Cloning and Molecular Evolution Analysis of NBS Class Resistance Gene Analogs in Black Bamboo (*Phyllostachys nigra*)

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Abstract

The nucleotide binding site (NBS) domain sequences were isolated from genomic DNA in black bamboo, using the degenerate primer designed according to the conserved motifs of the NBS resistance gene. The expected size of the PCR product was about 700 bp. Among 55 positive clones, the amino acid sequence alignment identified 33 black bamboo resistance gene analogs (RGAs) that contain the NBS conserved motifs. All of the 33 RGAs ORFs were constructed in an NJ (Neighbor-joining) tree, and divided into 10 groups. This analysis demonstrated the diversity of the NBS class RGA in black bamboo. The maximum likelihood estimates of various evolutionary models were analyzed; the result showed that 2 groups with a total of 10 sequences and 12 sites demonstrated statistically significant positive selection. Most of the positive selected sites were not located in the NBS conserved motifs. Two groups of gene conversion events had been discovered, which provide a material basis and research direction in isolating black bamboo *R* genes.

Keywords: *Black bamboo; NBS; RGA; Positive selection; Gene conversion*

Introduction

Many disease resistance (R) proteins in plants detect the presence of disease-causing bacteria, viruses, or fungi by recognizing specific pathogen effector molecules that are produced during the infection process (Gregory et al. 2003). Duplication, sequence variation by mutation, disparity in the length and structure of leucine rich repeats etc., causes tremendous variations within and among *R* genes in a plant thereby developing diverse recognition specificity suitable for defense against diverse new pathogens (Joshi and Nayak 2013). New technologies arising from the genomics and proteomics revolution will greatly expand our ability to investigate the role of R proteins in plant disease resistance. Since the first resistance gene *Hml* was isolated using the transposon tagging method in 1992 (Johal and Briggs 1992), more than 70 *R genes* have been cloned from different plant species (Liu et al. 2007). Later several R-genes such as *N, M, RPF, Gpa2* and others have been cloned in different plants like eggplant and chilli (Reddy et al. 2015, P. Naresh M et al. 2017). Most of them include a nucleotide binding site (NBS) conserved motif. The NBS class of resistance genes exist in many eukaryotes (Pan et al. 2000), including the *Pseudomonas syringae* bacteria resistance gene *RPS2* in *Arabidopsis thaliana* (Bent et al.1994), the flax rust resistance gene *L6* (Lawrence et al. 1995), the tobacco mosaic virus resistance gene *N* (Whitham et al. 1994), and the bacterial blight-resistance gene *Xa1* in rice (Yoshimura et al. 1998).

The NBS gene family is classified into two classes, which are TIR-NBS and non-TIR-NBS subfamily proteins (Hulbert et al. 2001). The two subfamilies of NBS class resistance gene protein are differentiated by the N-terminal domain, which influences the signaling pathway that will be activated upon effect or recognition (Aarts et al. 1998). However, TIR-NBS genes are rare in monocots according to D Ellen's report (Tarr and Alexander 2009). Designing degenerate primers according to *R gene* encoding protein functionally conserved domains, and amplifying resistance gene analogs by PCR, has become a new promising way of cloning resistance gene (Kanazin et al. 1996).

Comparing homologous coding DNA sequences from several species, it is possible to infer selection from the ratio of divergence rates at nonsynonymous (Ka) and silent (Ks) sites. If purifying selection is eliminating most amino acid replacements before they fix in the population and become

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substitutions, then Ka/Ks will be much less than one, while positive selection at many codones should cause this ratio to exceed unity. At present, positive selection has been found in the evolutionary process of terrestrial plants and algae. The positive selection genes found in terrestrial plants are mainly related to reproduction and disease resistance (Yuan et al. 2008). ω is a molecular evolutionary measure of selection, which is the ratio of the nonsynonymous to synonymous (Kimura and Ota 1974). ω>1 were considered as positive selection. In contrast, ω<1 is consistent with purifying selection (Nei and Kumar 2000). Gene conversion can generate diversity by reasserting variants among paralogs (Slightom). Whether there is a link between gene conversion and positive selection is still not clear (Parniske and Jones 1999; Mondragón-Palomino and Gaut 2005).

