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RESEARCH PAPER

Characterization of a canola C2 domain gene that interacts with PG, an effector of the necrotrophic fungus *Sclerotinia sclerotiorum*

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Abstract

Sspg1d, one of endopolygalacturonases, is an important fungal effector secreted by the necrotrophic fungus *Sclerotinia sclerotiorum* during early infection. Using sspg1d as bait, a small C2 domain protein (designated as IPG-1) was identified by yeast two-hybrid screening of a canola cDNA library. Deletion analysis confirmed that the C-terminus of IPG-1 is responsible for its interaction with sspg1d in the yeast two-hybrid assay. The sspg1d/IPG-1 interaction was further confirmed in plant cells by a biomolecular fluorescence complementation (BiFC) assay. A transient expression assay showed that the IPG-1–GFP fusion protein was targeted to the plasma membrane and nucleus in onion epidermal cells. Following treatment with a Ca^{2+} ionophore, it was distributed throughout the cytosol. Real-time PCR assay demonstrated that *IPG-1* was highly induced by *Sclerotinia sclerotiorum* in canola leaves and stems. Southern blot analysis indicated the presence of about five homologues of *IPG-1* in the canola genome. Two additional members of the *IPG-1*gene family were isolated by RT-PCR. Their sequence similarity with *IPG-1* is as high as 95%. However, they did not interact with sspg1d in the yeast two-hybrid assay. Possible roles of IPG-1 and its association with sspg1d in the defence signalling pathway were discussed.

Key words: BiFC, C2 domain, endo-PG, PG, *Sclerotinia sclerotiorum*.

Introduction

The necrotrophic fungal pathogen *Sclerotinia sclerotiorum* exhibits little host specifity and has a range of more than 400 plant species among 75 families, primarily dicotyledons, including many economically important crops such as the grain legumes (soybean, pea, and bean) and oilseeds (canola and sunflower) (Boland and Hall, 1994). Stem rot caused by the fungus is an important disease of canola in China. There is a lot of evidence to show that complete resistance to this pathogen has not been identified in canola germplasm, although partial resistance or tolerance to the pathogen in different breeding lines has been reported (Liu *et al.*, 1991; Chen *et al.*, 1993).

Sclerotinia sclerotiorum secretes several types of effector proteins such as polygalacturonases (PGs) during its development and plant infection. These PGs can be classified into endo-polygalacturonases (endo-PGs) and exo-polygalacturonases (exo-PGs) (Li *et al.*, 2004). Each of them has different expression levels under pathogenic conditions; one of the endo-PGs, named sspg1d, was highly expressed during early infection and thus may play an important role in pathogen development and pathogenicity (Li *et al.*, 2004; Hegedus and Rimer, 2005). Zuppini *et al.* (2005) reported that an endo-PG from *Sclerotinia sclerotiorum* can induce calcium-mediated signalling and programmed cell death in soybean cells.

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Abbreviations: BiFC, biomolecular fluorescence complementation; PG, polygalacturonase; endo-PG, endo-polygalacturonase; GFP, green fluorescent protein; YFP, yellow fluorescent protein; CaMV, cauliflower mosaic virus.

