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RESEARCH ARTICLE

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Initial function determination for the open reading frame (ORF) region of *Pib* gene via rice transformation

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Abstract Three plant binary expression vectors—pNAR501, pNAR502 and pNAR503—were constructed, carrying fragments of exon2-exon3, 5′partial deletion exon1 and 5′partial deletion exon1-exon2-exon3 of *Pib* gene driven by 35S. These three vectors were transformed into the japonica rice variety *Nipponbare* through agrobacterium-mediated transformation. More than 30 transgenic rice plants were obtained and confirmed by polymerase chain reaction (PCR), Southern hybridization and the hygromycin resistance test in seed germination of their progeny. A rice blast resistance test for in vitro leaves of T_0 transgenic plants in the tillering stage showed higher resistance to the races of E1, F1 and G1 of rice blast than that of the control *Nipponbare*. However, results of rice blast resistance test for seedlings of T_1 transgenic plants in the 3- to 4-leaf stage were different. All T_1 transgenic seedlings had a lower level of resistance to E1, F1and G1 races than that of the control *Nipponbare*.

Keywords rice, rice blast, *Pib* gene

1 Introduction

Cloning of the disease-resistant gene (*R* gene) and related functional genome researches are a hot field in crop gene engineering; with more than 40 plant disease-resistant genes already cloned (Ellis et al., 2000; McDonld and Linde, 2002; Gu et al., 2005). An analysis of molecular structure of *R* genes revealed that their donors and resistance to pathogens were different, but with some similar conservative structures in DNA sequence. Most cloned *R* genes were also found carrying 1eucine-rich repeats (LRRs), nucleotide-binding site (NBS) and putative amino—terminal signaling domain (Hammond and Parker, 2003; Youssef et al., 2004).

Rice blast (*Magnaporthe grisea*) is caused by a fungus which is a virulence differentiated pathogen. Based on studies of classical rice genetics, there are one to four major genes and some minor genes for rice blast resistance (McDonld and Linde, 2002). *Pib* gene is the first map-base cloned major gene for rice blast resistance. This is a 10.3 kb DNA fragment including a 3.6 kb fragment upstream ATG code, a 5 kb open reading frame fragment and a 3.6 kb downstream fragment. Sequence analysis for ORF of the *Pib* gene indicated that it is a split gene with four introns and three exons inside (Wang et al., 1999). Because this clone was isolated from a genomic library, its DNA sequence maintained its native state. This clone makes it possible to understand the interrelation between the molecular structure of *Pib* gene and the blast resistance of rice plants.

Wang et al. (1999) transformed the entire 10.3 kb genomic clone DNA into *Nipponbare*, whose transgenic plants showed good resistance to rice blast and whose resistant spectrum to pathogen races were the same as their donor cultivar. This result confirmed that this genomic clone contained a *Pib* major gene. However, there is still no research on the open reading frame (ORF) function of the gene. Based on the analysis for coding sequence characteristics of a *Pib* structure gene, three plant binary expression vectors were constructed and embedded into the different length coding regions of the gene and driven by 35 S. These three vectors were then transformed into *Nipponbare* using agrobacterium-mediated transformation. According to the expression of these transgenic rice plants, some data may help understand the relationship between the molecular structure of rice blast resistant genes and their functions.

2 Materials and methods

2.1 Plant materials

Dehusked mature seeds of japonica rice variety *Nipponbare* were used for callus induction and surface sterilized by

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commercial 84 sterilization solutions (Ni et al., 2002). N_{62} agar medium (N_6 basic medium +2 mg/L 2, 4-D) was used for callus inducing. Three-week-old embryogenic calluses were then collected for agrobacterium-mediated transformation.

2.2 Plasmids and strains

Binary plasmids pPZP2H3a (+/−) and strain EHA101 of *Agrobacterium tumefaciens* were donated by Masahiro Yano (Fuse et al., 2001). Rice blast pathogen (*Magnaporthe grisea)* strains were provided by the Plant Protection Institute of Jiangsu Academy of Agricultural Sciences.

2.3 Transformation and regeneration

Agrobacterium-mediated rice transformation and plant regeneration were conducted according to Bu et al. (2006).

2.4 PCR test for the transgenic rice plants

All polymerase chain reactions were conducted in a 20 µL volume system. Reaction conditions were: 94°C-5 min for pre-denaturation, followed by 35 cycles of repeating 94°C-1 min, 58°C-1 min, 72°C-1 min, then 72°C-10 min for post-extension. The PCR products were extracted by electrophoresis in 0.7% (w/v) agarose gel. Polymerase chain reaction primers were synthesized by TaKaRa Co., Dalian, China. Primers *Hpt-F* and *Hpt-R* were used to check the *Hpt* gene. Exon1-F and Exon1-R were used to verify the first exon, while Exon3-F and Exon3-R were used to test the third exon of the *Pib* structure gene (Table 1).

