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ORIGINAL PAPER

The identification of candidate rice genes that confer resistance to the brown planthopper (*Nilaparvata lugens*) through representational difference analysis

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Abstract The development of rice varieties (*Oryza sativa* L.) that are resistant to the brown planthopper (BPH; *Nilaparvata lugens* Stål) is an important objective in current breeding programs. In this study, we generated 132 BC_5F_5 near-isogenic rice lines (NILs) by five backcrosses of *Samgangbyeo*, a BPH resistant *indica* variety carrying the *Bph1* locus, with *Nagdongbyeo*, a BPH susceptible *japonica* variety. To identify genes that confer BPH resistance, we employed representational difference analysis (RDA) to detect transcripts that were exclusively expressed in one of our BPH resistant NIL, *SNBC61*, during insect feeding. The chromosomal mapping of the RDA clones that we subsequently isolated revealed that they are located in close proximity either to known quantitative trait loci or to an introgressed SSR marker from the BPH resistant donor parent *Samgangbyeo*. Genomic DNA gel-blot analysis further revealed that loci of all RDA clones in *SNBC61* correspond to the alleles of *Samgangbyeo*. Most of the RDA clones were found to be exclusively expressed in *SNBC61* and could be assigned to functional groups involved in plant defense. These RDA clones therefore represent candidate defense genes for BPH resistance.

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Introduction

The brown planthopper (BPH), *Nilaparvata lugens* Stål, is one of the most destructive insect pests for rice crops (*Oryza sativa* L.), and susceptible rice varieties often suffer severe yield losses annually from BPH infestations (Khush 1979; Sogawa et al. 2003). The feeding behavior of these insects is highly specific to phloem sucking, as the phloem contains sucrose, potassium, and amino acids as its main constituents (Hayashi and Chino 1990). Such feeding activity causes considerable physiological damage to the rice plants by removing nutrients and disrupting physiological processes, and consequently affects the growth and development of the plant (Sogawa 1982; Watanabe and Kitagawa 2000). Moreover, feeding by large numbers of BPH results in drying of the leaves and wilting of the tillers, which is referred to as a hopper-burn condition. BPH also causes indirect damage by transmitting viruses such as the rice tungro virus and grassy stunt virus, which results in severe disease (Heinrichs 1979). The damage and frequent outbreaks of BPH, along with the hazardous effects of using pesticides to protect rice crops, has now prompted researchers to identify BPH resistant germplasms, utilize these resistant lines in breeding programs, and attempt to isolate resistance genes.

Rice resistance to BPH is recognized as both a major (qualitative) and a minor (quantitative) trait. To date, 19 major BPH resistance genes have been identified in both *indica* varieties and wild rice species (Ishii et al. 1994; Hirabayashi et al. 1998; Huang et al. 2001; Liu et al. 2001;

Yang et al. 2004; Chen et al. 2006; Jena et al. 2006). Quantitative trait loci (QTLs) have been found to confer durable BPH resistance in the varieties IR64 (Alam and Cohen 1998) and Teqing (Xu et al. 2002). It has also been proposed that moderate and/or polygenic resistance to insect pests, including BPH, provides a more durable level of resistance than any single major gene (Heinrichs 1986; Bosque-Pérez and Buddenhagen 1992).

The molecular mechanisms underlying plant resistance to herbivores have been mainly studied by examining interactions between host plants and chewing insects (Moran and Thompson 2001). It has also been shown that both the feeding mode of a particular herbivore, and the degree of plant tissue injury that results from this, exercise considerable influence on the gene expression patterns at the feeding site (Walling 2000). Chewing insects usually cause extensive tissue damage and thus activate wound-signaling pathways in which jasmonic acid (JA) plays an important role (Baldwin and Preston 1999; Rojo et al. 1999). Reduced JA production in the tomato appears to increase susceptibility to herbivores (Howe et al. 1996). Consistently, the expression of genes associated with JA signaling was found to be up-regulated in *Nicotiana attenuata* injured by the herbivore *Manduca sexta* (Hui et al. 2003). Furthermore, it has been reported that an attack by chewing herbivores results in dramatic changes to the gene expression profiles of plants. Examples of this are evident for both photosynthesis-related genes that are significantly down-regulated in response to stress, wounding and pathogens, and genes involved in carbon- and nitrogen-based defense pathways which are up-regulated under these conditions (Hermsmeier et al. 2001).

In contrast to studies of chewing herbivores, very little information is currently available regarding the plants response mechanisms to sucking insects. Although phloem-feeding insects including BPH produce little damage to plant foliage, these minor injuries are still effectively perceived as pathogenic by the defense response pathways of the host plant (Moran et al. 2002). Moreover, the plant defense mechanisms that are activated in response to herbivorous phloem-feeding insects have been found to principally involve the salicylic acid (SA) and the JA signaling pathways. For example, SA- and JA-dependent genes were shown to be induced in sorghum (*Sorghum bicolor*) in response to a greenbug (*Schizaphis graminum*) attack (Zhu-Salzman et al. 2004). A JA-independent pathway may also be involved in such insect defense responses in sorghum as some transcripts that are exclusively activated by this invading greenbug were found to be independent of either the JA- or SA-regulated pathways (Zhu-Salzman et al. 2004). Consistent with this notion that JA-independent pathways are involved in plant defense against phloem-feeding insects, no differences in the gene expression levels of lipoxygenase, a key enzyme in JA synthesis, were observed in a previous study of rice plants infested with BPH (Zhang et al. 2004).

