The Asian and Australasian Journal of Plant Science and Biotechnology ©2010 Global Science Books

Development and Evaluation of SCAR Marker for Blast Resistance in Finger Millet (*Eleusine coracana* (L.) Gaertn)

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ABSTRACT

Blast disease is caused by the fungus *Pyricularia grisea* and is a widespread disease in India and other countries affecting different aerial parts of the plant at all stages of its growth starting from seedling to grain formation. Specific DNA technology, such as the use of SCAR (sequence characterized amplified region) markers, can be used to differentiate between resistant and susceptible species of finger millet (*Eleusine coracana* (L.) Gaertn). SCAR markers were created by sequencing a single RAPD (random amplified polymorphic DNA) band and designing primers to amplify the band of specific size. PCR analysis was done using two F_2 bulks (one resistant and another susceptible) and two resistant F_3 bulk populations. RAPD primer OPA20 amplified bands of 250 and 750 bp in resistant bulks and absent in susceptible bulks. The fragments were cloned and converted into SCAR markers. Two SCAR primer pairs were designed. Testing was done with 3 resistant cultivars and one susceptible cultivar; single unique bands were correctly amplified for resistant cultivars by SCAR primer pairs. The availability of DNA markers linked to genes for resistance to this disease would accelerate breeding programs, particularly when other traits are also being evaluated.

Keywords: PCR, primer designing, RAPD, sequencing

INTRODUCTION

Finger millet (*Eleusine coracana* (L.) Gaertn) sub-species *coracana* belongs to the Poaceae family, Eragrostideae tribe. Finger millet, commonly known as *ragi* (India), *bulo* (Uganda), *wimbi* (Kenya) and *tellebun* (Sudan), is an important cereal crop for subsistence agriculture in dry areas of Eastern Africa, India and Sri Lanka. World finger millet production is about 4.5 mt of which 2.5 mt are produced in Africa only (ICRISAT and FAO 1996). In India, finger millet is grown on an area of 1.6 million ha with a production of 2.1 mt and a productivity of 1306 kg ha^{-1} (Anonymous 2002). Finger millet has an excellent nutritional value as its seeds contain protein ranging from 7 to 14% and is particularly rich in methionine, iron and calcium (Barbeau and Hilu 1993; Vadivoo *et al*. 1998). Even though finger millet is known to be one of the hardiest crops, it is affected by a number of diseases like blast, foot rot, smut, streak and mottling virus (Govindu *et al*. 1970). Blast of finger millet is a major disease caused by the fungus *Pyricularia grisea* (Cooke) Sacc. (formerly *Pyricularia oryzae* Cavara.), an anamorph of *Magnaporthe grisea* (Hulbert *et al.* 2001) Barr. (Rossman *et al.* 1990) that causes blast disease in rice. It is a heterothallic, filamentous fungus pathogenic to almost 40 plant species in 30 genera of the Poaceae including *Eleusine*. Blast caused by the fungus *P. grisea* is the most devastating disease affecting different aerial parts of the plant at all stages of its growth starting from seedling to grain formation. Yield loss due to blast may be around 28% (Vishwanath *et al.* 1986). In spite of a great deal of research on the pathogen and the disease, blast still remains a serious constraint to finger millet production in areas with conducive environments where the high yielding cultivars grown become susceptible. Incorporation of genes for resistance in breeding populations, varieties and hybrids is the most effective means to manage diseases such as blast. There are very few reports on molecular work in finger millet, protein nutrition of elite finger millet genotypes (Shashi 2005) or RAPD analysis (Fakrudin *et al.* 2006). The genetic map of finger millet was constructed by Dida *et al.* (2007). The amino acid, fatty acid and mineral content of black finger millet cultivated in Nigeria were assessed by Glew *et al.* (2008). Development of EST-SSRs (simple sequence repeats from expressed sequence tags) in finger millet and their transferability to pearl millet (*Pennisetum glaucum*) was done by Arya *et al.* (2009). A set of 31 SSR markers were used for PCR analysis of 11 elite germplasm lines of finger millet of Indian and African origin, amplification products were obtained for 17 primer pairs. These 17 SSR primer pairs were also tested for amplification in three varieties of pearl millet and 11 could be transferred to pearl millet.

DNA markers have revolutionized the field of genetics by their wide applications and also by increasing the pace of genetic analysis. In modern studies of plant genetics and breeding, molecular markers have become important and efficient tools for genetic diversity assessment, QTL and/or gene mapping, variety protection, and marker-assisted selection. The common molecular markers are RAPD (random amplified polymorphic DNA), SSR (simple sequence repeat or microsatellite), AFLP (amplified fragment length polymorphism), RFLP (restriction fragment length polymorphism) and SNP (single nucleotide polymorphism). Identification of diverse germplasm using diversity studies by the use of markers will help in current breeding programmes and MAS programmes.

