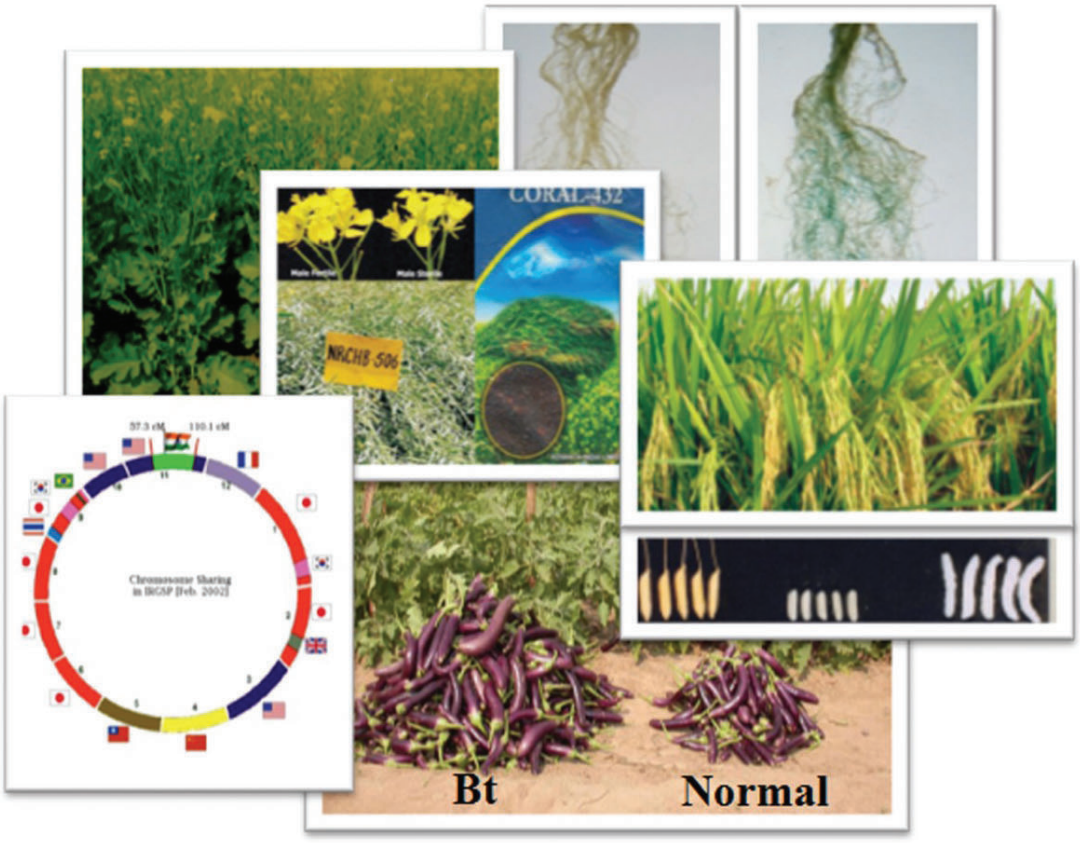
Department of Biotechnology
Gov. of India

birac
Ignite Innovate Incubate

भाकृअप
ICAR

BIRAC-DBT Sponsored Training Programme
on
Application of Marker Assisted Selection and Transgenic Development for Crop Improvement

(18th - 20th February, 2016)



Course Director
Dr TR Sharma

Course Coordinators
Dr Amolkumar U Solanke
Dr SV Amitha CR Mithra

ICAR-National Research Centre on Plant Biotechnology
Lal Bahadur Shastri Building, Pusa Campus, New Delhi- 110012

सत्यमेव जयते
Department of Biotechnology
Ministry of Science & Technology

ICAR-NATIONAL RESEARCH CENTRE ON PLANT BIOTECHNOLOGY PUSA CAMPUS, NEW DELHI

भाकृअनुप
ICAR

राष्ट्रीय पादप जैव प्रौद्योगिकी अनुसंधान केन्द्र
NRCPB

Training Manual for

A short term training course on

"Application of Marker Assisted Selection and Transgenic Development for Crop Improvement"

(February 18-20, 2016)

Funded by

birac
Ignite Innovate Incubate

Edited by

Dr. Amolkumar U Solanke

Dr. SV Amitha CR Mithra

भा.कृ.अं.प.-राष्ट्रीय पादप जैव-प्रौद्योगिकी अनुसंधान केन्द्र
ICAR-National Research Centre on Plant Biotechnology
लाल बहादुर शास्त्री केन्द्र, पूसा परिसर, नई दिल्ली–110012, भारत
Lal Bahadur Shastri Centre, Pusa Campus, New Delhi-110012, India
(An ISO 9001:2008 Certified Institute)

डॉ. तिलक राज शर्मा
परियोजना निदेशक
Dr. T.R. Sharma
FNA, FNASc, FNAAS
JC Bose National Fellow
Project Director

Foreword

Agricultural biotechnology has been at the forefront in recent past because of the development of various transgenics crops for different traits. Though, India could reap the benefit of GM crops in case of cotton, with increased cotton production for the past many years, the commercial status of rest of the GM crops is yet not clear. There are many tools of biotechnology which can be used for developing new cultivars or other useful products. Biotech products developed by molecular breeding and transgenic approaches in agriculture have proved to be important tools for the benefit of resource poor farmers in India. Though only Bt cotton is the cultivated transgenic crops in India, there are other products in the pipeline like golden rice, Bt brinjal, barnase/barstar based hybrid system in brassica and many herbicide and insect resistance crops. These products are much awaited in Indian markets. There are some considerations which need to be followed to encourage these new technologies as well as science-led agriculture. Without the intervention of Industry such changes are not feasible. In this context, there is a need to sensitize all the stakeholders about the technological aspects and achievements of biotechnology in crop improvement. The present training on "***Application of Marker Assisted Selection and Transgenic Development for Crop Improvement***" has been planned to give an over view of different types of DNA markers and their applications in crop improvement and development and characterization of different transgenic events to the real stake holders from the industries and academia.

I am sure the participants will take full advantage of getting useful information from the recourse persons who are experts in their own areas. I extend my best wishes to the coordinators, Dr. Amol K. Solanke and Dr. Amitha Mithra Sevanthi in successful organization of this training programme.

(T R Sharma) 16-2-16

Ph. : 91-11-25848783
: 91-11-25841787 Ext. 200
Fax : 91-11-25843984
E-mail : pdnrcpb@gmail.com
pd@nrcpb.org
Website : www.nrcpb.org

Preface

Agricultural biotechnology is one of the potential tools to bring in evergreen revolution and provide food and nutritional security to the burgeoning population in our country. Biotechnology in commercial agriculture in India began nearly three decades ago with micropropagation or tissue culture based clonal multiplication of superior genotypes of fruit, ornamental and other horticultural species. ICAR-NRCPB has the distinction of releasing one of the first plant biotech products in India, Pusa Jai Kisan, a somaclonal variant from Indian mustard variety Varuna. This was followed by use of DNA markers in crop improvement, esp., in marker assisted backcross breeding (MABB). After a decade of experience with MABB, marker assisted selection has become a norm in almost all major crops across the National Agricultural Research and Educational System (NARES). Over a dozen varieties have been developed using this technology and many more are in pipeline. Tremendous success obtained with transferring major genes using DNA markers has enabled extending this technology to transferring major QTLs. In the transgenic front, Bt cotton, the only transgenic allowed for commercial cultivation in India, increased farm income from 14.6 million USD in 2001-02 to 2100 million USD in 2012-13. Successful cultivation of Bt cotton over one and half decades by India's marginal farmers thus stands testimony to the importance of this technology. This success is also expected to bring in favourable policy environment for transgenic crops so that the transgenic products in pipeline in the country can serve the farming community.

To harness the real potential of agricultural biotechnology, it is imperative that Industry and the academic institutions work hand in hand. Biotechnology Industry Research Assistance Council (BIRAC) set up by DBT, Government of India facilitates industry-academia interface so as to strengthen and empower the emerging Biotech enterprise thus addressing nationally relevant product development needs. The present training programme, sponsored by BIRAC for industrial counterparts, on "**Application of Marker Assisted Selection and Transgenic Development for Crop Improvement"** and the manual designed exclusively for this training course, endeavours to strengthen the capability of the participants by providing exposure to agricultural biotechnology from experts in their respective area.

We are thankful to Project Director, NRCPB for his guidance and support in bringing out the manual in time and in organizing the training course. We sincerely thank BIRAC for funding support. We place on record our special thanks to Dr. Renu Swarup, Senior Advisor, DBT and Managing Director, BIRAC, Dr Sanjay Saxena, Head-Investment and Dr Amita Joshi, Manager-Technical, BIRAC for their constant support in conducting this training programme. We are indebted to Dr JS Sandhu DDG (CS) and Dr JS Chauhan (Seeds) for their unstinting support.

Dr Amolkumar U Solanke NRCPB, New Delhi
Dr SV Amitha CR Mithra Feb 16, 2016

Programme Schedule

Date	Time	Lecture/Demonstrations	Resource persons
18.02.16	9: 30 AM	**Pre-Evaluation of trainees**	Dr. Amitha/ Dr. Amolkumar
	10:00 AM	**Inauguration**	
	10:30 AM	Map based cloning of disease resistance genes/QTLs and their applications	Dr. T R Sharma
	11:15 AM	Molecular markers: Basic concepts, mapping populations and applications	Dr. AK Singh
	12:15 PM	Significance of foreground and background selection in molecular breeding	Dr. Feroz Hossain
	1:15 PM	**LUNCH**	
	2:00 PM	Next generation DNA markers: Development and their applications	Dr. NK Singh/ Dr. S. Gopalakrishnan
	3:00 PM	Molecular characterization of transgenic events	Dr. Rohini Sreevatsa
	4.00 PM onwards	**Demonstration:** PCR amplification with SSR and ISSR markers and gel electrophoresis of the amplicons to show polymorphism and selection in a population	Dr. SV Amitha Mithra; Dr. Deepak Bisht
19.02.16	10:00 AM	Role of tissue culture in crop improvement	Dr. SR Bhat
	11:00 AM	Tea Break	
	11:15 AM	Relevance of transgenic plants	Dr. Srinivasan
	12:15 PM	Transgenic development for insect pest resistance	Dr. RC Bhattacharya
	1:15 PM	**LUNCH**	
	2:00 PM onwards	**Demonstration:** Gene amplification; Cloning into binary vector; Confirmation using PCR; Agrobacterium mediated transformation	Dr. Amolkumar Solanke; Dr. Monika Dalal
	4:30 PM	Introduction to NGS technologies	Dr. Kishor Gaikwad

20.02.16	9:30 AM	Molecular cloning of plant promoters and their applications	Dr. P K Jain
	10:30 AM	Application of RNAi and genome editing technologies	Dr. D Pattanayak
	11:30 AM	Tea Break	
	11:45 AM	**Demonstration:** Confirmation of transgenic events using PCR	Dr. Jasdeep Padaria
	1: 15 PM	**LUNCH**	
	2:00 PM	**Demonstration:** Southern and Northern blot analysis	Dr. Rekha Kansal
	3:30 PM	**Valedictory**	
	4:15 PM	**Demonstration:** Bioassay	Dr. Rohini Sreevatsa
	5:00 PM	**Post Evaluation of trainees**	Dr. Amitha/ Dr. Amolkumar

CONTENTS

Map Based Cloning of Disease Resistance Genes and QTLs and their applications

TR Sharma

Director

ICAR-National Research Centre on Plant Biotechnology

Pusa Campus, New Delhi -12

Email: trsharma@nrcpb.org

Introduction

Management of plant diseases has been a high priority area of research worldwide. In India, during the post 'Green Revolution' era, productivity of high yielding cultivars of various crops has been seriously affected by pest and diseases which can only be sustained by the use of better plant protection strategies. Chemical management of plant diseases, though, quite easy and effective option, it has various inherent problems. The most important problem associated with this method is environmental pollution and health hazards caused by toxic chemicals used for the disease management. With the increased awareness and health consciousness among the peoples, produce of organic farming is being preferred for consumption in the developed world. Precise identification of disease causing organisms is the basic requirement of deciding various disease management options. Disease management through host resistance involves characterization of pathogen diversity for the effective and prolonged life of R - genes under natural conditions. Biotechnological approaches are now being used to facilitate the existing disease management strategies.

DNA markers like restriction fragment length polymorphism (RFLPs), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and sequence tagged sites (STS) are commonly being used for the molecular tagging of disease resistance genes. Besides, molecular markers are also used in marker assisted selection, gene pyramiding and map-based cloning of R-genes. Various R-genes have already been mapped and cloned by using this strategy. Uses of molecular markers in molecular tagging and map based cloning of disease resistance genes in plants will be discussed in the following paragraphs. These strategies are also applicable to vegetable crops.

An overview of molecular markers

Variation at genetic level can be detected by their phenotypic expression in terms of different characters under varied environment. Transmission of characters from parents to progenies, through genetic material and expression of those differentiating characters in the progenies are called genetic markers. Three types of markers can detect the genetic differences at chromosomal level.

1. **Morphological markers**: Different plant characters like plant height, leaf shape and pathogen characteristics like culture growth, pigmentation, pathogenecity and other visual identifiable characters are known as morphological markers.
2. **Biochemical markers**: These includes, analysis of proteins and enzymes like isozymes or allozymes extracted from the tissues. Proteins and isozymes are used as a markers after distinguishing the polymorphisms in the electrophoretically separated and specifically stained protein bands.
3. **DNA markers**: Analysis of DNA in the form of individual bands or restriction fragments is the direct method of estimating large number of differences at genetic level.

   Morphological and biochemical markers are the ultimate result of gene expression, which are influenced by the environment, as well as developmental stages of the organism under study. Besides, these markers are a few in numbers and do not represent the whole genome of an organism hence cannot be used for the construction of a saturated genetic map. However, DNA markers are enormous in numbers and stably inherited in Mendelian fashion. These are also not affected by environment or developmental stages of the plants. Variation at DNA level is detected by DNA polymorphisms survey by various molecular techniques. Polymorphisms at the DNA level are the result of simple point mutation, insertion or deletion of DNA segments etc.

Types of DNA markers

Natural variation at DNA level can be detected by a range of molecular biology techniques developed in the recent past. The choice of their use solely depends on nature of the study and crop species in question. Different DNA markers have been classified into three categories on the basis of molecular biology techniques used.

1. Hybridization based markers: The most important hybridization based techniques is RFLPs (Botstein *et al*., 1980). In this technique, DNA is first digested with restriction enzymes, separated on an agarose gel and Southern transferred to membrane filter. DNA

fixed on the filters is then hybridized with locus specific DNA probes labelled with radioactive or non-radioactive substance and DNA fingerprints are obtained by autoradiography. Alternatively, hybridization can be performed with microsatellite specific DNA probes. Such types of DNA markers are then termed as variable number tandem repeats (VNTR) and oligonucleotide fingerprinting.

2. **Sequence targeted and single locus PCR based markers:** Though DNA sequence information can be rapidly detected by the use of DNA markers, it gives incomplete information. Moreover, some of the PCR based DNA markers does not provide allelic information. Such problems can be overcome by the use of PCR primers flanking to a specific locus. Most ecological and evolutionary studies are based on DNA markers derived from the sequences of DNA. However, it is a labour intensive and expensive technique and sometimes difficult to use in the detection of polymorphisms at species or race level. Thus PCR based technique like cleaved amplified polymorphic sequence (CAPS) can prove better. The technique, which provides useful easy-to-screen markers, is also known as PCR-RFLP . Since, in this technique PCR products are first digested with restriction enzymes before their electrophoresis on agarose gels. For the specific and sensitive detection of locus other PCR techniques like, allele specific oligonucleotide ligation assay (OLA) and after cloning and sequencing of simple sequence repeats (SSR), PCR primers flanking to the region can be produced. These are known as sequence tagged microsatellite site (STMS) markers.

3. **PCR based markers:** Amplification based DNA markers have been proved versatile and easy because there is no need of difficult and lengthy steps of Southern blotting. All the genetic markers based on PCR are generated by the modifications of original method of PCR developed by Prof. Kerry Mullis and his group of the United States of America in 1985 (Saiki *et al*., 1985). The technique is very sensitive and allows specific amplification of DNA fragments from the genomic DNA of all the organisms. PCR is a simple chemical synthesis of DNA molecule with the help of DNA polymerase and primers by using genomic DNA as template. The technique can be automated for large-scale applications and devoid of radioactivity used for labelling DNA probes. Some of the PCR based markers are, Arbitrary primed PCR (AP-PC), amplified fragment length polymorphism (AFLP), Random amplified polymorphic DNA (RAPD), multiple arbitrary amplicon profiling (MAAP), DNA amplification fingerprinting (DAF), arbitrary amplified DNA (AAD), simple sequence repeats (SSR), RNA arbitrary primed PCR (RAP-PCR).

Molecular Mapping of Disease Resistance Genes

The process of locating genes of interest via linkage to markers is referred as gene tagging. The tagging of disease resistance genes with molecular markers involves the evaluation of classical phenotype for resistance and molecular marker genotype on the same individuals and the data is analysed to determine, if any of the markers co segregates with the target phenotype (resistant phenotype). A molecular marker closely linked to a resistance gene can be used for indirect selection of the genes in breeding programme. There are various methods of developing DNA markers linked to the R-genes. The basic requirement of tagging plant disease resistance genes with molecular markers is the development of a mapping population. NILs differing for single gene for resistance are considered a very good material for use in R-gene mapping experiments (Muehlbauer *et al*, 1988). NILs are developed by crossing resistant individuals with universally susceptible plants and back crossed for 6-7 generations with the susceptible recurrent parent to get homozygous resistant lines, which differs only for one gene for resistance. The NILs which differs by the presence or absence of a specific R-gene are crossed. The F_2 segregating population is analysed for detecting DNA polymorphism using one of the DNA markers technology. Polymorphic bands produced between resistant and susceptible lines are analysed for their linkages with the R-gene(s). In those cases where NILs are not available, RILs are used. RILs can be produced by making crosses between resistant and susceptible lines by using single seed descent method in seven or more generations. Plant disease resistance many times controlled by multiple genes. These genes are difficult to detect in F_2 populations of NILs. Therefore, for such analysis of resistance, fixed populations of doubled haploids are produced by using anther culture technique, and thus plants with 100% homozygocity can be produced after a single generation.

In those cases where NILs are not available, bulk segregant analysis (BSA) is the method of choice (Michelmore *et al*, 1991). In this method polymorphisms survey is conducted in the DNA bulks made from resistant and susceptible F_2 plants. Each resistant and susceptible bulks consist of individuals homozygous for all the characters, which differ genetically for the regions containing R-genes. The R-gene tightly linked to the markers produced polymorphic band between R-bulk and S-bulk. While, unlinked regions give rise

to monomorphic banding pattern. There are many reports available on molecular mapping of R-gene in different host-pathogen interactions (Mohan *et al*, 1997; Sharma *et al*., 1999).

Investigation on molecular tagging and cloning of blast resistance genes and resistance gene analogues from rice line Tetep which possesses durable resistance to the *M. oryzae* population of north western Himalayan region of India are in progress (Sharma *et al*, 2002 unpublished). A mapping population by crossing blast resistant line Tetep with susceptible rice line HP2216 has been developed. F_2 population was uniformly inoculated at seedling stage with an isolate of *M. oryzae* collected from north- western Himalayan region. A total of 205 F_2 plants of mapping population have been maintained and further screened with *M. oryzae* in F_3 plant progeny rows to identify the heterzygotes. RAPD marker, $S129_{700}$ has been mapped at a distance of 2.1 cM (Shanker, 2002) and an AFLP marker, $AFLP_{75}$ mapped at 15.1 cM on the linkage map (Singh, 2002).

Map based Cloning of R-genes

Various strategies have been reported for the cloning of disease resistance genes in different systems (Ellis *et al*, 1988). Among these, map based cloning and transposon tagging are being used for cloning disease resistance genes where gene product is not known (Sharma *et al*, 2002e). Transposon tagging was first demonstrated in maize by Fedoroft *et al* (1984). Since then, modified transposons from maize have been used for introduction in other plant species to facilitate gene tagging (Ellis *et al*, 1988).

Map-based cloning approach (Tanksley et al, 1989) is preferred over other alternatives because, it does not require any information regarding the gene product and thus each step can be monitored very systematically. Since it is labour intensive and time consuming, it cannot be used to clone a gene residing in the complex locus of the genome. Various steps involved in map based cloning are briefly explained as follows.

i. Fine Mapping of R-genes

Fine mapping refers to the identification of markers genetically tightly linked to the target gene. As explained earlier, molecular tagging of R- genes require a number of different approaches starting from the identification of different R-genes effective against a pathogen population of a particular geographical region, developing mapping population and using molecular markers for the tagging of R-genes. Once the DNA markers tightly linked to the target gene are identified, a fine saturated genetic map is often made. Fine mapping is very important to generate information regarding the orientation of tightly linked markers related

to each other. Such type of information is important for physical mapping and chromosome walking. Therefore, a large segregating population (more than 1000 individuals) is analyzed with the DNA markers and recombinant individuals are identified at targeted site.

ii. Physical Mapping of R-genes

Physical mapping is the determination of relationship between genetic and physical distances of DNA markers in the target region of a chromosome. Physical mapping helps in determining the distance between two markers flanking to R-genes in terms of number of nucleotides. It is basically performed by separating large DNA molecules with PFGE. Various electrophoretical techniques like CHEF and field inversion gel electrophoresis (Carle *et al*, 1986) are capable of separating large DNA molecules ranging from 100kb to 10 Mb in size (Chu *et al*, 1988). After this, genomic libraries of large inserts are constructed in various cloning vector systems. However, vectors like plasmids, phages and cosmids can only be used primarily in case of prokaryotes where genome size is small. In case of plants, different vector systems like YACs (Burke *et al*, 1987) and BACs (Shizuya *et al*, 1992) have been used for preparing genomic libraries of large DNA segments.

Integration of a physical map with the genetic map is necessary to place some BAC clones onto a DNA marker map (Yang *et al*, 1997). These BAC clones then become landmarks, which can be generated either by STS mapping or colony hybridization with RFLP markers. If the STS and RFLP markers are linked to disease resistance genes, the landmarks provide the initial points for chromosome walking and eventual cloning of target genes (Yang *et al*, 1997).

iii. Chromosome walking

Once a physical map of the genome near a target gene is constructed, this region can then be cloned by chromosome walking. It begins by identifying genomic clones that overlap the initial RFLPs, which is accomplished by probing a complete genomic library with radiolabelled nucleic acid probes synthesized from the initial RFLPs. The newly identified genomic clones are then isolated and ends of these clones become the starting points for next step in the chromosome walk. It is repeated many times until clones covering a large segment of contiguous genomic DNA are obtained. It stretches from one of the flanking RFLP markers to the other with the target gene located at one of the clones in between the RFLPs. Once the insert with target gene is cloned it can be transferred to the appropriate susceptible plants along with a suitable promoter. There are many examples of molecular cloning of

disease resistance genes in different host- pathogen systems by using map based cloning. It shows the power of this technique and its future implications for isolating more number of R-genes in plants.

Cloning of disease resistance genes

In recent years, the plant disease resistance genes have become a focus of attention of plant molecular biologists to understand their structure, and mechanisms of evolution and disease resistance. Efforts are being made to clone them. The lack of knowledge of disease resistance gene product has been a major bottleneck for cloning of these genes. However, with the development of transposon tagging and map based cloning as strategies for cloning of genes with unknown products in early 1980's, the cloning of disease resistance genes has become a reality. By now, many plant genes for resistance to a diverse range of pathogens including fungi, bacteria and viruses have been cloned using these two strategies. A brief description of the strategies used for cloning of disease resistance genes is given below:

Trasposon tagging of disease resistance genes

In principle, this method involves recovery of a susceptible mutant of a resistant plant which is known to be caused by the insertion of transposable element into the resistance gene. The clone of transposable DNA can be used as a probe to isolate genomic clones that contains both the transposon and the flanking sequences of the disrupted resistance gene. Subcloning of the flanking sequences will then provide a probe that can be used for the recovery of intact resistance gene from the genomic library of a resistant plant. The gene *Hm1* of maize for resistance to *Helminthosporium carbonum* was the first gene to be cloned by transposon tagging. Since then a number of disease resistance genes including L^6 and *M* conferring resistance to linseed rust (*Melampsora lini*) have been cloned by this strategy.

Map-based cloning of disease resistance genes

By this method cloning of genes of unknown biochemical functions is done by chromosome walking involving isolation of overlapping chromosomal DNA fragments. Since the chromosomal location of the gene is used as basis of cloning, the technique is also known as positional cloning. Detailed description of various steps used in map-based cloning is given by Sharma (2002). Briefly, map-based cloning involves the identification of markers linked to the target gene. Closely linked RFLP or RAPD markers are identified either by a mapping population. The next step in map-based cloning is the construction of physical map

near to the target gene on which the distance between markers is calculated in nucleotides, rather than recombination frequency. The physical map so produced provides basis for chromosome walking. Chromosome walking involves the identification of DNA sequences progressively further from the linked DNA markers by a series of overlapping cloning steps. If DNA markers flanking the gene of interest are known, then walking from one of the flanking marker towards other marker on the other side is performed. It will result in cloning of the entire DNA between two markers, including the target gene. The last step in map-based cloning is the identification of target gene among the overlapping DNA clones identified during chromosome walking. This is accomplished by transformation of the susceptible host with sub-clones containing putative R-gene. A sub-clone, which confers resistance to the transformant, contains the gene of interest. The gene *Pto* of tomato for resistance to *Pseudomonas syringae pv. tomato* was the first to be cloned by this technique (Martin *et al.*, 1993). Since then the technique has been successfully used to clone more resistance genes. However, there is no report on cloning of disease resistance genes from oilseed brassicas. These species have been refractory to map-based cloning due to large genome size and highly repetitive nature of their genome. The recent reports of a high degree of collinearity (conservation of gene structure and order) across the genomes of *Brassica* spp. and model plant *Arabidopsis thaliana* (Cavell *et. al.*, 1998; Lagercrantz, 1998) have, however, suggested the possibility of isolation of resistance genes from *Brassica* species. One of the most significant examples of map based cloning of disease resistance genes includes identification, mapping and cloning of a Rice Blast resistance gene *Pi54* (*Pi-k^h)* and its orthologues from wild rice. We identified the rice blast resistance gene resistant against pathogen population of rice blast pathogen (*Magnaporthe oryzae)* present in different part of India (Sharma et al, 2002). Then molecular mapping and cloning of a dominant gene *Pi54* (*Pi-k^h)* present in the rice line Tetep (Sharma et al., 2005a&b) and its functional validation was successfully achieved (Rai et al., 2011) by him. Using microarrays it has been shown that the single Pi54 gene activates complex defense mechanism in rice (Gupta et al. 2012a) and its protein is a unique zinc finger protein (Gupta et al. 2012b). Other orthologues of this gene from wild species of rice i.e., *Pi54rh* (Das et al., 2012) and *Pi54of* (Devanna et al, 2014) have been cloned and characterized.

Gene Pyramiding

Gene pyramiding is the technique of combining more than one gene for resistance in a common genetic background by the repeated back crossing and selection with the virulent races of the pathogen. The technique is highly effective in getting durable resistance to the target pathogen. Using conventional methods, it is really very difficult to pyramid 2-3 genes in a cultivar through crossing and normal screening procedures, since, presence of one gene in the plant masks the effect of others. Secondly, there should be specific pathogen races (isolates) to discriminate both the R-genes separately so that screening of both the genes can be done in segregating populations. In such cases, DNA markers highly linked to the R-gene can be used for detecting the presence or absence of both the genes in each plant after molecular analysis.

Gene pyramiding is being used in many host-pathogen systems. Recently, Hittalmani *et al* (2000) pyramided three rice blast resistance genes by using MAS. First, they performed fine mapping of three major blast resistance genes *Pi-1, Piz-5* and *Pita* on chromosomes 11, 6 and 12, respectively by RFLP markers. These RFLP markers were then used for gene pyramiding. Plants carrying 2 and 3 R-gene combinations were also identified from the resistant phenotypes by inoculation with *M. grisea*. They have used the isogenic lines containing *Pi1*, *Piz-5* and *Pi ta* genes for pyramiding in the background of susceptible cultivar Co 39 which is not being used for commercial cultivation. They are now transferring them to the commercial cultivars of rice. Pyramiding of 5 bacterial blight resistance genes *Xa1, Xa3, Xa4, xa5* and *xa10* have also been achieved by MAS (Yoshimura *et al*, 1995). Huang *et al* (1997) pyramided four bacterial blight resistance genes *Xa4, xa5, xa13* and *Xa21* using RFLP and PCR based markers.

The gene mapped and cloned by the nominee has already been used for the development of four rice lines, HPR2722 (*Pi1+ Pikh*), HPR2723 (*Pi1+Pikh+Pita*), HPR2739 (*Pi1 +Pikh*) and HPR2745 (*Pi-1+Pita*) at HPKV, Palampur which are under multiplications & testing for blast resistance through DDR, Hyderabad. These are being developed under ICAR projects. DNA makers tightly linked to *Pi-k*^{h} gene (Sharma et al., 2005a&b) which are being used by the rice breeders for transferring *Pi54* gene along with other genes for blast resistance for gene pyramiding using marker assisted selection in Improved Pusa Basmati (Singh et al., 2011) and parental lines of Basmati Hybrid RH-10 at IARI, New Delhi and in the background of non basmati type varieties, Swarna and BPT5204 at the DRR Hyderabad.

