Australian Journal of Crop Science
3(4):201-206 (2009)
ISSN: 1835-2707

Differential cDNA-AFLP screening of transcripts associated with brown planthopper resistance in rice (*Oryza sativa* L.)

N. S. Gamalath, P.N. Sharma, N. Mori and *C. Nakamura

Laboratory of Plant Genetics, Graduate School of Agricultural Science, Kobe University, Kobe, 657-8501, Japan

**Corresponding author: nakamura@kobe-u.ac.jp*

Abstract

We have previously constructed a pyramid line, in which two major BPH resistance genes, *Bph1* and *Bph2* on the long arm of rice chromosome 12, were introgressed into a susceptible *japonica* cultivar, Tsukushibare. To identify transcripts that are derived from the introgressed chromosomal segments carrying the resistance genes, we analyzed mRNA profiles using amplified fragment length polymorphism-based mRNA fingerprinting (cDNA-AFLP). After this differential screening using 423 primer combinations, 41 polymorphic fragments were selected that were specific either to the pyramid line or Tsukushibare. Among them, 10 fragments expressed only in the pyramid line were cloned and sequenced. According to the genome database, at least two were from the proximity of the introgressed *Bph1* locus. Our results suggest that cDNA-AFLP screening of differentially expressed fragments is a useful means to identify BPH resistance and/or infestation associated genes.

Keywords: *Nilaparvata lugens* Stål; RFLP; transcripts ; cDNA; resistance

Introduction

Rice is the second most important staple food crop cultivated in the world. Both biotic and abiotic stresses cause heavy losses in rice production. Beside diseases caused by microbial pathogens, insect pests are the serious problems in rice cultivation. Among insect pests, brown planthopper (BPH) is most destructive and widely distributed in almost all rice growing areas. Frequent and severe BPH outbreaks were often reported in South, Southeast, and East Asia and Oceania. In Japan, there are numerous records of BPH outbreaks, many covering large areas and some causing severe famine (Miyashita *et al*., 1963; Kishimoto *et al.*, 1968). Both direct feeding of phloem saps and transmitted viral diseases cause considerable yield losses in rice production. At least eighteen natural BPH resistant genes including quantitative trait loci have so far been identified, among which fine linkage maps have been constructed for at least five major resistance genes, *Bph1* (Sharma *et al*., 2003), *Bph2* (Murata *et al*., 1998; Murai *et al.*, 2001), *Bph15* (Yang *et al.,* 2004), *Bph18* (Jena *et al*., 2006) and *bph19* (Chen *et al.,* 2006). However, the molecular mechanism of BPH resistance remains largely unknown. Cloning of these resistant genes is necessary to understand the structure, expression and function of the natural BPH resistant genes and ultimately to understand the BPH resistance mechanism in rice.

Screening based on cDNA-amplified fragment length polymorphism (cDNA-AFLP) has been used as an efficient method for isolation of differentially expressed genes (Bachem *et al*., 1996, 1998; Jones and Harrower, 1998; Fukuda *et al*., 1999; Hartings, 1999; Durrant *et al*., 2000; Qin *et al*., 2000; Cooper, 2001; Milioni *et al*., 2001; Kashkush *et al*., 2002; Simões-Araújo *et al*., 2002). cDNA-AFLP was previously employed to identify the differentially expressed genes in BPH nymphs feeding on rice varieties (Yang *et al.,* 2006). A major objective of the present study was to apply this differential screening method using cDNA constructed from resistance and susceptible rice seedlings under

Table 1. Molecular characterization of cDNA fragments obtained by cDNA-AFLP screening.

