Dot blot assay technology: Low cost, non gel-based, high throughput technology for marker-assisted selection in cereals

A primary focus of the Generation Challenge Program (GCP) is the development of gene-based markers for drought tolerance using genomics and comparative biology. It is important that GCP works with end-users from an early stage to foster the integration of low-cost application of gene-based marker-aided selection (MAS) technologies, focusing on other target traits (bacterial blight resistance *Xa*-genes and quality protein maize *Opaque2* genes) as initial pilot projects concerning the proof-of-concept for technology product delivery pathways associated with gene-based MAS tools. One of the methods being developed under GCP Project 18 is the dot blot based-genotyping assay. This method uses nylon membrane filters, and is therefore a gel-free and low-cost MAS application for cereals. To maximize the usefulness of this technique, we have increased the throughput of DNA templates that could be spotted on the nylon membrane, which would also contribute to cost reduction. The overall objective of the project is to develop, refine and validate allele-specific gel-free assays for biotic stress and quality traits in cereals that are low-cost assays and require low capital set-up and unit costs for NARES and small and medium enterprises. We have developed and validated this method using SNP markers based on the bacterial blight resistance gene *Xa21* in rice and the *Opaque 2* gene for quality protein maize.

Materials and Methods

Rice and Maize germplasm

Recipient germplasm as source material for sequence comparison of the *Xa21* alleles and alleles at the *opaque2* locus were provided by NARES partners:

- Seeds and DNA of recipient susceptible rice varieties from Indonesia (9, *M. Bustamam*), Philippines (2, *E. Redoña*), China (4, *J. Wu*), India (5, *P. Kadirvel*), and Africa (2, *V. Verdier*).
- QPM donor sources (8) from diverse agroecological zones (mainly tropical lowland and subtropical) and non-QPM recipient sources (9) from tropical highland, tropical lowland and sub-tropical regions.

Rice lines for validation of dot-blot assay

- Basmati-derived F9 recombinant inbred lines (62 lines)
- IRBB21 and IR24 as sources of resistant (R) and susceptible (S) *Xa21* alleles, respectively.

Primer and Probe design

For Rice

- Sequences of susceptible *Xa21* allele from IR24, Vandana, Moroberekan and from other donor and recipient materials were aligned and compared with the *Xa21* R allele from IRBB21 (Fig. 2).

Figure 1. A portion of *Xa21* sequence showing an indel (yellow bar) in the kinase domain of *Xa21* R allele from IRBB21 and S allele from recipient varieties. SNPs discriminating R and S alleles are indicated by arrows.

- Two groups of oligonucleotide probes were designed based on: (a) diverged regions and indels, and (b) SNPs between R and consensus S allele sequences.
- Only one of the probes based on SNPs has been used so far (Table 1). The probes were sent for commercial synthesis and labeling (Proligo).

Xa21 R allele	*Xa21* S allele	Substitution	Sequence site
5'-ACACGTAAaTTTACATAA-3'	5'-ACACGTAAtTTTACATAA-3'	A/T	161-178
5'-CATTGTTTgGCTGCTTAG-3' SNPDB_513-530_*Xa21*R	5'-CATTGTTTcGCTGCTTAG-3' SNPDB_513-530_*Xa21*S	G/C	513-530
5'-GTTATGTCTcGTAATGTT-3'	5'-GTTATGTCTtGTAATGTT-3'	C/T	668-685

Table 1. SNP-based oligonucleotide markers designed from the sequences of IRBB21 and IR24. Highlight shows the probe pair that was used in this experiment.

For Maize:

- Reference sequence of *Opaque 2* in Genbank and maizegdb was used to design primers to amplify larger regions for sequence comparisons.
- Several potentially useful SNPs and indels were found that were polymorphic between the donors and some or all of the recipients.
- Three polymorphisms have been selected for further study; they are:
  - SNP1 (315) C/T
    - -perfectly cosegregates with *o2* phenotype
    - -C found in 7/7 donors and T in 9/9 recipients
  - SNP2 (612/613) AT/TC/AC
    - -AT cosegregates with 7/7 donors and 1/9 recipients.
  - SNP/INDEL 3
    - -7 base insertion present in 6 of 8 recipients.

Dot blotting of PCR amplified DNA

The protocol used to perform dot blotting in rice also worked well with maize with a few minor modifications. Here are the general steps:

1. DNA from the test lines were amplified using *Xa21* primers for rice and the *o2* primers for maize to generate the alternate alleles for both
2. PCR products were diluted in water and denatured at 95˚C for 5 minutes.
3. Then 1-2µl were spotted using a single or multi-channel (96 sample blot) pipet onto positively charged nylon membrane (Fig.2A). Two blots were prepared to genotype both alleles (R allele and S allele for *Xa21* in rice; Opaque2 and normal allele in maize).
4. The spots were air dried and then UV-crosslinked (Fig.2B).
5. The membranes were hybridized with the probe at 42˚C overnight using the DIG Easy Hyb hybridization buffer (Fig.2C). High and low stringency wash concentrations were 0.5X SSC + 0.1% SDS at 60°C and 2X SSC + 0.1% SDS at room temperature, respectively.
6. Washing and blocking steps were performed using Roche's DIG Wash and Block Buffer Set while chromogenic detection was performed using Roche's DIG Nucleic Acid Detection Kit according to the manufacturer's recommendations.