Genetics of QTLs

In crop plants the majority of quantitative traits are controlled by polygenes. Most of these polygenes have minor effect and only occasionally some have major effect (Falconer 1989). These loci are described as QTLs and can be detected with the help of molecular markers, which should ideally segregate in a Mendelian manner. With the advent of DNA markers, QTL mapping has become a routine strategy for the discovery of genes involved in complex quantitative traits. Thousands of QTLs have been mapped for important agronomical traits including disease resistance traits in rice (http://www.gramene.org). Map-based or positional cloning has been successful in isolating genes underlying QTLs in several plant species, including rice (Yano et al. 2000, Ashikari et al. 2005, Song et al. 2007). A number of independent studies have reported the incidence of clustered QTLs for traits that are functionally related, including yield and yield components (Xiao et al. 1998, Moncada et al. 2001, Thomson et al. 2003)

For instance Sheath blight caused by *Rhizoctonia solani* is one of the important diseases of rice for which only quantitative resistance is involved. Despite extensive search on rice germplasm, the major gene(s), which gives complete resistance to fungus, have not been identified (Zhikang et al. 1995). Only partial resistance is available for sheath blight in rice germplasm (Pan et al. 1999). Plant germplasm provides the possibilities of an extensive solution to disease management in crops (Hammond-Kosack and Jones, 1997). Development of rice cultivars resistant to sheath blight has been hampered by limited sources of resistance in existing cultivars and wild species (Amante et al. 1990). However, it has been known for sometimes that there are significant differences in sheath blight resistance among rice varieties (Khush, 1977). Also breeding effort to increase resistance to *R. solani* has been largely unsuccessful because of complexity of the resistance and a lack of detailed knowledge about the loci involved in controlling this trait (Li et al. 1995b). On the contrary, genetic studies on quantitative resistance to *R. solani* in rice have shown to be both polygenic and major gene inheritance (Sha 1987, Xie et al. 1990). Resistance to sheath blight is a typically quantitative trait controlled by polygenes (Sha and Zhu, 1989). The partial resistance available for sheath blight has been considered to be polygenic (Pan et al. 1999).

The quantitative resistance in the most resistant varieties, such as 'Tetep' and Taducan' can offer excellent protection against the pathogen under field conditions (Kikua et al 1983, Groth and Nowick 1992). Tetep, a primitive cultivar from Vietnam has been

identified as one of the cultivars most resistance to sheath blight resistance (Marshal and Rush, 1980). Resistance to sheath blight in Tetep has been reported to be controlled by QTLs under different environments (Channamallikarjuna, 2007). The resistant cultivars, Jasmine 85 and Tequing possess a nonallelic dominant major gene. The tall and late flowering growth habit, reduced number of infection and production of oxidized phenolic compounds of Tetep contributed its resistance to sheath blight (Groth and Nowick 1992).

Prerequisite of the identification of realistic QTLs are segregating mapping population, genetic linkage map covering entire genome, precise phenotyping and computational aids to interpret collective phenotypic and genotypic data.

Mapping populations for QTL analysis

Mapping populations including F_2, recombinant inbred lines (RILs) and double haploid lines (DHs) have been widely used for QTL mapping in rice (Li et al. 1995a, Yu et al. 1997, Marri et al. 2005). These mapping populations can be classified into two categories: temporary populations and permanent populations. In a temporary population such as F_2 or backcross (BC), each individual in the population will segregate after self-pollination. In contrast, in a permanent population such as DH and RIL, each individual in the population is genetically homozygous, and the genetic construction will not change through self-pollination. Thus, in permanent populations the phenotypic value of complex quantitative traits can be measured repeatedly through a replicated experimental design, and same genotype can be tested under different environments, allowing the study of genotype x environment interaction. Therefore, with permanent populations the random environmental errors can be better controlled and the precision of QTL mapping can be improved. QTL can be localized to a genomic region rather than a locus in those populations. Following the primary mapping population, advanced populations such as near isogenic lines (NILs) and chromosome segment substitution lines (CSSL) can be used to map QTLs to a locus as a Mendalian factor by reducing the noise of genetic background (Lin et al. 2002, Zhang et al. 2006, Wang et al. 2006). Based on this strategy several QTLs have been isolated in rice (Yano et al. 2000, Takahashi et al. 2001, Li et al. 2003, Channamalikarjuna et al. 2010, Deshmukh et al. 2010). The common aspect in all the QTL cloning work is to exploit the significance of high quality mapping populations.

Populations that can be permanently maintained are preferred because they provide unlimited seed supplies for repeated experiments in multiple years and locations; thus

accurate estimates for QTL effects are produced. RILs are derived from cross between inbred lines by single seed descent method (Dudley, 1993). The use of RILs has many advantages for QTL studies, but it takes a long time to develop such populations (Burr et al. 1998). RILs are produced by repeated inbreeding or selfing of the F_2 progeny derived from a single cross of two well-established progenitor inbreds to achieve homozygosity. Each RIL thus becomes fixed for short linkage blocks of parental alleles (Burr et al. 1988, Burr and Burr, 1991). In addition to the individual functional effects of the genes, RILs also allow us to study the gene interaction effects. However, as the individuals have a homogenous genotype at most of the loci, this kind of population is not suitable for genetic analysis involving dominant genetic effects because of the lack of heterozygosity. For dominant gene/QTLs, most genetic analysis and linkage mapping projects have been conducted in early segregating generations, such as the F_2, $F_{2\text{-}3}$ and the BC_1F_2 generations. However, when these populations are used, it is not possible to make repeated observations at the level of the individual or block or to carry out multiple trials. There are two alternative strategies which allow the repeated detection of non-additive effects through the creation of heterozygotes from permanent populations. The first is to create heterozygotes by testcrosses (TC) or backcrosses (BC) from a recombinant inbred population (Li et al. 2001, Luo et al. 2001, Mei et al. 2003). The second is to develop what is called an ‘‘immortalized F_2’’ population which is generated from intermating between the RILs (Hua et al. 2002, 2003).

The DH lines are usually derived from culturing the pollens of F_1 plants from crosses between inbred lines. The advantage of DH lines is that they reach homozygosity after a single generation. Since the lines are genetically homozygous, they can be propagated without further segregation. This feature allows precise measurement of the quantitative traits by repeated trials and for elimination of the environmental effect. A shortcoming associated with both types of populations is that they can be used only for detecting additive and epistatic types of genetic effects (Hua et al. 2002). An F_2 population from cross between two inbred lines provides for theoretically the most complete and most efficient genetic analysis (Allard, 1956). For a polymorphic locus, it has all three genotypes present in a proportion of 1:2:1, thus allowing for estimation of both additive and dominance effects of the locus. However, it is difficult to use F_2 for genetic analysis of quantitative traits as each distinct genotype is represented by only a single individual. Like F_2, backcross populations are also difficult to replicate in order to obtain accurate phenotypic values for precise QTL mapping

(Lu et al. 1997). The mapping populations are used both for recording the data on the character of interest (phenotyping) and for genotyping of individual plants/lines of the mapping population using polymorphic molecular markers. These two sets of data are then used for conducting gene/QTL analysis, whether it is the single–marker regression approach or the more sophisticated interval mapping approach. Once QTLs are mapped, the linked markers can be used in marker assisted selection for crop improvement.

Selected References

Botstein B, White R L, Skolnick M, & Davis R W, 1980. Construcation of a genetic linkage map in man using restriction fragment length polymorphism. Am. J. Hum. Genet., 32, 314-331.

Das Alok, D. Soubam , P. K. Singh, S. Thakur, N. K. Singh and T R Sharma*. 2012. A novel blast resistance gene, Pi54rh cloned from wild species of rice, *Oryza rhizomatis* confers broad spectrum resistance to *Magnaporthe oryzae.* Funct Integr Genomics 12:215–228

Devanna NB, Vijayan J, TR Sharma* (2014) The Blast Resistance Gene *Pi54of* Cloned from *Oryza officinalis* Interacts with *Avr-Pi54* through Its Novel Non-LRR Domains. PLoS ONE 9(8): e104840. doi:10.1371/journal.pone.0104840

Gupta S.K, A.K. Rai, S.S. Kanwar, D. Chand, N.K. Singh, TR Sharma*. 2012. The single blast resistance gene *Pi54* activates complex defense mechanism in rice. J Exp. Bot. 63 (2)757-772.

Gupta S.K, A.K. Rai, S.S. Kanwar, TR Sharma. 2012. Comparative Analysis of Zinc Finger Proteins Involved in Plant Disease Resistance. PLoS One 7(8): e42578

International Rice Genome Sequencing Project. 2005. The map-based sequence of the rice genome. Nature 436, 793-800

Khanna A, V. Sharma, R.K. Ellur , A. B. Shikari, S. Gopala Krishnan, U. D. Singh, G. Prakash, T. R. Sharma, R. Rathour, M. Variar, S. K. Prashanthi, M. Nagarajan, K. K. Vinod, P. K. Bhowmick, N. K. Singh, K. V. Prabhu, B. D. Singh, AK. Singh . 2015. Development and evaluation of near-isogenic lines for major blast resistance gene(s) in Basmati rice Theor Appl Genet . DOI 10.1007/s00122-015-2502-4

Michelmore R W, Paran I, & Kesseli R V, 1991. Identification of markers linked to disease resistance genes by bulked-segregant analysis: a rapid method to detect markers in

specific genomic regions by using segregating populations. Proc. Natl Acad Sci USA, 88, 9828-9832.

Mohan M, Nair S, Bhagwat A, Krishna T G, Yano M, Bhatia C R, & Sasaki T, 1997. Genome mapping, molecular markers and marker-assisted selection in crop plants. Molecular Breeding, 3, 87-103.

Saiki R K, Gelfand D H, Stiffel S, Higuchi R H, Horn G T, Mullis K B, & Erlich H A, 1985. Primer directed enzymatic amplification of DNA with a thermostaable DNA polymerase, Science 239, 487-492

Sharma T R, Prachi, & Singh B M, 1999. Applications of polymerase chain reaction in phytopathogenic microbes. Indian J. Microbiol., 39, 79-91.

Sharma T.R., M.S. Madhav, B.K. Singh, P. Shanker, T.K. Jana, V. Dalal, A. Pandit, A. Singh, K. Gaikwad, HC Upreti and N.K. Singh.2005b. High-resolution mapping, cloning and molecular characterization of the gene of rice, which confers resistance to rice blast. Mol Genet. Genomics. 274:569-578.

Sharma, TR , RS Chauhan, BM Singh, R. Paul, V. Sagar and R. Rathour. 2002. RAPD and pathotype analyses of *Magnaporthe grisea* population from North-Western Himalayan Region of India. J. Phytopathology (Germany): 150: 649 –656.

Sharma, TR, P Shanker, BK Singh, TK Jana, MS Madhav, K Gaikwad, NK Singh, P Plaha, R. Rathour. 2005a. Molecular mapping of rice blast resistance gene *Pik*^{h} in the rice variety Tetep. J Plant Biochem. Biotech: 14-127-133.

Singh A, V K. Singh, S. P. Singh, R. T. P. Pandian, Ranjith K. Ellur, D. Singh, P.K. Bhowmick, S. Gopala Krishnan; M. Nagarajan; K. K. Vinod; U. D. Singh; K. V. Prabhu; TR Sharma; T. Mohapatra; A. K. Singh.2012. *Molecular breeding for the development of multiple disease resistant Basmati rice* AoB Plants 2012; doi: 10.1093/aobpla/pls029

Singh S, Chand S, Singh NK, TR Sharma* (2015) Genome-Wide Distribution, Organisation and Functional Characterization of Disease Resistance and Defence Response Genes across Rice Species. PLoS ONE 10(4): e0125964. doi:10.1371

Singh WH, RK Kapila, TR Sharma and R Rathour 2015. Genetic and physical mapping of a new allele of *Pik* locus from *japonica* rice 'Liziangxintuanheigu. Euphytica. 10.1007/s10681-015-1437-9.

Singh, AK, S Gopalakrishnan, V P Singh, KV Prabhu, TMohapatra, NK Singh,T R Sharma, M. Nagarajan,K. K. Vinod et al. 2011. Marker assisted selection: a paradigm shift in Basmati breeding. Indian J. Genet :71:1-9

Thakur, S, P K Singh, A. Das, R Rathour, M Variar, S K Prashanthi, A K Singh, U D Singh, D Chand, N K Singh and T R Sharma*. 2015. Extensive sequence variation in rice blast resistance gene *Pi54* makes it broad spectrum in nature Frontiers in Plant Science (doi:10.3389/fpls.2015.00345).

The Rice chromosome 11 and 12 sequencing consortium. 2005. The sequence of rice chromosome 11 and 12, rich in disease resistance genes and recent gene duplications. BMC Biology. 3:20 .

Development and Use of Mapping Populations in Crops: Genetic Considerations

A.K. Singh and Gopala Krishnan S.

Division of Genetics, Indian Agricultural Research Institute, New Delhi – 110012

Introduction

The development of molecular marker technology has caused renewed interest in genetic mapping. An appropriate mapping population, suitable marker system and the software for analyses of data are the key requirements for a molecular mapping and molecular breeding programme. Genetic map construction requires that the researchers: (i) select the most appropriate mapping population(s); (ii) calculate pair wise recombination frequencies using these population; (iii) establish linkage groups and estimate map distances; and (iv) determine map order.

Since large mapping populations are often characterized by different marker systems, map construction has been computerized. Computer software packages, such as Linkage1 (Suiter et al., 1983), GMendel (Echt et al., 1992), Mapmaker (Lander and Botstein, 1986; Lander et al., 1987), Mapmanager (Manly and Eliot, 1991) and Joinmap (Stam, 1993), have been developed to aid in the analysis of genetic data for map construction. These programmes use data obtained from the segregating populations to estimate recombination frequency that are then used to determine the linear arrangement of genetic markers.

Mapping Populations

A population used for gene mapping is commonly called a mapping population. Mapping populations are usually obtained from controlled crosses. Decisions on selection of parents and mating design for development of mapping population and the type of markers used depend upon the objectives of experiments, availability of markers and the molecular map. The parents of mapping populations must have sufficient variation for the traits of interest at both the DNA sequence and the phenotype level. The variation at DNA level is essential to trace the recombination events. The more DNA sequence variation exists, the easier it is to find polymorphic informative makers. When the objective is to search for genes

controlling a particular trait, genetic variation of trait between parents is important. If the parents are greatly different at phenotypic level for a trait, there is a reasonable chance that genetic variation exists between the parents, although uncontrolled environmental effects could create large phenotypic variation without any genetic basis for the effects. However, lack of phenotypic variation between parents does not mean that there is no genetic variation, as different sets of genes could result in same phenotype.

Selection of parents for developing mapping population

Selection of parents for developing mapping population is critical to successful map construction. Since a map's economic significance will depend upon marker-trait association, as many qualitatively inherited morphological traits as possible should be included in the genetic stocks chosen as parents for generating mapping population. Consideration must be given to the source of parents (adapted vs exotic) used in developing mapping population. Chromosome pairing and recombination rates can be severely disturbed (suppressed) in wide crosses and generally yield greatly reduced linkage distances (Albine and Jones, 1987; Zamir and Tadmar, 1986). Wide crosses will usually provide segregating populations with a relatively large array of polymorphism when compared to progeny segregating in a narrow cross (adapted x adapted). To have significant value in crop improvement programme, a map made from a wide cross must be collinear (i.e. order of loci similar) with map constructed using adapted parents.

Types of mapping populations

Different types of mapping populations that are often used in linkage mapping are: (i) F_2 population; (ii) F_2 derived F_3 (F_2:F_3) populations; (iii) Backcross Inbred Lines (BILs); (iv) Doubled haploids (DHs); (v) Recombinant Inbred Lines (RILs); (vi) Near-isogenic Lines (NILs) and (vii) Chromosomal Segment Substitution Lines (CSSLs). The development, characterization and utilization of different mapping populations is given in Fig.1.

The characteristic features, merits and demerits of each of these populations are briefly presented below:

***F_2 population*:**

- Produced by selfing or sib mating of the F_1 individuals generated by crossing the selected parents.

- F_2 individuals are products of single meiotic cycle
- Ratio expected for dominant marker is 3:1 and for codominant marker is 1:2:1

Merits

- Best population for preliminary mapping
- Requires less time for development
- Can be developed with minimum efforts, when compared to other populations

Demerits

- Linkage established using F_2 population is based on one cycle of meiosis
- F_2 populations are of limited use for fine mapping
- Quantitative traits cannot be precisely mapped using F_2 population as each individual is genetically different and cannot be evaluated in replicated trials over locations and years. Thus, the effect the G x E interaction on the expression of quantitative traits cannot be precisely estimated.
- Not a long-term population; impossible to construct exact replica or increase seed amount

***F_2 derived F_3 ($F_{2:3}$) population*:**

- $F_{2:3}$ population is obtained by selfing the F_2 individuals for a single generation
- Suitable for specific situations like
    - Mapping quantitative traits
    - Mapping recessive genes
- The $F_{2:3}$ family can be used for reconstituting the genotype of respective F_2 plants, if needed, by pooling the DNA from plants in the family

Demerit

Like F_2 population, it is not 'immortal'

***Backcross Mapping Population*:**

- Backcross populations are generated by crossing the F_1 with either of the parents. Usually in genetic analysis, backcross with recessive parent (testcross) is used.

- With respect to molecular markers, the backcross with dominant parent (B_1) would segregate in a ratio 1:0 and 1:1 for dominant and codominant markers, respectively. However, backcross with recessive parent (B_2) or testcross would segregate in a ratio of 1:1 irrespective of the nature of marker.

Merits

- Like an F_2 population, the backcross populations require less time to be developed, but are not 'immortal'. However, the recombination information in case of backcrosses is based on only one parent (the F_1).
- The specific advantage of backcross populations is that, the populations can be further utilized for marker-assisted backcross breeding.

***Doubled Haploids (DHs)*:**

- Chromosome doubling of anther culture derived haploid plants from F_1 generates DHs. The suitability of doubled–haploid progenies for mapping project has been demonstrated in by Lefebvre et al. (1995) in pepper.
- DHs are also products of one meiotic cycle, and hence comparable to F_2 in terms of recombination information.
- The expected ratio for the marker is 1:1, irrespective of genetic nature of marker (whether dominant or codominant).

Merits

- DHs are permanent mapping population and hence can be replicated and evaluated over locations and years and maintained without any genotypic change
- Useful for mapping both qualitative and quantitative characters
- Instant production of homozygous lines, thus saving time

Demerits

- Recombination from the male side alone is accounted.
- Since it involves *in vitro* techniques, relatively more technical skills are required in comparison with the development of other mapping populations

- Often suitable culturing methods / haploid production methods are not available for number crops and different crops differ significantly for their tissue culture response. Further, anther culture induced variability should be taken care of.

***Recombinant Inbred Lines (RILs)*:**

- RILs are produced by continuous selfing or sib mating the progeny of individual members of an F_2 population until complete homozygous is achieved
- Single Seed Descent (SSD) method is best suited for developing RILs. Bulk method and pedigree methods without selection can also be used
- RILs also equalize marker types like DHs, the genetic segregation ratio for both dominant and co dominant marker would be 1:1
- RILs developed though brother-sister mating require more time than those developed through selfing. The number of inbred lines required is twice, in case they are developed through brother–sister mating compared to selfing particularly, when linkage is not very tight.

Merits

- Once homozygosity is achieved, RILs can be propagated indefinitely without further segregation
- Since RILs are immortal population, they can be replicated over locations and years and therefore are of immense value in mapping QTLs
- RILs being obtained after several cycles of meiosis, are very useful in identifying tightly linked makers
- RIL populations obtained by selfing have twice the amount of observed recombination between very closely linked markers as compared to population derived from a single cycle of meiosis

Demerits

- Requires many seasons / generations to develop
- Developing RILs is relatively difficult in crops with high inbreeding depression

Immortalized F_2 Population:

- Immortalized F_2 populations can be developed by paired crossing of the randomly chosen RILs derived from a cross in all possible combinations excluding reciprocals.
- The set of RILs used for crossing along with the F_1s produced, provide a true representation of all possible genotype combinations (including the heterozygotes) expected in the F_2 of the cross from which the RILs are derived.

Merits

- The RILs can be maintained by selfing and required quantity of F_1 seed can be produced at will by fresh hybridization. This population therefore provides an opportunity to map heterotic QTLs and interaction effects from multilocation data.

***Near-Isogenic Lines (NILs)*:**

- NILs are generated either by repeated selfing or backcrossing the F_1 plants to the recurrent parents.
- NILs developed through backcrossing are similar to recurrent parent but for the gene of interest, while NILs developed though selfing are similar in pair but for the gene of interest (however, differ a lot with respect to the recurrent parent)
- Expected segregation ratio of the markers is 1:1 irrespective of the nature of marker

Merits

- Like DHs and RILs, NILs are also 'immortal mapping population'
- Suitable population for tagging the trait, wherever such population is available
- NILs are quite useful in functional genomics

Demerits

- Require many generations for development
- Directly useful only for molecular tagging of the gene concerned, but not for linkage mapping
- Linkage drag is a potential problem in constructing NILs, which has to be taken care of.

Chromosomal Segment Substitution Lines (CSSLs) :

- CSSLs are series of plants that possess chromosome segments of the donor parent in the recurrent parental chromosome background. These lines are produced by repeated backcrossing with a recurrent parent in combination with systematic MAS (Fig.2).
- The backcrossed lines contain overlapping donor chromosome segments for each of the chromosome in the genome under consideration. These lines can be considered similar to a genomic library with a huge genome insert.
- Phenotypic characterization of each line can reveal which chromosome fragment from the donor has the gene(s) associated with an interesting trait.

Merits

- CSSLs can be used for the detection of QTLs and particularly QTLs with small additive effects that are masked by QTLs with larger effects in populations such as F_2 and RILs. Identifying QTLs using CSSLs does not require linkage map construction or statistical analysis.
- Further, each CSS line can be directly used as plant material for mapping and cloning QTL genes and as a mother line for breeding. Once developed, these lines can be easily propagated by self-pollination and are repeatedly available for evaluating any trait.

Demerit

- The main disadvantage of CSSLs is that they might have undesirable traits linked to the target gene(s) because the large introgressed chromosomal segment.

Bulk Segregant Analysis

Besides the above-mentioned populations, Bulk Segregant Analysis (BSA) approach, using any one of the above-mentioned populations (except NILs) is frequently used in gene tagging. BSA is based on the principle of isogenic lines. In BSA, two parents (say a resistant and susceptible), showing high degree of molecular polymorphism and contrast for the target trait are crossed and F_1 is selfed to generate F_2 population. In F_2, individual plants are phenotyped for resistance and susceptibility. Usually, the DNA isolated from 10 plants in each group is pooled to constitute resistant and susceptible bulks. The resistant parent, susceptible parent, resistant bulk and susceptible bulk, are surveyed for polymorphism using

polymorphic markers. A marker showing polymorphism between parents as well as bulks is considered putatively linked to the target trait, and is further used for mapping using individual F_2 plants. Conceptually, the genetic constitution of the two bulks is similar, but for the genomic region associated with the target trait. Hence, they serve the purpose of isogenic lines in principle.

It has been observed over experiments that when 10 plants are sampled in each group for constituting the bulk, the probability of a polymorphic marker (between parents as well as bulks) not being linked to the target trait is extremely low (10^{-19}). Hence, usually 10 plants are used for constituting the bulks. However, this number may vary depending upon the types of mapping populations used. In absence of isogenic lines, the BSA approach provides a very useful alternative for gene tagging (Michelmore et al., 1991).

Combining Markers and Populations

The genetic segregation ratio at maker locus is jointly determined by the nature of marker (dominant / codominant) and types of mapping populations (Table 1). Therefore, a thorough understanding of the nature of markers and mapping population is crucial for any mapping projects. Markers such RFLPs, microsatellites and CAPS etc. are codominant in nature, while AFLP, RAPD, ISSR are often scored as dominant markers. Mapping populations such as RILs and DHs equalize marker type because of fixation of parental alleles at marker locus in homozygous condition. These population results in 1: 1 segregation ratio at marker locus irrespective of genetic nature of markers, while an F_2 population segregates in 1: 2: 1 ratio for a codominant marker and in 3:1 ratio for dominant marker. Depending upon the segregation pattern, statistical analysis of marker data will vary.

Table 1: Genetic segregation ratio at marker locus in different marker–population combinations.

Marker	**Nature**	**Genetic Segregation Ratio**					
		F_2	**RILs**	**DHs**	**NILs**	**Backcross Popn.**	
						B_1	**B_2**
RFLP	Co-dominant	1 : 2 : 1	1 : 1	1 : 1	1 : 1	1:1	1:1
RAPD	Dominant	3 : 1	1 : 1	1 : 1	1 : 1	1:0	1:1
AFLP	Dominant	3 : 1	1 : 1	1 : 1	1 : 1	1:0	1:1

Microsatellites	Co-dominant	1: 2: 1	1 : 1	1 : 1	1 : 1	1:1	1:1

Characterization of Mapping Populations

Precise molecular and phenotypic characterization of mapping population is vital for success of any mapping project. Since the molecular genotype of any individual is independent of environment, it is not influenced by G x E interaction. However, trait phenotype could be influenced by the environment, particularly in case of quantitative characters. Therefore, it becomes important to precisely estimate the trait value by evaluating the genotypes in multilocation testing over years using immortal mapping populations to have a valid marker-trait association.

Segregation Distortion of Markers in Linkage Mapping

Significant deviation from expected segregation ratio in a given marker-population combination is referred to as segregation distortion. There are several reasons for segregation distortion, including: gamete/zygote lethality, meiotic drive/preferential segregation, sampling/selection during population development and differential responses of parental lines to tissue culture in case of DHs. Segregation distortion can also be specific with respect to some markers in an otherwise normal mapping population. It is therefore important that the 'goodness of fit' of segregation ratio must be tested for individual marker locus and if necessary, the marker showing high degree of segregation distortion be eliminated from the analysis.

Choice of Mapping Populations

It is evident from the foregoing discussion that the short-term mapping populations such as F_2, backcross and conceptual near isogenic lines developed through BSA approach can be a good starting point in molecular mapping, while long-term mapping populations such as RILs, NILs, CSSLs and DHs must be developed and characterized properly with respect to the traits of importance for global mapping projects. As a matter of fact, the development and phenotypic characterization of mapping populations should become an integral part of the ongoing breeding programmes in important crops. At this point, the role of geneticists and plant breeders becomes crucial to reap the benefits of molecular plant breeding.

Suggested Readings

Burr, B., Burr, F.A. 1991. Recombinant inbred lines for molecular mapping in maize. Theor. Appl. Genet. 85: 55-60.

Eshed, Y., Zamir, D. 1995. An Introgression Line Population of *Lycopersicon pennellii* in the Cultivated Tomato Enables the Identification and Fine Mapping of Yield-Associated QTL Genetics 141: 1147-1 162.

Kaeppler, S.M., Philips, R.L., Kim, T.S. 1993. Use of near-isogenic lines derived by backcrossing or selfing to map quantitative traits. Theor. Appl. Genet. 87: 233-237.

Lefebvre, V., Palloix, A., Caranata, C., Pochard, E. 995. Construction of an intraspecific linkage map of pepper using molecular markers and doubled-haploid progenies. Genome 38:112-121.

Michelmore RW, Paran I, Kesseli RV. 1991. Identification of markers linked to disease resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl Acad. Sci. USA 88: 9829-9832.

Simpson, S.P. 1989. Detection of linkage between quantitative trait loci and restriction fragment length polymorphisms using inbred lines. Theor. Appl. Genet. 77: 815-819.

Tamulonis, J.P., Luzzi, B.M., Hussey, R.S., Parrot, W.A., Boerma, H.R. 1997. DNA marker analysis of loci conferring resistance to root-knot nematode in soybean. Theor. Appl. Genet. 95: 664-670.

Xi, Z.Y., He F.H., Zeng, R.Z., Zhang Z.M., Ding X.H., Li W.T., Zhang G.Q. 2006. Development of a wide population of chromosome single-segment substitution lines in the genetic background of an elite cultivar of rice (*Oryza sativa* L.) Genome 49: 476–484.

