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mRNA extraction, cDNA synthesis and tillering specific gene isolation from BLB resistant rice BR14

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ABSTRACT

Tillering or the production of lateral branches (i.e. culms), is an important agronomic trait that determines shoot architecture and grain production in grasses like rice. Due to its agronomic and biological importance, tillering has been widely studied and numerous works demonstrate that the control of AXM initiation, bud development and tillering in the grasses is via a suite of genes, hormones and environmental conditions. The cDNA rice plant was constructed by an improved RT-PCR technique from a template cDNA library derived from the total RNAs extracted from the young plant leaves of a *Oryza sativa* rice to identify genes *pi55*(t) involved in the control of rice tillering. The identification of tillering specific gene in BR14 rice germplasm will help in accelerating the production rate of rice in future, including bacterial leaf blight (BLB) disease resistant genes in different varieties. BLB caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) is a major biotic stress in the irrigated rice belts. Genetic resistance is the most effective and economical control for bacterial blight disease. Branching capacity is the most important. Molecular survey was conducted to identify the presence of tillering specific gene in BLB resistant BR14 rice. Reproducible means of BR14 rice at 21 days was designed based on the timing of full expression of the leaf. Gel documentation showed that the size of the synthesized cDNA was 250 bp and tillering specific gene was 201bp long. PCR with specific primers RM5493 for tillering specific gene was used in the study. On the basis of the result of PCR, it was concluded that after PCR, the band that was found in 201bp was indicated tillering specific gene *pi55* (t).

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food for more than half of the world's population and it occupies almost one-fifth of the total land area covered under cereals (Bhuiyan and M.A.R. 2005). It is considered as a model cereal crop due to its relatively small genome size (2n-24), vast germplasm collection, enormous studies on molecular genetics resources and efficient transformation system (Prashant, *et al.*, 2006). To achieve a substantial improvement in grain yield in a limited period, a second green revolution based on advanced plant biotechnology and plant genomics is needed (Conway and Toenniessen, 1999). The production of rice is affected by a number of biotic and abiotic stresses. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causes bacterial blight (BB, formerly called bacterial leaf blight, BLB) is one of the major diseases of rice *Oryza sativa* L. in the world. Bacterial leaf blight attacks on leaves and leaf sheath of rice plant at tiller and booting stage (Ou, 1985; Arif *et al*., 2003; Chunming *et al.*, 2005). The BLB of rice, leads to crop losses of up to 50%. The BLB resistant genomic sequences of BR14 rice are not publically available, which limits the application of functional genomics to leading variety of rice in Bangladesh. Tillering in rice (*Oryza sativa* L.) is an important agronomic trait for grain production, and a model system for the study of branching in monocotyledonous plants. 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). If it is possible to introduce BLB resistance and high branching gene in local variety then the rate of rice production will grow higher. BR14 rice contains both genes and by identifying and isolating those genes from that rice, it will be possible to transfer the both genes into local variety to increase the production level of rice. Considering the above facts the present study was designed with the aims to construction of cDNA library from mRNA of BLB resistant rice, and to isolation of tillering specific gene from cDNA of BLB resistant BR14 rice.

MATERIALS AND METHODS

The experiment was carried out at Biotechnology laboratory, Department of Biotechnology; and Microbiology laboratory, Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh. The study was conducted from July to December 2013 to extract mRNA, synthesis of cDNA and isolate tillering specific gene pi55 (*t*).

Collection of plant materials and BR14 rice seeds

On the basis of previous research works based on phenotypic and genotypic evaluation, BR14 rice variety was selected as BLB resistant rice for isolation and identification of tillering specific gene. BR14 rice seeds were collected from Bangladesh Rice Research Institute (BRRI). Young rice leave samples were prepared under liquid nitrogen condition and RNA was extracted using one-step RNeasy kit of QIAGEN (Germany) according to manufacturer's instruction and 1st strand cDNA was synthesized by using Thermo Scientific DyNAmo cDNA synthesis kit followed by detection by RT-PCR. RM5493 primer was used to detect tillering specific gene from BLB

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resistant rice. Molecular works were done in Microbiology lab of Bangladesh Agricultural University.

Germination of seeds

BR14 seeds were germinated and seedling in Biotechnology tissue culture lab of BAU, Mymensingh. These samples are kept for germination for 4-5 days in tissue culture laboratory of Biotechnology department under 22-25°C.