Black Bamboo (*Phyllostachys nigra*) originated in China, which has been widely cultivated elsewhere in China and introduced in many other countries (Wu and Peter 2006). Spot disease, leaf lust disease, and bamboo mosaic virus disease are the common disease in black bamboo (Xu et al. 2006), which affect its ornamental characteristics and survival ratio. Therefore, isolate RGAs in black bamboo has a guiding significance in production and breeding of plant disease resistance in bamboo and even other monocots. The purpose of this study is to explore the selection pressure and gene conversion event, predict the potential resistance genes by RGA isolation, sequence analysis, phylogenetic tree construction, adaptive evolution analysis and gene conversion analysis. Learning the evolutionary process of bamboo resistance genes as well as the promoting regularity of genetic variation has an important meaning of separating new functional *R genes* and improving breeding efficiency.

Materials and Methods

Plant material and genomic DNA extraction

The plant material used for this study was derived from black bamboo(Phyllostachys nigra Munro). Genomic DNA was isolated from fresh leaf tissues, using the DNA Quick Plant System (TIANGEN BIOTECH).

PCR amplification and sequencing

The NBS primer pair design was based on the consensus sequence of the P-loop (forward primer) and RNBS-D (reverse primer) (Pan et al. 2000; Joseph and David 2001; Hanan et al. 2009). Primers corresponding to the conserved domains of known *R genes* are as follows, the forward primer S1: GGN GGN RTN GGN AAN ACN AC; the reverse primer NBS6: YTC NGG RAA NAR NGC RCA RTA(R=A or G, Y=C or T, N =A, C, G or T).

PCR was performed in a total volume of 25 μl containing 2.5 μl 10 × Taq DNA polymerase buffer (plus Mg2+)(PINPIN), 2 μl dNTP (2.5 mM), 0.5 μl each primer(100 μM), 1.0 μl of genomic DNA (Concentration?), 0.2 μl Taq polymerase(5 u/μl), [1]8.3 μl dd H_2O. The reaction conditions were 2 min at 95˚C, followed by 35 cycles of denaturing at 95 ˚C for 30 s, the annealing temperature gradient was 40 ˚C -60 ˚C, the annealing time was for 30 s, and elongating at 72 ˚C for 50 s, and a final extension step at 72°C for 7 min.

The expected 700bp size fragments were excised from the gel and purified using TIAN Gel Midi Purification Kits (TIANGEN, China). The purified PCR products were cloned into the pMD18-T vector (TAKARA, China), using standard procedures. Recombinant plasmid DNA was transferred into *Escherichia coli* BL21 cells treated by calcium chloride method (Meyers et al.1999), and plated on LB (Luria Broth) agar medium with IPTG and X-Gal. The white clones, which indicate successful insertion of the

DNA fragment, were selected. PCR amplification was used to identify the expected insert fragment. Primers were designed according to the vector sequence. The forward primer PUCRV: GAA GCT ATG ACC ATG ATT AC ACA; the reverse primer PUCM1: GTT TTC CCA GTC ACG ACG TTG. The annealing temperature was 58 ˚C. Positive clones were screened and used for further testing by the original S1 and NBS6 primers. The sequences were determined by ABI 3730xl DNA sequencer.

Sequence analysis

Sequences were edited using BioXM2.6(Huang and Zhang, 2004), to remove the vector sequences, and translate into amino acid sequences. Database searches were performed using the National Center for Biotechnology Information GenBank (https://www.ncbi.nlm.nih.gov/genbank/) to search for the NBS-encoding *R gene* protein and RGA homologous protein sequences. The sequences which did not result in open reading frame were defined as "pseudogenes". The amino acid sequences with ORFs were aligned using DNAMAN6.0 (Zhang et al. 2016). Amino acid sequences RPS2 (GenBank: AAA21874) from *Arabidopsis thaliana*, N (GenBank: AAA50763) from tobacco, L6 (GenBank: AAA91022) from flax and Xa1 (GenBank: BAA25068) from rice were used in the alignment. The NJ (Neighbor-joining) tree was constructed based on the nucleotide sequences using Mega5.0 (Yin et al. 2014).

