Asian J. Med. Biol. Res. 2015, 1 (2), 265-270; doi: 10.3329/ajmbr.v1i2.25621

Asian Journal of Medical and Biological Research
ISSN 2411-4472 (Print) 2412-5571 (Online)
www.ebupress.com/journal/ajmbr

Article

mRNA extraction, cDNA synthesis and tillering specific gene isolation from BLB resistant Binashail rice

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Received: 23 July 2015/Accepted: 31 August 2015/ Published: 30 September 2015

Abstract: Bacterial Leaf Blight (BLB) caused by *Xanthomonas oryzaepv. oryzae (Xoo)* bacteria, is a major biotic stress in the irrigated rice belts. Genetic modification is the most effective and economical control for bacterial blight disease. For high yielding, branching capacity is most important. Molecular survey was conducted to identify the presence of tillering specific gene in BLB resistant Binashail rice. Reproducible means of Binashail rice at 21 days was designed based on the timing of full expression of the leaf. PCR with primers specific for tillering specific gene was used in the study. The cDNA of Binashail rice synthesized by an improved RT-PCR technique, derived from the total mRNAs extracted from the plant leaf. Gel documentation showed that the size of the synthesized cDNA was 250bp and tillering specific gene was 201bp in length long. The identification of tillering specific gene in Binashal rice germplasm will help in accelerating the elite breeding program in future.

Keywords: BLB; tillering; PCR; biotic stress

1. Introduction

Rice (*Oryza sativa L.*) serves as an important carbohydrate source for nearly half of the world's population and 66 % of the protein sourse (Zahidul, 2003) of the people in Bangladesh. Rice production is decreased because of two main reasons. First of all is abiotic stress and the second one is biotic stress. Due to heavy monsoon and warm climate which provide the most favorable agro ecological situation for developing disease and that is why the production of rice is low in Bangladesh. 32 diseases of which 2 viral, 3 bacterial, 22 fungal and 5 nemic diseases have been listed in rice and bacterial leaf blight (BLB) is the most important (Hopkins *et al.*,1992) damaging and destructive disease, yield loss ranging from 20-30% (Rao *et al.*, 2002) among all of Bacterial disease (Miah and Shahjahan, 1987). Ishiyama (2002) stated that BLB caused by the short rod–shaped bacterium with round ends 1-2.0×8-1 µm, monotrichous flagellum 6-8µm.The damage of rice crop from BLB was first reported in 1963 in Pathum Thani Province (Eamchit and Mew, 1982). BLB of rice is caused by *Xanthomonas oryzaepv. oryzae (Xoo)* throughout the world (Mew *et al.*, 1992).The bacteria are yellow(Swing *et al*. 2000) slime-producing, motile, gram-negative rod with a polar flagellum and the pathogen enters into the host through wounds or natural openings(Mew *et al.*, 1993). It reaches the vascular tissue, specifically into the xylem (Alvarez *et al.*, 1989), from where it multiplies itself and spreads throughout the plant. Symptoms are located at the tillering stage (Zaragosa *et al.*, 1979), disease incidence increases with growth stage, peaking at the flowering stage. The disease is incurring severe crop loss up to 50%.The faviorable temperature for BLB is

26-30 °C (Tagami *et al.*, 1962). At tillering stage BLB causes 10-20% reduction of rice yiels (Ou, 1985). Irrigation water is considered to contribute the spread of BLB disease, (Islam, 2004). Identifying and characterizing of major genes for qualitative resistance and polygenic factors controlling quantitative resistance have contributed a great deal to the success of resistant cultivars. Gene transformation is the most eco-friendly and economical method of BLB controls (Devadath, 1989). It might be reduced cost and at the same time it would be maintained food crisis. Tillering in rice (Oryza sativa L.) is an important agronomic trait for grain production. Rice tiller is a specialized grain-bearing branch that is formed on the unelongated basal internode and grows independently of the mother stem (culm) by means of its own adventitious roots (Li *et al.*, 1979). Rice tillering occurs in a two-stage process: the formation of an axillary bud at each leaf axil and its subsequent outgrowth (Hanada *et al.*, 1993). A new plant with high tillering and huge numbers of high-density grains per panicle should be developed (Peng *et al*., 1999; Vergara *et al*., 1990).

2. Materials and Methods

Binashail plant seeds were collected from Bangladesh Institute of Nuclear Agriculture. RM5493primer was used to detect tillering specific genome in the sample. Molecular works was done in Microbiology lab of Bangladesh Agricultural University. Binashail seeds were germinated and seedlings in Biotechnology tissue culture lab of Bangladesh Agricultural University.