The interaction between rice and BPH has the potential to serve an excellent model system for understanding the genetic basis of plant defense against phloem-feeding insects. To date, a number of BPH-responsive genes have been isolated in rice plants (Ren et al. 2004; Yuan et al. 2004, 2005; Zhang et al. 2004; Wang et al. 2005) and most have been assigned to functional groups for signaling pathways, oxidative stress/apoptosis, wound-response, drought-inducible and pathogen-related proteins. Moreover, the expression levels of many of these genes in insect susceptible varieties of rice were, in most cases, lower than that in resistant varieties. It has also been found that genes involved in macromolecule degradation and plant defense are up-regulated in BPH susceptible rice varieties during an insect attack, whereas those involved in photosynthesis and cell growth are down-regulated, suggesting that leaf senescence is activated in these plants upon damage by insect feeding (Yuan et al. 2005).

To further elucidate the defense mechanisms in rice infested with BPH, we postulated that it would be of great value to identify the genes that were specifically expressed in a resistant variety during insect feeding. In this regard, representational difference analysis (RDA) is a powerful and sensitive tool that can identify genes differentially expressed between two cDNA populations (Hubank and Schatz 1994; Pastorian et al. 2000). Upon completion of the RDA procedure, only the differentially expressed products remain. A similar cDNA subtraction method, suppression subtractive hybridization (SSH), includes a normalization step in the subtraction procedure (Diatchenko et al. 1996). In rice, RDA has been utilized previously to analyze genomic differentiation by isolating transposable elements (Panaud et al. 2002), and to detect alien introgression into the rice genome (Vitte et al. 2003) using genomic DNA. In this study, we employed RDA to identify BPH-responsive genes in a resistant rice variety using cDNAs. The use of the appropriate plant materials and the determination of the optimal subtraction strategy are key factors for success in these experiments. Hence, to facilitate the identification of genes that are unique to the BPH resistant rice variety by RDA, we developed a near-isogenic line (NIL) by crossing a BPH resistant *indica* variety with a susceptible *japonica* variety which was then used as the recurrent parent. Our RDA experiments were performed by comparing this BPH resistant NIL with the susceptible background *japonica* variety. Significantly, the RDA clones that we subsequently isolated mapped to rice chromosomes with known QTLs and harboring other markers associated with BPB resistance. This information regarding our identified clones could therefore be useful for marker-assisted breeding of

BPH resistant varieties and in the cloning of the genes that control BPH resistance.

Materials and methods

Plant materials

Two rice varieties, a BPH resistant line *SNBC61* and a BPH susceptible *japonica* variety *Nagdongbyeo*, were used in this study. The *SNBC61* resistant line was selected from a panel of 132 BC_5F_5 near-isogenic lines (NILs) that had been generated by five backcrosses using *Samgangbyeo* as the donor parent and *Nagdongbyeo* as the recurrent parent. *Samgangbyeo* is a BPH resistant *indica* variety harboring *Bph1*, a major locus of resistance that maps to chromosome 12 (Huang et al. 1997; Lee et al. 2005).

Rice plant growth and BPH feeding experiments

Forty seeds of *SNBC61*, *Nagdongbyeo* and *Samgangbyeo* were grown in plastic pots (25 cm × 45 cm in length), and 3-week-old seedlings were subsequently placed inside clear plastic cages with the insects. Second or third instar nymphs of BPH were maintained in these cages at a density of approximately 10 insects per seedling over a period of 2 days during the feeding experiments.

Genotype analysis of *SNBC61*

Genomic DNA was extracted from the leaves of rice seedlings (Stacey and Isaac 1994). In order to identify the introgression region from a donor parent, the *SNBC61* was genotyped using 336 polymorphic SSR markers covering the 12 rice chromosomes (Supplementary Table A). For PCR, the temperature cycling conditions were 4 min at 94°C, followed by 40 cycles of 94°C for 30 s; 55°C or 60°C for 30 s; and 72°C for 1 min, and a final extension at 72°C for 7 min. The amplification products were separated using either a 4% denatured polyacrylamide gel or a 3% agarose gel and visualized by silver staining or EtBr.

Representational difference analysis

After exposure to BPH insects for 2 days, culms with fully emerged third or fourth leaves were collected for total RNA extraction using RNA pure reagent (GenHunter Co., Nashville, TN, USA; http://www.genhunter.com/) according to the manufacturer's instructions. mRNA was then prepared using the PolyATtract mRNA isolation system (Promega Co., Madison, WI, USA; http://www.promega.com/). Eluted mRNA was precipitated with 3 M sodium acetate (pH 5.2) and isopropanol, followed by centrifugation at 12,000×*g* for 10 min, and cDNAs were systhesized using the cDNA Synthesis System (Roche Co., Basel, Switzerland; http://www.roche.com/l).