The maximum likelihood estimates of various evolutionary models were calculated using the program codeml, a constituent of the PAML package (Version 4.3), and its notation is described here: the Ka/Ks ratio is termed, and model names are M7 and M8. Most codons in most genes are under purifying selection and have a Ka/Ks ratio < 1, while some genes may have few codons under positive selection (with Ka/Ks ratio > 1). Using models allowing for several classes of codons with separate ratios (Swanson et al. 2003; Yang et al. 2000), it is possible to test for the variation of selective pressures across the codons and for the presence of codons under positive selection. When posterior probability was greater than 0.99, this indicated a significant positive selection. The test can be more reliably performed by a likelihood ratio test that compares the log-likelihood values under the two models, $2\Delta\ell(\Delta\ell=\ell1-\ell0)$, while the degree of freedom was the difference between the NP of a pair of two models. The significance of the LRT was evaluated by comparing it to χ^2. The results were considered very significant at a P value < 0.01.

Gene conversion detection was performed using the GENECONV1.81 exchange program, parameters were setto default, and the results were considered significant at a P value < 0.05.

Results

Isolation and sequence analysis of NBS class RGAs

The NBS sequences were isolated from genomic DNA in black bamboo, using the degenerate primer pair designed according to the NBS resistance gene conserved motifs (Fig1). The expected size of the PCR product was about 700bp. The band of 40 product was clear and blight. The product was purified and cloned into the pMD18-T vector. 231 clones were selected randomly from LB agar medium. The results of the empty vector test showed that there were 55 of 231 clones (23.8 %) had a fragment inserted. The result of the single primer test showed that, all of the 55 clones obtained an insert fragment which amplified by two different primers. These 55 clones were sequenced.

Fig1

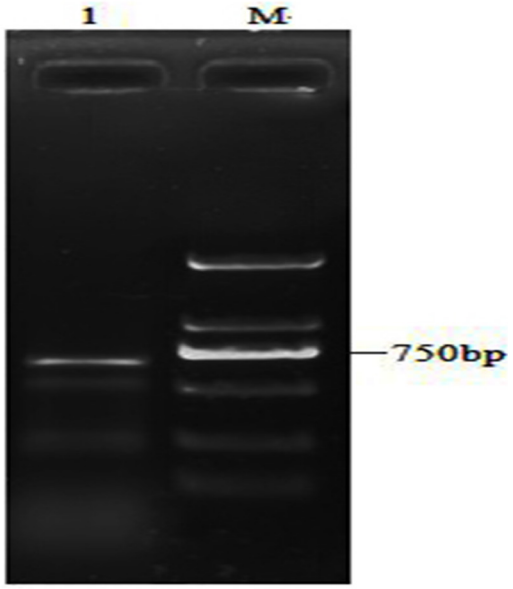

Figure 1
Amplification results of resistance gene analogs
1: the PCR product under the annealing temperature was 40°C; M: 2000bp DNA Marker

After editing, of 55 positive clones, we obtained a total of 41 (74.5 %) NBS class resistance gene analogs, of which 36 (87.8 %) of the sequences gave ORFs, while 5 (12.2 %) of the sequences were pseudogenes. Of the 36 ORFs, 3 sequences were redundant t other sequences, while the other 33 sequences were not, which accounts for 80.5 % of the 41 RGAs, named Z-1 to Z-33. The accession numbers were assigned (Genebank: JQ929586 to JQ929618). The length range of these 33 sequences were from 678bp to 729bp. The sequences were translated into amino acid sequences, then blasted in the GenBank protein datbase. The blast results showed that these 33 resistance gene analogs have high homology with resistance genes or resistance gene analogs cloned in monocots like rice and dicots like potato.

