

# Low-Cost Marker Technologies in Major Cereal Crops: Development of low-cost gene-based trait assay technologies in cereals

Generation Challenge Program  
Commissioned Research # 18



# Partner Institutions and Collaborators

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SME breeding company





# Proof-of-concept for technology product delivery pathways

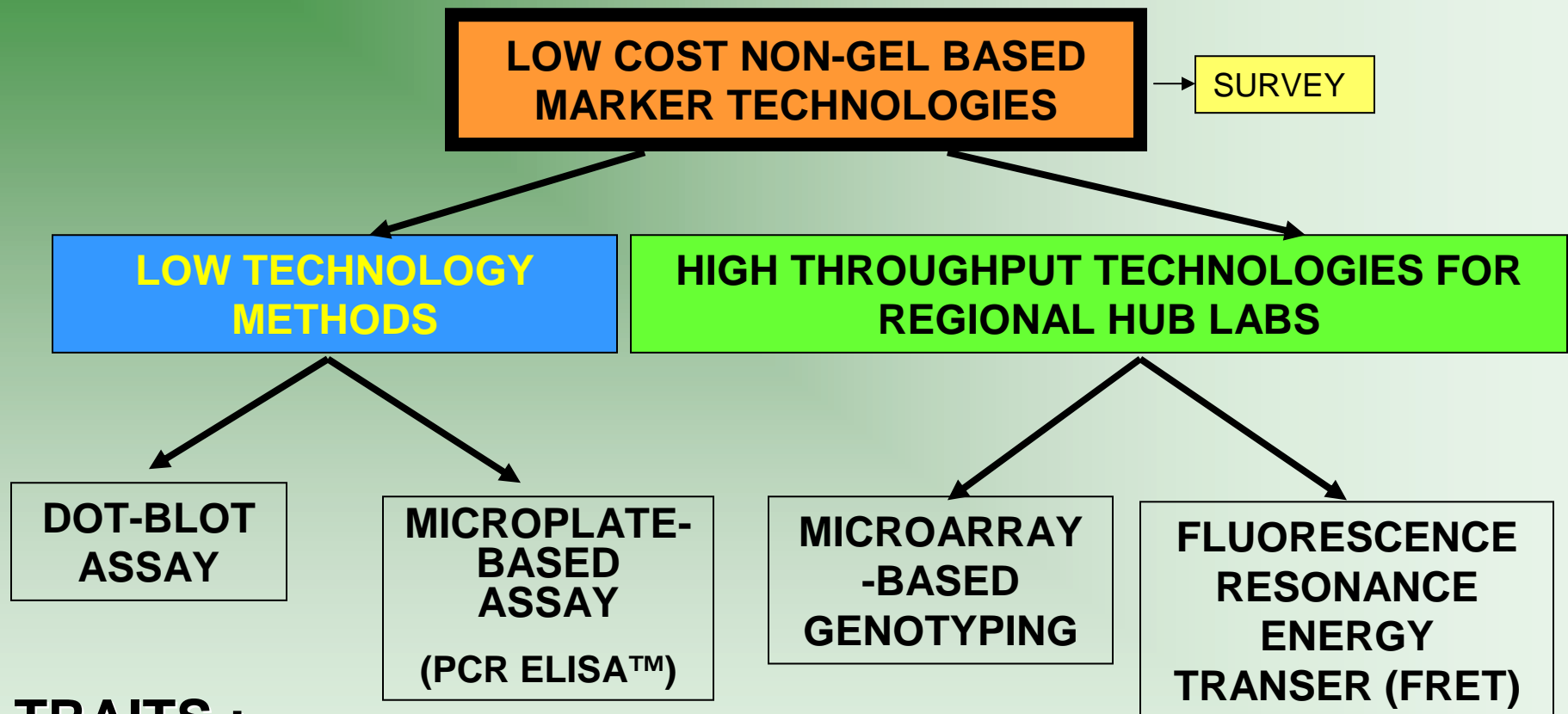
- Goals:
  - To generate MAS technologies that are attractive, cost effective and useful for a range of molecular breeding applications targeting a variety of traits.
  - To validate the product delivery pathway for GCP outputs (particularly gene-based markers) confirming the cost-effectiveness and efficient transfer to various types of end-users with different needs and capacities.
- Targeting traits with a somewhat simpler genetic basis than drought tolerance

# Objective:

- To develop, refine and validate allele-specific gel-free assays for biotic stress and quality trait in cereals that are:
  - ❑ low cost, low technology assays that require low capital set-up and unit costs for NARS and small and medium enterprises, and
  - ❑ low cost, high throughput assays for shuttle genotyping regional hubs in collaboration with national partners



# APPROACHES



## TRAITS :

- Bacterial blight (rice) resistance & *Xa* genes
- QPM (maize) & Opaque2 (O2) alleles



# Gel-based STS markers available for BB *Xa*-genes

Gene	Chrom	Linked marker	Distance (cM)	References
<i>Xa4</i>	11	Npb181	1.7	Ma Bo-Jun et al, 1999
<i>Xa5*</i>	5	RG556	0-1	McCouch et al, 1991
		TFIIA $\gamma$	0	Iyer and McCouch, 2004
<i>Xa7</i>	6	P5	0	Porter et al, 2000
<i>xa13</i>	8	RG136	3.8	Zhang et al, 1996
<i>Xa21*</i>	11	pTA248, Kinase domain	0-1, 0	Ronald et al, 1992; Song et al, 1995



## F5 Basmati-derived line (IR71730-51-2/IRBB60) carrying *Xa4*, *xa5*, *xa13* and *Xa21* using MAS

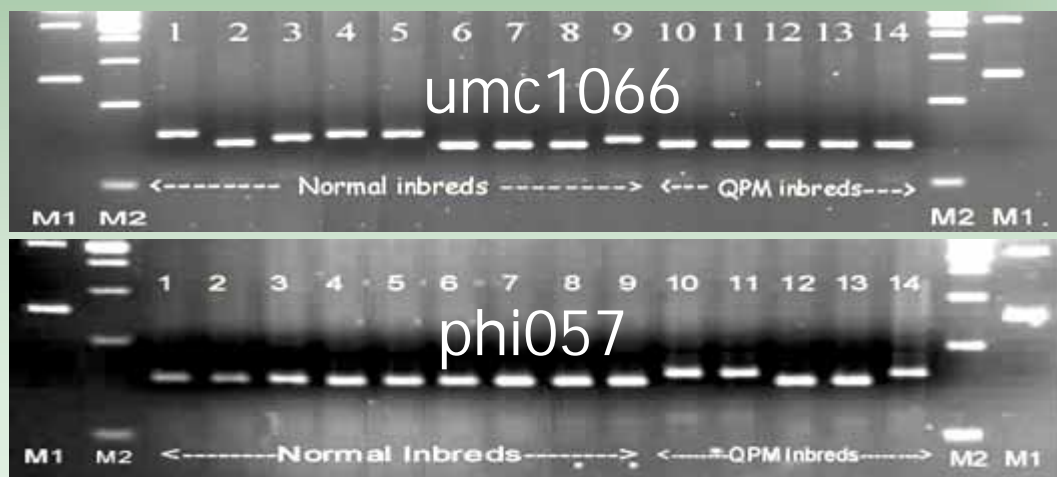


Begum, Virk, et al.