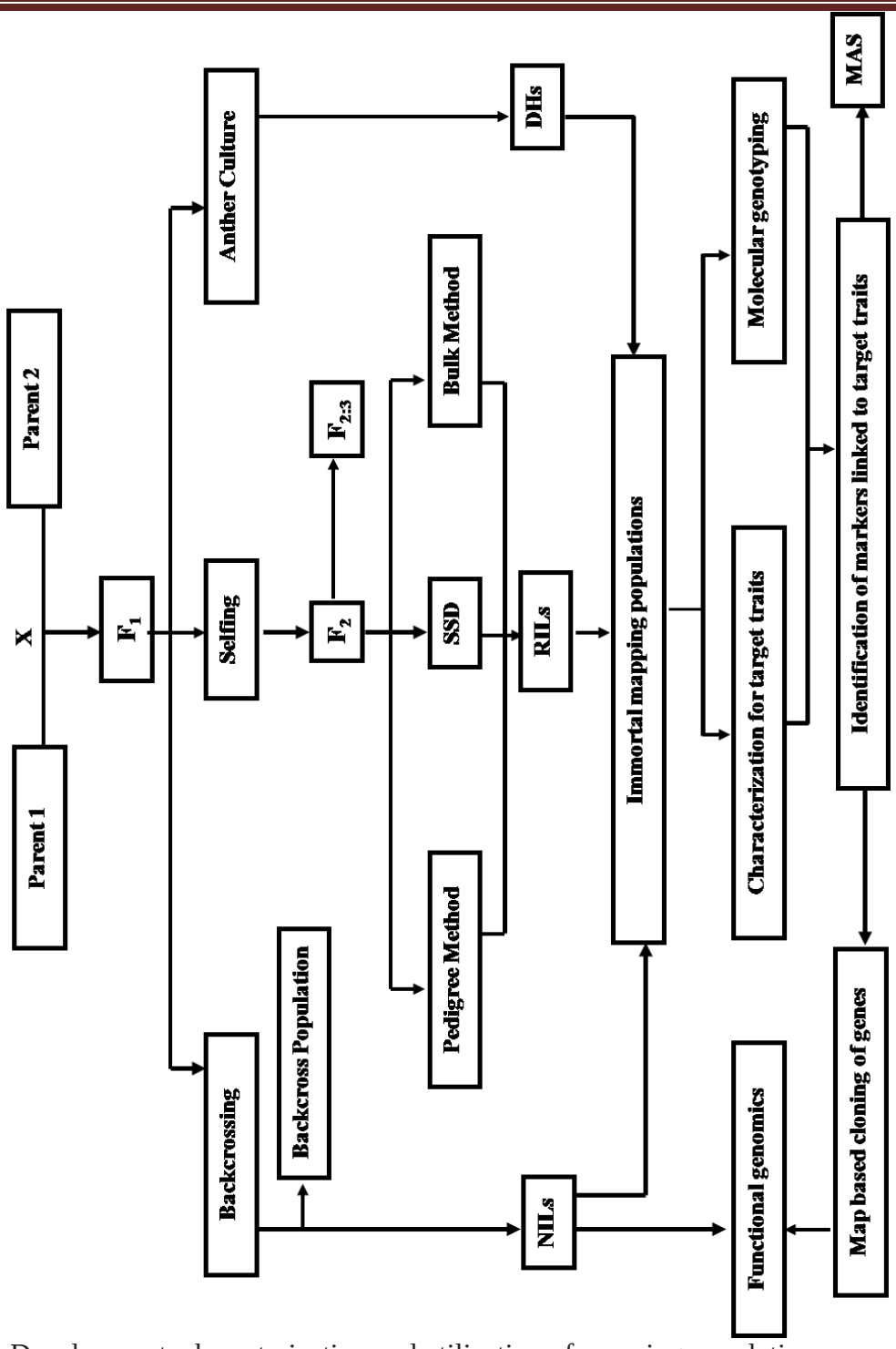


Figure 1. Development, characterization and utilization of mapping populations.

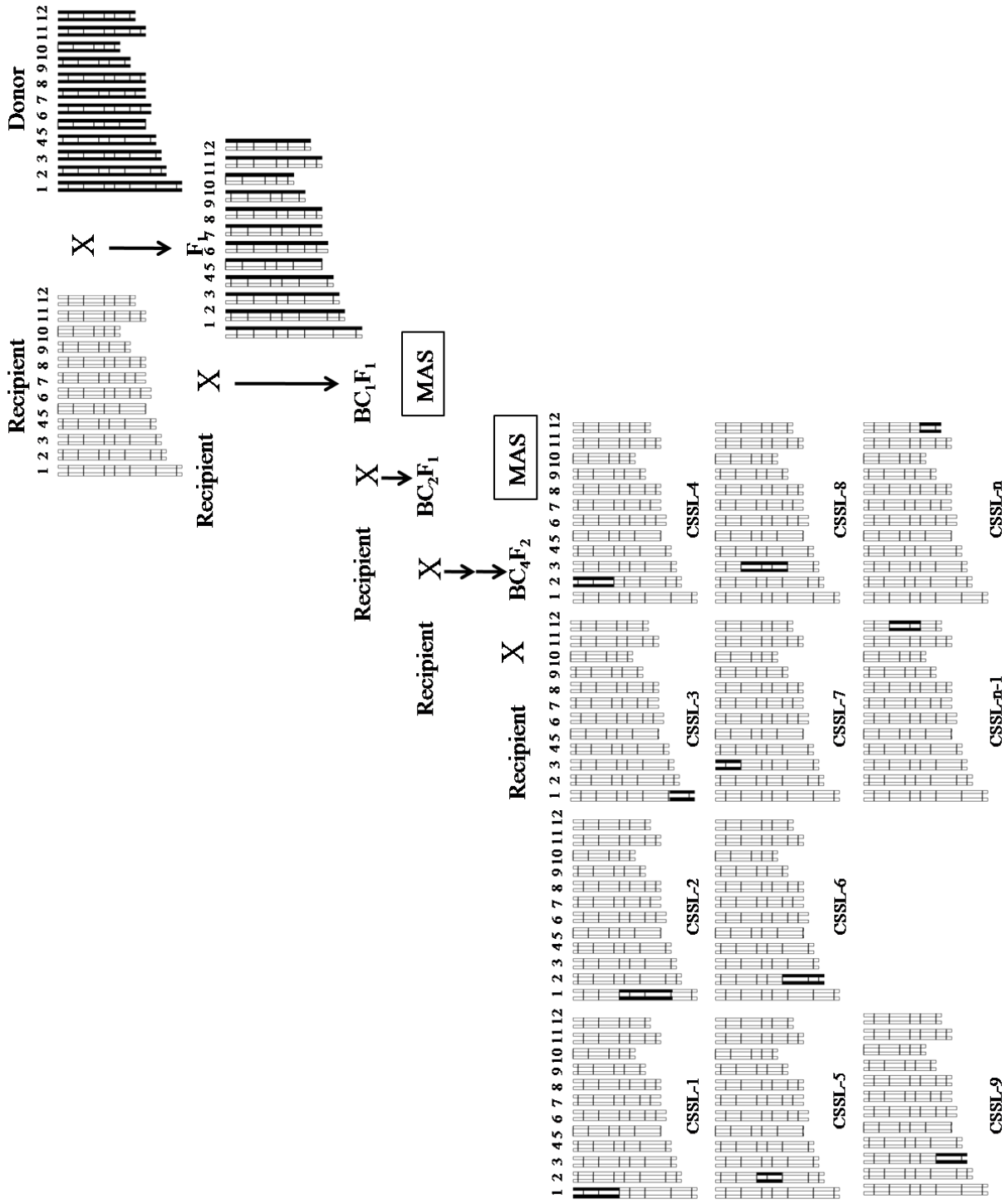


Figure 2. Development of Chromosomal Segment Substitution Lines (CSSLs).

Genetic Enhancement of Nutritional Quality in Maize using Marker Assisted Selection

Firoz Hossain*, Vignesh Muthusamy, Jayant S. Bhat, Abhijit Das, Konsam Sarika, Rajkumar Zunjare, Ashish K. Vishwakarma, Neha Pandey, Aanchal Baveja, Rajat Goswami and Hema Singh Chauhan

Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi-110012

*Corresponding author: *fh_gpb@yahoo.com*

Maize as staple:

Maize (*Zea mays* ssp. *mays* L., 2n=20), considered as '*queen of the cereals*', has emerged as one of the most important staple crops worldwide. It is utilized as human food and livestock feed, and provides over 20% of total calories in human diets in 21 countries, and over 30% in 12 countries that are home to a total of more than 310 million people (Shiferaw et al. 2011). Globally, annual maize production is about 1016.73 million metric tonnes, of which Asia alone produces 304.31 million metric tonnes (FAOSTAT 2014). India is the second-most important maize growing country in Asia, and is the world's sixth largest producer and the fifth-largest consumer of maize. During 2013-14, India produced 24.35 million metric tonnes of maize from 9.4 million hectares (www.indiastat.com). Nearly 23% of the maize produced in India, is used for human food and around 63% is utilized as poultry- and animal- feed (Yadav et al. 2015). It is a staple food crop in many tropical African and Asian countries.

Micronutrient deficiency:

Micronutrient deficiency is a global challenge as nearly 50% of the world's population suffer from diseases caused by chronic insufficient supply of vitamins and minerals (Zhu et al. 2007). Among various micronutrients, deficiency of vitamin A, and essential amino acids such as lysine and tryptophan result in profound effects in humans (Gupta et al. 2015). Vitamin A is required for normal functioning of the visual system, maintenance of cell function for growth, epithelial integrity, red blood cell production, immunity and reproduction (Sommer and West 1996). Vitamin A deficiency (VAD) affects 190 million preschool children and 19 million pregnant and/or lactating women globally,

mostly in Africa and South Asia (WHO 2009). It further contributes to predisposition of several major diseases, such as anaemia, diarrhoea, measles, malaria and respiratory infections (West 2000). India figures in the world map with severe form of VAD (www.harvestplus.org). Lysine and tryptophan, besides role in protein synthesis, serve as precursors for several neuro-transmitters and metabolic regulators, and their deficiency leads to reduced appetite, delayed growth, impaired skeletal development and aberrant behaviour (Gupta et al. 2015). Globally, nearly 200 million children younger than five years are undernourished for protein, leading to a number of health problems, including stunted growth, weakened resistance to infection and impaired intellectual development. It is estimated that India loses 2.5% of the national GDP on account of micronutrient deficiency (FAO 2013). The challenge therefore is to deliver nutritious, safe and affordable food to reduce the impact of nutritional-insecurity.

Ways forward:

A number of methods such as food fortification, supplementation and dietary diversification are practised all over the world for alleviating the micronutrient deficiencies. However, these measures in general have not been found viable in the long run, as they are expensive and inaccessible for the poor in developing countries, thereby reducing their efficiency and application (Lieshout and Pee 2005). Biofortification, a process in which micronutrient density in crops is increased by plant breeding, is proposed as a sustainable and cost-effective mean for providing the required levels of micronutrients in the diet to alleviate malnutrition in humans (Pfeiffer and McClafferty 2007). Crop biofortification, therefore is an economically and socially sound way to address the global challenge of micronutrient deficiency. Traditional maize is deficient in provitamin A, lysine and tryptophan, and enrichment of maize with these micronutrients would provide significant benefit to human health. Availability of natural mutants for these desired traits in maize and robust molecular marker system (Table 1) provide great opportunity to develop micronutrient rich maize through marker assisted breeding strategy (Hossain et al. 2013).

Table 1: Details of genes and markers associated with nutritional quality traits in maize

S. No.	Trait	Genes	Chr.	Marker	Type	Reference(s)
1.	Lysine/ tryptophan	*opaque2*	7	*umc1066, phi057*	Gene-based SSR	Gupta et al. 2013
2.	Lysine	*opaque16*	8	*umc1141, umc1149*	Linked-SSR	Yang et al. 2005
3.	β-carotene	*crtRB1*	10	3'TE-based marker	Gene-based *InDel*	Yan et al. 2010
4.	β-carotene	*lcyE*	8	5'TE-based marker	Gene-based *InDel*	Harjes et al. 2008

Marker assisted breeding:

DNA based markers facilitates the detection of presence of favourable allele of the genes underlying the traits with increased efficiency and precision (Collard and Mackill 2008). It helps in selecting an individual plant without any progeny testing. Marker assisted backcross breeding (MABB) facilitates the introgression of favourable allele of a target gene from 'donor' parent into the background of an otherwise well adapted 'recipient' parent. It significantly reduces number of breeding cycles required to reconstitute the recurrent parent genome (Ribaut et al. 2010). A typical MABB programme comprises of (i) foreground selection, (ii) recombinant selection and (iii) background selection.

(i) *Foreground selection:* Selection of the target allele of the gene in the segregating generation(s) is referred to as 'foreground selection' (Hospital and Charcosset 1997). In BC_nF_1 (n= no. of backcross generation), individuals heterozygous for the target gene are selected, while in BC_nF_2 generation, individual plants having the target allele in homozygous state is selected for further evaluation. Marker-based foreground selection is preferable over phenotypic evaluation, where (a) it is governed by recessive inheritance, (b) estimation of expression of trait is expensive, (c) the expression of trait occurs at later stages of life cycle, (d) estimation of trait leads to loss of the plants/seeds, and (e) assessment of phenotypic expression is time consuming and labour intensive. The effectiveness of foreground selection depends on the number of genes involved in the selection, and genetic distance between the

marker and target gene. Closer distance between the marker and gene ensures greater efficiency of target allele since low chance for recombination exists between the gene and marker. With the increasing distance between the gene and the marker, frequency of recombination increases. Under such situation, it is preferable to have linked flanking markers, as the frequency of occurrence of double cross over is less than the frequency of single cross over. The best strategy however is to have functional or gene-based marker which is most precise as no chance of recombination exists (Lau et al. 2015).

(ii) *Recombinant selection:* It deals with the elimination of the donor genome flanking the target allele (Young and Tanksley 1989). Many undesirable genes linked to the target gene from the donor parent may negatively affect crop performance, and it is referred to as 'linkage drag' (Hospital 2005). Recombinant selection minimizes linkage drag, which is otherwise difficult to eliminate in a conventional breeding programme (Salina et al. 2003). In case of inter-specific crosses especially involving a wild species, the possibility of having linkage drag becomes very high, and recombinant selection becomes an essentiality. In case, where donor is a cultivated/well-adapted/elite line of the same species (as that of recurrent parent), and no gene having unfavourable effect is located near the target allele, recombinant selection may be avoided.

(iii) *Background selection:* Here the genomic regions of the recurrent parent are recovered, and the genomic regions of the donor parent are eliminated except for target locus under introgression (Hospital and Charcosset 1997). Background selection is performed among the foreground positive plants, and selection for the marker alleles distributed throughout the genome of recurrent parent facilitates the rapid recovery of recurrent parent genome (Tanksley et al. 1989; Young and Tanksley 1989). The efficiency of background selection depends on density of molecular marker in the genetic map and level of line conversion desired (Ribaut et al. 2002). In essentially derived varieties (EDVs), the recurrent parent genome (RPG) in the introgressed progeny should be as close as possible to the recurrent parent. On the other hand, recovery of RPG of lower magnitude provides opportunity to derive improved and new genotype through recombination of genome from both recurrent and donor parents. Moreover, larger population size is essential for identifying the recombinants with more number of recurrent parent alleles. Two to three backcrosses coupled with background selection have the potential to develop individuals with high degree of

phenotypic resemblance to the recurrent parent. Young and Tanksley (1989) presented the theory of graphical genotypes which allows graphical representation of the genotypes and populations, which helps breeders to easily identify the desired genotype. Van-Berloo (1999) had also came-up with GGT (Graphical GenoTyping), a free software package, for visualization of molecular marker data by simple chromosome drawing where marker data serve as an input file. GGT is freely available and can be downloaded from http://www.dpw.wageningen-ur.nl/pv/pub/ggt.

Phenotypic selection:

During the selection process of individuals in MABB scheme, it is beneficial to select progenies with difference in one or few easily visible characteristics that differentiate introgressed progenies from the respective recurrent parent. Choudhary et al. (2014) could select some of the MABB-derived introgressed progenies (having *crtRB1* allele) with difference in silk- and glume base- pigmentation (compared to respective recurrent parent). However, this would depend on the careful selection of donors and recipients possessing the easily visible contrasts. This difference is highly useful for registration of introgressed genotypes. Besides, these traits also act as morphological marker(s) to unambiguously differentiate the introgressed genotype from the original genotype in the field, especially during large scale seed production and certification processes.

Enhancement of protein quality:

Protein content of common maize generally varies from 9-10%, however maize protein is deficient in two essential amino acids, lysine (~2.0% in protein) and tryptophan (~0.4% in protein) (Mertz et al. 1964). Monogastric animals such as poultry birds and human cannot synthesize these amino acids in their body and has to be provided externally. Of the various kernel mutations, *opaque2* possessing significantly higher lysine (4.0% in protein) and tryptophan (~0.8% in protein) has been utilized the most in breeding programme for enhancement of kernel quality (Vivek et al. 2008). Sustained breeding efforts at CIMMYT, Mexico and University of Natal, South Africa could successfully accumulate desirable endosperm modifiers in *opaque2* genetic background that finally led to the development of nutritionally enriched vitreous maize, popularly phrased as quality protein maize (QPM) (Vasal et al. 1980). The biological value of QPM is 80% as compared to 45% of normal maize. Only 37% of common maize protein intake is utilized compared to 74% of the same

amount of QPM maize protein. The nitrogen balance index for skim milk and *opaque2* maize protein is 0.80 and 0.72, respectively, which indicates that the protein quality of QPM is 90% of that of milk (Vasal et al. 1980). The other nutritional benefits of QPM include higher niacin availability due to a higher tryptophan and lower leucine content, higher calcium and carbohydrate, and carotene utilization. Decrease in leucine is considered particularly desirable as it makes leucine/isoleucine ratio more balanced, which in turn helps to liberate more tryptophan for niacin biosynthesis, and thus, helps to combat pellagra.

The *opaque2* gene located on chromosome 7L produces leucine-zipper (bZIP) protein that acts as a transcriptional factor for expression of zein family of storage protein genes, especially 22-kDa α-zeins (Ueda et al. 1992). The mutant protein causes reduction in synthesis of zein protein by 50-70% primarily due to its less affinity of binding to the promoter regions (Kodrzycki et al. 1989). The enhancement of nutritional quality in *opaque2* mutant is mainly due to reduction of lysine deficient zein proteins followed by enhanced synthesis of lysine-rich non-zein proteins (Habben et al. 1993). Recessive *opaque2* also significantly reduces transcription of lysine keto-reductase (LKR), the enzyme that degrades lysine in maize endosperm, thereby enhancing the concentration of lysine (Kemper et al. 1999). Further, *opaque2* is involved in regulation of various metabolic pathways and causes enhanced synthesis of various lysine-rich proteins and enzymes (Jia et al. 2013). *Opaque2* gene was cloned using a transposon tagging strategy with the maize mobile genetic elements, *Spm* and *Ac*.

In India, though a dozen of QPM hybrids have been developed, there is an urgent need to develop diverse QPM hybrids across maturity groups with adaptability to different agro-ecologies. Availability of gene based SSR markers (*umc1066* and *phi057*) for *opaque2* gene facilitates the adoption of marker-assisted conversion of normal maize hybrids into QPM version (Prasanna et al. 2010). The first example of 'proof of concept' on application of molecular markers for improvement of nutritional quality in India, was the introgression of *opaque2* allele from a white kernel QPM inbred, CML176 into an elite yellow inbred, V25 using MABB strategy (Babu et al. 2005). This research effort at VPKAS, Almora led to the increase of tryptophan in endosperm protein from 0.41% to as high as 0.82% among marker-assisted selection (MAS)-derived inbreds. Background selection employed was effective to recover >90% of recurrent parent genome in just two generations of backcrossing. Utilizing the same strategy at VPKAS, parental inbreds (CM145 and CM212) of Vivek Hybrid-9 were improved for endosperm lysine and tryptophan; and it led to the development and

commercial release of Vivek QPM-9 (Gupta et al. 2013). Vivek QPM-9 possesses 41% more tryptophan and 30% more lysine over the original hybrid, with similar grain yield potential of Vivek Hybrid-9. Stringent selection of endosperm modification helped in having high degree of vitreous kernels in the reconstituted version of parental inbreds and hybrids. Vivek QPM-9 earned the distinction of being the first MAS-based maize cultivar released for commercial cultivation in India. Vivek QPM-21, developed through marker-assisted introgression of *opaque2* allele into Vivek Hybrid-21 was yet another QPM hybrid released in 2012 for Uttarakhand state (Gupta et al. 2015). Currently, a large number of single cross normal maize hybrids have been targeted for conversion to QPM using accelerated strategy of marker-aided foreground and background selection at various institutions in India *viz.,* IARI, New Delhi; VPKAS, Almora; ICAR-NEH, Barapani; ANGRAU, Hyderabad; HPKV, Palampur; JNKVV, Jabalpur and PAU, Ludhiana. Parental lines *viz.,* HKI-323, HKI-1105 and HKI-1128 have been improved for tryptophan level in endosperm under the biofortification programme at IARI, New Delhi (Hossain et al. 2014). The improved versions of HM-4, HM-8, HM-9, HM-10 and HM-11 have been evaluated, and were found to possess higher protein quality with grain yield at par with the original hybrids. Stringent selection of endosperm modification helped in having high degree of vitreous kernels in the reconstituted version of parental inbreds and hybrids.

Endosperm modifier loci can potentially enhance the nutritive value in *opaque2* genetic background to a limited extent (Pandey et al. 2015). A recessive *opaque16* mutant isolated from Robertson's Mutator (Mu) stock was found to be associated with higher nutritional value in maize (Yang et al. 2005). *Opaque16* was mapped on chromosome 8L, and *umc1141* and *umc1149* were identified as closely linked markers. It further reported that *opaque16* in combination with *opaque2* can cause an average increase of 30% lysine with as high as 60% over *o2o2* genotype. In our programme, pyramiding of *opaque2* and *opaque16* in parents of elite hybrids is currently being undertaken. Our results suggest that *opaque16* does not have much influence on endosperm opaqueness (Sarika et al. 2015).

Enhancement of provitamin A:

Though yellow maize possesses tremendous natural variation for carotenoids (Tiwari et al. 2012; Sivaranjani et al. 2013), it is predominated by lutein and zeaxanthin – fractions that do not possess provitamin A activity (Vignesh et al. 2012; 2013; Choudhary et al. 2015;

Muthusamy et al. 2015a, b, c). Provitamin A carotenoids such as β-carotene is present in low amount (<2 µg/g) in most of the tropical germplasm compared to target level of 15 µg/g (Bouis et al. 2011). The carotenoid metabolic pathway has been well researched in model species, and key genes governing critical steps have been identified. The key regulatory step of the pathway involves the condensation of two geranyl geranyl pyrophosphate (GGPP) to form 15-cis-phytoene that is further converted to all-translycopene (a red pigment) by four desaturation reactions and by an isomerization reaction. The carotenoid biosynthesis pathway has two major branches that occur after the biosynthesis of the linear carotenoid, all-trans-lycopene (DellaPenna and Pogson 2006). Lycopene may be cyclized to form two β rings, as found in β-carotene and its derivatives, β-cryptoxanthin and zeaxanthin. Alternatively, lycopene may be cyclized to form one β ring and one ε ring, as found in α-carotene and its derivatives, zeinoxanthin and lutein.

In maize, three genes have been proposed to play crucial roles in the final accumulation of provitamin A carotenoids in the endosperm. *Phytoene synthase1* (*Y1* or *Psy1*) catalyses the first committed step in the pathway leading to formation of phytoene from GGPP, and is primarily responsible for the shift from white to yellow maize (Li et al. 2010). Two genes, *lycopene epsilon cyclase* (*lcyE*) and *β-carotene hydroxylase 1* (*crtRB1*) have been shown to regulate the accumulation of provitamin A compounds. Natural *lcyE* converts lycopene into ζ-carotene and eventually to α-carotene through the action of other associated genes. Favourable *lcyE* allele forces pathway flux towards β-carotene branch and its non-provitamin A derivatives (Harjes et al. 2008). Though the favourable *lcyE* allele increases the proportion of β-carotene in the pathway but a large amount is hydroxylated to produce β-cryptoxanthin (with 50% provitamin A activity) and zeaxanthin (0% provitamin A activity). *CrtRB1* is a hydroxylase gene that converts β-carotene into β-cryptoxanthin. However, naturally available favourable *crtRB1* allele blocks the process of hydroxylation of β-carotene in to further components, thereby leading to the increase of concentration of β-carotene in the kernel (Yan et al. 2010). Thus *lcyE* and *crtRB1* are the two crucial genes responsible for the accumulation of higher β-carotene in maize kernels (Harjes et al. 2008; Vallabhaneni et al, 2009; Yan et al. 2010). However, the frequency of the favourable allele of *crtRB1* and *lcyE* is extremely low (<4.0%) in the available maize germplasm (Muthusamy et al. 2015c).

We have successfully introgressed *crtRB1* favourable allele into parental inbreds of four hybrids, Vivek QPM-9, Vivek Hybrid-27, HM-4 and HM-8 using MABB approach (Muthusamy et al. 2014). Newly developed introgressed inbreds possessed mean kernel β-

carotene of 14.1 µg/g as compared to 1.4 µg/g across original inbreds with a mean increase of 10.3-fold over recurrent parent. Mean kernel β-carotene among the MAS-derived hybrids was 17.5 µg/g, while the same among the original hybrids was 2.1 µg/g. The reconstituted hybrids also showed high degree of similarity for most of the yield- and yield attributing- traits. These β-carotene rich maize inbreds can be effectively used as donor for high β-carotene in the breeding programme, while the improved hybrids can be readily used in the alleviating the VAD. This is the first-ever demonstration of conversion of elite maize hybrids into β-carotene-rich version using MABB approach (Muthusamy et al. 2014).

Multinutrient maize:

We developed three *crtRB1*-introgressed versions of Vivek QPM-9, and kernel β-carotene ranged from 17.8 to 21.5 µg/g, compared to the original hybrid Vivek QPM-9 (2.1 µg/g). An average of 9.6-fold enhancement of kernel β-carotene was observed among the introgressed hybrids. The improved hybrids also recorded a similar concentration of tryptophan (0.81%) compared to the original hybrid (0.83%), as MAS for *opaque2* was also undertaken during the backcross breeding program. The improved hybrids also exhibited a similar grain yield potential and other yield-attributing characters compared to the original hybrid Vivek QPM-9. The β-carotene enriched MAS-derived hybrids recorded a grain yield of 5.6-6.0 tons/hectare at Delhi, and 5.7-6.1 tonnes/hectare at Dharwad, compared to the original hybrid Vivek QPM-9 (6.1 and 5.1 tonnes/hectare at Delhi and Dharwad, respectively). Improved version of Vivek QPM-9 having high β-carotene coupled with higher lysine and tryptophan, thus provides multi-nutrients to humans through maize-based diet. This is the first successful example of combination of nutrients, *viz.*, provitamin A and QPM (Muthusamy et al. 2014). Parental lines of the hybrids HM-4, HM-8 and HM-9 have been introgressed with *opaque2* and *crtRB1* (Gupta et al. 2015). Further, parental lines of popular QPM hybrids have been introgressed with favourable alleles of both *crtRB1* and *lcyE* using marker-assisted selection (Zunjare et al. 2015). These inbreds possess >20 µg/g of provitamin A as compared to 2-3 µg/g in original parents, and are rich in lysine and tryptophan. Improved inbreds resembled their respective recurrent parent with high degree of similarity for plant- and ear- characteristics. Maize breeders at IARI have improved a number of popular hybrids for provitamin A, lysine and tryptophan in different maturity groups with adaptability in diverse agro-ecologies (Choudhary et al. 2014; Muthusamy et al. 2014; Goswami et al. 2015; Gupta et al. 2015). These multinutrient maize genotypes would help in

providing a holistic approach towards alleviation of micronutrient malnutrition in a sustainable manner.

References:

Babu R., Nair S.K., Kumar A., Venkatesh S., Sekhar J.C., Singh N.N., Srinivasan G. and Gupta H.S. (2005). Two-generation marker-aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). *Theor. Applied. Gen.* 111: 888-897.

Bouis H.E., Hotz C., McClafferty B., Meenakshi J.V. and Pfeiffer W.H. (2011). Biofortification: a new tool to reduce micronutrient malnutrition. *Food Nutr. Bull.* 32: S31-S40.

Choudhary M., Hossain F., Muthusamy V., Thirunavukkarasu N., Saha S., Pandey N., Jha S.K. and Gupta H.S. (2015). Microsatellite marker-based genetic diversity analyses of novel maize inbreds possessing rare allele of *β-carotene hydroxylase* (*crtRB1*) for their utilization in β-carotene enrichment. *J. Plant Biochem. Biotechnol.* DOI: 10.1007/s13562-015-0300-3.

Choudhary M., Muthusamy V., Hossain F., Thirunavukkarasu N., Pandey N., Jha S.K. and Gupta H.S. (2014). Characterization of β-carotene rich MAS-derived maize inbreds possessing rare genetic variation in *β-carotene hydroxylase* gene. *Indian J. Genet.* 74 (Suppl.): 620-623.

Collard B.C.Y. and Mackill D.J. (2008). Marker-assisted selec- tion: an approach for precision plant breeding in the twenty-first century. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 363: 557-572.

DellaPenna D. and Pogson B.J. (2006). Vitamin synthesis in plants: Tocopherols and carotenoids. *Ann. Rev. Plant Biol.* 57: 711-738.

FAO. 2013. State of Food and Agriculture: 99.

FAOSTAT (2014). www.faostat.org: accessed on 01-09-2015.

Goswami R., Hossain F., Khan S., Muthusamy V., Baveja A., Zunjare R., Sarika K., Thirunavukkarasu N., Saha S. and Gupta H.S. (2015). Marker-assisted introgression of *crtRB1*-favourable allele into QPM version of an elite maize inbred-HKI1128 for enrichment of kernel β-carotene. In: National symposium on Germplasm to genes: Harnessing Biotechnology for Food Security and Health, 9-11, August 2015, ICAR-NRCPB, New Delhi, Abstract, pp.86.

Gupta H.S., Babu R., Agrawal P.K., Mahajan V., Hossain F. and Nepolean T. (2013). Accelerated development of quality protein maize hybrid through marker-assisted introgression of *opaque-2* allele. *Plant Breed.* 132: 77-82.

