*, 2008).

Although the antioxidant and physiological properties of CO have been studied, the role of CO in wounding is still poorly understood. *IPO*, a wound-inducible gene from sweet potato (*Ipomoea batatas*), is induced by the application of MJ, ethylene, and mechanical wounding, and is further regulated by Ca²⁺, H₂O₂, and NO (Imanishi *et al.*, 1997; Chen *et al.*, 2003, 2008; Jih *et al.*, 2003). In this study, *IPO* was used as an indicator to certify the relationship between CO and the wounding response.

Materials and methods

Plant materials and treatments

Sweet potato (*I. batatas* cv. Tainung 57) and tobacco (*Nicotiana benthamiana*) plants were grown in a controlled environment (16h/25 °C day; 8h/22 °C night; humidity 70%; light 40 μmol photons m⁻² s⁻¹). Plants with six to eight fully expanded leaves were used in this study. Sweet potato was used for gene isolation, gene expression assay, determination of CO and H₂O₂, enzyme activity assay, and ERK phosphorylation analysis; tobacco was used in co-immunoprecipitation experiments. *Arabidopsis thaliana* (Col-0) was grown at 22 °C under a 16h light/8h dark photoperiod with cool fluorescent light at 100 μmol photons m⁻² s⁻¹, and 15-d-old plants were used in bimolecular fluorescence complementation (BiFC) assays.

Chemical treatments were performed based on the procedure described by Chen *et al.* (2008) and Lin *et al.* (2012). In this study, chemical reagents were purchased from Sigma-Aldrich, and tanks with 99.5% pure CO gas were purchased from Ciao Chong Gaseous Corporation in Taipei, Taiwan. All results in this study were repeated at least three times, and the similar gene expression patterns were obtained.

CO solution preparation

CO solution preparation was performed based on the method described by Kong *et al.* (2010). CO gas was passed through 20ml water in an open tube for 30min to reach saturated solution. The saturated CO in water was treated as 100% CO solution.

RNA isolation and quantitative real-time reverse transcription (qRT)-PCR

Total RNA was isolated from leaves that were ground in liquid nitrogen using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA isolated from plants was treated with DNase I (Ambion) before the reaction of Moloney murine leukemia virus reverse transcriptase (Invitrogen) with primer T₂₅VN (Supplementary Table S1 at JXB online) at 37 °C for 90min. The cDNAs were further amplified by quantitative PCR with primer sets IPO F/IPO R, IbHO1 F/IbHO1 R, and IbActin F/IbActin R (Supplementary Table S1) to detect the expression levels of *IPO*, *IbHO1*, and *IbActin*, respectively. The amplification reactions contain 1× SYBR Green Supermix (Bio-Rad), 125nM primers, and 100ng cDNA. Data were normalized by the expression levels of the *IbActin* gene and are shown as relative expression levels. The error bars indicate standard deviation (SD) from at least three biological assays.

Isolation of *IbHO1*, *IbMEK1*, and *IbMAPK*

The conserved domains of the *HO1*, *MEK1*, and *MAPK* genes from *Arabidopsis*, tobacco, tomato, and rice were used to search the *Ipomoea* EST and WGS databases from the NCBI to obtain putative *IbHO1*, *IbMEK1*, and *IbMAPK* genes of sweet potato. The full-length sequences of *IbHO1* and *IbMEK1* genes were then obtained using a BD SMARTTM RACE cDNA Amplification kit (Clontech) with primer sets *IbHO1* F/*IbHO1* R and *IbMEK1* F/*IbMEK1* R (Supplementary Table S1 at *JXB* online), respectively. *IbMAPK* is SPMAPK (GenBank accession no. AAD37790) and was amplified by PCR with primer sets *IbMAPK* F/*IbMAPK* R (Supplementary Table S1).

Multiple sequences alignment and phylogenetic analyses

The protein sequences of *IbHO1*, *IbMEK1*, and *IbMAPK* were aligned with the related protein sequences from plants using ClustalX2. The phylogenetic trees were reconstructed using the neighbour-joining method with the MEGA5.1 program. A bootstrap test of phylogeny was performed with 1000 replicates.

CO determination

Detection of CO content was performed based on the method described by Chalmers (Chalmers, 1991; Kong *et al.*, 2010) with minor modifications. Leaves (0.5 g) were ground in liquid nitrogen, added to 5 ml of H₂O, and sealed in tubes. After centrifuged at 3000g for 5 min, supernatants (0.5 ml) of samples were mixed with 0.5 ml of 1 mg ml⁻¹ haemoglobin (Hb), which was dissolved in 0.24 M ammonia solution. After 0.1 ml of 0.2 g ml⁻¹ freshly prepared sodium dithionite solution was added, the solution was stood for 10 min and then analysed. The absorbance at 595 nm of the solution was measured to calculate protein concentration, and absorbance at 420 and 432 nm was measured for CO content determination as described previously (Chalmers, 1991; Kong *et al.*, 2010). The CO content in the wounded leaves was represented as the values relative to those of the unwounded leaves.

H₂O₂ determination

H₂O₂ content was quantified by the titanium chloride method as described previously (Jana and Choudhuri, 1982).

APX, CAT, and peroxidase (POX) activity assays

APX, CAT, and POX activity assays were performed based on the method described by Lin *et al.* (2011) with minor modifications. Total protein was extracted with an extraction buffer [50 mM sodium phosphate buffer (pH 6.8) and 1% Protease Inhibitor Cocktail (Sigma)]. In the APX activity assay, 33 µl of extracted protein was added to 967 µl of reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA, and 1 mM H₂O₂. In the POX activity assay, 30 µl of extracted protein was added to 2.97 ml of reaction mixture containing 50 mM sodium acetate buffer (pH 5.6), 5.4 mM guaiacol, and 15 mM H₂O₂. In the CAT activity assay, 30 µl of extracted protein was added to 2.97 ml of reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 10 mM H₂O₂. These reaction mixtures were monitored at 290, 470, and 240 nm for 1 min for the APX, POD, and CAT assays, respectively. The enzyme activity was calculated as U min⁻¹ µg⁻¹ of protein. The enzyme activity of the H₂O-treated leaves at the time point zero was treated as a value of 1 for determining the relative ratios of other reactions. Data are represented as mean ± SD.

Protein extraction and immunoblot analysis

Total protein was extracted with an extraction buffer [100 mM HEPES (pH 7.5), 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 50 mM glycerophosphate, 10 mM Na₃VO₄, 10 mM dithiothreitol, 1 mM

phenylmethylsulphonyl fluoride, 5 µg ml⁻¹ of leupeptin, 5 µg ml⁻¹ of aprotinin, 1% Protease Inhibitor Cocktail, and 10% glycerol]. Protein concentration was determined using a Bio-Rad protein assay kit. Total proteins (50 µg) were separated by 12% SDS-PAGE, and then transferred to Immobilon polyvinylidene difluoride membranes (Millipore). The membrane was probed with an anti-pERK antibody (Santa Cruz) followed by horseradish peroxidase-conjugated sheep anti-mouse IgG, and detected using an Immobilon TM Western Chemiluminescent HRP Substrate kit (Millipore).

Protein expression and purification

The coding regions of *IbMEK1* and *IbMAPK* isolated from the cDNA library of sweet potato were constructed into pGEX6p-1 or pET21a to become pGEX6p-1-*IbMEK1*, pET21a-*IbMEK1*, and pET21a-*IbMAPK* by PCR with primer sets BamHI-*IbMEK1* F/EcoRI-*IbMEK1* R, BamHI-*IbMEK1* F/HindIII-*IbMEK1* R, and BamHI-*IbMAPK* F/HindIII-*IbMAPK* R (Supplementary Table S1), respectively. These constructs were transformed into the *Escherichia coli* Rosetta(DE3)pLysS strain to express *IbMEK1* fused with a glutathione S-transferase (GST) or His tag and *IbMAPK* fused with a His tag. The recombinant protein GST-*IbMEK1* and GST were purified using a GSTrapTM FF affinity column (GE Healthcare). The recombinant proteins *IbMEK1*-His and *IbMAPK*-His were purified using a HiTrapTM TALON[®] Crude column (GE Healthcare).

In vitro phosphorylation analysis

Total protein from leaves of sweet potato was extracted with extraction buffer [20 mM Tris/HCl (pH 7.2) and 1% Protease Inhibitor Cocktail]. The extracted protein was incubated with reaction mixture containing 20 mM Tris/HCl (pH 7.2), 10 mM MgCl₂, 0.5 mM CaCl₂, 0.5 mM ATP, 2 mM dithiothreitol, and 10 µg of purified *IbMAPK*-His. After incubation at 30 °C for 1 h, ProBondTM Nickel-Chelating Resin (Invitrogen) was added to purify *IbMAPK*-His. After washing five times, the bound proteins were eluted and separated by 12% SDS-PAGE, and detected by immunoblotting using an anti-pERK antibody (Santa Cruz) and an anti-His antibody (LTK Biolaboratories).