The immediate response of plant cells to pathogen attack is the increase in cytosolic Ca^{2+} concentration, which can be decoded by various Ca^{2+}-binding proteins (Ca^{2+} sensors) (Reddy, 2001). Once activated by Ca^{2+} ions, these Ca^{2+} sensors interact with downstream effectors that modulate the numerous biochemical and cellular functions involved in defence responses (Lecourieux *et al.*, 2006). Many of these Ca^{2+}-binding proteins contain C2 domains (Ca^{2+}-regulatory domains) (Kretsinger, 1980). In mammals, C2 domains have been found in more than 100 proteins, most of which are involved in lipid metabolism, signal transduction or membrane trafficking (Rizo and Südhof, 1998). In response to Ca^{2+} being imported, the C2 domains of phospholipases, synaptotagmin I, and protein kinase C were shown to bind Ca^{2+} and migrate from the cytosol to the plasma membrane, thereby transducing the foreign signal into the cells (Clark *et al.*, 1991; Davletov and Sudhof, 1993; Edwards and Newton, 1997; Lomasney *et al.*, 1999; Pepio and Sossin, 2001; Ananthanarayanan *et al.*, 2002; Teruel and Meyer, 2002). In plants, copine and phospholipase D, two proteins that contain C2 domains, have been shown to be involved in defence responses (Young *et al.*, 1996; Laxalt *et al.*, 2001; Laxalt and Munnik, 2002; Jambunathan and McNellis, 2003). In rice, the small protein OsERG1 contains a single C2 domain and is induced by treatment with a fungal elicitor resulting in protein migration from the cytosol to the plasma membrane in a Ca^{2+}-dependent manner (Kim *et al.*, 2003). In mung beans (*Vigna radiata* L.), the C2 domain of V3-PLC3 plays an important role in the translocation of the protein to the membrane in response to abiotic stress (Kim *et al.*, 2004). However, only a few C2 domain-containing proteins have been identified or investigated in plants.

Using sspg1d as bait, a C2 domain protein, designated IPG-1, was identified by screening a canola cDNA library with the yeast two-hybrid assay (Wang *et al.*, 2008). In the current report, the sspg1d/IPG-1 interaction was confirmed in living plant cells by bimolecular fluorescent complementation (BiFC) assay (Walter *et al.*, 2004). The C-terminus of IPG-1 was demonstrated to be responsible for its interaction with sspg1d. In addition, the dynamic subcellular localization of IPG-1 protein was analysed in response to Ca^{2+}. Finally, the expression of IPG-1 in different tissues of canola in response to *Sclerotinia sclerotiorum* inoculation was characterized and the copy number of IPG-1 in the canola genome was investigated. The possible roles of PG and IPG-1, and their interaction in defence signalling, are discussed.

Materials and methods

Construction of IPG-1 *deletion mutants and the two-hybrid assay*

Two *IPG-1* mutants, IPG-1/del-C (contains residues 1–87 aa) and IPG-1/del-N (contains residues 89–168 aa) were produced. The coding sequences of each of the two mutants were fused with the GAL4 activating domain in the pGADT7 vector and individually transformed into yeast AH109 cells. The full-length coding sequence of *sspg1d* was fused with the GAL4 DNA binding domain in the pGBKT7 vector to generate the pGBKT7–sspg1d construct and it was transformed into yeast Y187 cells. Yeast mating between AH109 and Y187 was used to test the IPG-1 domain that interacts with sspg1d.

BiFC aasay

The vectors (PUC-pSPYNE, PUC-pSPYCE) used in the BiFC assay were kind gifts of Jorg Kudla. For BiFC analysis, the full-length coding sequence of sspg1d was fused with the N-terminal fragment of YFP in PUC-pSPYNE vector to form the YFP^N–sspg1d construct. The full-length coding sequence of IPG-1 was cloned into PUC-pSPYCE as a fusion with the C-terminal fragment of YFP to form the YFP^C–IPG-1 construct. The plasmids YFP^N–sspg1d and YFP^C–IPG-1 were cotransformed into onion epidermal cells by bombardment. YFP fluorescence was analysed 16 h later using a Leica TCS SP2 laser confocal scanning microscope.

Dynamic subcellular localization of IPG-1 in plant cells in response to Ca^{2+}

The full-length coding region of IPG-1was cloned into pJIT166GFP vector as a fusion with the N-terminus of GFP to form the IPG-1–GFP construct. The IPG-1–GFP construct was transformed into onion epidermal cells by bombardment, and after GFP was detected, the cells were treated with the calcium ionophore (5 mM Ca^{2+} and 10 μM ionomycin) and incubated in the dark at 22 °C for 5–8 h and then the GFP fluorescence was monitored using a Leica TCS SP2 laser confocal scanning microscope.

