

Ectopic expression of a rice protein phosphatase 2C gene *OsBIPP2C2* in tobacco improves disease resistance

Xuebo Hu · Huijuan Zhang · Guojun Li ·
Yuxia Yang · Zhong Zheng · Fengming Song

Received: 26 May 2008 / Revised: 12 February 2009 / Accepted: 31 March 2009 / Published online: 19 April 2009
© Springer-Verlag 2009

Abstract Protein phosphatase 2Cs (PP2Cs) have been demonstrated to play critical roles in regulation of plant growth/development, abscisic acid signaling pathway and adaptation to environmental stresses. Here we report the cloning and molecular characterization of a novel rice protein phosphatase 2C gene, *OsBIPP2C2* (*Oryza sativa* L. BTH-induced protein phosphatase 2C 2). *OsBIPP2C2* has three alternatively spliced transcripts and the largest transcript *OsBIPP2C2a* encodes a 380 aa protein containing all 11 conserved catalytic subdomains of PP2Cs. Expression of *OsBIPP2C2a* was significantly induced by benzothiadiazole (BTH), one of defense-related signal molecules in plants. Expression of *OsBIPP2C2a* was induced by infection with the blast fungus, *Magnaporthe grisea*, and the pathogen-induced expression of *OsBIPP2C2a* in BTH-treated rice seedlings was much earlier and stronger than those in water-treated seedlings. Overexpression of *OsBIPP2C2a* in transgenic tobacco plants resulted in increased disease resistance against tobacco mosaic virus and *Phytophthora parasitica* var. *nicotianae*. Importantly, the *OsBIPP2C2a*-overexpressing transgenic tobacco plants showed constitutive expression of defense-related genes. These results

suggest that *OsBIPP2C2a* may play an important role in disease resistance through activation of defense response.

Keywords Disease resistance response · *Oryza sativa* L · *Magnaporthe grisea* · *OsBIPP2C2* · Protein phosphatase 2C

Abbreviations

ABA	Abscisic acid
BTH	Benzothiadiazole
CaMV	Cauliflower mosaic virus
PP2C	Protein phosphatase 2C
TMV	Tobacco mosaic virus

Introduction

Protein phosphatases play critical roles in balance of protein dephosphorylation by removing the phosphor-groups from their phosphorylated targets. Among them, protein phosphatase 2Cs (PP2Cs) have been studied extensively for their biochemical functions in plant growth, development and responses to hormones and abiotic stresses (Schweighofer et al. 2004). In Arabidopsis, 76 genes were identified to encode putative PP2C-type phosphatases (Kerk et al. 2002). Implication of PP2Cs in regulation of abscisic acid (ABA) response is well established in Arabidopsis. Biochemical and genetic analyses have resulted in the identification of at least four PP2Cs, including ABI1, ABI2, PP2CA, and HAB1 (formerly named AtP2C-HA), as negative regulators of ABA signal transduction (Meyer et al. 1994; Stone et al. 1994; Leung et al. 1997; Gosti et al. 1999; Merlot et al. 2001; Tahtiharju and Palva 2001; Gonzalez-Garcia et al. 2003; Saez et al. 2004; Kuhn et al.

Communicated by A. Atanassov.

X. Hu and H. Zhang contributed equally to this study.

The nucleotide sequences of *OsBIPP2C2a* and *OsBIPP2C2c* have been deposited in GenBank under accession numbers of AY831394 and AY831395, respectively.

X. Hu · H. Zhang · G. Li · Y. Yang · Z. Zheng · F. Song (✉)
State Key Laboratory of Rice Biology,
Department of Plant Protection, Institute of Biotechnology,
Zhejiang University-Huajiaochi Campus,
310029 Hangzhou, Zhejiang, People's Republic of China
e-mail: fmsong@zju.edu.cn

2006; Yoshida et al. 2006; Nishimura et al. 2007). Functions of ABI1 and ABI2 appear to be highly overlapped, including ABA-regulated seeds dormancy, guard cell closure, and acclimation to various stresses (Pei et al. 1997; Leung et al. 1997; Gosti et al. 1999; Chak et al. 2000; Merlot et al. 2001; Saez et al. 2006). Other identified Arabidopsis PP2Cs include kinase-associated protein phosphatase (KAPP) and POL, which regulate the normal developmental process of tissues and organs (Stone et al. 1998; Shah et al. 2002; Yu et al. 2003).

A number of PP2Cs identified from other plant species have also been shown to play roles in growth and response to various abiotic stresses. *FsPP2C1* and *FsPP2C2*, two PP2C genes from beech (*Fagus sylvatica*) seeds, were induced by ABA in dormant seeds (Lorenzo et al. 2001, 2002). Results from transgenic Arabidopsis overexpressing *FsPP2C1* gene indicate that negative regulation of ABA signaling by *FsPP2C1* is a factor contributing to promote the transition from seed dormancy to germination during early stage of stratification (Gonzalez-Garcia et al. 2003). Interestingly, constitutive expression of *FsPP2C2* in transgenic Arabidopsis under the cauliflower mosaic virus 35S promoter resulted in enhanced sensitivity to ABA and osmotic stress in seeds and vegetative tissues as well as dwarf and delayed flowering, suggesting that *FsPP2C2* is a positive regulator of ABA (Reyes et al. 2006). Thus, *FsPP2C2* is different from other identified plant PP2Cs that act as negative regulators of ABA signaling. *MP2C*, a wound-induced alfalfa PP2C gene, was found to be activated within 10 min and gradually decreased to non-detectable levels after 20 min wounding treatment (Meskiene et al. 1998). *NtPP2C1*, a tobacco PP2C gene, was strongly activated by drought, but repressed by oxidative stress and heat shock, suggesting that *NtPP2C1* may operate at the junction of drought, heat shock and oxidative stress (Vranova et al. 2000). Moreover, a lotus PP2C gene, *LiNPP2C1*, was shown to be expressed specifically during establishment of root nodules with *Mesorhizobium*, but not in the early stage (Kapranov et al. 1999), indicating the involvement of PP2Cs in plant-microbe interactions.

Compared with the extensive studies on the functions of MAP kinases, little attention has been paid on the involvement of PP2Cs in plant biotic stress responses. Recently, several lines of evidence suggest a role for PP2Cs in regulation of plant defense responses. Tobacco DBP1 contains a typical catalytic domain of PP2C at its C terminus and a transcription factor domain at the N terminus and was found to modulate transcription of a defense gene, *CEVII*, whose expression was up-regulated only in compatible interaction between citrus exocortis viroid and tobacco, but not in incompatible interaction (Carrasco et al. 2003). 14-3-3G is directly involved in regulating DBP1

function by promoting nuclear export and subsequent cytoplasmic retention of DBP1 under conditions that in turn alleviate DBP1-mediated repression of target gene expression (Carrasco et al. 2006). Arabidopsis AP2C1 is a novel stress signal regulator that inactivates the stress-responsive MAPKs. Mutant *ap2c1* plants produce higher amounts of jasmonic acid and are more resistant to phytophagous mites. Plants overexpressing *AP2C1* gene display reduced ethylene production and defense-related gene expression, and compromised innate immunity against the necrotrophic pathogen *Botrytis cinerea* (Schweighofer et al. 2007).

We are interested in the function of protein phosphorylation/dephosphorylation, which is modulated by protein kinases and phosphatases, respectively, in regulation of plant defense response. We previously found that two MAP kinase genes, *OsBIMK1* and *OsBIMK2*, and a PP2C gene, *OsBIPP2C1*, play roles in rice disease resistance responses (Song and Goodman 2002a; Song et al. 2006; Hu et al. 2006). Here we report the isolation and identification of a new rice PP2C gene, *OsBIPP2C2*. Among the predicted three alternatively spliced transcripts of *OsBIPP2C2* gene, accumulation of *OsBIPP2C2a* transcript was increased in rice seedlings after treatment with benzothiadiazole, a disease resistance signal molecule and infection by *Magnaporthe grisea*, a fungal pathogen causing rice blast disease. Overexpression of *OsBIPP2C2a* in transgenic tobacco plants resulted in enhanced disease resistance and constitutive expression of defense-related genes. Our results suggest that *OsBIPP2C2a* may play an important role in disease resistance through activation of defense response.