Table 1 Nucleotide sequences of the PCR used primers

Primer	Sequence	Length of the PCR products/bp
Hpt-F	5′-ACACAGCCATCGGTCCAGAC-3′	589
Hpt-R	5′-ATCTTAGCCAGACGAGCGGG-3′	
Exon1-F	5′-CAAAGCAGGACAATCAG-3′	523
Exon1-R	5′–TCGTAGAATGCGTAAAGG-3′	
Exon3-F	5′-TGTCGTAAGGTGGGATGT-3′	986
Exon3-R	5′-CAGGTAATGATGGG1TrGG-3′	

2.5 Southern hybridization test for transgenic rice plants

After the PCR test, only the positive putative T_0 transgenic rice plants were tested again by Southern hybridization. Twenty µg of genomic DNA per plant was digested with restriction endonuclease overnight. After eight hours of electrophoresis, the target DNA was transferred onto a nylon membrane, and the Southern hybridization was performed according to the 'User Manual of DIG High Primer Labeling and Detection Starter Kit I, Roche Co.' without any modification.

2.6 Hygromycin resistance test for transgenic rice seeds

After breaking off dormancy, the T_1 seeds from transgenic T_0 plants were soaked in 50 mg/L of hygromycin water solution at 28°C with 16-h lighting for germination. The germination rates were collected on the seventh day.

2.7 Detection of blast resistance of transgenic plants

Rice blast resistance tests of in vitro leaves for T_0 transgenic plants were done at their tiller stage (Lu and Zhang, 1994). The "Scale Rating System Referred to Standard Evaluation System for Rice (IRRI 1996)" was used for scoring blast resistance.

The rice blast resistance test for T_1 transgenic plants was carried out during seedling stage. Seeds of T_1 were germinated in a hygromycin solution for seven days and washed with water on the eighth day, and then the cleaned seedlings were transplanted into plastic pots. *Nipponbare* seeds soaked in water for two days were sowed in pots at the same time and used as non-transgenic control. And *Lijianxintuoheigu*, a susceptive Chinese local cultivar, was used as a control for blast pathogen vigor. Inoculation of blast pathogen was conducted in the 3–4 leaf stage by spraying blast spore suspensions. The seedlings were maintained under a closed chamber at 28°C for 24 h to keep humidity high, then moved outdoors and water sprayed every day. Incidence was investigated on the 8th day after inoculation with two replicates for each pathogen race.

3 Results

3.1 Construction of plant expression binary vectors for the *Pib* structure gene region

Restriction enzyme cleavage was used to isolate expected segments of the *Pib* structure gene and avoid possible base mutation in PCR.

According to computer analyzed results in *Pib* gene sequence, a *Bam* H I fragment (5 769–10 322 bp) containing exon2-exon3 from the genomic clone of *Pib* gene was inserted into multiple cloning site (MCS) of binary plasmid pPZP2H3a (+) and recombined with a plasmid pNAR501. An *Xho* I+*Bam* H I segment (3 879–5 769 bp), segment of partial deleted exon1 in its 5'end, was inserted into MCS of binary plasmid pPZP2H3a (−) to construct a plasmid pNAR502. The plasmid pNAR503 was constructed by inserting the 5 769–10 322 bp *Bam* H I segment (Exon2–Exon3) to the *Bam* H I site in pNAR502. The plasmid pNAR503 contained Exon1-Exon2-Exon3 (3 869–10 322 bp) (Fig. 1). The insert in recombinant plasmid pNAR503 involved most of the *Pib* structure gene sequence. Because it was not a full-length *Pib* structure gene, this segment did not have the ATG code and its downstream 296 bp of exon1.