Clone.no	Length (bp)	*MseI*/*Eco*RI Adapter	Accession no.	Selected protein similarities	Gene name/ Genomic location	Similarity percentage	E value
Me1602	328	ACC/AAG	AK102391	DNA gyrase subunit B	Os01g0268300 Chromosome 1	98%	2e - 74
Me1605	289	ACC/AGA	AK067787	Small G-protein-like protein (ARH1)	Chromosome 11	97%	1e – 90
Me1607	240	ACC/AGT	AK068943	Integrins alpha chain family protein	Os02g0100700 Chromosome 2	97%	1e - 74
Me1612	251	ACC/ATC	AK104841	Conserved hypothetical protein	Os09g0541700 Chromosome 9	97%	5e - 108
Me1617	331	ACC/GAA	AK110939	Hypothetical protein, F-box protein	Os01g0379400 Chromosome 1	99%	9e - 157
Me2007	485	GAC/AGT	AK066543	Ankyrin repeat containing protein	Os03g0213900 Chromosome 3	98%	5e - 101
Me2014	511	GAC/ACG	AK065775	Disease resistance protein family protein	Os12g0565100 Chromosome 12 23218661p-23218942bp	97%	0e
Me2027	483	GAC/GTT	AK068501	No apical meristem (NAM) protein domain containing protein	Os09g0493700 Chromosome 9	99%	1e - 162
Me2042	459	GAC/TTG	AK104628	1.Conserved hypothetical protein	Os12g0186600 Chromosome 12 4340976bp-4341406	97%	0e
				2.Unidentified	22017852bp-22017566	81%	
Me2044	500	GAC/TTC	AK072557	Histone deacetylase superfamily protein	Os12g0182700 Chromosome 12 4196313bp-4196565	100%	2e - 330

infestation condition for identifying BPH resistance-associated transcripts. Ten putatively resistance-associated transcripts were detected, cloned and sequenced, among which at least two were from the proximity of the *Bph1* locus on the long arm of rice chromosome 12.'

Materials and methods

Plant materials, maintenance and infestation conditions of BPH

A pyramid line #64-6 was used as a resistant source, in which two major BPH resistance genes, *Bph1* and *Bph2*, were pyramided by the marker-assisted selection (Sharma *et al*. 2004). These resistance genes are located 10 cM apart on the long arm of rice chromosome 12. Briefly, to produce the pyramid line, crosses were made between *Bph1Bph1* and *Bph2Bph2* homozygous carriers that were derived from Tsukushibare/Norin-PL3 and Tsukushibare/ Norin-PL4 crosses, respectively. Norin-PL3 and Norin-PL4 are authentic *japonica* introgression lines in which *Bph1* and *Bph2* were introgressed from the *indica* donors Mudgo and IR1154-243, respectively. Tsukushibare is a susceptible *japonica* cultivar. From the previously constructed high-resolution linkage maps for *Bph1 (*Sharma *et al*., 2003), and *Bph2* (Murata *et al*., 1998; Murai *et al.*, 2001), tightly linked markers were selected for marker-assisted pyramiding of these genes.

Brown planthopper (BPH, *Nilaparvata lugens* Stål, Homoptera: Delphacidae) was maintained on susceptible rice cultivar "Nipponbare" in an incubator at 25°C with 80% relative humidity under a 16 h photoperiod. In bioassay for BPH resistance of rice lines, the 2nd and the 3rd instar nymphs were released for infestation at a density of 10-12 nymphs per one-week-old seedling. Resistance or susceptibility was judged during the eight to ten days after infestation according to Sharma *et al*. (2004).

AFLP based mRNA fingerprinting (cDNA-AFLP) analysis

For BPH infestation, seedlings of Tsukushibare and the pyramid line were grown for 15 days after germination under the same conditions as for BPH maintenance. Infestation was performed by feeding 15 to 20 adult BPH per seedling for 6 h. After this infestation period, seedlings were collected and immediately frozen in liquid nitrogen for RNA extraction.

