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Research Article

Efficient separation of PCR products of SSR markers linked with brown plant hopper resistance in rice using high-resolution metaphor agarose

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ABSTRACT

Detecting polymorphism is necessary for developing and mapping SSR markers, efficient selection of parents for crossing, and progenies carrying the gene of interest in segregating generations for an efficient backcross breeding program. Brown plant hopper resistance breeding studies in rice involve SSR marker-based screening and PCR analysis. In this study, metaphor agarose is compared with normal agarose gel electrophoresis for separating similar fragments or small difference bands. Rice SSR marker was analyzed in a susceptible and resistant variety and their F_1 progenies. PCR products were distinctly separated on metaphor agarose gel electrophoresis and showed parental polymorphism and F_1 hybridity confirmation with a high resolution of bands even within a 20bp difference compared to normal agarose gel. The results confirmed that Metaphor Agarose is an effective substitute for normal agarose and other electrophoresis techniques when compared on basis of cost, time, and need for specialized equipment and technology.

***Keywords*:** Brown plant hopper, metaphor agarose, PCR product, and SSR marker

Rice is the staple food for two-thirds of the world population which is ever-increasing and the demand for rice increases globally every year (Chacko *et al*., 2023; Adarsh and John, 2020). Along with the increasing population in the world, the constraints in crop production also increases such as depletion in cultivable land, and other resources, biotic and abiotic stress such as the incidence of pest and diseases, and drastic changes in climatic factors posing serious challenges. Among the biotic stress, pest incidence is a serious challenge that needs to be addressed (Sasmal *et al*., 2018). Changes in insect behaviour, unpredictable increase in pest incidence, evolving biotypes by adapting to the resistant cultivars, and continuous use of various pesticides results in complex insect-plant interactions. Modern breeding approaches viz., transgenic breeding, plant-mediated RNA interference technology, and genome editing have facilitated the development of varieties with durable and multiple resistance against insect pests. The recent advancements in gene/QTL mapping and functional genomic studies revealing the molecular basis of inheritance play a key role in novel insect pest-resistant variety development (Shi *et al*., 2023).

The brown plant hopper (*Nilaparvata lugens* Stal) is a typical phloem sap-feeding pest of rice resulting in hopper-burn symptoms and its role as a vector of rice grassy stunt virus and ragged stunt virus have aggravated the threat posed by the insect (Kumar *et al*., 2022). When a variety with a major gene becomes susceptible due to selection for a new biotype, another variety with new major genes for resistance should be released. Traditional varieties and crop wild relatives are the major sources of genes for brown plant hopper (BPH) insect resistance for varietal development and the effectiveness of genes is confirmed from stability analysis over multi locations (Chacko *et al*., 2018., George *et al*., 2021). This suggests that the breeding program should focus on using the most effective genes (Sharma and Ortiz, 2000; Chacko *et al*., 2023).

Classical markers were employed for species identification (Shahiba *et al*., 2022), and genotypic selection based on cytology or biochemical properties.

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With the introduction of DNA-based molecular markers, indirect selection of target traits, and mapping, the identification of desired genes is more efficiently accomplished rather than using morphological, biochemical, or cytological markers. A series of DNA markers are available now for the efficient selection of our gene of interest. RFLP (Restriction fragmented length polymorphism) triggered the development of molecular markers. Further, the development of PCR (Polymerase Chain Reaction) led to the development of PCR-based markers such as RAPD (Randomly Amplified Polymorphic DNA), ISSR (Inter-Simple Sequence Repeats), AFLP (Amplified Fragment Length Polymorphism), Simple Sequence Repeats (SSR), Sequence Characterized Amplified Regions (SCAR), CAPS (Cleaved Amplified Polymorphic Sequences), and significantly more. ESTs (Expressed Sequence Tags) and SNPs (Single Nucleotide Polymorphism) are third-generation molecular markers.