Real-time quantitative RT-PCR

The eukaryotic translation elongation factor 1-α (EF-1α) was used as the internal control in real-time quantitative RT-PCR. The sequences of the forward and the reverse primers were 5′-AGACCACCAAGTACTACTGCAC-3′ and 5′-CCACCAATCTTGTACACATCC-3′, respectively. The primers used to amplify IPG-1 gene were 5′-GAG CCT CGC CAT CAG AGA TA-3′ and 5′-GTC CTC ATG GAC TTG CAC ACT-3′. PCR was performed using Multicolor Real-Time PCR Detection System, iQ™5 (Bio-Rad).

Results

Full-length IPG-1 and its C-terminal region interact with sspg1d in yeast cells

IPG-1 was a small C2 domain protein containing 168 amino acid residues (Wang *et al.*, 2008). BLAST analysis in Genbank predicted that the 1–87 aa is the C2 domain in which there are five conserved aspartic acid residues (D) (Fig. 1), the potential Ca^{2+}-binding sites. The 88–168 aa is

the C-terminal region that may be involved in protein–protein interaction. To verify this hypothesis, two deletion mutants were constructed: IPG-1-del-N, which contained the C-terminal domain (89–168 aa) and IPG-1-del-C, which contained the N-terminal domain (C2 domain, 1–87 aa). The coding sequence of the two mutants and the full-length IPG-1 were individually fused with an activating domain (AD) in the pGADT7 vector and transformed into yeast strain AH109 separately. The pGBKT7–sspg1d construct was transformed into yeast strain Y187. The domain of IPG-1 necessary for its interaction with *sspg1d* was determined by yeast mating between Y187 and AH109 yeast cells.

Yeast cells with pGBKT7–sspg1d and IPG-1-del-N plasmids, and cells with pGBKT7–sspg1d and pGADT7–IPG-1 plasmids were able to grow on SD/-Ade/-His/-Leu/-Trp and turned blue with the X-αGal overlay assay, but yeast cells with pGBKT7–sspg1d and IPG-1-del-C plasmids were not (Fig. 2). The experiments were replicated three times and each obtained the same results. It means that the full-length IPG-1 and the C-terminal region of *IPG-1* can interact with sspg1d, while the C2 domain was not necessary for the interaction.

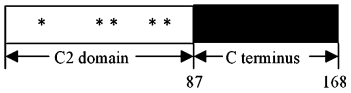

Fig. 1. The structure of the IPG-1 protein. The domain consisting of residues 1–87 is the C2 domain, in which the asterisks represent the five conserved aspartatic residues (the positions are 22, 73, 75, 80, and 81, respectively). The C-terminus is shown in black box consisting of residues 88–168.

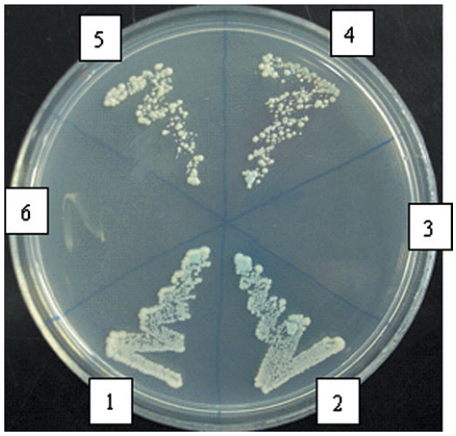

Fig. 2. sspg1d/IPG-1 interaction and the domain of IPG-1 necessary for its interaction with sspg1d analysed by yeast two-hybrid assay. 1, sspg1d/IPG-1 interaction; 2, positive control (pGBKT7-53/ pGADT7-RecT interaction); 3, negtive control (pGBKT7-Lam/pGADT7-RecT interaction); 4, 5, sspg1d/IPG-1-delN interaction; 6, sspg1d/IPG-1-delC interaction. Yeast cells transformed with combinations of various AD and BD constructs were subjected to β-galactosidase overlay activity assay. The blue colour of the yeast cells and yeast growth on SD/-Ade/-His/-Leu/-Trp media indicate the activation of reporter genes and therefore a positive protein–protein interaction.