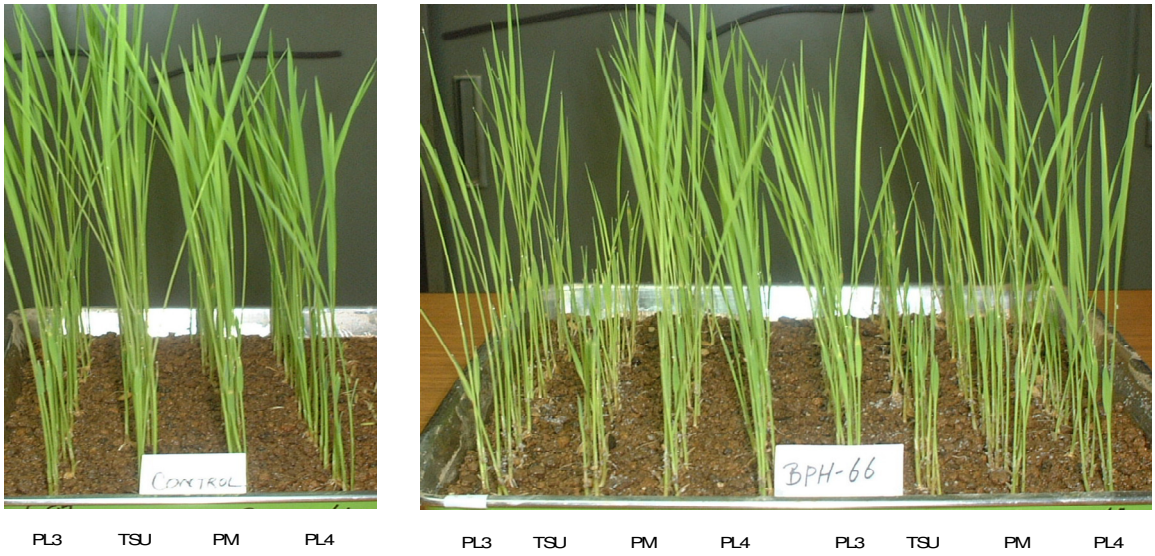

Fig 1. Bioassay for BPH resistance. The single gene introgression lines (PL3 and PL4), the pyramid line (PM) and "Tsukushibare" (TSU) were infested with biotype 1. Left panel shows the non-infested control and right the infested seedlings.

Total RNA was extracted using Cephasol RNA-I (Nacalai Tesque, Kyoto Japan). Poly(A)$^+$RNA was purified by the PolyATtract mRNA purification kit (Promega, USA). The first and second strand cDNA was synthesized using Just cDNA™ Double-Stranded cDNA Synthesis Kit (Stratagene, USA). The template for amplification was prepared according to Vos *et al.* (1995) and Bachem *et al*. (1996, 1998) with minor modifications. The double strand cDNA was first digested with *MseI* and *EcoRI* for 2 h at 37°C and then ligated to *MseI* and *EcoRI* adapters. Pre-amplification was done with 20 cycles at 94°C for 30 s, 56°C for 1 min, 72°C for 1 min using primers corresponding to *MseI* and *EcoRI* adapters. The pre-amplified products were diluted 50-fold and subjected to selective amplification using 38 cycles including 16 touchdown cycles comprising a decrease of annealing temperature from 68°C to 56°C, at 0.7°C per step, which was then continued at 56°C for the remaining 22 cycles. All amplification reactions were performed in a Gene Amp PCR System 9700 Thermocycler. (Applied Biosystems, USA). Sequences of the primers and adapters used for cDNA-AFLP reactions were as follows: *EcoRI* adapter for top strand, 5'-CTC GTA GAC TGC GTA CC-3'; *EcoRI* adapter for bottom strand, 3'-CAT CTG ACG CAT GGT TAA-5'; *MseI* adapter for top strand, 5'-GAC GAT GAG TCC TGA G-3'; *MseI* adapter for bottom strand, 3'- TA CTC AGG ACT CAT-5'; *EcoRI* pre-amplification primer, 5'-GAC TGC GTA CCA ATT C-3'; and *MseI* pre-amplification primer, 5'-GAT GAG TCC TGA GTAAC-3'.

Selectively amplified fragments were separated by 6 % polyacrylamide gel electrophoresis and detected by silver staining. Silver staining was performed according to Silver Sequence™ DNA Sequencing System (Promega, USA).

Isolation and sequence analysis of differentially expressed transcript-derived fragments

Through comparison of cDNA profiles derived from transcripts of Tsukushibare and the pyramid line after BPH infestation, cDNA fragments showing differential patterns of expression, i.e. appearance, disappearance or presence with different intensities, were detected by high-resolution polyacrylamide gel electrophoresis. These transcript-derived fragments were excised and re-amplified with the same primer sets and the same PCR program used for the selective amplification. PCR products thus obtained were further purified by Microspin S-400 column (Amersham, UK) and used for ligation. Among the polymorphic fragments, possibly resistance-associated ones from the pyramid line were ligated into pGEM-T vector (pGEM-T Vector System I, Promega, USA) and transformed to *Escherichia coli* JM109 competent Cells (TaKaRa, Japan). Plasmid extraction was according to alkali-SDS method. The cloned fragments were PCR amplified with universal M13 (forward) and RV (reverse) primers for 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min.

