Physiological and Molecular Plant Pathology (2002) **61**, 31–40
doi:10.1006/pmpp.2002.0414, available online at http://www.idealibrary.com on IDEAL

Molecular cloning and characterization of a rice phosphoinositide-specific phospholipase C gene, *OsPI-PLC1*, that is activated in systemic acquired resistance

FENGMING SONG* and ROBERT M. GOODMAN†

Department of Plant Pathology, 1630 Linden Drive, University of Wisconsin, Madison, WI 53706, U.S.A.

(Accepted for publication 27 May 2002)

Phospholipid signaling is an important component in eukaryotic signal transduction pathways; in plants, it plays a key role in growth and development as well as in responses to environmental stress. We cloned and characterized a gene from rice encoding phosphoinositide-specific phospholipase C (*OsPI-PLC1*, *Oryza sativa* L. phosphoinositide-specific phospholipase C1). *OsPI-PLC1* encodes a 599 amino acid protein containing the catalytic X and Y domains, as well as a C2-like domain, characteristics of this class of enzymes. Expression of *OsPI-PLC1* was induced by various chemical and biological inducers of plant defence pathways, including benzothiadiazole (BTH), salicylic acid (SA), dichloroisonicotinic acid, probenazole (PB), jasmonic acid (JA) and its methyl ester, *Pseudomonas syringae* pv. *syringae*, and wounding. All of these treatments were shown to induce resistance in rice against blast disease caused by *Magnaporthe grisea*. *OsPI-PLC1* was activated within 6 h after inoculation with the blast fungus in BTH-treated rice seedlings and in the incompatible interaction between a resistant genotype of rice and *M. grisea*, whereas the expression in the BTH-untreated seedlings and in the compatible interaction was only induced to a relatively low level at later time points (30 h) after inoculation, indicating that expression of *OsPI-PLC1* is associated with the resistance response and/or the incompatible interaction. In addition, BTH treatment also induced systemic expression of *OsPI-PLC1*. These results suggest that *OsPI-PLC1* may be involved in the signaling pathways leading to disease resistance in rice.

© 2002 Elsevier Science Ltd. All rights reserved.

Keywords: benzothiadiazole; *Magnaporthe grisea*; phosphoinositide-specific phospholipase C (*PI-PLC*); rice (*Oryza sativa* L.); *OsPI-PLC1*; systemic acquired resistance.

INTRODUCTION

Upon perception of pathogens or chemical signals, a complex signaling network that involves a series of early signaling events and subsequently diverse signaling pathways is required for the activation of the defence responses in plants. Biochemical and physiological studies, through analysing the effects of specific inhibitors or activators on defence response in cultured cells or intact plants that have been challenged with pathogens or purified elicitors, have identified a number of such early signaling events that are involved in activation of defence responses [2, 27]. These early signaling events include ion fluxes, occurrence of an oxidative burst, Ca^{2+} flux, activation of G proteins, generation of nitric oxide, phospholipase, and protein phosphorylation [8, 10, 13–15, 22, 45, 48].

Most of the early signaling events involved in activation of the defence responses in plants, such as oxidative burst, G-protein and Ca^{2+}, are associated with the plasma membrane. It is now well recognized that phospholipids are not just basic constituents of membranes, but also dynamic informational elements at the interface between cells and the environment. In animal systems, phospholipids or phospholipid-derived metabolites have been shown to have critical cellular functions particularly as mediators or second messengers in signal transduction [11].

* Current address: Department of Plant Protection, College of Agriculture and Biotechnology, Zhejiang University-Huajiachi Campus, Hangzhou, Zhejiang 310029, People's Republic of China

† To whom all correspondence should be addressed. E-mail: rgoodman@facstaff.wisc.edu

Nucleotide sequence data for *OsPI-PLC1* have been deposited in the GenBank database as accession number AF332874.

Abbreviations used in text: ABA, abscisic acid; BTH, benzothiadiazole; DEPC, diethyl pyrocarbonate; DIG, digoxigenin; INA, dichloroisonicotinic acid; JA, jasmonic acid; MeJA, methyl jasmonate; *OsPI-PLC1*, *Oryza sativa* L. phosphoinositide specific phospholipase C1; ORF, open reading frame; PB, probenazole; PI-PLC, phosphoinositide-specific phospholipase C; PLC, phospholipase C; RACE, rapid amplification of cDNA ends; SA, salicyclic acid; SAR, systemic acquired resistance; SSH, suppression subtractive hybridization.

0885-5765/02/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.

Three classes of phospholipases, phospholipase C, D and A_2, are key enzymes capable of generating lipids that act as second messengers in signal transduction. In plants, phospholipase D and A_2 have been shown to play an important role in the signaling pathways leading to activation of defence responses [3, 38, 50] and in response to environmental stress, hormones, wounding, and senescence [28, 29].

Phosphoinositide-specific phospholipase C (*PI-PLC*; also called phospholipase C, PLC; EC 3.1.4.11) specifically hydrolyses phosphatidylinositol 4,5-bisphosphate producing two second messengers: Inositol 1,4,5-trisphosphate is a compound soluble in the cytosol that triggers transient increases of the cytosolic Ca^{2+}, and diacylglycerol is a lipid that stays within the plasma membrane and activates protein kinase C [28]. Inositol 1,4,5-trisphosphate and diacylglycerol have both been implicated in hormone responses, guard-cell signaling, root hair deformation and in responses to environmental stress, such as osmotic stress [5, 7, 9, 12, 24, 25, 30, 31, 33, 42, 44]. Fungal elicitors prepared from *Mycosphaerella pinodes* or *Phytophthora nicotianae* both stimulated increased levels of inositol 1,4,5-trisphosphate, which in turn were required for induction of defence responses in pea and tobacco cells [21, 46]. In soybean cells, PLC activation was correlated with the induction of oxidative burst upon treatment with fungal elicitor [26], but the *PI-PLC* enzyme activity and the inositol 1,4,5-trisphosphate level were reduced upon infection by *Pseudomonas syringae* pv. *glycinea* [40]. Protein kinase C, which may be activated by diacylglycerol, was also found to participate in the elicitor-induced defence response in potato [43]. These results suggest that the PLC-mediated signaling pathways also play important roles in activation of defence responses.

Molecular study of the PLC-mediated signaling pathway in plants has recently been approached by the cloning and characterization of several PI-PLC genes from various plant species including potato, soybean, *Arabidopsis thaliana*, and tobacco [18–20, 23, 32, 39, 49]. Expression of PI-PLC encoding genes was found to be induced by a number of stresses such as low temperature, high salinity, wounding, abscisic acid (ABA), dehydration and osmotic stress [19, 20, 23]. These results suggest that the *PI-PLCs* play a role in environmental stress signaling.