To overcome the reproducibility problem associated with RAPD, RAPD markers have been converted into sequence-characterized amplified regions (SCAR). SCAR markers have been developed for several crops, including lettuce (Paran and Michelmore 1993), rice (Naqvi and Chattoo 1996), common bean (Adam-Blondon *et al.* 1994) and

Received: 4 March, 2010. Accepted: 30 April, 2010.

Research Note

apple (Evans and James 2003).

Evaluation of a finger millet crop is a time-consuming process, which requires expertise for the precise distinction between susceptible and resistant plants. The problems which arise can be better managed in breeding programs by identifying and using DNA markers linked to resistance genes. DNA markers are abundant and essentially independent from environmental conditions (Keim *et al.* 1989) and several research groups have been using this tool in breeding programs (Young and Kelly 1996; Young *et al.* 1998). In this paper, we report on the identification of DNA markers linked to finger millet loci responsible for resistance to blast in finger millet cultivars.

MATERIALS AND METHODS

Genetic material and crosses

Four bulks were used in the present study. Two bulks each from F_2: a susceptible bulk obtained from the cross of (PR202 X GPU28) X GE5421 and a resistant bulk obtained from the cross of (GE4971 X GPU26) X GE5579 and two F_3 resistant bulks obtained from each cross of (GPU28 X GE4931) and (GPU48 X GE4931).

DNA amplification

Total genomic DNA was extracted by the CTAB (2%) method (Sambrook *et al*. 1989) with modifications. RAPD analysis was performed with 200 decamer primers (primer sets: A, B, C, D, E, F, G, H, I, J) of arbitrary sequence obtained from Operon Technologies Inc. PCR was performed in 10 µl reaction volume containing 1 µl 10X buffer having 15 mM $MgCl_2$, 1 µl of each forward and reverse primer (5 pm/µl) , 1 µl of 2 mM dNTPs, 0.1 µl of 5 U of *Taq* DNA Polymerase (Invitrogen, USA) and about 10 ng of template DNA. PCR reactions were carried out in a PTC-100 thermal cycler (MJ Research, USA). Thermocycler parameters were: Initial denaturation step at 94°C for 3 min, followed by 45 thermal cycles of 92°C for 30 sec, annealing at different 36°C for 45 sec, 2 min extension at 72°C, a final extension at 72°C for 7 min and a continuous hold of 10°C at the end. PCR products of four bulks were size separated in adjacent lanes by electrophoresis in a 1.5% TAE agarose gel, stained with ethidium bromide and visualized under UV light. PCR was repeated thrice to check for the reproducibility of bands.

Cloning of RAPD fragments

Two bright and highly reproducible RAPD bands were selected to develop SCAR markers. DNA from RAPD markers associated with resistant pools was recovered from agarose gels using the QIAquick Gel Extraction method. The recovered DNA fragments were ligated once into pGEM Easy (Promega, USA) vector cloning kit as per the manufacturer's protocol. Ligation was carried out at 14°C for 16 h.

Competent cells of *E. coli* DH10B strain were prepared using 10% glycerol (Sambrook *et al.* 1989) and bacterial transformation was carried by electroporation. Colony PCR was carried out for the confirmation of DNA inserts in the selected putative recombinant clones. PCR was performed in a total reaction volume of 30 µl with M13 universal forward and reverse primers. PCR was carried out in a PTC-100 thermal cycler (MJ Research, USA) and the parameters were: Initial denaturation step of 95°C for 5 min followed by 30 thermal cycles of {95°C for 1 min, 55°C for 1 min and 72°C for 1 min}, with a final extension step of 10 min. PCR products were verified for cloned inserts by resolving the DNA fragments on a 1.5% TAE agarose gel.

Sequencing and analysis

Plasmids were isolated by alkaline lysis using the PEG precipitation method (Sambrook and Russell 2001) and were sequenced twice to check if there was any difference between the sequences. BLAST searches (Altschul *et al.* 1997) were performed *via* the National Centre for Biotechnology Information (NCBI) (http://www. blast.ncbi.nlm.nih.gov). The sequence was vector screened using Vec screen from the NCBI database. Primers were designed using Primer3 software. Primers were synthesized with MWG-BIOTECH Pvt. Ltd, Bangalore.