2.1. Collection of samples, planting prepartion and RNA extraction

Seeds of varieties Binashail was obtained from BINA. These samples were kept for germination for 21 days. RNA was extracted from fresh seedling leaves of Binashail. Two hundred grams of rice seed was rinsed with tap water and soaked for 24 hours to stimulate germination. Seeds were planted in separate seedbeds. After 21 days in the nursery, leafs were formed. RNA extracted from 100mg of plant sample using RNA was extracted using one-step RNeasy kit of QIAGEN (Germany) according to manufacturer's instructions.

2.2. cDNA synthesis using RevertAid H Minus First Strand cDNA Synthesis Kit

1st strand cDNA was synthesized by using Thermo Scientific DyNAmo cDNA Synthesis Kit. After thawing, mixed and centrifuged the components of the kit and stored on ice. Template RNA (2µg), Oligo (dt) primer (1 µg), Water, nuclease-free (12 µg), reagents were added into a sterilenuclease-free tube on ice in the indicated order and total volume (25µg) were used. The RNA template contained secondary structure, was mixed gently and centrifuged briefly and then it was incubated at 65°C for 5 min. It was chilled on ice, then was spin down and was placed the vial back on ice.5X Reaction Buffer (4 µl), RiboLockRNase Inhibitor (20µl) (1µl), 10 mMdNTP Mix (2µl), RevertAid H Minus M-MuLV Reverse Transcriptase (200 u/µl) (1 µl) was mixed gently and centrifuged. Oligo (dT)18 for cDNA synthesis was added and then incubated for 60 min at 42°C.The reaction was terminated by heating at 70°C for 5 min.The reverse transcription reaction product was directly used in PCR applications or stored at -20°C for less than one week.

2.3. PCR amplification of first strand cDNA

The PCR cocktail including cDNA had total volume of 25 µl. For the preparation of PCR cocktail (for each sample), at first 12.5 µl master mix were taken in a PCR tube. Then 1 µl of primer forward and 1 µl of primer reverse were added. Nuclease free water 12.5 µl was added. 2-µl template cDNA was added with PCR cocktail. The mixture was then vortexed. 2 µl of cDNA template samples were pipette into the wells of the PCR tubes for PCR amplification. Reaction was placed in the PCR tubes and run in the cDNA thermal cycler. RM5493 is a SSR marker (Forward primer 5'-CACGACGTTGTAAAACGACGCGGTAACAAACCAACCAACC-3', Reverse primer 5'-AAAGCAGGACACAGTCACACAGG-3') used for RT-PCR of tillering specific gene (QingHua *et al*., 2012) and annealing temperature is 58 and it shows bands at 201 bp.

2.4. DNA sample preparation, electrophoresis and documentation of the cDNA samples

Loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol and 1 mM EDTA) and DNA marker (20bp) were used.6 µl 1xTBE buffer was placed on a piece of aluminum foil paper and 2 µl loading dye was added to it using 0.5-10 µl adjustable micropipette. Finally, 2 µl extracted DNA was added to it and mixed well using same micropipette. The samples were then added slowly to allow them to sink to the bottom of the wells. The gel was placed in the gel chamber containing 1xTBE buffer. The final level of buffer was5mm above the gel. The power supply was then connected and turned on to move the DNA from negative to positive

electrode (black to red). Electrophoresis was carried out at 120v for about 11/2 hour. After the bromophenol blue dye had reached three-fourths of the gel length, the electrophoresis was stopped and the power supply was disconnected. After electrophoresis, the gel was taken out carefully from the gel chamber and the gel gently washed in running water and placed on the UV transilluminator in the 254 nm range in the dark chamber of the Image Documentation System. The image was viewed on the monitor, focused, acquired and saved in a diskette, as well as printed on paper.

3. Results

3.1. Screening for RNA

Total RNA was extracted from 21 days seedlings of Bangladeshi cultivated rice Oryza sativa L. japonica varieties, BLB resistant Binashail rice which was collected from BINA; the poly (A) mRNA from the RNA was used to generate cDNA. The cDNA was processed and used to produce cDNA library. We used 100mg purely green leaf extract and all procedure was run under highly sterile condition. We used Ladder of 1kb which put on lane L, RNA1 is containing Band of RNA of BR14 rice and RNA2 is containing RNA band of Binashail rice. Total RNA was nearly 700 kb (Figure 1).