Materials and methods

Plant growth and treatments

Rice (*Oryza sativa* L. subsp. *indica* cv. Yuanfengzao) seedlings were grown under greenhouse conditions at 22/27°C (night/day) and 3-week-old seedlings were used for all experiments. Rice seedlings were treated by foliar spraying with 0.3 mM BTH solution (Novartis Crop Protection Inc., Research Triangle Park, NC, USA) or with sterilized distilled water as a control. At 3 days after BTH treatment, the seedlings were inoculated with spore suspension (5×10^5 spores per ml in 0.05% Tween-20) of *Magnaporthe grisea* (strain 85-14B1 of race ZB1) and then the inoculated seedlings were kept at 100% relative humidity in darkness for 36 h (Luo et al. 2005a). Samples were collected at different time points as indicated after treatment and stored at -80°C until further use.

Cloning of *OsBIPP2C2*

Two differentially expressed clones (BNHN-n22 and BNHN-n23), obtained previously from screenings of suppression subtractive hybridization libraries (Song and Goodman 2002a), show high level of similarity to plant PP2C genes. To obtain the full-length cDNA of this putative PP2C gene, the 5'- and 3'-missing ends were amplified by PCR using phage DNA prepared from a rice cDNA library (Song and Goodman 2002a) as template. To amplify the 3'-end, a gene-specific primer, BNHN-n22-1F (5'-GGA CGG GTA TAG CAA ACA GA-3') and a vector anchored universal reverse primer, T7 (5'-GTA ATA CGA CTC ACT ATA GGG C-3') were used for amplification of the 3'-end; a gene-specific primer, BNHN-n22-1R (5'-CTT CCT CGT GGC TTC CTT TA-3'), and another vector anchored forward universal primer, T3 (5'-AAT TAA CCC TCA CTA AAG GGA-3') were used for amplification of the 5'-end. The full-length cDNAs were amplified using a gene-specific primer, BNHN-n22-2F (5'-GCT TGT GTT GGA TCG GAG T-3'), located in the 5'-UTR, and the vector universal primer T7. A pair of primers, BNHN-n22-3F (5'-CAT ATG TTG AGG GCG GTG GC-3', underlined is an *NdeI* site) and BNHN-n22-3R (5'-GAA TTC TAC CAA CCG GAG CC-3', underlined is an *EcoRI* site) was used for cloning of the ORF sequences. All PCR products were cloned into pUCm T-vector (Sangon, Shanghai, China) by T/A cloning and confirmed by sequencing. The correct clones for the full-length cDNAs were designated as pUCm-OsBIPP2C2.

DNA sequencing and analysis

DNA sequencing was performed on both strands on the MegaBACE 1000 DNA Analysis System (Amersham Biosciences, UK) at the Center of Analysis and Measurement in Zhejiang University. Sequence edits and ORF prediction were carried out using DNASTAR software (LaserGene, Madison, WI, USA). Similarity searches were done using BLAST at the NCBI GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequence alignments were conducted using ClustalW (<http://www.ebi.ac.uk/clustalw/>).

Total RNA extraction and Northern blotting

Total RNA was extracted from rice and tobacco leaf samples using a standard acid-guanine method (Sambrook and Russell 2001). Twenty (rice) or ten (tobacco) micrograms of total RNA were separated on 1% agarose-formaldehyde gel, and transferred onto Hybond N⁺ (Amersham Biosciences, UK) nylon membrane in 20 × SSC by

capillary action overnight. A 3'-end 700 bp fragment of *OsBIPP2C2* was prepared from plasmid pUCm-OsBIPP2C2 by digesting with *PstI*. Fragments for tobacco *PR-1a* and *Sar8.2b*, two defense-related genes were prepared as previously described (Song and Goodman 2002b; Luo et al. 2005b). cDNA fragments were labeled with [α -³²P]-dCTP by the random priming method using a Random Primed DNA Labeling Kit (Takara, Dalian, China). Pre-hybridization was performed at 42°C for 30 min in ULTRAhyb buffer (Ambion Inc., Austin, USA) and hybridization was carried out overnight at 42°C in the same hybridization buffer with the [α -³²P]-labeled probe. After hybridization, the blots were washed twice with 2 × SSC, 0.1%SDS and 1 × SSC, 0.1%SDS for 5 min each at 42°C, and autoradiographed by exposure to X-ray film (Lucky Film Corporation, Baoding, China) for 2 days at −80°C.

Construction of binary vector and generation of transgenic tobacco

The ORF sequence of *OsBIPP2C2* was released by digestion with *XbaI/PstI* from plasmid pUCm-OsBIPP2C2 and ligated into *XbaI/PstI* sites of plant binary vector CHF3, in which the *OsBIPP2C2* gene was placed under the control of the CaMV 35S promoter in the sense orientation. The resulting plasmid, CHF3-OsBIPP2C2, was introduced into *Agrobacterium tumefaciens* EHA105 by electroporation. *Agrobacterium* were grown at 28°C with shaking (200 rpm) in YEP broth containing 100 μg/ml streptomycin and 100 μg/ml spectinomycin and bacterial suspension of OD₆₀₀ = 0.80 was used for transformation. Transformation of tobacco (cv. Xanthi nc) was performed using *Agrobacterium*-mediated leaf disc transformation method. Transgenic lines with single copy of *OsBIPP2C2a* gene were selected and T3 homozygous seeds were used for study. The seeds of the transgenic tobacco lines were germinated on 1/2 MS medium containing 200 μg/ml kanamycin in a growth chamber under 16 h day/8 h night at temperature of 22–25°C for 3–4 weeks and the surviving plants were transferred into natural soil and grown in the growth room for another 3–4 weeks.

Genomic DNA of transgenic plants was extracted by CTAB protocol (Luo et al. 2005b). Approximately 100 ng of genomic DNA was used for detection of the transgene in transgenic plants by PCR with primers BNHN-n22-3F and BNHN-n22-3R. Genomic DNA was digested with *HindIII*, separated on agarose gel, and transferred onto Hybond N⁺ (Amersham Biosciences, UK) nylon membrane by capillary action. Southern blotting analysis was performed with the same protocol as Northern blotting analysis.

Disease resistance assays

Inoculation of the tobacco plants with TMV was performed using standard mechanical rubbing method. For preparation of the inoculums, TMV-infected tobacco leaves were ground in a mortar with a pestle in 5 ml of potassium phosphate buffer (50 mM, pH 7.0). Fully expanded leaves were dusted with dry carborundum and inoculated by gently rubbing the upper leaf surface with 100 μ l of the viral suspension inoculums, followed by rinsing with deionized water immediately. Control plants were also dusted with carborundum and mock inoculated with sample volume of potassium phosphate buffer. Following inoculation, plants were maintained at 23–26°C under continuous illumination provided by cool-white fluorescent lamps. The lesion numbers on the inoculated leaf of at least six plants were counted 5 days after inoculation.