Gupta H.S., Hossain F. and Muthusamy V. (2015). Biofortification of maize: An Indian perspective. *Indian J. Genet.* 75: 1-22.

Habben I.E., Kirleis A.W. and Larkins B.A. (1993). The origin of lysine-containing proteins in *opaque-2* maize endosperm. *Plant Mol. Biol.* 23: 825-838.

Harjes C.E., Rocheford T.R., Bai L., Brutnell T.P., Kandianis C.B., Sowinski S.G., Stapleton A.E., Vallabhaneni R., Williams M., Wurtzel E.T., Yan J. and Buckler E.S. (2008). Natural genetic variation in *lycopene epsilon cyclase* tapped for maize biofortification. *Science* 319: 330-333.

Hospital F. (2005). Selection in backcross programmes. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 360: 1503-1511.

Hospital F. and Charcosset A. (1997). Marker-assisted introgression of quantitative trait loci. *Genetics* 147: 1469-1485.

Hossain F., Nepolean T., Pandey N., Vishwakarma A.K., Saha S., Vignesh M., Agrawal P.K., Reddy S.S., Pattanayak A., Lata S., Tiwari S., Prasanna B.M. and Gupta H.S. (2014). Development of quality protein maize hybrids through marker-assisted breeding strategy. 12th Asian Maize Conference and Expert Consultation on 'Maize for Food, Feed and Nutritional Security' held in Bangkok, Thailand. 30 October - 01 November, 2014. Abstracts. pp. 65.

Hossain F., Nepolean T., Vishwakarma A.K., Panday N., Prasanna B.M. and Gupta H.S. (2013). Mapping and validation of microsatellite markers linked to *sugary1* and *shrunken2* genes in maize. *J. Plant Biochem. Biotechnol.* DOI 10.1007/s13562-013-0245-3.

Jia M., Wu H., Clay K.L., Jung R., Larkins B.A. and Gibbon B.C. (2013). Identification and characterization of lysine-rich proteins and starch biosynthesis genes in the *opaque2* mutant by transcriptional and proteomic analysis. *BMC Plant Biol.* 13: 60. doi: http:// www.biomedcentral.com/ 1471-2229/13/60

Kemper E. L., Neto C.G., Papes F., Moraes M.K.C. and Leite A. (1999). The role of *opaque2* in the control of lysine-degrading activities in developing maize endosperm. *Plant Cell* 11: 1981-1993.

Kodrzycki R., Boston R. S. and Larkins B. A. (1989). The *opaque-2* mutation of *maize* differentially reduces zein gene transcription. *Plant Cell* 1: 105-114.

Lau W.C. P., Rafii M.Y., Ismail M.R., Puteh A., Latif M.A. and Ramli A. (2015). Review of functional markers for improving cooking, eating, and the nutritional qualities of rice. *Front. Plant Sci.* doi: 10.3389/fpls.2015.00832.

Li Q., Farre G., Naqvi S., Breitenbach J., Sanahuja G., Bai C., Sandmann G., Capell T., Christou P. and Zhu C. (2010) Cloning and functional characterization of the maize carotenoid isomerase and β-carotene hydroxylase genes and their regulation during endosperm maturation. *Transgenic Res.* 19: 1053-1068.

Lieshout M.V. and Pee S.D. (2005). Vitamin A equivalency estimates: understanding apparent differences. *Am. J. of Clinical Nutr.* 81: 943-945.

Mertz E.T., Bates L.S. and Nelson O.E. (1964). Mutant gene mutant that changes the protein composition and increases the lysine content of maize endosperm. *Science* 145: 279-280.

Muthusamy V., Hossain F., Nepolean T., Saha S., Agrawal P.K., Guleria S.K. and Gupta H.S. (2015a). Genetic variability and inter-relationship of kernel carotenoids among indigenous and exotic maize (*Zea mays* L.) inbreds. *Cereal Res. Comm.* http://dx.doi.org/10.1556/0806.43.2015.012

Muthusamy V., Hossain F., Thirunavukkarasu N., Choudhary M., Saha S., Bhat J.S., Prasanna B.M. and Gupta H.S. (2014). Development of β-carotene rich maize hybrids through marker-assisted introgression of *β-carotene hydroxylase* allele. *PLOS One* 9(12): e113583. doi:10.1371/ journal.pone.0113583.

Muthusamy V., Hossain F., Thirunavukkarasu N., Pandey N., Vishwakarma A.K., Saha S. and Gupta H.S. (2015b). Molecular characterization of exotic and indigenous maize inbreds for biofortification with kernel carotenoids. *Food Biotechnol.* 29: 276-295.

Muthusamy V., Hossain F., Thirunavukkarasu N., Saha S. and Gupta H.S. (2015c). Allelic variations for *lycopene ε- cyclase* and *β-carotene hydroxylase* genes in maize inbreds and their utilization in β-carotene enrichment programme. *Cogent Food Agri.* doi: 10.1080/23311932.2015.1033141.

Pandey N., Hossain F., Kumar K., Vishwakarma A.K., Nepolean T., Vignesh M., Manjaiah K.M., Agrawal P.K., Guleria S.K., Reddy S.S. and Gupta H.S. (2015). Microsatellite marker-based genetic diversity among quality protein maize (QPM) inbred lines differing for kernel iron and zinc. *Mol. Plant Breed.* 6: 1-10.

Pfeiffer W. H. and McClafferty B. (2007). HarvestPlus: Breeding crops for better nutrition. *Crop Sci.* 47: S88-S105.

Prasanna B.M., Pixley K.V., Warburton M. and Xie C. (2010). Molecular marker-assisted breeding for maize improvement in Asia. *Mol. Breed.* 26: 339-356.

Ribaut J.M., de Vicente M.C. and Delannay X. (2010). Molecular breeding in developing countries: challenges and perspectives. *Curr. Opin. Plant Biol.* 13: 1-6.

Ribaut J.M., Jiang C. and Hoisington D. (2002). Simulation experiments on efficiencies of gene introgression by backcrossing. *Crop Sci.* 42: 557-565.

Salina E., Dobrovolskaya O., Efremova T., Leonova I. and Roder M.S. (2003). Microsatellite monitoring of recombination around the *Vrn-B1* locus of wheat during early backcross breeding. *Plant Breed*. 122: 116-119.

Sarika K., Hossain F., Muthusamy V., Zunjare R., Baveja A., Thirunavukkarasu N., Goswami R. and Gupta H.S. (2015). Marker-assisted pyramiding of *opaque2* and *opaque16* alleles for the enhancement of lysine and tryptophan in maize endosperm. In: National symposium on Germplasm to genes: Harnessing Biotechnology for Food Security and Health, 9-11, August 2015, ICAR-NRCPB, New Delhi, Abstract. pp. 87.

Shiferaw B., Prasanna B., Hellin J. and Banziger M. (2011). Crops that feed the world. 6. Past successes and future challenges to the role played by maize in global food security. *Food Security* 3: 307-327.

Sivaranjani R., Prasanna B.M., Hossain F. and Santha I.M. (2013). Genetic variability for total carotenoid concentration in selected maize inbred lines. *Indian J. Agric. Sci.* 83: 431-436.

Sommer A. and West K.P. (1996). Vitamin A deficiency: Health survival and vision. New York, Oxford University Press.

Tanksley S.D., Young N.D., Patterson A.H. et al. (1989). RFLP mapping in plant breeding: new tools for an old science. *Biotechnol.* 7: 257-263.

Tiwari A., Prasanna B.M., Hossain F. and Guruprasad K.N. (2012). Analysis of genetic variability for kernel carotenoid concentration in selected maize inbred lines. *Indian J. Genet.* 72: 1-6.

Ueda T., Waverczak W., Ward K., Sher M., Ketudat M., Schmidt R.J. and Messing J. (1992). Mutation of 22 and 27-kD zein promoters affect transactivation by the *opaque-2* protein. *Plant Cell* 4: 701-709.

Vallabhaneni R., Gallagher C.E., Licciardello N., Cuttriss A.J., Quinlan R.F. and Wurtzel E.T. (2009). Metabolite sorting of a germplasm collection reveals the *hydroxylase3* locus as a new target for maize provitamin: A biofortification. *Plant Physiol.* 151: 1635-1645.

Van-Berloo R. (1999). GGT: software for display of graphical genotypes. *J. Hered.* 90: 328-329.

Vasal S.K., Villegas E., Bajarnason M., Gelaw B. and Geirtz P. (1980). In: Improvement of Quality Traits for Silage Use. ed, Martinus Nijhoff, The Hague, Netherlands, pp. 37-71.

Vignesh M., Hossain F., Nepolean T., Saha S., Agrawal P.K., Guleria S.K., Prasanna B.M. and Gupta H.S. (2012). Genetic variability for kernel β-carotene and utilization of *crtRB1* 3'TE gene for biofortification in maize (*Zea mays* L.). *Indian J. Genet.* 72: 189-194.

Vignesh M., Nepolean T., Hossain F., Singh A.K. and Gupta H.S. (2013). Sequence variation in 3′UTR region of *crtRB1* gene and its effect on β-carotene accumulation in maize kernel. *J. Plant Biochem. & Biotech.* DOI 10.1007/s13562-012-0168-4. 22: 401-408.

Vivek B.S., Krivanek A.F., Palacios-Rojas N., Twumasi-Afiriye S. and Diallo A.O. (2008). Breeding quality protein maize (QPM) cultivars: protocols for developing QPM cultivars. CIMMYT, Mexico.

West C.E. (2000). Vitamin A and measles. *Nutr Rev.* 58: 546-554.

WHO (2009). Global prevalence of vitamin A deficiency in populations at risk 1995-2005. http://www.who.int/nutrition/publications/ micronutrients/vitamin_A deficiency.

Yadav O.P. , Hossain F., Karjagi C.G., Kumar B., Zaidi P.H., Jat S.L., Chawla J.S., Kaul J., Hooda K.S., Kumar O., Yadava P. and Dhillon B.S. (2015). Genetic improvement of maize in India: retrospect and prospects. *Agric. Res.* 4: 325-338. doi 10.1007/s40003-015-0180-8.

Yan J., Kandianis B.C., Harjes E.C., Bai L., Kim H.E., Yang X., Skinner D.J., Fu Z., Mitchell S., Li Q., Fernandez G.S.M., Zaharoeva M., Babu R., Fu Y., Palacios N., Li J., DellaPenna D., Brutnell T., Buckler S.E., Warburton L.M. and Rocheford T. (2010). Rare genetic variation at *Zea mays crtRB1* increases β-carotene in maize grain. *Nat. Genet.* 42: 322-327.

Yang W., Zheng Y., Zheng W. and Feng R. (2005). Molecular genetic mapping of a high-lysine mutant gene (*opaque-16*) and the double recessive effect with *opaque-2* in maize. *Mol. Breed.* 15: 257-269.

Young N.D. and Tanksley S.D. (1989). RFLP analysis of the size of chromosomal segments retained around the Tm-2 locus of tomato during backcross breeding. *Theor. Appl. Genet.* 77: 353-359.

Zhu C., Naqvi S., Gomez-Galera S., Pelacho A.M., Capell T. and Christou P. (2007) Transgenic strategies for the nutritional enhancement of plants. *Trends in Plant Sci.* 12: 548-555.

Zunjare R., Hossain F., Muthusamy V., Baveja A., Thirunavukkarasu N., Saha S. and Gupta H.S. (2015). Marker-aided pyramiding of *β-carotene hydroxylase* (*crtRB1*) and *lycopene-ε-cyclase* (*lcyE*) genes for provitamin-A enrichment in quality protein maize (QPM) inbreds. In: National symposium on Germplasm to genes: Harnessing Biotechnology for Food Security and Health, 9-11, August 2015, ICAR-NRCPB, New Delhi, Abstract. pp.74.

High Throughput Markers –Discovery, Genotyping and Applications in Crops

Gopala Krishnan S[1], NK Singh[2] and AK Singh[1]

[1]Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi 110 012

[2]ICAR-NRC on Plant Biotechnology, ICAR-IARI, New Delhi 110 012

Introduction

Genetic variations in the DNA have been the basis for development of a vast array of molecular markers, from Restriction Fragment Length Polymorphism (RFLP) to Simple Sequence Repeats (SSR), for use in genetic analysis. Recent improvements in whole-genome shot gun sequencing has drastically reduced the cost of whole genome sequencing, providing low cost coverage of any genome generating a sequence resource for molecular marker and gene discovery, comparative genomics and genome assembly (Gopala Krishnan et al. 2012). Coupled with the developments in bioinformatics to assemble short reads, next generation sequencing technologies enables the discovery of millions of DNA polymorphisms such as SNPs and InDels by comparing the whole genome sequences of several individuals within a species (Henry et al. 2012). SNPs and InDels are becoming the preferred markers in molecular breeding due to multiple advantages such as high frequency, stability, high throughput capability and cost effectiveness over other DNA markers (Henry and Edwards, 2009). SNPs are being employed in breeding programs for marker-assisted and genomic selection, association and QTL mapping, positional cloning, haplotype and pedigree analysis, seed purity analysis and variety identification (Singh et al., 2015; McCouch et al., 2010).

Discovery of genome-wide variations

Advances in sequencing technologies have played a pivotal role in discovery of genome-wide variations (SNPs and InDels) in crops. The discovery of high throughput markers using NGS platforms can be accomplished through sequencing involving different approaches (Henry et al. 2012) namely,

(i) *Amplicon sequencing*, where a gene(s)/ genomic region(s) of interest are sequenced from an individual/ a population.

(ii) *Transcriptome sequencing*, where the sum total of the transcripts from the whole organism/ tissue(s) are sequenced from a genotype/ a set of genotypes.

(iii) *Gene enriched genome sequencing*, where the gene-rich regions of a genome are sequenced for discovering polymorphisms within genes of an individual, using genic probes, which could be associated with the phenotype.

(iv) *Whole genome sequencing*, where the whole genome of the genotype/ populations are re-sequenced. Whole genome sequencing accelerates the rate of gene and molecular marker discovery. High levels of homology and gene synteny allows knowledge of gene function to be applied across species by comparison to high quality reference genome sequences.

(v) *Organelle sequencing*, where the organellar (chloroplast and mitochondrial) genomes from different organisms are sequenced especially useful in phylogenetic studies.

(vi) *Bisulfite sequencing*, where the methylation pattern of different tissues of genotypes can be understood through sequencing of DNA pre-treated with sodium bisulphite.

(vii) *Reduced representation sequencing*, wherein only a fraction (usually 0.1 to 10%) of the variation in the genome is sequenced through the use of restriction enzyme digestion.

High Through-put Genotyping

Although the earlier SNP genotyping techniques relied on gel based assays, rapid advances in technology, automation and data analysis has helped in development of genotyping techniques, popularly known as high throughput genotyping.

High throughput genotyping can be defined as genotyping of many SNPs (hundreds to millions in multiplexed fixed arrays) in many individuals (hundreds to thousands), and especially focus on the technologies aimed at large-scale association studies (Tsuchihashi and Dracopoli, 2002).

For genotyping SNP markers in low to medium-throughput approaches, more than 30 assays are currently available. These high-throughput genotyping methods is a viable combination of the allele-discrimination techniques (allele-specific hybridisation, single nucleotide primer extension, oligonucleotide ligation and enzymatic cleavage), reaction formats (solution-phase, solid-phase, and gel electrophoresis, next-generation sequencing) and detection method (colorimetry, spectrometry, fluorescence, fluorescence resonance energy transfer, fluorescent polarization, chemiluminescence) (Kwok, 2000).

Some of the widely used SNP genotyping platforms include (1) BeadXpress™, GoldenGate™ and Infinium® from Illumina (http://www.illumina.com); (2) GeneChip™ and GenFlex™ Tag array from Affymetrix (http://www.affymetrix.com); (3) SNaPshot™ and TaqMan™ from the Applied Biosystems (http://www.appliedbiosystems.com); (4) SNPstream™ from Beckman Coulter (http://www.labwrench.com); (5) SNPWave™ from KeyGene (http://www.keygene.com); (6) iPLEX Gold™ Assay and Mass-ARRAY™ from Sequonome (http://www.sequenom.com/iplex); (7) Pyrosequencing from Royal Institute of Technology (Sweden); (8) molecular inversion probes from ParAllele Biosciences; and (9) competitive allele-specific PCR (currently called Kompetitive Allele Specific PCR, or KASP™) from KBioscience or LGC Genomics (http://www.lgcgenomics.com). From the array of choices available for high throughput genotyping, the choice would vary based on the application, cost per sample/ data point, throughputness, turnaround time, ease of handling, performance (sensitivity, reliability, reproducibility, and accuracy), flexibility (genotyping few samples with many SNPs or many samples with few SNPs), number of markers generated per run (uniplex versus multiplex assay capability) and assay development requirements (Semagn et al. 2014). For example, High-resolution SNP-detection platforms such as Infinium®/ GeneChip™ will be useful for assaying SNP diversity in a germplasm collection while the low resolution assays such as BeadXpress™/ GoldenGate™ are generally custom-designed for assay of large number of individuals within a short time window including QTL analysis, NIL development and backcross conversion, varietal identification, quality control in the market or as part of germplasm management, to ensure outcrossing or inbreeding in a hybrid rice program, or to fine map a target region in a gene-discovery program (Tung et al. 2010).

In addition to the above genotyping methods, Genotyping By Sequencing (GBS) is gaining importance as a cost-effective tool for genomics-assisted breeding applications ranging from low-cost tool to genotype breeding populations to implement GWAS, to study genomic diversity, for genetic linkage analysis, molecular marker discovery, and genomic selection (GS). It is a simple highly multiplexed system for constructing reduced representation libraries for the Illumina platform, which generates millions of SNPs for use in genetic analyses and genotyping in crops (He et al., 2014). However, a major challenge with GBS is the considerable investment needed for bioinformatics support to properly analyze,

curate and store the massive amounts of sequence data obtained from running GBS on large populations (Thompson, 2014).

Applications of high throughput markers in crops

High-throughput genotyping technologies have a range of applications instrumental for unravelling the genetic basis of complex traits, for genetic diversity, phylogeny and population structure analysis, for marker-assisted and genomic selection in plant and animal breeding, for fingerprinting and genetic diagnostics.

Conclusion

Next generation sequencing has played a vital role in creating resources which has enhanced our capability to resolve genetic variants which in turn has helped in understanding of the molecular basis underlying various traits in crop plants. The combination of advanced sequencing technologies, high throughput genotyping technologies, genome-wide association studies, novel mapping populations (Morrell et al. 2012) and tools to analyse the huge volume of data generated using these techniques will improve our ability to connect phenotypes and genotypes, and application of these techniques for crop improvement.

References

Gopala Krishnan S, Waters DLE, Katiyar SK, Sadananda AR, Satyadev V and Henry R (2012) Genome-wide DNA polymorphisms in elite indica rice inbreds discovered by whole-genome sequencing. Plant Biotech J. 10(6): 623-634.

He J, Zhao X, Laroche A, Lu ZX, Liu H and Li Z (2014) Genotyping-by-sequencing (GBS), An ultimate marker-assisted selection (MAS) tool to accelerate plant breeding. Front Plant Sci. 5: 484.

Henry RJ and Edwards K (2009) New tools for single nucleotide polymorphism (SNP) discovery and analysis accelerating plant biotechnology. Plant Biotech J. 7: 311.

Henry RJ, Edwards M, Waters DLE, Gopala Krishnan S, Bundock P, Sexton TR, Masouleh AR, Nock CJ and Pattiemore J (2012) Application of large scale sequencing to marker discovery in plants. J. Biosci. 37(5): 829-841.

McCouch SR, Zhao K, Wright M, Tung CW, Ebana K, Thomson M, Reynolds A, Wang D, DeClerck G, Ali ML, McClung A, Eizenga G and Bustamante C (2010) Development of genome-wide SNP assays for rice. Breed. Sci. 60: 524–535.

Morrell PL, Buckler ES, Ross-Ibarra J (2012) Crop genomics: advances and applications. *Nat. Rev. Genet.* **13**: 85-96.

Semagn K, Babu R, Hearne S and Olsen M (2014) Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. Mol. Breed. 33(1): 1-14.

Singh N, Jayaswal PK, Panda K, Mandal P, Kumar V, Singh B, Mishra S, Singh Y, Singh R, Rai V, Gupta A, Sharma TR and Singh NK (2015) Single-copy gene based 50 K SNP chip for genetic studies and molecular breeding in rice. Sci. Rep. 5:11600.

Thomson MJ (2014) High-throughput SNP genotyping to accelerate crop improvement. Plant Breed. Biotech. 2: 195–212.

Tsuchihashi Z and Dracopoli NC (2002) Progress in high throughput SNP genotyping methods. Pharmacogenomics J. 2: 103–110.

Tung C, Zhao K, Wright LM, Ali ML, Jung J, Kimball J, Tyagi W, Thomson MJ, McNally K, Leung H, Kim H, Ahn S, Reynolds A, Scheffler B, Eizenga G, Bustamante C and McCouch SR (2010) Development of a research platform for dissecting phenotye-genotype associations in rice (*Oryza* spp.). Rice 3: 205–217.

Foreground and Background selection in a breeding population using PCR based markers: Functional, SSR and ISSR markers

Amitha Mithra SV and Deepak Singh Bisht

ICAR-National Research Centre on Plant Biotechnology,

Pusa Campus, New Delhi-12

Marker assisted backcross breeding (MABB) has become a norm in the past decade using which more than a dozen varieties have been released in rice, pearl millet and maize in India. Many more are in pipeline and a sample list of varieties released in the country using MABB is given in Table 1.

Foreground selection: For selection of the trait of interest in a breeding population, either gene based functional markers or linked SSR markers are used. Selection can be practiced for either specific genes or QTLs in a population using these markers. Identification of such tagged DNA markers is a project in itself and most of the academic institutions and some of the private institutions actively pursue this in various crop species. In the practical session, we will be using a recently identified SSR marker linked to fertility restoration in *Brassica juncea* harbouring the *Moricandia* CMS cytoplasm.

Background selection: Once it is ensured that the trait of interest is introgressed in the recipient parent's genome, it is equally important to ensure that most, if not all of the recipient parent's genome, is recovered in the progeny. For this, any random marker system equally covering the genome can be used. The most popular marker system for this is SSR. Of late, high density SNP chips are also being used for this purpose. However, in crops where genome-wide SSRs are not experimentally known, anonymous marker systems like ISSRs can be used.

SSRs: Simple sequence repeat (SSR) markers are excellent for plant breeding applications, population genetics studies and mapping endeavours. SSR markers also called microsatellites; short tandem repeats (STRs) or sequence-tagged microsatellite sites (STMS). These are random tandem repeats of short nucleotide sequences (2-6 bp long), i.e. di,tri and

tetranucleotide repeats, for e.g. (CA)n, (GT)n, (AAT)n and (GATA)n, which are widely distributed throughout genomes of plants and animals. The SSR polymorphism across genoypes results due to the presence of variable number of repeat units within the microsatellite structure. **Thus, microsatellites are flanked by unique regions and primers are developed from these regions enabling the amplification of intervening repeat sequences.**They are valuable genetic markers due to high level of allelic variation (multi-allelic) in microsatellite loci. They are co-dominant, relatively abundant, reproducible, robust and have good genome coverage. SSR markers have also been useful for integrating the genetic, physical and sequence based physical maps in plant species.

ISSRs: ISSRs (Inter simple sequence repeats) are DNA fragments of about 100-3000 bp located between adjacent, oppositely oriented microsatellite regions. These are dominant markers with high polymorphic information content. The main advantage of ISSRs is that no sequence information required, low quantity of DNA and these are randomly distributed throughout the genome.

Table 1: Varieties Developed through MAS in India*

Crop	Trait	Variety	Status	Remarks
Rice	Resistance to BLB	Improved Pusa Basmati1	Released in 2008	*Xa21* and *xa13* (**IARI+NRCPB**)
	Resistance to BLB	Imp Samba Mahshuri	Released in 2008	*Xa21*, *Xa5* and *xa13* (**DRR+CCMB**)
	Resistance to BLB	Punjab Basmati3	Released	*Xa21* and *xa13* (PAU)
	Resistance to BLB	Pusa 1592	Identified for release	*Xa21* and *xa13* (**IARI**); developed in PS5 background
	Resistance to BLB	Improved Lalat	Released in 2012	**CRRI**
	Resistance to BLB	Improved Tapaswini	Released in 2012	**CRRI**
	Resistance to Blast	Pusa 1612	Released in 2013	*PiZ* and *Pi54* (**IARI**); developed in PS5 background
	Resistance to Blast	Pusa 1609	Identified for release	*PiZ5* and *Pi54* (**IARI**);

				developed in Basmati background
	Resistance to Blast	BPT5204	Under testing	*PiZ5* and *Pi54* (**DRR**)
	Drought tolerance	MAS 946-1 (Sharada)	Released in 2007	MAS946-1 (**UAS, Bangalore**); QTLs from Azucena in IR 64
	Drought tolerance	PMK(R) 4 (PM 01 011)	Released in 2009	**TNAU**
	Brown plant hopper resistance	CB (MAS) 20001	Under trial	**TNAU**
	Resistance to gall midge	2 varieties	In pipeline	**TNAU and ANGRAU**
Pearl millet	Downey mildew resistance	HHB 67-2	Released in 2005	**ICRISAT**
Cotton	Resistance to cotton boll worm	More than 23 hybrids - transferring event Mon 531 through MAS	-	All private players using this event
Maize	Protein quality	Vivek QPM9	Released in 2009	**VPKAS, Almora**
	Protein quality	2 hybrids	In pipeline	**IARI**
	VitA enrichment	3 hybrids	In pipeline	**IARI**
Wheat	Leaf, stem and stripe rust	-	In pipeline	**IARI** and **DWR**
Mustard (B. juncea)	Oil quality (double zero)	In station trial	-	**IARI**

*as on Sept 2014

Because of these benefits, they can be used in background selection even in crops where genomic information is scarce. To avoid stuttering, ISSR primers can be anchored to unique genomic sequences flanking the repeat either in the 5' or 3'. 3'-anchoring give better results than 5'-anchoring. Repeat polymorphisms within the SSR do not influence the chance for ISSR polymorphisms.

PCR and gel electrophoresis for foreground selection using SSR marker:

Materials Required:

1. Genomic DNA (30 ng/µl) of multiple individuals from population
2. Thermal cycler
3. PCR tubes or plates

4. Micro pipettes
5. Taq DNA polymerase and buffer
6. dNTPs (10mM/ µl)
7. Mg Cl_2 (if not included in the buffer, 50 mM/ µl)
8. *Brassica juncea* microsatellite primers (20 ng/µl)
9. Nuclease free water
10. Metaphor Agarose (3.5%)
11. TBE buffer
12. Electrophoresis unit
13. Loading dye
14. Ethidium Bromide
15. Gel documentation system

Procedure:

1. Prepare a master mix with forward and reverse primer, dNTPs, Taq DNA polymerase and buffer containing $MgCl_2$ and nuclease free water.
2. Add 1.5 µl of genomic DNA in each well of PCR plate.
3. Dispense 8.5 of master mix in each well.
4. Seal the plate carefully with appropriate sealer.
5. Vortex the plate gently and spin down.
6. Subject it to PCR following the steps given below:
    i. Heating the lid – 99 ^oC
    ii. Denaturation - 95 ^oC – 5 min
    iii. Start cycle – 35 times
    iv. Denature – 95 ^oC – 30 seconds
    v. Primer annealing – 55 ^oC – 30 seconds
    vi. Primer Extension – 72 ^oC – 2 min
    vii. End cycle
    viii. Final extension - 72 ^oC – 7 min
    ix. End
7. Resolve the amplicons in 3.5% metaphor agarose gel.
8. Document your results.

PCR and gel electrophoresis for background selection using SSR and ISSR marker:

Requirements for 25 µl PCR reaction

1. Several SSR/ISSR markers

2. High quality DNA of working standard: 50 ng/µl
3. Taq DNA polymerase (1 unit/reaction)
4. dNTPs
5. Components of gel electrophoresis

PCR reaction mix

• Buffer	2.5 µl
• dNTPs (2.5 mM each)	2.0 µl
• Primer (200-500 pg)	1.0 µl
• DNA template (~50 ng)	1.0 µl
• Taq DNA polymerase (1U)	0.2 µl
• Nuclease free water	18.3 µl
	25.0 µl

PCR conditions

• 95^0C	5 min	
• 95^0C	30 s	35 cycles
• 45-50 ^{0}C	30 s	
• 72 ^{0}C	2 min	
• 72 ^{0}C	10 min	
• 4 ^{0}C	∞	

Gel electrophoresis of PCR products

1. Select appropriate gel casting tray and matching comb, fix into slots
2. Weigh 2.0 g agarose into 500 ml flask containing 200 ml 1X TAE and boil to dissolve agar completely
3. After cooling agarose gel to around 50 $^{\circ}$C add 10 µl EtBr and mix gently so that no bubbles would form
4. Pour agarose into sealed casting tray slowly in order to bubble formation and let it solidify for 30-45 min
5. Remove the sealing and keep the tray in electrophoresis tank containing 1X TAE buffer sufficient enough to dip the gel

6. Mix 25 µl DNA sample with loading dye (5 µl) and pipette the mix into the wells carefully to avoid floating out of sample. Also load 4 µl DNA ladder of known base pairs, preferably 1 kb.
7. Place the lid back on the running tank and connect the power supply. Run the apparatus at 60-80 volts for 1h or till the front bromophenol blue dye travel around 3/4 of the gel.
8. At the end take put the tray along with gel and capture the image under UV light using gel documentation unit.