Planting and collection of sample

Two hundred grams of rice seed (cv. IR 64, susceptible to Xoo race IV) was rinsed with tap water and soaked for 24 hours to stimulate germination. Seeds were planted in Petri dishes. After 4-5 days, seedlings were uprooted. Seedlings grown from seeds were directly transplanted to soil. Young fresh green leaves were collected from 21 days rice plants by using scissor and forceps under liquid nitrogen condition and leaves powder were formed by using mortar and pestle.

RNA extraction from rice leaves

Total RNA was extracted with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Twenty micro liter mercapto ethanol was added with 1 ml of RLT stock solution to make working solution. One part RPE stock solution was added with four parts of pure alcohol to make working solution. One hundred milligram of leave powder was used as sample for extraction of RNA. The sample leaves (tissue) was immediately placed in liquid nitrogen, and was grinded thoroughly with a mortar and pestle. Leaves powder and liquid nitrogen were decanted into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube. The liquid was allowed nitrogen to evaporate, but did not allowed the tissue to thaw. Step 3 was immediately proceeded. 450 µl Buffer RLT was added to 100 mg tissue powder and was vortex vigorously. Short 3min incubation at 56°C was helped to disrupt the tissue. β-ME was added to Buffer RLT. The lysate was transferred to a QIAshredder spin column (lilac) placed in a 2 ml collection tube and centrifuged for 2 min at full speed. The supernatant was carefully transferred of the flow-through to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. Only this supernatant was used in subsequent steps. The end of the pipet tip was cut off to facilitate pipetting of the lysate into the QIAshredder spin column. Centrifugation through the QIAshredder spin column was removed cell debris and simultaneously homogenized the lysate. While most of the cell debris was retained on the QIAshredder spin column, very small amounts of cell debris was passed through and formed a pellet in the collection tube. This pellet was not disturbed when transferring the lysate to the new microcentrifuge tube. 0.5 Volume of ethanol (96–100%) was added to the cleared lysate, and was mixed immediately by pipetting and was not centrifuged. Step 6 was preceded immediately. The sample (usually 650 µl) was transferred, including any precipitate that was formed, to an RNeasy spin column (pink) was placed in a 2 ml collection tube (supplied). The lid was closed gently, and was centrifuged for 15 s at ≥8000 x g (≥10,000 rpm). The flow-through was discarded. The flow-through was discarded after each centrifugation.700 µl Buffer RW1 was added to the RNeasy spin column. The lid was closed gently, and was centrifuged for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. The flow-through was discarded. The collection tube was reused in step 8. 500 µl Buffer RPE was added to the RNeasy spin column. The lid was closed gently, and was centrifuged for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. The flow-through was discarded. Then 500 µl Buffer RPE was added to the RNeasy spin column. The lid was closed gently, and was centrifuged for 2min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. The long centrifugation was dried the spin column membrane, ensuring that no ethanol was carried over during RNA elution. The RNeasy was placed spin column in a new 1.5 ml collection tube (supplied). 30–50 µl RNase-free water was added directly to the spin column membrane. The lid was closed gently, and was centrifuged for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA. The RNA yield would be 15–30% less than that obtained. Second volume of RNase-free water was used to get the final higher concentration of RNA.

cDNA synthesis

After thawing, mix and was briefly centrifuged the components of the kit and stored on ice. Following reagents were added into a sterile, nuclease-free tube on ice in the indicated order and the total volume of these reagents were 12g. Template RNA 2 µg, Oligo(dt) primer 1 µg, Water, nuclease-free 12 µg. The RNA template contained secondary structures, 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. The following components as 5X Reaction Buffer 4 µl, RiboLockRNase Inhibitor (20 u/µl) 1µl, 10 mMdNTP Mix 2 µl, RevertAid H Minus M-MuLV Reverse Transcriptase (200 u/µl) 1 µl were added in the indicated order and the total volume of these components were 20 µl. The solution was mixed gently and then centrifuged. $Oligo(dT)_{18}$ for cDNA synthesis was 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. For longer storage, 70°C was recommended.

Synthesis of rice cDNA library template by RT-PCR

The rice cDNA library template was synthesized directly from the total RNA of rice young leaves using RT-PCR according to the manufacturer's protocol (Invitrogen, USA) of cDNA Synthesis kit. First strand cDNAs were synthesized in a reaction volume of 12µL containing 3 µgRNase-free DNase I treated total RNA, 2µl Oligo(dT)18, 1 mM dNTPs, 10 mM dithiothreitol, and 200 units of SuperScript™ III reverse transcriptase. 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.