Verification of sspg1d–IPG-1 *interaction in living plant cells*

BiFC was used to test weather sspg1d and IPG-1 can associate in plant cells. *sspg1d* and *IPG-1* was fused with the N-terminal 154 amino acid or C-terminal 84 amino acids of yellow fluorescent protein (YFP), respectively, driven by the CaMV 35S promoter. The two constructs were co-bombarded into onion epidermal cells. YFP fluorescence was monitored using laser confocal scanning microscopy.

As shown in Fig. 3, YFP fluorescence could be detected in onion epidermal cells co-transformed with YFP^N–sspg1d and YFP^C–IPG-1. No YFP fluorescence was detected in the negative controls (i.e. transformed with YFP^C–IPG-1/YFP^N, YFP^N–IPG-1/YFP^C) (data not shown). These results confirm that sspg1d interacts with IPG-1 in living plant cells.

Dynamic subcellular localization of IPG-1 in response to Ca^{2+}

It is known that C2 domain proteins play a role in Ca^{2+}-dependent spatio-temporal targeting in different regulatory signal transduction chains (Evans *et al.*, 2004). Furthermore, it has been shown that the small rice C2 domain protein OsERG1 is translocated to the plasma membrane of plant cells in a Ca^{2+}-dependent manner (Kim *et al.*, 2003). To determine whether the newly identified C2 domain protein IPG-1 also exhibits calcium-dependent subcellular localization, the IPG-1–GFP construct driven by the CaMV 35S promoter was introduced into onion epidermal cells by bombardment for transient expression. As shown in Fig. 4, IPG-1 protein is mainly targeted to the plasma membrane and nucleus. After treatment with ionomycin, a calcium ionophore, for 5 h, the IPG-1–GFP signals was observed distributing throughout the cytosol (Fig. 5). It means that the Ca^{2+} ionophore treatment induced translocation of the green fluorescence signal emitted from the IPG-1-GFP from the plasma membrane and the nucleus to the cytosol.

IPG-1 *is highly induced by* Sclerotinia sclerotiorum

Semi-quantitative PCR revealed that *IPG-1* was highly induced by *Sclerotinia sclerotiorum* in canola leaf and stem (Wang *et al.*, 2008). Here, real-time PCR was used to analyse the expression of *IPG-1* in leaf, stem, and flower organs of canola following inoculation with *Sclerotinia sclerotiorum.* As shown in Fig. 6, the expression of *IPG-1* in flowers is about three times higher than that in leaves and stems before inoculating with *Sclerotinia sclerotiorum,* whereas the expression level of *IPG-1* in leaves and stems was about 2–3 times higher than in flowers following inoculation with *Sclerotinia sclerotiorum*. The experiment again provided evidence that *IPG-1* is significantly induced in leaves and stems by *Sclerotinia sclerotiorum.*

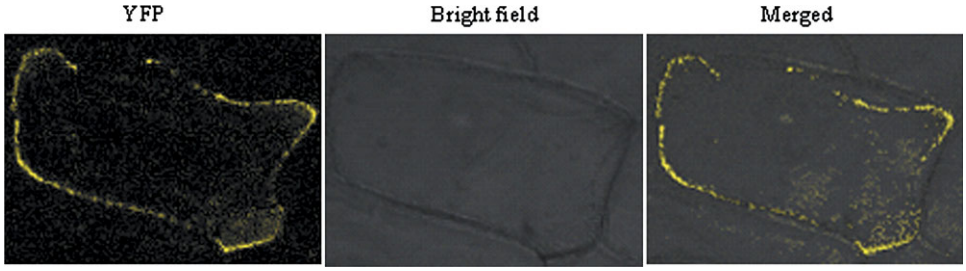

Fig. 3. Bimolecular fluorescence complementation assays in onion epidermal cells. The reconstitute YFP signals show that IPG-1 and sspg1d can associate in plant cells.