BIFC

IbMEK1 fragments were isolated from the sweet potato cDNA library using PCR with primer sets BamHI-*IbMEK1* F/SacI-*IbMEK1* R and XbaI-*IbMEK1* F/BamHI-*IbMEK1* R (Supplementary Table S1), and inserted into pBI221 to obtain pBI221-*IbMEK1* and pBI221-*IbMEK1* Δstop codon, respectively. *IbMAPK* fragments were also isolated from the sweet potato cDNA library using PCR with primer sets BamHI-*IbMAPK* F/SacI-*IbMAPK* R and XbaI-*IbMAPK* F/BamHI-*IbMAPK* R (Supplementary Table S1), and inserted into pBI221 to obtain pBI221-*IbMAPK* and pBI221-*IbMAPK* Δstop codon, individually. The N terminus (YN) of yellow fluorescent protein (YFP) was inserted into pBI221-*IbMEK1* and pBI221-*IbMAPK* via the *XbaI* and *BamHI* sites to become pBI221-YN-*IbMEK1* and pBI221-YN-*IbMAPK*, and the C terminus (YC) of YFP was inserted into pBI221-*IbMEK1* Δstop codon and pBI221-*IbMAPK* Δstop codon by *BamHI* and *SacI* sites to obtain pBI221-*IbMEK1*-YC and pBI221-*IbMAPK*-YC, respectively.

Arabidopsis protoplast isolation and transformation were performed according to Yoo *et al.* (2007), and incubated at room temperature for 16 h. Confocal laser-scanning microscopy was used to visualize the fluorescent signal from the protoplasts. The protoplasts co-transformed with plasmids encoding YN and YC were used as negative controls.

GST pull-down assays

GST pull-down assays were performed according to Yu *et al.* (2011). The recombinant protein GST-*IbMEK1* or GST was co-incubated

with IbMAPK–His and GST•Bind™ Resin (Millipore). After washing five times, the bound proteins were eluted and separated by 12% SDS-PAGE, and detected by immunoblotting using an anti-His antibody (LTK Biolaboratories).

Co-immunoprecipitation

Transient expression of GST, GST–IbMEK1, and IbMAPK–His in *N. benthamiana* leaves was performed based on the method described by Lin *et al.* (2012, 2013). The GST fragment was isolated from pGEX6p-1 (Clontech) by PCR using primer set BamHI–GST F/ SacI–GST R (Supplementary Table S1), and inserted into pBI221 to obtain pBI221–GST, whose 35S–GST–terminator fragment was then cloned into the *Hind*III and *Eco*RI sites of pCambia1300. IbMEK1 and IbMAPK fragments were obtained from the sweet potato cDNA library by PCR with primer sets XbaI–IbMEK1 F/ BamHI–IbMEK1 R and BamHI–IbMAPK F/ SacI–IbMAPK R (Supplementary Table S1), respectively. IbMEK1 fragment was inserted into pBI221–GST to obtain pBI221–IbMEK1–GST, whose 35S–IbMEK1–GST–terminator fragment was then cloned into pCambia1300. The IbMAPK fragment was inserted into pBI221 to get pBI221–IbMAPK. The His tag of pBI221–HC was then cloned into the *Xba*I and *Bam*HI sites of pBI221–IbMAPK to become pBI221–His–IbMAPK, whose 35S–His–IbMAPK–terminator fragment was further cloned into pCambia1300 for assays.

Total proteins from infiltrated leaves were extracted with an extraction buffer [50mM Tris/HCl (pH 7.4), 150mM NaCl, 2mM MgCl₂, 1mM dithiothreitol, 20% glycerol, and 1% Protease Cocktail Inhibitor], and incubated with GST•Bind™ Resin. After washing five times, the bound proteins were eluted and separated by 12% SDS-PAGE, and detected by immunoblotting using an anti-His antibody (LTK Biolaboratories).

Results

Reduction of CO content and *IbHO1* transcripts by wounding

To gain insights into the regulatory roles of CO under wounding, the CO content of the unwounded and wounded leaves was analysed. CO is strongly bound by the ferrous haem in Hb. Hence, the percentage of Hb with CO was used to estimate CO content in cells. The results indicated that CO concentrations in leaves were reduced significantly at 1 h and went back to normal levels at 6 h after wounding (Fig. 1A). Hence, wounding may reduce CO levels in leaves temporarily.

HO is the main enzyme producing CO in plants (Terry *et al.*, 2002), and its full-length gene was obtained by rapid amplification of cDNA ends (RACE). The sequence of the *IbHO1* protein showed that it is homologous to the HO1 proteins from *Arabidopsis*, *Brassica juncea*, and *Oryza sativa* (Supplementary Fig. S1A at JXB online), and a phylogenetic analysis of these proteins was performed (Supplementary Fig. S1B). The haem-binding pocket residues and the axial haem iron ligand of *IbHO1* were also identified to the conserved residues of other HO1 in plants (Supplementary Fig. S1A). In addition, the expression of *IbHO1* transcripts upon wounding was examined by qRT-PCR in sweet potato. The expression levels of *IbHO* were decreased at 0.5 and 1 h, and went back to normal levels at 3 h after wounding (Fig. 1B). Taken together, the above results showed that wounding might reduce *IbHO1* expression and then decrease CO in leaves.

Effects of *IbHO1* on *IPO* expression

To analyse the effects of endogenous CO on wounding responses, wound-inducible *IPO* was used as an indicator. The HO-specific activator hemin (Hm) was supplied to elevate the endogenous CO levels (Xuan *et al.*, 2007). In sweet potato, Hm can increase the CO content in leaves (Supplementary Fig. S2 at JXB online). Leaves of sweet potato were pre-treated without or with various concentrations of Hm for 12 h, and wounded for another 6 h. The *IPO* expression induced by wounding was inhibited when the solution contained 1 or 10 μ M Hm (Fig. 2A). To further confirm the involvement of HO in *IPO* regulation, a potential HO inhibitor, zinc protoporphyrin IX (ZnPP), was used to inhibit the HO activity. ZnPP can decrease the endogenous CO in the leaves of sweet potato (Supplementary Fig. S2). Leaves were supplied with various concentrations of ZnPP for 6 h to analyse *IPO* expression, showing that *IPO* expression was elevated in a ZnPP concentration-dependent manner (Fig. 2B). These results indicated that the activity of HO might influence *IPO* expression.

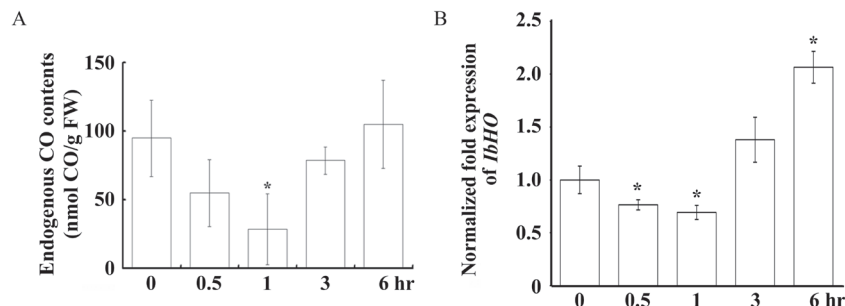


Fig. 1. Levels of endogenous CO and haem oxygenase transcripts in sweet potato upon wounding. The third fully expanded leaves of sweet potato were wounded by forceps and collected at 0, 0.5, 1, 3, and 6 h later. These leaves were used to analyse the endogenous CO contents (A) and the expression levels of the haem oxygenase gene (*IbHO*) (B). CO contents were detected by haemoglobin binding. *IbHO* expression levels were analysed by qRT-PCR. *IbActin* expression was used as an internal control. Statistic differences between unwounded and wounded sweet potato plants are marked with asterisk when $P < 0.01$ according to Student's *t*-test. The error bars are indicated as SD for at least three biological assays.