No molecular evidence has been obtained so far supporting the biochemical evidence for a role of PI-PLC in signaling pathways leading to activation of defence responses. Here we report the molecular cloning and characterization of a rice *PI-PLC*, *OsPI-PLC1* (*Oryza sativa* L. phosphoinositide-specific phospholipase C1), from experiments in which suppression subtractive hybridization (SSH) was used to detect gene expression associated with induction of systemic acquired resistance (SAR) in rice. Expression of *OsPI-PLC1* was activated by treatment with various inducers of disease resistance and by infection in an incompatible interaction between *Magnaporthe grisea*, the blast pathogen, and blast-resistant rice. Our results indicate that PLC-mediated signaling may be involved in the signaling pathways leading to activation of SAR as well as resistance gene-mediated disease resistance in rice.

MATERIALS AND METHODS

Growth of rice seedlings

Rice (*O. sativa* L.) cv. Yuanfengzao and a pair of near-isogenic lines (H8R and H8S) were used in this study. Seeds were planted into Redi-earth plug and seedling mix (Scotts-Sierra, Horticultural Products Co., Marysville, OH, U.S.A.) in pots (10 cm in diameter and 9 cm in height). Seedlings were grown in a growth chamber with 16 h photoperiod per day at 27°C in light and 22°C in the dark. Light (244 μE m^{-2} s^{-1} at the soil level) was provided by Cool White fluorescent bulbs. Each pot was seeded with 12–15 seeds. Three-week-old seedlings with four fully expanded leaves were used in this study unless indicated otherwise in the text.

Treatments with inducers of disease resistance

Rice seedlings were treated by foliar spraying with solutions of benzothiadiazole (BTH), dichloroisonicotinic acid (INA), salicylic acid (SA, pH 6·5), probenazole (PB) and jasmonic acid (JA) in water or with sterile water as control. Treatment with methyl jasmonate (MeJA) was performed by placing the seedlings in a sealed glass container and pipetting pure MeJA onto a cotton ball inside of the container (approx. 0·5 μl l^{-1} air). The third and the fourth leaves were collected for analysis of gene expression.

For treatment with *P. syringae* pv. *syringae* DC3000, the fourth leaves of 4-week-old rice seedlings were treated by infiltration at five locations with 20 μl of the bacterial suspension (5×10^8 colony forming U ml^{-1}) in 10 mM Mg_2SO_4 or with solution of 10 mM Mg_2SO_4 using a 1 ml syringe without a needle. Wounds were applied to the fourth leaf by squeezing the leaf blade with forceps at 10 locations. For analysis of the systemic effect of BTH on gene expression, the fourth leaves of 4-week-old seedlings were injected at five locations with 20 μl of 300 μM BTH solution or with water as control. In these cases, the treated fourth leaves and the untreated fifth leaves were collected separately.

Inoculation of rice seedlings with M. grisea

The rice seedlings were inoculated with a spore suspension (5×10^5 spores ml^{-1} in 0·05 % Tween 20) of *M. grisea* (race ZB1, isolate 85-14B1) or with sterile water

(containing 0·05 % Tween 20) 3 days after treatment with the inducers of disease resistance. The inoculated and the uninoculated rice seedlings were kept in a plastic box to ensure 100 % relative humidity and darkness for 36 h and then returned to the growth chamber under the conditions described above.

Extraction of total RNA

Total RNA was extracted using a hot-phenol method [47]. Homogenized leaf tissues were extracted in a preheated 1 : 1 mixture of phenol and extraction buffer (100 mM LiCl, 100 mM Tris–HCl, pH 8·0, 10 mM EDTA, 1 % SDS) at 80°C and the samples were then mixed after addition of 0·5 volume of chloroform. After centrifugation, the supernatants were transferred to a new tube and the RNA was precipitated overnight at 4°C in 2 M LiCl. RNA pellets were dissolved in diethyl pyrocarbonate (DEPC)-treated water, and again precipitated by adding 0·1 volume of sodium acetate (pH 5·2) and two volumes of ethanol. RNA was resuspended in DEPC-treated water.

Suppression subtractive hybridization

Suppression subtractive hybridization (SSH) was performed using the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, U.S.A.). Two populations of double-stranded cDNAs (tester cDNA and driver cDNA) were synthesized and digested with *Rsa*I. The digested cDNAs were divided into two samples and ligated with two different adaptors, followed by two rounds of hybridization. An excess of driver cDNA was added to the diluted adaptor 1-ligated tester or adaptor 2R-ligated tester cDNA and allowed to hybridize for 9 h at 68°C. These two samples were then mixed in the presence of freshly denatured driver cDNA and hybridized for 20 h at 68°C. Two rounds of PCR were performed to amplify exponentially the differentially expressed sequences and the PCR products were cloned into pGEM T-Easy vector (Promega, Madison, WI, U.S.A.) by T/A cloning. These libraries of differentially expressed cDNAs were subsequently screened by two rounds of differential screening using the PCR-Select Differential Screening Kit (Clontech, Palo Alto, CA, U.S.A.) and a third round of reverse Northern analysis. The remaining clones were sequenced and similarity searches were performed as described below.

Rapid amplification of cDNA ends

Rapid amplification of cDNA ends (RACE) (GIBCO BRL, Gaithersburg, MD, U.S.A.) was performed with 1 μg of RNase-free DNase-treated total RNA from *M. grisea*-infected BTH-treated rice leaves. PLC-specific primer PLC 51-1 (5′-TACCATCTGAGCACCATGCA-3′) and the poly(dT) primer were used for 5′ and 3′ cDNA synthesis, respectively, and the 5′ cDNA was tailed with poly(dC) according to the manufacturer's instructions (GIBCO BRL, Gaithersburg, MD, U.S.A.). A nested 5′-RACE primer PLC 51-3 (5′-TTCTTCCATAACCACAGCC-3′) and an abridged anchor primer were used in 5′-RACE, with the following parameters, in a Robocycler 40 (Stratagene, La Jolla, CA, U.S.A.): 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 2·5 min, and a final extension at 72°C for 5 min. Thirty cycles of PCR using the same parameter as the 5′-RACE except for 2 min of extension at 72°C were used for 3′-RACE with primer PLC 31 (5′-AAGCTAATGGTGGCTGTGGT-3′) and the abridged anchor primer. One μl of 1 : 50 dilution of the first PCR product was used for secondary PCR with nested primer PLC 31n (5′-CCAGATTTCTTAATGCAAAC-3′) and the abridged anchor primer under the same parameters. The 25 μl reactions contained 1 μl of the cDNAs, 1 μl primer (10 μM), 2·5 μl 10× PCR buffer, 1 μl dNTP mix (10 mM) and 0·5 μl *Taq* DNA polymerase (2·5 U, Promega, Madison, WI, U.S.A.). Purified PCR products were cloned into pGEM T-Easy vector using T/A cloning (Promega, Madison, WI, U.S.A.).

