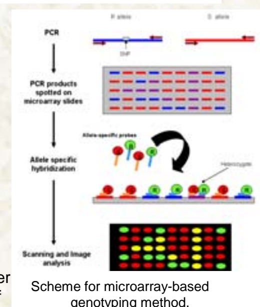
Development of gene-based marker technologies for high throughput genotyping

We are developing three alternative genotyping methods for marker-assisted selection (MAS) application in a collaborative project between IRRI, CIMMYT, and NARES partner institutions. Two methods, microarray- and FRET-based genotyping, are being developed as high-throughput and cost-effective techniques that could be utilized in regional laboratory hubs/core genotyping institutes while two other methods, dot blot-based genotyping (see accompanying poster) and PCR-ELISA, are being developed as low-cost approaches that can be adopted in laboratories with limited resources. We are validating these methods using previously developed markers and characterized or cloned bacterial blight resistance genes (*xa5, Xa7,* and *Xa21*) in rice.

Materials and Methods

Microarray-based genotyping (MBG)

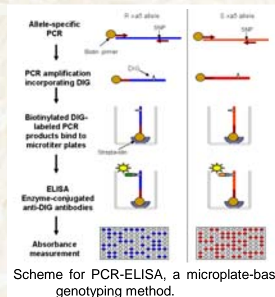
- ***DNA samples.*** A standard set of 96 DNA samples of rice accessions was prepared for validation using *xa5*. This set included numerous control accessions with and without *xa5* genes, popular rice varieties and a segregating recombinant inbred population for four bacterial blight genes and quality rice.
- ***Microarray slide printing.*** Microarray slides were printed using a GeneTac G3 Arrayer. Synthetic oligo nucleotides (single-stranded DNA) that are perfect complementary matches for the *xa5* detector probes were spotted on slides as positive controls for hybridization along with the replicated 96 standard DNA set
- ***Hybridization.*** Fluorescently labelled detector probes 12- and 26-mer specific to the R and S *xa5* alleles were hybridized at temperatures of 35° and 40°C (12-mer) and 58°C (26-mer) were done. Competitive hybridization using unlabeled probes specific for the S allele was done to decrease cross-hybridization.



Scheme for microarray-based genotyping method.

PCR-ELISA

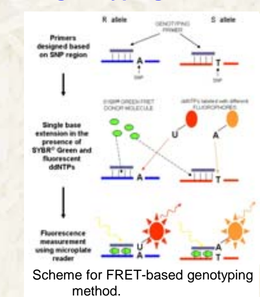
- ***PCR and Dig-labelling.*** New PCR primers were tested for *xa5*-allele specificity using 96 priority lines. PCR reaction conditions (especially annealing temperature) were optimized until PCR primers discriminated between *xa5* resistant and susceptible lines. PCR products were labeled with digoxigenin-11-dUTP during PCR (dig-11-dUTP was obtained from Roche Diagnostics).
- ***ELISA.*** Enzyme immunoassay was performed using rice varieties IR24 and IRBB55 as susceptible samples, IRBB5, IRBB54 and IRBB56 as resistant samples, and no template control (NTC) PCR product as negative control. Phosphate-buffered saline (PBS), used to dilute the PCR products, was also used as a negative control.
- ***Digoxigenin titration.*** To determine the optimum concentration of DIG-label to reduce non-specific binding, DIG-labelling was done during PCR using different ratios (1:3 and 1:20) of dNTPs:DIG-dUTP.



Scheme for PCR-ELISA, a microplate-based genotyping method.

Fluorescence Resonance Energy Transfer (FRET)-based genotyping

- ***Control test using synthetic oligonucleotides.*** Control test was performed using synthetic oligonucleotides as single-stranded DNA template for the single base extension (SBE). This control was used to ensure that SBE and FRET could successfully occur using single-stranded template, in the absence of contamination caused by the dNTPs and primers which were carried over from PCR.
- ***Generation of single stranded DNA (ssDNA) template.*** PCR products were treated with different concentrations (0.25 to 10 U) of Lambda Exonuclease to degrade double-stranded DNA (dsDNA) and generate ssDNA.
- ***Validation of FRET assay using Xa21 SNP.*** Primers for PCR and SBE based on the sequence of the *Xa21* resistant lines (Hui333, Hui593 and Hui811) and *Xa21* susceptible lines (IR24 and Hui161) were synthesized. SBE was done following the method of Takatsu, et al (2004) with one modification. Gradient test was made to modify annealing temperature (Ta) so that single copy PCR products were generated. Fluorescence intensity was quantified using a FLUOstar OPTIMA microplate fluorometer (BMG LabTechnologies Ltd.).



Scheme for FRET-based genotyping method.