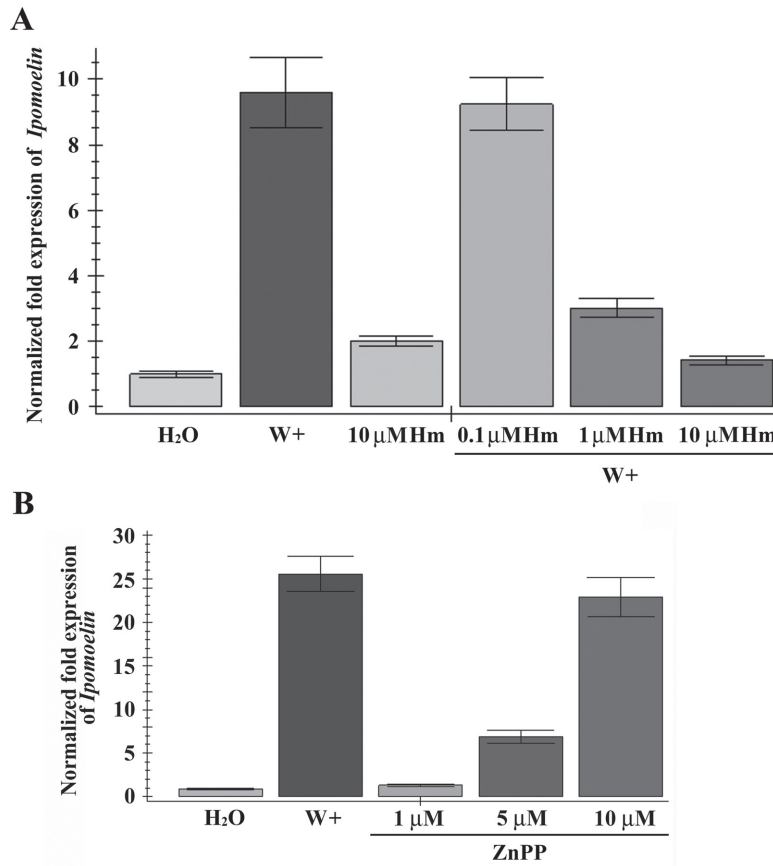


Fig. 2. Effects of HO on *IPO* expression upon wounding. (A) Effects of the HO activator Hm on *IPO* expression. Leaves with petiole cuts of sweet potato were immersed in water for 12h and then treated with various concentrations (0, 0.1, 1, and 10 μM) of the HO activator Hm for another 12h. These leaves were unwounded or wounded (W+) by tweezers. After 6h, the total RNAs from these leaves were analysed by qRT-PCR to detect *IPO* expression. (B) Effects of the HO repressor ZnPP on *IPO* expression. The petiole cuts of leaves were immersed in water for 24h. Then, the total RNAs of leaves were isolated at 6h after wounding (W+) or the addition of various concentrations (0, 1, 5, and 10 μM) of HO repressor ZnPP. The *IPO* expression levels were analysed by qRT-PCR. The *IbActin* expression was used as an internal control. The error bars are indicated as SD for at least three biological assays.

Wounding-, MJ-, or H₂O₂-induced *IPO* expression is repressed by CO

Saturated CO water, prepared from bubbling CO gas through water for 30 min, was used to supply CO in this study. When the leaves were pre-treated with CO water, the wound-induced *IPO* expression was inhibited in the presence of 3, 5, or 10% CO (Fig. 3A). Based on the CO level in Fig. 1A, the CO content in the leaves of sweet potato upon wounding for 1 h was 29 μM when leaves contained 92% water. Because the solubility of CO in water at 25 °C is about 0.025 g l⁻¹ (http://www.engineeringtoolbox.com/gases-solubility-water-d_1148.html), 5% CO water was 46 μM CO. The effect of CO on sweet potato was then studied in the presence of 5% CO combined with the endogenous CO in the following studies.

In the signalling pathway of *IPO* induction, MJ and H₂O₂ also act as important transducers (Chen *et al.*, 2003). To monitor the function of CO in the *IPO* induction pathway, *IPO* expression in the response of MJ or H₂O₂ was examined in the presence of CO. The leaves were pre-treated with 5% CO for 12h, and then MJ or H₂O₂ was supplied for another 6h before analyses (Fig. 3B). The expression levels of *IPO* were induced by MJ or H₂O₂ but could not be elevated in the presence of CO (Fig. 3B). These results indicated that the

presence of CO prohibited the *IPO* expression induced by not only wounding but also MJ or H₂O₂.

CO reduces wound-induced H₂O₂ through antioxidants

In sweet potato, wounding stimulates the production of H₂O₂ from NADPH oxidase, and generates both local and systemic signals to induce *IPO* expression (Jih *et al.*, 2003). HO, which can product CO, participates in reactive oxygen species scavenging (Balestrasse *et al.*, 2008a, b; Zilli *et al.*, 2009). To investigate the relationship between CO and H₂O₂ in wounding responses, H₂O₂ content upon wounding was examined in the present of CO (Fig. 4A). The level of H₂O₂ was induced after the sweet potato was wounded, whereas it was significantly reduced by pre-supplying the leaves with 5% CO solution.

Previous studies have indicated that CO can activate various antioxidant enzymes (Han *et al.*, 2008; Cui *et al.*, 2012; Xie *et al.*, 2012). The activities of APX, CAT, and POX were then analysed to study how CO inhibits the accumulation of the wound-induced H₂O₂. The results showed that CO could influence the activities of APX, CAT, and POX (Fig. 4B–D). Without wounding, CO could significantly increase the CAT activity (time 0 in Fig. 4C) and slightly induce the POX

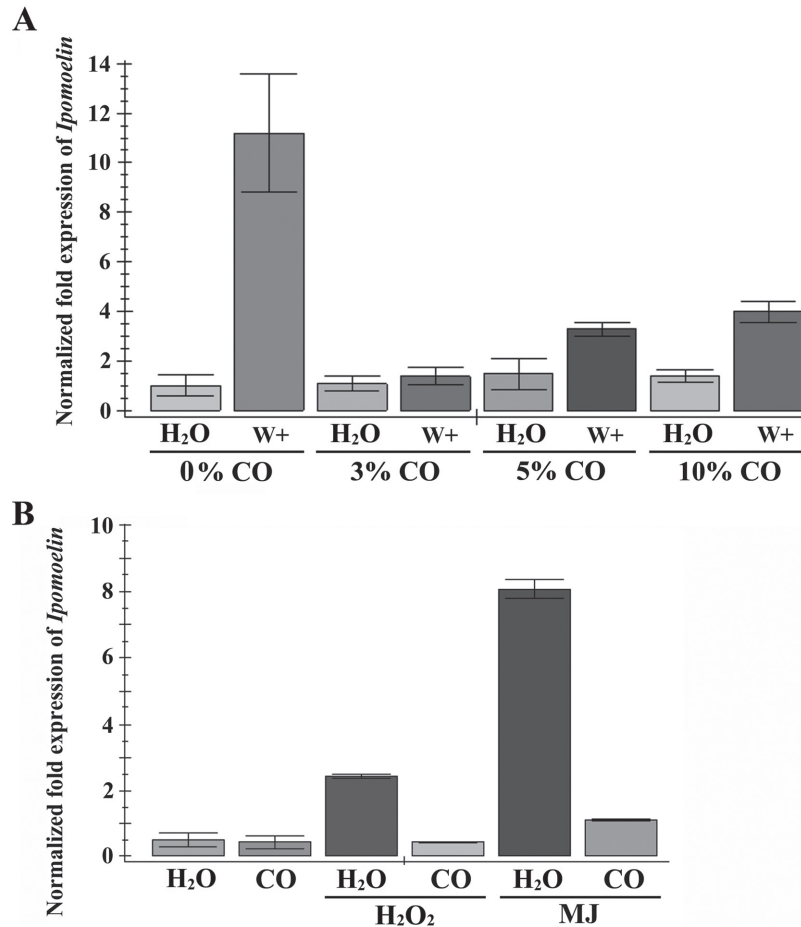


Fig. 3. Effects of CO on *IPO* expression induced by wounding, H₂O₂, or MJ. (A) Effects of CO on the *IPO* expression upon wounding. Leaves with petiole cuts of sweet potato were immersed in water for 12 h, and then treated with various concentrations (0, 3, 5, and 10%) of CO solution for another 12 h. These leaves were then unwounded (H₂O) or wounded (W+) by tweezers. After 6 h, the total RNAs from these leaves were analysed by qRT-PCR to detect *IPO* expression. (B) Effects of CO on *IPO* expression induced by H₂O₂ or MJ. Leaves with petiole cuts were immersed in water for 12 h and then some of these leaves were treated with 5% CO solution for another 12 h. These leaves were then treated with 20 mM H₂O₂ or 50 μM MJ for 6 h. The *IPO* expression levels of these leaves were analysed by qRT-PCR. The *IbActin* expression level was used as an internal control. The error bars are indicated as SD for at least three biological assays.

activity (time 0 in Fig. 4D). The activities of APX, CAT, and POX were quickly and intensely induced at 1 h after wounding in the present of CO (Fig. 4B–D). Interestingly, APX activity remained higher in the wounded leaves with CO than with water (Fig. 4B). Therefore, CO elevated the activities of APX, CAT, and POX to decompose the H₂O₂ induced by wounding and further interfered in *IPO* expression.

CO suppresses the phosphorylation of ERK induced by wounding

In animals, the phosphorylation of ERK1/2 can be influenced by CO (Song *et al.*, 2002; Kim *et al.*, 2005; Basuroy *et al.*, 2011; Schallner *et al.*, 2012). MAPK cascades are also involved in the *IPO* induction pathway (Chen *et al.*, 2008). Hence, the potential inducer staurosporine (STA) and inhibitor PD98059 of ERK1/2 phosphorylation were used to examine the effects of CO on ERK phosphorylation. STA induces ERK phosphorylation (Xiao *et al.*, 1999; Cho *et al.*, 2003; Zhao *et al.*, 2013). STA also increases *IPO* expression, whereas PD98059 counteracts STA response for *IPO* induction (Chen

et al., 2008). In Fig. 5A, CO blocked the expression of *IPO* induced by STA. In addition, PD98059 inhibited the expression of *IPO* induced by HO repressor ZnPP (Fig. 5B). These results indicated that CO may regulate ERK phosphorylation in sweet potato. Using anti-pERK antibody as a probe in immunoblotting analysis, wounding and STA elevated ERK phosphorylation (Fig. 5C). By contrast, ERK phosphorylation was repressed by CO and PD98059 (Fig. 5C). These results indicated that CO could decrease ERK phosphorylation, and then inhibit *IPO* induction. In wounding responses, therefore, CO contents were decreased to enhance ERK phosphorylation, and then *IPO* expression was induced.