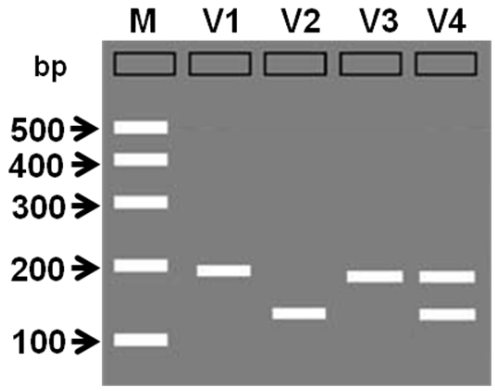


Fig. 1: Schematic representation of SSR polymorphism across four varieties. Note that SSR is a co-dominant and multiple allelic marker system. After polymorphism survey in the respective parents, only polymorphic markers will be used in the breeding population. M: 100 bp DNA ladder, V1-V4: Varieties. Note their multiple allelic nature.

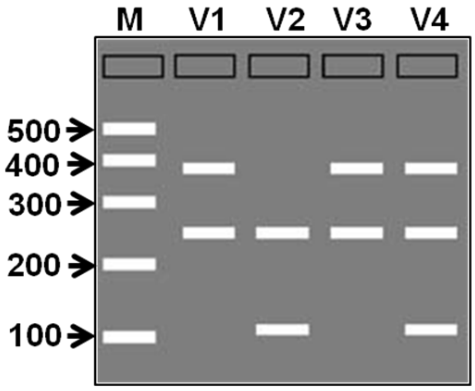


Fig. 2: Schematic representation of IISR polymorphism across four varieties. Note that ISSR is a multi-locus dominant marker system. After polymorphism survey in the respective parents, only polymorphic markers will be used in the breeding population. M: 100 bp DNA ladder, V1-V4: Varieties.

Generation, Analysis and Relevance of Transgenic Plants

Srinivasan

ICAR-National Research Centre on Plant Biotechnology,

Pusa Campus, New Delhi-12

srinivasan53@gmail.com

Our ability to generate a variety of transgenic plants, which carry and express foreign genes, has profoundly affected biology and agriculture. Plant transformation is increasingly being used as an important experimental tool for investigations into various aspects of plant biology such as genetics, physiology, biochemistry, pathology, developmental biology, molecular biology etc. This revolution in plant biology has become possible because of rapid developments in the techniques for: i) isolation and manipulation of genes; ii) the introduction of DNA into plant cells; iii) selection of the transformed plant cells and v) regeneration of the transformed cells into fertile plants.

In the first part of my discussion, I would like to introduce the term transgenic plants(GM plants)and discuss the concept and the techniques employed for the transformation of plants. The second part of our discussion would pertain to the significance of transgenic plants. A number of techniques for introduction of DNA into plant cells are available (Draper and Scott, 1991; Songstad et al., 1995, Table 1). These techniques could be divided into two broad categories; one that exploits a biological agent as a vector and another, which employs physical/chemical agents to introduce DNA into plant, cells (Srinivasan et al., 1990; Rivera et al., 2012).

Table1. List of techniques employed for plant transformation

Biological
Agrobacterium mediated
Virus mediated
Physicochemical
Biolistics
Diret DNA uptake
Electroporation
Lipofection
Laser mediated
Silicone Fibre mediated
Microinjection
Macroinjection
Use of Nanoparticles

Since amongst the transformation techniques, *Agrobacterium* and microprojectile bombardment based methods are the most extensively used methods (Veluthambi et al., 2003) and the advancements in the transgenic technology has been largely associated and dependent on the developments in the field of *Agrobacterium*-plant interactions, we would discuss these aspects in detail.

In order to understand and appreciate the technology about transgenic plants it would be worthwhile to know some history and about the methods used for the introduction of DNA into plant cells. Since the developments have been very intimately associated with the studies conducted on the biology of a plant pathogen, *Agrobacterium* and a number of concepts and techniques were developed while elucidating the molecular processes underlying *Agrobacterium*-plant interactions, I would briefly dwell on the story of *Agrobacterium*.

Mechanism of *Agrobacterium* mediated transformation

Agrobacterium tumefaciens, a soil bacterium causes tumor/gall formation at the site of infection in several dicotyledonous plants. Two major differences were observed between the crown gall and the normal plant tissues. While the normal plant cells, in axenic cultures, require phytohormone for their growth, the crown gall cells are capable of phytohormone independent growth. The crown gall cells produce novel compounds called opines which are generally not present in plant cells. Agrobacteria utilize opines as carbon and nitrogen source for their growth and development. Subsequent work by various groups of scientists to understand the molecular basis of *Agrobacterium*-plant interaction, during 70's and 80's revealed a wealth of information leading to the exploitation of *Agrobacterium* as a plant transformation vector (Zupan and Zambryski, 1995; Hanson and Chilton, 1999). A very interesting and informative account of the initial work on the molecular aspects of *Agrobacterium* -plant interaction has been provided by Chilton (2001) and Van Montagu(2011). All virulent strains of agrobacteria possess a large plasmid called tumor-inducing (Ti) plasmid. A part of this plasmid is transferred (T-DNA) from the bacteria to plant cells. Genes present on the T-DNA are responsible for the production of phytohormones and opines in the crown gall cells. Detailed molecular and genetic analysis revealed that the process of tumor formation and T–DNA transfer could be delinked. Thus disarmed strains of agrobacteria, from which oncogenes have been deleted, have been developed which are capable of transferring DNA but do not cause formation of tumors.

The three important genetic components which are involved in the transfer of T-DNA are:

i) chromosomal virulence (*Chv*) loci involved in mediating the attachment of bacteria to plant cells ;

ii) 24 base pair border sequences which flank the T-DNA and are required for the processing of T-DNA, and

iii) virulence (*vir*) region which encodes several polypeptides involved in eventual processing, packaging and delivery of the T -DNA into plant cell nucleus. Table 2 lists some of the *vir* genes along with their proposed roles in Agrobacterium –plant interaction.

While the *chv* and *vir* genes can function either in *cis* or in *trans*, the border sequences must be present in *cis* configuration to the T -DNA. Based on the mechanistic details of the T –DNA transfer two types of vectors (co-integrative and binary) have been developed.

Some plant genes, which play an important role in the integration and incorporation of T-DNA, have also been identified (Gelvin, 2000; Tzfira et al., 2002).

Although plants are the naturally susceptible hosts for *Agrobacterium*, this bacteria has also been made to transfer DNA into several other eukaryotic species like yeast, mushrooms, filamentous fungi, phytopathogenic fungi and human cells (Tzfira and Citovsky, 2003). Valentine's review article provides an excellent information on the molecular mechanism of *Agrobaceterium* mediated plant transformation (Valentine, 2003).

Understanding the molecular mechanism of *Agrobacterium* mediated gene transfer has helped Broothaerts et al. (2005) to develop plant transformation system based on several plant symbiotic bacterial bacteria outside the Agrobacterium genus. *Rhizobium* species NGR234, *Sinorhizobium meliloti* and *Mesorhizobium loti* were suitably modified for gene transfer by acquisition of both a disarmed Ti plasmid and a binary vector. This alternative to *Agrobacterium*-mediated transformation system for plants might become a versatile 'open source' platform for plant transformation and is likely to result in exploitation of natural symbiotic bacteria–plant interactions to achieve plant transformation (Gelvin, 2005).

Microprojectile bombardment

Another important method popularly known as BIOLISTIC (**Bio**logical bal**listic**) or the so-called Gene gun employs a system, which accelerates microprojectiles, coated with DNA for transformation of plants (Sanford et al., 1993). In this procedure, small metal (gold or tungsten) particles (< 4μm diameter) coated with DNA are accelerated and allowed to hit

at a high velocity, the target plant tissue. In some plant cells, the DNA thus introduced gets incorporated into the nuclear or organelle genome. The acceleration of the micro projectiles could be achieved by any of the following: i) using gunpowder charge; ii) sudden release of a compressed gas or iii) generation of shock waves by a high voltage electric discharge in a drop of water. Whereas the application of the *Agrobacterium* mediated process is limited by the host range of bacteria for the plants, no such restriction applies to the biolistic mediated process. Using biolistic approach, the production of fertile transplastomic plants has been reported for tobacco, tomato, petunia, soybean, cotton and *Lesquerella fendleri* (Maliga, 2004). Wherever regeneration and isolation of protoplasts is feasible, DNA could be introduced into the protoplasts via polyethylene glycol (PEG) treatment or by subjecting the protoplasts to high voltage electric pulses. Both these processes create in the plant cell membrane transient pores, through which DNA gains entry inside the cell. Plastid-transformed in lettuce has also been reported using a polyethylene glycol-mediated transformation system (Lelivelt et al., 2005).

In recent years, nanoparticles have also been used to directly deliver DNA into plants (Torney et al., 2007; Corredor et al., 2009) without the use of a gun. However, generation of transgenic plants using these particles is yet to be reported.

Strategies for selection and identification of transformants

Since gene transfer is a low frequency process, a system/strategy is required to identify the cell possessing the introduced gene(s). Only a few out of the several hundred cells subjected to transformation actually get transformed. Thus strategies have been devised to identify and select the transformants. In order to evaluate the transformation protocol and to get an idea about the frequency of transformation, a majority of transformation vectors have marker genes linked to the genes of interest. The marker gene product helps either selective growth of transformed cells on a specific selection medium (selectable marker) or visual identification of transformed cells (scorable marker). Typically genes imparting antibiotic or herbicide resistances are used as selectable markers, e.g., genes imparting resistance to kanamycin, hygromycin or Basta (Table 2).

Another group of markers like β-glucuronidase or GFP, which are used to monitor and measure the expression of genes, are called scorable markers (Table 3). One can examine the cells or tissues following 24-48 hours of transformation and estimate the number of cells or groups of cells (spots) showing marker gene expression. With selectable markers,

transformation becomes apparent only when colonies of cells or green shoots emerge from the treated cells or explants.

Table 2. Some commonly used selectable markers for plant transformation

Selectable marker gene	**Abbreviation**	**Selection Agent**
Acetolactate synthase	*als*	Sulfonylurea
Bromoxynil nitrilase	*bxn*	Bromoxynil
Dihydrofolate reductase	*dhfr*	Methotrexate
Neomycin phosphotransferase II	*nptII*	Neomycin, kanamycin, G418
Phosphoinothricin acetyltransferase	*Bar/pat*	Basta, glufosinate, bialophos, phosphoinothricin
Hygromycin phosphotransferase II	*hptII*	Hygromycin
Mannose6-phosphate isomerase*	*pmi/manA*	Mannose
Xylose isomerase*	*xylA*	Xylose

* Markers used for positive selection

Table 3. Some commonly used scorable/screenable marker genes

Scorable/Screenable genes	**Abbreviation**
Chloramphenicol acetyl transferase	*Cat*
β-glucuronidase	*gus/uidA*
Green fluorescent protein	*Gfp*
Luciferase	*luxA/luxB/luc*

An excellent review article by Birch (1997) not only provides a generalized approach for developing transgenic plants but also describes in detail the problems and prospects associated with various plant transformation strategies.

Employing plant transformation techniques several transgenic plants carrying novel traits of economic and agronomic importance have been generated (Sharma et al., 2002; Moeller and Wang, 2008). In recent years, transgenic plants have revolutionized the world agriculture. Insect-resistant and herbicide-tolerant crops, in corn, soybean and cotton have been successfully commercialized and the same are being grown worldwide. Transgenic crops carrying herbicide tolerance, insect resistance are presently cultivated on 148 million hectares in 29 countries, worldwide [James, C., 2011]. Transformation technology has resulted in the generation of plants carrying novel traits, plants which can withstand a variety of biotic and abiotic stresses, to produce pharmaceuticals, nutraceuticals and novel products. Transgenic crops tolerant to biotic stresses such as viruses, fungi, bacteria and insects are

being developed in several crops. A variety of strategies are being evaluated to generate transgenic crops tolerant to abiotic stresses such as high temperature, drought, cold and salinity. Transgenic plants with improved nutritional quality are now a reality. Plant systems that produce proteins for pharmaceutical applications ('Molecular Pharming') are being developed. The potential role of transgenic plants in meeting the international development targets set out in the eight Millennium Development Goals (MDGs) for the year 2015 have been lucidly described by Yuvan et al. (2011).

Besides being exploited for commercial purposes, transgenics are also widely used for basic studies in plant biology. Plant transformation is an important tool in the hands of molecular biologists to understand the organization and regulation of eukaryotic genes. Transgenics are widely used for the cloning and characterization of genes and promoters (Radhamony et al., 2005). Similarly, transgenics find application in almost every branch of biology such as genetics, physiology, biochemistry, developmental biology, virology, environmental sciences etc. (Raikhel, 2001). A number of functional genomic approaches require creation and analysis of transgenic plants.

In all such cases, where transformation protocols are established, researchers from a variety of disciplines generate and exploit transgenic plants to find answers to specific questions. A word of caution is in order here. Although, to most of the beginners the cause and effect relations in transformation experiments might appear simple and the results obtained are directly attributed to transformation but in practice, all effects observed are not directly attributable to the transgene introduced through transformation.

Variations are common among transgenic plants obtained from the same set of experiments. These variations may arise because of:

i) the method of transformation and regeneration,

ii) the effect of introduced DNA, or,

iii) a combined effect of all the above.

The present methods of transformation can deliver DNA into an individual cell which has to be multiplied and differentiated to obtain a complete transformed plant (Puonti-Kaerlas et al., 1992, Schroeder et al., 1991, 1993; Pigeare, 1997). With the exception of the model plant *Arabidopsis thaliana*, transgene insertion usually involves a tissue culture step (Zhang et al., 2006). Tissue culture (as used in plant transformation) induces dedifferentiation of plant tissues in the presence of hormones and antibiotics or other selective agents and allows

selection and regeneration of an intact plant from a single genetically modified cell containing the transgene. Tissue culture is known to cause heritable and non-heritable changes (somaclonal variation) (Larkin and Scowcroft, 1981). Hence, some of the variations observed among transgenics may arise from tissue culture (Sala et al., 2000; Labra et al., 2001, 2004). In some cases the ploidy level of the plant may also change. For example, all pea transgenics recovered in a study were tetraploids (Puonti-Kaerlas et al., 1992). Similarly, Imai et al. (1993) reported a high frequency of tetraploids in transgenics derived from *Agrobacterium*-mediated transformation of potato tuber discs.

Current methods of transformation lead to insertion of foreign DNA at random locations within the host chromosomes (Barakat et al., 2000). Since the expression of transgene is not only controlled by its promoter but also influenced by the flanking host genomic region, individual transgenics may exhibit variation due to 'position effect' (Dean et al., 1988). Further, the number of insertions and the integrity of the introduced DNA sequences are variable among independent transgenics, and thus contribute to variation (Perrin et al., 2000; Aragao, 1999; De Block, 1993). Depending upon the method of transformation and the regeneration protocols, the putative transgenic plant may be chimeric in nature (Christou, 1990; Schroeder et al., 1991). Therefore, analysis of variation among transgenic plants assumes importance to separate transgene effects from other associated effects. Plants homozygous for the transgene might also exhibit altered phenotype, in case the transgene is inserted within a functional host gene (a case of insertional mutagenesis).

The strategy for analysis of transgenic plants would depend upon the purpose of the experiment. Usually researchers involved in generating transgenics are concerned about the following:

1. Whether transformation has occurred and, if so, the frequency of transformation.
2. Whether the foreign gene has been incorporated into the host genome. If so,
   i) What is the number of copies introduced?
   ii) What is the site(s) of integration?
   iii) Is the introduced DNA intact?
3. Whether the introduced gene is expressed? If so, what is the specificity and levels of expression?
4. Is the product biologically functional?
5. Is the foreign gene inherited and expressed as per expectation?
6. Whether the changes observed are due to transgene or due to other causes?

A variety of genetic and molecular techniques are used to analyze and evaluate the transgenic plants (Bhat and Srinivasan, 2002). PCR and Southern blot analysis is used to detect the presence of the gene. Whereas, northern and RT-PCR analysis is carried out to determine the m-RNA, the techniques like ELISA and western are employed to detect and assay the protein product of the gene.

Polymerase chain reaction (PCR) based techniques are widely used to screen shoots recovered on a selection medium. Another technique used for a dependable assessment of transformation is reverse transcriptase polymerase chain reaction (RT-PCR) (D'Haullin et al., 1990). Since marker genes are generally constitutively expressed, RNA isolated from test samples can be used for RT-PCR amplification of the marker gene.

Southern hybridization technique is considered is the ultimate method to confirm integration of foreign DNA into the host genome. Since considerable amount of DNA is required for preparing a Southern blot, this can only be undertaken when large amount of tissue is available for sampling. Choice of probe and restriction enzyme combination is very important and crucial for arriving at a meaningful interpretation of results about the integration, copy number and DNA rearrangement in the transferred DNA. A single Southern blot can be probed at least 2-3 times with different probes and can thus be used to derive different information pertaining to foreign gene integration. Thus, care should be taken while selecting the restriction enzymes and probe combination for Southern hybridization.

Thus one or more shoots may arise from a single point, which may originate from the same event or from different events. Schroeder et al., (1991) observed variations in Southern patterns among plants regenerated from the same callus. Such variations may arise from somaclonal variation or due to several independent transformation events occurring in neighboring cells that give rise to a callus. Therefore, it is desirable to maintain a precise record of the lineage of each shoot during *in vitro* multiplication and rooting stages. Only after establishing their identity based on Southern pattern, independent events can be unambiguously identified and carried forward.

A variety of transgenic plants also serve as important resources for functional genomic studies (Alonso, et al., 2003). T-DNA mutagenized plants have resulted in the identification and characterization of a large number of genes and promoters in plants (Radhamony et al., 2005). The impact of this technology has been equally significant in the field of plant molecular biology. In particular, use of transgenic plants has unraveled several novel facets of expression and regulation of genes in plants (Raikhel, 2001).

Transgenic plants have become an integral part of modern plant biology and judicious and innovative use of transgenics would continue to offer not only novel solutions to several problems in the area of crop productivity, health, nutrition and environmental security (Farre, et al., 2010) but would also provide newer and useful information in all branches of plant biology for all times to come. There have been a few concerns regarding safety of GM crops. A recent article that has critically evaluated the scientific literature published in the last ten years on various aspects concerning the safety of transgenic plants comes to the conclusion that these crops are quite safe (Nicolia et al., 2013). The article also brings out the need for a fact based effective communication system to remove misconceptions and improve the public perceptions about the utility and safety of GM crops. Another recent article by Peter Raven very categorically brings out the fact that there is nothing unnatural about GM crops and they should be an important and integral component of crop development strategies (Raven, 2013). In recent years, another powerful versatile technology, CRISPR-Cas employed for manipulating genomes is set to revolutionize the field of plant biotechnology (Woo et al., 2015). This technology might fall outside the definition of transgenic plants and thus remove a lot of apprehensions and misgivings about GM technology.

References:

Alonso, J.M., et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* 301:653-657.

Barakat, A., Gallois, P.,. Raynal, M, Mestre-Ortega, D., Sallaud, C., Guiderdoni, E., Delseny, M. and Bernardi, G. (2000). The distribution of T-DNA in the genomes of *Arabidopsis* and rice. *FEBS Letters* 471:1612-164.

Bhat, S. R., and Srinivasan. (2002) Molecular and genetic analyses of transgenic plants: Considerations and approaches. Plant Science, **163**, 673-681.

Birch, R.G. (1997) Plant transformation: Problems and strategies for practical application. Ann. Rev. PI. Physiol. Plant Mol. Bioi. **48**,297-326.

Broothaerts, W., Mitchell, H. J., Weir, B., Kaines, S., Smith, L. M. A., Yang, W., Mayer, J. E., Rodrýguez, C. and Jefferson., R. A. (2005) Gene transfer to plants by diverse species of bacteria. Nature, **433**, 629-633.

Chilton, M-D. (2001) *Agrobacterium*. A memoir. Plant Physiol., **125**,914.

Christou, P. (1990). Morphological description of transgenic soybean chimeras created by the delivery, integration and expression of foreign DNA using electric discharge particle acceleration. *Annals of Botany* 66:379-386.

Corredor, E., Testillano, P.S., Coronado, M-J.,González-Melendi, P., Fernández-Pacheco, R., Marquina, C.,

Ibarra, M. R., Fuente, J. M., Rubiales, D., Pérezde-Luque, A. and Risueño, M-C.(2009). Nanoparticle penetration and transport in living pumpkin plants: *in situ* subcellular identification. *BMC Plant Biology* 2009, 9:45

De Block, M. (1993). The cell biology of plant transformation: current state, problems, prospects and implications for plant breeding. *Euphytica*. 71:1-14.

Dean, C., Jones, J., Favreau, M., Dunsmuir, P. and Bedbrook, J. (1988). Influence of flanking sequences on the variability in expression levels of an introduced gene in transgenic tobacco plants. *Nucleic Acids Research* 16:9267-9283.

Draper, J. and Scott, R. (1991). Gene transfer to plants. In: Plant Genetic Engineering, Vol. **1**, D.Grierson (Ed.) Blackie Glasgow pp. 39-81.

Farre, G., Ramessar, K., Twyman, R. M., Capell,T. and Christou, P. (2010) The humanitarian impact of plant biotechnology: recent breakthroughs vs bottlenecks for adoption. Current Opinion in Plant Biology, **13**:219–225.

Feldmann,, K.A. (1991) T-DNA insertion mutagenesis in *Arabidopsis*: mutational spectrum. *The Plant Journal* 1:71-82.

Gelvin, S.B. (2000) *Agrobacterium* and plant genes involved in T -DNA transfer and integration. Ann. Review Plant Physiol. Plant Mol. Biol. **51**, 223-256.

Gelvin, S.B. (2005) Gene exchange by design. Nature, **433**, 583-584.

Hanson, G and Chilton, M.D. (1999) Lessons in gene transfer to plants by a gifted microbe. In: Plant Biotechnology. Hammon. J, McGarvey. P. & Yusibov. V. (Eds.) Springer Berlin pp.21-57.

Imai, T. Aida, R. and Ishige, T. (1993) High frequency of tetraploidy in *Agrobacterium*-mediated transformants regenerated from tuber discs of diploid potato lines. Plant Cell Reports 12: 299-302

James, C. **(**2012) Global Status of Commercialized Biotech/GM Crops: 2011 ISAAA Briefs, ISAAA, Ithaca, NY. http://www.isaaa.org/resources/publications/briefs/42/default.asp

Labra, M., Savini,C., Bracale, M., Pelucchi,F., Colombo, L., Bardini, M. and Sala, F. (2001) Genomic changes in transgenic rice (*Oryza sativa* L.) plants produced by infecting calli with *Agrobacterium tumefaciens*. *Plant Cell Reports* 20:325-330.

Labra, M., Vannini, C. , Grassi, F., Bracale, M., Balsemin, M., Basso, B. and Sala, F. (2004) Genomic stability in Arabidopsis thaliana transgenic plants obtained by floral dip. *Theoretical Applied Genetics* 109:1512-1518.

Larkin, P.J., and. Scowcroft, W.R. (1981) Somaclonal variation: a novel source of variability from cell cultures for plant improvement. *Theoretical Applied Genetics* 60:197-214.

Lelivelt, C. L. C., McCabe, M. S., Newell, C. A., C. Bastiaan de Snoo, C. B., van Dun, K.M. P., Birch-Machin, I., Gray. C., Kingston H.G. Mills, K. H. G. and Jacqueline M. Nugent, J. M. (2005) Stable plastid transformation in lettuce *(Lactuca sativa* L.). Plant Mol. Biol. **58**:763-774.

Maliga, P. (2004) Plastid transformation in higher plants. Annu. Rev. Plant Biol. 55:289–313.

Moeller, L. and Wang, K. (2008) Engineering with Precision: Tools for the New Generation of Transgenic Crops BioScience 58, 391-401

Nicolia, A., Manzo, A., Veronesi, F., and Rosellini, D. (2013) An overview of the last 10 years of genetically engineered crop safety research. Critical Rev. Biotecnology, 1549-7801 (electronic) http://informahealthcare.com/bty

Perrin, Y., Vaquero, C., Gerrard, I.,. Sack, M, Drossard, J., Stoger, E. Christou, P., and Fischer, R. (2000) Transgenic pea seeds as bioreactors for the production of a single-chain Fv fragment (scFV) antibody used in cancer diagnosis and therapy. *Molecular Breeding* 6:345-352.

Puonti-Kaerlas, J., Eriksson, T., and Engström, P. (1992) Inheritence of a bacterial hygromycin phosphotransferase gene in the progeny of primary transgenic pea plants. *Theoretical and Applied Genetics* 84:443-450.

Radhamony, R. N., Prasad, A. M. and Srinivasan (2005) T-DNA insertional mutagenesis in *Arabidopsis* : a tool for functional genomics. Electronic Journal of Biotechnology Vol.8 No.1, This paper is available online at http://www.ejbiotechnology

Raikhel, N.V. (2001) Plant Physiology: Past, Present, and Future, *Plant Physiology*, 125, 1–3.

Raven, P. H. (2013) GM crops, the environment and sustainable food production. Transgenic Res., DOI 10.1007/s11248-013-9756-x

Rivera, A. L., Gómez-Lim, M., Fernández, F., and Loske, A. M.(2012) Physical methods for genetic plant transformation. *Physics of Life Reviews* 9, 308–345.

Sala, F., Arencibia, A., Castiglione, S., Yifan, H., Labra, M., Savini, C., Bracale, M. and Peluchi, N. (2000) Somaclonal variations in transgenic plants. *Acta Horticulturae* 530:411-419.

Sanford, J.C., Smith, F.D. and Russel, J.A. (1993) Optimizing the biolistic process for different biological applications. Methods Enzymol. **217**,483-509.

Schroeder, H.E., Khan, M.R.I., Knibb, W.R., Spencer, D., and Higgins, T.J.V. (1991) Expression of a chicken ovalbumin gene in three Lucerne cultivars. *Australian Journal pf Plant Physiology* 18:495-505.

Schroeder, H.E., Schotz, A.H., Wardley-Richardson, T., Spencer, D., and Higgins, T.J.V. (1993) Transformation and regeneration of two cultivars of pea (*Pisum sativum* L.). *Plant Physiology* 101:751-757.

Sharma H.C., Crouch, J.H., Sharma, K.K., Seetharama, N. and Hash, C.T. (2002) Applications of biotechnology for crop improvement: prospects and constraints. Plant Science, **163**, 381-/395.

Songstad, D.O. Somers, D.A. and Griesbach, R.J. (1995) Advances' in alternate DNA delivery techniques. Plant Cell and Tissue Organ Culture **40**, 1-15.

Srinivasan, Gupta, N. and Chopra, V.L. (1990) Engineering better plants through transformation. Proc. lnd. Natl. Sci. Acad. **B56**, 177-194.

Torney, F., Trewyn, B.G., Lin V. S.-Y., & Wang. K., (2007) Mesoporous Silica Nanoparticles deliver DNA and chemicals into plants, Nature Nanotechnology 2, 295 - 300

Tzfira, T. and Citovsky, V.(2003) The Agrobacterium-Plant Cell Interaction. Taking Biology Lessons from a Bug. Plant Physiol.**133**, 944-947.

Tzfira, T., Vaidya, M. and Citovsky, V. (2002) Increasing plant susceptibility to *Agrobacterium* infection by over expression of the *Arabidopsis* nuclear protein VIP1. PNAS (USA) **99**, 10435-10440.

Valentine, L. (2003) *Agrobacterium tumefaciens* and the Plant: The David and Goliath of Modern Genetics. Plant Physiol. **133**, 948-955.

Van Montagu, M. (2011) It is a long way to GM agriculture. Annu. Rev. Plant Biol. **6**2, 1-23.

Veluthambi, K., Gupta A,K. and Sharma, A. (2003) The current status of plant transformation technologies. Current Science, **84**, 368-380.

Woo JW, Kim J, Kwon SI, Corvalán C, Cho SW, Kim H, Kim SG, Kim ST, Choe S, Kim JS (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotechnol., 33(11):1162-4

Yin, Z., Plader, W., and Malepszy, S. (2004) Transgene inheritance in plants. *Journal of Applied Genetics* 45:127-144.

Yuan, D.,…….. Christou, P. (2011) The potential impact of plant biotechnology on the Millennium Development Goals. Plant Cell Rep., **30**:249–265.

Zhang, X., Henriques, R., Lin,S., Niu**,** Q. & Chua**,** N-H.(2006) *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. Nature Protocols **1**, - 641 - 646

Zupan, J.R and Zambryski, P. (1995) Transfer of T -DNA from *Agrobacterium* to the plant cells. Plant Physiol. **107**, 1040-1047.