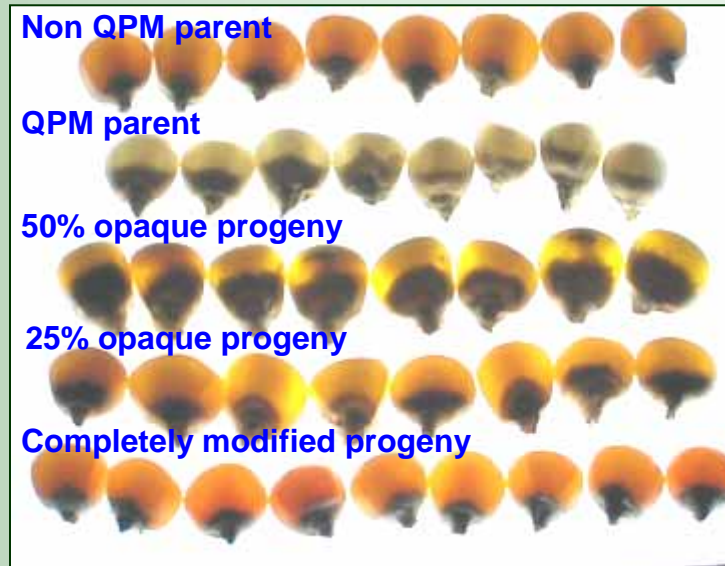
# Quality Protein Maize (QPM)

- Normal maize protein deficient in lysine and tryptophan
- *opaque2* maize contains twice the amount of lysine and tryptophan but poor in yield & keeping quality.
- Recessive suppressors of the opaque phenotype and altered amino acid content have been reported.
- *opaque2* + Kernel modifiers = QPM. The lysine value of QPM is 3.5 g/100g of protein

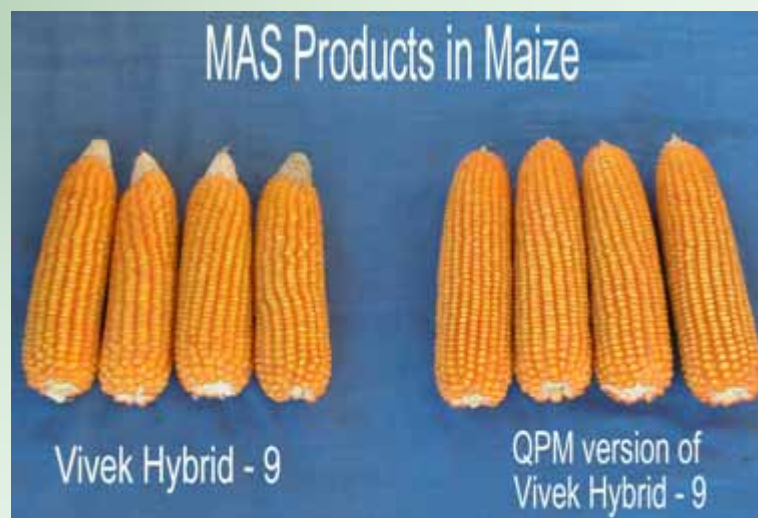
## Gel-based marker analysis for *opaque2*



Three SSR markers: *phi 057*, *phi 112* and *umc 1066* are utilized in gel-based assays to select *opaque-2* gene responsible for high lysine & tryptophan. To select the line carrying *o2* mutation, the parents (QPM and non-QPM) are analyzed with all three SSRs.



Phenotypic Selection for Endosperm Modification



R Babu and HS Gupta, India



# QPM donors and recipient sources

## ■ QPM sources

- Tropical lowland, white – CML 159, CML 144, CLQ 4203, CLQ–RCW Q01, CLQ–RCW Q50
- Tropical lowland, yellow – CML 161, CML 165
- Sub-tropical environment, white – CML 176
  - Important QPM sources in Asia, Africa and Latin America
  - Contain excellent kernel modifying capacity and have been extensively used in line conversion activities.
  - No available sources of QPM for tropical highlands.

## ■ Recipient sources from diverse agro-ecological zones

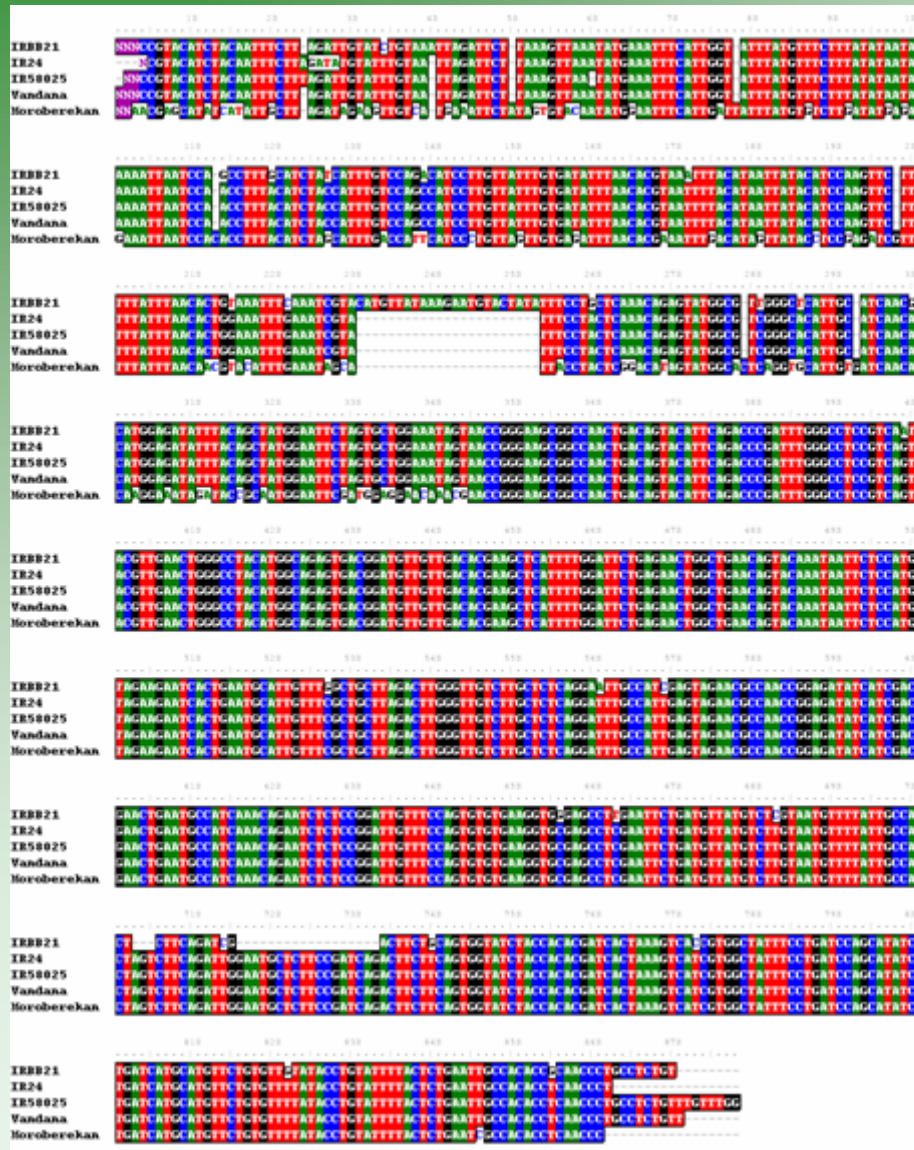
- Tropical white – CML RCW 22, CML 343, CML 254, RCW01
- Tropical yellow – CML 348, CML 451
- Highland white – CML 244, CML 349
- Ethiopian white – F7237, A-7018
  - These normal maize materials have been selected based on their use and importance in Asia, Latin America and East African highland regions.
  - Selected based on adaptability and superior performance in respective regions.