The amino acid sequence alignment showed that the 33 black bamboo resistance gene analogs contain the NBS conserved motifs: P-loop (GGVGKTT), RNBS-A (FDLx-AWVCVSQxF), Kinase-2a (LLVLDDVW), kinase-3a (GS/NR/KILVTTR), hydrophobic domain (GLPL), and RNBS-D (CFLYCALFPE) (Fig2a-d). According to related reports, the RNBS-A conserved motifs were FLENIRExSKKHGLEHLQKKLLSKLL in the TIR-NBS class resistance genes, but FDLxAWVCVSQxF in the non-TIR-NBS class resistance genes. Kinase-2a sequences were LLVLDDVD in the TIR-NBS class of resistance genes, but LLVLDDVW in the non-TIR-NBS class resistance genes. RNBS-D sequences were FLHIACFF in the TIR-NBS class resistance genes, but CFLYCALFPED in the non-TIR-NBS class resistance genes (Deyoung and Innes 2006). The RNBS-A, Kinase-2a and RNBS-D motifs of all the black bamboo resistance gene analogs were consistent with non-TIR-NBS class disease resistant gene characteristics. Therefore all the 33 sequences should be classified as non-TIR-NBS class resistance gene analogs. However, substitutions were found in these conserved motifs.

Fig2a

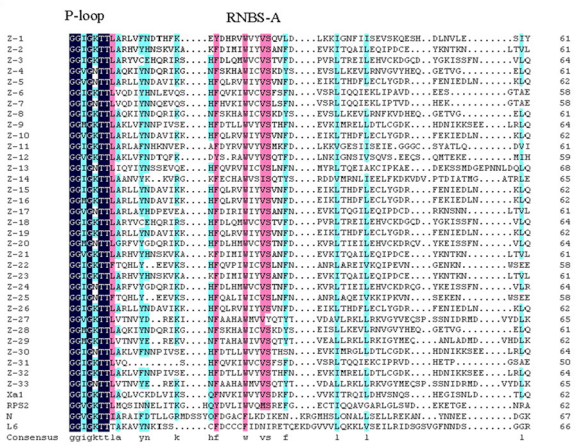

Fig2b

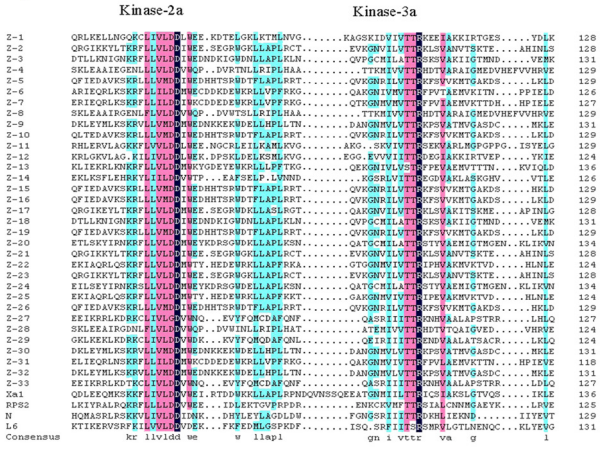

Fig2c

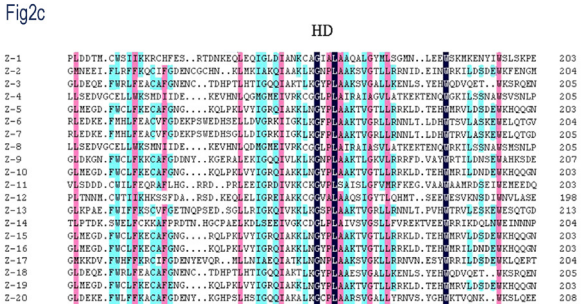

Fig2d

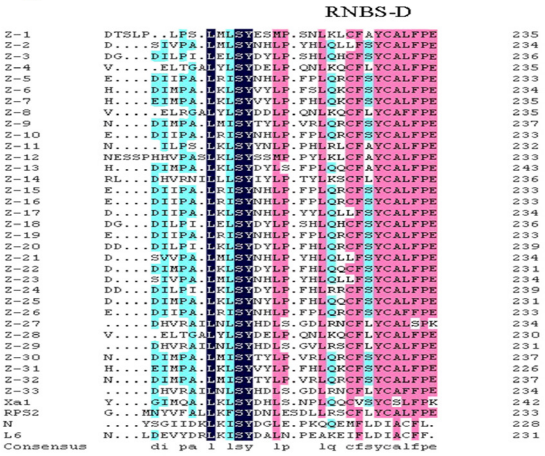

Figure 2
Amino acid sequence alignment of NBS disease resistance gene analogs in black bamboo and reported disease resistance genes a-d: a. the NBS conserved motifs: P-loop (GGVGKTT), RNBS-A (FDLx-AWVCVSQxF);b. Kinase-2a (LLVLDDVW),and kinase-3a (GS/NR/KILVTTR); c. hydrophobic domain (GLPL); d. hydrophobic domain RNBS-D (CFLYCALFPE)

Phylogenetic analysis

All of the 33 RGAs with ORFs were constructed in an NJ tree, and divided into 10 groups, and named group 1 to group 10 (Fig3). The genetic distance between groups was >0.400. This demonstrated the diversity of NBS class RGA in black bamboo. The number of sequences in different groups were significantly different. The range was from 1 to 6. The three largest groups were group 1, group 4 and group 7, each with 6 sequences respectively, 16 % of total. The smallest groups contained only 1 sequence, such as groups 6 and 9. The genetic distance between different groups was divergent, but within each group there was convergence. Because PAML software and RDP require a minimum of 3 sequences per group, there were 8 groups, a total of 35 (94.6 %) sequences could be used for further analysis, which were groups 1, 2, 3, 4, 5,7, 8 and 10.