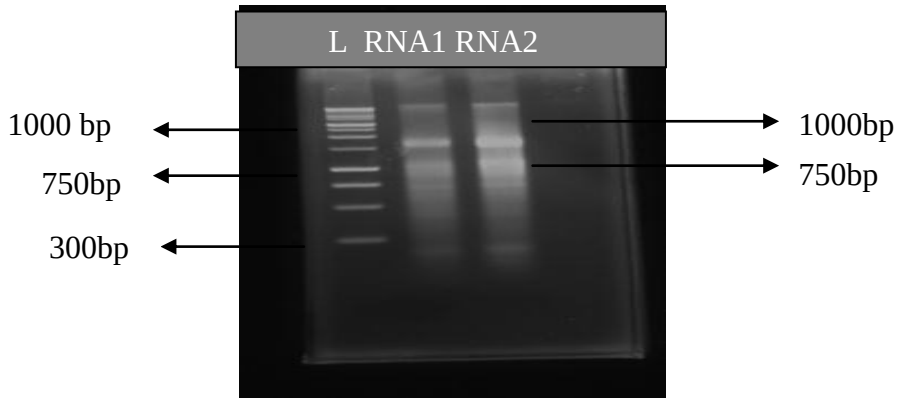

Figure 1. Detection of RNAof BR14 rice leaves. Lane L=Ladder, (1kb), RNA1= RNA band of BR14 rice leaves from 1st replication, RNA2= RNA band of Binashail rice leaves from 1st replication.

3.2. cDNA synthesis and quantitative RT-PCR analysis

Total RNAs were extracted using RLT, RPE and RW1 buffer according to the manufacturer's protocol (Invitrogen, USA). First strand cDNAs were synthesized in a reaction volume of 12µL. Quantitative RT-PCR (qRT-PCR) was carried out. One replicate was performed for the analysis of gene and for determining the relative expression levels. The PCR products were separated on agarose gel electrophoresis. They appeared as smear, the molecular weight of the cDNAs was ranged from 0.8 kb to 4.0 kb, but mostly were between 2–3 kb. It indicated that a relatively complete cDNA library could be synthesized using this improved RT-PCR technique. It was quite favorable to get genes having longer coding regions such as that of the tillering specific genes. DEPH water was used as an internal control for normalization. cDNA preparation and library construction for Illumina sequencing. Two deferent thermal profile was used in the protocol which was provided by RevertAid H Minis First cDNA synthesis kit. Genomic DNA was digested using DNase, and total RNA was isolated using the TRIzol reagent. The OligoTex mRNA mini kit (Qiagen) was used to purify poly (A) mRNA from the total RNA samples. In gel run process, Lane L=Ladder, (1kb), cDNA1= cDNA band of BR14 rice leaves from 1st replication, cDNA2= cDNA band of Binashail rice leaves from 1st replication. The first cDNA strand was synthesized usingOligo (dT) 18 primers. For high-throughput sequencing, the sequencing library could be constructed by following the manufacturer's instruction. cDNA of 250bp were excised. As Oligo (dT) 18 was binded with poly A tail so the band of cDNA was shown at the lower side of the gel on the gel documentation machine (Figure 2).

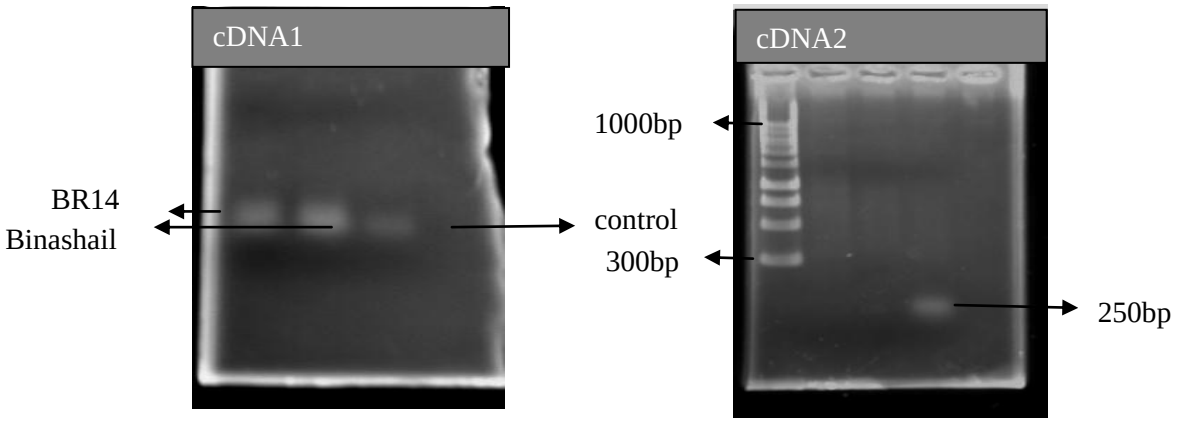

Figure 2. Detection of cDNA of Binashail rice leaves. Lane L=Ladder, (1kb), cDNA1= cDNA band of BR14 rice leaves from 1st replication, cDNA2= cDNA band of Binashail rice leaves from 1st replication.