Phage DNA was extracted from a rice cDNA library (see below) using QIAGEN's Lambda DNA Extraction kit (QIAGEN Inc., Valencia, CA, U.S.A.) and used for amplification of the PLC 5′-end sequence. A vector-specific primer, T3 (5′-AATTAACCCTCACTAAAGGG-3′), and a PLC-specific primer PLC 51-5 (5′-GGTGGTGGGCGT CCTGCCAT-3′) were used in 25 cycles of primary PCR amplification. One μl of 1 : 50 dilution of the primary PCR product was used in 20 cycles of secondary PCR amplification with the T3 primer and a nested PLC-specific primer PLC 51-6 (5′-AGCCGCTCCATGTCCACGTC-3′). The PCR reaction parameters were the same as the 5′-RACE.

Construction and screening of a rice cDNA library

Rice leaf samples were harvested from uninoculated BTH-treated and untreated rice seedlings 4 days after treatment and from inoculated BTH-treated and untreated rice seedlings 1 day after inoculation. One mg of total RNA from each leaf sample was pooled and used for poly$(A)^+$ RNA isolation with Promega's PolyATtract mRNA Isolation System (Promega, Madison, WI, U.S.A.). A cDNA library was constructed with Stratagene's ZAP Express cDNA Construction Kit according to the manufacturer's recommendation (Stratagene, La Jolla, CA, U.S.A.).

Screening of the cDNA library was carried out to isolate a full-length clone of the putative PI-PLC cDNA. The 405 bp insert of the SSH clone BIHN-w13, which

showed 82 % similarity to potato StPLC2 [23], was amplified and digested with *Rsa*I, *Eag*I and *Sma*I to remove the adaptors from both ends of the cDNA. The digested PCR products were purified with QIAQuick PCR Purification Kit (QIAGEN, Valencia, CA, U.S.A.) and labelled with digoxigenin (DIG)-dUTP using the random primer labelling system from Boehringer Mannheim (Indianapolis, IN, U.S.A.).

Preparation of plaque lifts was performed following Stratagene's protocol (Stratagene, La Jolla, CA, U.S.A.). Prehybridization was carried out in 10 ml of aqueous hybridization solution [0·5 mol l^{-1} $NaHPO_4$ (pH 7·2), 1 % BSA, 1 mmol l^{-1} EDTA and 7 % SDS] containing 50 μl of Blocking Solution (10 mg ml^{-1} sheared salmon sperm DNA) for 2 h at 65 °C. The hybridization was performed overnight with approx. 20 ng ml^{-1} DIG-labelled probe at 65 °C. The membranes were washed with 10 ml 2×SSC/0·5 % SDS (4 × 20 min) and then with 10 ml 0·2×SSC/0·5 % SDS (2 × 20 min) at 65 °C. Immunodetection of DIG-labelled probes was done by the chemiluminescence method using CSPD according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN, U.S.A.). The cDNA inserts of the positive clones were excised *in vivo* as phagemids following Stratagene's protocol (Stratagene, La Jolla, CA, U.S.A.).

DNA sequencing and sequence analysis

DNA sequencing was performed on both strands using the BigDye sequencing reagent. Sequencing reactions were analysed at the Biotechnology Center of University of Wisconsin-Madison using an ABI 377 automatic DNA Sequencer (Perkin–Elmer, Foster City, CA, U.S.A.). Nucleotide sequence similarity searches were carried out at the National Center for Biotechnology Information using the BLAST network service (http://www.ncbi.nlm.nih.gov/BLAST/) [1]. Protein sequences of plant PI-PLCs were retrieved from GenBank and alignments were performed using DNAStar software (DNAstar, Madison, WI, U.S.A.). A phylogenetic tree was constructed by the Clustal method using the DNAstar program (DNAstar, Madison, WI, U.S.A.) based on the genetic distance of the protein sequences.

Northern analysis

Total RNA (20 μg) was fractionated on 1·2 % agarose–formaldehyde gel and transferred by capillary action overnight to a Magnagraph nylon membrane (Micron Separation Inc., Westboro, MA, U.S.A.) using 10×SSC. The RNA on the membrane was fixed by baking at 80 °C for 2 h. The purified 515 bp insert cDNA was labelled with [α-^{32}P]-dCTP (3000 Ci mM^{-1}) by the random priming labelling system (Amersham Pharmacia, Piscataway, NJ, U.S.A.). Prehybridization was performed at 42 °C for 1 h in UltraHyb hybridization buffer (Ambion, Austin, TX, U.S.A.) and hybridization was carried out overnight at 42 °C in the same hybridization buffer with the [^{32}P]-labelled probe. After hybridization, the blots were washed four times with 2×SSC, 0·1 % SDS and 0·2×SSC, 0·1 % SDS for 10 min each at room temperature. The hybridization signal was detected by exposure of the membranes to Kodak X-OMAT film (Rochester, NY, U.S.A.) for 2 h.

RESULTS

Cloning of OsPI-PLC1, *encoding a rice phosphoinositide-specific phospholipase C*

Pre-treatment of rice seedlings by foliar spraying or soil-drenching with BTH induced SAR against blast disease [Song and Goodman, unpublished work]. To elucidate the molecular biology of BTH-induced SAR in rice, differentially expressed cDNA libraries were constructed by SSH and 276 differentially expressed cDNA clones were obtained from library screening. Sequencing and BLAST searches revealed that one clone, BIHN-w13, contained a 405 bp insert from a differentially expressed cDNA library obtained by subtracting cDNAs from a BTH-treated and pathogen-inoculated leaf sample with cDNA from water-treated and uninoculated sample. The BIHN-w13 clone showed 82 % identity at the nucleotide sequence level to the potato phosphoinositide-specific phospholipase C gene, *StPLC2* (GenBank accession X94183) [23].

To obtain the full-length cDNA of this putative PI-PLC gene, 5′-RACE and 3′-RACE were performed to amplify the cDNA ends using primers designed according to the sequence of clone BIHN-w13. Two fragments of 760 and 750 bp were amplified in 5′-RACE and 3′-RACE, respectively. After sequencing, the *PI-PLC* gene was found to be 1765 bp long but still lacked its 5′ end, as no starting codon was found in the sequence. Therefore, a rice cDNA library was screened using a labelled insert of clone BIHN-w13 as probe. Five positive clones ranging in insert size from 1·6 to 2·0 kbp were recovered from screening of approx. 6 × 106 plaques. Sequencing of the longest clone revealed it was not full-length. A rice cDNA library in phage DNA was used as template to PCR-amplify the lacking sequence at the 5′ end using a vector primer and nested primers of the known sequence of the *PI-PLC* gene. The putative rice *PI-PLC* gene, designated as *OsPI-PLC1* for *Oryza sativa* L. phosphoinositide-specific phospholipase C, contained 2207 nucleotides with an 1800 bp open reading frame (ORF), flanked by 5′- and 3′-non-coding sequences of 146 and 261 bp, respectively.