Phytophthora parasitica var. *nicotianae* was cultivated on PDA medium (potato, 200 g/L; dextrose 20 g/L; agar 15 g/L; pH 6.5) at 25°C for 4–6 days. The mycelia were collected and suspended in water to OD₆₀₀ \approx 0.5 for inoculation. Six two-month-old tobacco plants were inoculated by pouring of mycelium suspension at the base of the stem (20 mL/plant) and were then incubated in growth room under the conditions described above. Disease severity was evaluated three weeks after inoculation on a four-scale standard as the follows: 0, the inoculated plants have no significant symptom; 1, lesions on stem base of the inoculated plants do not encircle the stem and the plants have no wilted leaves; 2, lesions on stem base encircle the stem and 2–3 lower leaves of the inoculated plants become yellowish and/or wilted; 3, lesions on stem base encircle the stem and the inoculated plants become wilted. Disease index was calculated by dividing sum of plants scored in each rating \times corresponding rating value with total number of plants \times 3.

All experiments were performed independently three times, and the data from these experiments were subjected to statistical analysis using DPS software (Beijing China-agri BRAINS Science & Technology Co. Ltd, <http://www.nbs.net.cn/yzjaa.htm>).

Results

Cloning of *OsBIPP2C2* and identification of two alternatively spliced transcripts

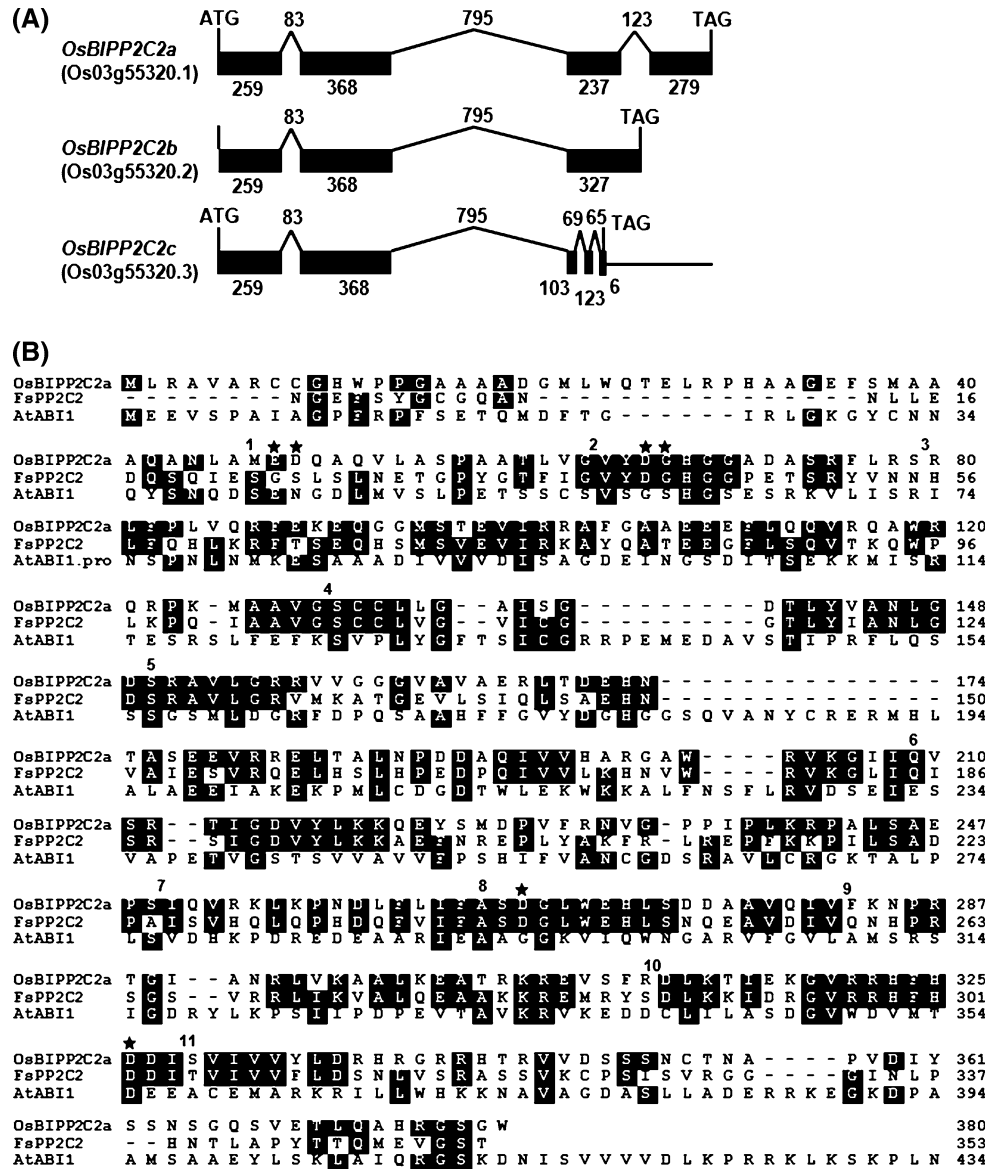
In our previous studies aimed at elucidation of molecular biology of rice disease resistance response, we isolated and characterized hundreds of differentially expressed cDNA clones (Song and Goodman 2002a). Among them, two differentially expressed clones, BNHN-n22 (GenBank

Accession No. BI118738) and BNHN-n23 (GenBank Accession No. BI118739), contain cDNA inserts of 344 bp and 399 bp, respectively, which show high level of similarity to genes encoding plant PP2Cs. A rice PP2C gene, *OsBIPP2C1* (*Oryza sativa* L. BTH induced protein phosphatase 2C 1) was previously identified (Hu et al. 2006), we thus designated this new PP2C gene as *OsBIPP2C2*. Rapid amplification of cDNA ends approach was used to amplify the missing 5'- and 3'-end sequences and a full-length cDNA was assembled. Database searches revealed that *OsBIPP2C2* corresponds to locus Os03g55320, which is annotated to have three alternatively spliced transcripts, Os03g55320.1, Os03g55320.2 and Os03g55320.3 (Fig. 1a). In amplification of *OsBIPP2C2* ORF, two fragments of \sim 1,140 and \sim 1,050 bp in size were amplified. Cloning and sequencing revealed that these two fragments were 1,143 and 1,040 bp in size, respectively, and had identical sequences of 696 bp at 5'-end. Alignments of these two transcripts with predicted Os03g55320 transcripts, indicate that the large (1,143 bp) and small (1,040 bp) transcripts correspond to Os03g55320.1 and Os03g55320.3, respectively. We designated the large transcript as *OsBIPP2C2a* and the short one as *OsBIPP2C2c*. *OsBIPP2C2a* is supported by two full-length cDNAs (GenBank Accession Nos. AK102620 and AK105764) isolated from rice subsp. *Japonica* by Knowledge-based *Oryza* Molecular biological Encyclopedia (<http://cdna01.dna.affrc.go.jp/cDNA/>); while *OsBIPP2C2c* lacks support of full-length cDNA available in public database. Another full-length cDNA, AK066083, confirms the predicted transcript of Os03g55320.2. The *OsBIPP2C2c* transcript seems to be transcribed from Os03g55320 locus with an advanced transcription stop due to an alternatively splicing event. This was the reason that the *OsBIPP2C2c* sequence was amplified using a pair of primers that were designated for amplification of the *OsBIPP2C2* ORF. Database searching and alignment analyses revealed that there are 3 nucleotide differences between the *OsBIPP2C2a* sequence and its corresponding rice genomic sequence; but these differences do not result in any changes in protein sequence. Because *OsBIPP2C2c* does not encode a typical PP2C, *OsBIPP2C2a* was chosen for further study.

Characterization of *OsBIPP2C2* protein

The full-length cDNAs of *OsBIPP2C2a* contains a predicted ORF of 1,143 bp, encoding a 380 aa protein with calculated molecular weight of 41.8 kD and isoelectric point of 9.4. *OsBIPP2C2a* contains all 11 conserved catalytic subdomains present in all PP2Cs (Rodriguez 1998) with six residues putatively involved in binding of the phosphate and metal ions (Das et al. 1996) (Fig. 1b).