All recombined binary plasmids were identified with restriction enzyme recut experiments (Fig. 2). A 4.6 kb *Bam*

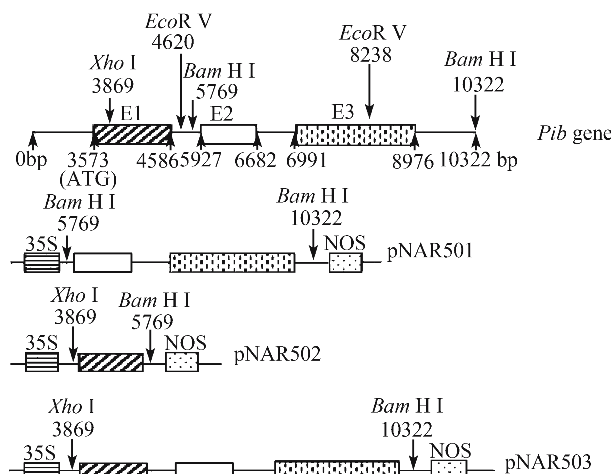


Fig. 1 Genomic DNA clone of *Pib* gene and constructions of expression vector pNAR501, pNAR502 and pNAR503

H I fragment and a 1.2 kb (*Apa* I+*Sal* I) fragment were obtained from pNAR501 (Fig. 2(a)). A 1.9 kb (*Xho* I +*Bam* H I) fragment was obtained from pNAR502 (Fig. 2(b)). From pNAR503A / *Eco* R V digestion, the 4 kb fragment was not available. These recut experimental results were the same as those expected from the physical map.

3.2 Rice transformation and plant regeneration

Cultured on hygromycin selective medium for six weeks, the hygromycin resistant calluses developed from the transferred mature seed calluses were subculture to differentiation medium I (Bu et al., 2006) containing hygromycin, cefotaxime and carbenicillin. The earliest shoot differentiation would be observed two weeks later. After three weeks of culture on the differentiation medium I, all the calluses were subcultured on differentiation medium II (Bu et al., 2006), in which only hygromycin was supplemented until the regenerated plantlets were removed from the dishes. We found that antibiotics in the regeneration medium performed negatively for plant regeneration. In our transformation procedure, the calluses—after co-culture with agrobacteria—must be water-washed more than ten times first before washing twice with an antibiotics solution and transferred to an antibiotics selection medium. The nine-week-old hygromycin resistant calluses were usually beyond the contamination problem with *Agrobacterium tumefaciens*. Cefotaxime and carbenicillin were thus deleted from the differentiation medium II to improve regeneration efficiency. Regenerated plantlets were heeled in vermiculite with a rice water culture nutrition solution for root development, then planted into soil. A total of 30 putative transgenic plants survived.

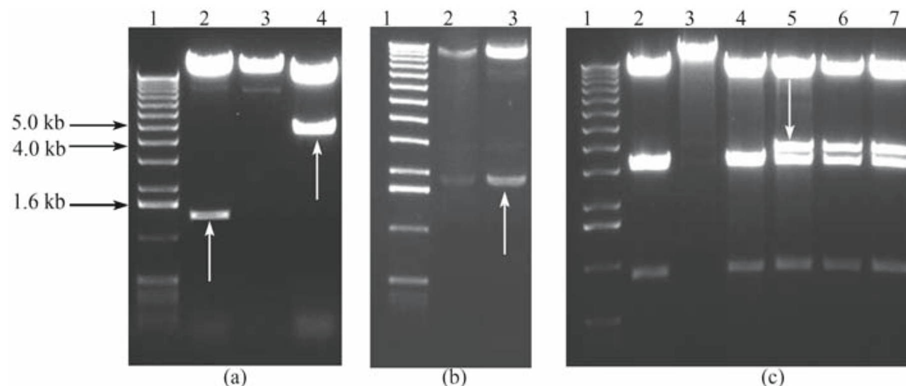
3.3 PCR and Southern verification for transgenic plants

Based on PCR with *Hpt* primers, all of the 30 transgenic plants were produced at the amplification band of 589 bp (Fig. 3(a)). Transgenic plants of pNAR502 and pNAR503 produced the 523 bp fragments in PCR using primers for Exon1 (Fig. 3(b)). Transgenic plants of pNAR501 and pNAR503 produced a 986 bp band with Exon3 primers, and pNAR502 had no amplification product with Exon3 primers in PCR reaction (Fig. 3(c)). All PCR test results matched inference from maps of the plasmids used for rice transformation.

The digoxigenin-labeled *Hpt* probe was hybridized with genomic DNA digested with *Eco*RV. There were positive hybridization signals in all 30 transgenic plants during Southern hybridization and 25 out of the 30 transgenic plants were single-copy insert. The results indicated that *Hpt* gene (and *Pib* gene together) had been integrated in the genome of these transgenic plants.