3.3. Molecular detection of tillering specific gene by RT-PCR

One step RT-PCR was performed with a specific of primer RM for detection of tillering specific gene. Initially RM primer was used to conduct uniplex PCR using 1 sample. Primer RM5493 binds with locus pi55 (*t*) of tillering specific gene *R* at chromosome 8. During detection of tillering specific gene from BLB resistant rice Binashail Lane 1 is 1kb ladder and *=PCR product (tillering specific gene). Tillering specific 201bp band was observed in the sapmle Binashail (Figure 3).

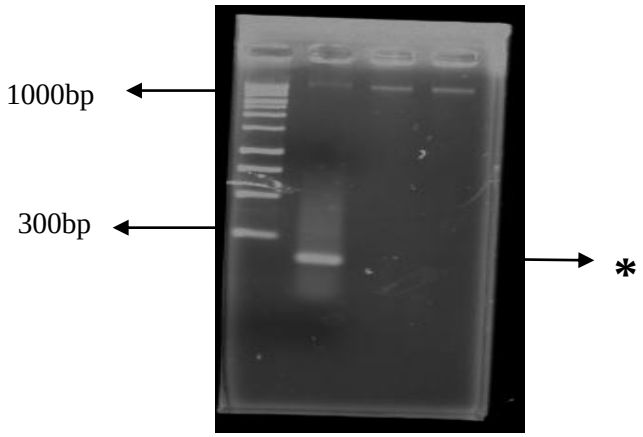

Figure 3. Detection of tillering specific gene by RT-PCR from BLB resistant rice Binashail. Lane 1=1kb ladder, *=PCR product (tillering specific gene)

So the sample was confirmed that it contained tilliering specific gene which is important for brnching capacity. Thus RT-PCR method can be successfully applied to detect tillering specific gene at field level.

4. Discussion

Gene isolation and cloning are considered to be the basic issues in molecular biology and genetic engineering (Xu *et al*. 2001). For cloning of cDNA with known sequences, RT-PCR is an usual technique, in which polyA$^+$ RNA is as template and oligo dT as primer. The first strand of the cDNA is synthesized by reverse transcription, then the target cDNA fragment is amplified using specific primers and Taq DNA polymerase, but the synthesized cDNA by this method is often relatively short.

Tillering is one of the most important agronomic traits because tiller number per plant determines panicle number, a key component of grain yield as a key regulator of tillering, tillering specific gene could make a significant contribution to future improvement of these crops.

Primer RM5493 showed band of 201bp at gel documentation and It bound with locus Pi55 (*t*) of tillering specific gene *R* which is present at chromosome 8 at location 26236354-26236335 and 26236154- 26236173. This gene is a recessive gene. (*Qing Hua et al*., 2012).

As RM5493 was used as a primer with the cDNA which was synthesized from BLB resistant Binashail rice and showed 201 bp band at gel documentation. So Binashail contains tillering specific gene *R* with Pi55 (*t*) locus and the location is 26236354-26236335 and 26236154- 26236173 at chromosome 8 . This DNA responsible for high numbers of tillering branching by how it can play an important role for better yield.

In present study, we synthesized a relatively complete cDNA library from the rice endosperm total RNA using a RT PCR cDNA Synthesis method, and most of the synthesized cDNAs were in the range of 2–3 kb. The synthesis operation was quite simple and can be accomplished in one day. The two cDNAs of the rice Binashail and BR14 we obtained, were both as long as 250bp, much longer than those obtained by oligo dT primer. It might be due to the SMART II oligo and CDS primer, which have contributed their effort during cDNA synthesis. We have performed the PCR after synthesis cDNA from the extracted RNA of the Binashail rice and found positive band at 250bp after electrophoresis on 2% agarose gel which was specific for Binashail rice. The uniplex PCR amplification product for tillering specific gene run on 2% agarose gel at 110volts for 30min. The product land for sample was confirmed and shown at the molecular weight 205bp for tillering specific gene of Binashail rice. So our sample was identified as for better branching capacity containing rice plant by molecular technique. On the basis of present studies it is clearly indicated that the sample of Binashail rice was mostly positive for tillering specific gene. From this study it is proved that the productivity of Binashail rice is sufficiently high.

5. Conclusions

From the study, it is concluded Rneasy plant mini ki and RevertAid H Minis First cDNA synthesis kit that can be sensitive, simple, efficient, powerful, highly informative, mostly mono locus, co-dominant, easily analyzed and cost effective tool for screening BLB resistant gene and develop cDNA library.

Conflict of interest

None to declare.

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