Fig. 1 Alternatively spliced transcripts of *OsBIPP2C2* gene and alignment of *OsBIPP2C2a* with other PP2Cs. **a** Exon/intron organizations, splicing patterns of the *OsBIPP2C2* gene. Exons are indicated by black boxes and the introns are indicated by thick inverted V-shape lines. For *OsBIPP2C2c* transcript, 3' end sequence after stop codon is also presented as bold line. Numbers of nucleotides are indicated above for introns and below for exon in bp. **b** Alignment analysis of and conserved domains in *OsBIPP2C2a* protein. The putative subdomains of PP2C are indicated by numbers above the sequences and residues putatively involved in binding metal ions are marked by filled stars. Plant PP2Cs used for alignment are *Arabidopsis thaliana* ABI1 (CAA54383) and *Fagus sylvatica* FsPP2C2 (CAB90634)



However, *OsBIPP2C2b* and *OsBIPP2C2c* lack domains 10–11 and domains 7–11, respectively. *OsBIPP2C2a* has a relatively shorter N-terminal extensions (44 aa) than those of other plant PP2Cs (110–140 aa).

Because PP2Cs family comprises of a large number of members in plants, we thus searched for possible function of *OsBIPP2C2a* by comparing phylogenetic relationship between *OsBIPP2C2a* and other previously studied plant PP2Cs with defined biological functions. Phylogenetic tree analysis revealed that *OsBIPP2C2a* shows a relatively low level of identity (<45%) to other reported plant PP2Cs (Fig. 2) at amino acid sequence level. *OsBIPP2C2a* is more close (45% identity) to a biochemically identified *Fagus sylvatica* *FsPP2C2* (Lorenzo et al. 2002), which has been shown to be involved in osmotic stress tolerance in seeds and vegetative tissues through regulating ABA sensitivity

(Reyes et al. 2006). However, *OsBIPP2C2a* has less than 25% of identity to other well-identified plant PP2Cs such as *Arabidopsis* ABI1 (Leung et al. 1994; Meyer et al. 1994), ABI2 (Leung et al. 1997; Rodriguez et al. 1998) and *AtPP2CA* (Kuromori and Yamamoto 1994), tobacco *NtPP2C1* and *NtPP2C2* (Vranova et al. 2000), and *Medicago sativa* *MP2C* (Meskiene et al. 1998). Furthermore, *OsBIPP2C2a* has only 21% of identity to *OsBIPP2C1*, previously identified rice PP2C that plays a role in disease resistance (Hu et al. 2006). Thus, *OsBIPP2C2a* may be a new stress-related PP2C.

Expression of *OsBIPP2C2a* in rice defense response

The differentially expressed clones BNHN-n22 and BNHN-n23, which contain cDNA inserts derived from

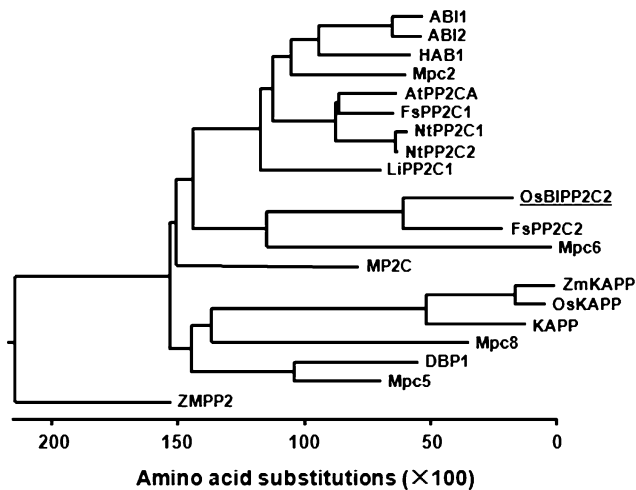


Fig. 2 Phylogenetic tree analysis of *OsBIPP2C2a*. Alignment was done using ClustalW program in DNASTar software and the PP2C proteins used are: *Arabidopsis thaliana* ABI1 (CAA54383), ABI2 (CAA70162), AtPP2CA (BAA07287), KAPP (AAB38148) and HAB1 (At1g72770); *Nicotiana tabacum* NtPP2C1 (CAC10358), NtPP2C2 (CAC10359) and DBP1 (AAM75346); *Fagus sylvatica* FsPP2C1 (CAB90633) and FsPP2C2 (CAB90634); *Lotus japonicus* LiPP2C1 (AAD17804); *Medicago sativa* MP2C (CAA72341); *Mesembryanthemum crystallinum*, Mpc2 (AAC36697), Mpc5 (AAC36698), Mpc6 (AAC36699) and Mpc8 (AAC36700); *Zea mays* ZMPP2 (AAG43835) and ZmKAPP (AAB93832); *Oryza sativa* OsKAPP (AAC26828)

OsBIPP2C2a, were obtained from suppression subtractive hybridization library constructed by subtraction of cDNAs from BTH-induced rice tissues with those from water control. This implied that *OsBIPP2C2* gene might be induced by BTH treatment in rice. We first analyzed whether expression of *OsBIPP2C2a* was induced by BTH treatment. Because *OsBIPP2C2* has multiple alternatively spliced transcripts, a 700 bp fragment at 3'-end of *OsBIPP2C2a* that covers the different sequence among the three alternatively spliced transcripts was used as probe. The specificity of the probe used was further confirmed by RT-PCR, in which only the *OsBIPP2C2a* transcript could be amplified as confirmed by sequencing the RT-PCR product. Northern blot analysis revealed that *OsBIPP2C2a* has a very low level of basic expression in healthy leaf tissues of rice seedlings grown under normal conditions (Fig. 3a). However, treatment of 3-week-old seedlings with BTH induced markedly the expression of *OsBIPP2C2a* (Fig. 3b). *OsBIPP2C2a* transcript accumulated significantly at 12 h, peaked at 24 h and maintained at high level until 48 h after treatments (Fig. 3b). The *OsBIPP2C2a* transcript returned to background level at 72 h after BTH treatment (Fig. 3b).

The expression pattern of *OsBIPP2C2a* in disease resistance response against the blast fungus, *M. grisea* was further analyzed. In this experiment, we treated the rice

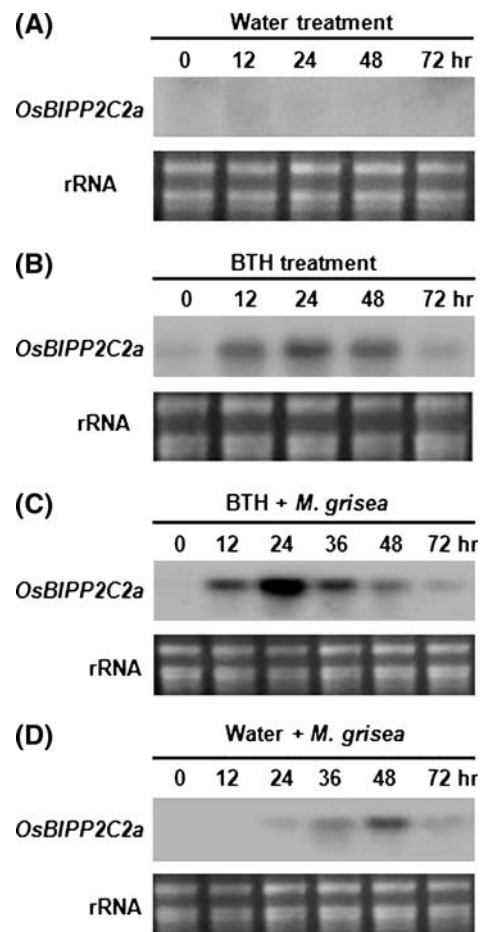


Fig. 3 Induction of *OsBIPP2C2a* expression in defense responses. **a** and **b** *OsBIPP2C2a* expression in 3-week-old rice seedlings after treatment by foliar spraying with 0.3 mM of BTH or water as control. **c** and **d** Expression of *OsBIPP2C2a* in response to infection by *Magnaporthe grisea*. Three-week-old seedlings were treated by foliar spraying 0.3 mM BTH solution and inoculated with *M. grisea* 3 days after treatment. Leaf samples were collected at various time points (h) as indicated after BTH treatment (**a** and **b**) or after inoculation (**c** and **d**). Twenty micrograms of total RNA were fractionated on 1.5% agarose formaldehyde gel and hybridized with the 32 P-labeled 700 bp fragment of *OsBIPP2C2a* cDNA as probe. The corresponding ethidium bromide gel image confirms equal loading of RNA samples