## Recipients for introgression of BB resistance in NARES rice breeding program

Country	Variety	Gene of interest		
		<i>xa5</i>	<i>Xa7</i>	<i>Xa21</i>
China	Hui 161	n*	y*	y
	Hui 333	n	y	y
	Hui 593	n	y	y
	Hui 811	n	y	y
Philippines	IR58025B	y	y	y
	IR68888B	y	y	y
Indonesia	Simacan	y	n	n
	Sintanur	n	y	n
	Setail	y	y	n
	BP364	y	n	y
India	IR58025B	n	y	y
	Pusa 6B	n	y	y
	Samba Mahsuri	y	y	y
	Swarna	y	y	y
Africa	Adny	y	y	y
	Kogony	y	y	y



# Sequence alignments of *Xa21*



- Sequencing of important breeding and recipient lines has been made in order to maximize the efficiency of probe designs

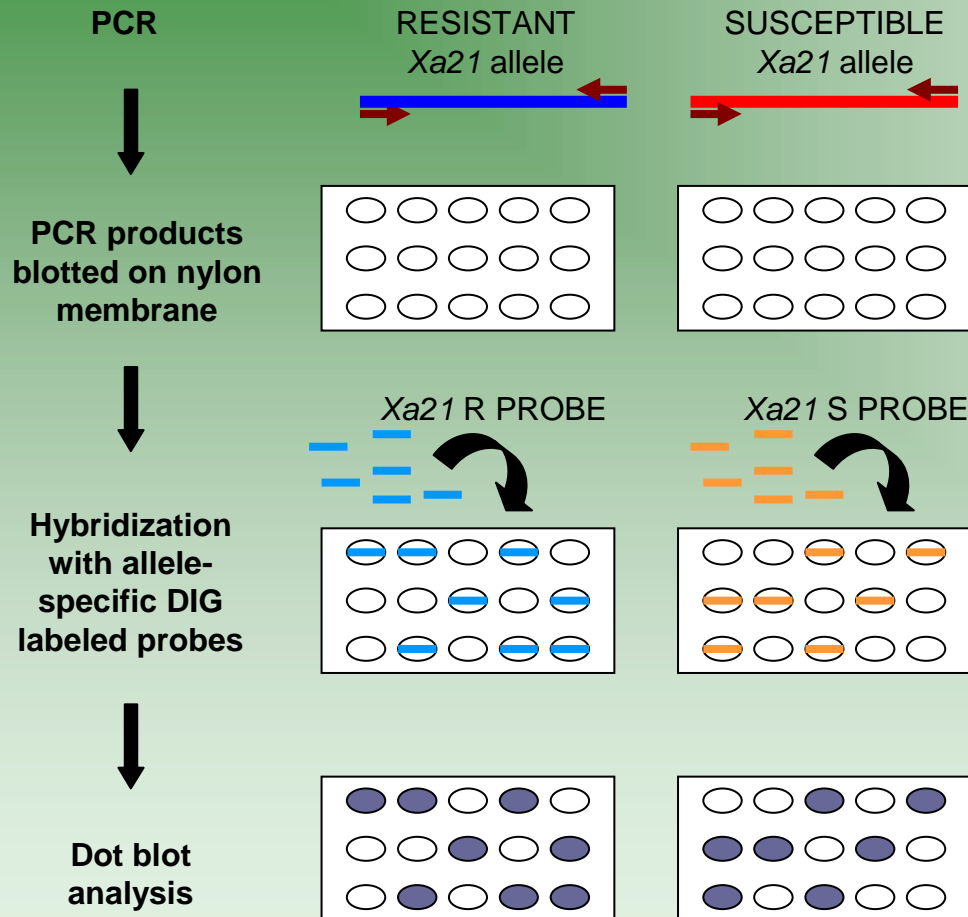


## SNP markers for genotyping *Xa21* R and S alleles designed from the sequences of IRBB21 and IR24

<i>Xa21</i> R allele	<i>Xa21</i> S allele	Substitution	Seq site
5' - ACACGTA <b>A</b> TTTAC ATAA-3'	5' - ACACGTA <b>T</b> TTTAC ATAA-3'	A/T	161-178
5' - CATTGTTT <b>g</b> GCTGC TTAG-3'	5' - CATTGTTT <b>c</b> GCTGC TTAG-3'	G/C	513-530
5' - GTTATGTCT <b>C</b> GTAA TGTT-3'	5' - GTTATGTCT <b>t</b> GTAA TGTT-3'	C/T	668-685



# Dot blot-based detection



- PCR products spotted on membranes
- Probed with allele-specific R and S alleles
- DIG-based detection on dot-blot
- Cost



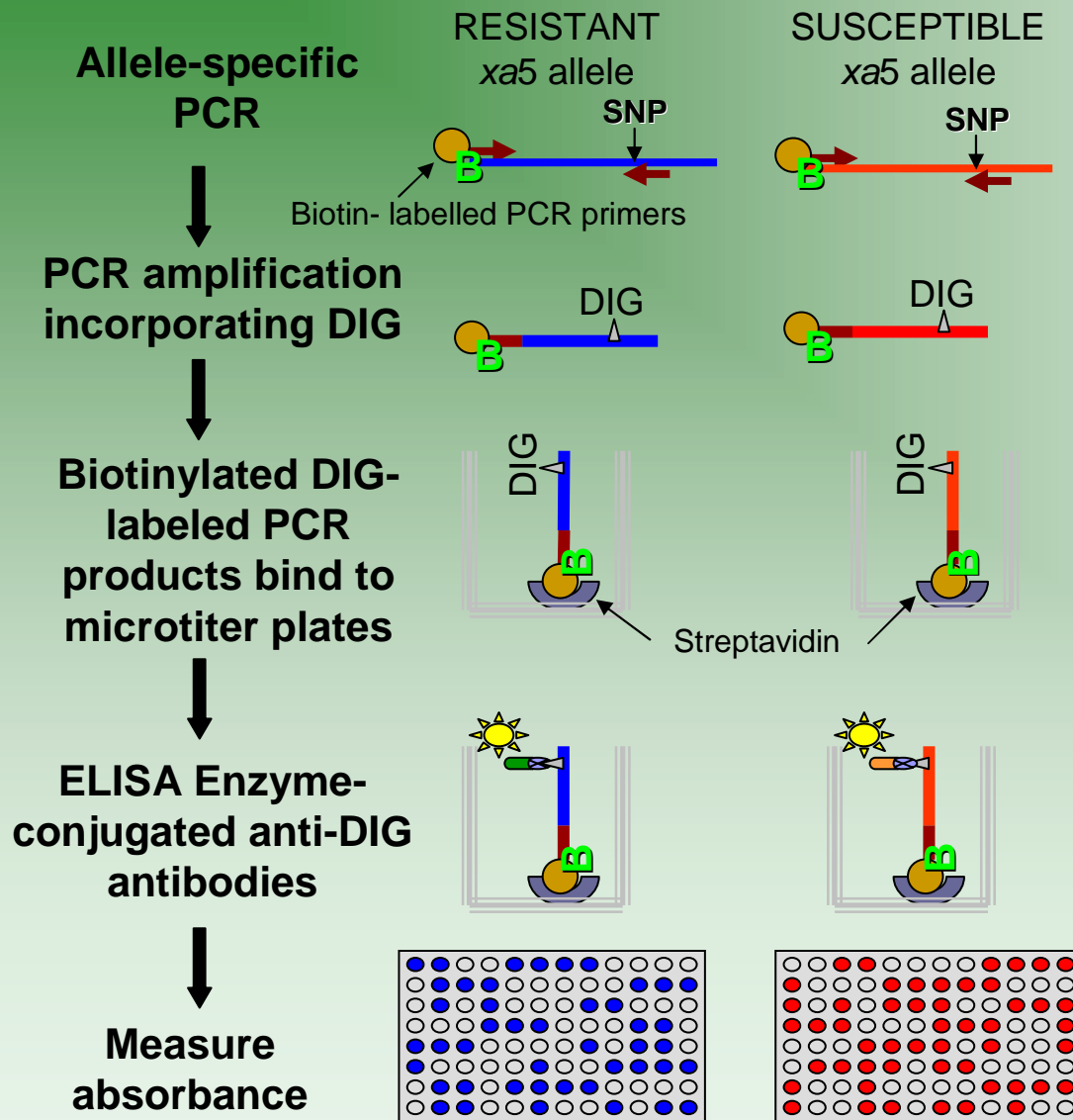
## Markers for genotyping xa5 R and S alleles designed from the sequences of IRBB5 and IR24