Identification of ERK of sweet potato

Anti-pERK antibody mainly detects a short amino acid sequence containing phosphorylated Tyr 204 of ERK 1 of human origin (<http://www.scbt.com/datasheet-7383-p-erk-e-4-antibody.html>), and the activation of ERK1/2 is inhibited in the presence of PD98059 (Alessi *et al.*, 1995; Gudesblat *et al.*, 2007). MPK3 and MPK6 of *Arabidopsis* can be

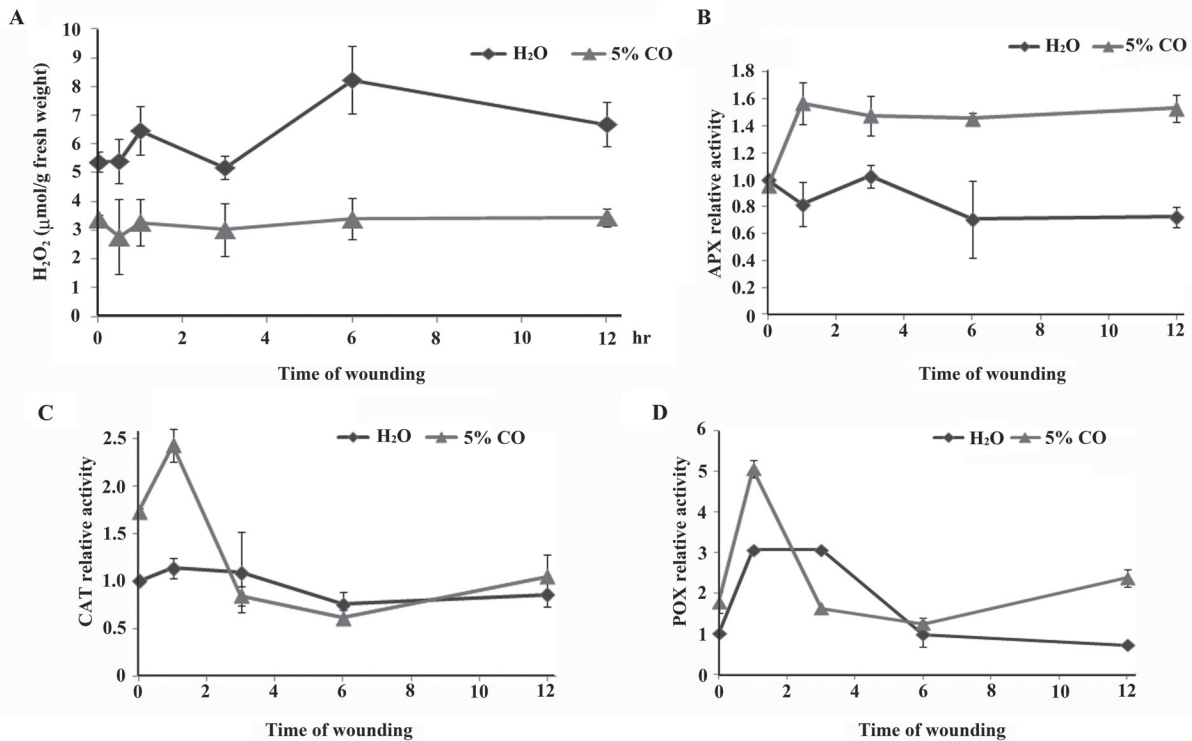


Fig. 4. Effects of CO on the production of H₂O₂ and the activities of APX, CAT, and POX. Leaves with petiole cuts of sweet potato were immersed in water for 12 h and then treated with water or 5% CO solution for another 12 h. These leaves were then wounded by tweezers. The leaves at the times indicated were collected to extract H₂O₂ and total proteins. H₂O₂ content was detected by TiCl₄ method (A). Total proteins were used for the activity assays of APX (B), CAT (C), and POX (D). The error bars are indicated as SD for at least three biological assays.

detected by anti-pERK antibody in immunoblotting analyses (Singh *et al.*, 2012; Gonzalez Besteiro and Ulm, 2013). Hence, ERK of sweet potato was searched using bioinformatics. An IbMAPK (GenBank accession no. AAD37790) was obtained by BLAST using the conserved domain of MPK3 and MPK6 as the search criterion, and was homologous to the MPK3 proteins from *Arabidopsis*, tomato, and tobacco (Supplementary Fig. S3A at *JXB* online). A phylogenetic analysis of these proteins was performed (Supplementary Fig. S3B). The recombinant protein of IbMAPK was further purified using a bacterial expression system. Phospho-IbMAPK could also be detected by anti-pERK antibody as a probe (Fig. 6). The total proteins from wounding leaves could elevate the phosphorylation of the recombinant IbMAPK protein, whereas those from CO- and PD98059-pre-treated wounding leaves could not (Fig. 6). Thus, this IbMAPK was the ERK involved in the wounding responses of sweet potato.

Interaction between IbMEK1 and IbMAPK in vivo and in vitro

PD98059 can inactivate MEK1/2, and further inhibits p-ERK activation. Hence, the IbMEK1 of sweet potato was also identified by bioinformatics using the conserved domain of MEK1/2 from other plants as the search criterion, and its full-length sequence was obtained by RACE. IbMEK1 was homologous to the MEK1/2 proteins from *Arabidopsis*, tomato, and rice (Supplementary Fig. S4A at *JXB* online), and a phylogenetic analysis of these proteins was performed

(Supplementary Fig. S4B). BiFC (Fig. 7A), GST pull-down assays (Fig. 7B), and co-immunoprecipitation (Fig. 7C) all showed that IbMEK1 could interact with IbMAPK, indicating the interaction between IbMEK1 and IbMAPK would occur both *in vivo* and *in vitro*. In wounding responses, taken together, IbMEK1 might activate the phosphorylation of IbMAPK.

Discussion

HO converts haem to biliverdin, CO, and free iron (Muramoto *et al.*, 2002; Terry *et al.*, 2002; Shekhawat and Verma, 2010). Biliverdin and CO participate in reactive oxygen species scavenging though activating antioxidant enzymes (Xie *et al.*, 2012). The addition of biliverdin or CO partially rescues the UV-C hypersensitivity responses in the *hy1* mutant (Xie *et al.*, 2012). CO also partially rescues the phenotypes of *SE5*-RNAi plants in the herbicide paraquat-induced oxidative stress (Xu *et al.*, 2012). The addition, HO products, especially CO, can mimic the responses of root development after treatment of Hm, salt, or polyethylene glycol (Cao *et al.*, 2011). CO also plays an important signal in seed germination (Dekker and Hargrove, 2002), iron homeostasis (Kong *et al.*, 2010), and various stress defences (Yannarelli *et al.*, 2006; Xie *et al.*, 2008; Shen *et al.*, 2011; Cui *et al.*, 2012). In this study, CO contents were decreased in sweet potato after wounding (Fig. 1A), indicating that CO might act as a negative physiological messenger to regulate downstream genes.