Determine the copy numbers of IPG-1 *in the canola genome*

The copy numbers of *IPG-1* in the canola genome were analysed by Southern blot of canola genomic DNA probed with *IPG-1* cDNA (Fig. 7). The results showed that *IPG-1* has about 2–5 copies in the canola genome, which means that the canola genome may have a C2 domain gene family. To test whether other members in the gene family interact with sspg1d, an additional two genes in the gene family were isolated by RT-PCR and designated as *BnC2d1* and *BnC2d2*. BLAST analysis indicated that the two genes also contain the C2 domain. Sequence comparison of the two genes with *IPG-1* revealed that their identity is as high as 95% in amino acids (Fig. 8). The coding sequences of *BnC2d1* and *BnC2d2* were individually fused into the GAL4 activating domain of the pGADT7 vector. Yeast mating was used to test the interaction between *sspg1d* and the two genes. To our surprise, the two genes did not interact with *sspg1d*, although the experiment was replicated several times. This means that the two *IPG-1* homologues may have evolved functions different from *IPG-1*. This may explain why only one gene that interacts with sspg1d in the canola cDNA library was isolated by yeast two-hybrid screening.

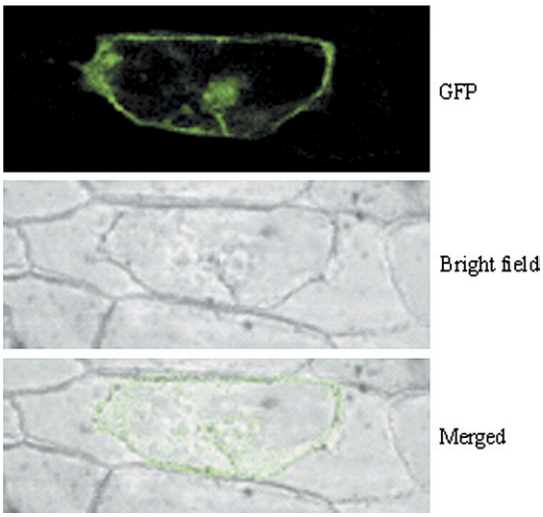

Fig. 4. Localization of IPG-1-GFP fusion in onion epidermal cell. Plant cell wall and membrane were separated by treatment with 20% sugar.

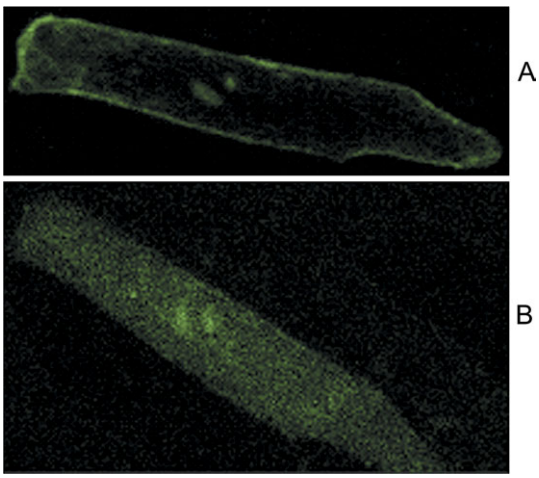

Fig. 5. Subcellular localization of IPG-1-GFP fusion transiently expressed in onion epidermal cells before (A) and after (B) treatment with Ca^{2+} ionophore.