seedlings by spraying BTH solution and inoculated with spores of *M. grisea* 3 days after BTH treatment. The BTH-treated seedlings showed an induced defense response against infection by the blast fungus. In the BTH-treated rice seedlings, *OsBIPP2C2a* transcript accumulated to a high level as early as 12 h and peaked at 24 h after inoculation with the fungus. High level of the *OsBIPP2C2a* transcript was maintained within first 36 h and declined to background level after 36 h of inoculation (Fig. 3c). In contrast, no significant accumulation of *OsBIPP2C2a* transcript was detected in water-treated control seedlings within the first 24 h after inoculation. However, a relatively low level of expression was also observed in water-treated control

seedlings during 36–48 h after inoculation (Fig. 3d). These results suggest that *OsBIPP2C2a* transcript could be induced by infection with the blast fungus and the expression level in the BTH-induced rice plants was much earlier and stronger than those in the uninduced plants, especially during the early stage of infection.

Generation and characterization of *OsBIPP2C2a* transgenic tobacco plants

To gain further insights into the biological function of the *OsBIPP2C2* gene, we performed a functional analysis in transgenic tobacco plants by overexpression of *OsBIPP2C2a* under control of the cauliflower mosaic virus (CaMV) 35S promoter. Sixteen independent transgenic lines were obtained and all of them contained the *OsBIPP2C2a* gene as confirmed by PCR detection of its cDNA sequence with genomic DNA as templates and sequencing of the amplified fragments (Fig. 4a). Copy number of the *OsBIPP2C2a* gene in transgenic lines was analyzed by Southern blotting and five transgenic lines with single copy of the transgene were identified (Fig. 4b). The transgene *OsBIPP2C2a* expressed in the transgenic lines, although the expression level varied to some extent (Fig. 4c). No significant abnormal morphological and growth/development phenotypes were observed in *OsBIPP2C2a* transgenic plants. Independent transgenic lines #03, #07, #33 and #36 were chosen for further studies as these lines contained a single copy of the transgene and showed high level of *OsBIPP2C2a* expression in transgenic plants.

OsBIPP2C2a-overexpressing plants enhanced disease resistance

To explore the possible role of *OsBIPP2C2a* in defense responses, we analyzed the disease resistance levels of *OsBIPP2C2a*-overexpressing tobacco plants against tobacco mosaic virus (TMV) and *P. parasitica* var. *nicotianae*. Under our experimental conditions, typical necrotic lesions were observed on the leaves of the wild-type and the *OsBIPP2C2a*-overexpressing transgenic plants 3–5 days after inoculation with TMV (Fig. 5). However, the lesion numbers in the leaves of the *OsBIPP2C2a*-overexpressing transgenic lines #03, #07, #33 and #36 were significantly reduced ($P \leq 0.05$) by 39–55% as compared with that of the wild-type plants (Fig. 5). Likewise, disease symptoms, typical wilting of the lower old leaves and rotting of the stem bases were seen on the transgenic and the wild-type plants 10 days after drench-inoculation with *P. parasitica* var. *nicotianae* (Fig. 6). Disease indexes at 10 and 20 days after inoculation in the transgenic lines #03, #07 and #36 were significantly reduced ($P \leq 0.05$) by 43–90 and 33–84%,

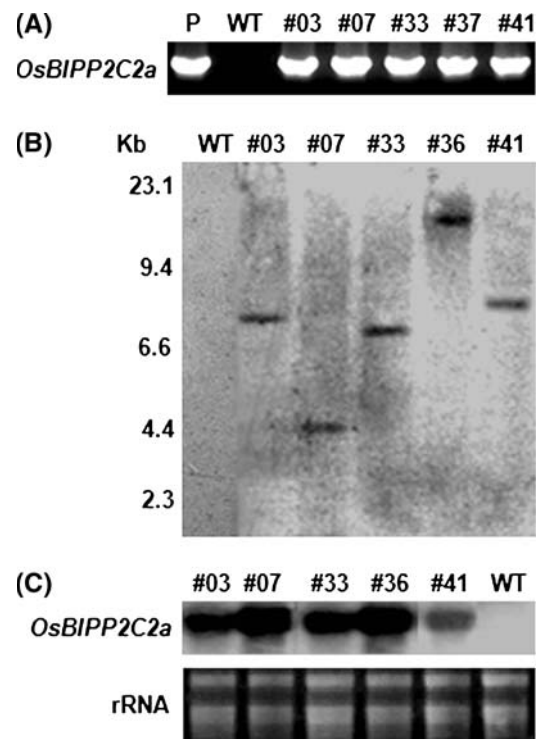


Fig. 4 Generation and characterization of *OsBIPP2C2a*-overexpressing transgenic tobacco plants. **a** Detection of *OsBIPP2C2a* in transgenic plants by PCR. **b** Southern blot analysis of copy number of the *OsBIPP2C2a* gene in the transgenic lines. Genomic DNA was digested with *Hind*III, separated on agarose gel, and transferred onto nylon membrane. The blot was hybridized with 32 P-labeled fragment as a probe. **c** Expression of *OsBIPP2C2a* in transgenic plants. Ten micrograms of total RNA extracted from leaf samples of 6-week-old plants were fractionated and hybridized with 32 P-labeled *OsBIPP2C2a* fragment as a probe. The corresponding ethidium bromide gel image shows the relative levels of RNA loaded for each sample. *P* Plasmid, *WT* untransformed wild-type plants; #03, #07, #33, #36 and #41, independent transgenic lines

respectively, as compared with those of wild-type plants (Fig. 6). These results suggest that overexpression of the rice *OsBIPP2C2a* in tobacco plants enhances disease resistance against TMV and *P. parasitica* var. *nicotianae*.

Constitutive expression of defense genes in *OsBIPP2C2a* transgenic plants

To further confirm the disease resistance phenotype observed in *OsBIPP2C2a*-overexpressing tobacco plants, we analyzed and compared expression levels of *PR-1a* and *Sar8.2b*, two defense-related genes, in *OsBIPP2C2a* transgenic plants with those in wild-type plants. As shown in Fig. 7, under the experimental conditions, no significant expression of *PR-1a* and *Sar8.2b* was detected in the wild-type plants. However, elevated expressions of these two *PR* genes were observed in *OsBIPP2C2a*-overexpressing plants in absence of pathogens or other chemical inducers