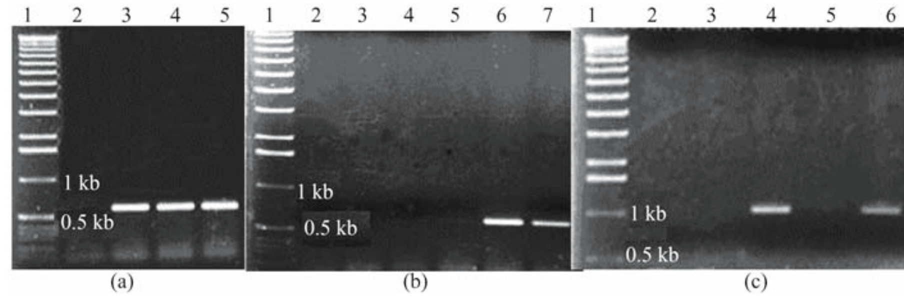
3.4 Germination rate of T_1 seeds in hygromycin solution

The seed set rate of T_0 transgenic plants was very low and no seed was obtained from eight of the thirty transgenic plants. The highest seed set rate of a single plant in T_0 transgenic plants was 6.7%. The germination rate of seeds from T_0 plants in hygromycin solution ranged from 44.1% to 91.8%. Test of significant differences for segregating hygromycin resistance/susceptibility in these germination experiments showed that most descendants of the transgenic plants segregated at a ratio of 3:1. This result also confirmed again that *Hpt* and *Pib* genes had been integrated into the host genome.



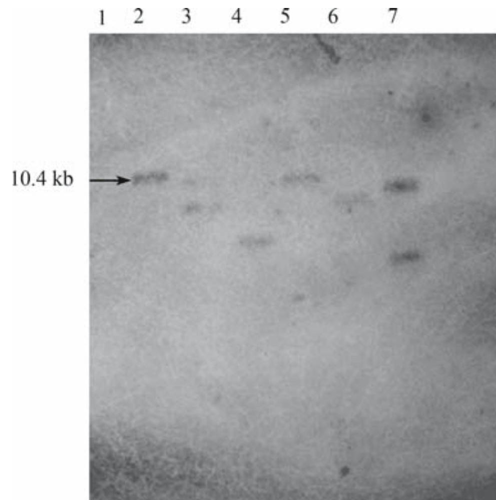
Note: (a) 1) Marker; 2) *Apa* I-*Sal* I; 3) *Sal* I; 4) *Bam* H I; (b) 1) Marker; 2) *Xho* I–*Bam* H I; 3) *Xho* I–*Bam* H I; (c) 1) Marker; 2–7) *Eco* RV.

Fig. 2 Restricted enzyme digest analysis for pNAR501(a), pNAR502(b) and pNAR503(c)



Note: (a) stands for primers of *Hpt*, lane 1: marker; lane 2: CK; lane 3: pNAR501; lane 4: pNAR502; lane 5: pNAR503; (b) stands for primers of the first exon of *Pib*, lane 1: marker; lanes 2 and 4: CK; lanes 3 and 5: pNAR501; lane 6: pNAR502; lane 7, pNAR503. (c) stands for primers of the 3rd exon of *Pib*, lane 1: marker; lane 2: CK; lane 3 and 5: pNAR502; lane 4: pNAR501; lane 6: pNAR503.

Fig. 3 PCR analysis for transgenic plants



Note: 1–7 stand for control plants, pPZP2H3a, pNAR501 transgenic line 501-1, pNAR502 transgenic line 502-1, pNAR502 transgenic line 502-2, pNAR503 transgenic line 503-2 and pNAR503 transgenic line 503-8, respectively.

Fig. 4 Southern hybridization for transgenic plants

3.5 Rice blast resistance test for transgenic plants

3.5.1 Rice blast resistance test for in vitro leaves of T_0 transgenic plants

Predominant races of rice blast in Jiangsu Province, E1 (001-33-1-3), F1 (001-187-1-3) and G1 (001-152-1-3), were used to verify blast resistance for transgenic plants. Leaves of T_0 transgenic plants were cut off and put into Petri dishes for an in vitro resistance test in incubators. All in vitro leaf segments were collected from the tillering stage transgenic plants in a greenhouse and only a well spread leaf was used to conduct the in vitro test. The results showed that all control *Nipponbare* leaves scored 7 (susceptible) to the three blast races, but transgenic materials scored 2–5 (resistant-medial susceptible) and the blast resistance of transgenic plants was found to be significantly higher than that of the controls. It is very interesting that the pNAR501, a construction of only exon2-exon3 without exon1 showed the highest blast resistance amount of all transgenic rice plants, and their resistance were all scored 2 to E, F and G races. Blast resistances in transgenic plants of pNAR503, a construction carrying an almost full three exons of *Pib* structure gene (only 296 bp was deleted from 5′end of exon1), and pNAR502, a construction of only a non-intact exon1 (296 bp was deleted from 5′end), were scored 5 (medial susceptible) to race G (Table 2). This result indicated that different exon in the *Pib* structure gene probably had a different function for blast resistance.