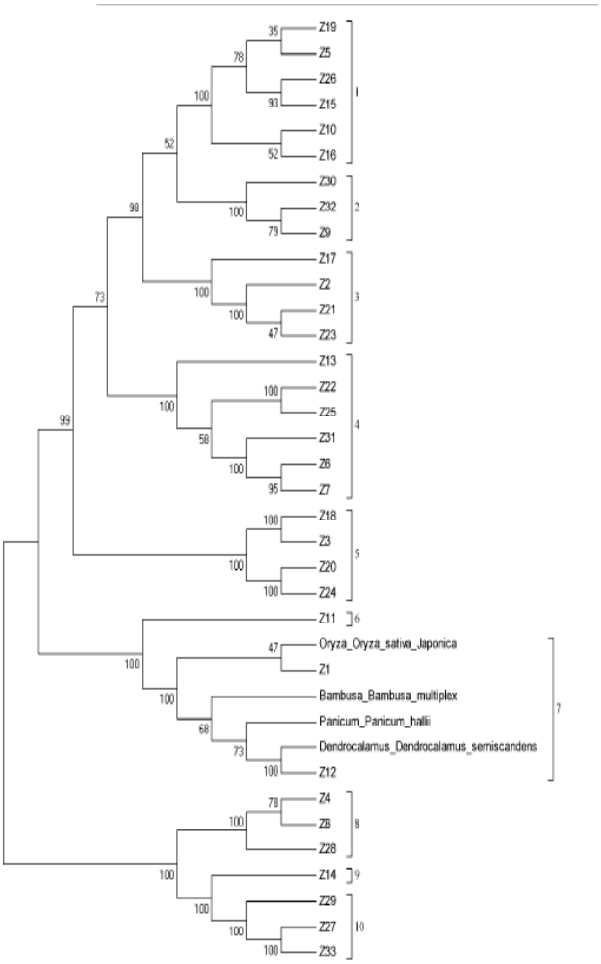

Figure 3
Phylogenetic tree of black bamboo RGAs based on NJ

Positive selection analysis

For the second class of codons, the 1 ratio exceeds unity for all the RGAs genes, suggesting that some codons in these genes may be under positive selection. To test whether adaptive selection is indeed acting in all or some of these genes, we have to demonstrate that the 1 ratios are significantly greater than unity for this class of codons. This can be done by comparing two nested models, one allowing for a class of codons free to take any Ka/Ks ratio > 1 and the other with this class having fixed Ka/Ks ratio<1. Models M7 and M8 implemented in PAML allow the Ka/Ks ratios below 1 to be distributed according to a beta distribution and thus may better account for the variation in purifying selection across the codons. The comparison of M7 and M8 by the LRTs demonstrated that model M8 fits the data significantly ($P < 0.05$), which suggested that positive selection was allowed in M8. The result showed that 2 groups (groups 4 and 5) with a total of 10 sequences and 12 sites had been detected as statistically significant positive selection (Tab. 1). The number of positively selected sites was different between these two groups. Only one site was detected in group 4, but 11 sites were detected in groups 5. The loci of the positively

selected sites in different groups were also different between groups.

Tab. 1
Positive selection sites and likelihood ratio test results of black bamboo RGAs

Group	N[1]	2Δℓ	M8 Parameters[2]	Positively selected positions[3]
1	6	4.94	ω= 2.65662, p= 0.06914	3M
2	4	0	ω=151.02890, p=0.00001	None
3	3	0	ω= 1.00000, p=0.00001	None
4	4	10.42	ω= 1.73931, p= 0.36058	20D、33N、36K、41I、53G、55K、101S、177R、184Y、194T、197N
5	6	13.12	ω= 4.12526, p=0.03938	37L
8	3	0.46	ω= 1.47223, p= 0.42257	None
10	3	0.44	ω= 1.15306, p=0.24810	None

[1] Number of the sequences in the group

[2] ω is dN/dS, estimated under model M8, p is the inferred proportion of positively selected sites.

[3] Positively selected positions based on alignments without gaps. The sites listed in the table were greater than 0.95 in posterior probability. The sequences in boldface mean LRT test P<0.01.

The positively selected sites of the group 4 and group 5 alignments amino acids refer to sequences Z-7 and Z-24, respectively (fig4, fig5). It could be seen from the figure that, most positively selected sites were not located in the NBS conserved motifs except 33N (9.1 % of 11 sites) which was located in a conserved RNBS-A motif (FDLx-AWVCVSQxF) in group 5. No positively selected sites were located in the NBS conserved motifs in group 4.