xa5 R allele	Seq site	xa5 S allele	Substitution
5' - <span style="border: 1px solid black; padding: 2px;">c</span> GCCATTCAAGTTCT TG <span style="border: 1px solid black; padding: 2px;">ag</span> -3' *	29-47	5' - <span style="border: 1px solid black; padding: 2px;">t</span> GCCATTCAAGTTCTT G <span style="border: 1px solid black; padding: 2px;">tc</span> -3' *	C/T, AG/TC
5' - AGTTCTT <span style="border: 1px solid black; padding: 2px;">g</span> agCAGTT TGATA-3'	38-57	5' - AGTTCTT <span style="border: 1px solid black; padding: 2px;">tc</span> CAGTTT GATA-3'	AG/TC
5' - CCCGGAGCT <span style="border: 1px solid black; padding: 2px;">c</span> GCCAT TCAAG-3'	20-39	5' - CCCGGAGCT <span style="border: 1px solid black; padding: 2px;">tc</span> GCCATT CAAG-3'	C/T

\*Deposited in the GenBank (Iyer and MacCouch, 2005)



# PCR ELISA-based detection



- Biotin forward primer designed from conserved regions
- Allele specific reverse primers for R and S alleles
- Cost



# Allele-Specific PCR-ELISA/Microtiter Plate Assay

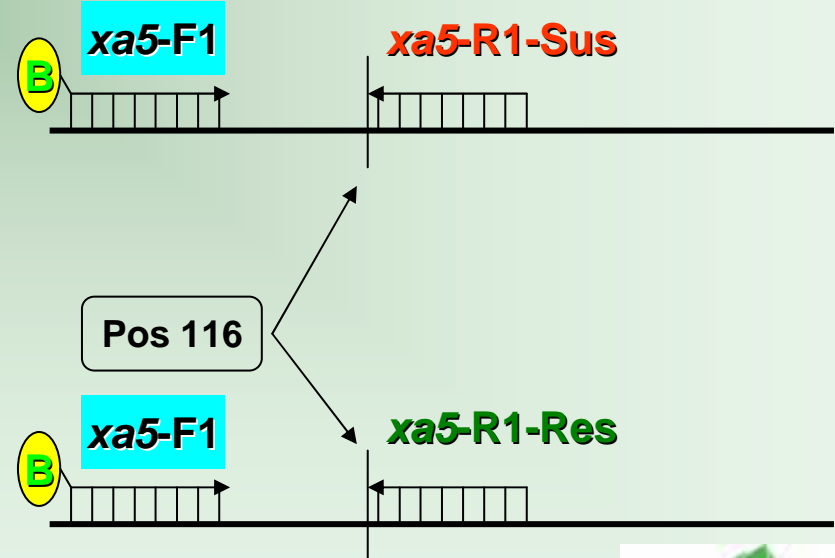
## Primer Design for *xa5*

- Utilized nucleotide polymorphisms at positions **116** & **117** (**GAG** / **GTC**) for reverse primers
- Forward primer is **biotin**-labeled at 5' end (capture probe)
- Expected amplicon size is **132bp**

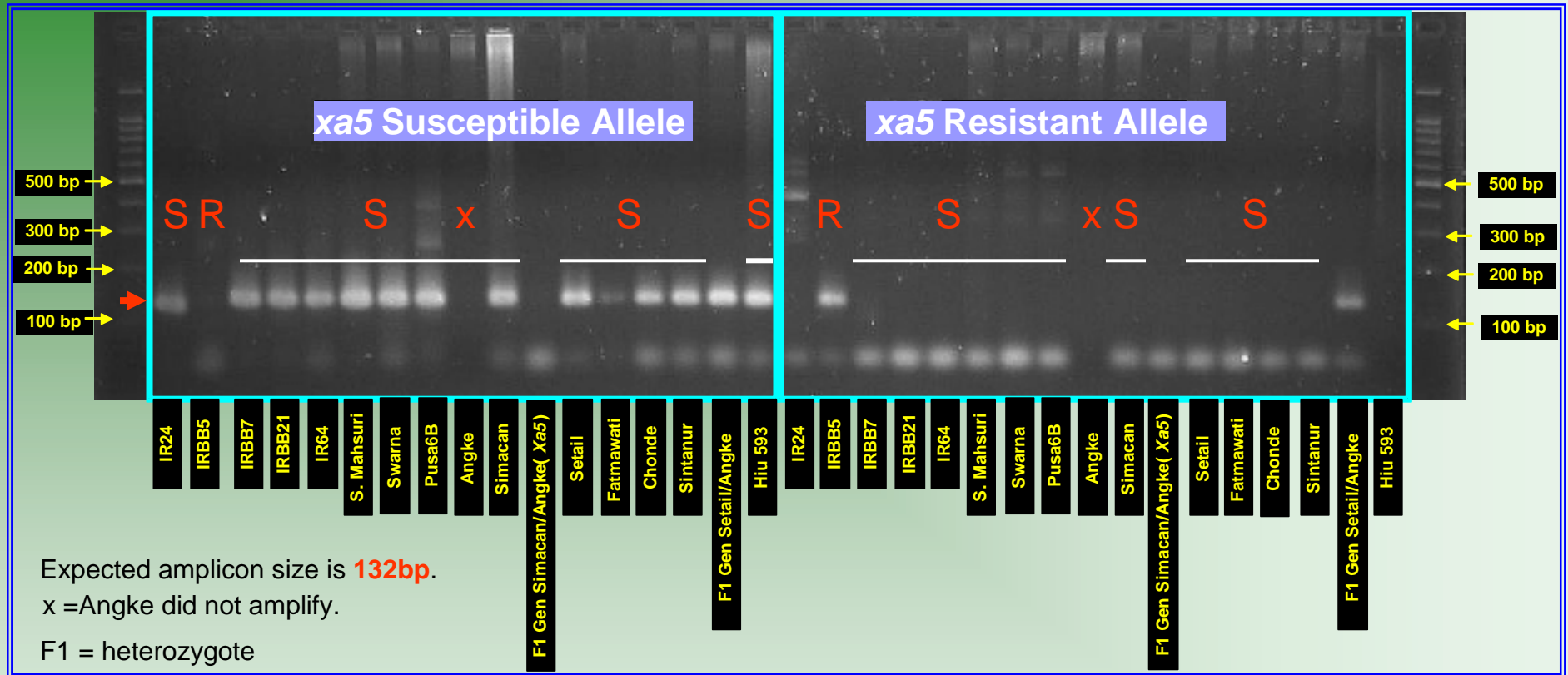
```
>xa5-For1
5' biotin-CTTCGAGCTCTACCGGAGGT 3'

>xa5-Rev1-Sus
5' CCGTCATAGACTTATCAAACTGga 3'

>xa5-Rev1-Res
5' CCGTCATAGACTTATCAAACTGct 3'
```