RDA was conducted according to the modified procedures described previously by Michael and Andrew (1997) and Pastorian et al. (2000). The oligonucleotides used in our RDA experiements were as follows: R-Dpn 24, 5′AGC ACTCTCCAGCCTCTCACCGCA3′; R-Dpn 12, 5′GATC TGCGGTGA3′; J-Dpn 24, 5′ACCGACGTCGACTATCC ATGAACA3′; J-Dpn 12, 5′GATCTGTTCATG3′; N-Dpn 24, 5′AGGCAACTGTGCTATCCGAGGGAA3′; N-Dpn 12, 5′GATCTTCCCTCG3′. To identify genes unique in the BPH resistant line, cDNA populations were prepared from BPH-fed *SNBC61* and *Nagdongbyeo* and used as the tester and the driver, respectively. Restriction of double strand (ds) cDNAs generated from the tester and the driver was performed with *Dpn*II (New England Biolabs, Co. Ipswich, MA, USA; http://www.neb.com/nebecomm/). Adapters, R-Dpn 24 and R-Dpn 12, were then ligated to these digested cDNA preparations using T_4 DNA ligase (New England Biolabs Co.). Amplicons were subsequently prepared by PCR using Taq DNA polymerase (Promega Co., Madison, WI, USA) and after purification, the R-Dpn adaptors were removed by *Dpn*II digestion, and replaced with J-Dpn adapter for the tester amplicons. After first round hybridizations (tester and driver at a 1:100 ratio), the tester:driver ratio was increased from 1:800 in the second round of hybridization, to 1:400,000 in the third round. The products of the differentially expressed genes were digested with *Dpn*II and ligated with an N-Dpn adapter in the second round of hybridization and with a J-Dpn adaptor in the third round.

Sequence analysis and in silico chromosomal mapping of RDA products

The differentially expressed products from the third round of hybridization in the RDA procedure were purified using the QIAEX II kit (Qiagen, Valencia, CA, USA; http://www1.qiagen.com/) and subcloned with the TOPO TA Cloning Kit for sequencing analysis (Invitrogen, Carlsbad, CA, USA; http://www.invitrogen.com/). The sequence information for the associated markers was obtained from NCBI (http://www.ncbi.nlm.nih.gov) and GRAMENE (http://www.gramene.org) databases.

Genomic DNA gel-blot analysis

Approximately 3 μg of genomic DNA from *Nagdongbyeo*, *Samgangbyeo*, and *SNBC61* were digested with *Eco*RI and *Hin*dIII, respectively, and then subjected to electrophoresis on a 0.8% agarose gel. Hybridization was carried out with [α-^{32}P] dCTP-labeled gene-specific probes according to

standard procedures under high-stringency hybridization conditions (Jeon et al. 2000). The blot was hybridized in a solution containing 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 1% (w/v) BSA, and 7% (w/v) SDS for 20 h at 60°C. After washing, the hybridization signals were recorded with a phosphorimager (Typhoon, Amersham Biosciences). All probes used were prepared by PCR using gene-specific primers designed on the basis of the sequences of each RDA fragment or the longest ORF obtained from homology analysis (Table 1).

Semi-quantitative RT-PCR

Total RNA was isolated from the culms of fully emerged third or fourth leaves of *Nagdongbyeo* and *SNBC61* during the period from 2 h to 8 days after BPH feeding. These isolated RNA preparations were then reverse-transcribed with an oligo-dT primer and a first strand cDNA synthesis kit for RT-PCR (Roche Co., Basel, Switzerland) and amplified with the gene-specific primers (Table 1). *Act1* control primers were used as internal standards for mRNA expression profiling (McElroy et al. 1990; Volkov et al. 2003). For PCR, the amplification program consisted of an initial denaturation at 94°C for 5 min followed by 28–35 cycles of 94°C, for 1 min; 56°C, for 1 min; 72°C, for 1 min, and a final extension at 72°C for 5 min. These experiments were repeated at least three times and all produced similar results. The control primers for *Act1* were 5′GGAACTGG TATGGTCAAGGC3′ and 5′AGTCTCATGGATACCC GCAG3′ (Cho et al. 2006).

Results

Development of the BPH resistant NIL *SNBC61* rice line

A total of 132 NILs, referred to as *SNBC*, were developed by five backcrosses between *Samgangbyeo* as the donor of the *Bph1* locus, and *Nagdongbyeo* as the recurrent parental line. In each generation, we selected a resistant line by bioassay which was then backcrossed with *Nagdongbyeo*. In the BC_5F_5 *SNBC* population, we selected the *SNBC61* line as a representative genotype as it maintained a high level of resistance to BHP biotype 1 in all of the bioassay experiments. After exposure to herbivorous BPH for 15 days, all of the parental *Nagdongbyeo* plants died (Fig. 1), indicating that this variety is highly susceptible to BPH. In contrast, the *SNBC61* genotype conferred a level of BPH resistance similar to *Samgangbyeo*, the donor BPH resistance parent (Fig. 1). The plant growth and development of *SNBC61* was indistinguishable from *Nagdongbyeo*, and indeed the *SNBC61* line appeared to be very similar to *Nagdongbyeo* in most characteristics, including heading date, grain shape, and chemical properties (Lee et al. 2005). These initial data suggested that *SNBC61* is an appropriate BPH resistant NIL for comparison with the susceptible *Nagdongbyeo*.