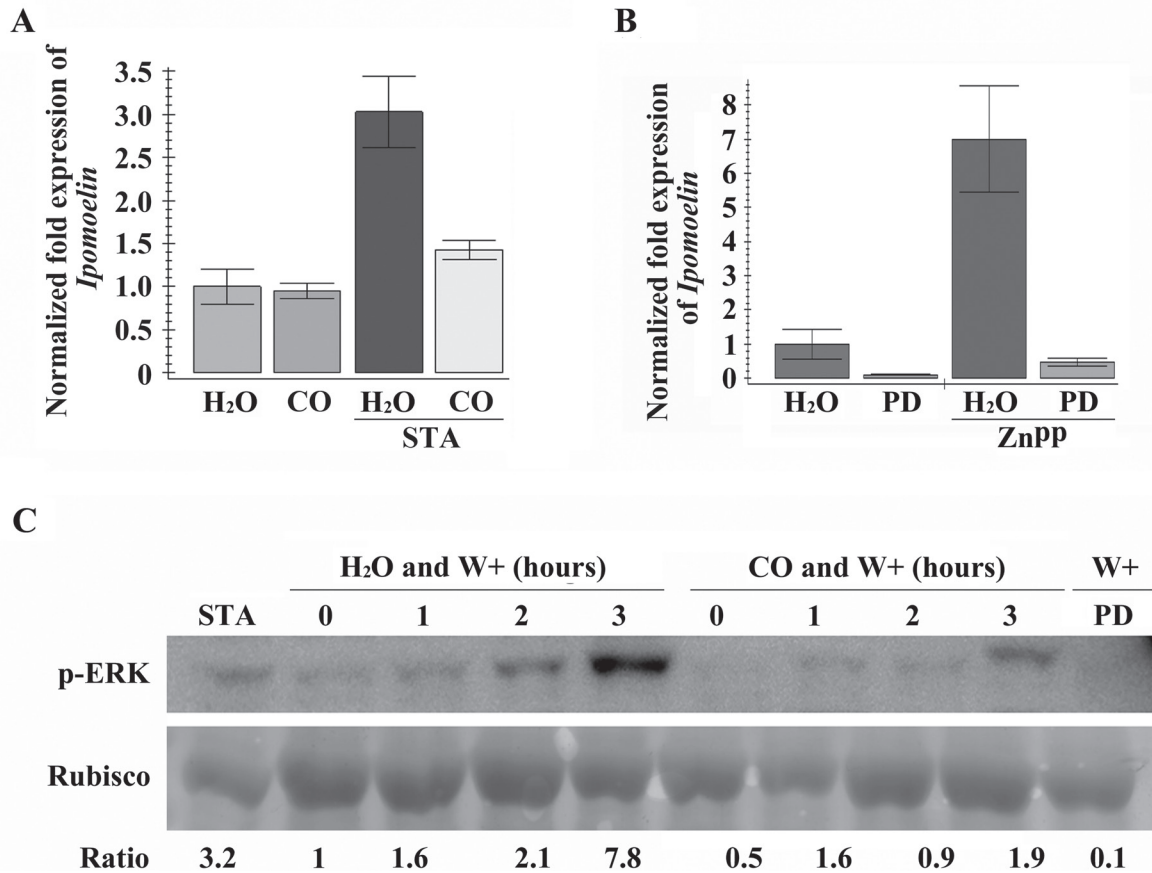


Fig. 5. Effects of CO on ERK phosphorylation. (A) Effects of CO on *IPO* expression induced by STA, an ERK1/2 phosphorylation inducer. Leaves with petiole cuts of sweet potato were immersed in water for 12 h and then treated with water or 5% CO solution for another 12 h. Some of them were treated with 1 μ M STA for 2 h. Total RNAs from these leaves were analysed by qRT-PCR to detect *IPO* expression. (B) Effects of PD980559, an ERK1/2 phosphorylation inhibitor, on the *IPO* expression induced by ZnPP. Leaves with petiole cuts were immersed in water for 12 h and then treated with water or 0.1 μ M PD980559 (PD) for another 12 h. Some of these leaves were then treated with 10 μ M ZnPP for 6 h. The *IPO* expression levels of these leaves were analysed by qRT-PCR. *lbActin* expression was used as an internal control. The error bars are indicated as SD for at least three biological assays for both (A) and (B). (C) Effects of CO on the phosphorylation of ERK (p-ERK) upon wounding. Leaves with petiole cuts were immersed in water for 12 h and then treated with water, 5% CO solution, or 0.1 μ M PD980559 for another 12 h. The leaves treated with water and CO were then wounded for 0, 1, 2, and 3 h. Leaves treated with PD980559 were further wounded for 6 h. Some leaves treated with water were incubated in 1 μ M STA for 2 h. The total proteins were analysed by western blot assays for the detection of p-ERK. Rubisco from the same amounts of total protein was separated by SDS-PAGE, and stained by Coomassie blue as a loading control.

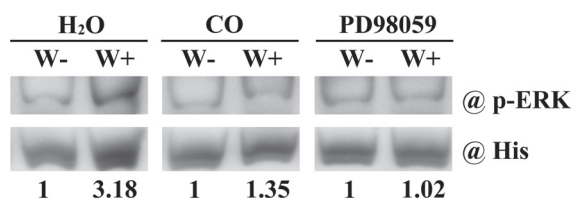


Fig. 6. *In vitro* phosphorylation of IbMAPK by anti-p-ERK antibody. Leaves with petiole cuts were immersed in water for 12 h, and then treated with water, 5% CO solution, or 0.1 μ M PD980559 for another 12 h. The leaves were then left unwounded (W-) or wounded (W+) by tweezers. The total proteins extracted from these leaves were incubated with recombinant IbMAPK-His at 30 $^{\circ}$ C for 1 h and purified by His resin. The bound proteins were eluted from the resin and detected by anti-p-ERK and anti-His antibody. Immunoblots using anti-His antibody were used as controls.

HO has been identified from different plants and proven to play important roles in plant developmental processes and stress responses. IbHO1 shared significant similarity with other known HO1 proteins in plants (Supplementary

Fig. S1a). In *Arabidopsis*, HO includes the HO1 and HO2 subfamilies based on sequence similarity. However, HO2 lacks the canonical HO activate site, a positionally conserved histidine, and is thus considered a fake HO (Snyder and Baranano, 2001; Gisk *et al.*, 2010). IbHO1 contained the conserved haem interaction residues and a His haem ligand (Supplementary Fig. S1). Based on the sequence similarity, IbHO1 is a member of the HO1 protein, which has been considered a stress response protein in plants. GmHO1 has been found to be significantly induced by UV-B irradiation and salinity stresses (Xie *et al.*, 2012). AtHO1 was induced by iron deficiency in *Arabidopsis* (Kong *et al.*, 2010). After wounding, IbHO1 was significantly repressed at the early intervals of 0.5 and 1 h (Fig. 1B), and the main reduction in CO was detected at 3 h after wounding (Fig. 1A). Therefore, IbHO1 and its product CO may participate in the wounding response.

IPO has been characterized as a defence-related protein in sweet potato. In the wounding response, IPO can be induced to inhibit silkworm growth and survival rates (Chen *et al.*, 2005).

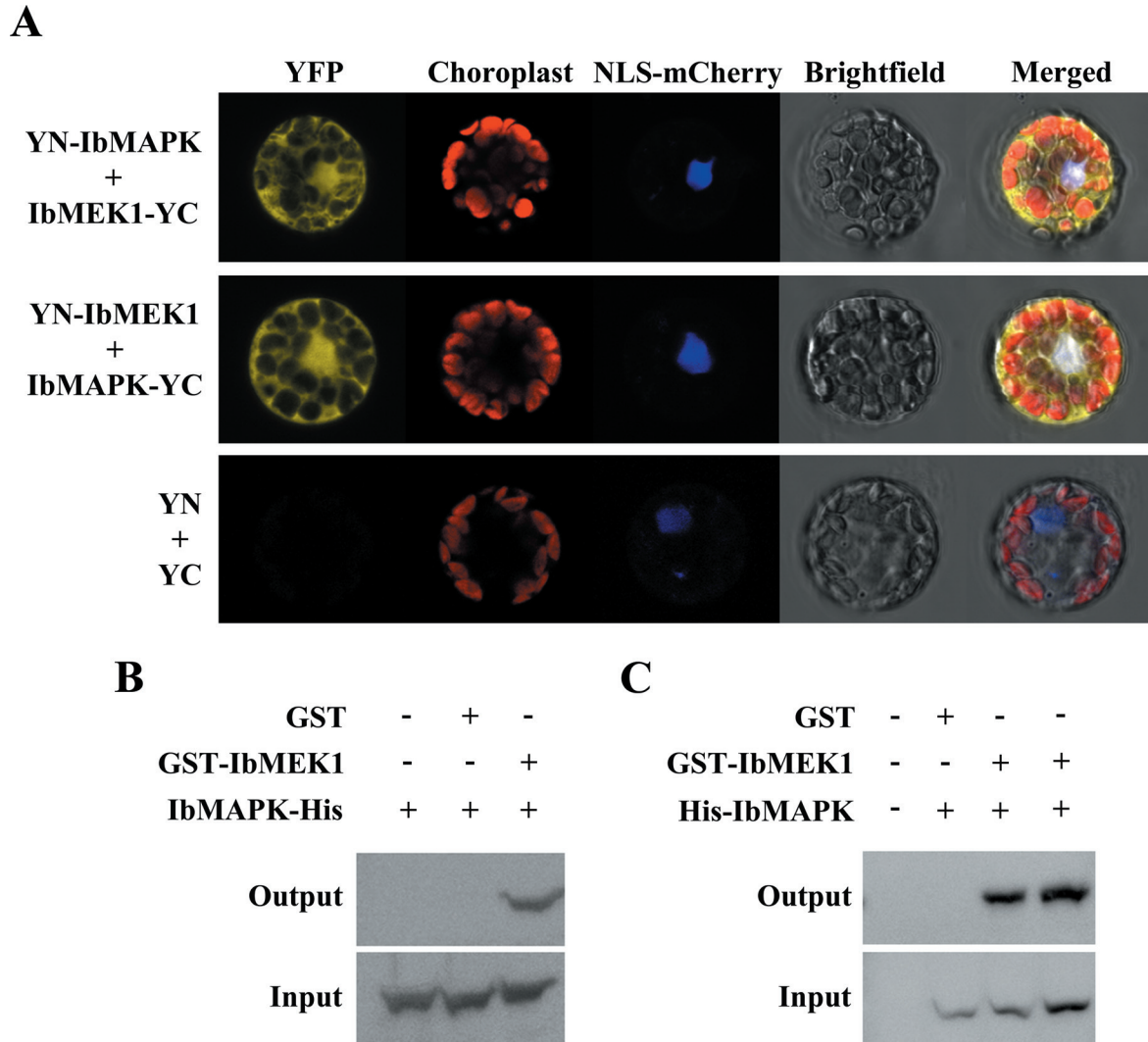


Fig. 7. IbMEK1 interacts with IbMAPK both *in vivo* and *in vitro*. (A) BiFC assays in *Arabidopsis* protoplasts for interaction between IbMEK1 and IbMAPK. Protoplasts were co-transformed with plasmids encoding IbMEK1 and IbMAPK fused with the YC and YN of YFP. These protoplasts were then visualized using a confocal microscope. Column 1 shows signals from YFP, column 2 shows chlorophyll autofluorescence, column 3 shows signals from NLS-mCherry as a nuclear marker, column 4 shows bright-field images, and column 5 shows merged images of columns 1–4. (B) GST pull-down assays for interaction between IbMEK1 and IbMAPK. GST-IbMEK1 or GST was incubated with IbMAPK-His and GST resin, and the bound proteins were then eluted from resin. These eluted proteins were detected with an anti-His antibody. (C) Co-immunoprecipitation assays in *N. benthamiana* leaves for interaction between IbMEK1 and IbMAPK. Tobacco leaves were infiltrated with agrobacteria carrying vectors containing 35S:GST (GST), 35S:GST-IbMEK1 (GST-IbMEK1), or 35S:His-IbMAPK (His-IbMAPK). After 4 d, total proteins extracted from these infiltrated leaves were incubated with GST resin, and the bound proteins were then eluted from the resin. These eluted proteins were detected with an anti-His antibody.