# Allele-specific PCR of DNA (*xa5*) from NARES partners

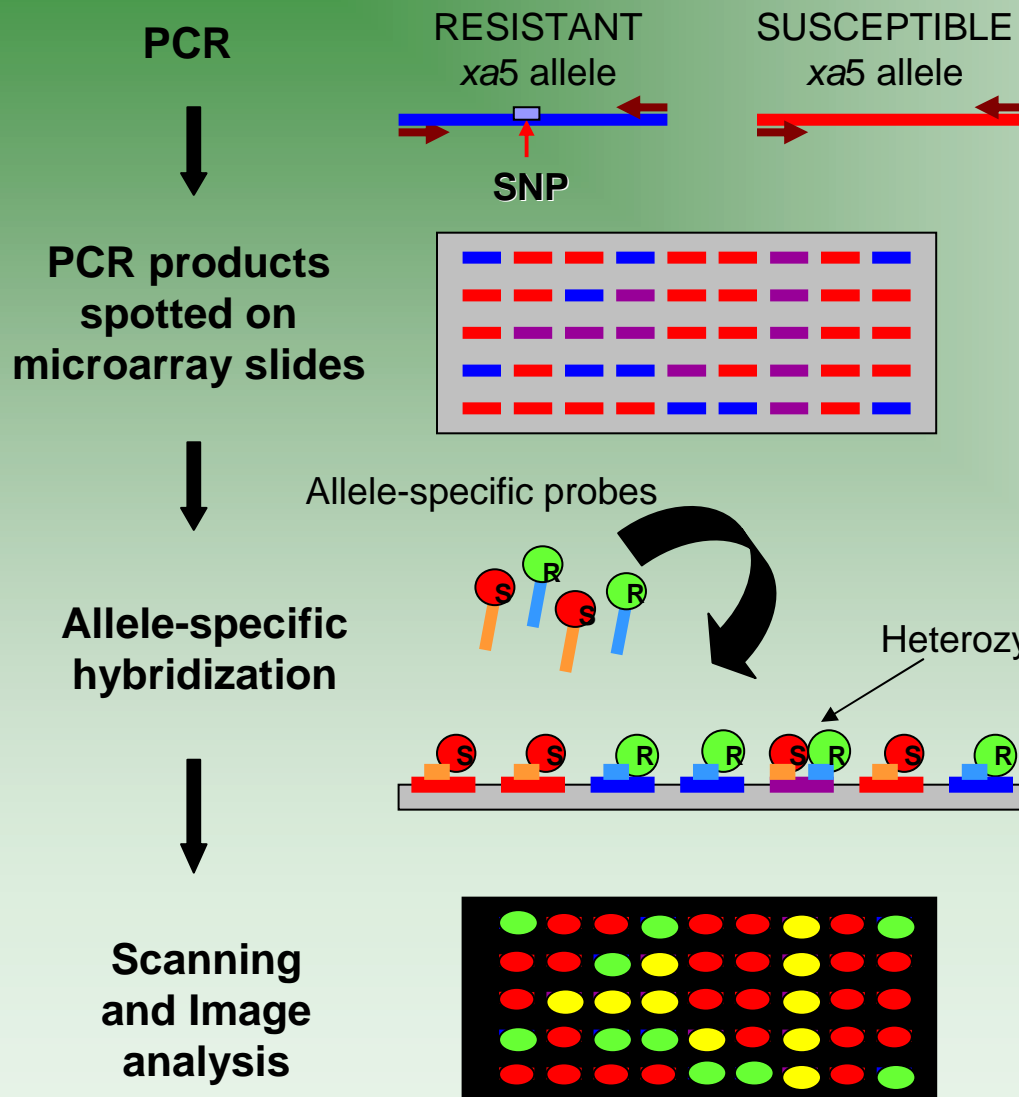


## Upcoming Activities:

- Further optimization of PCR conditions
- Sequencing of PCR products
- PCR-ELISA of amplicons using commercially-available test kit, until detection
- Primer design for *Xa7* and *Xa21* following protocol established for *xa5*



# Microarray-based genotyping

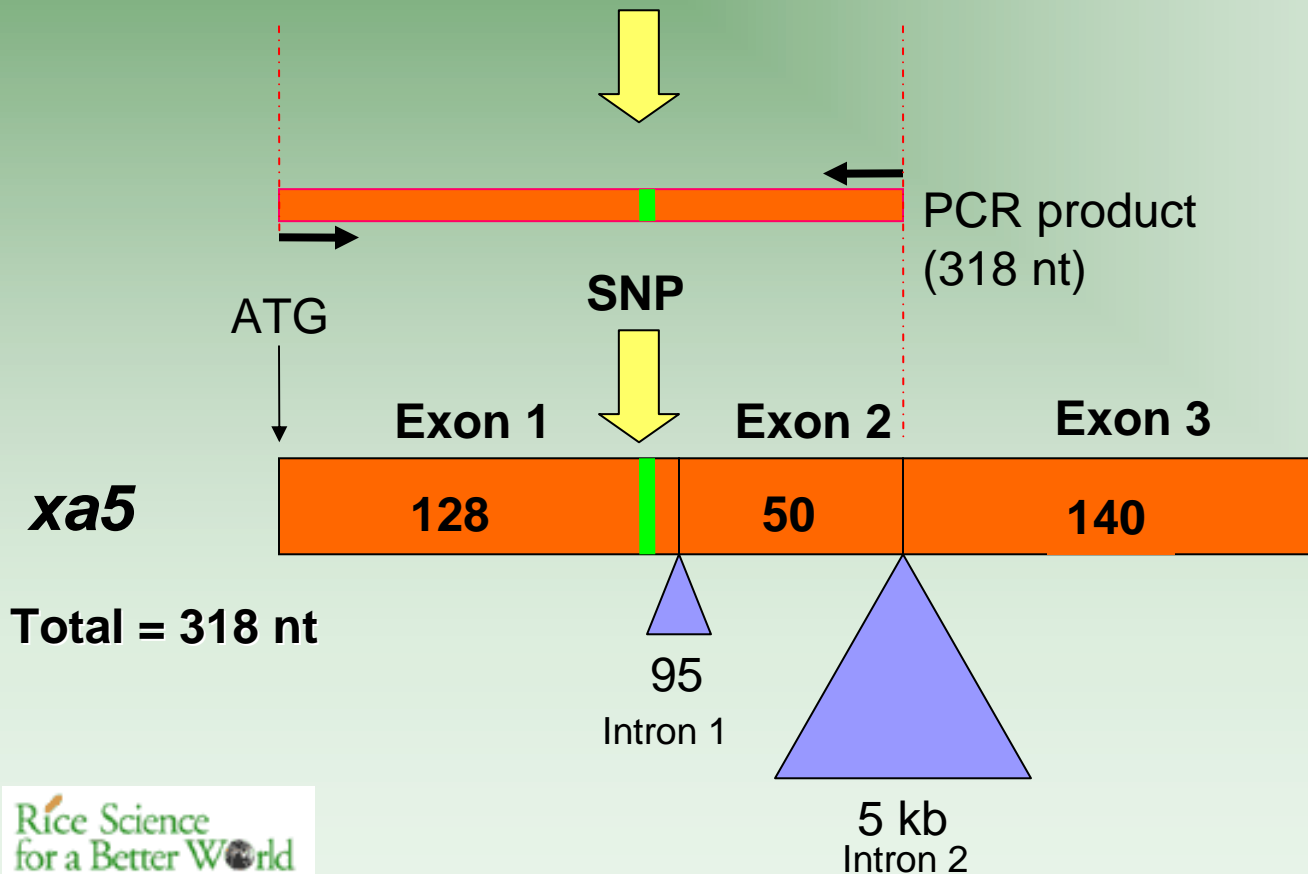


- Microarray-based method for scoring 1000s of DNAs for co-dominant markers
- PCR products spotted onto slides
- Hybridized with fluorescent tags attached to allele specific probes
- Cost