Results

Microarray-based genotyping

- Scanning analysis indicated that hybridization did not occur when 40° C was used; a partial hybridization occurred when using a hybridization temperature of 35° C, however the signal was extremely weak and effects of uneven hybridization were observed.
- Results with 37° C manual hybridization for 14 hrs with 12-mer R probe showed good discrimination between the R and S complementary oligos spotted on the slide; no hybridization was observed on the S oligos. However, cross hybridization was observed with the PCR products from S genotypes, probably due to length of the probe in relation to the template DNA and DNA purity.

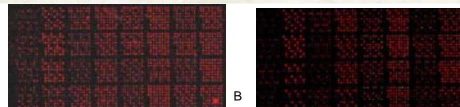


Fig. 1. Microarray slides: (A) hybridized with 100uM 26-mer R probe labeled with AlexaFluor 647, and (B) hybridized with 50uM 26-mer R probe labeled with AlexaFluor 647

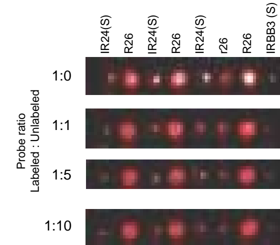


Fig. 2. Results of competitive hybridization. Unlabeled 26-mer oligonucleotide complementary to S allele was hybridized along with labeled R probe at different ratios. Hybridization temp = 58°C. Samples spotted: IR24, R26 (26-mer complementary to R probe, 2uM), r26 (26-mer complementary to R probe, 0.2uM) and IRBB3. Labeled probe = 10uM.

- Cross-hybridization was observed but was less as compared to previous experiments using a 12-mer detector probe (hybridization temperature at 37°C). No detectable difference was observed in hybridization strength when using 50uM and 100uM probe concentrations.
- 26-mer probe concentrations of 50, 25, 15 and 10uM were also used. Slight hybridization was still present (Fig. 1).
- Competitive hybridization (Shirazawa, 2006) using unlabeled S probes at two-, five- and ten-fold volumes decreased intensities of unspecific spots (Fig. 2).

PCR-ELISA

- Background readings were still present in the "no-DIG" set-up which might suggest that the anti-DIG-POD conjugate binds to the wells at some extent.
- Increasing the number of washes to six or seven might decrease color development in these wells.
- The ratio of DIG concentrations (1:3 vs. 1:20) gave no significant difference in their optical density values (Fig. 3).

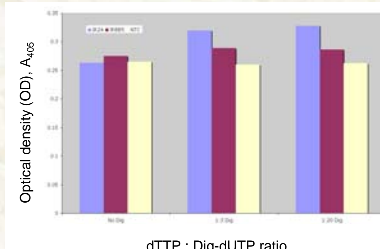


Fig. 3. Optical densities Dig-labeled susceptible allele at different levels of incorporated Dig-dUTP.

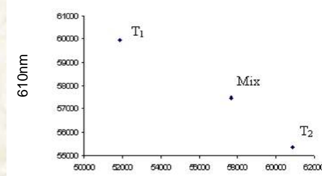


Fig 4. Fluorescence intensity ratios of *Xa21*-resistant (T1), susceptible (T2) and heterozygous lines. ROX®-ddATP and Cy5®-ddUTP were used as labeled ddNTPs for T1 and T2, respectively.

FRET-based genotyping

- SBE and FRET were successful because a higher fluorescence compared to the background was observed at 610 nm and 670 nm when Rox-ddATP and Cy5-ddGTP were used, respectively. Single stranded DNA (ssDNA) was derived from the phosphorylated PCR product. ssDNA is the template for SBE. 10 U of Lambda Exonuclease was necessary to generate ssDNA
- Results from the validation experiment indicated that FRET occurred and while there were moderate levels of background fluorescence, resistant and susceptible lines could be distinguished (Fig. 4).

Plans/Recommendations

- ***Microarray-based genotyping.*** **Hybridization stringency will be increased, including increasing concentration of competitive probe**
- ***PCR-ELISA.*** **Additional blocking step after immobilization of biotin-labelled PCR product will be done to decrease unspecific binding of antibody to the wells. Also, lower DIG-dUTP concentrations of 1:50 during Dig- labelling will be used in future experiments**
- ***FRET.*** **Validation experiments with improved PCR clean up procedures will be done, aimed at reducing background reading.**
- **Options for cost reduction will be identified for each technology while maximizing output.**
- **Evaluation, refinement, and deployment of technology with NARS and SMEs in Asia and Africa and for regional hub laboratories will be linked with the activities of existing networks (e.g. ARBN and AMBIONET) in Asia and Africa.**

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Acknowledgements

Funding from the Generation Challenge Program is gratefully acknowledged.

E. Mercado[1], M.Y. Reveche[1], J. Chen[2], D. Skinner[3], G. Carrillo[1], B.C.Y. Collard[1], M. Bernardo[1], M. William[3], Y. Xu[3] and C.M. Vera Cruz[1]

[1]International Rice Research Institute, Metro Manila, Philippines; [2]China National Rice Research Institute, Hangzhou, China; [3]CIMMYT, Mexico