Testing of SCAR primer

PCR was performed (Paran and Michelmore 1993) using the primers designed and synthesized in a total reaction volume of 10 µl having 50 ng DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.5 ρmol of forward and reverse primers, 0.1 U *Taq* Polymerase (all Invitrogen, USA) were run in a PTC-100 thermal cycler. Thermocycler parameters were: initial denaturation step at 94°C for 4 min, followed by 35 thermal cycles of 94°C for 1 min, annealing at 60°C for 1 min, 1.30 min extension at 72°C, a final extension at 72°C for 7 min and a continuous hold of 10°C at the end. PCR products were separated by size in adjacent lanes by electrophoresis in a 1.5% TAE agarose gel, stained with ethidium bromide and visualized under UV light. PCR was repeated twice to check for the reproducibility.

RESULTS AND DISCUSSION

This investigation combined the use of classical breeding value prediction with selective DNA pooling using RAPD molecular markers, screening to detect markers associated with resistance. The approach used here to build the markers linked to resistance.

Once DNA were collected, we used RAPD screening to find bright and consistent bands specific to resistant bulks. From this data, one primer, OPA20 which amplified bright and consistent bands consistently among selected bulks (F_3 and F_2 resistant bulks and absence of the band in susceptible F_2 bulk), was chosen. Two bright and reproducible bands, approximately 250 and 750 bp in size, were amplified with the RAPD primer OPA20 only in resistant bulks. Both bands, indicated by arrows, were selected for conversion into SCAR markers (**Fig. 1**).

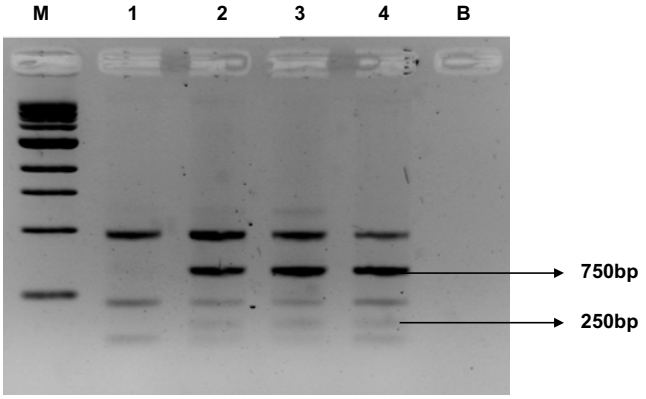

Fig. 1 Agarose gel electrophoresis pattern of bulks with Operon A20 primer. PCR was carried out as described in the Materials and Methods and the amplicons were separated on 1.5% TAE agarose gel and viewed under UV light after ethidium bromide staining. Lane M- 1 Kb ladder (NEB). Lane 1- F2 susceptible bulk - (PR202 X GPU28) X GE5421; Lane 2- F2 resistant bulk-(GE4971 X GPU26) X GE5579; Lane 3- F3 bulk - (GPU28 X GE4931); Lane 4- F3 bulk -(GPU48 X GE4931); Lane B- Control (without DNA).

Cloning selected RAPD bands

The PCR bands were cloned and sequenced; Three transformed white colonies from each excised band representing 250 and 750 bp in size (plasmid isolated) were selected for sequencing. The sequences of colonies were compared for each sample, and no differences were observed. The 750 bp band showed homology to NBS-LRR resistance genes from *Helianthus* and *Oryza sativa* (**Table 1**) whereas the 250 bp fragment did not show homology to any known resistance genes. In **Table 2**, the sequences of the designed primers are

Table 1 Blast homology of the 700 bp scar sequence.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF560158.1	*Helianthus tuberosus* NBS-LRR resistance-like protein RGC977 pseudogene, partial sequence	30.1	30.1	3%	9.3	90%
EF560119.1	*Helianthus paradoxus* NBS-LRR resistance-like protein RGC938 pseudogene, partial sequence	30.1	30.1	3%	9.3	90%
EF559751.1	*Helianthus deserticola* NBS-LRR resistance-like protein RGC570 gene, partial cds	30.1	30.1	3%	9.3	90%
DQ285630.1|	*Oryza sativa* (indica cultivar-group) putative nitrate-induced NOI protein (75-1-127BAC12.1), putative NBS-LRR disease resistance protein (Nbs1-Pi9),putative NBS-LRR disease resistance protein (Nbs3-Pi9), and NBS-LRR disease resistance protein(Pi9) genes,complete cds; putative NBS-LRR disease resistance protein (Nbs4-Pi9) pseudogene, complete sequence; putative NBS-LRR disease resistance protein (Nbs5-Pi9) gene, complete cds; putative NBS-LRR disease resistance protein (Nbs6-Pi9) gene, partial cds	30.1	30.1	2%	6.3	87%

BLAST searches (Altschul *et al.* 1997) were performed via the National Centre for Biotechnology Information (NCBI) web site.