Among the molecular markers, SSR markers were the most preferred by plant breeders, geneticists, and molecular biologists due to the codominant nature of inheritance, highly polymorphic, reproducible, multi-allelic, extensive, and extensive genomic coverage, and high-throughput genotyping capacity (Amiteye, 2021; Jayalekshmy *et al.,* 2022; Indraja *et al*., 2022). SSRs are tandem repeat motifs of which dinucleotide repeats are the most frequent. These sequences are distributed almost evenly in plant genomes with a lesser number of repetitions but higher polymorphism. Unique sequences flanking the SSR locus are used to design a specific pair of primers for complementarily annealing the DNA template in the forward and reverse directions. Based on the genome sequence available or restriction/insert fragments the SSR primers are designed which may vary from one species to another. From a genomic library, SSR motifs can be identified using data mining software like FASTA, or from the *Unigenes* database (Jayalekshmy *et al.,* 2022). Computer-based programs such as Primer3.0 are often employed in designing SSR primers (Untergasser *et al.,* 2012). The amplification of sample DNA is obtained by the polymerase chain reaction (PCR) with the help of PCR-based SSR markers. The PCR technique produces multiple copies (2^n copies) of DNA from even a single copy of desired or target DNA fragment at the end of the '*n'* number of PCR cycles. The amplified PCR product or the SSR PCR fragment separation is done by gel electrophoresis (Singh and Singh, 2015).

PCR amplification product analysis is the key step to assess the quality and quantity of the amplified DNA target. PCR fragment separation in SSR marker analysis is accomplished by electrophoresis in agarose or polyacrylamide gels. Agar composes of two fractions namely, agaropectin (high amount of sulfate and carboxyl groups) and almost neutral fraction agarose (Jeppson *et al*., 1979). Agarose is one of the principal components of agar, which is extracted from certain red seaweed genera *Gracilaria* and *Gelidium*. Purification of agar by removing the agaropectin component yields neutral agarose which is structurally composed of repeating units of alpha-1,3 linked D-galactose and beta-1,4 linked 3,6-anhydro-L-galactopyranose by glycosidic bonds (Arnott *et al*., 1974). A supercoiled structure with a radius of 20 to 30 nm is formed by the aggregation of helical fibres, each of which contains about 800 galactose molecules. Depending on the agarose concentration, the fibres can be any length and are quasi-rigid, the fibres solidify to form a three-dimensional mesh of channels with an average pore size of more than 50 nm. The higher the agarose concentration smaller will be the pore size. As the three-dimensional structure is kept together by hydrogen bonds, boiling it back to liquid form has the potential to damage it. Agarose melts at a relatively high temperature of 85-90°C and on cooling at around 35°C, it forms the gel. This melting transition and gel setting includes the process of helix conversion, nucleation and helix-helix aggregation as the rare limiting step (Normand *et al.,* 2000).

Agarose gel electrophoresis (AGE) technique is efficient in separating, identifying, and purifying 0.1 to 25kb DNA (Aaij and Borst, 1972). The efficient separation of DNA fragments is mainly depended on the concentration of agarose used for gel preparation. Usually, 0.8 to 2% gels are prepared for the separation of fragments of size 0.2-3 kb. EtBr (Ethidium bromide) is the commonly used dye for staining the DNA fragments in the gel and observing under UV illumination (Bandyopadhyay *et al.,* 2022). Other than agarose concentration, the factors such as applied voltage (V/cm), electrophoretic buffer (Tris-Acetate or Tris-Borate), agarose type, DNA conformation, EtBr presence and tracking dye (bromophenol blue/xylene cyanol) affect the DNA migration in the gel (Lee *et al.,* 2012). The limitation of agarose gels lies in the separation of low-molecular weight nucleic acids and proteins. For separating fragments of DNA less than 100 bp, PAGE (Poly Acrylamide Gel Electrophoresis) and Capillary gel electrophoresis is preferred over AGE.