Endogenous Defense in Plant for Insect-Pest resistance

R.C. Bhattacharya

ICAR-National Research Centre on Plant Biotechnology

Pusa Campus, New Delhi-110012

Like any other organism, plants are also equipped with self-immunity and endogenous defense response to counter the adverse consequences of diverse kind of biotic and environmental stresses that it encounters. In case of abiotic stresses altered physiological parameters and inorganic factors imposed by the stresses are perceived as signals whereas the biotic stresses are recognized by specific recognition of the invading organisms in molecular interaction. A primary event in the recognition process involves ligand-receptor interaction in which 'Microbe-associated molecular patterns' (MAMPs) released by invading organisms are specifically recognized by host pattern recognition receptors (PRRs) to trigger the host immune responses. Several MAMP–PRR interactions have been identified in plants, including the bacterially derived peptide MAMPs flg22, elf18 and Ax21 which bind the FLS2, EFR and Xa21 receptors respectively. In the similar way molecular patterns associated with herbivores (HAMPs) are specifically recognized and trigger plant herbivory-defenses. A few of the non-protein HAMPs have been identified which include glutamine–fatty acid conjugates such as volicitin, derived from insect oral secretions that induce emission of defensive volatiles in corn seedlings, and bruchins, long-chain a,ω-diols found in oviposition-fluid of weevils.

In addition to MAMPs from invading organisms host-derived defense elicitors that are released due to injury and/or infection, play an important role in triggering and amplifying the host-defense responses. Identification of endogenous signal elicitors related to defense, has always attracted a lot of research in quest of a possible clue to understand endogenous defense system in plants. Different types of elicitors have been identified some of which are now proven to occur universally across the plant species. These include reactive oxygen species (ROS), oligosaccharide fragments and protein fragments. The enzymes responsible for the release of these signals are usually activated by wound or other defense related signals. Among the several classes of endogenous elicitors that have been identified in higher plants, our interest limits to peptide elicitors and delineating their downstream

signalling mechanisms. A few aspects that will be dealt in this section are: discovery and examples of defense peptides, properties and downstream signalling pathways activated by them.

Systemin, the first signalling peptide was discovered in tomato. The 18 amino acid long systemin is derived from the C-terminus of a 200 amino acid precursor protein, prosystemin. Analyses using plants treated with systemin or transgenic plants that overexpress or suppress the prosystemin gene revealed that systemin regulates many defense responses in tomato in addition to promoting accumulation of protease inhibitors and other anti-nutritive proteins. Systemin also provide indirect defense by increasing the release of plant volatiles that attract natural enemies of insect herbivores. Prosystemin accumulates in the cytosol of vascular phloem parenchyma cells, and upon wound-induced processing, active systemin induces jasmonic acid (JA) synthesis in the vascular bundle, leading to systemic protease inhibitor induction. In the following years, systemin was found to be present across many of the solanaceous plants except tobacco. Interestingly, in tobacco another class of small signal peptide, called hydroxyproline rich systemin (HypSys) was found to mediate protease inhibitor accumulation against insect-herbivors. Systemin or HypSys defense signals have been identified in many other plant species within and outside the solanaceae family using a cell suspension based alkalinization assay. The small (18-20 amino acids) HypSys glycopeptides, like systemin, are derived from larger precursor proteins (proHypSys) and until recently were thought to function only in protection from herbivore attack. However, HypSys glycopeptides isolated from petunia induced the defensin gene, known for its involvement in pathogen defense. More recently, a HypSys glycopeptide was isolated from sweet potato, a member of the Convolvulaceae family and found to induce the sporamin gene which codes for the major storage protein in tubers with trypsin inhibitor activity. These recent discoveries expand the function and range of the HypSys family of glycopeptides and establish these unique inducible signalling molecules as potential components of defense pathways across the plants. In potato, for the first time it was demonstrated that HypSys glycopeptides could activate insect as well as pathogen defense genes indicating more universal role of these defense peptides. In future research it remains interesting to fully understand the peptide signal mediated defense in plants, the associated cascades and the downstream defense genes so that the signal-peptide genes can be utilized in crop

biotechnology as a novel avenue to engineer broad spectrum resistance in plants. More studies including more number of plant species are required for progress on this aspect.

References for further readings:

Pearce G, Strydom D, Johnson S, Ryan CA: A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. Science 1991, 253:895-897

Pearce G, Moura D S, Stratmann J, Ryan CA: Production of multiple plant hormones from a single polyprotein precursor. *Nature* 2001, *411:* 817-820.

Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JD, Felix G, Boller T: Bacterial disease resistance in Arabidopsis through flagellin perception. Nature 2004, 428:764-767.

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JDG, Boller T, Felix G: Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell 2006, 125:746-760.

Pearce G, Siems WF, Bhattacharya R, Chen YC, Ryan CA: Three hydroxyproline-rich glycopeptides derived from a single petunia polyprotein precursor activate defensin I, a pathogen defense response gene. J Biol Chem 2007, 282:17777-17784.

Pearce G, Bhattacharya R, Chen YC, Barona G, Yamaguchi Y, Ryan CA: Isolation and characterization of hydroxyproline-rich glycopeptides signals in black nightshade leaves. Plant Physiol 2009, 150:1422-1433.

Lee SW, Han SW, Sririyanum M, Park CJ, Seo YS, Ronald PC: A type I-secreted, sulfated peptide triggers XA21-mediated innate immunity. Science 2009, 326:850-853.

Koramutla MK, Kaur A, Negi M, Venkatachalam P, Bhattacharya RC: Elicitation of jasmonate-mediated host defense in Brassica juncea (L.) attenuates population growth of mustard aphid *Lipaphis erysimi* (Kalt.). Planta 2014, 240:177-94

(Disclaimer: The above write up is merely for the purpose of supplementing oral lecture and no way should be considered as any publication or reporting.)

Gene amplification by PCR and cloning into binary vector

Monika Dalal

ICAR-National Research Centre on Plant Biotechnology

Pusa Campus, New Delhi-110012

Polymerase chain reaction, or PCR, is a laboratory technique for *in vitro* amplification or making multiple copies of a segment of DNA. The method of PCR was developed by Kary Mullis who received Nobel Prize in chemistry in 1993 for this work. PCR is a very specific and sensitive technique which can amplify billions of copies of a desired segment of DNA from a small amount of template DNA or cDNA in short period of time.

Reaction Components

Template: It can be genomic DNA, plasmid or cDNA which contains the sequence of DNA to be amplified (target DNA). The source of template can be from animals, plants, viruses, or bacteria.

Enzyme: The enzyme used is DNA Polymerase which adds a nucleotide onto a preexisting 3'-OH group. The original method used Klenow DNA polymerase which is thermolabile and had to be replenished after every cycle. However it was replaced by thermo stable DNA polymerase from *Thermos aquaticus* which can catalyze multiple rounds of amplification at high temperatures. Now a day many different types of recombinant DNA polymerases are being used for different purposes.

Primers: These are small ~18-26 nucleotides long segments of single-stranded DNA, which hybridize to a specific region on either side of the target DNA sequence on opposite strands. It is required for initiation of amplification of the target DNA by DNA polymerase. Please note that the primers are custom made depending on the sequence to be amplified.

Nucleotides: The four nucleotides that is dATP, dGTP, dCTP and dTTPs are used as mixture (dNTPs or deoxynucleotide triphosphates) and are the building blocks for synthesis of new DNA strands.

Buffer: It is an ionic solution with specific pH that helps to maintain the enzyme activity, stabilize the DNA and other components of the reaction. Magnesium ion which is a co factor for DNA polymerase is one of the main components of the buffer.

PCR reaction

The composition of a typical PCR reaction mix is as follows

Components	Final Concentration	Volume
10X Buffer	1X	5 µl
dNTP (2mM)	0.2M	5 µl
Primer (F+R) (10µM)	0.4 µM	2µl
Template DNA	10pg-100ng*	1 µl
Enzyme (Taq Polymerase) (5 U/ µl)	1 U/ µl	0.2 µl
Sterile Distilled Water	1X	36.8 µl
	Total Volume	50 µl

* The concentration of template varies for plasmid (pg or ng) and genomic DNA (ng).

PCR is carried out in automated thermocycler which carries out following steps:

1. **Denaturation** The DNA is heated to 94° C. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate creating single stranded DNA.

2. **Primer Annealing**

   The mixture is cooled to temperature from 50-68° C. This allows the primers to bind (anneal) to their complementary sequence in the template DNA. The annealing temperature depends on the Tm of the primer and it is usually kept 2-5 degrees below the Tm of the primers.

3. **Extension**

   The reaction is then heated to 72° C, which allows DNA polymerase to extend the primers by adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

These three steps are repeated to amplify the desired DNA segment. The time required for these three individual steps is determined by the processivity of the DNA polymerase enzyme and the size of the fragment to be amplified (amplicon size). In PCR, amplification is exponential because for each cycle, the DNA made in the previous cycles can also serve as template (Fig. 1).

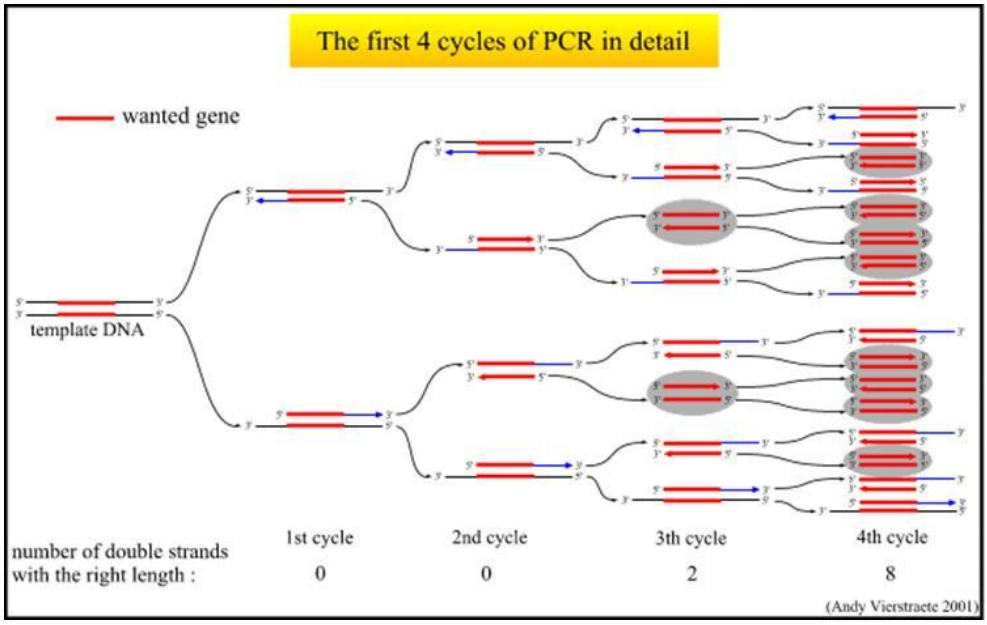


Fig. 1 Schematic representation of first four cycle of PCR showing exponential amplification of DNA

Visualization of the amplified product

The PCR amplified product (amplicon) can be visualized by gel electrophoresis where the product is resolved in a 1% agarose gel. The % of the gel depends on the size of the amplicon (Fig. 2).

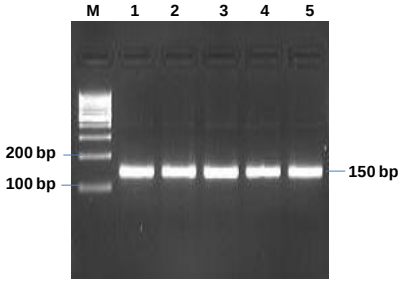


Fig. 2 Visualization of PCR amplified DNA fragment. M, Molecular weight marker; 1-5 amplified product (150bp)

Application

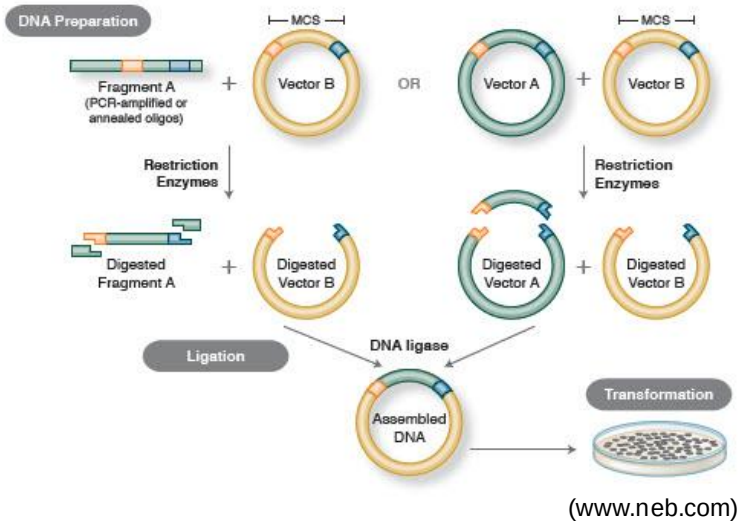
The DNA amplified by PCR can be used in a number of laboratory and clinical techniques such as for amplification of gene, DNA fingerprinting, detection of viruses and bacteria, for diagnosis of diseases and genetic disorders etc.

Cloning of amplified product in a binary vector

Cloning of double-stranded DNA (dsDNA) molecules into plasmid vectors is routinely used techniques in molecular biology. The most common method of cloning and subcloning of DNA fragments rely on restriction enzyme digestion and ligation. However in recent years, Gateway® cloning technology (Invitrogen Co.) has developed a fast and reliable alternative cloning methodology based on bacteriophage λ site-specific recombination. A general procedure based on restriction enzyme is described as follows

Cloning by cohesive end joining

Restriction endonucleases recognize and cleave dsDNA at highly specific nucleotide sequences. The type II restriction enzymes are most commonly used in cloning experiments. These restriction enzymes generally produce short, staggered ends (with 5′ or 3′ overhangs) that can be rejoined by a DNA ligase. The requirement for restriction digestion based strategy is that the DNA fragment to be cloned and the vector to which it is to be cloned should be restricted with the same set of restriction enzyme. For this purpose, while designing primers the specific sequence for restriction site is added at the 5' end of the primers. These primers are used for amplifying the desired DNA fragment so that restriction site gets incorporated at both the ends of the amplified DNA fragment. For example to clone a DNA fragment in *Bam*HI and *Hind*III site of a vector, *Bam*HI site is added in the forward primer and *Hind*III site is added in the reverse primer. Please note that these two restriction site should be in the multiple cloning site of the vector and should not be present elsewhere neither in vector nor in the amplified product (except at the ends of amplicon). After amplification the amplified product (Insert) is digested with *Bam*HI and *Hind*III restriction enzyme. Same way vector is also restricted with these two enzymes but in a separate reaction (Fig. 3). If subcloning of a desired DNA fragment or gene is required, then the gene or fragment can be restricted from that plasmid and sub cloned into another vector (Fig.3)



(www.neb.com)

Fig. 3 Schematic presentation of cohesive end cloning in plasmid

A typical restriction reaction is shown as follows

Components	**Volume**
10X Buffer	1 µl
DNA (Vector or amplicon)	Up to 8 µl (depending on concentration and requirement)
Enzyme (HindIII or BamHI) (10 U/ µl)	0.5 to 1 µl
Sterile Distilled Water	Made up the volume to 10 µl
Total volume	10 µl
Add the enzyme in the last. Mix gently by pipetting the solution up and down. Incubate the reaction at 37°C for 1-4 hr.	

The restriction can be single digestion i.e. one enzyme at a time or double digestion with two enzymes added in the same reaction.

Following digestion, both insert and vector are purified to remove all enzymes. For purification the restricted products are resolved in the agarose gel, DNA fragment or insert and vectors are cut from the gel and purified by gel extraction. There are several kits available for gel extraction. Subsequently, the insert is ligated into the plasmid, and the ligated plasmid is transformed into *E. coli.*

The typical ligation reaction is given as follows

Components	**Volume**
10X ligase buffer	2 µl
Digested vector DNA (50 ng/µl)	2 µl
Digested Insert DNA	the appropriate amount of digested insert DNA *
T4 DNA ligase (20 NEB units/µl)	1 µl
Sterile Distilled water	Add water to a volume of 20 µl

∗ Digested insert to vector DNA in a molar ratio of **1:1, 2:1**, and **3:1**.

Add the ligase last. Mix gently by pipetting the solution up and down. Incubate the reaction mixtures at 16°C for 2 h to overnight. Transform the ligation reaction in to competent *E. coli* cells.

Suggested reading

Sambrook and Russell (2001) *Molecular Cloning: A Laboratory Manual*,Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Agrobacterium-mediated Transformation of Tobacco Plants

Ch. Ramakrishna, Sonam and Amolkumar U. Solanke*

ICAR-National Research Centre on Plant Biotechnology,

Pusa Campus, New Delhi -110012

Agrobacterium-mediated tobacco transformation comprises the development of *in vitro* plant regeneration and genetic transformation studies in tobacco.

Sterilization methods

a) Steam sterilization (Heating in autoclave):

Exposure of microorganisms to saturated steam under pressure in an autoclave achieves their destruction by the irreversible denaturation of enzymes and structural proteins. The temperature at which denaturation occurs varies inversely with the amount of water present. Sterilization in saturated steam thus requires precise control of time, temperature, and pressure. As displacement of the air by steam is unlikely to be readily achieved, the air should be evacuated from the autoclave before admission of steam. The recommendations for sterilization in an autoclave are 15 psi for 20 minutes at 121-124 °C.

b) Dry-heat sterilization (hot air oven):

Sterilization of glass wares and metallic instruments are carried out using dry heat for 1-2hrs at 160-180^{O}c.

c) Filtration:

Some growth factors, amino acids and vitamins are heat labile and get destroyed during autoclaving. Therefore filter sterilization is employed for such thermolabile solutions. Membrane filter having 0.45-0.22µm.

d) Alcohol sterilization:

It is necessary that hands of the worker have to be kept aseptic during manipulation of the work. A wash with an antibacterial detergent followed by spraying 70% ethanol on hands is quite effective. The laminar air flow cabinets should also be sprayed with 70% ethanol and wiped before use.

e) Flame sterilization:

This method is employed for instruments that are continuously used during work. Instruments are soaked in 70-80% alcohol followed by flaming on a burner in the laminar air flow hood.

Plant material:

The aseptic leaves of tobacco (*Nicotiana tabacum* L. var. Petit Havana) are used for genetic transformation studies.

Bacterial strain and plasmid:

Agrobacterium mediated genetic transformation of the tobacco is carried out using the EHA105 strain of *Agrobacterium tumefaciens* harbouring the binary plasmid construct pBI121 containing gene of interest and selectable marker *npt-II.*

Preparation of culture medium:

One of the most widely employed media is the medium developed by Toshio Murashige and Folke Skoog (Murashige and Skoog, 1962). The Murashige and Skoog medium is so well known to the scientific community that it if often abbreviated simply as the MS medium.

For 1L composition:

1. Two 1000mL conical flasks were taken, sterilized properly
2. One 1000mL measuring cylinder was taken, washed and rinsed properly.
3. MS media sachet for 1L was taken.
4. In one of the conical flask, 700mL of double distilled water was added then the media sachet contents were added.
5. The flask was kept for stirring using magnetic stirrer.
6. pH was checked and adjusted to 5.8
7. 300mL of water was added and the media solution was equally distributed into the 2 conical flasks, of 500mL each.
8. 4g of agar was added into each flask
9. Non-absorbant cotton was plugged into the flasks and covered with a paper.
10. The flasks were then autoclaved at 121°C for 20 mins.

Media	Composition
Preculture Media	MS+NAA+2BAP
Co-Culture Media	MS+NAA+2BAP
Inoculation Media	YEM+KAN+RIF
Selection Media-I	MS+NAA+2BAP+CEF+KAN
Selection Media-Ii	MS+NAA+1BAP+CEF+KAN
Rooting Media	MS+CEF+KAN

Table1: Different compositions of MS media used in *Agrobacterium* mediated genetic transformation.

Plant Hormones:

Phytohormones or plant growth regulators may be added to the basal medium to stimulate the growth and development of the explant in a particular fashion. The growth hormones included in culture media involve (i) auxins and (ii) cytokinins. The auxins as mainly used to facilitate cell division and root differentiation. Commonly used auxins for the work here is NAA (napthaleneacetic acid). The cytokinins facilitate the cell division and differentiation. Cytokinin used was BAP (benzylamino purine).

Antibiotics:

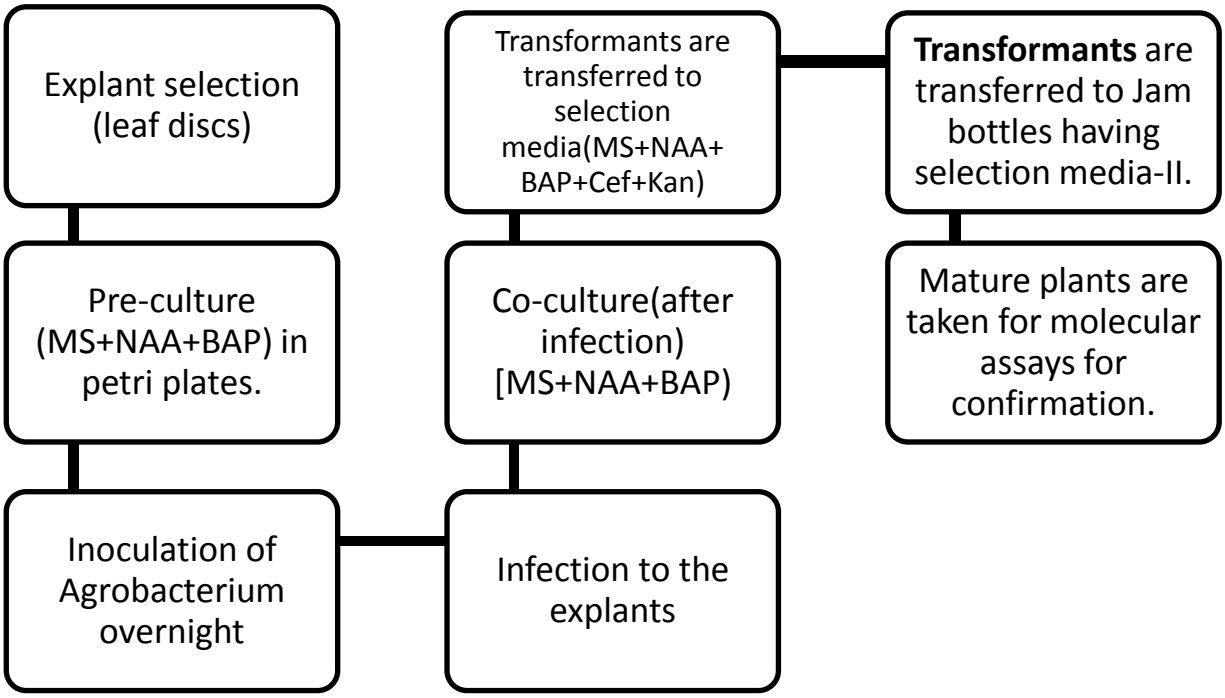
Kanamycin and cefotaxime both antibiotics were used in the present study at concentrations of 100mg/l and 500mg/l respectively. These antibiotics were dissolved in sterile distilled water and filter-sterilised and stored in -20^{0}C. Rifampicin has also been used in the present study for maintaining of *Agrobacterium* at the concentration of 10mg/l, which has been dissolved in methanol then filter sterilized. For the maintenance of *Agrobacterium* Yeast Extract Mannitol (YEM) medium was used.

Components	Stock	Working
NAA	1mg/ml	0.1mg/L
BAP	5mg/ml	2mg/L
Kanamycin	100mg/ml	500mg/L
Cefatoxime	200mg/L	500mg/L
Rifampicin	10mg/ml	10mg/L

Table 2: Concentrations of hormones and antibiotics required for different media preparation.

GENETIC TRANSFORMATION OF TOBACCO

OUTLINE:



DAY-1:

Preparation of *Agrobacterium* strain EHA 105 culture harboring binary vector:

In this experiment, the strain was streaked for the transformation purpose using loop in YEM Solid medium containing Kanamycin (50mg/L) and Rifampicin (10mg/L). The plates were incubated for 2-3 days at 28°C.

DAY-3

Preculturing:

In the following step, aseptic leaf discs of the tobacco plant (1 month old) was cut and placed on the MS medium having required amount of BAP and NAA (also called BN medium) for 2 days. Also *Agrobacterium* strain EHA 105 that harbor the binary plasmid pBI121 was grown in 5ml YEM liquid culture.

DAY-4

Inoculation of *Agrobacterium:*

Agrobacterium tumefaciens strain containing a binary vector carrying gene of interest under the control of CaMV35S promoter was used in the present study. *A. tumefaciens* was grown in about 50 ml of YEM liquid medium containing Kanamycin (100mg/l) and Rifampicin (10mg/l) for 20-24 hours at 28^0C on a shaker at the rate of 220 rpm. After incubation, the inoculum was centrifuged at 10K rpm to obtain the bacterial pellet which was resuspended in MS (Liquid) medium at the ratio of 1:20. The MS (Liquid) was then used to carry out infection.

DAY-5

Agro infection:

About 35-40 explants were put in *Agrobacterium* consisting 1/2MS Plain (Liquid) in the conical flask or petriplate were gently stir by hand for 10 to 15 minutes. After this, the explants (leaf discs) were blotted dry using sterile filter papers to avoid excess growth of *Agrobacterium*. The explants were then co-cultivated with *Agrobacterium* on BN medium for 48 hours at 28^0C. Of the remaining 20 leaf discs, 10 each were kept as control in Pre-culture medium.

DAY-7

Transfer to selection medium:

After the period of incubation, the co-cultivated explants were transferred to selection medium consisting of Kanamycin (200mg/l) and Cefotaxime (500mg/l) in MS medium, with hormones BAP (2.5 mg/l) and NAA (0.1mg/l). The explants were sub-cultured after every two week on the same medium to avoid excess growth of *Agrobacterium*. The callusing of the explants started after 20-25 days after the infection. With this, the BAP concentration for which the plants are subjected is gradually lowered (selection media-I), bringing up to zero (selection media-II).

DAY-31

Transfer to rooting medium:

After continuous sub culturing with gradual decrease of BAP concentration in the selection medium, the regenerated plantlets from the callus were transferred to culture bottles consisting MS medium with antibiotics but without any hormones (Rooting media), for complete plant generation.

Acclimatization to green house:

Once the complete plant was generated in the culture bottle, the entire plant was shifted to small pots containing soil rite. The pot containing plant was covered with plastic poly bag for maintaining humidity. After seven days of this initial acclimatization, poly bags were removed from the plants and plants were shifted to green house for acclimatization to ambient temperature.

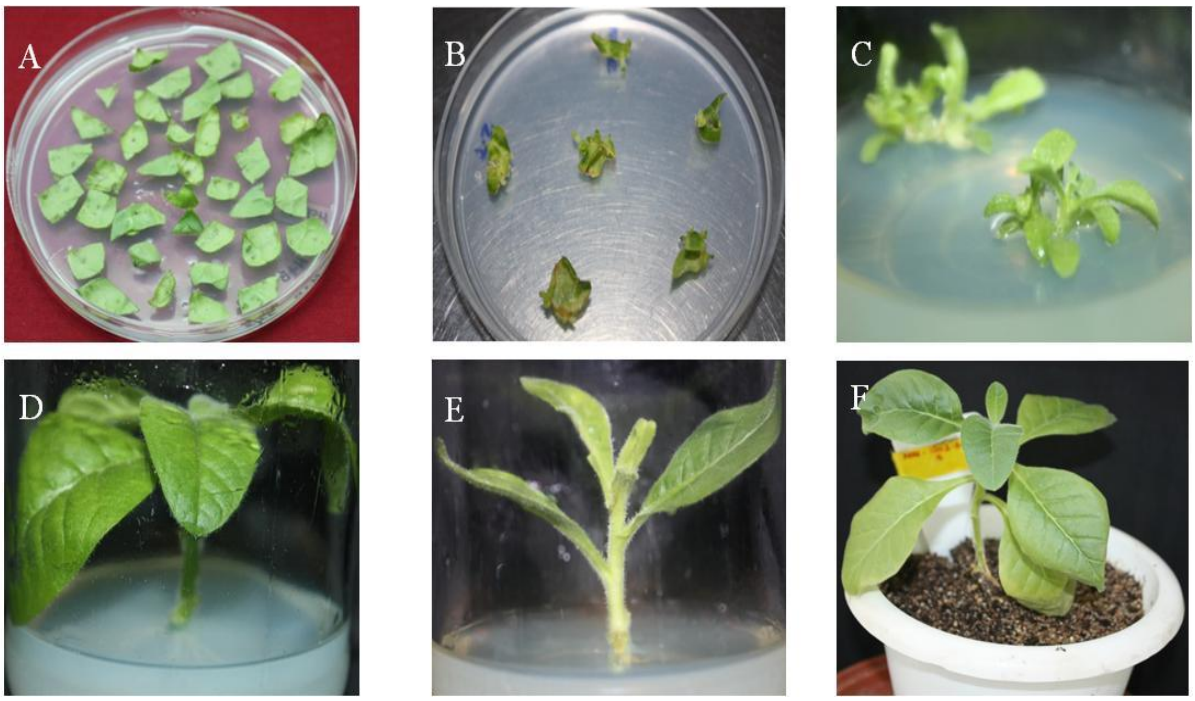


Figure: Schematic representation of tobacco transformation. (A: Pre culture Explants, B: Callus Induction in selection medium, C: Shoot Initiation, D: Plant on rooting media, E : Plant with established roots, F : Plants for hardening).