Fig4

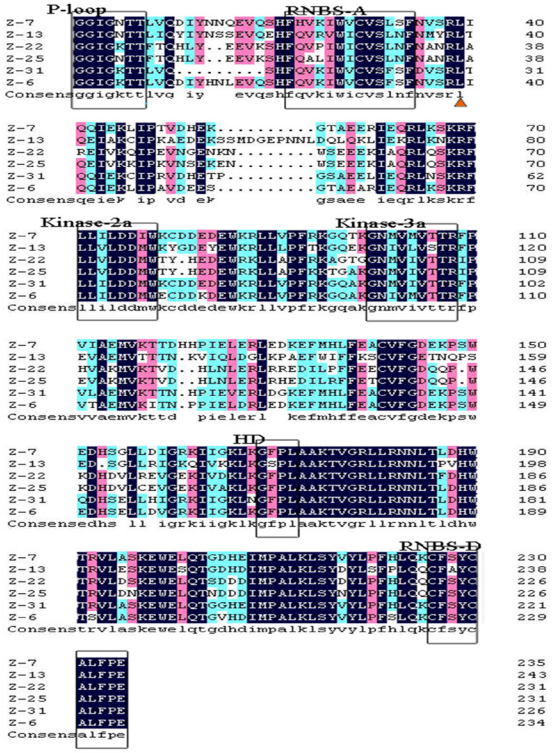

Fig4
Positive selection sites of black bamboo group 4

Fig5

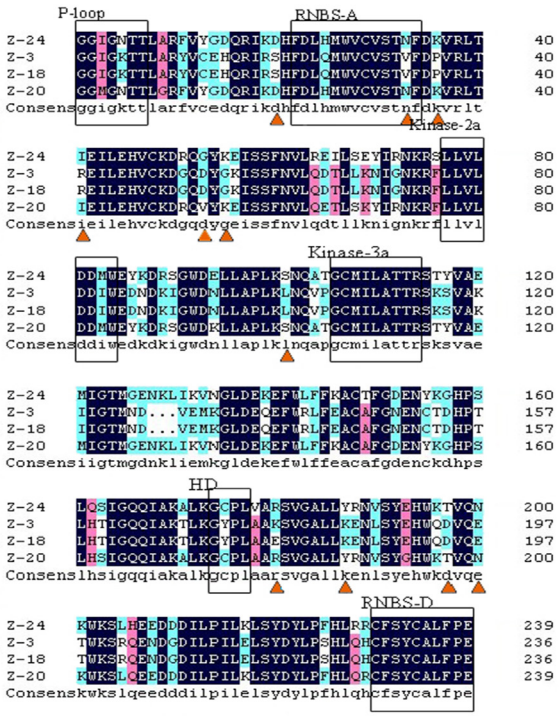

Fig5
Positive selection sites of black bamboo group 5

Gene conversion analysis

Gene conversion analyses were run on the groups which contain more than 3 sequences. The results showed that there were two groups (group 8, group 10) that demonstrated statistically significant gene conversion events (Tab. 2). Each group contained a single event between two genes, and conversion lengths were 147bp and 24bp, respectively. Thus, the quantity of gene conversion in black bamboo RGAs was low.

Tab. 2
Gene conversion testing results of black bamboo groups

Group	Sequence name	Length	Gene conversion		
			Events	Length	Gene Number
1	Z-5,Z-10,Z-15,Z-16,Z-19,Z-26	699	0	0	0
2	Z-2,Z-17,Z-21,Z-23	702	0	0	0
3	Z-9,Z-30,Z-32	702	0	0	0
4	Z-3,Z-18,Z-20,Z-24	717	0	0	0
5	Z-6,Z-7,Z-13,Z-22,Z-25,Z-31	735	0	0	0
8	Z-4,Z-8,Z-28	705	1	147	2
10	Z-27,Z-29,Z-33	702	1	24	2

Discussion

The NBS domain is one of the most important conserved domains of plant disease resistance genes, and plays an important role in plant hypersensitivity responses (He et al. 2001). However, there is no certain connection between the NBS domain and the gene's disease-resistant function. Genes containing the NBS domain might not necessarily be a disease resistance genes (Mondragón-Palomino et al. 2002). Therefore, the sequences we cloned were just candidate resistance genes. This hypothesis needs to be confirmed by further experimentation to determine whether these RGAs are *R genes*.