Poly Acrylamide Gel Electrophoresis (PAGE) is considered an alternative to AGE for PCR amplification product analysis by separating products of similar sizes with high resolution. The gel preparation is comparatively complex in PAGE as it is a free-radicle catalyzed reaction and

the presence of atmospheric oxygen can interfere with the polymerization (Davis and Ornstein, 1959; van Pelt-Verkuil *et al*., 2008).

Metaphor agarose gel electrophoresis (MAGE) is another electrophoretic technique that works on the same working principle and experimental setup as that of the AGE technique. The wide acceptance of this method over AGE and PAGE is due to its high efficiency in separating the similar-sized PCR products within less period of time and its easiness in preparation and handling of gels. PAGE is done on a vertical gel running unit whereas, MAGE can be done with ease using a horizontal gel electrophoresis system (Cheng *et al.,* 2012). The PCR-amplified products for waxy and aroma genes in rice were well differentiated in metaphor-based gel electrophoresis, the results of which were comparable with the polyacrylamide gel run method (Cheng *et al*.,2015). In rice, SSR markers or microsatellite DNA markers can be used for the detection of parental polymorphism, enabling plant breeders to select plants with the gene of interest. PCR-amplified products can be separated using diverse electrophoretic techniques such as AGE, PAGE, or capillary electrophoresis effectively. Easy separation of PCR products with very small differences is possible using the metaphor agarose gel electrophoresis (Akagi *et al*., 1996). Metaphor agarose has greater molecular sieving effects compared to other electrophoretic gels and can be reused 3-4 times but for the initial gene validation or parental polymorphism checking, whereas a fresh gel is recommended for better resolution (Asif *et al.,* 2008., Tomioka *et al.,* 2022).

The resolving power of an electrophoretic system is its ability to separate DNA molecules or amplified target DNA or other products of similar size (Ribeiro and Sutherland, 1993). PCR product resolution is often measured in base pairs. Resolving power is comparatively higher in capillary gel electrophoresis and PAGE compared to the AGE (Weiss *et al.,*1995). Polyacrylamide gels do not scatter light as they are transparent and clear whereas agarose gels are semi-opaque which scatters the light. But the gel properties of Metaphor agarose provide a comparable resolution as that of polyacrylamide gels and a better resolution than normal agarose (Cheng *et al*., 2015).

More than 40 genes have been mapped, out of which 11 are cloned and characterized. The majority of the genes are tracked using SSR markers for foreground, recombinant, and background selection in the segregating generations in order to develop or improve a variety with 3 to 4 backcrosses (Tanksley *et al.,*1989., Hu *et al.,* 2016). Detecting polymorphism is necessary for developing and mapping SSR markers, efficient selection of parents for crossing, and the selection of individuals/progenies carrying the gene of interest in segregating generations for an efficient backcross breeding program (Jayalekshmy *et al.,* 2020). But base pair difference of 2-5bp is not detected using the agarose gels due to its low resolution (McCouch *et al.,*2002). Instead, the PAGE technique is recommended for better resolution which is more difficult to prepare and handle, and time-consuming in gel preparation. Use of toxic monomers, leakage of gels, and no possibility of re-usage of gels are the other limitations of PAGE. In rice, the BPH resistance-linked markers are of similar size or with small differences which require fine separation for detecting parental polymorphism and further screening (Kristamtini *et al.,* 2016., Liu *et al.,* 2016., Shabanimofrad *et al.,* 2015). In order to cope with the difficulties and limitations of PAGE and AGE, this study is put forth for better resolution of PCR products with a small difference in size or similar size by using Metaphor agarose.

MATERIALS AND METHODS

Plant materials

In this experiment, two-parent genotypes namely, Jyothi (BPH susceptible rice variety) and PTB33 (resistant donor) along with their twelve F_1 populations were screened using SSR marker 'RM588' linked with BPH resistance.

Extraction of genomic DNA and quantification

Genomic DNA was isolated using the CTAB DNA extraction method (Doyle, 1991).

