Carbon monoxide regulates the expression of the wound-inducible gene \textit{ipomoelin} through antioxidation and MAPK phosphorylation in sweet potato

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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, \textit{ipomoelin} (\textit{IPO}), of sweet potato (\textit{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 \textit{IbHO} expression level were significantly reduced in leaves. \textit{IPO} expression upon wounding was prohibited by the HO activator hemin, whereas the HO inhibitor zinc protoporphyrin IX elevated \textit{IPO} expression. The \textit{IPO} expression induced by wounding, \textit{H2O2}, or methyl jasmonate was inhibited by CO. CO also affected the activities of ascorbate peroxidase, catalase, and peroxidase, and largely decreased \textit{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 \textit{IbHO} expression and CO production, induced \textit{H2O2} generation and ERK phosphorylation, and then stimulated \textit{IPO} expression.

Key words: Carbon monoxide, ERK phosphorylation, \textit{H2O2}, \textit{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
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 hyl1-100 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), parquat (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 (Ca^{2+}) (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 Deuff 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 H_{2}O_{2} 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 AtMPK3, and SA-induced protein kinase, the orthologous of AtMPK6, 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 Ca^{2+}, H_{2}O_{2}, 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 H_{2}O_{2}, 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 Cao 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 T_{2}VN (Supplementary Table S1) at 37 °C for 90 min. 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 SMART™ 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 (0.5g) 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 ammonium 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 POD 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 mg−1 of protein. The enzyme activity of the H2O2-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 Na2VO4, 10 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 5 μg ml−1 of leupeptin, 5 μg ml−1 ofafiprotein, 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-1-1-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 GSTrap™ FF affinity column (GE Healthcare). The recombinant proteins IbMEK1–His and IbMAPK–His were purified using an HiTrap™ 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, ProBond™ 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 Biobaterialories).

**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 Astropodon, 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 Astropodon, 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 Astropodon and pBI221-IbMAPK Astropodon 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 HindIII and EcoRI 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-IHC was then cloned into the XbaI and BamHI 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 [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 2 mM MgCl₂, 1 mM 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.

**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 mM 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.
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Wounding-, MJ-, or H$_2$O$_2$-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 H$_2$O$_2$ 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 H$_2$O$_2$ was examined in the presence of CO. The leaves were pre-treated with 5% CO for 12 h, and then MJ or H$_2$O$_2$ was supplied for another 6 h before analyses (Fig. 3B). The expression levels of IPO were induced by MJ or H$_2$O$_2$ 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 not only by MJ or H$_2$O$_2$.

CO reduces wound-induced H$_2$O$_2$ through antioxidants

In sweet potato, wounding stimulates the production of H$_2$O$_2$ from NADPH oxidase, and generates both local and systemic signals to induce IPO expression (Jih et al., 2003). HO, which can produce CO, participates in reactive oxygen species scavenging (Balestrasse et al., 2008a, b; Zilli et al., 2009). To investigate the relationship between CO and H$_2$O$_2$ in wounding responses, H$_2$O$_2$ content upon wounding was examined in the present of CO (Fig. 4A). The level of H$_2$O$_2$ 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 H$_2$O$_2$. 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...
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 H$_2$O$_2$ 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...
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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.
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. 5.](image)

**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 μM STA for 2 h. Total RNAs from these leaves were analysed by qRT-PCR to detect IPO expression. (B) Effects of PD98059, 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 μM PD98059 (PD) for another 12 h. Some of these leaves were then treated with 10 μM ZnPP for 6 h. The IPO expression levels of these leaves were analysed by qRT-PCR. IbActin 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 μM PD98059 for another 12 h. The leaves treated with water and CO were then wounded for 0, 1, 2, and 3 h. Leaves treated with PD98059 were further wounded for 6 h. Some leaves treated with water were incubated in 1 μM 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.](image)

**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 μM PD98059 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 °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.
Carbon monoxide involved in wounding responses

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). 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, H$_2$O$_2$ 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 H$_2$O$_2$ from NADPH oxidase and generates both local and systemic signals to induce IPO expression (Jih et al., 2003). MJ also stimulates the production of H$_2$O$_2$ (Orozco-Cardenas et al., 2001), and further induces IPO expression (Jih et al., 2003). The addition of CO inhibited MJ- or H$_2$O$_2$-induced IPO expression (Fig. 3B), and significantly decreased H$_2$O$_2$.

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.
CO could induce the activities of CAT, APX, and POX to regulate to inhibit IbMAPK through IbMEK1. Furthermore, in the wheat aleuronic layer, gibberellin stimulates H$_2$O$_2$ production and further gives rise to programmed cell death. The application of exogenous CO significantly decreases the production of H$_2$O$_2$ 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 H$_2$O$_2$ 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 have been identified to be associated with 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 AtMPK3, 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 H$_2$O$_2$ 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.

**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.

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