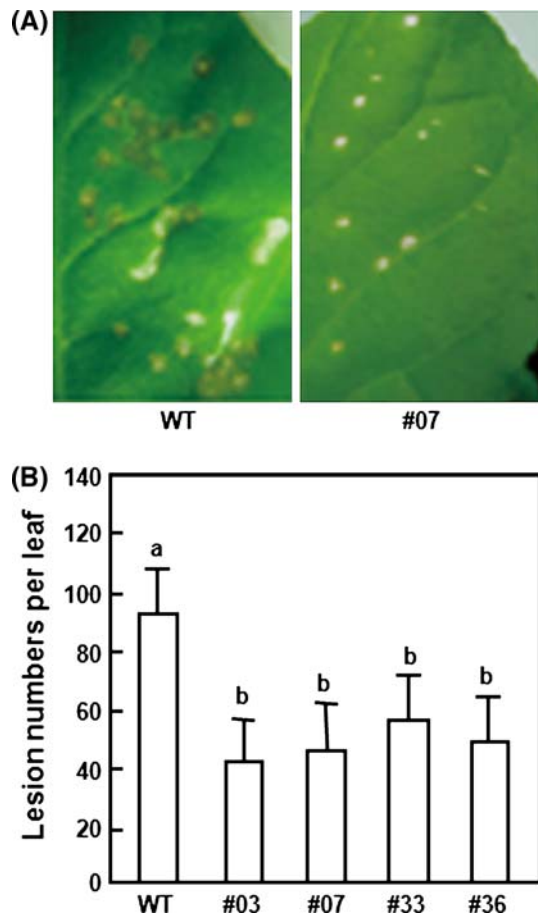


Fig. 5 Enhanced disease resistance against tobacco mosaic virus in *OsBIPP2C2a*-overexpressing tobacco plants. **a** Representative disease symptom on transgenic and wild-type plants. **b** Disease severity as measured by lesion numbers. Data presented are the means (standard deviations from three independent experiments). The bars with the same letter are not significantly different at $P \leq 0.05$. Photographs were taken 7 days after inoculation. *WT* untransformed wild-type plants; #03, #07, #33 and #36, independent transgenic lines

(Fig. 7). The increased levels of *PR-1a* and *Sar8.2b* expression varied among the transgenic lines tested. These results indicate that overexpression of the rice *OsBIPP2C2a* in transgenic tobacco plants results in constitutive activation of defense responses, which in turn confers enhanced disease resistance against TMV and *P. parasitica* var. *nicotianae*.

Discussion

Protein phosphatase 2Cs have been extensively studied for their involvement in regulation of plant growth and development as well as in the ABA-responsive signaling pathway and in adaptation to environmental stresses (Rodriguez et al. 1998; Schweighofer et al. 2004). However, our

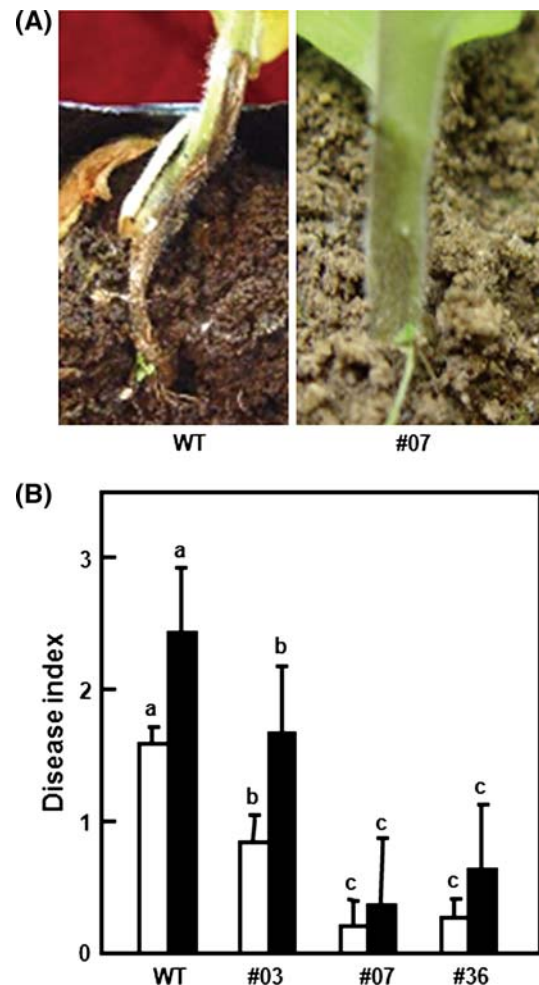


Fig. 6 Enhanced disease resistance against *Phytophthora parasitica* in *OsBIPP2C2a*-overexpressing tobacco plants. **a** Representative disease symptom on transgenic and wild-type plants. **b** Disease index on the transgenic and wild type plants 10 and 20 days after inoculation with *P. parasitica*. Open and solid columns represent the disease indexes at 10 and 20 days after inoculation, respectively. Data presented are the means (standard deviations from three independent experiments). The bars with the same letter are not significantly different at $P \leq 0.05$. Photographs were taken 20 days after inoculation. *WT* untransformed wild-type plants; #03, #07 and #36, independent transgenic lines

knowledge on the involvement and function of PP2Cs in regulation of defense responses in plants is very limited. Recently, it was demonstrated that the Arabidopsis AP2C1 plays a key role in regulation of innate immunity against *Botrytis cinerea* (Schweighofer et al. 2007). We previously identified a rice PP2C gene, *OsBIPP2C1* and found that overexpression of *OsBIPP2C1* in tobacco led to enhanced disease resistance (Hu et al. 2006). In the present study, we identified a new PP2C gene, *OsBIPP2C2*, whose expression was induced in rice defense response. Our functional analysis by ectopic expression of *OsBIPP2C2a*, one of three alternatively spliced transcripts of *OsBIPP2C2* gene, in

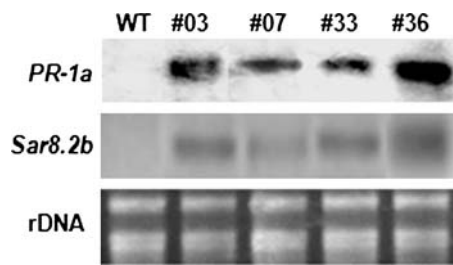


Fig. 7 Expression of *PR-1a* and *Sar8.2b* in *OsBIPP2C2a*-overexpressing tobacco plants. Leaf samples were collected from six-week-old tobacco plants of different transgenic lines. Ten micrograms of total RNA were fractionated on agarose formaldehyde gel and hybridized with the ^{32}P -labeled fragments of *PR-1a* and *Sar8.2b* cDNAs. The corresponding ethidium bromide gel image shows the relative levels of RNA loaded for each sample. WT untransformed wild-type plants; #02 and #07, independent transgenic lines

transgenic tobacco plants suggest that *OsBIPP2C2* may function in regulation of defense responses through activation of defense-related gene expression.

The *OsBIPP2C2* gene (Os03g55320) is predicted to have three alternatively spliced transcripts. Our cloning of *OsBIPP2C2a* and *OsBIPP2C2c*, and the available full-length cDNAs in GenBank database, confirm the existence of these three alternatively spliced transcripts in rice. The *OsBIPP2C2a* transcript encodes a typical PP2C containing all 11 conserved subdomains of the PP2C catalytic domain, while the other two transcripts encode C-terminal truncated putative PP2C proteins lacking subdomains 8–11. This is similar to the situation for rice *OsMAPK5*, whose large transcript *OsMAPK5a* encodes a functional MAPK with typical kinase activity, but the small transcript *OsMAPK5b* encodes a truncated MAPK with no kinase activity (Xiong and Yang 2003). The *OsBIPP2C2a* transcript was accumulated in rice tissues in response to BTH treatment and pathogen attack. We tried to detect the *OsBIPP2C2c* transcript in rice by RT-PCR using a pair of specific primers located in the flanking sequences of the alternative splicing sites. However, we were unable to detect the *OsBIPP2C2c* transcript in healthy rice seedlings and its accumulation, like the *OsBIPP2C2a* transcript, in response to BTH treatment and pathogen infection (data not shown). Therefore, it is likely that *OsBIPP2C2a* transcript is the prevalent transcript of the *OsBIPP2C2* gene. However, the biological role of the other two alternatively spliced transcripts of the *OsBIPP2C2* gene or the biochemical activity of their products, if any, remain to be explored further.