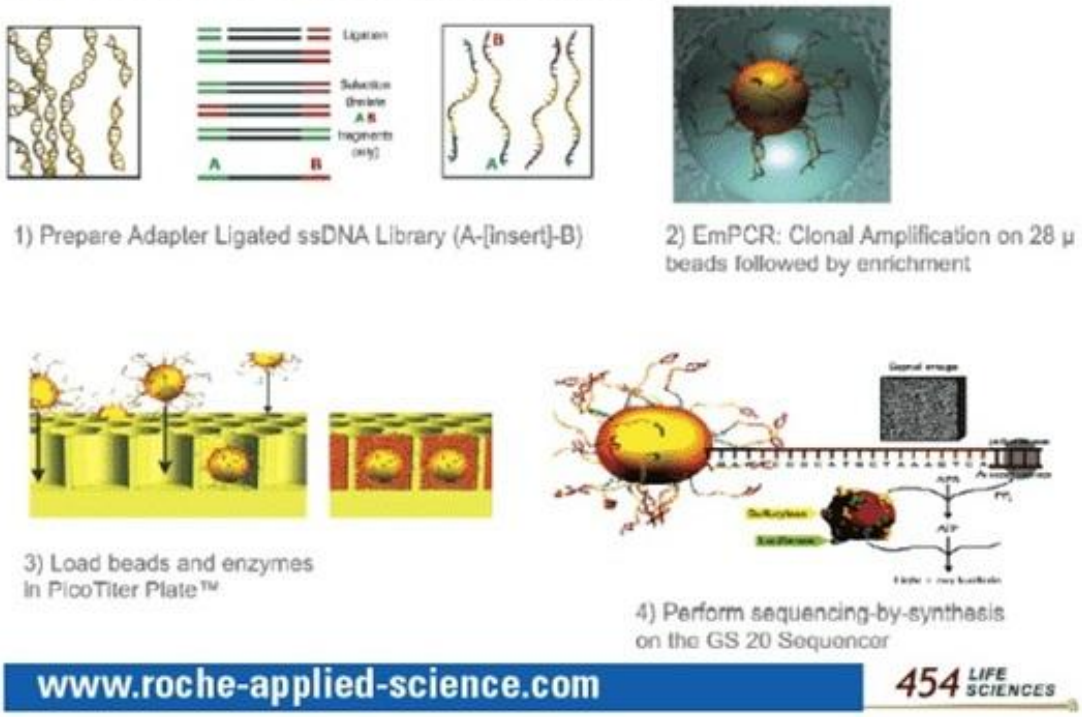
Rapid Library Preparation Method for Roche 454 GS-FLX

Kishor Gaikwad, Amolkumar U. Solanke and Swati

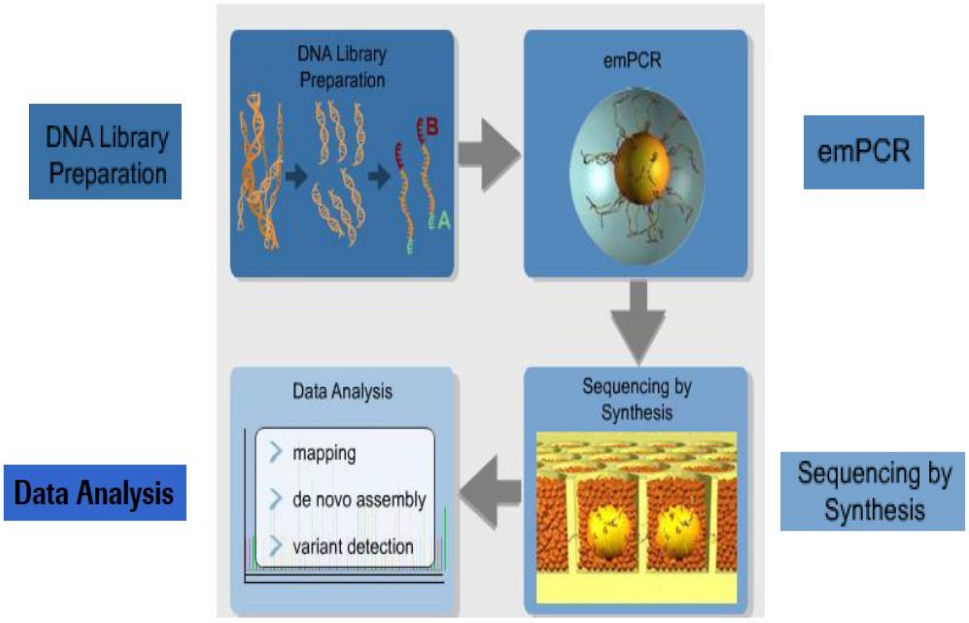
ICAR-National Research Centre on Plant Biotechnology

Pusa Campus, New Delhi -110012

Figure 1. Overview of the 454 sequencing system



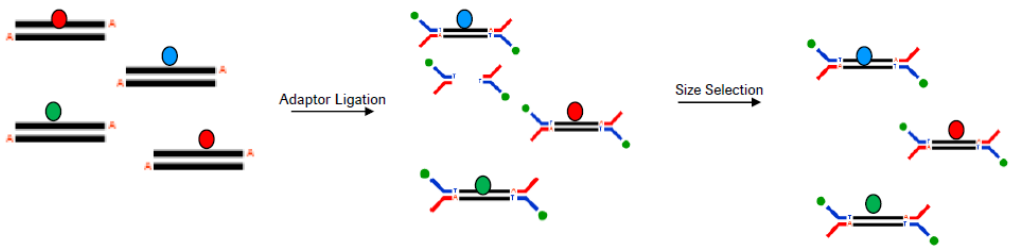
Sequencing Workflow

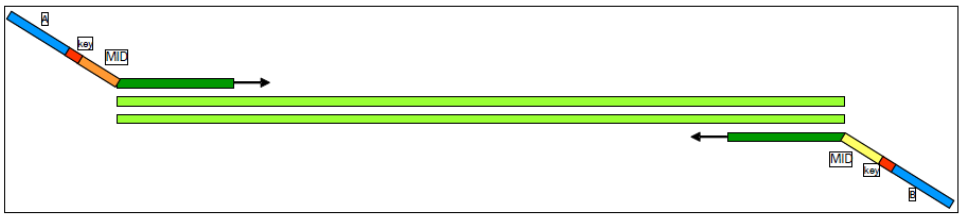


454 SEQUENCING

Library Preparation

Adaptor Ligation and amplicon sequencing



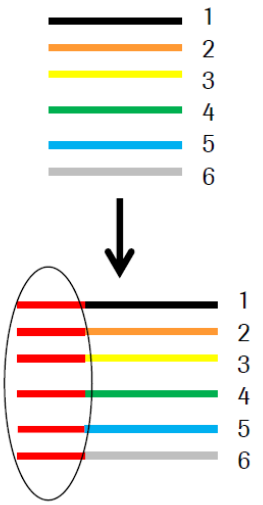


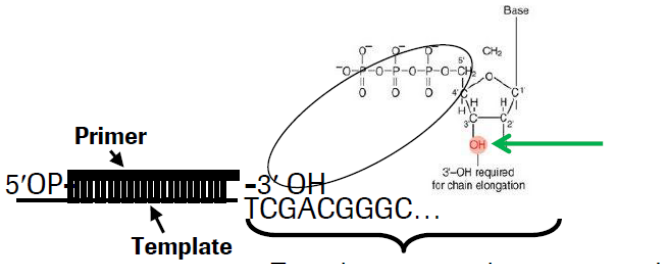
454 SEQUENCING

Process Steps

Emulsion PCR

Library Preparation

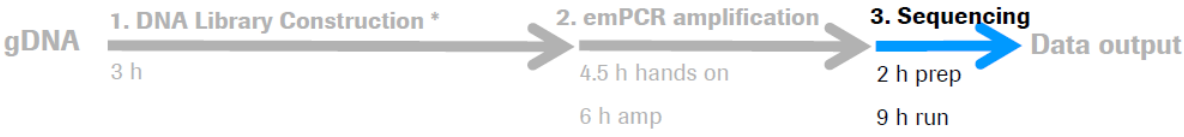


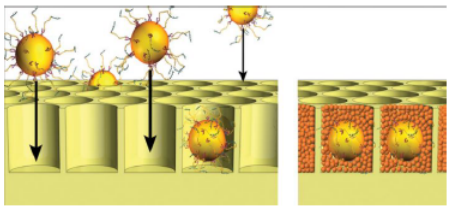


Library is prepared to introduce Universal primer Binding site

Process Steps

Chemistry: Sequencing Run Setup





- A single clonally amplified DNA bead is deposited per well.
- Layers of packing, enzyme and PPiase Beads are deposited
- Plate is loaded into instrument for sequencing

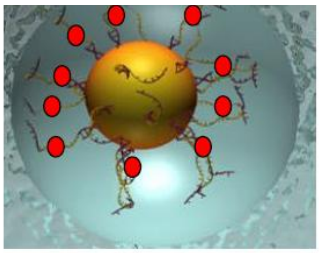
Amplified sstDNA library beads → **PTP device ready for sequencing**

www.454.com

C) Amplify

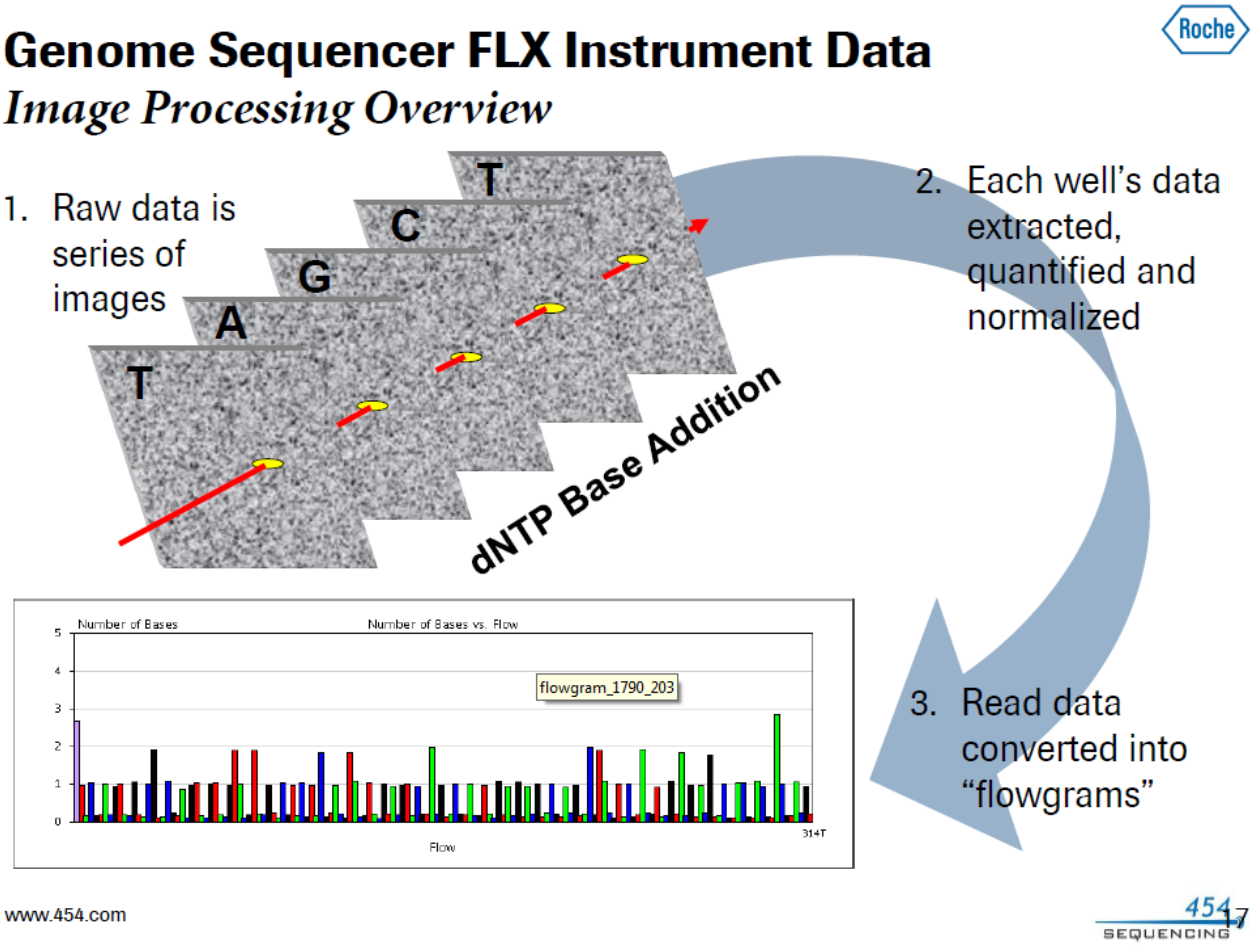
D) Break Microreactors

E) Enrich for DNA positive beads



After PCR

www.454.com



GS FLX Titanium Series

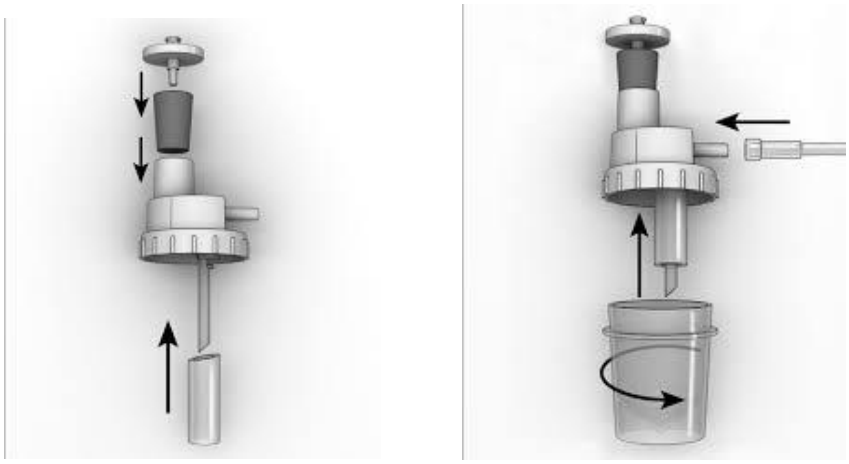
1. Workflow: The DNA is fragmented, ligated to adaptors and taken up for EmPCR before loading into the picotiter plate (PTP). Each bead then carries out sequencing of individual strand of DNA and millions of beads provide sequence information.

2. Sample Requirements:Sample DNA should be:

- double-stranded genomic or cDNA
- OD260/280 ≥ 1.8
- concentration ≥ 5 ng/µl
- fragment size > 1.5 kb

3. Procedure:

3.1 DNA Fragmentation by Nebulization (provide with the kit or separately available with Invitrogen) Fig. 2

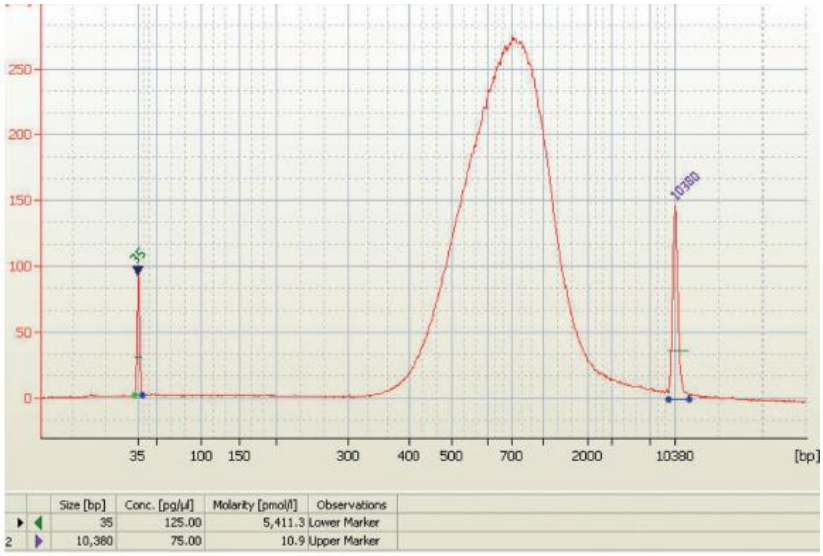


Attach to Nitrogen/Argon gas cylinder. Place DNA sample mixture at base

Start with 500 ng of sample DNA in a 1.7 ml microcentrifuge tube.

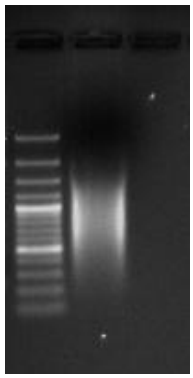
1. Add TE Buffer to a final volume of 100 µl.
2. Using sterile gloves, affix a Nebulizer Condensor tube around the Aspiration tube. To ensure proper function, make sure to push the Condensor tube all the way down around the base of the Aspiration tube, being careful not to rotate the Aspiration tube, and press the vented cap into the Nebulizer top Set the assembled Nebulizer top, with the aspiration tube pointing upwards, making sure that the inside parts do not contact any contaminated surfaces (counter top, hands etc).
3. Pipet the 100 µl DNA sample in the Nebulizer cup.Add 500 µl of Nebulization Buffer, pipet up and down to mix. Keep in ice.
4. Screw the Nebulizer top to the cup, transfer the cup to the external vented hood and connect the tubing to the nitrogen tank. Apply 30 psi (2.1 bar) of nitrogen for 1 minute.
5. Disconnect the tubing and remove the cup from the hood. Remove the Nebulizer top from the cup.
6. Add 2.5 ml of PBI Buffer. Pipette up and down to mix.

7. Purify the nebulized DNA sample on a column from the Qiagen MinElute PCR Purification kit, as follows, with all centrifugation steps carried out at 13,000 rpm in a tabletop centrifuge:



a) Load 750 µl of the nebulized DNA at a time into a single column.
b) Centrifuge for 15 seconds and discard the flow-through.
c) Repeat steps a and b three more times, using the same column.
d) Centrifuge for 1 minute. Discard all the flow-through.
e) Add 750 µl of PE Buffer, centrifuge for 1 minute, and discard the flow-through.
f) Centrifuge 1 minute, rotate the column 180º, centrifuge 1 minute.
g) Elute in new tube with 16 µl of TE Buffer by centrifuging for 1 minute.
h) Transfer the sample to a 200 µl PCR tube.

Check on agarose gel and Bioanalyzer (Fig.3)



Nebulized DNA in 500-1000bp size range

3.2 Fragment End Repair

1. In a 1.7 ml microcentrifuge tube, prepare the End Repair mix, as follows.
    - 2.5 µl RL 10× PNK Buffer
    - 2.5 µl RL ATP
    - 1 µl RL dNTP
    - 1 µl RL T4 Polymerase
    - 1 µl RL PNK
    - 1 µl RL Taq Polymerase

    9 µl Total volume
2. Pipet up and down to mix, and add the 9 µl of End Repair mix to the DNA sample:
3. Vortex for 5 seconds, then spin for 2 seconds in a mini centrifuge.
4. Run the End Repair program on a thermocycler, with the heated lid on:

    25ºC for 20 min

    72ºC for 20 min

    4ºC on hold
5. While the program is running, prepare the Agencourt AMPure beads as described in Section 3.3, below.

3.3 AMPure Bead Preparations

1. Vortex the AMPure bead bottle for 20 second or until the beads are completely resuspended.
2. Aliquot 125 µl of AMPure beads in a 1.7 ml microcentrifuge tube
3. Place the tube on the Magnetic Particle Concentrator (MPC,Invitrogen/NEB)
4. When the beads have completely pelleted on the side of the tube, carefully remove and discard all supernatant, without disturbing the beads.
5. Aadd 73 µl of TE Buffer to the beads and vortex 5 seconds.
6. Add 500 µl (of Sizing Solution to the beads, vortex for 5 seconds and spin for 10 seconds in a mini centrifuge.
7. Keep the tube on ice, until further use as described in Section 3.5.
8. Prepare 5 ml of 70% ethanol, by adding 3.5 ml of 100% ethanol to 1.5 ml Molecular Biology Grade Water, and vortex.

3.4 Adaptor Ligation

1. Once the End Repair program has completed from Section 3.2, add 1 µl of RL Adaptor or of RLMID Adaptor to the reaction tube.
2. Add 1 µl of RL Ligase to the reaction tube.
3. Vortex 5 seconds, then centrifuge for 10 seconds in a mini centrifuge.
4. Incubate at 25ºC for 10 minutes in a heating block/thermal cycler.

3.5 Small Fragment Removal

1. Add the sample to the AMPure beads prepared in Section 3.3. Vortex for 5 seconds and spin for 5 seconds in a mini centrifuge.
2. Incubate at room temperature for 5 minutes.Place the tube on the MPC.
3. When the beads have fully pelletted on the wall of the tube, carefully remove and discard the supernatant.
4. Add 100 µl of TE Buffer. Vortex for 5 seconds and add 500 µl of Sizing Solution. Vortex for 5 seconds. Incubate at room temperature for 5 minutes.
5. Place the tube on the MPC. When the beads have fully pelleted on the wall of the tube, carefully remove and discard the supernatant.
6. Repeat steps 5 to 9, once.
7. Keeping the tube on the MPC, wash the beads twice, as follows:Add 1 ml of 70% ethanol. Completely remove and discard the ethanol.
8. Keeping the tube on the MPC, uncap the tube and air dry the pellet at room temperature for 2 minutes.
9. Remove the tube from the MPC.Add 53 µl of TE Buffer. Vortex for 5 seconds and spin for 5 seconds in a mini centrifuge.
10. Place the tube on the MPC, wait for the beads to pellet on the wall of the tube and transfer 50 µl of the **SUPERNATANT, containing the library**, to a new, labeled 1.7 ml micro centrifuge tube. Make sure not to carry-over any beads in this process as they will cause incorrect readings during library quantitation.

3.6 Library Quantitation

Use either a single cuvette or a 96-well plate fl uorometer to quantitate the DNA library. The recommended instruments are TBS 380 Fluorometer (Turner Biosystems) for single use cuvette.

3.6.1 Preparing the Standard

To generate the standard curve, begin by labeling 8 tubes, 1 to 8.

1. In tube 1, prepare a 2.5 × 109 molecule/µl solution of the RL Standard by mixing 90 µl of the RL Standard (orange cap) with 90 µl of TE Buffer.
2. Fill the remaining 7 tubes (tubes 2 to 8) with 60 µl of TE Buffer.
3. Transfer 120 µl from tube 1 into tube 2.
4. Vortex for 5 seconds and spin for 5 seconds in a mini centrifuge.
5. Change pipet tip and transfer 120 µl of tube 2 into tube 3.
6. Vortex for 5 seconds and spin for 2 seconds in a mini centrifuge.
7. Proceed with the same serial dilution (transferring 120 µl of one tube into the next, vortexing for 5 seconds, and changing pipet tip between each dilution) for the remaining 5 tubes.

3.6.2 Using the TBS 380 Fluorometer

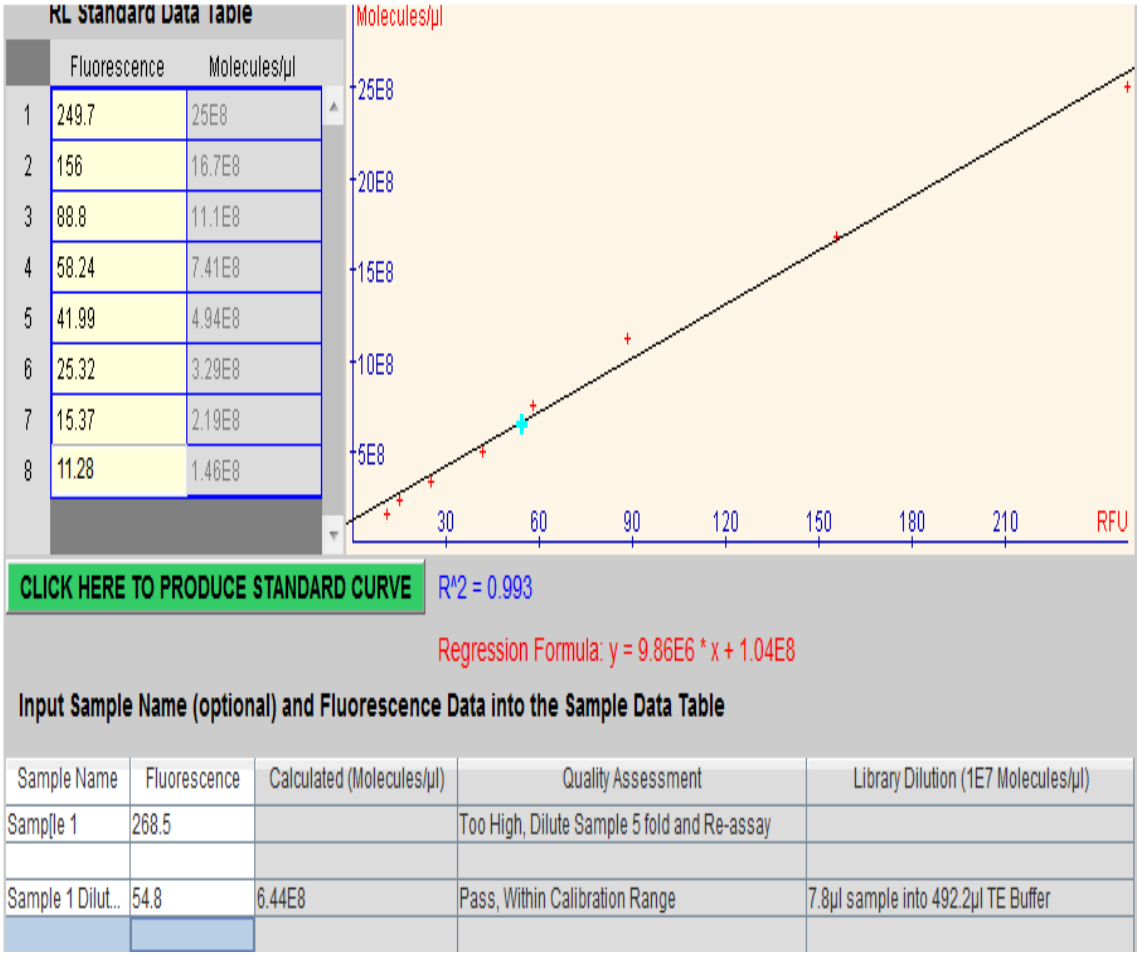
1. Transfer 50 µl of the 8 dilutions of the RL Standard into 8 cuvettes.
2. To generate a blank, transfer 50 µl of TE Buffer in a cuvette.
3. Set the TBS 380 fl uorometer on the Blue channel with the Blue cuvette holder insert. Set the standardvalue (Std Val) to 250.
4. Calibrate the fluorometer with the blank and the 2.5 × 109 molecule/µl solution RL Standard.
5. Read and record the relative fluorescence units (RFU) of each dilution.
6. Transfer 50 µl of the sample library in a cuvette. Read and record the RFU.

   DO NOT DISCARD THE SAMPLE.

7. Transfer the sample library back to its tube with a 20 µl pipet tip.

3.6.3 Generating a RL Standard Curve and Calculating the Sample Concentration

To generate a standard curve of fluorescence readings and calculate the library sample concentration, use either the Rapid Library Quantitation Calculator, or Excel software, selecting XY (scatter), creating a linear trend line, and selecting for the mathematical equation of the linear regression. An example of a RL Standard Curve is in Section 4.1. It is also possible to use software supplied by your fluorometer manufacturer to generate a standard curve and calculate the concentration of the sample (Fig. 4).



Sample Name	Fluorescence	Calculated (Molecules/µl)	Quality Assessment	Library Dilution (1E7 Molecules/µl)
Sample 1	268.5		Too High, Dilute Sample 5 fold and Re-assay	
Sample 1 Dilut...	54.8	6.44E8	Pass, Within Calibration Range	7.8µl sample into 492.2µl TE Buffer

Fig4: Flourometer Readings and the standard graph for the library.

3.6.3.1 Using the Rapid Library Quantitation Calculator

Go to the Web page www.454.com/my454 to access the Rapid Library Quantitation Calculator.

Explanations on how to use the Calculator can be found on the web page.

3.6.3.2 Using Excel Software

1. Create an XY (scatter) plot in Excel using the fluorescence readings as the x axis and the RL Standard concentrations (molecules/µl) as the y axis.
2. Right click any of the data points in the plot and select add trend line. Set regression type to linear. Select options tab then choose display equation on chart as well as display R-squared value on chart. R2, the correlation coefficient for the linear regression, must have a minimum value of 0.9. If the sample RFU falls above the standard curve, dilute the sample by adding 50 µl of TE Buffer and read the RFU again. The total DNA library amount should be at minimum 7.3 × 109 molecules which correspond to a concentration of ≥1.46 × 108 mol/µl.