Hm functioned in a concentration-dependent manner to inhibit the *IPO* expression induced by wounding (Fig. 2A). In wheat and rapeseed, CO contents were elevated through the HO activated by Hm (Cao *et al.*, 2007; Xuan *et al.*, 2007). The treatment of CO or Hm showed similar functions in various phenotypes including root development and antioxidant activation (Cao *et al.*, 2011; Xu *et al.*, 2012). In addition to the effect of Hm, *IPO* induction was also repressed in the presence of CO (Fig. 3A). ZnPP, an HO inhibitor, was used in cucumber root, broad bean, and wheat aleuronic layers to inhibit HO activity and further decrease the production of CO (Song *et al.*, 2008; Xuan *et al.*, 2008b; Wu *et al.*, 2011). ZnPP was also used here to inhibit HO activity to decrease CO production. In addition, the more ZnPP was added, the more *IPO* expression was observed (Fig. 2B). In conclusion,

the *IPO* expression was inhibited by CO, which was generated from HO. After wounding, HO activity was decreased, the amount of CO was reduced, and *IPO* was then activated.

In plants, H₂O₂ is involved in redox signalling to regulate local and distal wound signals (Orozco-Cardenas and Ryan, 1999; Orozco-Cardenas *et al.*, 2001), and it also activates wound-induced genes to protect plants from pathogen and insect attacks (Moloi and van der Westhuizen, 2006; Choi *et al.*, 2007). In sweet potato, wounding stimulates the production of H₂O₂ from NADPH oxidase and generates both local and systemic signals to induce *IPO* expression (Jih *et al.*, 2003). MJ also stimulates the production of H₂O₂ (Orozco-Cardenas *et al.*, 2001), and further induces *IPO* expression (Jih *et al.*, 2003). The addition of CO inhibited MJ- or H₂O₂-induced *IPO* expression (Fig. 3B), and significantly decreased H₂O₂

contents (Fig. 4A). Hence, CO might regulate H₂O₂ contents to affect *IPO* induction. The excess production of H₂O₂ acts as an oxidative stress to mediate chlorophyll decay, lipid peroxidation, ion leakage, and DNA and protein modification (Halliwell and Gutteridge, 1984; Mittler, 2002; Yang *et al.*, 2006). Thus, the balance of H₂O₂ production in cells is very complex and important to ensure that organisms and cellular components work well (Foyer and Noctor, 2005). Furthermore, in the wheat aleuronic layer, gibberellin stimulates H₂O₂ production and further gives rise to programmed cell death. The application of exogenous CO significantly decreases the production of H₂O₂ and prevents gibberellin-induced programmed cell death (Wu *et al.*, 2011). In cadmium-induced oxidative stress, CO induces various antioxidant activities, including SOD, CAT, APX, and POX, to scavenge reactive oxygen species (Cui *et al.*, 2012). In soybean, HO1 activates SOD, CAT, and APX to protect plant cells against UV-C irradiation (Xie *et al.*, 2012). The activities of CAT, APX, and POX were also elevated by CO in wounding responses (Fig. 4B). Therefore, CO could activate APX, CAT, and POX to scavenge the H₂O₂ induced by wounding, and further interfered in *IPO* expression.

MAPK cascades are the major pathways to drive extracellular stimuli to multiple intercellular responses in mammals, yeast, and plants. The kinases in MAPK cascades of plants also share significant similarity with the kinase families found in animals (Colcombet and Hirt, 2008). In *Arabidopsis*, 20 MAPKs, 10 MAPKKs, and 80 MAPKKKs have been found based on the genomic sequence databases (Colcombet and Hirt, 2008). Among the MAPKs, AtMPK3 and AtMPK6 can be detected by anti-pERK antibody in immunoblotting analyses (Lee and Ellis, 2007; Singh *et al.*, 2012). ERK1/2 activation in mammalian cells has been reported to trigger cell death (Stanciu *et al.*, 2000). MPK3 and MPK6 have also been shown to be associated with the stress-induced cell death in plants (Lee and Ellis, 2007; Colcombet and Hirt, 2008), and regulate the levels of jasmonic acid and SA in various stresses (Yang *et al.*, 2001; Menke *et al.*, 2004; Seo *et al.*, 2007). MAPK cascades play important roles in the induction of *IPO* expression (Chen *et al.*, 2008). In animals, CO affects the phosphorylation of ERK1/2 (Song *et al.*, 2002; Kim *et al.*, 2005; Basuroy *et al.*, 2011; Schallner *et al.*, 2012). The phosphorylation of IbMAPK, the orthologue of AtMAPK3, was also affected by CO in wounding responses (Figs 5C and 6). PD98059 can prevent MEK1 activity and then block its downstream activity (Burnett *et al.*, 2000; Xing *et al.*, 2008; Li *et al.*, 2012), and blocked IbMAPK phosphorylation in the wounding response (Fig. 5). These results indicated that MEK1 may activate IbMAPK. BiFC, co-immunoprecipitation, and GST pull-down assays also identified the interaction between IbMEK1 and IbMAPK (Fig. 7). Taken together, CO might prevent IbMAPK from IbMEK1 activation. Wounding could decrease CO content to activate the phosphorylation of IbMAPK through IbMEK1.

Conclusively, CO produced by HO may act as a negative regulator to inhibit *IPO* expression in sweet potato (Fig. 8). CO could induce the activities of CAT, APX, and POX to reduce the H₂O₂ production (Fig. 4A–D). The phosphorylation of IbMAPK was also inhibited by CO (Fig. 5C). When leaves were wounded, the HO and CO contents were reduced

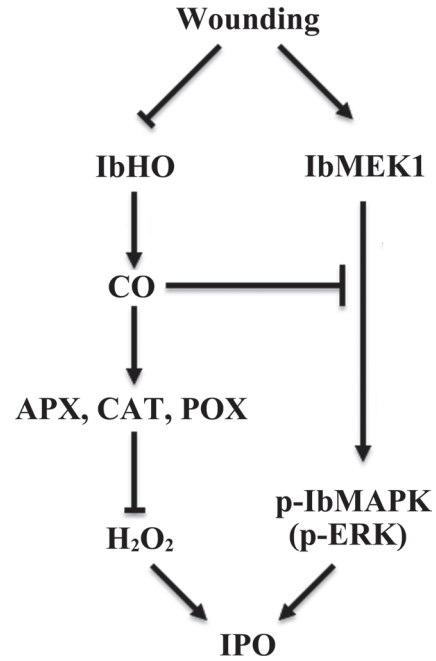


Fig. 8. Schematic representation of the role of CO in the signal transduction pathway inducing *IPO* expression. The signal transducers, including H₂O₂ and MAPK cascades, induced by wounding for stimulating *IPO* expression have been reported previously (Chen *et al.*, 2003, 2008). In sweet potato, CO could increase the activities of antioxidants APX, CAT, and POX and decrease the accumulation of H₂O₂ and the phosphorylation of ERK. Upon wounding, the expression of *IbHO* was repressed, and further decreased the contents of CO. Subsequently, the content of H₂O₂ and the phosphorylation of ERK were induced, and the wound-inducible gene *IPO* was then expressed.

(Fig. 1A, B). H₂O₂ is then produced, IbMAPK phosphorylation is elevated, and *IPO* expression is finally induced (Fig. 8).

Supplementary data

Supplementary data is available at *JXB* online.

Supplementary Table S1. Primers for this study.

Supplementary Fig. S1. Protein sequence comparisons and the phylogenetic analyses of haem oxygenase (HO).

Supplementary Fig. S2. CO contents in the leaves of sweet potato treated with Hm or ZnPP.

Supplementary Fig. S3. Protein sequence comparisons and the phylogenetic analyses of MAPK.

Supplementary Fig. S4. Protein sequence comparisons and the phylogenetic analyses of MEK.

Acknowledgements

This work was supported by the National Science Council under grants 102-2628-B-002-002-MY3 and 102-2311-B-002-033-MY3 and by the National Taiwan University under grants 103R892004 and 102R892004 to S-TJ.

References

Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *Journal of Biological Chemistry* **270**, 27489–27494.