Our genotyping results indicate that the *SNBC61* line has the donor parent *Samgangbyeo* allele for the eight SSR markers (RM5, RM3061, RM163, RM459, RM1103, RM5609, RM6693, and RM28493) which are located on chromosomes 1, 5, and 12 (Fig. 2) suggesting that 97.6% of

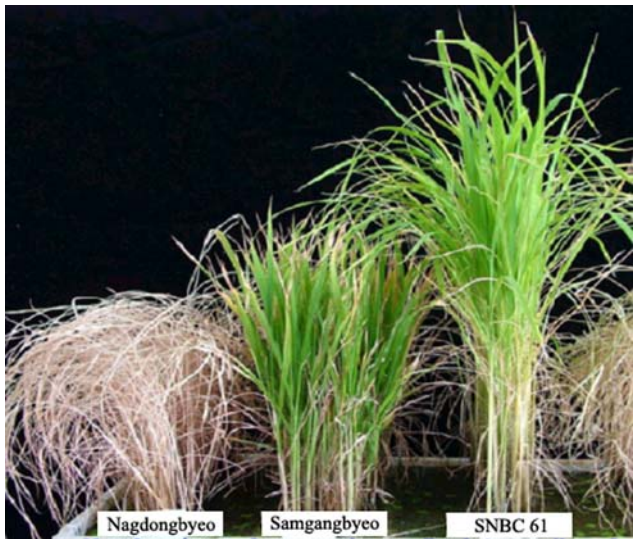

Fig. 1 The levels of resistance to BPH in *Nagdongbyeo*, a susceptible recurrent line (*left*), *Samgangbyeo*, a resistant donor line (*middle*), and in our NIL *SNBC61* variant (*right*). The plants have been exposed to BPH feeding for 15 days

Table 1 Primers used in the PCR analyses

Clone	Forward primer	Reverse primer
OsBphi1	5′GACAAGTCATCTTCTGATATAGGCG3′	5′TAGGATACAACCGTCTGATGGCTC3′
OsBphi8	5′AAATGTGGCTTACTTCCAGGTCC3′	5′CTCTTGGTAGAGCTACTGTTGCG3′
OsBphi11	5′AAGCAACCACCTAAAGGATCAGC3′	5′CAAATCAACCACTAGACATGTCTTCC3′
OsBphi25	5′GTACAGTGTTCTAACACC3′	5′ATGGTAACGTATGGTTTGCTTGC3′
OsBphi66	5′GTGGTGCCAGATTTCTTACACG3′	5′CGTCATCCTTGTATCTTACGGTCC3′
OsBphi71	5′TCAAACGCTAGAAGATGCTATTGG3′	5′GGGAGAACAAACTGATTATTGTGAC3′
OsBphi76	5′AAATCTAAACCTTCCATCAATTGC3′	5′TTACACCAATGTCCATACGATAGG3′
OsBphi96	5′AATCAAGATAATATGGGCCAATGG3′	5′CCATCTTTTAAAGTGTAAGGGTGC3′

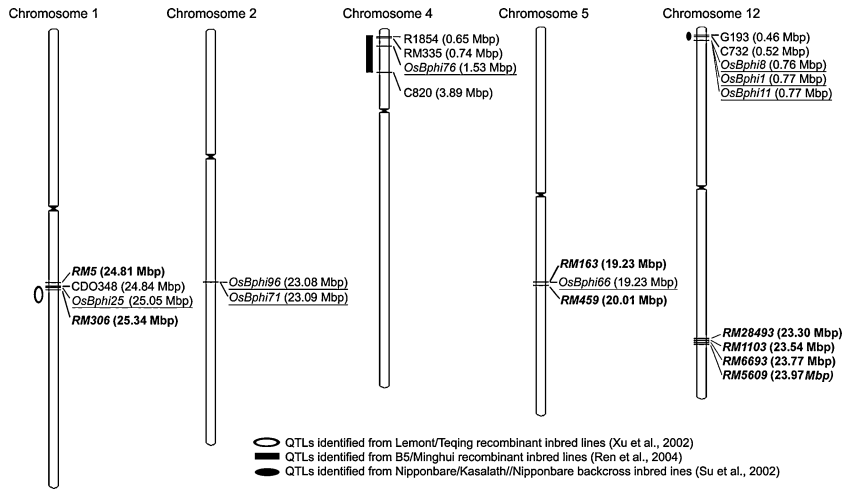

Fig. 2 Chromosomal location of BPH responsive *OsBphi* genes identified in *SNBC61*, and QTLs associated with BPH resistance from previous studies, using the BGI RISe Information System (http://www.rice.genomics.org.cn). *Bold italics* indicate the introgressed SSR markers of the donor parent *Samgangbyeo* in the NIL *SNBC61* plant with a *Nagdongbyeo* background

the genome of *SNBC61* is recovered from the recurrent parent.

RDA of BPH responsive genes

To isolate genes that are specifically up-regulated in the resistant NIL *SNBC61*, RDA was performed using *Dpn*II-digested cDNAs from this clone as the tester and from *Nagdongbyeo* as the driver. In our RDA experiments, the hybridization conditions were modified by increasing the initial ratio of tester and driver to 1:100 to increase the probability for trapping the unique genes expressed in the tester strain, and by decreasing the number of hybridization rounds from four (Pastorian et al. 2000) to three. The RDA products were subcloned following three rounds of hybridization and transformed into *Escherichia coli*.

The cloned RDA cDNA fragments were between ~250 and 350 bp in length, and 200 of these products were randomly selected for further analysis by BLASTN searches from the publicly available databases. Only eight RDA clones were found to be unique genes, and their sequence data were registered in the GenBank database with the accession numbers DV080732 for *OsBphi1* (*Oryza sativa* BPH-inducible gene 1), DV080733 for *OsBphi8*, DV080734 for *OsBphi11*, DV080735 for *OsBphi25*, DV080736 for *OsBphi66*, DV080737 for *OsBphi71*, DV080738 for *OsBphi76*, and DV080739 for *OsBphi96*. Remaining clones were identical to one of the selected eight RDA clones.