The ORF of *OsPI-PLC1* encodes a 599 amino acid polypeptide with a calculated molecular weight of 67·65 kDa and isoelectric point of 6·61. Alignment of the

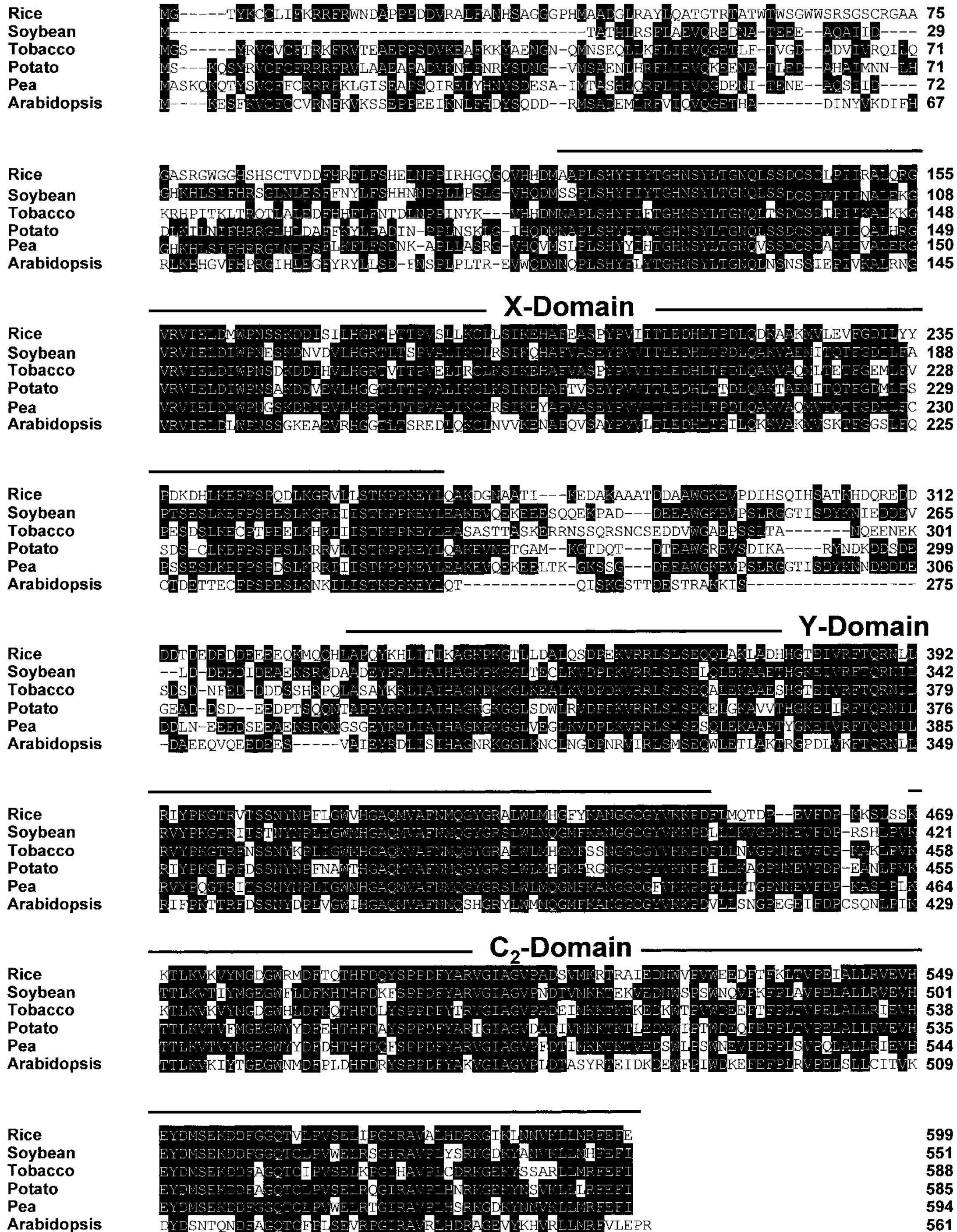

FIG. 1. Alignment of the deduced protein of the rice *OsPI-PLC1* gene with PI-PLCs from other plant species and the conserved domains. The PI-PLCs used for alignment were: Arabidopsis AtHATPLC1 (D38544) [21]; soybean P25 (U41475); pea PsPLC (Y15253); aztec tobacco NrPHOSPLC (X95877) [33]; and potato StPLC3 (X94289) [24].

deduced protein sequence of OsPI-PLC1 with PI-PLCs from other plant species indicated that OsPI-PLC1 shows a high level of identity at the amino acid level with known plant PI-PLCs and contains three conserved characteristic domains (X-, Y- and C_2-domains) (Fig. 1). A phylogenetic tree was constructed according to the amino acid sequences of OsPI-PLC1 and other plant PI-PLCs retrieved from GenBank (Fig. 2). OsPI-PLC1 is most closely related to

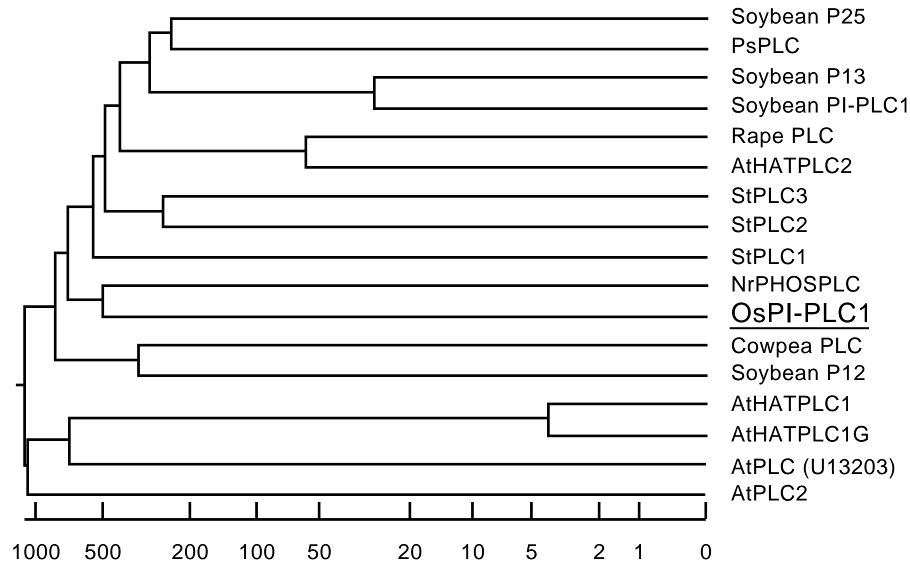

FIG. 2. The phylogenetic relationships of OsPI-PLC1 with PI-PLCs from other plant species. Phylogenetic tree based on the genetic distance of the protein sequences was constructed by the Clustal method using DNAStar software. The protein sequences used for construction of the phylogenetic tree are listed in the GenBank database under the following accession numbers: AtHATPLC1, D38544 [*21*]; AtHATPLC1G, U76423 [*19*]; AtHATPLC2, D50804 [*20*]; AtPLC2, X85973; AtPLC-U13203, U13203 [*50*]; cowpea PLC, U85259; soybean P12, U41473; soybean P13, U41474; soybean P25, U41475; soybean *PI-PLC*, U25207 [*40*]; StPLC1, X93564 [*24*]; StPLC2, X94183 [*24*]; StPLC3, X94289 [*24*]; PsPLC, Y15253; NrPHOSPLC, X95877 [*33*]; rape PLC, AF108123.