- Balestrasse KB, Yannarelli GG, Noriega GO, Batlle A, Tomaro ML.** 2008a. Heme oxygenase and catalase gene expression in nodules and roots of soybean plants subjected to cadmium stress. *Biometals* **21**, 433–441.
- Balestrasse KB, Zilli CG, Tomaro ML.** 2008b. Signal transduction pathways and heme oxygenase induction in soybean leaves subjected to salt stress. *Redox Report* **13**, 255–262.
- Basuroy S, Tcheranova D, Bhattacharya S, Leffler CW, Parfenova H.** 2011. Nox4 NADPH oxidase-derived reactive oxygen species, via endogenous carbon monoxide, promote survival of brain endothelial cells during TNF- α -induced apoptosis. *American Journal of Physiology Cell Physiology* **300**, C256–C265.
- Bose J, Xie Y, Shen W, Shabala S.** 2013. Heme oxygenase modifies salinity tolerance in *Arabidopsis* by controlling K⁺ retention via regulation of the plasma membrane H⁺-ATPase and by altering SOS1 transcript levels in roots. *Journal of Experimental Botany* **64**, 471–481.
- Burnett EC, Desikan R, Moser RC, Neill SJ.** 2000. ABA activation of an MBP kinase in *Pisum sativum* epidermal peels correlates with stomatal responses to ABA. *Journal of Experimental Botany* **51**, 197–205.
- Cao ZY, Xuan W, Liu ZY, Li XN, Zhao N, Xu P, Wang Z, Guan RZ, Shen WB.** 2007. Carbon monoxide promotes lateral root formation in rapeseed. *Journal of Integrative Plant Biology* **49**, 1070–1079.
- Cao Z, Geng B, Xu S, Xuan W, Nie L, Shen W, Liang Y, Guan R.** 2011. BnHO1, a haem oxygenase-1 gene from *Brassica napus*, is required for salinity and osmotic stress-induced lateral root formation. *Journal of Experimental Botany* **62**, 4675–4689.
- Cao Z, Huang B, Wang Q, Xuan W, Ling T, Zhang B, Chen X, Nie L, Shen W.** 2007. Involvement of carbon monoxide produced by heme oxygenase in ABA-induced stomatal closure in *Vicia faba* and its proposed signal transduction pathway. *Chinese Science Bulletin* **52**, 2365–2373.
- Capiati DA, Pais SM, Tellez-Inon MT.** 2006. Wounding increases salt tolerance in tomato plants: evidence on the participation of calmodulin-like activities in cross-tolerance signalling. *Journal of Experimental Botany* **57**, 2391–2400.
- Chalmers A.** 1991. Simple, sensitive measurement of carbon monoxide in plasma. *Clinical Chemistry* **37**, 1442–1445.
- Chen YC, Chang HS, Lai HM, Jeng ST.** 2005. Characterization of the wound-inducible protein ipomoelin from sweet potato. *Plant, Cell & Environment* **28**, 251–259.
- Chen YC, Lin HH, Jeng ST.** 2008. Calcium influxes and mitogen-activated protein kinase kinase activation mediate ethylene inducing ipomoelin gene expression in sweet potato. *Plant, Cell & Environment* **31**, 62–72.
- Chen YC, Tseng BW, Huang YL, Liu YC, Jeng ST.** 2003. Expression of the ipomoelin gene from sweet potato is regulated by dephosphorylated proteins, calcium ion and ethylene. *Plant, Cell & Environment* **26**, 1373–1383.
- Cho JY, Katz DR, Chain BM.** 2003. Staurosporine induces rapid homotypic intercellular adhesion of U937 cells via multiple kinase activation. *British Journal of Pharmacology* **140**, 269–276.
- Choi HW, Kim YJ, Lee SC, Hong JK, Hwang BK.** 2007. Hydrogen peroxide generation by the pepper extracellular peroxidase CaPO₂ activates local and systemic cell death and defense response to bacterial pathogens. *Plant Physiology* **145**, 890–904.
- Colcombet J, Hirt H.** 2008. *Arabidopsis* MAPKs: a complex signalling network involved in multiple biological processes. *Biochemical Journal* **413**, 217–226.
- Cui W, Li L, Gao Z, Wu H, Xie Y, Shen W.** 2012. Haem oxygenase-1 is involved in salicylic acid-induced alleviation of oxidative stress due to cadmium stress in *Medicago sativa*. *Journal of Experimental Botany* **63**, 5521–5534.
- Davis SJ, Bhoo SH, Durski AM, Walker JM, Vierstra RD.** 2001. The heme-oxygenase family required for phytochrome chromophore biosynthesis is necessary for proper photomorphogenesis in higher plants. *Plant Physiology* **126**, 656–669.
- Dekker J, Hargrove M.** 2002. Weedy adaptation in *Setaria* spp. V. Effects of gaseous environment on giant foxtail (*Setaria faberii*) (Poaceae) seed germination. *American Journal of Botany* **89**, 410–416.
- El-kereamy A, El-sharkawy I, Ramamoorthy R, Taheri A, Errampalli D, Kumar P, Jayasankar S.** 2011. *Prunus domestica* pathogenesis-related protein-5 activates the defense response pathway and enhances the resistance to fungal infection. *PLoS One* **6**, e17973.
- Foyer CH, Noctor G.** 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell* **17**, 1866–1875.
- Galletti R, Ferrari S, De Lorenzo G.** 2011. *Arabidopsis* MPK3 and MPK6 play different roles in basal and oligogalacturonide- or flagellin-induced resistance against *Botrytis cinerea*. *Plant Physiology* **157**, 804–814.
- Gisk B, Molitor B, Frankenberg-Dinkel N, Kotting C.** 2012. Heme oxygenases from *Arabidopsis thaliana* reveal different mechanisms of carbon monoxide binding. *Spectrochimica Acta Part A: Molecular & Biomolecular Spectroscopy* **88**, 235–240.
- Gisk B, Yasui Y, Kohchi T, Frankenberg-Dinkel N.** 2010. Characterization of the haem oxygenase protein family in *Arabidopsis thaliana* reveals a diversity of functions. *Biochemical Journal* **425**, 425–434.
- Gonzalez Besteiro MA, Ulm R.** 2013. ATR and MKP1 play distinct roles in response to UV-B stress in *Arabidopsis*. *The Plant Journal* **73**, 1034–1043.
- Gudesblat GE, Iusem ND, Morris PC.** 2007. Guard cell-specific inhibition of *Arabidopsis* MPK3 expression causes abnormal stomatal responses to abscisic acid and hydrogen peroxide. *New Phytologist* **173**, 713–721.
- Guo K, Kong WW, Yang ZM.** 2009. Carbon monoxide promotes root hair development in tomato. *Plant, Cell & Environment* **32**, 1033–1045.
- Halliwel B, Gutteridge JM.** 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemical Journal* **219**, 1–14.
- Han Y, Zhang J, Chen X, Gao Z, Xuan W, Xu S, Ding X, Shen W.** 2008. Carbon monoxide alleviates cadmium-induced oxidative damage by modulating glutathione metabolism in the roots of *Medicago sativa*. *New Phytologist* **177**, 155–166.
- Howe GA, Jander G.** 2008. Plant immunity to insect herbivores. *Annual Review of Plant Biology* **59**, 41–66.
- Imanishi S, Kito-Nakamura K, Matsuoka K, Morikami A, Nakamura K.** 1997. A major jasmonate-inducible protein of sweet potato, ipomoelin, is an ABA-independent wound-inducible protein. *Plant Cell & Physiology* **38**, 643–652.
- Jana S, Choudhuri MA.** 1982. Glycolate metabolism of three submersed aquatic angiosperms during ageing. *Aquatic Botany* **12**, 345–354.
- Jih PJ, Chen YC, Jeng ST.** 2003. Involvement of hydrogen peroxide and nitric oxide in expression of the ipomoelin gene from sweet potato. *Plant Physiology* **132**, 381–389.
- Kim HP, Wang X, Nakao A, Kim SI, Murase N, Choi ME, Ryter SW, Choi AM.** 2005. Caveolin-1 expression by means of p38 β mitogen-activated protein kinase mediates the antiproliferative effect of carbon monoxide. *Proceedings of the National Academy of Sciences, USA* **102**, 11319–11324.
- Kong WW, Zhang LP, Guo K, Liu ZP, Yang ZM.** 2010. Carbon monoxide improves adaptation of *Arabidopsis* to iron deficiency. *Plant Biotechnology Journal* **8**, 88–99.
- Le Deunff E, Davoine C, Le Dantec C, Billard JP, Huault C.** 2004. Oxidative burst and expression of germin/oxo genes during wounding of ryegrass leaf blades: comparison with senescence of leaf sheaths. *The Plant Journal* **38**, 421–431.
- Lee JS, Ellis BE.** 2007. *Arabidopsis* MAPK phosphatase 2 (MKP2) positively regulates oxidative stress tolerance and inactivates the MPK3 and MPK6 MAPKs. *Journal of Biological Chemistry* **282**, 25020–25029.
- Li Z, Yue H, Xing D.** 2012. MAP Kinase 6-mediated activation of vacuolar processing enzyme modulates heat shock-induced programmed cell death in *Arabidopsis*. *New Phytologist* **195**, 85–96.
- Lin CC, Jih PJ, Lin HH, Lin JS, Chang LL, Shen YH, Jeng ST.** 2011. Nitric oxide activates superoxide dismutase and ascorbate peroxidase to repress the cell death induced by wounding. *Plant Molecular Biology* **77**, 235–249.
- Lin JS, Lin CC, Li YC, Wu MT, Tsai MH, Hsing YI, Jeng ST.** 2013. Interaction of small RNA-8105 and the intron of IbMYB1 RNA regulates IbMYB1 family genes through secondary siRNAs and DNA methylation after wounding. *The Plant Journal* **75**, 781–794.