Chromosomal localization of the *OsBphi* clones

The sequence of each *OsBphi* clone was aligned with the genomic sequences deposited in the NCBI database. Two fully sequenced rice genomes of the *indica 93-11* (Yu et al. 2002) and the *japonica Nipponbare* (International Rice Genome Sequencing Project 2005) were used in these BLASTN searches. From these analyses, most of the *OsBphi* clones exhibited relatively high sequence similarity to sequences of the *indica* variety rather than the *japonica* variety (Table 2), suggesting that the loci of the isolated *OsBphi* clones most likely originated from the *indica* parental variety *Samgangbyeo*. In particular, two *OsBphi* clones, *OsBphi25* and *OsBphi66*, were found to be localized close to the introgressed SSR markers of the resistant donor parent (Fig. 2). On chromosome 1, *OsBphi*25 mapped to a 25 Mbp region containing introgressed SSR markers RM5 and RM306. Similarly, *OsBphi*66 mapped to chromosome 5 near the introgressed SSR markers RM163 and RM459. These results support the reliability of our RDA experiments.

OsBphi1 shows the highest similarity to a sequence with the accession no. AAAA01005583 on chromosomes 12 in the *93-11* genome (Fig. 2). Notably, *OsBphi1* also showed a high level of homology (less than with the region of *93-11* chromosome 12) with a *Nipponbare* sequence, accession no. BX000497 on chromosome 11, suggesting that the *OsBphi1* locus is likely to be located on different chromosomes in the *japonica* and *indica* varieties. The chromosomal localization of *OsBphi71* in the *indica* genome was also found to differ from that in the *japonica*. In contrast, the *OsBphi8* and *OsBphi96* clones did not match any regions of the *japonica Nipponbare* genome, suggesting the absence of both genes in this variety and in the *japonica* variety *Nagdongbyeo*. The remaining four RDA products, *OsBphi11*, *OsBphi25*, *OsBphi66*, and *OsBphi76*, appeared to map to the same locus in the *japonica* and *indica* genomes. The loci for the RDA clones *OsBphi1*, *OsBphi8*, *OsBphi11*, *OsBphi25*, and *OsBphi76*, were found to be in close proximity to previously identified BPH resistance loci (Fig. 2). In addition, two BPH responsive genes *OsBphi*71 and *OsBphi*96 and three BPH responsive genes *OsBphi1*, *OsBphi8*, and *OsBphi11*were found to be clustered on chromosomes 2 and 12, respectively (Fig. 2, Table 2).

Table 2 BLASTN homology analysis of the cDNAs isolated from the *SNBC61* rice line

Clone	Length (bp)	*Indica*			*Japonica*		
		Accession no.	Expect	Ch.	Accession no.	Expect	Ch.
OsBphi1	209	AAAA01005583	1e-103	12	BX000497	8e-39	11
OsBphi8	242	AAAA01005583	1e-120	12	–	–	–
OsBphi11	265	AAAA01005583	1e-172	12	BX000505	1e-169	12
OsBphi25	238	AAAA01000835	1e-119	1	AP002844	2e-79	1
OsBphi66	212	AAAA01009771	8e-45	5	AC132492	1e-19	5
OsBphi71	303	AAAA01001070	2e-79	2	AC098835	1e-16	5
OsBphi76	258	AAAA01015698	1e-162	4	BX842608	2e-71	4
OsBphi96	274	AAAA01001070	1e-122	2	–	–	–

Amino acid sequence analysis of the *OsBphi* clones

To predict a putative function for each *OsBphi* clone, we identified proteins that are homologous to the likely products of each of these BPH responsive RDA genes. Sequences from our cloned RDA products were analyzed against the protein databases using both BLASTX and BLASTP searches (Table 3). In this analysis, *OsBphi11* exhibited a 46% sequence identity at the amino acid level with a hypothetical putative protein carrying an ankyrin domain of rice (accession no. AK603302). *OsBphi25* was found to be perfectly matched to the *Arabidopsis* zinc finger (C3HC4-type RING finger)-like protein (accession no. BAD52533.1). *OsBphi66* showed 89% amino acid identity with a putative endo-1,3;1,4-beta-D-glucanase (accession no. AAU10802.1) of the *93-11 indica* rice genome. *OsBphi76* shared a 55% amino acid sequence homology with the rice putative subtilisin-like serine proteinase (accession no. BAD29425.1). *OsBphi96* exhibited a 48% amino acid identity with a rice HGWP repeat-containing protein (accession no. BAD36117.1). *OsBphi1*, *OsBphi8*, and *OsBphi71* did not show any significant homology with known protein sequences in the databases.