NrPHOSPLC (GenBank accession X95877) [*33*], a gene from *Nicotiana rustica*, and shows 42·7–64·1 % identity at the amino acid level to other plant PLCs.

OsPI-PLC1 *is activated upon induction by various chemical and biological SAR inducers*

In untreated and/or uninfected healthy rice seedling, the level of constitutive expression of *OsPI-PLC1* was very low. Expression of *OsPI-PLC1* was activated by BTH treatment. The induction of *OsPI-PLC1* by BTH treatment was found to be dose-dependent; expression of *OsPI-PLC1* was activated at the lowest concentration of BTH tested (0·3 μM) [Fig. 3(A)]. In a time-course experiment, expression of *OsPI-PLC1* was detected at the earliest time point tested (1 day after treatment with 0·3 mM BTH) and the expression levels remained unchanged over the experimental period [Fig. 3(B)].

It has been shown that SA, INA, probenazole, JA and its methyl ester MeJA, wounding, and *P. syringae* can induce resistance against blast disease and activate expression of defence-related genes in rice [*34–37*, *41*]. Therefore, we tested whether expression of *OsPI-PLC1* can be induced by these SAR inducers. As shown in Fig. 3(C), treatment of rice seedlings with SA, INA, PB, JA and MeJA induced varying levels of expression of *OsPI-PLC1*. Compared with that in control leaves that were infiltrated with Mg_2SO_4 only, infiltration of rice leaves with *P. syringae* pv. *syringae* DC3000 in Mg_2SO_4 induced high levels of expression of *OsPI-PLC1* both in the local leaves and the upper uninfiltrated systemic leaves [Fig. 3(C)]. Mechanical wounding also activated expression of *OsPI-PLC1* in the wounded leaves as well as in the upper unwounded leaves [Fig. 3(C)]. These results indicated that the *OsPI-PLC1* gene is expressed in response to various chemical and biological SAR inducers, all of which can induce resistance in rice to blast disease.

Expression of OsPI-PLC1 *is activated in resistance responses*

To determine whether induced expression of OsPI-PLC1 is associated with disease resistance in rice, we analysed and compared the expression kinetics of *OsPI-PLC1* between BTH-treated and water-treated rice seedlings and between incompatible and compatible interactions of rice and *M. grisea* (Fig. 4). Rice seedlings of cv. Yuanfengzao used in this study are highly susceptible to blast fungus infection, normally forming typical large susceptible-type lesions with a gray sporulation center and surrounded by chlorotic tissues 3–4 days after inoculation. Treatment of 3-week-old seedlings by foliar spraying with BTH significantly reduced blast disease severity by 65–80 %. Small dark-brown lesions (resistant-type lesions) less than 1·0 mm in diameter were seen in BTH-treated seedlings 2–3 days after inoculation and

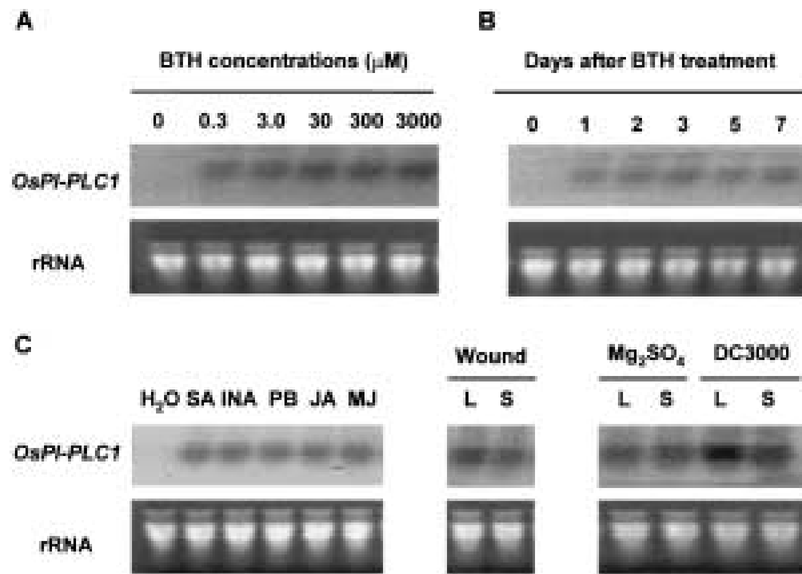

FIG. 3. Induction of *OsPI-PLC1* expression by various chemical and biological inducers. (A and B) Dosage and time-course of *OsPI-PLC1* gene expression induced by BTH. Three-week-old rice seedlings were treated by spraying with different concentrations of BTH solutions (A) or 300 μM (B). Leaf samples were collected 3 days after treatment (A) or as indicated (B). (C) Expression of *OsPI-PLC1* induced by SA, dichloroisonicotinic acid (INA), PB, JA, MeJA, wounding and *P. syringae* pv. *syringae*. Rice seedlings were treated by spraying with 2 mM SA, 0·3 mM INA, 0·5 mM PB, 1 mM JA or water. Rice seedlings were also treated with volatile MeJA at a concentration of 0·3 μl l^{-1} air in a sealed container. The fourth leaves of 4-week-old rice seedlings were wounded by squeezing with blunt-ended forceps at 10 locations or infiltrated with *P. syringae* pv. *syringae* DC3000 in 10 mM Mg_2SO_4 (5 × 108 cfu ml^{-1}) or 10 mM Mg_2SO_4. Leaf samples were collected 3 days after treatments except in the case of wound treatment, in which samples were harvested 2 days after wounding. The fourth and the fifth leaves were collected separately as local and systemic samples, respectively, in the experiments of wounding or *P. syringae* pv. *syringae*. L, local-the fourth leaf; S, systemic-the fifth leaf; NI, not inoculated; I, inoculated.