PCR Amplification of first strand cDNA

The PCR mixture including cDNA had total volume of 25 µl/ reaction was placed in the PCR tubes and run in the cDNA thermal cycler. The amplification of CDNA was

performed by PCR using the primers 5′-CACGACGTTGTAAAACGACGCGGTAACAAACCAACC AACC-3′ and 5′- AAAGCAGGACACAGTCACACAGG- 3′. PCR thermal condition was as follows: initial denaturation at 94°C for 3min, followed by denaturation at 94°C for 30sec, annealing at respective temperature of individual primer at 60°C for 30sec, polymerization at 74°C for 30sec, concluded by an extension step at 74°C for 3 min.

The PCR product was resolved in 1% agarose gel (450 ml) and stained with ethidium bromide (10mg/ml) solution for 25min. The image was viewed by gel documentation system and recorded in computer.

RESULTS AND DISCUSSION

The total product of RNA was confirmed through gel electrophoresis. Two bands of total RNA was shown nearly 700bp and 750 bp in the gel documentation (Figure1). The molecular weight of the cDNAs was 250bp (Fig 2).

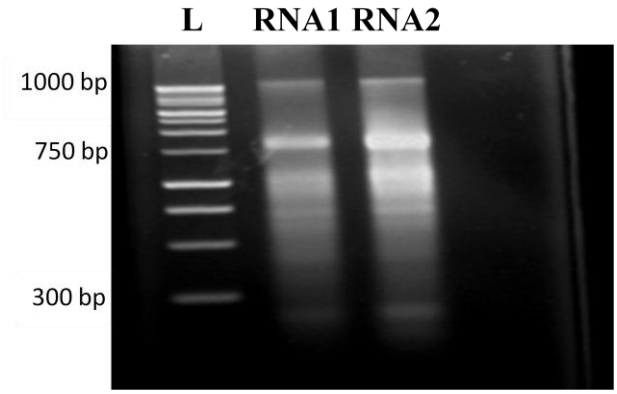
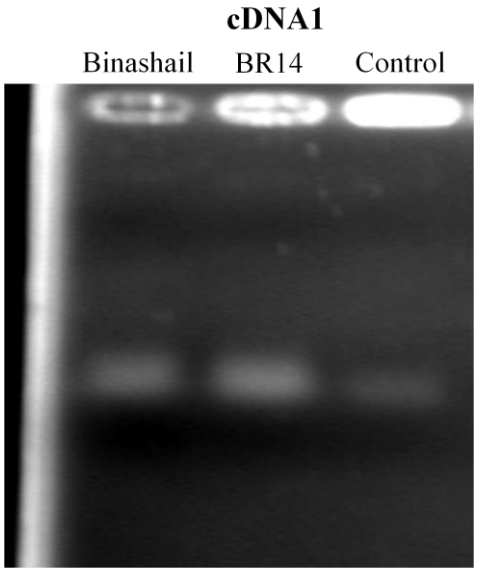


Figure1. Detection of RNA of BR14 rice leaves. Lane L=Ladder, (1kb), RNA1= RNA band of Binashail rice leaves, RNA2= RNA band of BR14 rice leaves.

Amplification of first strand cDNAs by PCR



By using the synthesized rice plant cDNA library as the template, the cDNA of tillering specific gene was amplified by PCR. The results showed that a specific band of about 250 kb was amplified in either PCR reaction, and the expected size of the amplified cDNAs was observed (Figure 2).

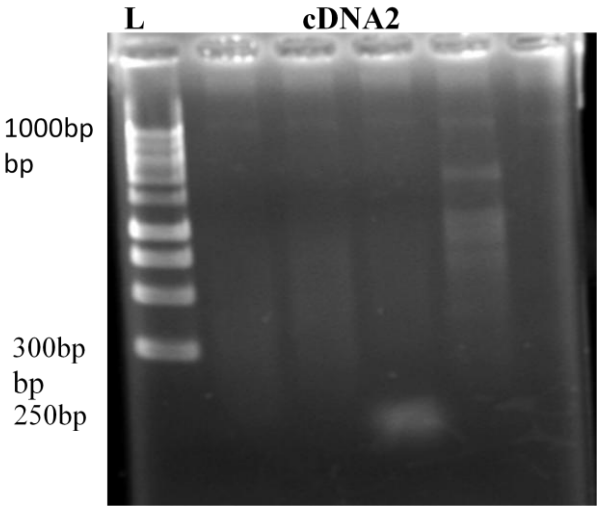


Figure2. Detection of cDNA of BR14 rice leaves. Lane L=Ladder, (1kb), cDNA1= cDNA band of BR14 rice leaves from first replication, cDNA2= cDNA band of Binashail rice leaves from first replication.