Software Analysis for DNA sequence of *Pib* structure gene indicated that in Exon1 and after the ATG code, there were more molecular elements correlating with promotion and regulation of genes. Finding out the actual reason for this different disease resistance to various plasmid transgenic plants—including intrinsic attributes for exon1 of the *Pib* gene and the partial sequence delete in exon1—need further research.

3.5.2 Rice blast resistance test for T_1 transgenic plants

In June of 2005, we evaluated blast resistance for T_1 transgenic plants, with seeds from T_0 transgenic plants germinated in hygromycin solution for a week. The hygromycin resistant seedlings were screened out and transplanted into small pots and inoculated with pathogen spore suspensions during the three to four leaf stages. Twenty days after spraying inoculation, the resistance degree to rice blast was scored (Table 3).

Table 2 Blast resistance for in vitro leaves of T_0 transgenic plants

Code of fungus	Race	*Lijiangxintuanheigu*	*Nipponbare*	T_0 transgenic line		
				pNAR501	pNAR502	pNAR503
001-33-1-3	E1	8 HS (60%)	7 S (40%)	2 R (<1%)	2 R (<1%)	2 R (<1%)
001-187-1-3	F1	8 HS (65%)	7 S (30%)	2 R (<1%)	2 R (<1%)	2 R (<1%)
001-152-1-1	G1	8 HS (60%)	7 S (35%)	2 R (<1%)	5 MS (9%)	5 MS (9%)

Note: R, S, MS, HS and (%) represent resistant, susceptible, moderately susceptible, highly susceptible and diseased leaf area, respectively.

Table **3** Blast resistance of T_1 transgenic plants

Code of fungus	Race	*Lijiangxintuanheigu*	*Nipponbare*	T_0 transgenic line		
				pNAR501	pNAR502	pNAR503
001-33-1-3	E1	8 HS (60%)	7 S (30%)	8 HS (55%)	8 HS (65%)	8 HS (55%)
001-187-1-3	F1	8 HS (65%)	7 S (30%)	8 HS (60%)	8 HS (60%)	8 HS (55%)
001-152-1-1	G1	8 HS (60%)	7 S (35%)	8 HS (60%)	8 HS (65%)	8 HS (60%)

4 Discussion

From the sequence analysis for rice genome, Wang et al. (1999, 2001) indicated that the *Pib* gene was a member of a small gene family. More than one copy of the gene sequence existed not only in *Pib* donor rice variety, but also in the blast susceptible rice variety. The *Pib* gene was not a pathogen-inducing gene but its expression was induced by environmental factors (darkness, temperature and some chemical substances for signal transduction such as jasmonic acid (JA), salicylic acid (SA) and ethylene-ethephon (E-E). The gene expression was also regulated by lighting and exhibited a diurnal rhythm (Wang et al., 1999; Wang et al., 2001). In our laboratory, the promoter region of *Pib* gene has been studied in a way similar to that for promoter-β-glucuronidase (GUS) fusion plasmids by rice transformation. From GUS qualitative and quantitative analysis for these transgenic rice plants, results showed that the *Pib* promoter was an inductive promoter and GUS activity in the roots was much higher both before and after inducing treatment. However, GUS activity was hardly detected in leaves before inducing treatment, but found multiple times after the treatment (Li et al., 2006).

Infected rice blast pathogen and diseased spots are seen on the leaves and other aboveground parts of rice plants. The functional target of the disease-resistance production coded by the *Pib* structure gene is in parts of rice above the ground, but not in its root system. In our experiments, the leaves from T_0 transgenic plants in tillering stage were found to be blast resistant, but the 3–4 leaf stage seedlings of their descendant were medial susceptible to the disease. Further studies are needed to understand the expression difference in transportation procedure (path) for some functional materials of the *Pib* gene.

Pib gene is an inductive structure gene with time and space specificity. However, in our transgenic events, the gene was driven by 35S promoter and set a constitutive expression. This deviated from the original synthesis and expression of *Pib*. The influence of the excessive mRNA to blast resistance for rice plants is another interesting subject for further study.

Moreover, the *Hpt* gene did not express the full hygromycin resistance in transgenic rice plants. Seeds of transgenic rice could slowly germinate in hygromycin solution and their seedlings growing up in the solution were weaker than those from control seeds in water. Hygromycin selection significantly suppressed seedling vigor. Further studies are being conducted in our lab to understand whether this selection could influence the following blast resistance tests during the 3–4 leaf period and the cause of higher incidence in the transgenic rice compared to the control.

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