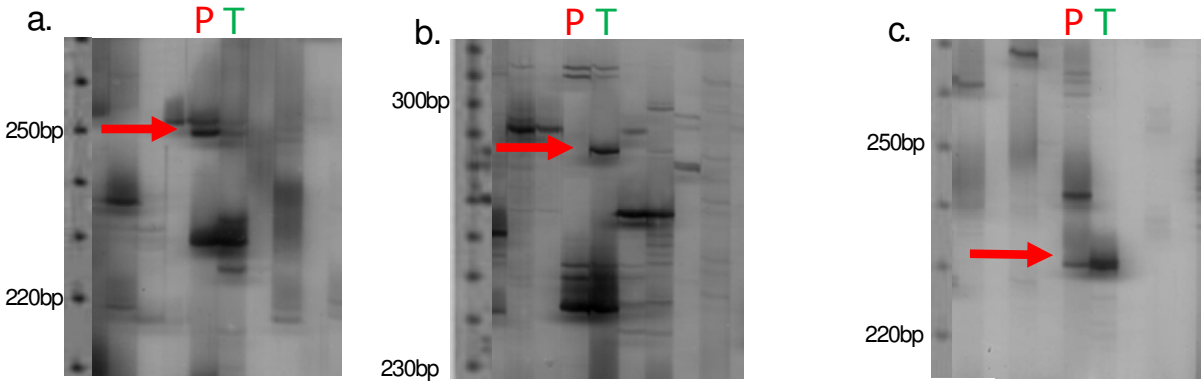

Fig 2. AFLP band patterns on the sequencing gel. On the gel, in each pair of bands, the left hand side bands are from the pyramided line and the right hand side bands are from Tsukushibare. Arrows indicate a pyramid line specific band (**a**), a Tsukushibare specific band (**b**) and a band showing different levels of expression (**c**).

The amplified fragments were sequenced using ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems) by automated fluorescence dye deoxy terminator cycle system. Sequenced fragments were analyzed by DNASIS and homology check was done by BLAST search NCBI.

Sequence characterization of the differentially expressed transcript-derived fragments

The sequences of differentially expressed transcript-derived fragments were compared with those in the GenBank database using BLASTN 2.2.18+ program.

Results and discussion

We first confirmed resistance of the pyramid line by the bioassay in comparison with the single gene introgression lines and a susceptible control, Tsukushibare. The pyramid line showed a comparable level of resistance against BPH infestation as the single gene introgression lines (Fig. 1).

We next employed cDNA-AFLP for identifying BPH resistance associated rice transcripts. We expected that BPH resistance genes introgressed into the pyramid line were either expressed constitutively or expressed after induction by BPH infestation and that BPH infestation could induce expression of some defense-related genes other than the introgressed BPH resistance genes. We therefore adopted the infestation condition for screening differential transcripts, which are possibly associated with BPH resistance and/or infestation from seedlings of the pyramid line in comparison with those of susceptible Tsukushibare.

In cDNA-AFLP, we examined 423 different primer combinations to select transcripts that showed clear amplification in the pyramid line (Fig 2). We then

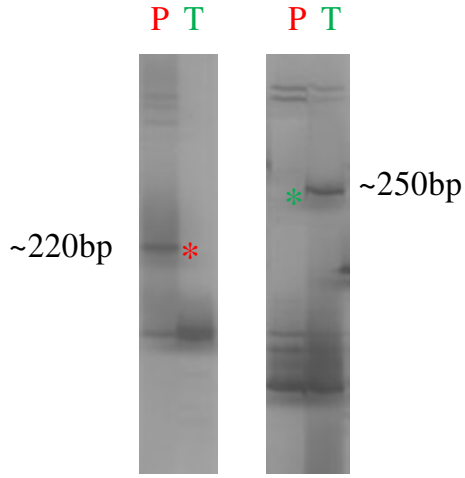

Fig 3. Polymorphic fragments specific to the pyramid line (#64-6) or "Tsukushibare". P and T represent the pyramid line and "Tsukushibare", respectively.

compared the transcript profiles between the pyramid line and Tsukushibare, and obtained 41 polymorphic fragments that were detected only in the pyramid line or Tsukushibare (Fig. 3). Among these differentially expressed transcript-derived fragments, 10 were expressed only in the pyramid line. According to the linkage map constructed for *Bph1* and *Bph2*, these two major BPH resistant genes are located ca. 10 cM apart on the long arm of chromosome 12 (Murai *et al*., 2001; Sharma *et al*., 2003, 2004). Since the genomic background of the pyramid line and Tsukushibare is mostly common except for the introgressed chromosomal segment carrying *Bph1/Bph2* genes and some undetected segments derived from the resistant donors (Sharma *et al*., 2004) we expected that fragments