Fig. 2 Steps during the dot blot process: (A) template spotted, (B) then UV crosslinked before (C) hybidizing with the probe at 42°C. (D) Blot shown right after detection.

To lower the cost of the whole process, IRRI and CIMMYT tried some cost saving measures like using homemade buffers and increasing the throughput.

For the 384 format, replications of undiluted PCR amplified DNA (from a 96 sample set) were denatured then spotted manually (Fig.3) using the High Density Replicating Tool (HDRT) of the Biomek® 2000 Laboratory Automation Workstation (Beckman Instruments, Inc.). Competitive hybridization was performed at a 10:1 ratio of unlabeled probe to labeled probe. Processing of the blot followed the same procedure as that for the 96 sample blot.

Figure 3. The process of spotting denatured PCR products onto a nylon membrane using the 384 pin Biomek® HDRT

Results and Discussion

Using the SNP-based probes SNPDB_513-530_Xa21R and SNPDB_513-530_Xa21S, discrimination was observed between the R and S alleles (Fig. 3). Since the RILs have been previously characterized using a polymorphic *Xa21* marker, we were able to map the expected results in comparison with the blots. The probes were able to distinguish between R and S alleles.

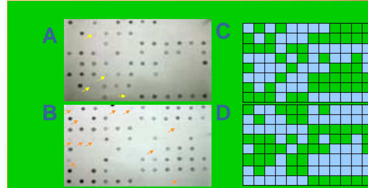

Fig. 4. Ninety-six samples blots and maps of the expected results. Yellow and orange arrows on the blots indicate background spots (A) R blot probed with SNPDB_513-530_*Xa21*R. (B) S blot probed with SNPDB_513-530_*Xa21S*. (C) and (D) are maps of the expected results of thr R blot and the S blot, respectively.

Increasing specificity of probe:allele hybridization

To reduce detection of background spots (Fig. 3A and 3B), competitive hybridization (Shirasawa et al, 2006) was employed in a ratio of 5:1 unlabeled probe to labeled probe. Adding the unlabeled probe of the alternate allele led to (1) increased specificity in hybridization, (2) increased detection of the desired allele, and (3) reduced background signal (Fig. 5).

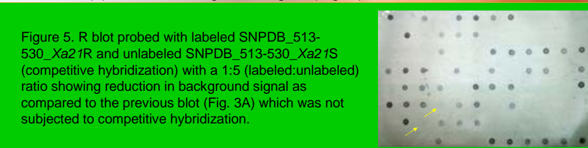

Figure 5. R blot probed with labeled SNPDB_513-530_*Xa21*R and unlabeled SNPDB_513-530_*Xa21*S (competitive hybridization) with a 1:5 (labeled:unlabeled) ratio showing reduction in background signal as compared to the previous blot (Fig. 3A) which was not subjected to competitive hybridization.

Eliminating sources of possible cross-hybridization

We hypothesized that the source of cross-hybridization could be due to the heat denaturation step at 95˚C. Thus, we used 0.4N NAOH (Shirasawa et al, 2006) for DNA denaturation spotted on the blot. However, allele detection took ~7 hrs with associated background in comparison to 45 min with 95˚C denaturation for the same intensity of chromogenic detection. Thus, heat denaturation at 95˚C is recommended.

Increasing throughput and reducing cost

- Increasing the number of data points on a blot (IRRI), and replacing reagents in the kit with homemade wash buffers (CIMMYT) reduced costs . Spots in the 384 blot spotted using the Biomek® HDRT were not consistent yet, but increasing the throughput is still possible with modifications for optimum results (Fig. 5).
- Homemade buffers -- Tris buffer and Maleic acid buffer-- were used to replace wash buffers in the kit. Use of homemade buffers resulted in distinct spots (Fig. 6).
- Separate cost estimates by IRRI and CIMMYT showed that increasing the throughput and using homemade buffers can reduce the cost (Table 2A, 2B and 2C).

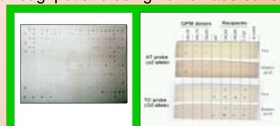

Figure 5A) Dot blot for the 384 format. 5B) Dot blots using Tris and maleic acid buffers

Table 3. Cost analysis for a) dot blot, b) gel electrophoresis and c) using homemade buffers

Summary

The dot blot assay can be an alternative method to traditional gel-based genotyping for MAS. It is sensitive enough to detect probe:allele hybrids even with single-base SNP. Increasing data points to 384 per blot and using homemade buffers can reduced the total cost for genotyping. We will continue to optimize the 384 dot blot platform as well as conduct a detailed cost analysis to reduce cost per data point while increasing throughput.

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Generation

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