Characterization of *OsBphi* genomic regions in *SNBC61*

To determine whether the *OsBphi* genomic regions in the BPH resistant *SNBC61* correspond to those of the BPH resistant donor *Samgangbyeo*, we carried out genomic DNA gel-blot experiments using *OsBphi* gene-specific probes (Fig. 3). The hybridization patterns indicated that the genomic regions for all *OsBphis* in *SNBC61* are identical to those in *Samgangbyeo*, but markedly different from those in the BPH susceptible variety *Nagdongbyeo*. Notably, each *OsBphi*, except for *OsBphi25* and *OsBphi76*, exhibited the dominant hybridizing bands in the BPH resistant genomes of *SNBC61* and *Samgangbyeo*. For each *OsBphi* we were able to amplify the identical PCR products from *SNBC61* and *Samgangbyeo* genomic DNAs using gene-specific primers, but failed to produce PCR products in *Nagdongbyeo*, except for *OsBphi25* (data not shown). For the *OsBphi25* genomic region, the PCR product in

Table 3 Protein sequence analysis of cloned cDNAs isolated from *SNBC61*

Clone	Longest homologous ORF in rice		Homologous protein in plant species			
	Accession no.	Expect	Putative function (accession no.)	Identity	Expect	Organism
OsBphi1	CB627818.1	3e-101	–	–	–	–
OsBphi8	CB634565.1	7e-56	–	–	–	–
OsBphi11	AK063302	5e-72	Hypothetical protein having ankyrin domain (AK063302)	46%	5e-72	*O. sativa* (*Japonica*)
OsBphi25	CB622502.1	2e-117	Zinc finger protein-like (BAD52533.1)	100%	3e-64	*A. thaliana*
OsBphi66	CB627994.1	5e-103	Putative endo-1,3;1,4-beta-D-glucanase (AAU10802.1)	89%	4e-113	*O. sativa* (*Japonica*)
OsBphi71	BI305430.1	3e-34	–	–	–	–
OsBphi76	BM420938.1	3e-93	Putative subtilisin-like serine proteinase (BAD29425.1)	55%	1e-10	*O. sativa* (*Japonica*)
OsBphi96	BAD36117.1	3e-15	HGWP repeat containing protein like (BAD36117.1)	48%	3e-15	*O. sativa* (*Japonica*)

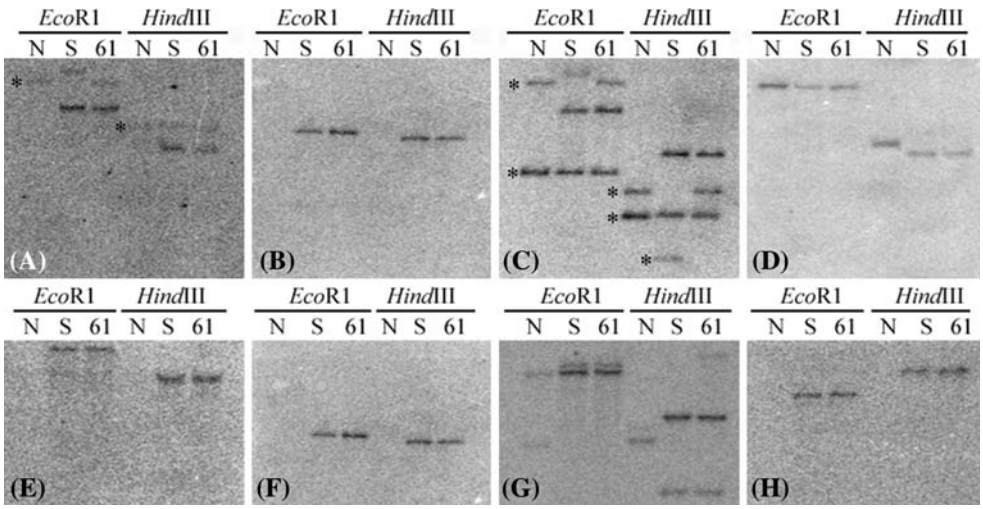

Fig. 3 Genomic DNA gel-blot analysis of *Nagdongbyeo* (N), *Samgangbyeo* (S) and *SNBC61* (61). DNAs digested with *Eco*RI and *Hind*III were electrophoresed, blotted and hybridized with the *OsBphi*-specific probes *OsBphi1* (**a**), *OsBphi8* (**b**), *OsBphi11* (**c**), *OsBphi25* (**d**), *OsBphi66* (**e**), *OsBphi71* (**f**), *OsBphi76* (**g**), *OsBphi96* (**h**), respectively, as probes. Non-specific signals are indicated by *asterisks* (*)

SNBC61 and *Samgangbyeo* (1417 bp) differed in length from that of *Nagdongbyeo* (1350 bp) (data not shown). In the hybridization experiments with *OsBphi1*- and *OsBphi11*-specific probes, non-specific signals were observed, probably due to cross-hybridization with *OsBphi1* and *OsBphi11* homologous sequences (Fig. 3a, c). These results suggest that the *OsBphi* genomic regions in *SNBC61* are identical to the alleles of the resistant parental line *Samgangbyeo*.

Expression profiling of *OsBphi* clones during BPH feeding

Our RT-PCR data showed that the *OsBphi1*, *OsBphi8*, *OsBphi11*, *OsBphi66*, *OsBphi71*, *OsBphi76*, *OsBphi96* clones are mainly up-regulated in the BPH resistant *SNBC61* line with maximum expression levels evident at 4 or 8 days after BPH feeding (Fig. 4). In contrast, these *OsBphi* genes did not respond to BPH feeding in the susceptible *Nagdongbyeo* variety. The *OsBphi25* expression was found to be slightly increased after BPH feeding in *Nagdongbyeo*, indicating that unlike other RDA clones this gene is up-regulated in response to BPH feeding in the susceptible variety. In *SNBC61*, the expression of *OsBphi25* was high in the control plants and increased during BPH feeding. Together with genomic DNA gel-blot analysis, the gene expression experiment suggests that the eight *OsBphi* genes may play an important role in the BPH resistance of *SNBC61*.