Table 2 Primers designed from of the eluted sequences.

Primer	Forward	Reverse	Annealing temp (°C)
BHFMSCAR1	CTCAATGGGACGCATTTCTT	CGGTTGTAAGGGCACTGTTC	60°C
BHFMSCAR2	ACACAACTCCCGAACCACTC	AAGAAATGCGTCCCATTGAG	60°C

Two primers were designed based on sequencing results using Primer3 software each for 250 and 750 bp fragments.

listed. The SCAR primer pair BHFMSCAR1 was named after band amplification at approximately 250 bp. Similarly, primer BHFMSCAR2 was named after band amplification at approximately 750 bp. The primer pairs were first screened on two resistant DNA samples to determine the optimal annealing temperature (**Table 2**).

Testing designed SCAR primers

The SCAR primers were then used to amplify the DNA of four finger millet species (3 resistant and one susceptible species). A single, distinct, and easily identify band at approximately 500 bp (**Fig. 2**) was observed in resistant cultivars but was absent in susceptible cultivars with both the primers. Further testing of more cultivars representing the resistant species will be needed. This should be done to reconfirm the validity of the designed SCAR markers.

Since finger millet is predominantly grown as a rain fed crop by small farmers, disease management by chemical means is economically unaffordable. Hence, it would be useful for the disease to be managed with the inherent capacity of the plant. Growing resistant varieties is not only economical for minimizing the losses caused by the disease, but it is also an environmentally friendly method. Though many markers are used for screening of finger millet like RAPDs (Fakrudin *et al.* 2006), SSRs (Dida *et al.* 2008) and EST-SSRs (Arya *et al.* 2009) there is still a need for developing more efficient markers for screening of finger millet genotypes. These SCAR markers can be used for marker-assisted selection in breeding programs aiming at the development of cultivars which are resistant to blast, particularly when other traits are also being evaluated because they are simple, fast and inexpensive, do not require the use of radioactive isotopes, and can be scaled up for analysis of high throughput applications. SCARs have several advantages i.e. the SCAR markers are generally more allele specific, the SCAR amplifications are more stable and reliable, and more easily reproduced in different laboratories with various thermal cyclers, codominant SCARs are more useful in high-resolution mapping and genetic studies (Paran and Michelmore 1993). Furthermore, it is useful to map whole F_2 populations without losing genetic information and it can discriminate between different alleles identifying homozygous and heterozygous plants in segregating populations. SCAR markers can be more advantageous in commercial breeding programs if a quick plus/minus assay can be developed to detect the presence or absence of the product (Paran *et al.* 1993). Dominant SCARs are efficiently used in a breeding program if a quick plus or minus assay is needed to identify a locus (Gu *et al*. 1995; Melotto *et al.* 1996). Since only a single fragment is amplified, post amplification electrophoresis can be eliminated because the PCR products can be detected directly by staining with ethidium bromide in a microtiter plate or by measuring DNA concentration with an ELISA reader or a spectrophotometer (Weeden 1994). This would decrease the cost and increase the speed of the analysis.

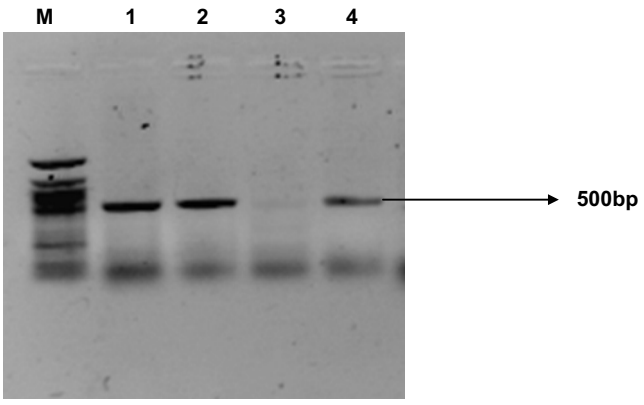

Fig. 2 Agarose gel electrophoresis pattern of individual plants with SCAR primers. PCR was carried out as described in the Materials and Methods and the amplicons were separated on 1.5% TAE agarose gel and viewed under UV light after ethidium bromide staining. Lane M- 1Kb ladder (NEB); Lane 1- GPU28; Lane 2- GPU48; Lane 3- PR202; Lane 4- VR847.

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