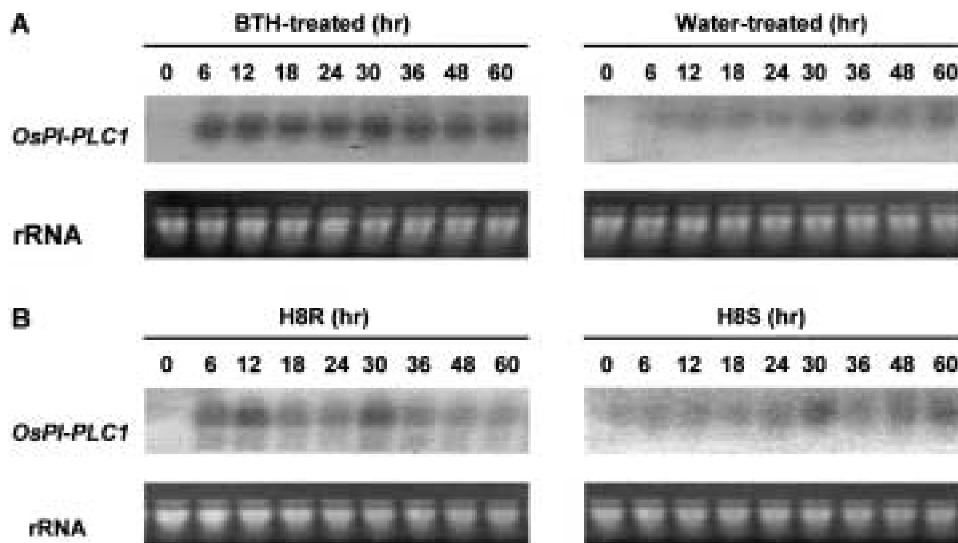

FIG. 4. Induction of *OsPI-PLC1* expression is associated with resistance responses. (A) Rice seedlings were treated by spraying with 300 μM BTH solution or water, and inoculated with *M. grisea* 3 days after treatment. (B) Three-week-old rice seedlings of H8R and H8S were inoculated with *M. grisea*. Leaf samples were collected at each time points (h) as indicated.

these lesions did not enlarge [Song and Goodman, unpublished work]. In BTH-treated rice seedlings, infection by *M. grisea* rapidly activated expression of *OsPI-PLC1*; expression of *OsPI-PLC1* was detected as early as 6 h after inoculation with the fungus. Relatively high levels of expression were maintained for 36 h after

inoculation and thereafter returned to the basal level [Fig. 4(A)]. In contrast, low levels of *OsPI-PLC1* expression were observed in water-treated rice seedlings during the early stage (6–30 h) after inoculation with the blast fungus.

A pair of near isogenic lines (H8R and H8S) was used to analyse the expression kinetics of *OsPI-PLC1* between incompatible and compatible interactions of rice and *M. grisea*. When infected with strain 85-14B1 of *M. grisea*, H8R shows an incompatible interaction, producing small brown-dark HR-like lesions 2–3 days after inoculation; H8S shows a compatible interaction with typical blast lesions on the leaves 5–7 days after inoculation. Expression of *OsPI-PLC1* was rapidly activated upon inoculation of H8R seedlings with *M. grisea* and reached its first peak 12 h after inoculation [Fig. 4(B)]. The second peak of *OsPI-PLC1* expression in blast-inoculated H8R seedlings was observed 30 h after inoculation. Compared with the expression in H8R, lower levels of expression were detected in H8S leaves during the first 24 h after inoculation. But a relatively high level of expression in H8S seedlings was detected 30 h after inoculation.

Expression of OsPI-PLC1 *is activated systemically by BTH*

We tested whether expression of *OsPI-PLC1* was activated systemically by BTH treatment. We infiltrated BTH solution into the intracellular spaces of the fourth leaves and analysed the expression levels of *OsPI-PLC1* in the upper (fifth) leaves. In the fifth leaves of the water-infiltrated control seedlings, low levels of *OsPI-PLC1* expression were detected in uninoculated rice seedlings or in inoculated seedlings (Fig. 5). Infiltration of BTH significantly induced expression of *OsPI-PLC1* in the fifth leaves, and the expression level increased further with inoculation of *M. grisea* (Fig. 5). This result suggests that the expression of *OsPI-PLC1* is activated systemically by BTH.

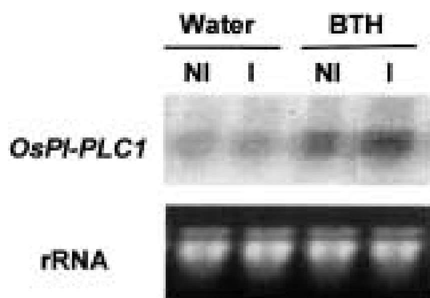

FIG. 5. Systemic induction by benzothiadiazole of *OsPI-PLC1* in rice seedlings. The fourth leaves of 4-week-old rice seedlings were infiltrated with 20 μl at five locations with 0·3 μM BTH solution or water. The rice seedlings were inoculated with *M. grisea* or, treated with water as a control, 3 days after infiltration. The fifth leaves were collected 1 day after inoculation for analysis. NI, not inoculated; I, inoculated.

DISCUSSION

Physiological and biochemical studies have demonstrated that second messages released by PI-PLC may be involved in the activation of defence responses in pea and tobacco cells and the induction of oxidative burst in soybean cells upon treatment with fungal elicitor [21, 26, 46]. However, no direct molecular evidence has so far implicated PI-PLC in the signaling pathways leading to activation of defence responses in plants. Here we have presented results suggesting that PI-PLC may play such a role in the signaling pathways leading to activation of defence responses. Expression of *OsPI-PLC1* was rapidly activated in response to treatment with various SAR inducers that induce disease resistance and in the incompatible interaction between rice and *M. grisea*; therefore, PI-PLC-mediated signaling may be involved in the signaling pathways leading to activation of systemic acquired resistance as well as resistance gene-mediated resistance in rice.

In animals, *PI-PLC* genes are classified into three subfamilies on the basis of sequence relatedness and mechanisms of activation; the subfamilies are designated as β, γ, and δ PLCs [28]. Based on amino acid sequence comparisons, plant PI-PLCs are most closely related to the δ PLC subfamily [20, 23, 39]. The PI-PLCs of the δ subfamily contain three conserved domains, X and Y, which together constitute the catalytic domain of the enzymes, and a C_2-like domain (Fig. 1). The X domain of *OsPI-PLC1* contains five conserved histidine residues, which have been shown to be essential for enzyme activity [4, 16]. The C_2-domains are Ca^{2+}-dependent protein–phospholipid interaction domains, which could mediate membrane attachment of the amphipathic PI-PLCs, and are present in a variety of enzymes involved in transmembrane signaling [6, 16].