Discussion

PGs produced by fungi belong to cell wall-degrading enzymes and some of them from several necrotrophic fungal pathogens have been implicated as potential virulence factors unrelated to their enzyme activity (Shieh *et al.*, 1997; Have *et al.*, 1998; Poinssot *et al.* 2003; Kikot *et al.*, 2008). They can cause Ca^{2+} elevation in the cell cytosol and subsequent cell death (Zuppini *et al.*, 2005). In order to identify host factors involved in PG signalling, sspg1d, one of the important PGs secreted by *Sclerotinia sclerotiorum* during its early development and the plant infection process, was used as bait to screen PG-interacting proteins in the canola cDNA expression library by using a yeast two-hybrid technique. A C2 domain protein was identified that interacts with sspg1d, and which was further confirmed in plant cells by BiFC. The C2 domain was first described in protein kinase C (PKC), representing a large family comprising the most studied of all protein kinases in animals. Animal PKC isoforms include 3–4 conserved domains, C1–C4, representing catalytic and regulatory

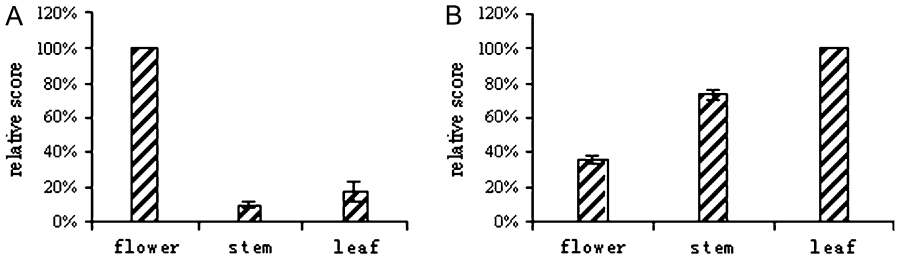

Fig. 6. Expression of *IPG-1* in different organs of canola analysed by real-time PCR with eukaryotic elongation factor 1-α (EF-1α) gene as internal control. (A) Without inoculation with *Sclerotinia sclerotiorum.* (B) Inoculation with *Sclerotinia sclerotiorum.*

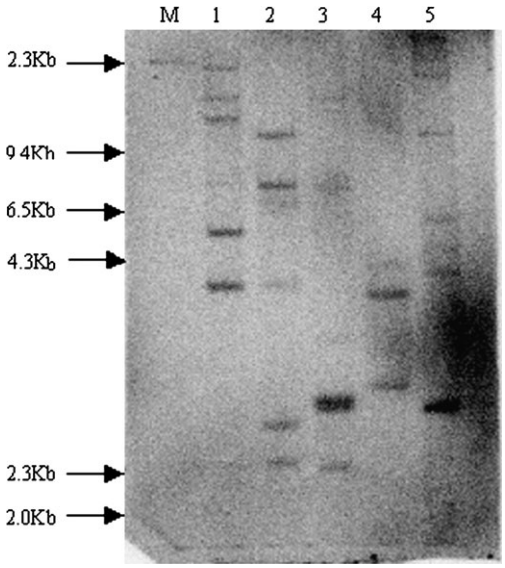

Fig. 7. Southern blot of canola genomic DNA using ^{32}P labelled IPG-1 cDNA as probe. Genomic DNA was digested with *Bam*HI (1), *Eco*RI (2), *Eco*RV (3), *Hin*dIII (4), and *Xba*I (5). M, molecular weight marker.

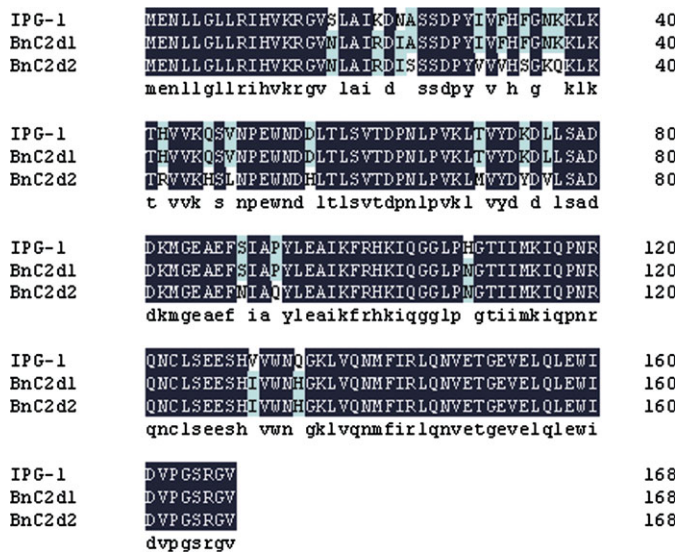

Fig. 8. Amino acid sequence alignment of IPG-1 with its homologues BnC2d1 and BnC2d2.