Discussion

There is accumulating evidence that a gene-for-gene type of plant defense against sucking insects exists in a number of plant species (Kaloshian et al. 1995; Brotman et al. 2002; Kaloshian 2004). For example, the tomato *Mi-1* gene

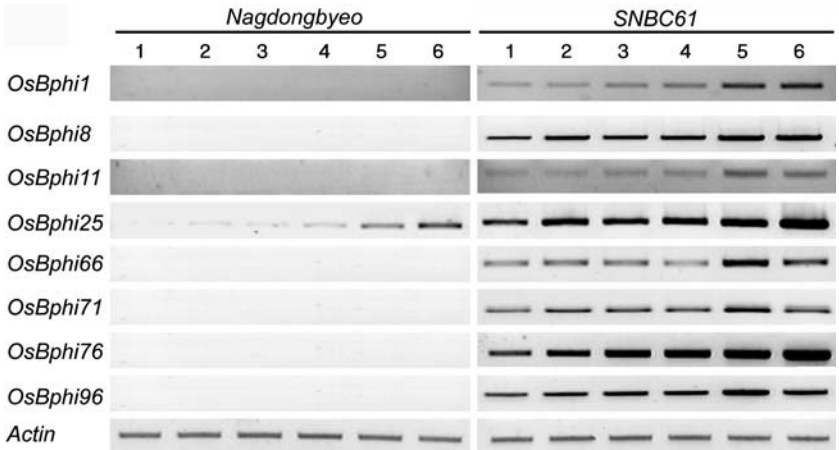

Fig. 4 RT-PCR analysis of the *OsBphi* genes in both the resistant and susceptible rice varieties in response to BPH feeding. Total RNA was extracted from rice seedlings after different BPH feeding time periods. *Lane 1* control, *Lane 2* 2 h, *Lane 3* 1 day, *Lane 4* 2 days, *Lane 5* 4 days, *Lane 6* 8 days after BPH feeding. The number of reaction cycles for each cDNA were as follows: *OsBphi1* 25 cycles, *OsBphi8* 32 cycles, *OsBphi11* 25 cycles, *OsBphi25* 35 cycles, *OsBphi66* 35 cycles, *OsBphi71* 35 cycles, *OsBphi76* 32 cycles, *OsBphi96* 35 cycles, and *Actin* 28 cycles. Primers for the rice *actin1* gene were used as the internal control reference

is known to confer resistance to potato aphid (*Macrosiphum euphorbiae*), whitefly (*Bemisia tabaci*), and root-knot nematodes (*Meloidogyne spp.*) (Kaloshian et al. 1995; Kaloshian 2004). Upon recognition of the invading pest, activation of plant defense is accompanied by an array of transcriptional reprogramming of pathogen responsive genes such as defense-related genes. In this study, we have described the isolation of a number of putative BPH responsive genes in a BPH resistant rice variety.

Identification of *OsBphi* genes by RDA

To better understand the molecular mechanisms underlying the host plant defense to BPH, we employed RDA to analyze cDNAs in a BPH resistant NIL *SNBC61* and its associated susceptible recurrent variety *Nagdongbyeo*. We subsequently identified seven *OsBphi* genes, *OsBphi1*, *OsBphi8*, *OsBphi11*, *OsBphi66*, *OsBphi71*, *OsBphi76*, and *OsBphi96*, which appeared to be present specifically in the genome of *SNBC61*. These genes were all found to be specifically responsive to the resistant rice line *SNBC61*. Further examination based on the sequence analysis indicated that the *OsBphi* genes more closely resemble the *indica* variety which is consistent with the fact that the BPH resistance phenotype in *SNBC61* is derived from the BPH resistant *indica* variety, *Samgangbyeo*.

Association between the chromosomal localization of our *OsBphi* clones and known BPH-resistant loci

The BPH resistant donor variety *Samgangbyeo* used in our study is known to carry the major locus *Bph1*, which confers resistance to BPH feeding (Lee et al. 2005). The quantitative resistance to BPH reported in the variety *IR64* is believed to be due to the presence of minor loci (QTLs) in addition to the *Bph1* locus (Khush 1989; Cohen et al. 1997; Huang et al. 1997). Previous studies using marker-based genetic analyses of mapping populations have also identified a number of major loci and QTLs that are associated with various resistance mechanisms of resistance to BPH (Huang et al. 1997; Alam and Cohen 1998; Su et al. 2002; Xu et al. 2002; Ramalingam et al. 2003; Ren et al. 2004; Yang et al. 2004; Chen et al. 2006; Jena et al. 2006).

In this study, we compared the predicted chromosomal locations for our cloned RDA amplicons with previously identified BPH resistant loci (Fig. 2). The location of *OsBphi25* appeared to be in close proximity to a known QTL for damage score in response to BPH that was identified from the *Lemont/Teqing* recombinant inbred lines (RILs) (Xu et al. 2002). On chromosome 4, *OsBphi76* mapped within a 1.53 Mbp region associated with a BPH resistance QTL located between R1854 and C820 from the *B5/Minghui* RILs (Ren et al. 2004). Furthermore, the three *OsBphi* loci, *OsBphi1*, *OsBphi8*, and *OsBphi11*, were all found to map within the region from 0.76 to 0.77 Mbp on chromosome 12, which is closely linked to a QTL identified from the *Nipponbare/Kasalath//Nipponbare* backcross inbred lines (Su et al. 2002). These findings raise the possibility that some of the identified *OsBphi* loci may indeed be allelic to known QTLs, but none of these fragments is closely linked to the *Bph1* locus on the long arm of chromosome 12. Hence, the chromosome locations for the remaining three *OsBphis* may be new minor QTLs for BPH resistance.