- Lin JS, Lin CC, Lin HH, Chen YC, Jeng ST. 2012. MicroR828 regulates lignin and H₂O₂ accumulation in sweet potato on wounding. *New Phytologist* **196**, 427–440.
- Manavella PA, Dezar CA, Bonaventure G, Baldwin IT, Chan RL. 2008. HAHB4, a sunflower HD-Zip protein, integrates signals from the jasmonic acid and ethylene pathways during wounding and biotic stress responses. *The Plant Journal* **56**, 376–388.
- Menke FL, van Pelt JA, Pieterse CM, Klessig DF. 2004. Silencing of the mitogen-activated protein kinase MPK6 compromises disease resistance in Arabidopsis. *Plant Cell* **16**, 897–907.
- Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* **7**, 405–410.
- Moloi MJ, van der Westhuizen AJ. 2006. The reactive oxygen species are involved in resistance responses of wheat to the Russian wheat aphid. *Journal of Plant Physiology* **163**, 1118–1125.
- Muramoto T, Tsurui N, Terry MJ, Yokota A, Kohchi T. 2002. Expression and biochemical properties of a ferredoxin-dependent heme oxygenase required for phytochrome chromophore synthesis. *Plant Physiology* **130**, 1958–1966.
- Orozco-Cardenas M, Ryan CA. 1999. Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. *Proceedings of the National Academy of Sciences, USA* **96**, 6553–6557.
- Orozco-Cardenas ML, Narvaez-Vasquez J, Ryan CA. 2001. 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.
- Pieterse CMJ, Van Loon LC. 2004. NPR1: the spider in the web of induced resistance signaling pathways. *Current Opinion in Plant Biology* **7**, 456–464.
- Ryan CA, Pearce G, Scheer J, Moura DS. 2002. Polypeptide hormones. *Plant Cell* **14** (Suppl.), S251–S264.
- Sa ZS, Huang LQ, Wu GL, Ding JP, Chen XY, Yu T, Shi C, Shen WB. 2007. Carbon monoxide: a novel antioxidant against oxidative stress in wheat seedling leaves. *Journal of Integrative Plant Biology* **49**, 638–645.
- Schallner N, Fuchs M, Schwer CI, Loop T, Buerkle H, Lagreze WA, van Oterendorp C, Biermann J, Goebel U. 2012. Postconditioning with inhaled carbon monoxide counteracts apoptosis and neuroinflammation in the ischemic rat retina. *PLoS One* **7**, e46479.
- Seo S, Katou S, Seto H, Gomi K, Ohashi Y. 2007. The mitogen-activated protein kinases WIPK and SIPK regulate the levels of jasmonic and salicylic acids in wounded tobacco plants. *The Plant Journal* **49**, 899–909.
- Shekhawat GS, Verma K. 2010. Haem oxygenase (HO): an overlooked enzyme of plant metabolism and defence. *Journal of Experimental Botany* **61**, 2255–2270.
- Shen Q, Jiang M, Li H, Che LL, Yang ZM. 2011. Expression of a *Brassica napus* heme oxygenase confers plant tolerance to mercury toxicity. *Plant, Cell & Environment* **34**, 752–763.
- Siegel SM, Renwick G, Rosen LA. 1962. Formation of carbon monoxide during seed germination and seedling growth. *Science* **137**, 683–684.
- Singh P, Kuo YC, Mishra S, et al. 2012. The lectin receptor kinase-VI.2 is required for priming and positively regulates Arabidopsis pattern-triggered immunity. *Plant Cell* **24**, 1256–1270.
- Sjostrand T. 1952. The formation of carbon monoxide by the decomposition of haemoglobin in vivo. *Acta Physiologica Scandinavica* **26**, 338–344.
- Snyder SH, Baranano DE. 2001. Heme oxygenase: a font of multiple messengers. *Neuropsychopharmacology* **25**, 294–298.
- Song R, Mahidhara RS, Liu F, Ning W, Otterbein LE, Choi AM. 2002. Carbon monoxide inhibits human airway smooth muscle cell proliferation via mitogen-activated protein kinase pathway. *American Journal of Respiratory Cell and Molecular Biology* **27**, 603–610.
- Song XG, She XP, Zhang B. 2008. Carbon monoxide-induced stomatal closure in *Vicia faba* is dependent on nitric oxide synthesis. *Physiologia Plantarum* **132**, 514–525.
- Stanciu M, Wang Y, Kentor R, et al. 2000. Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. *Journal of Biological Chemistry* **275**, 12200–12206.
- Terry MJ, Linley PJ, Kohchi T. 2002. Making light of it: the role of plant haem oxygenases in phytochrome chromophore synthesis. *Biochemical Society Transactions* **30**, 604–609.
- Wu M, Huang J, Xu S, Ling T, Xie Y, Shen W. 2011. Haem oxygenase delays programmed cell death in wheat aleurone layers by modulation of hydrogen peroxide metabolism. *Journal of Experimental Botany* **62**, 235–248.
- Xiao YQ, Someya K, Morita H, Takahashi K, Ohuchi K. 1999. Involvement of p38 MAPK and ERK/MAPK pathways in staurosporine-induced production of macrophage inflammatory protein-2 in rat peritoneal neutrophils. *Biochimica et Biophysica Acta* **1450**, 155–163.
- Xie Y, Ling T, Han Y, et al. 2008. Carbon monoxide enhances salt tolerance by nitric oxide-mediated maintenance of ion homeostasis and up-regulation of antioxidant defence in wheat seedling roots. *Plant, Cell and Environment* **31**, 1864–1881.
- Xie Y, Xu D, Cui W, Shen W. 2012. Mutation of Arabidopsis HY1 causes UV-C hypersensitivity by impairing carotenoid and flavonoid biosynthesis and the down-regulation of antioxidant defence. *Journal of Experimental Botany* **63**, 3869–3883.
- Xing Y, Jia W, Zhang J. 2008. AtMKK1 mediates ABA-induced CAT1 expression and H₂O₂ production via AtMPK6-coupled signaling in Arabidopsis. *The Plant Journal* **54**, 440–451.
- Xu S, Wang L, Zhang B, et al. 2012. RNAi knockdown of rice SE5 gene is sensitive to the herbicide methyl viologen by the down-regulation of antioxidant defense. *Plant Molecular Biology* **80**, 219–235.
- Xuan W, Huang L, Li M, Huang B, Xu S, Liu H, Gao Y, Shen W. 2007. Induction of growth elongation in wheat root segments by heme molecules: a regulatory role of carbon monoxide in plants? *Plant Growth Regulation* **52**, 41–51.
- Xuan W, Xu S, Yuan X, Shen W. 2008a. Carbon monoxide: a novel and pivotal signal molecule in plants? *Plant Signal and Behavior* **3**, 381–382.
- Xuan W, Zhu FY, Xu S, Huang BK, Ling TF, Qi JY, Ye MB, Shen WB. 2008b. The heme oxygenase/carbon monoxide system is involved in the auxin-induced cucumber adventitious rooting process. *Plant Physiology* **148**, 881–893.
- Yang JD, Yun HY, Zhang TH, Zhao HL. 2006. Presoaking with nitric oxide donor SNP alleviates heat shock damages in mung bean leaf discs. *Botanical Studies* **47**, 129–136.
- Yang KY, Liu Y, Zhang S. 2001. Activation of a mitogen-activated protein kinase pathway is involved in disease resistance in tobacco. *Proceedings of the National Academy of Sciences, USA* **98**, 741–746.
- Yannarelli GG, Noriega GO, Batlle A, Tomaro ML. 2006. Heme oxygenase up-regulation in ultraviolet-B irradiated soybean plants involves reactive oxygen species. *Planta* **224**, 1154–1162.
- Yoo SD, Cho YH, Sheen J. 2007. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature Protocols* **2**, 1565–1572.
- Yu CW, Liu X, Luo M, Chen C, Lin X, Tian G, Lu Q, Cui Y, Wu K. 2011. HISTONE DEACETYLASE6 interacts with FLOWERING LOCUS D and regulates flowering in Arabidopsis. *Plant Physiology* **156**, 173–184.
- Zhao C, Yin P, Mei C, et al. 2013. Down-regulation of DNA methyltransferase 3B in staurosporine-induced apoptosis and its mechanism in human hepatocarcinoma cell lines. *Molecular and Cellular Biochemistry* **376**, 111–119.
- Zilli CG, Santa-Cruz DM, Yannarelli GG, Noriega GO, Tomaro ML, Balestrasse KB. 2009. Heme oxygenase contributes to alleviate salinity damage in *Glycine max* L. leaves. *International Journal of Biochemistry and Cell Biology* **2009**, 848516.