modules. Domains C1, C3, and C4 are present in all PKC isoforms, whereas the C2 domain is unique to the Ca^{2+}-dependent isoforms PKCα, PKCβ, and PKCγ, thus identifying the C2 domain as a potential Ca^{2+}-regulatory motif (Hug and Sarre, 1993). C2 domains interact with phospholipids in a Ca^{2+}-dependent manner and thereby modulate a diverse range of cellular actions. They mediate the Ca^{2+}-dependent translocation of soluble proteins to membranes, the Ca^{2+}- and phospholipid-dependent activation of enzymes, Ca^{2+}- and phospholipid-dependent interaction between proteins, or promote Ca^{2+}-triggered self-association (Kopka *et al.*, 1998). The C2 domain proteins found in human and animals generally consist of 1–3 C2 domains (Nalefski and Falke, 1996). Only a few C2 domain proteins are discovered in plants, and many of these proteins contain only single C2 domain, known as small C2 domain protein. The functions of already-described plant small C2 domain proteins are not yet very clear. In pumpkin, a small C2 domain protein has been reported to increase the size of mesophyll plasmodesmata to enable transport of cellular materials, including RNA molecules, from cell to cell (Xoconostle-Cazares *et al.*, 1999). In *Arabidopsis*, the C2-domain protein BAPl negatively regulates defence responses (Yang *et al.*, 2006). In rice, two small C2-domain rice proteins named OsERG1a and OsERG1b, are significantly induced by a fungal elicitor (Kim *et al.*, 2003), implicating a functional role in defence signalling systems in plant cells. The HvC2d, a C2-domain protein identified in barley (*Hordeum vulgare* L.) was induced by exposure to different heavy metals and its mRNA was accumulated during leaf senescence (Ouelhadj *et al.*, 2006). In this study, the novel C2 domain protein identified, IPG-1, was first shown to interact with sspg1d, a fungal effector of *Sclerotinia sclerotiorum*. It is closely related in sequence to a C2-domain protein in *Arabidopsis* (NP198590) with unknown functions (Wang *et al.*, 2008). The expression studies (Fig. 6) showed that the mRNA of this gene was highly induced by *Sclerotinia sclerotiorum* inoculation. The subcellular localization of IPG-1–GFP fusion protein is dynamic in response to Ca^{2+} elevation. These results implicate a role of IPG-1 in Ca^{2+}-dependent defence signalling.

Localization of proteins to distinct subcellular compartments, including membranes, is a critical event in multiple cellular pathways. The C2 domain has been identified in many cellular proteins involved in signal transduction or