PCR amplification of SSR marker linked with BPH resistance

The SSR marker 'RM588' which is linked with the gene '*Bph3*' was analyzed in this study (Table 1). The reaction was executed in a reaction mixture of 25 microlitres consisting 20ng DNA template, 2 µl of dNTP mix, 2.5 µl of PCR buffer, 2.5 µl of $MgCl_2$ and 0.3 µl Taq DNA polymerase, and both forward and reverse primers (1 µl). Amplification compassed in an Eppendorf master cycler nexus PCR. Following PCR conditions were used: First denaturation at 94ºC for 5 minutes, denaturation at 94 º C for 30 seconds, the annealing temperature of 55.3 º C for 30 seconds, extension at 72 º for 1 minute for 35 cycles, and 8 minutes of final extension at 72°C.

PCR products separation using different gel electrophoresis methods

Agarose gel electrophoresis (AGE)

Agarose gel electrophoresis was carried out to resolve the PCR product in a horizontal gel electrophoresis unit of BIORAD. Agarose gel of

3% was used for PCR product separation in 1X TBE buffer. 3g of agarose powder (Genei) was added to 100 ml of the 1X TBE buffer taken in a 500 ml conical flask upon swirling so that the agarose will get mixed up in the solution easily and kept allowed to soak over 5-10 minutes. The conical flask was covered with aluminum foil and the agarose was melted at around 90°C in a microwave oven for 1 min initially followed by 1-2 mins melting for the complete dissolving of agarose. After a few minutes of cooling, 5 µl EtBr was added and mixed thoroughly. The solution was then poured into the casting tray for solidification and kept at 4°C for 10-15 min for better resolution. The comb was removed after the solidification of the gel and transferred into the BIORAD gel electrophoresis unit. PCR products were loaded into each well (8µl PCR product+2µl loading dye). A voltage of 60V for 2-3 hours was supplied for the electrophoresis of the PCR product. The gel was then visualized under UV light using the SYNGENE gel documentation system in order to analyze the SSR marker amplification.

Metaphor Agarose Gel Electrophoresis (MAGE)

Metaphor Agarose Gel Electrophoresis (MAGE) was also carried out in a horizontal gel electrophoresis unit of BIORAD for the PCR product analysis. Compared to normal agarose, the metaphor agarose has a low melting temperature (75°C), gelling temperature of less than 35°C, and gel strength of more than 300 g/cm^2. Metaphor Agarose gel of 4% was used for PCR product separation in 1X TBE buffer. 4g of metaphor agarose powder (Lonza, Rockland, USA) was added to 100 ml of the 1X TBE buffer taken in a 500 ml conical flask upon swirling so that the agarose would get mixed up in the solution easily and kept allowed to soak over 5-10 minutes. The conical flask was covered with aluminum foil with a small hole for aeration and the agarose was melted in a microwave oven for 1 min initially followed by cooling for 15 mins and 1-2 mins melting for the complete dissolving of Metaphor agarose. After a few minutes of cooling, 5 µl EtBr was added and mixed thoroughly. The solution was then poured into the casting tray for solidification, cooled at room temperature, and kept at 4°C for 10-15 min for better resolution. The comb was removed after the solidification of the gel and transferred into the BIORAD gel electrophoresis unit. PCR products were loaded into each well (8µl PCR product+2µl loading dye). A voltage of 60V for 2-3 hours was supplied for the electrophoresis of the PCR product. The gel was then visualized under UV light using the SYNGENE gel documentation system in order to analyze the SSR marker amplification. Proper care should be taken while preparing the gel and documentation as the gel is very sensitive to breakage.