Molecular detection of tillering specific gene by RT-PCR

One-step RT-PCR was performed with a specific of primer RM5493that is a SSR marker and its annealing temperature 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. Tillering specific 205bp band was observed in the sample BR14 (Figure 3). So the sample was confirmed that it contained tilliering specific gene, which is important for branching capacity.

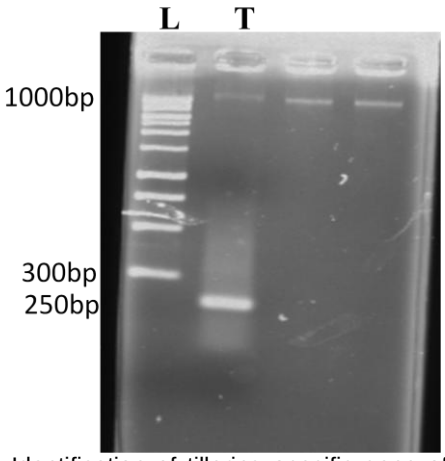


Figure3. Identification of tillering specific gene of BR14 rice leaves. Lane L=Ladder, (1kb), T = tillering specific gene of BR14 rice leaves from first replication, cDNA of BR14 rice leaves.

The research work was undertaken for isolation and identification of tillering specific gene from BLB resistant rice BR14. Tillering is a complex process in which expression of many genes must be fine-tuned and it 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. A tiller bud is normally formed at each leaf axil, but only those formed on the unelongated basal internodes can grow out into tillers and those formed on the elongated upper internodes become arrested.

Gene isolation and cloning are considered as the basic issues in molecular biology and genetic engineering. Several techniques have been developed and reported for gene cloning, for example construction of a cDNA library and then screening and identification of the expected genes. However, this activity was costly, laborious and time consuming (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. 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. As RM5493 was used as a primer with the cDNA, which was synthesized from BLB resistant BR14 rice and showed 201 bp band at gel documentation. So BR14 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 and branching and it can play an important role for better yield.

In present study, a relatively complete cDNA library was synthesized from the rice endosperm total RNA using a SMART PCR cDNA Synthesis method, and most of the synthesized cDNAs were in the range of 250-300bp. The synthesis operation was quite simple and can be accomplished in one day. The two cDNAs of the rice SBE we obtained, were both as long as 2.5 kb, 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.

The PCR was performed after synthesis cDNA from the extracted RNA of the BR14 rice and found positive band at 250bp after electrophoresis on 1% agarose gel, which was specific for BR14 rice. The uniplex PCR amplification product for tillering specific gene runs on 1% agarose gel at 110volts for 30min. The product band of tillering specific gene of BR14 rice for sample was confirmed and shown at the molecular weight 205bp. So this sample was identified as for better branching capacity containing rice plant by molecular technique.

PAN QingHua *et al*. (2012) used RM5493 primer to identify tillering specific gene *Pi55* (t) in *Magnaporthe oryzae* variety of rice. After PCR, they got band 201 bp at gel documentation. The same band after PCR was found in BR14 rice in this research. On the basis of present studies it is clearly indicated that the sample of BR14 rice was mostly positive for tillering specific gene. From this study, it is proved that the productivity of BR14 rice is sufficiently high. RT-PCR technique is very sensitive method of tillering specific gene isolation and identification.

Finally, the overall performance of RT-PCR method is very sensitive and quick which might be applied for the detection of economically important tillering specific gene of BR14 rice at field condition. On the basis of the findings of the present study, further studies are required for improvement and modification of the newly developed molecular method (RT-PCR) to make it more popular and economic for the detection of molecular gene at laboratory.

CONCLUSIONS

This work demonstrated the potential applying of genetic analyses in rice research and it was conducted for isolation and identification of tillering specific gene from BLB resistant rice. The results of this study may be utilized as the local source of gene *x21*gene for elite molecular breeding program and it can be used as a base for improvement of rice varieties in Bangladesh. More varieties that are resistant will improve yield of the crops and help address the food security issue facing the developing world. Considering the above information, it could be concluded that BR14 rice is the best source sample for isolating and identifying of tillering specific novel recessive gene pi 55 (*t*) gene. RT-PCR is more effective method for isolating and detecting mRNA, cDNA and tillering specific gene. BR14 rice is the resistant plant against BLB pathogen.

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