membrane trafficking. A majority of C2 domains bind the membrane in a Ca^{2+}-dependent manner and thereby play an important role in Ca^{2+}-dependent membrane targeting (Nalefski and Falke, 1996; Stahlen and Cho, 2001). Analyses of mammalian C2 proteins, for example, phospholipases, synaptotagmin I and protein kinase C, also showed that these proteins migrate after binding of Ca^{2+} from the cytosol to the plasma membrane and thus are able to transduce foreign signals into the cell (Pepio and Sossin, 2001; Ananthanarayanan *et al.*, 2002; Teruel and Meyer, 2002). By using GFP constructs, Kim *et al.* (2003) showed that the small C2 domain protein OsERG1 is translocated to the plasma membrane of plant cells by treatment with a Ca^{2+} ionophore and also by a fungal elicitor. Immunocytochemical analyses with the other known small C2 domain protein from pumpkin (CmPP16-1) also suggested an association with the plasma membrane (Xoconostle-Cazares *et al.*, 1999). Interestingly, there are several reports showing that such calcium-binding proteins are not only localized in the cytoplasm but also in the nucleus. Among these proteins with nuclear localization are calcium-dependent protein kinases (Dammann *et al.*, 2003; Chehab *et al.*, 2004), a novel calmodulin-binding protein (Perruc *et al.*, 2004), and a novel C2 domain protein HvC2d1 found in barley (Ouelhadj *et al.*, 2006). In addition, the calcium-dependent protein kinase McCPK1 from the ice plant was shown to undergo a reversible change in subcellular localization from the plasma membrane to the nucleus, endoplasmic reticulum, and actin filaments of the cytoskeleton in response to environmental stimuli (Chehab *et al.*, 2004). In the present study, another novel protein was identified with a calcium-binding C2 domain-like motif that was shown to be located in a calcium-dependent manner in the cytosol and also in the nucleus. Our data indicate that IPG-1 plays a role in cytosolic and nuclear localized calcium signalling processes in response to external stressors.

It has been reported that the *Arabidopsis* C2 domain proteins, BAP1 and BAP2, are general inhibitors of programmed cell death (PCD) (Yang *et al.*, 2007). Overexpression of BAP1 or BAP2, with their partner BON1, inhibits PCD induced by pathogens. Thus, the BAP genes function as general negative regulators of PCD induced by biotic stimuli. The BAP1 and BON1 molecules might become targets of pathogen effector proteins because of their ancestral role in cell death control during the evolution of plant innate immune system (Jones and Dangl, 2006). Considering that an endo-PG of *Sclerotinia sclerotiorum* could induce PCD in plant cells (Zuppini *et al.*, 2005), and here, IPG-1 is shown to be the target of an endo-PG of *Sclerotinia sclerotiorum*, implicating IPG-1 in the PCD process in plant cells. It has been suggested that effective defence against biotrophic pathogens is largely due to PCD in the host, whereas necrotrophic pathogens benefit from host cell death; they can utilize dead tissue and are not limited by cell death (Govrin and Lexine, 2000; Glazebrook, 2005). Plants expressing animal PCD inhibitor genes, such as the human *Bcl-2* and *Bcl-xl* and the nematode *CED-9*, confer resistance to several necrotrophic fungal pathogens including *Sclerotinia sclerotiorum* (Dickman *et al.,* 2001). Considering that the plant material used here is susceptible to the fungus, it is postulated that the PG/IPG-1 interaction may interfere with the binding of IPG-1 with Ca^{2+} and the subsequent Ca^{2+}-dependent signal transduction might be involved in PCD processes in the host. Overexpression of IPG-1 might inhibit PCD and produce resistance to *Sclerotinia sclerotiorum*.

At present, there are two models explaining pathogen perception by plants: the Guard Model and the recently proposed Decoy Model (van der Hoorn and Kamoun, 2008). The Guard Model proposes that pathogen effectors may have a common target; the plant R-protein indirectly perceives the presence of the pathogen effector by monitoring the state of the effector target that associates with the R-protein. In the absence of the R-protein, the effector target will enhance pathogen fitness in plants. The Decoy Model implies that some host targets of fungal effectors have evolved to act as a 'decoy' to trap the pathogen into a recognition event. These decoys only function in the perception of pathogen effectors without contributing to pathogen fitness in the absence of its cognate R-protein in plant. What types of effector target the IPG-1 belongs to is an interesting question to be explored. Whether IPG-1 associates with plant R-proteins remains to be investigated.

Nowadays, numerous laboratories are determining the enzymatic functions of pathogen effectors as well as their host targets (Chisholm *et al.*, 2006), which will benefit elucidation of the molecular basis of plant resistance or susceptibility. These works are of great importance for both basic and applied research. This study lays the foundation for further investigation of the functions of PG, IPG-1, and the roles of PG–IPG-1 interaction in plant defence responses, which may also involve other factors such as calcium, phospholipids, and other proteins.

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