RESULTS AND DISCUSSION

Agarose gel electrophoresis (AGE)

The SSR marker analysis of RM588 using agarose gel electrophoresis of 3% gave a band resolution at around 200bp for all the samples including the parents Jyothi (S-susceptible) and PTB33 (R-resistant) (Fig. 1). The presence of similar bands for the parental genotypes and progenies in 3% agarose gel hinders the detection of parental polymorphism between parents, Jyothi (S-susceptible) and PTB33 (R-resistant) and the hybridity of F_1 could not be confirmed. The SSR markers were widely used for the distinguishing the parental types and amplification of the specific bands in the hybrids. But the separation of PCR products of similar size or with small differences is poor in agarose gel electrophoresis technique (Barril and Nates, 2012). Higher the concentration of agarose gels, smaller the pore size. The normal agarose gels are effective in separation of bands ranged between 100 bp to 25 kb. Bands with less than 100 bp size is usually separated using a vertical gel electrophoresis system, the polyacrylamide gel electrophoresis (PAGE) technique (Chang *et al.*, 2023).

Metaphor agarose gel electrophoresis (MAGE)

Metaphor agarose gel of 4% was used for SSR marker analysis and the use of MAGE obtained better resolution of bands between the parents. RM588 showed polymorphism between the two parents, Jyothi (S) and PTB33 (R) which produced bands at 180 and 200bp respectively. A band size difference for 20bp was easily distinguished using 4% gel. The F_1 hybrids (Samples 1-6, and 10) were identified with the heterozygous band produced confirming the hybridity. Samples 7, 8, 9, 11 and 12 were having susceptible bands at 180bp depicting that these were selfed plants (Fig. 2). Parental polymorphism between two indica rice cultivars was assessed using SSR markers and 3% metaphor agarose clearly differentiated the PCR amplified products of two parents and the polymorphic markers were used for hybridity confirmation of F_1, foreground and background screening in marker-assisted backcrossing (Hangloo *et al.*, 2022). Pandita *et al.* (2023) used 2.5% metaphor agarose gel for the separation of PCR-amplified products in SSR marker analysis for genetic diversity study in fifty genotypes of rice. Similar results were obtained in the validation of SSR markers in grain amaranth genetic diversity analysis done using 4% metaphor agarose gel for efficient separation of final amplified PCR products (Vats *et al.*, 2023).

Table 1: Forward and reverse primer and sequence of SSR marker RM588

Primer	Sequence	Annealing temperature
RM588 F	5′-GTTGCTCTGCCTCACTCTTG-3′	55.3 º C
RM588 R	5′-AACGAGCCAACGAAGCAG-3′	

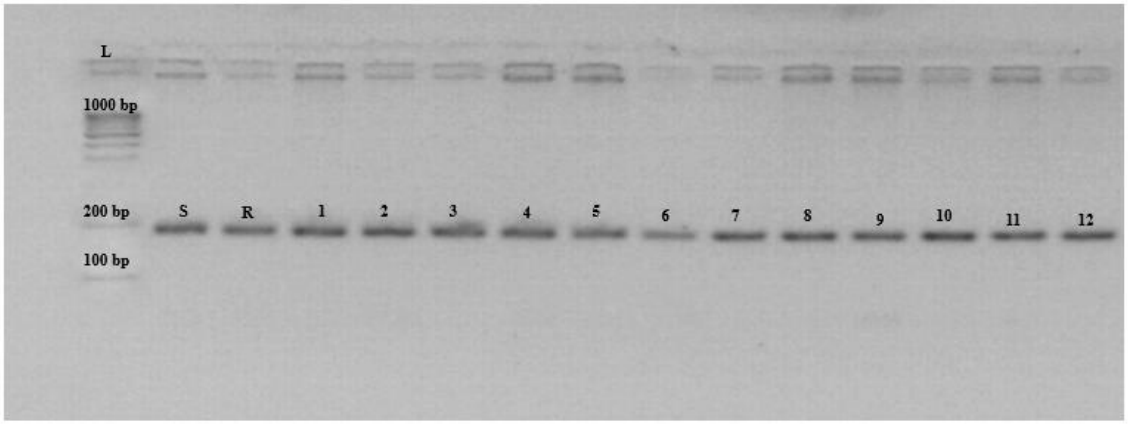

Fig. 1: PCR amplification profile in 3% agarose gel (Susceptible variety (S)- Jyothi, Resistant variety (R)-PTB33, 1-12 denotes the F_1 hybrids)