Benzothiadiazole has been previously shown to be an effective inducer of induces defense response in rice against blast, bacterial leaf blight and sheath blight diseases (Gorlach et al. 1996; Song et al. 2001; Ge et al. 1999; Zhang et al. 2003). In this study, we observed that treatment of rice seedlings with BTH induced expression of *OsBIPP2C2*

gene, resulting in accumulation of the *OsBIPP2C2a* transcript. This is supported from the other side by the fact that the two differentially expressed clones that contain partial cDNA of *OsBIPP2C2* gene were isolated from BTH-treated rice seedlings. Moreover, accumulation of *OsBIPP2C2a* transcript was also induced by *M. grisea*. Thus, it is likely that *OsBIPP2C2* gene is responsive and inducible to chemical inducers of defense response, including BTH, and pathogen infection. *OsBIPP2C1*, a rice PP2C gene that we identified previously, and the Arabidopsis *AP2C1* were also shown to be up-regulated by different biotic and abiotic stress factors, including pathogen infection (Hu et al. 2006; Schweighofer et al. 2007). Another feature of *OsBIPP2C2* expression is that the accumulation kinetics of *OsBIPP2C2a* transcript showed different patterns in the BTH-induced rice plants and the uninduced plants after infection with *M. grisea*, and this pathogen-induced expression of *OsBIPP2C2a* was much earlier and stronger in the BTH-induced rice plants than those in the uninduced plants. It is likely that expression of *OsBIPP2C2* gene, especially the *OsBIPP2C2a* transcript, can be induced during early stage of the disease resistance response in rice against *M. grisea* and thus *OsBIPP2C2a* might be involved in regulation of defense response.

Direct involvement of *OsBIPP2C2a* in disease resistance response was further studied through functional analysis by ectopic overexpression in transgenic tobacco plants. The transgenic tobacco plants overexpressing the *OsBIPP2C2a* transcript of *OsBIPP2C2* gene showed enhanced disease resistance against TMV and *P. parasitica* var. *nicotianae*, leading to a significant reduction in disease severity. This is similar to our previous observation that overexpression of *OsBIPP2C1* in transgenic tobacco resulted in enhanced disease resistance (Hu et al. 2006). Importantly, coincided with the enhanced disease resistance in *OsBIPP2C2a*-overexpressing plants is the constitutive expression of defense-related genes including *PR-1* and *Sar8.2b*. Such relationship between enhanced disease resistance and elevated levels of defense gene expression has been already documented previously for some defense-related transcriptional factors, e.g. constitutive expression of genes encoding for rice *OsBIHD1* or *OsBIERF3* in tobacco and *AtWRKY70* or *AtWRKY18* in Arabidopsis resulted in increased disease resistance and elevated levels of *PR* gene expression (Luo et al. 2005b; Cao et al. 2006; Chen and Chen 2002; Li et al. 2004). The coincidence of the enhanced disease resistance with elevated *PR* gene expression suggests that *OsBIPP2C2a* in transgenic tobacco plants acts as a positive regulator of defense response by modulating *PR* gene expression. This is different from the Arabidopsis *AP2C1*, which is a negative modulator of innate immunity because plants with increased *AP2C1* levels compromised disease resistance

against *B. cinerea* and expression of *PDF1.2* gene (Schweighofer et al. 2007). Taken together, it is likely that plant PP2Cs have much diverse biological functions and relatively narrower substrate specificity than those in other organisms, probably due to the large number of PP2Cs in plants as compared with the numbers in yeast and human (Kerk et al. 2002; Stark 1996; Cheng et al. 2000).

In conclusion, our studies present evidence supporting that OsBIPP2C2a plays an important role in disease resistance; however, the mechanism by which OsBIPP2C2a regulates defense response remains to be explored. Further functional genomics studies with knockout/knockdown mutants and/or overexpression in transgenic rice are underway and results from these studies will provide new insights into the biological function and the mechanism of OsBIPP2C2 in rice.

Acknowledgments We are grateful to Dr. Xueping Zhou, Institute of Biotechnology, Zhejiang University for TMV samples and Mr. Rongyao Chai (Zhejiang Academy of Agricultural Science) for the *Magnaporthe grisea* isolate 85-14B1. This study was supported by projects from National Natural Science Foundation of China (grants no. 30771399), National 863 Project (2007AA10Z140) and the Ph.D. Programs Foundation of Ministry of Education of China (No. 20070335111).

References

- Cao Y, Wu Y, Zheng Z, Song F (2006) Overexpression of the rice EREBP-like gene *OsBIERF3* enhances disease resistance and salt tolerance in transgenic tobacco. *Physiol Mol Plant Pathol* 67:202–211
- Carrasco JL, Ancillo G, Mayda E, Vera P (2003) A novel transcription factor involved in plant defense endowed with protein phosphatase activity. *EMBO J* 22:3376–3384
- Carrasco JL, Castello MJ, Vera P (2006) 14-3-3 mediates transcriptional regulation by modulating nucleocytoplasmic shuttling of tobacco DNA-binding protein phosphatase-1. *J Biol Chem* 281:22875–22881
- Chak RK, Thomas TL, Quatrano RS, Rock CD (2000) The genes *ABI1* and *ABI2* are involved in abscisic acid- and drought-inducible expression of the *Daucus carota* L. Dc3 promoter in guard cells of transgenic *Arabidopsis thaliana* (L.) Heynh. *Planta* 210:875–883
- Chen C, Chen Z (2002) Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen-induced *Arabidopsis* transcription factor. *Plant Physiol* 129:706–716
- Cheng A, Kaldis P, Solomon MJ (2000) Dephosphorylation of human cyclin-dependent kinases by protein phosphatase type 2C alpha and beta isoforms. *J Biol Chem* 275:34744–34749
- Das AK, Helps NR, Cohen PT, Barford D (1996) Crystal structure of the protein serine/threonine phosphatase 2C at 2.0 Å resolution. *EMBO J* 15:6798–6809
- Ge X, Song F, Zheng Z (1999) Systemic acquired resistance to *Magnaporthe grisea* in rice induced by BTH. *Acta Agri Zhejiangensis* 11:311–314
- Gonzalez-Garcia MP, Rodriguez D, Nicolas C, Rodriguez PL, Nicolas G, Lorenzo O (2003) Negative regulation of abscisic acid signaling by the *Fagus sylvatica* FsPP2C1 plays a role in seed dormancy regulation and promotion of seed germination. *Plant Physiol* 133:135–144
- Gorlach J, Volrath S, Knauf-Beiter G, Hengy G, Beckhove U, Kogel K-H, Oostendorp M, Staub T, Ward E, Kessmann H, Ryals J (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell* 8:629–643
- Gosti F, Beaudoin N, Serizet C, Webb AA, Vartanian N, Giraudat J (1999) ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. *Plant Cell* 11:1897–1910
- Hu X, Song F, Zheng Z (2006) Molecular characterization and expression analysis of a rice protein phosphatase 2C gene, *OsBIPP2C1*, and overexpression in transgenic tobacco conferred enhanced disease resistance and abiotic tolerance. *Physiol Plant* 127:225–236
- Kapranov P, Jensen TJ, Poulsen C, de Bruijn FJ, Szczyglowski K (1999) A protein phosphatase 2C gene, *LjNPP2C1*, from *Lotus japonicus* induced during root nodule development. *Proc Natl Acad Sci USA* 96:1738–1743
- Kerk D, Bulgrien J, Smith DW, Barsam B, Veretnik S, Gribskov M (2002) The complement of protein phosphatase catalytic subunits encoded in the genome of *Arabidopsis*. *Plant Physiol* 129:908–925
- Kuhn JM, Boisson-Dernier A, Dizon MB, Maktabi MH, Schroeder JI (2006) The protein phosphatase AtPP2CA negatively regulates abscisic acid signal transduction in *Arabidopsis*, and effects of abh1 on AtPP2CA mRNA. *Plant Physiol* 140:127–139
- Kuromori T, Yamamoto M (1994) Cloning of cDNAs from *Arabidopsis thaliana* that encode putative protein phosphatase 2C and a human Dr1-like protein by transformation of a fission yeast mutant. *Nucleic Acids Res* 22:5296–5301
- Leung J, Bouvier-Durand M, Morris PC, Guerrier D, Chefdor F, Giraudat J (1994) *Arabidopsis* ABA response gene ABI1: features of a calcium-modulated protein phosphatase. *Science* 264:1448–1452
- Leung J, Merlot S, Giraudat J (1997) The *Arabidopsis* *ABSCISIC ACID- INSENSITIVE2* (*ABI2*) and *ABI1* genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. *Plant Cell* 9:759–771
- Li J, Brader G, Palva ET (2004) The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *Plant Cell* 16:319–331
- Lorenzo O, Rodriguez D, Nicolas G, Rodriguez PL, Nicolas C (2001) A new protein phosphatase 2C (FsPP2C1) induced by abscisic acid is specifically expressed in dormant beechnut seeds. *Plant Physiol* 125:1949–1956
- Lorenzo O, Nicolas C, Nicolas G, Rodriguez D (2002) Molecular cloning of a functional protein phosphatase 2C (*FsPP2C2*) with unusual features and synergistically up-regulated by ABA and calcium in dormant seeds of *Fagus sylvatica*. *Physiol Plant* 114:482–490
- Luo H, Song F, Goodman RM, Zheng Z (2005a) Up-regulation of *OsBIHDI*, a rice gene encoding BELL homeodomain transcriptional factor, in disease resistance responses. *Plant Biol* 7:459–468
- Luo H, Song F, Zheng Z (2005b) Overexpression in transgenic tobacco reveals different roles for the rice homeodomain gene *OsBIHDI* in biotic and abiotic stress responses. *J Exp Bot* 56:2673–2682
- Merlot S, Gosti F, Guerrier D, Vavasseur A, Giraudat J (2001) The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. *Plant J* 25:295–303
- Meskiene I, Bogre L, Glaser W, Balog J, Brandstotter M, Zwerger K, Ammerer G, Hirt H (1998) MP2C, a plant protein phosphatase 2C, functions as a negative regulator of mitogen-activated