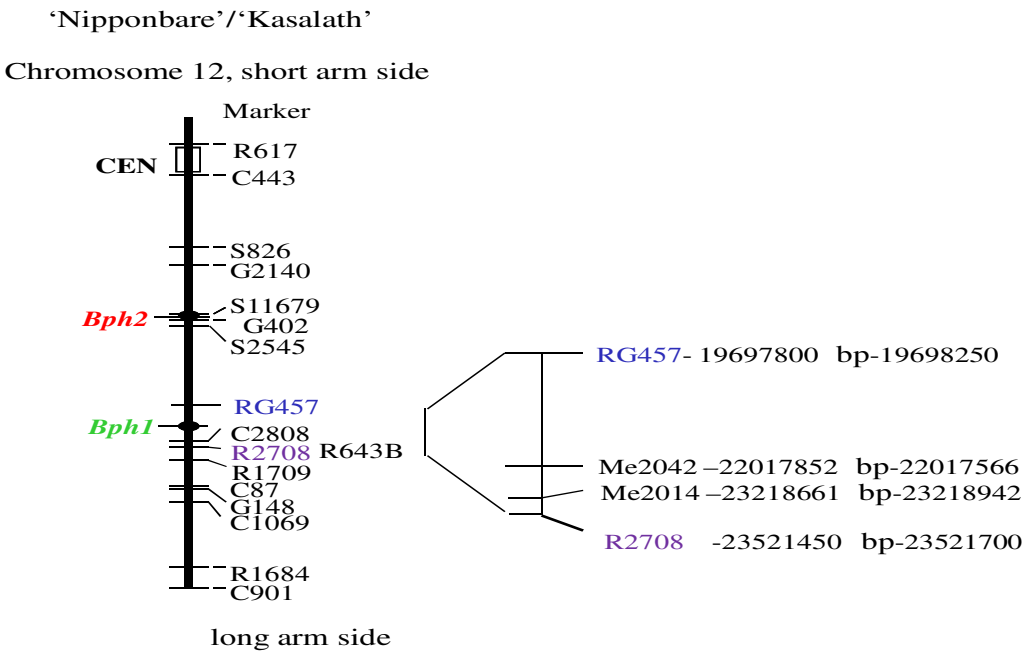

Fig 4. A map showing the chromosomal location of *Bph1* and *Bph2* and the two fragments detected by cDNA-AFLP screening.

unique to the pyramid line in comparison with the control Tsukushibare under the infestation condition should include ones derived from transcripts from the introgressed chromosomal segment. Hence, these 10 differentially expressed fragments were cloned and sequenced. Based on the homology search, these fragments showed high similarity with genes that were reported to be involved in disease resistance, signal transduction, metabolism and regulation of topological links between two DNA strands and also those with unknown function (Table 1). Among the genes corresponding to three of the 10 fragments (clones Me2014, Me2042 and Me2044), two were located in the region covering the two BPH resistant genes in the long arm of chromosome 12 (Fig. 4). It was thus suggested that these two fragments were derived from the introgressed segment on the long arm of rice chromosome 12 at the location close to the *Bph1* locus. Since we did not study transcript profiles under non-infestation conditions, however, if they represent infestation specific transcripts or constitutive ones remains unknown. The clone Me2014 locating at the proximity of the *Bph1* locus encodes disease resistance protein (RPP8-like protein; Recognition of *Peronospora parasitica* 8) in *Arabidopsis thaliana*. After confirmation of the specificity to BPH resistance, these clones can be used as markers in the further work for fine mapping and cloning at least of *Bph1*.

References

Bachem CWB, Oomen RJFJ, Visser RGF (1998) Transcript Imaging with cDNA-AFLP : A Step-by-Step Protocol. Plant Mol Biol Rep 16: 157–173.

Bachem CWB, van der Hoeven RS, de Bruijn SM, Vreugdenhil D, Zabeau M,. Visser RGF (1996) Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: Analysis of gene expression during potato tuber development. Plant J 9:745–753.

Chen WJ, Wang L, Pang XF, Pan QH (2006) Genetic analysis and fine mapping of a rice brown planthopper (*Nilaparvata lugens* Stål) resistance gene *bph19* (t). Mol Genet Genom 275: 321-329

Cooper B (2001) Collateral gene expression changes induced by distinct plant viruses during the hypersensitive resistance reaction in *Chenopodium amaranticolor*. Plant J 26: 339–349.