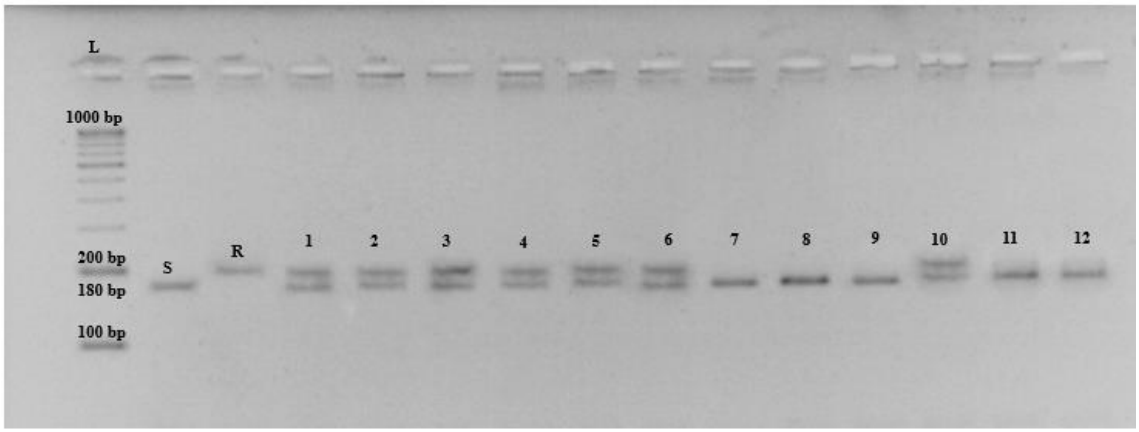

Fig. 2: PCR amplification profile in 4% metaphor agarose gel (Susceptible variety (S)- Jyothi, Resistant variety (R)-PTB33, 1-12 denotes the F_1 hybrids)

In concordance with our results, distinct separation of PCR amplified products of SSR markers were obtained using 3% Metaphor agarose gel in QTL mapping for BPH resistance in a cross between Rathu heenati (BPH resistant variety) and MR276 (BPH susceptible variety) (Shabanimofrad *et al.,* 2017).

Most of the SSR markers linked with brown plant hopper (BPH) resistance have small differences in size (base pairs) and the amplified products were easily separated on 4% Metaphor agarose gel with high resolution compared to normal agarose separation in this study. High gel strength and low electroendosmosis level of Metaphor agarose are its special characteristics making it the finest medium for PCR product analysis.

CONCLUSION

To summarize, the use of metaphor agarose gel electrophoresis (MAGE) in the present study could efficiently separate the PCR products of SSR markers linked with brown plant hopper (BPH) resistance in the parental genotypes, Jyothi (S-susceptible) and PTB33 (R-resistant) and F_1 hybrids. The highly resolved bands with a size difference of around 20 bp were precisely obtained in metaphor agarose gel electrophoresis. This was comparable with the normal agarose gel where all the bands resolved were of similar size and couldn't detect any parental polymorphism or hybridity. The high resolution of bands in MAGE makes it suitable to compare the product yield of PCR amplification of SSR markers linked with BPH resistance, provided the amplicons are of

similar fragment length or with small base pair differences. The field of molecular genetics uses Metaphor agarose for efficient PCR amplified product resolution in various studies viz., QTL mapping, SNP analysis, genetic diversity analysis, detection of genetically modified crops, mapping bio-fortification traits, RT-PCR assays, association mapping studies, and so on. Undoubtedly, Metaphor agarose is a boon to plant molecular biologists.

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