In our previous studies, we have shown that the treatment of rice seedlings by spraying or soil-drench with BTH can induce systemic acquired resistance against rice blast disease [17, Song and Goodman, unpublished work]. Here we reported that BTH treatment, even at very low concentration, can induce expression of *OsPI-PLC1*, and when BTH-treated plants were infected with *M. grisea* the expression of *OsPI-PLC1* was more rapidly activated than was the case in infected seedlings not treated with BTH. Thus, expression of *OsPI-PLC1* appears to be BTH induced. In BTH-treated rice seedlings, a high level of *OsPI-PLC1* gene expression was observed during the experimental period after inoculation with the blast fungus [Fig. 4(A)]. This may be associated with the relatively long time that BTH acts

in the seedlings since a comparable high level of *OsPI-PLC1* gene expression was also seen 7 days after induction treatment [Fig. 3(B)]. Enhanced expression of *OsPI-PLC1* during the early stage was only detected in the incompatible interaction but not in the compatible interaction between rice and the blast fungus. However, an increase in *OsPI-PLC1* gene expression was also detected at later stages of infection in both the compatible and incompatible interactions [Fig. 4(B)]. This increase in *OsPI-PLC1* gene expression may be associated with disease development. Moreover, certain chemical and biological inducers, including SA, INA, PB, JA, MeJA, wound and *P. syringae* pv. *syringae* DC3000, which are capable of inducing resistance against rice blast disease [*34–37*, *41*], were also able to induce expression of *OsPI-PLC1* (Fig. 3). Therefore, it is possible that an *OsPI-PLC1*-mediated signaling plays a role in the signaling pathways leading to disease resistance in rice.

However, direct evidence for a role of *OsPI-PLC1*-mediated signaling in rice disease resistance signaling pathways remains to be found. It has been demonstrated that the activity of PI-PLC is mainly regulated by heterotrimeric G-protein [*5*, *26*] and is also associated with Ca^{2+} signaling [*23*, *42*]. PI-PLCs require Ca^{2+} for their enzymatic activity [*23*]; but PI-PLCs are also involved in the production of the Ca^{2+} signal [*42*]. In another experiment, we found that BTH treatment and/or infection with *M. grisea* activated expression of two cDNAs, showing high levels of similarity to G protein and calmodulin, respectively [Song and Goodman, unpublished work]. The relationships between the *OsPI-PLC1*-mediated signaling and these two components in the signaling pathway leading to disease resistance in rice need to be studied further. Functional analysis by up- or down-regulated expression of the *OsPI-PLC1* gene through functional genomic approaches will be helpful to further clarify the role of the *OsPI-PLC1*-mediated signaling in rice disease resistance signaling pathways.

The authors thank Novartis Crop Protection, Inc., Research Triangle Park, North Carolina, U.S.A. and The McKnight Foundation for financial support.

REFERENCES

1. **Altschul SF, Madden TL, Schäffer AA, Zhang J Zhang Z, Miller W, Lipman DJ.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389–3402.
2. **Blumwald E, Aharon GS, Lam BC-H.** 1998. Early signal transduction pathways in plant–pathogen interactions. *Trends in Plant Science* **3**: 342–346.
3. **Chandra S, Heinstein PF, Low PS.** 1996. Activation of phospholipase A_2 by plant defense elicitors. *Plant Physiology* **85**: 679–683.
4. **Cheng HF, Jiang MJ, Chen CL, Liu SM, Wong LP, Lomasney JW, King K.** 1995. Cloning and identification of amino acid residues of human phospholipase Cδ1 essential for catalysis. *Journal of Biological Chemistry* **270**: 5495–5505.
5. **Cho MH, Shears SB, Boss WF.** 1993. Changes in phosphatidylinositol metabolism in response to hyperosmotic stress in *Daucus carota* L. cells grown in suspension culture. *Plant Physiology* **103**: 637–647.
6. **Clark JD, Lin LL, Kriz RW, Ramesha CS, Sultzman LA, Lin AY, Milonia N, Knopf JL.** 1991. A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. *Cell* **63**: 1043–1051.
7. **Coursol S, Giglioli-Guivarch N, Vidal J, Pierre JN.** 2000. An increase in phosphoinositide-specific phospholipase C activity precedes induction of C4 phosphoenolpyruvate carboxylase phosphorylation in illuminated and NH_4Cl-treated protoplasts from *Digitaria sanguinalis*. *The Plant Journal* **23**: 497–506.
8. **Delledonne M, Xia Y, Dixon RA, Lamb C.** 1998. Nitric oxide signal functions in plant disease resistance. *Nature* **394**: 585–588.
9. **den Hartog M, Musgrave A, Munnik T.** 2001. Nod factor-induced phosphatidic acid and diacylglycerol pyrophosphate formation: a role for phospholipase C and D in root hair deformation. *The Plant Journal* **25**: 55–65.
10. **Desikan R, Hancock JT, Coffey MJ, Neill SJ.** 1996. Generation of active oxygen in elicited cells of Arabidopsis thaliana is mediated by a NADPH oxidase-like enzyme. *FEBS Letters* **382**: 213–217.
11. **Divecha N, Irvine RF.** 1995. Phospholipid signaling. *Cell* **80**: 269–278.
12. **Drobak BK, Watkins PA.** 2000. Inositol(1,4,5)trisphosphate production in plant cells: an early response to salinity and hyperosmotic stress. *FEBS Letters* **481**: 240–244.
13. **Durner J, Klessig DF.** 1999. Nitric oxide as a signal in plants. *Current Opinion in Plant Biology* **2**: 369–374.
14. **Durner J, Wendehene D, Klessig DF.** 1998. Defense gene induction in tobacco by nitric oxide, cyclic GMP and cyclic ADP ribose. *Proceedings of the National Academy of Sciences of the U.S.A.* **95**: 10328–10333.
15. **Dwyer SC, Legendre L, Low PS, Leto TL.** 1996. Plant and human neutrophil oxidative burst complexes contain immunologically related proteins. *Biochemica et Biophysica Acta* **1289**: 231–237.
16. **Ellis MV, Katan SU, Katan M.** 1995. Mutations within a highly conserved sequence present in the X region of phosphoinositide-specific phospholipase C-δ_1. *Biochemical Journal* **307**: 69–75.
17. **Essen LO, Perisic O, Cheung R, Katan M, Williams RL.** 1996. Crystal structure of a mammalian phosphoinositide-specific phospholipase Cδ. *Nature* **380**: 595–602.
18. **Hartweck LM, Llewellyn DJ, Dennis ES.** 1997. The *Arabidopsis thaliana* genome has multiple divergent forms of phosphoinositol-specific phospholipase C1. *Gene* **202**: 151–156.
19. **Hirayama T, Mitsukawa N, Shibata D, Shinozaki K.** 1997. *AtPLC2*, a gene encoding phosphoinositide-specific phospholipase C, is constitutively expressed in vegetative

and floral tissues in *Arabidopsis thaliana*. *Plant Molecular Biology* **34**: 175–180.