- protein kinase pathways in yeast and plants. *Proc Natl Acad Sci USA* 95:1938–1943
- Meyer K, Leube MP, Grill E (1994) A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. *Science* 264:1452–1455
- Nishimura N, Yoshida T, Kitahata N, Asami T, Shinozaki K, Hirayama T (2007) ABA-Hypersensitive Germination1 encodes a protein phosphatase 2C, an essential component of abscisic acid signaling in *Arabidopsis* seed. *Plant J* 50:935–949
- Pei ZM, Kuchitsu K, Ward JM, Schwarz M, Schroeder JI (1997) Differential abscisic acid regulation of guard cell slow anion channels in *Arabidopsis* wild-type and *abi1* and *abi2* mutants. *Plant Cell* 9:409–423
- Reyes D, Rodriguez D, Gonzalez-Garcia MP, Lorenzo O, Nicolas G, Garcia-Martinez JL, Nicolas C (2006) Overexpression of a protein phosphatase 2C from beech seeds in *Arabidopsis* shows phenotypes related to abscisic acid responses and gibberellin biosynthesis. *Plant Physiol* 141:1414–1424
- Rodriguez PL (1998) Protein phosphatase 2C (PP2C) function in higher plants. *Plant Mol Biol* 38:919–927
- Rodriguez PL, Leube MP, Grill E (1998) Molecular cloning in *Arabidopsis thaliana* of a new protein phosphatase 2C (PP2C) with homology to ABI1 and ABI2. *Plant Mol Biol* 38:879–883
- Saez A, Apostolova N, Gonzalez-Guzman M, Gonzalez-Garcia MP, Nicolas C, Lorenzo O, Rodriguez PL (2004) Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C HAB1 reveal its role as a negative regulator of abscisic acid signalling. *Plant J* 37:354–369
- Saez A, Robert N, Maktabi MH, Schroeder JI, Serrano R, Rodriguez PL (2006) Enhancement of abscisic acid sensitivity and reduction of water consumption in *Arabidopsis* by combined inactivation of the protein phosphatases type 2C ABI1 and HAB1. *Plant Physiol* 141:1389–1399
- Sambrook J, Russell DW (2001) *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Schweighofer A, Hirt H, Meskiene I (2004) Plant PP2C phosphatases: emerging functions in stress signaling. *Trends Plant Sci* 9:236–243
- Schweighofer A, Kazanaviciute V, Scheikl E, Teige M, Doczi R, Hirt H, Schwanninger M, Kant M, Schuurink R, Mauch F, Buchala A, Cardinale F, Meskiene I (2007) The PP2C-type phosphatase AP2C1, which negatively regulates MPK4 and MPK6, modulates innate immunity, jasmonic acid, and ethylene levels in *Arabidopsis*. *Plant Cell* 19:2213–2224
- Shah K, Russinova E, Gadella TW Jr, Willemse J, De Vries SC (2002) The *Arabidopsis* kinase-associated protein phosphatase controls internalization of the somatic embryogenesis receptor kinase 1. *Genes Dev* 16:1707–1720
- Song FM, Goodman RM (2002a) *OsBIMK1*, a rice MAP kinase gene involved in disease resistance responses. *Planta* 215:997–1005
- Song F, Goodman RM (2002b) Cloning and identification of the promoter of the tobacco *Sar8.2b* gene, a gene involved in systemic acquired resistance. *Gene* 290:115–124
- Song F, Ge X, Zheng Z, Xie Y (2001) Benzothiadiazole induces systemic acquired resistance in rice against bacterial leaf blight. *Chin J Rice Sci* 15:323–326
- Song D, Chen J, Song F, Zheng Z (2006) A novel rice MAPK gene, *OsBIMK2*, is involved in disease-resistance responses. *Plant Biol* 8:587–596
- Stark MJ (1996) Yeast protein serine/threonine phosphatases: multiple roles and diverse regulation. *Yeast* 12:1647–1675
- Stone JM, Collinge MA, Smith RD, Horn MA, Walker JC (1994) Interaction of a protein phosphatase with an *Arabidopsis* serine/threonine receptor kinase. *Science* 266:793–795
- Stone JM, Trotochaud AE, Walker JC, Clark SE (1998) Control of meristem development by CLAVATA1 receptor kinase and kinase-associated protein phosphatase interactions. *Plant Physiol* 117:1217–1225
- Tahtiharju S, Palva T (2001) Antisense inhibition of protein phosphatase 2C accelerates cold acclimation in *Arabidopsis thaliana*. *Plant J* 26:461–470
- Vranova E, Langebartels C, van Montagu M, Inze D, van Camp W (2000) Oxidative stress, heat shock and drought differentially affect expression of a tobacco protein phosphatase 2C. *J Exp Bot* 51:1763–1764
- Xiong L, Yang Y (2003) Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. *Plant Cell* 15:745–759
- Yoshida T, Nishimura N, Kitahata N, Kuromori T, Ito T, Asami T, Shinozaki K, Hirayama T (2006) ABA-hypersensitive germination3 encodes a protein phosphatase 2C (*AtPP2CA*) that strongly regulates abscisic acid signaling during germination among *Arabidopsis* protein phosphatase 2Cs. *Plant Physiol* 140:115–126
- Yu LP, Miller AK, Clark SE (2003) *POLTERGEIST* encodes a protein phosphatase 2C that regulates CLAVATA pathways controlling stem cell identity at *Arabidopsis* shoot and flower meristems. *Curr Biol* 13:179–188
- Zhang W, Ge X, Song F, Zheng Z (2003) Systemic acquired resistance of rice against sheath blight disease induced by benzothiadiazole. *Acta Phytologica Sinica* 30:171–176