# PCR product and probe design for microarray-based genotyping

## ALLELE-SPECIFIC PROBES



- Currently optimizing PCR conditions for *xa5* primers and testing on recipient lines
- 12-mer allele-specific R and S *xa5* probes (labelled with Alexa Fluor 546/647 dyes) have been designed



# Markers available for rice blast *Pi*- genes

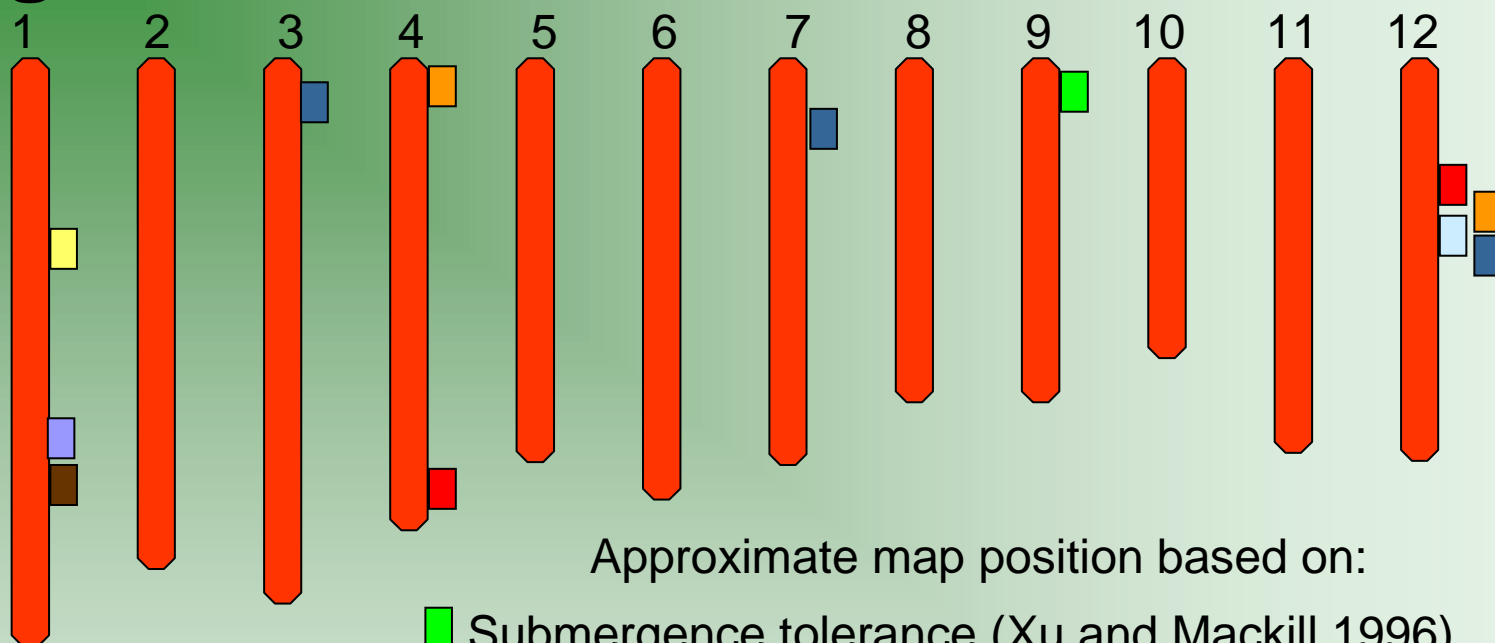
R-gene tagged	Chromosome	Linked marker	Distance (cM)
<i>Pi1</i>	11	r10	-
<i>Pi2</i>	6	RG64 Pto Kinase	2.8 -
<i>Pi9</i>	6	RG16	-



## Candidate genes potentially involved in disease resistance

Gene	Bioprocess	Functional evidence
Oxalate oxidase/germin like protein	Oxidative burst, signaling, structure	Gene cluster as a disease resistance QTL
Aspartyl protease (Esi-18)	Early salt-induced	As disease resistance QTL
14-3-3	Signal cascade	As disease resistance QTL
PR-1	Stress response	As disease resistance QTL
PBZ (PR10a)	Could have ribonuclease activity	Pathogen-induced defense gene
Rice peroxidase 22.3	Oxidation of organic and inorganic substrates at the expense of H <sub>2</sub> O <sub>2</sub>	Pathogen-induced defense gene
Heat shock protein 90	Stress response	Pathogen-induced defense gene
Putative 2-dehydro-3-deoxyphosphoheptonate aldolase	Aromatic amino acid synthesis	Pathogen-induced defense gene
Thaumatin-like pathogenesis related protein	Binding to D-glucans of the type commonly found in fungal cell walls	Stress response
Glyoxalase 1 ( <i>Oryza sativa</i> )	Detoxification of the cytotoxic metabolite methylglyoxal that can be produced by increased levels of glycolysis under conditions of stress	Stress response
S-adenosyl L-homocystein hydrolase	Cytokinin-binding protein CBP57 (cytokinin-mediated signal transduction)	Pathogen-induced defense gene

# Target QTLs for Abiotic Stress Tolerance

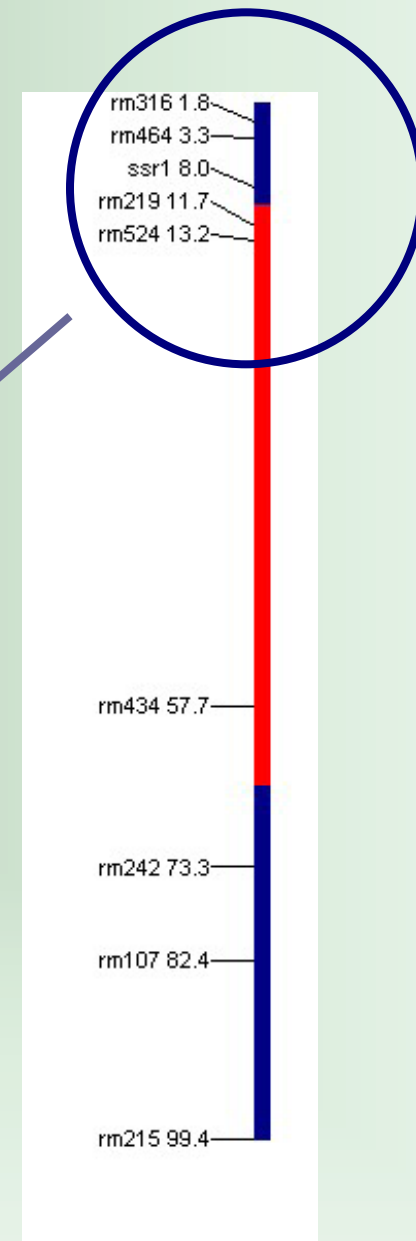
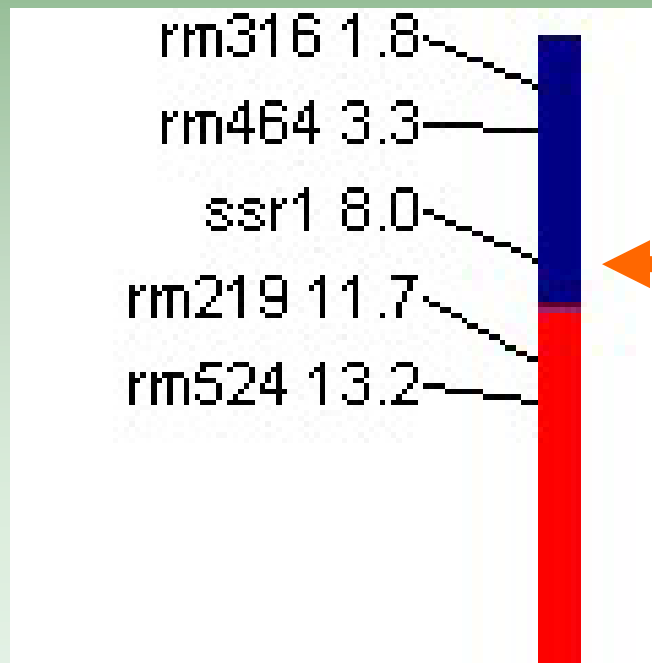