Putative functions of the *OsBphi* genes

OsBphi25 expression in response to BPH feeding was observed to be highly increased at 2 h in *SNBC61* but only marginally so in *Nagdongbyeo*. The sequence of *OsBphi25*, which encodes a putative zinc finger RING domain protein, is identical to OSIIEa09E16, an EST clone identified from a blast inoculated cDNA library (accession no. CB622502.1; Jantasuriyarat et al. 2005). Previous database searches have identified a total of 469 predicted RING domain-containing proteins in *Arabidopsis* (Stone et al. 2005), the majority of which are active in in vitro ubiquitination assays, suggesting that they function as ubiquitin E3 ligases. It has also been suggested that the presence of such a large and diverse number of RING domain-containing proteins enables target-specific proteolysis to occur in response to complex and significant stress conditions (Stone et al. 2005). Recent discoveries also suggest that ubiquitination may play an important role in plant disease resistance (Devoto et al. 2003). Moreover, although no ubiquitin ligase targets that are associated with disease resistance have still been identified in plants, it is evident that the protein modification systems regulate plant defense responses against pathogens.

OsBphi66 is predicted to encode a putative endo-1,3;1,4-ß-D-glucanase which has been reported to play a role in plant development, whereas 1,3-glucanase functions in both plant defense and development (Hoson 1993; Romero et al. 1998). An endo-1,3;1,4-ß-D-glucanase was similarly up-regulated in wheat plants infested with Hessian fly larvae (Sardesai et al. 2005). During seed germination, 1,3;1,4-ß-D-glucanase is secreted into the endosperm where it degrades the cell walls and facilitates the entry of other hydrolases that then mobilize stored reserve materials (Briggs 1992). The production of the 1,3;1,4-ß-D-glucanase enzyme and the breakdown of 1,3;1,4-ß-D-glucans are also prominent features of the cell walls in growing tissues throughout the cereal plant species (Inouhe et al. 1997). The predicted amino acid sequence of the *OsBphi66* clone in the present study has diverged from the 1,3;1,4-ß-D-glucanase genes previously reported in rice (Romero et al. 1998), suggesting that it may have a distinct role during a BPH attack in the resistant plants. The corresponding EST

clones (accession nos. CB627818.1 and CB634565.1) for *OsBphi1* and *OsBphi8*, which encode proteins of unknown function, were also present in a blast inoculated cDNA library (Jantasuriyarat et al. 2005). These findings suggest that these four BPH defense associated genes (*OsBphi1*, *OsBphi8*, *OsBphi25*, and *OsBphi66*) are probably involved in a general resistance response.

OsBphi11 is predicted to encode a hypothetical protein with an ankyrin domain. Ankyrin repeats are present in a great variety of proteins in eukaryotes, prokaryotes and some viruses, and function in both disease resistance and antioxidation metabolism through protein–protein interactions (Cao et al. 1997; Yan et al. 2002; Becerra et al. 2004; Keyan et al. 2005). The *Arabidopsis NPR1* gene encoding an ankyrin repeat-containing protein is also known to control the onset of systemic acquired resistance (SAR) to a broad spectrum of pathogens after a primary exposure to their avirulent counterparts (Cao et al. 1997). A gene encoding ankyrin protein (accession no. DR831511) was similarly up-regulated in a strong resistance sorghum line infested with greenbug (*S. graminum*) (Park et al. 2005). Therefore, it is most probable that *OsBphi11* also functions during the molecular interactions between rice plants and BPH insects.

The *OsBphi71* and *OsBphi96* clones exhibit sequence similarity to a novel cDNA clone from *indica* rice that was found to be induced during drought stress (accession no. BI305430.1) and to an HGWP repeat containing protein (BAD36117.1), respectively. The short conserved HGWP motif repeat is found in a number of rice proteins but the function of this group of genes is largely unknown. Our data suggest, however, that this domain may function in the defense response to invading insects. *OsBphi76* encodes a putative subtilisin-like serine proteinase (subtilase), and the first serine protease to be identified in rice, RSP1, was found to be expressed in the seeds and shoots of seedlings and function during specific proteolytic events (Yamagata et al. 2000). Although subtilisin-like serine proteinases are known to be widespread and possess diverse functions, their physiological functions remain unclear. Our current findings suggest that *OsBphi76* plays a specific role in BPH resistance which is the first such indication for this group of proteins.

Many genes isolated from BPH-fed rice plants in other studies have been observed to be expressed in both resistant and susceptible varieties during BPH feeding (Zhang et al. 2004; Wang et al. 2005; Yuan et al. 2005). In contrast, seven *OsBphi* genes identified in this study appeared to be unique only to the BPH resistant line *SNBC61* and to be up-regulated during BPH feeding. Notably, the seven *OsBphi* genes also appeared to be considerably expressed in *SNBC61* plants without BPH treatment. Therefore, the constitutive function of these *OsBphis* might be essential for resistance to BPH. Further experiments, including the production of rice plants with *OsBphi* cDNAs, are thus needed to more precisely elucidate the roles of these BPH responsive genes during the host–pathogen interactions. The *OsBphi* clones could therefore be utilized not only as linked markers to isolate QTLs, but also as genetic markers for developing varieties of rice that are more durably resistant to BPH.

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