According to the experimental analysis in *Arabidopsis thaliana*, all the *R genes* fall into groups in which positive selection was detected, while groups with no positive selection detected contain no reported *R gene* (Ji et al. 2007). According to the experimental analysis in rice, the results showed that frequent gene conversion events had been detected in groups within *R genes* (Dangl and Jones 2001). It can be inferred that, there might be disease resistance genes in groups 4, 5, 8 and 10 in which positive selection were detected. This evidence provides a material basis and research direction in cloning black bamboo R genes.

Positive selection is ubiquitous in plant NBS-LRR resistance genes (Dangl and Jones 2001). In this study, we had cloned 33 RGAs, among which 29 RGAs underwent positive selection detection, and were detected in 10 RGAs. 12 positively selected sites had been detected. Although fewer in number, this outcome corresponds to Dangl's research (Dangl and Jones 2001), which demonstrated that positively selected sites were located in LRR domain much more than in the NBS domain. This difference in selection site location might be because the function of LRR domain is often related to virus identification (Mondragón-Palomino and Gaut 2005), which determines the specificity of disease resistance. Therefore, this region has undergone a strong positive selection pressure with extensive amino acid polymorphism. According to the location of the positive selected sites in identified by alignment in group 4 and group 5, the positive selected sites were rare in NBS conserved motifs.

Presumably the NBS conserved motif might be related to disease resistance function. The substitutions of amino acid in the NBS conserved motif, might lead to the loss of disease-resistant functions, leading to gene elimination in the long-term evolutionary process.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interest.

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