Approximate map position based on:

- Submergence tolerance (Xu and Mackill 1996)
- Deepwater elongation (Sripongpangkul et al. 2002)
- Drought (Babu et al. 2003)
- Al toxicity (Nguyen et al. 2003; Wu et al. 2000)
- P uptake (Wissuwa et al. 1998)
- Salt tolerance (Bonilla et al. 2002)
- Cold tolerance tolerance (Andaya and Mackill 2003)
- Fe toxicity tolerance (Wan et al 2003)

# Submergence tolerance

Chromosome 9  
of individual #242



# QTL mapping of Maize Streak Virus

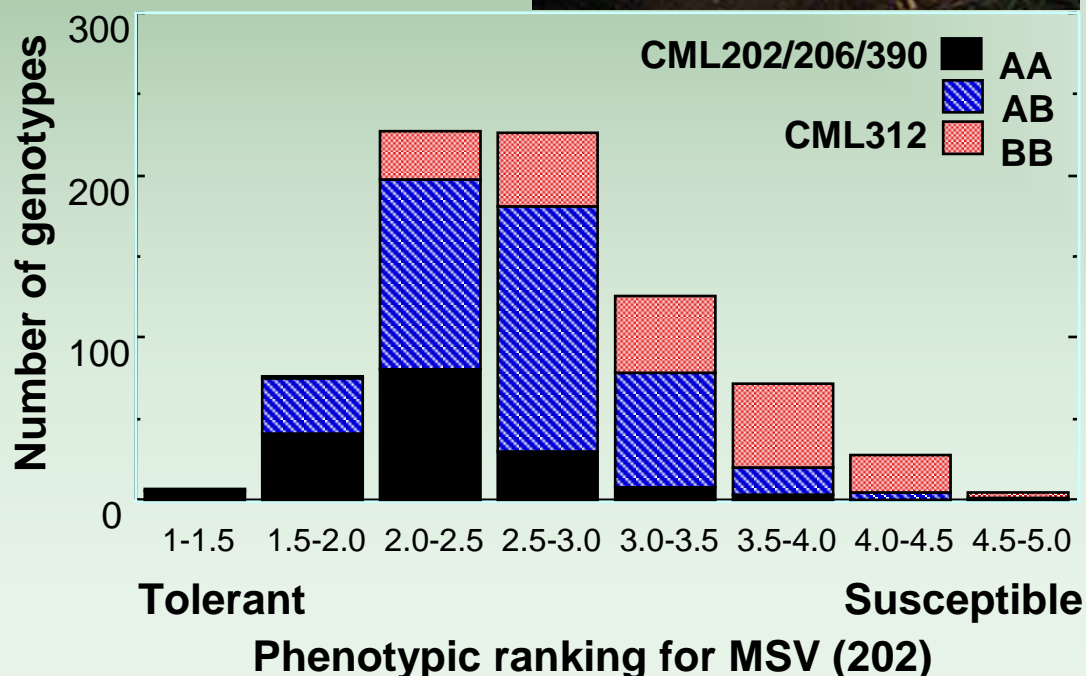
## Chromosome 1

bin 3  
bin 4  
bin 5

bin 3: 13 SSRs

bin 4: 7 SSRs

bin 5: 9 SSRs



## Studies

### A. Schechert

Cross: CML202xLo951

Chr 1S: 59%

Chr 2, 3 and 4 minor QTLs

### D. T. Kyetere

Cross: Tzi4xHi34

Chr 1S: 76%

Chr 1L and 9 minor QTLs

### A. Pernet

Cross: D211xB73

Chr1S: 60%

Chr 3 and 10 minor QTLs

### A. Pernet

Cross: CIRAD390xB73

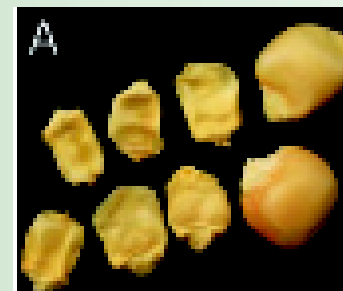
Chr 1S: 50%

Chr 6 and 10 minor QTLs

M. William, CIMMYT

## MAS for specialty traits in maize

Trait	Gene(s)	Location	Markers
Multiple aleurone (Increased mineral content)	<i>Mal</i> <i>sal1-1</i> <i>sal1-2</i>	Chr.2	SSR/STS
Brown mid-rib (reduced lignin content)	<i>bm1</i> <i>bm3</i> <i>bm3-1</i>	Chr.5	SSR/STS
Elevated sucrose	<i>su1+se1</i> <i>sh2/bt2</i>	Chr.4&Chr.2 Chr3 / Chr4	SSR/STS
Low phytic acid (high Fe and Zn bioavailability)	<i>lpa1-1</i> <i>lpa2-1</i>	Chr.2	SSR/STS



Expression of *sal1-1*



Expression of *bm1*



Expression of *su1*

# Marker implementation in wheat at CIMMYT

1. *Cre 1* – cereal cyst nematode (*T.tauschii* derived)
2. *Cre 3* - cereal cyst nematode (Aus. land race)
3. BYDV – TC14 derived
4. *Ph1b* deletion
5. VPM segment – *Aegiolops ventricosa* – *Lr*, *Yr*, *Ccn*
6. *Bo-1* – major qtl (7B) Boron tolerance
7. *Rlnn-1* root lesion nematode – linked
8. Sumai-3 derived scab resistance - QTL

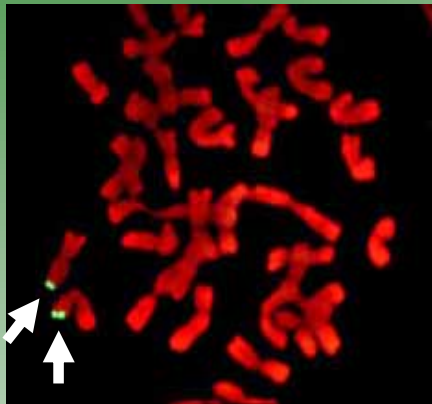
- ✓ On average, >10,000 assays performed per growing cycle.
- ✓ *Cre 1* & *Cre3* – unable to screen at CIMMYT without markers
- ✓ BYDV – field evaluation for resistance not reliable