Durrant WE, Rowland O, Piedras P, Hammond-Kosack KE, Jones JDG (2000) cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. Plant Cell 12: 963–977.

Fukuda T, Kido A, Kajino K, Tsutsumi M, Miyauchi Y, Tsujiuchi T, Konishi Y, Hino O (1999) Cloning of differentially expressed genes in highly and low metastatic rat osteosarcomas by a modified cDNA-AFLP method. Biochem Biophys Res Commun 261:35–40.

Hartings H (1999) High resolution fingerprinting of transcribed genes by means of a modified cDNA-AFLP method. Maydica 44: 179–181.

Jena KK, Jeung JU, Lee JH, Choi HC, Brar DS (2006) High-resolution mapping of a new brown planthopper (BPH) resistance gene, *Bph18(t)*, and marker-assisted selection for BPH resistance in rice (*Oryza sativa* L.). Theor Appl Genet 112: 288-297.

Jones JT, Harrower BE (1998) A comparison of the efficiency of differential display and cDNA-AFLPs as tools for the isolation of differentially expressed parasite genes. Fund Appl Nematol 21: 81–88.

Kashkush K, Feldman M, Levy AA, (2002) Gene loss, silencing and activation in a newly synthesized wheat allotetraploid. Genetics 160: 1651-1659.

Kishimoto R (1968) Studies on the polymorphysm and its role playing in the population growth of the brown planthopper, *Nilaparvata lugens* Stål. Plant Protect Res 1: 111-115.

Milioni D, Sado PE, Stacey NJ, Domingo C, Roberts K, McCann MC (2001) Differential expression of cell-wall-related genes during the formation of tracheary elements in the Zinnia mesophyll cell system. Plant Mol Biol: 221–238.

Miyashita K (1963) Outbreaks and population fluctuations of insects, with special reference to agricultural insect pests in Japan, Bulletin of National Institute of Agricultural Science, Japan. Series C 15: 99-170.

Murai H, Hashimoto Z, Sharma PN, Shimuzu T, Murata K, Takumi S, Mori N, Kawasaki S, Nakamura C (2001) Construction of a high-resolution linkage map of rice brown planthopper (*Nilparvata lugens* Stål) resistance gene *bph2*. Theor Appl Genet 103: 526-532.

Murata K, Fujiwara M, Kaneda C, Takumi S, Mori N, Nakamura C (1998) RFLP mapping of a brown planthopper (*Nilaparvata lugens* Stål) resistance gene *bph2* of *indica* rice introgressed into a *japonica* breeding line 'Norin-PL4'. Genes Genet Syst 73: 359-364.

Qin L, Overmars B, Helder J, Popeijus H, van der Voort JR, Groenink W,. van Koert P, Schots A, Bakker J, Smant G (2000) An efficient cDNA-AFLP-based strategy for the identification of putative pathogenicity factors from the potato cyst nematode *Globodera rostochiensis*. Mol Plant-Microb Interact: 830–836.

Sharma PN, Ketipearachchi Y, Murata K, Torri A, Takumi S, Mori N, Nakamura C (2003) RFLP/AFLP mapping of a brown planthopper (*Nilparvata lugens* Stål) resistance gene *Bph1* in rice. Euphytica 129: 109-117.

Sharma PN, Tori A, Takumi S, Mori N, Nakamura C (2004) Marker-assisted pyramiding of brown planthopper (*Nilaparvata lugens* Stål) resistance genes *Bph1* and *Bph2* on rice chromosome 12. Hereditas 140: 61-69.

Simões-Araújo JL, Rodrigues RL, Gerhardt LBA, Mondego JMC (2002) Identification of differentially expressed genes by cDNA-AFLP technique during heat stress in cowpea nodules., FEBS Lett 515: 44-50.

Vos P, Hogers R, Bleeker M, Reijans M, Lee TVD, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucl Acid Res 23: 4407-4414.

Yang H, You A, Yang Z, Zhang F, He R, Zhu L, He G (2004) High-resolution genetic mapping at the *Bph15* locus for brown planthopper resistance in rice (*Oryza sativa* L.). Theor Appl Genet 110: 182-191

Yang Z, Zhang F, Zhu L, He G (2006) Identification of differentially expressed genes in brown planthopper Nilaparvata lugens (Hemiptera: Delphacidae) responding to host plant resistance. Bull Entomol Res 96: 53–59