20. **Hirayama T, Ohto C, Mizoguchi T, Shinozaki K.** 1995. A gene encoding a phosphatidylinositol-specific phospholipase C is induced by dehydration and salt stress in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the U.S.A.* **92**: 3903–3907.
21. **Kamada Y, Muto S.** 1994. Stimulation by elicitor of inositol phospholipid turnover in tobacco suspension culture cells. *Plant and Cell Physiology* **35**: 397–404.
22. **Keller T, Damude HG, Werner D, Doemer P, Dixon RA, Lamb C.** 1998. A plant homolog of the neutrophil NADPH oxidase gp91^{phox} subunit gene encodes a plasma membrane protein with calcium binding motifs. *The Plant Cell* **10**: 255–266.
23. **Kopka J, Pical C, Gray JE, Muller-Rober B.** 1998. Molecular and enzymatic characterization of three phosphoinositide-specific phospholipase C isoforms from potato. *Plant Physiology* **116**: 239–250.
24. **Lee Y, Assmann SM.** 1991. Diacylglycerols induced both ion pumping in patch-clamped guard-cell protoplasts and opening of intact stomata. *Proceedings of the National Academy of Sciences of the U.S.A.* **88**: 2127–2131.
25. **Lee Y, Choi YB, Suh S, Lee J, Assmann SM, Joe CO, Kelleher JF, Crain RC.** 1996. Abscisic acid-induced phosphoinositide turn-over in guard cell protoplasts of *Vicia faba*. *Plant Physiology* **110**: 987–996.
26. **Legendre L, Yueh YG, Crain R, Haddock N, Heinstein PF, Low PS.** 1993. Phospholipase C activation during elicitation of the oxidative burst in cultured plant cells. *Journal of Biological Chemistry* **268**: 24559–24563.
27. **McDowell JM, Dangl JL.** 2000. Signal transduction in the plant immune response. *Trends in Biochemical Science* **25**: 79–82.
28. **Munnik T, Irvine RF, Musgrave A.** 1998. Phospholipid signaling in plants. *Biochemica et Biophysica Acta* **1389**: 222–272.
29. **Pappan K, Wang X.** 1999. Molecular and biochemical properties and physiological roles of plant phospholipase D. *Biochemica et Biophysica Acta* **1439**: 151–166.
30. **Parmar PN, Brearley CA.** 1993. Identification of 3- and 4-phosphorylated phosphoinositides and inositol phosphates in stomatal guard cells. *The Plant Journal* **4**: 255–263.
31. **Parmar PN, Brearley CA.** 1995. Metabolism of 3- and 4-phosphorylated phosphatidylinositols in stomatal guard cells of *Commelina communis* L. *The Plant Journal* **8**: 425–433.
32. **Pical C, Kopka J, Muller-Rober B, Hetherington AM, Gray JE.** 1997. Isolation of two cDNA clones for phosphoinositide-specific phospholipase C from epidermal peels and guard cells of *Nicotiana rustica* (PGR97-086). *Plant Physiology* **114**: 748.
33. **Reggiani R, Laoreti P.** 2000. Evidence for the involvement of phospholipase C in the anaerobic signal transduction. *Plant and Cell Physiology* **41**: 1392–1396.
34. **Sakamoto K, Tada Y, Yokozeki Y, Akagi H, Hayashi H, Fujimura T, Ichikawa N.** 1999. Chemical induction of disease resistance in rice is correlated with the expression of a gene encoding a nucleotide binding site and leucine-rich repeats. *Plant Molecular Biology* **40**: 847–855.
35. **Schweizer P, Buchala A, Dudler R, Metraux J-P.** 1998. Induced systemic resistance in wounded rice plants. *The Plant Journal* **14**: 475–481.
36. **Schweizer P, Buchala A, Metraux J-P.** 1997. Gene-expression patterns and levels of jasmonic acid in rice treated with the resistance inducer 2,6-dichloroisonicotinic acid. *Plant Physiology* **115**: 61–70.
37. **Schweizer P, Schlagenhauf E, Schaffrath U, Dudler R.** 1999. Different patterns of host genes are induced in rice by *Pseudomonas syringae*, a biological inducer of resistance, and the chemical unducer benzothiadiazole (BTH). *European Journal of Plant Pathology* **105**: 659–665.
38. **Sekizawa Y, Haga M, Kanoh Y.** 1990. α-Linolenate releasing enzyme, phospholipase A_2 of rice blade infected by blast fungus. *Annals of Phytopathology Society of Japan* **56**: 561–564.
39. **Shi J, Gonzales RA, Bhattacharyya MK.** 1995. Characterization of a plasma membrane-associated phosphoinositide-specific phospholipase C from soybean. *The Plant Journal* **8**: 381–390.
40. **Shigaki T, Bhattacharyya MK.** 2000. Decreased inositol 1,4,5-trisphosphate content in pathogen-challenged soybean cells. *Molecular Plant-Microbe Interaction* **13**: 563–567.
41. **Smith JA, Metraux J-P.** 1991. *Pseudomonas syringae* pv. *syringae* induces systemic resistance to *Pyricularia oryzae* in rice. *Physiological and Molecular Plant Pathology* **39**: 451–461.
42. **Staxen II, Pical C, Montgomery LT, Gray JE, Hetherington AM, McAinsh MR.** 1999. Abscisic acid induces oscillations in guard-cell cytosolic free calcium that involve phosphoinositide-specific phospholipase C. *Proceedings of the National Academy of Sciences of the U.S.A.* **96**: 1779–1784.
43. **Subramaniam R, Despres C, Brison N.** 1997. A functional homolog of mammalian protein kinase C participates in the elicitor-induced defense response in potato. *The Plant Cell* **9**: 653–664.
44. **Takahashi S, Katagiri T, Hirayama T, Yamaguchi-Shinozaki K, Shinozaki K.** 2001. Hyperosmotic stress induces a rapid and transient increase in inositol 1,4,5-trisphosphate independent of abscisic acid in Arabidopsis cell culture. *Plant and Cell Physiology* **42**: 214–222.
45. **Torres MA, Onouchi H, Hamada S, Machida C, Hammond-Kosack KE, Jones JDG.** 1998. Six *Arabidopsis thaliana* homologues of the human respiratory burst oxidase (*gp91^{phox}*). *The Plant Journal* **14**: 365–370.
46. **Toyoda K, Shiraishi T, Yamada T, Ichinose Y, Oku H.** 1993. Rapid changes in polyphosphoinositide metabolism in pea in response to fungal signals. *Plant and Cell Physiology* **34**: 729–735.
47. **Verwoerd TC, Dekker BMM, Hoekema A.** 1989. A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acid Research* **17**: 2362.
48. **Xing T, Higgin VJ, Blumwald E.** 1997. Race-specific elicitors of *Cladosproium fulvum* promote translocation of cytosolic components of NADPH oxidase to the plasma membrane of tomato cells. *The Plant Cell* **9**: 249–259.
49. **Yamamoto YT, Conkling MA, Sussex IM, Irish VF.** 1995. An Arabidopsis cDNA related to animal phosphoinositide-specific phospholipase C genes. *Plant Physiology* **107**: 1029–1030.
50. **Young SA, Wang X, Leach JE.** 1996. Changes in the plasma membrane distribution of rice phospholipase D during resistant interaction with *Xanthomonas oryzae* pv. *oryzae*. *The Plant Cell* **8**: 1079–1090.