**MAS is jointly done with plant breeders**

M. William, CIMMYT



# Assay for *Agropyron intermedium* derived BYDV resistance developed at CIMMYT



FISH - Sharma et al. (1995).  
Genome 38(2):406-413

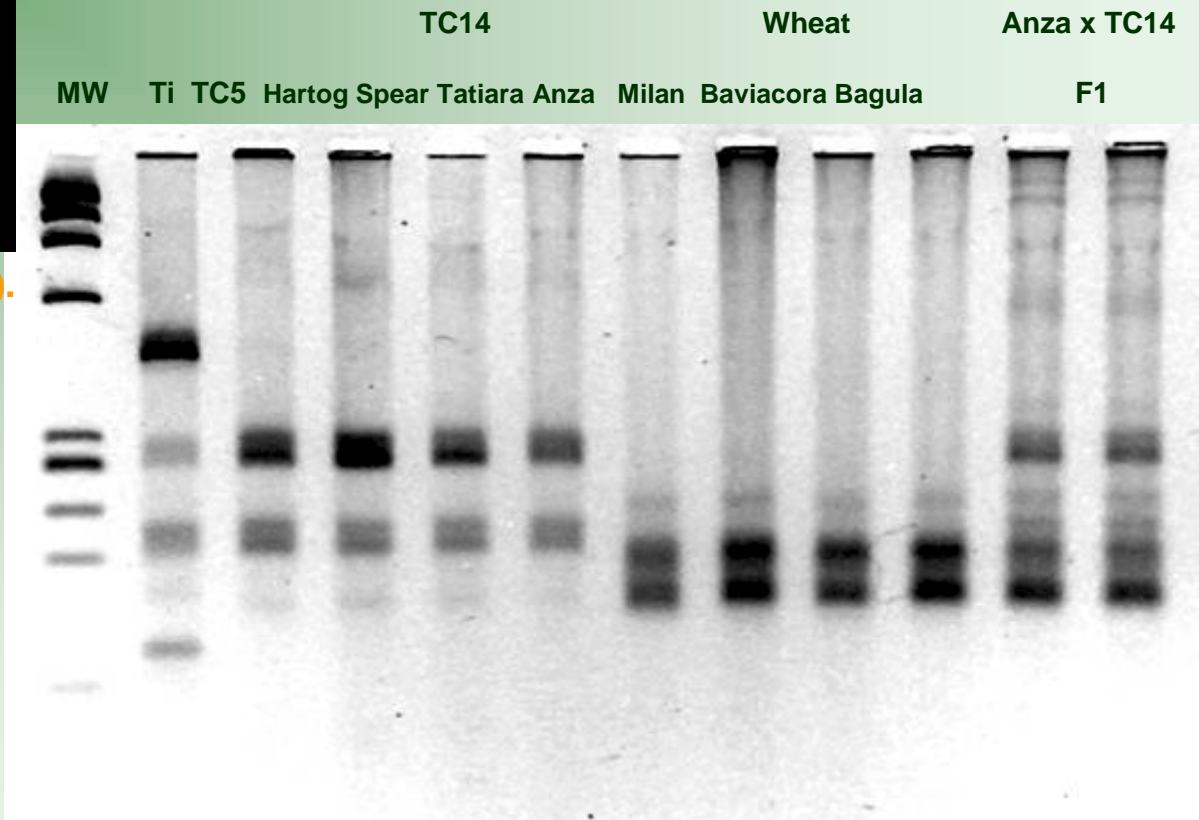
## Chromosome

7D/7Ai

 *T. aestivum*

 *Th. intermedium*

 *Bdv2*



M. William, CIMMYT

# Barley: adaptation to Syrian dryland condition



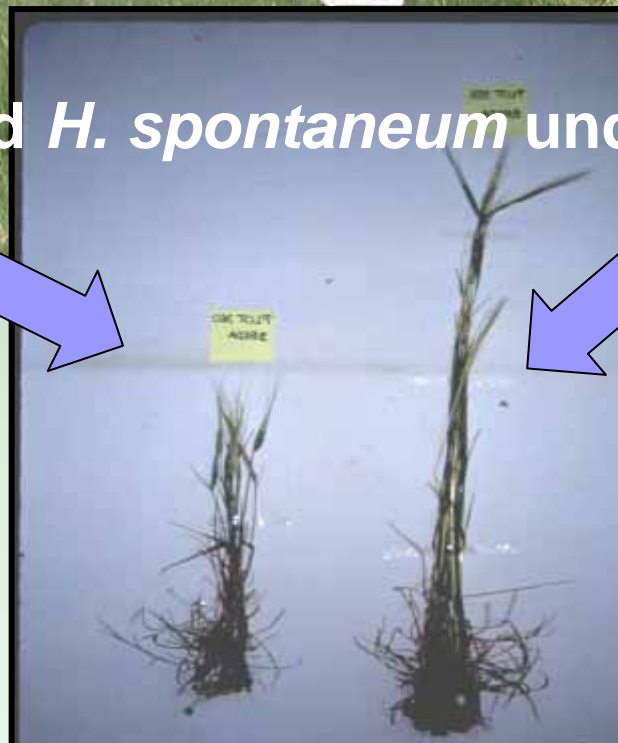
Arta



*Hordeum spontaneum*



Arta and *H. spontaneum* under drought



Arta/*H. spontaneum* 41-1 QTLs

- Plant height
- Yield characters
- Straw QTLs

M. Baum, ICARDA



## Examples of Marker-Assisted Selection for Disease R in Barley

- Scald (*Rhynchosporium secalis*) resistance gene *Rrs1* on chromosome 3H in barley (Arta x *H. spontaneum*) population
- Screening for Yd2 (virus) resistance

## Candidates for MAS for drought in barley \*

- Earliness (days-to heading) – *Ppd1*
  - Cold tolerance – *Fr1*
  - Plant height under drought - *denso*
  - Early growth vigor
  - Growth habit
  - Tiller number
- (need to be tested and verified)

### F2 Population

P<sub>1</sub> = Arbyan-01/C107117-9/Deir-Alla106/6/As46/Ath7/

P<sub>2</sub> = Sutter//sutter\*2/Numar

P<sub>1</sub> P<sub>2</sub>

S R S R S R R R R R R S R S R R R S R R R R R S R R S R R R R S R R R R R R S

### F2 Population

P<sub>1</sub> = Sutter//sutter\*2/Numar/3/

P<sub>2</sub> = IPA265

P<sub>1</sub> P<sub>2</sub>

S R R S R R R R S R R R R R S R R S R R R R S R R R R R S

# PAGE-gel gene-based marker development in sorghum and pearl millet

Crop	Trait/Function	Gene/QTL	Marker
Sorghum	Stay green	Stay green QTLs	EST-SSRs
Pearl millet	Proline synthesis	Delta-1-pyrroline-5-carboxylase synthase	TRAP*
	Antioxidant enzymes	Glutathione reductase, Superoxide dismutase	TRAP*
	Drought tolerance QTL	Brown midrib 1 (Bm1) Brown midrib 3 (Bm3)	Bm1- & Bm3-based TRAPs
	Drought tolerance QTL	Teosinte branched 1 (tb1)	tb1-based TRAP

\*ESTs from pearl millet, rice, sorghum, and barley

**Tom Hash, Rupashree Mukhopadhyay, V. Rajaram,  
Punna Ramu, Kassahum Bannte, ICRISAT**

# Survey

- Purpose: to identify candidate gene markers for abiotic and biotic stresses across GCP mandate crops (cereals, legumes, clonal crops)
- Assist to enable these new technologies to be adopted for MAS on a wider scale
- Target respondents will be scientists from CGIAR centers, ARIs, NARES and SMEs
- SP3 project: “Creating a database of public and private sector applications of MAS”



## Next steps ...

- Collect seeds/DNA of the recipient germplasm for designing specific primers and probes for QPM alleles.
- Optimize the dot-blot assay using the new oligoprobes designed from *Xa* and QPM R and S alleles of donor and recipient rice and maize cultivars, respectively.
- With the availability of new probes, optimize and refine the dot-blot and microplate-based assay technologies.
- Validate the modified TAM approach and FRET
- Shuttle researcher from CNRRI will join IRRI this month to develop other techniques (FRET)
- A consultant with experience in high throughput genotyping techniques is expected to join the project at CIMMYT in September.
- On-site training at NARES partners' labs