3.7 Library Quality Assessment

1. Run a 1 µl aliquot of the DNA library on an Agilent Bioanalyzer High Sensitivity DNA chip, to assess the quality of the library.
2. Assess the quality of the DNA library for the characteristics listed below:

Library Characteristics	**Expected Results**
Average fragment length:	Between 600 bp and 900 bp
Lower size cut-off :	<10% below 350 bp

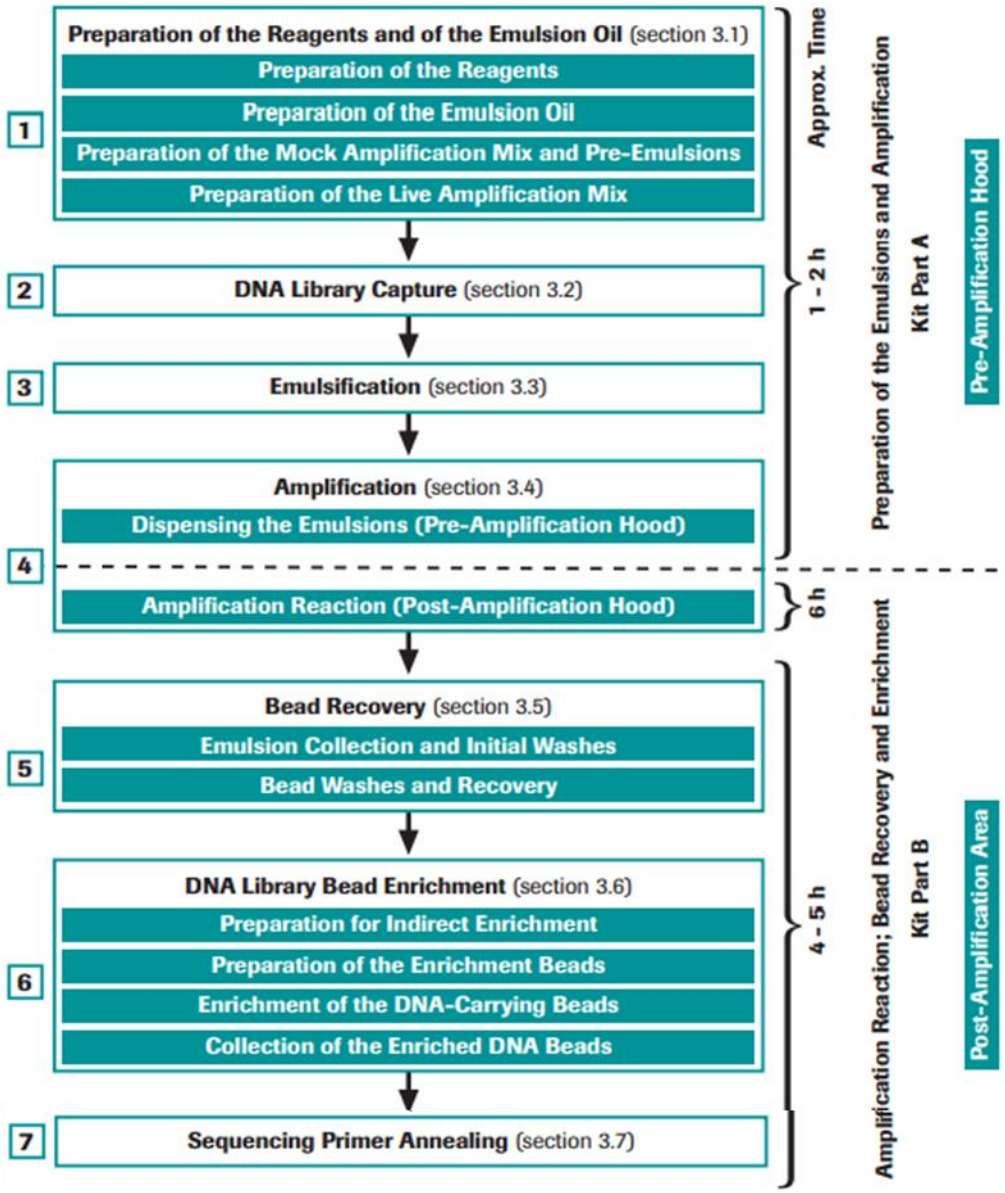
3.8 Preparing Working Aliquots

1. Dilute an aliquot of the DNA library to a working stock of 1 × 107 molecules/µl, in TE Buffer.
2. Transfer the working stock in 25–100 µl aliquots, and store these at -15 to -25ºC, for up to 2 months.

Immediately before emPCR amplification, heat denature the Rapid library sample at 95°C for 2 minutes, using a thermocycler with heated lid on.

The emulsion-based clonal amplification (emPCR amplification) of a DNA library sample involves 7 major steps, summarized in Figure below. Variants of this procedure exist to accommodate different types of DNA libraries and different number of beads requirements for the experiment. This step is critical for loading samples onto a PTP for final pyrosequencing process.

EmPCR workflow



Apical meristem-directed *in planta* transformation strategy for the development of transgenic plants

Rohini Sreevathsa

ICAR-National Research Centre on Plant Biotechnology, LBS Centre,

Pusa Campus, New Delhi

Transgenic technology has emerged as a powerful tool being used by scientists globally in plant research. Various techniques and strategies are used with and without involving the bacterium involved, *Agrobacterium tumefaciens.* Indirect DNA transfermethodology which is the most commonly used method for obtaining transgenic plants involves the introduction of T-DNA, harbouring gene(s) of interest into plant cells by a pathogenic bacterium *Agrobacterium tumefaciens*or *A. rhizogenes*. So far, a number of successful protocols are available both in crop plants and tree species. In plant species that are amenable to transformation and regeneration by *A. tumefaciens*, T-DNA-directed gene transfer remains the method of choice because of its ease, efficient transgenedelivery and integration into the host genome. *Agrobacteriumtumefaciens-mediated gene transformation depends on host susceptibility. Based on the utility of tissue culture and plant regeneration, A.tumefaciens-mediated genetic transformation is further segregated into a) plant transformation methods that exploit the plant regeneration and in vitro tissue culture techniques, and b) transformation methods that avoid the tissue culture regeneration. Hence, they are referred to as tissue culture dependent (in vitro) and independent (in planta) A.tumefaciens-mediated plant transformation strategies, respectively.*

Compared to direct DNA transfer methods, *Agrobacterium*-mediated transformation is a more sophisticated and established method for most of the higher plants. In addition, this method does not demand specialized equipment or associated consumables for transformation. However, an efficient tissue culture system is a prerequisite for *in vitro* transformation studies.In addition somaclonal variation/mutationsand DNA methylationcaused due to either epigenetic effects or chromosomal rearrangements are certain serious hurdleswith regeneration-based transformations.

In particular, imbibition, vacuum infiltration and floral dip method of *Arabidopsis* have contributed greatly to the functional characterization of gene products in the *omics* era. The floral dip method of transformation has also been used to generate transgenic plants in *Medicagotruncatula, Raphanussativus, Brassica napus,Triticumaestivum*among others. Alternatively, soybean transgenicswere obtained by targeting *Agrobacterium* T-DNA to wounded mesocotyl of germinating seeds based on the fact that, there are undifferentiated cells in plumule, cotyledonary node, or adjacent regions of the germinating seed which later differentiate into germ cells. Various laboratories (Table 1) pursued and improved this method by directing either apical meristem or meristems of axillary buds.

As the *in planta* transformation evades tissue culture or regeneration steps, the associated concerns can be overcome. Starting from successful transformation by vacuum infiltration at the early stages of *Arabidopsis* floweringfollowed by many refinements by several laboratories has improved the *in planta* transformation method dramatically in the recent years. This methodology is advantageous as it is genotype-independent and avoids tissue culture steps. In addition, time taken for development of independent transgenic plants is short. Nevertheless, highly stringent screening strategies are required to select putative transformants among the large number of primary transformants.

For more than two decades, our laboratory was involved in the development of an apical meristem-targeted *in planta* transformation. We could establish proof of concept in a wide array of crop plants like horticultural (bell pepper[Manoj Kumar et al., 2009]), leguminous (pigeon pea [Sankara Rao et al., 2008; Ramu et al., 2012], field bean[Keshamma et al., 2012], chick pea[GowriNeelima et al., 2009], groundnut[Rohini and Rao, 2000; Keshamma et al., 2008; Sundaresha et al., 2010]), oilseed (sunflower[Manoj Kumar et al., 2011], safflower[Manoj Kumar et al., 2009], castor[Manoj Kumar et al., 2011]), fiber (cotton[Keshamma et al., 2008]) and cereals (rice[Prashantkumar et al., 2011]) using a variety of traits (genes) introduced to combat abiotic and biotic stress. The strategy essentially involves *in planta* inoculation of embryo axes (plumule) of germinating seeds and allowing them to grow into seedlings *ex vitro* (Figure 1). The selection of the putative transformants is carried out in the T1 generation.

Table 1: *Agrobacterium tumefaciens*-mediated transformation by *in planta* approach in various crops.

***Agro* strain**	**Plant species**	**Infection method**	**%Transformation**	**Reference**
C58-Z707	*Glycine max*	plumule/ cotyledonary node	0.72	Chee et al. 1989
LBA4404	*Oryza sativa*	apicalmeristem	40-43	Supartana et al. 2005
	Triticumaestivum	apicalmeristem	29-38	Supartana et al. 2006
	Fugopyrumesculentum	apicalmeristem	36-60	**Kojima et al. 2000**
	Hibiscus cannabinus	apical meristem	-	**Kojima et al. 2004**
	Corchorusolitorius	shoot tip	13.11-48	
	Gossypiumhirsutum	pistildip	0.46-0.93	Tianzi et al. 2009
	Kalanchoe pinnata	leaf crenate margins	77-84	10
	Notocactus scopa	Leaf crenate margins	67-100	Seol et al. 2008
GV3101	*Zea mays*	via pistil filaments	6.8	Chumakov et al. 2006; Mamontova et al. 2010
EHA 105	*Solanum lycopersicum*	fruit injection and floral dip	17-21	Abida et al. 2008

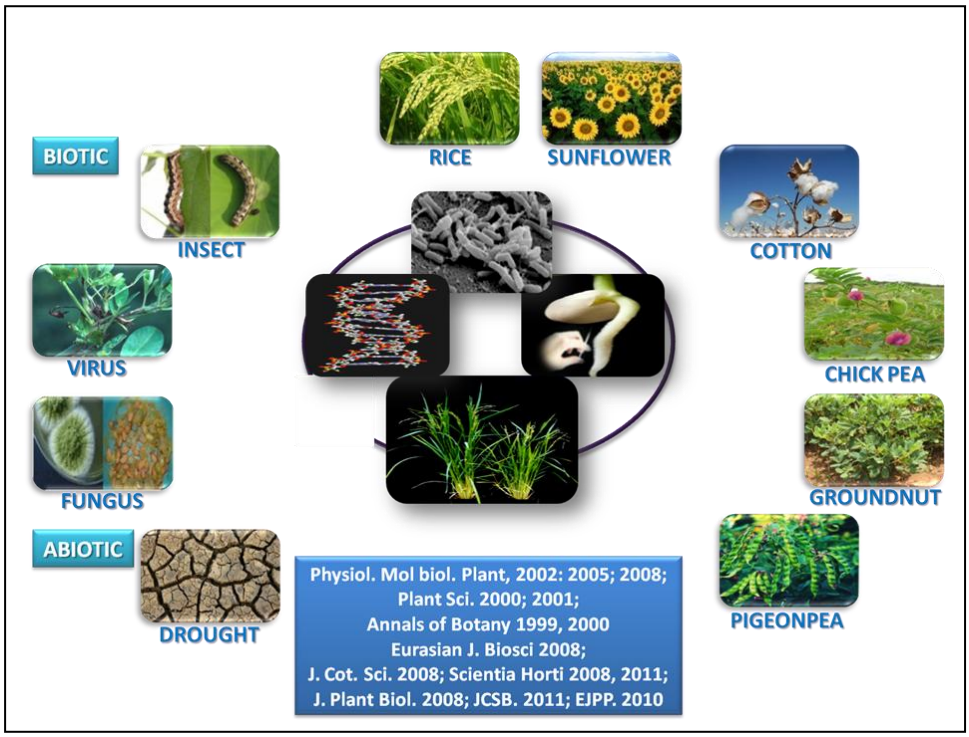


Figure 1. Apical meristem-based *in planta* transformation strategy.

Identification of transformants at initial stages

The feasibility of the transformation strategy can be ascertained by the utility of screenable markers like GUS and GFP (**Figure 2**)

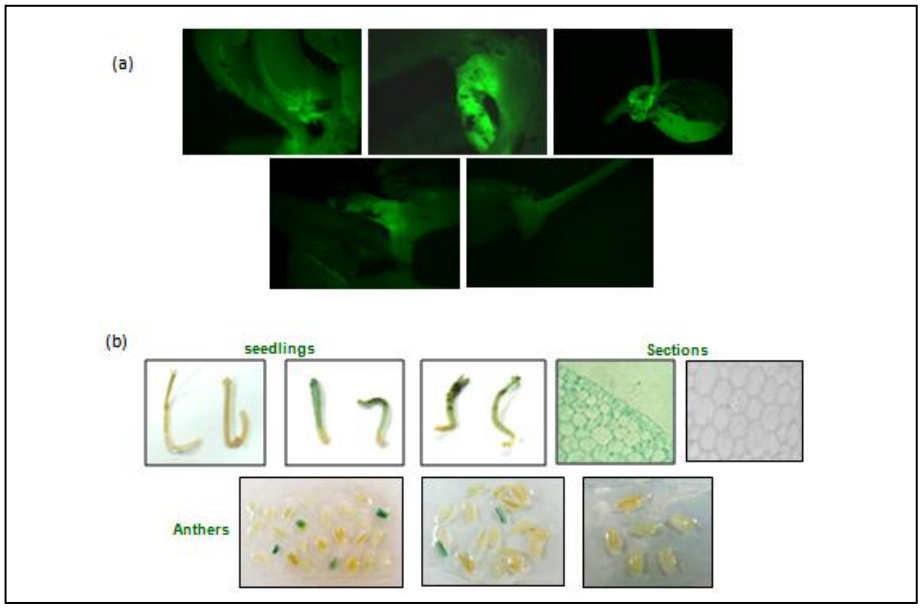


Figure 2. Assessment of transformability by *in planta* strategy by using screenable markers, (a) GFP and (b) GUS

The plants developed in the T_0 generation are chimeric, and stable transformants are obtained in the T_1 generation. We developed crop-specific protocols for transformation with suitable selectable markers for efficient screening of T1 plants to identify stable transformants. The major determinant of the strategy is the stringet screening that is required in the T1 generation for the selection of putative transformants. This can be realized by the use of right selectable markers in the binary vector to be used for transformation like *nptII*, *epsps*, etc. stringent protocols have been standardized for the selection (Figure 3)

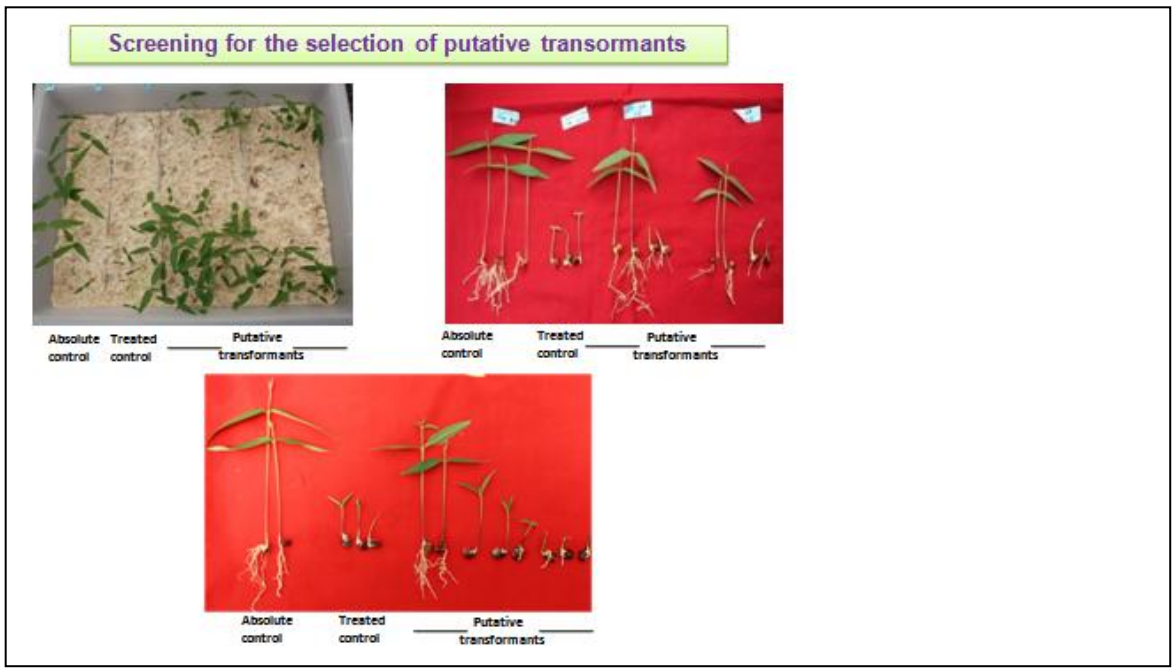


Figure 3. Stringent sand-based screening on the selection agent

The strategy has been used to develop transgenics for various agronomic traits to address both biotic and abiotic constraints like pests, viruses, fungal pathogens, salt and drought (Figure 4).

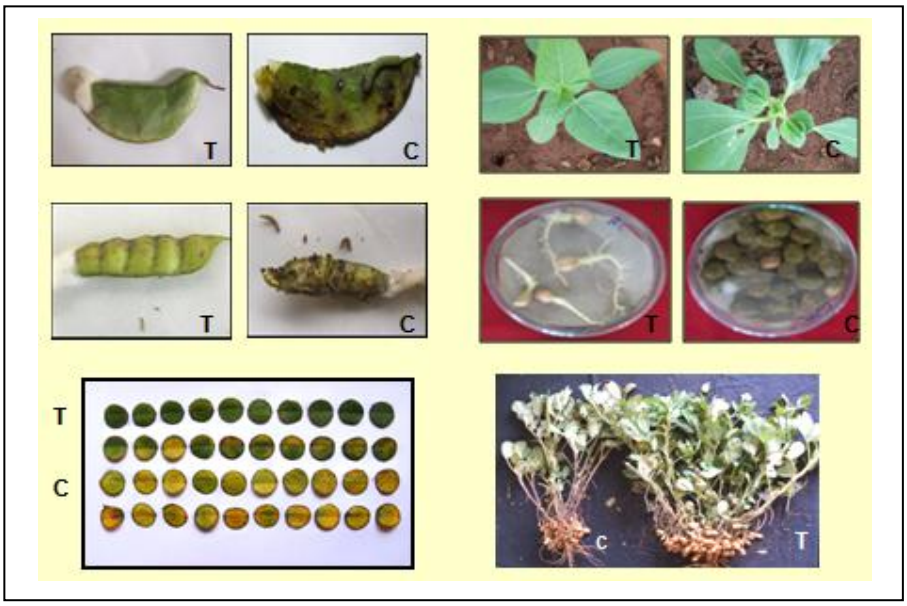


Figure 4. Utility of the in planta transformation strategy in the development of transgenic plants for resistance to both biotic and abiotic stress

The strategy forms an important and useful component of transgenic technology because of its genotype-dependent nature and can be exploited in a crop-independent manner.

References

Manoj-Kumar, A., Kalpana-NanjaReddy, Manjulatha, M.,Elizabeth Sauceda Arellano, E.S.Sreevathsa, R. &Ganeshan, G. A rapid and high-throughput identification of putative bell pepper transformants generated via *in planta* approach. *Scientia Horticulture*. 129, 898-903 (2011).

Sankara-Rao, K. & Rohini, V.K. *Agrobacterium*-mediated transformation of sunflower (*Helianthus annuus* L.): a simple protocol. *Ann. Bot*. **83**, 347-354 (1999).

Manoj-Kumar, A, Kalpana-NanjaReddy,Sreevathsa, R.,Ganeshan, G.&Udayakumar, M. Towards crop improvement in bell pepper (*Capsicum annuum* L.): transgenics (*uid*

A::*hpt* II) by a tissue culture independent *Agrobacterium*-mediated *in planta* approach. *Scientia Horticulture*. **119**, 362-370 (2009).

Sankara-Rao, K., Rohini, S., Pinakee, D., Sharma, Keshamma, E.&Udayakumar, M. *In planta* transformation of pigeon pea: a method to overcome recalcitrancy of the crop to regeneration *in vitro.Physiol. Mol. Biol. Plants***14**, 321-328 (2008).

Ramu, S.V., Shanmugam, N.B., Manoj-Kumar, A., Keshamma, E., Gowri, N., Keshava-Reddy, G., Rohini, S. &Udayakumar, M. Transgenics in pigeonpea: *A cry*1 AABC-mediated protection against *Helicoverpaarmigera*. *National seminar on Interventions for Environmental Moderations* on 8th-10th January, Pp: 78 (2008).

Keshamma, E., Rohini, S., Manoj-Kumar, A., Kalpana-NanjaReddy, Manjulatha, M., Shanmugam, N.B., Kumar, A.R.V. &Udayakumar, M. *Agrobacterium*-mediated *in planta* transformation of field bean (*Lablab purpureus* L.) and recovery of stable transgenic plants expressing the *cry*1AcF gene. *Plant MolBiol Rep*. (2011). In press DOI: 10.1007/s11105-011-0312-7

Gowri, N.M., Ramu, S.V., Rohini, S., Asha-Rani, B.M., Kumar, A.R.V. &Gayatri, M.C. *In planta* transformation strategy to generate transgenic plants in chickpea: Proof of concept with a *cry* gene. *J. Plant Biol*. **35**, 201-206 (2009).

Rohini, V.K. &Sankara-Rao, K. Transformation of peanut (*Arachishypogeae* L.): a non-tissue culture based approach for generating transgenic plants. *Plant Sci*. **150**, 41-49 (2000).

Sundaresha, S. Manoj-Kumar, A., Rohini, S., Math, S.A., Keshamma, E., Chandrashekar, S.C. &Udayakumar, M. Enhanced protection against two major fungal pathogens of groundnut, Cercosporaarachidicola and Asperigillusflavus in transgenic groundnut over-expressing a tobacoo ß 1-3 gluconase.*Eur J Plant Pathol.***126**, 497-508 (2010).

Manoj-Kumar, A., Sundaresha, S. & Rohini, S. Transgenic sunflower (*Helianthus annuus*L.) withenhanced resistance to a fungal pathogen *Alternariahelianthi*. *Transgenic Plant Journal*. **5**, 50-56 (2011).

Manoj-Kumar, A, Sundaresha, S. & Rohini, S. Resistance to *Alternaria*leaf spot disease in transgenic safflower (*Carthamustictorius*L.) harboring a rice chitinase gene. *Transgenic Plant Journal*. **3**, 113-118 (2009).

Manoj-Kumar, A., Rohini, S.,Kalpana-NanjaReddy, Prasad, T.G. &Udayakumar, M. Amenability of castor to an *Agrobacterium*-mediated *in planta* transformation strategy: Evidence for transformability using a *cry*1AcF gene for insect tolerance. *J. Crop Sci. Biotech.***14**, 125-132 (2011).

Keshamma, E., Rohini, S., Sankara-Rao, K., Madhusudhan, B. &Udayakumar, M. Tissue culture-independent *in planta* transformation strategy: an *Agrobacterium tumefaciens*-mediated gene transfer method to overcome recalcitrance in cotton (*Gossypiumhirsutum* L.). *Journal of Cotton Science***12**, 264-272 (2008).

Prashantkumar, S. H., Sashidhar, V.R., Sushma, M.A. & Rohini, S. Improvement of Salt Tolerance in Putatively Transgenic Rice Plants Overexpressing *AVP*1, a Vacuolar H+-Pyrophosphatase. *Transgenic Plant Journal***5**, 43-49 (2011).

Keshamma, E., Rohini, S., Manoj-Kumar, **A.**, Ananda-Kumar, P., Kumar, A.R.V., Madusudhan, B. &Udayakumar, M. A chimeric *cry1X* gene imparts resistance to *Spodopteralitura*(Fabricus) and *Helicoverpaarmigera*(Hubner) in transgenic groundnut. *EurAsian J Bioscien*. **2**, 53-65 (2008).

Manjunatha, S.B., Suma, T.C., Rohini, S., Devendra, R.,Udayakumar, M.& Prasad, T.G. Evaluation of advanced generation transgenic groundnut lines resistant to herbicide-glyphosate. *Indian Journal of Weed Science* **40**,162-165(2008).

Gowri N.M., Rohini, S., Kumar, A.R.V. &Gayatri, M.C. *Agrobacterium*-mediated *in planta* transformation of chickpea: utility of EPSPS as a selectable marker. *J. Plant Biol*. **36**, 55-63 (2008).

Sowmya, S., Rohini, S., Nagaveni, V., Ravikumar, K. &Sashidhar, V.R. Genetic engineering of rice for heavy metal stress tolerance: Initial physiological screening of diverse rice genotypes and stringent evaluation test (SET) of putative transformants. Journal of Plant Science Research **25**,21-37 (2009).

Cloning of plant promoters and their applications

Navin C Gupta, SR Bhat, Srinivasan and Pradeep K Jain*

ICAR-NRC on Plant Biotechnology, IARI Campus, New Delhi

Gene expression is regulated, both qualitatively and quantitatively, by promoter sequences. The promoter region is usually the key *cis*-acting regulatory region that controls the transcription of adjacent coding region(s) into messenger ribonucleic acid (mRNA). A typical eukaryotic promoter consists of a stretch of DNA sequence known as 'core promoter' and regulatory elements called proximal and distal elements, which in concert control the transcription of adjacent coding region.Based on the degree of expression, site of expression or depending upon the capability of a promoter to express under induction of external as well as inducible stimuli, promoters are broadly categorized into four types namely,i) constitutive promoters, ii) inducible promoters, iii) tissue specific promoters and iv) synthetic promoters.Skillful use of genetic engineering techniques depends upon our ability to make an organism produce a gene product at the required expression level, in specified cells or tissues at appropriate time. This can only be achieved by properly fusing appropriate promoter elements with the gene.

In plants the availability of the insertional mutagens like T-DNA and transposons have significantly aided the identification and isolation of genes and promoters of desirable interest. The insertional mutagens (T-DNA or transposon) containing reporter gene constructs, are classified based on their application in three basic forms namely, enhancer trap, promoter trap and gene trap. The major difference among such trapping systems lies in the use of chimeric gene construct of reporter gene and that are usually β-glucuronidase (*gus*) or green fluorescent protein (*gfp*) or luciferase (*luc*) with *cis*-acting regulatory element. Promoter trapping is the random insertion of a promoterless reporter gene in the genome followed by screening for reporter gene expression in tissue/condition-specific manner. This technique can be used for isolation of different types of promoter.Once a promoter is tagged with T-DNA and the reporter gene expression pattern specifies the promoter activity, the T-DNA tag becomes a potential target for cloning of the flanking sequence as promoter element. Several strategies are available to isolate and clone the T-DNA flanking genomic

DNA sequences. One of the following methods can be employed to clone the upstream sequence of the T-DNA insertion site consisting of the putative promoter elements:

i) Screening of the genomic DNA library constructed from the mutant plant

ii) Plasmid rescue

iii) Inverse PCR (IPCR)

iv)The thermal asymmetric interlaced PCR (TAIL-PCR)

v) Genome Walking

Arabidopsis, with its so many advantages, is an ideal source for mining of different plant promoters. We have developed promoter trap lines which have been very useful for screening and identification of different promoters. We have isolated and characterized several tissue specific promoters, namely anther-, trichome-, root-,lateral organ junction-specific and wound-responsive. The promoter region of Peroxidase gene, Fatty acyl-CoA reductase gene and Ethylene Response factor gene was involved in anther, wound and trichome-specific expression, respectively. Recently we have isolated and characterized nematode-responsive root-specific (NRRS) promoters which show preferential expression upon nematode infection, exclusively in the root in one case and in the galls in other. All these promoters have several applications and their appropriate utilization towards need based expression will be immensely useful.

Selected Publications/Patents:

PK Jain, AtulKakrana, Anil Kumar, Anil Sirohi and Srinivasan (2015) Polynucleotide fragments for directing expression of genes in plant roots in response to pathogens (complete patent application).2246/DEL/2015.

PK Jain, AtulKakrana, Anil Kumar, Anil Sirohi and Srinivasan (2015) Polynucleotide fragments for directing expression of genes in plant roots in response to pathogens and wounding (provisional patent application). 2245/DEL/2015.

Srinivasan, Vajindra, DhirajThakare, SR Bhat and PK Jain. (2009) Novel Trichome specific Promoter (complete patent application). (2251/DEL/2009).

Prasad, A. M., Sivanandan, C., Resminath, R., Thakare, D. R., Bhat, S. R., and Srinivasan (2005). Cloning and characterization of a pentatricopeptide protein encoding gene (LOJ) that is specifically expressed in lateral organ junctions in *Arabidopsis thaliana*. *Gene*,**353**: 67-79Sivanandan, C., Sujatha, T. P., Prasad, A.M., Resminath, R.,

Thakare, D. R., Bhat, S.R. and Srinivasan, R. (2005). Tagging and characterization of a cryptic root-specific promoter in *Arabidopsis*. *Biochim. Biophys. Acta.***1731**: 202-208.

Gupta, N. C., Jain, P. K., Bhat, S. R. and Srinivasan, R. (2012). Upstream sequence of fatty acyl-CoA reductase (FAR6) of Arabidopsis thaliana drives wound-inducible and stem-specific expression. *Plant Cell Rep*.**31**:839-850.

Kumar, V., Thakare, D. R., Saha, D. N. Jajoo, A., Jain, P. K., Bhat, S. R. and Srinivasan, R. (2012). Characterization of upstream sequences of the peroxidase gene, Atprx18 of *Arabidopsis thaliana*. *J. Plant Biochem. Biotech*. **21**(1):121–127.

Confirmation of Transgenic events by Polymerase Chain Reaction

Arul Prakash, Geetika C Gambhir and J. C. Padaria

ICAR-National Research Centre on Plant Biotechnology, New Delhi

Introduction

An organism which contains a gene or genes that have been artificially inserted instead of the organism aquiring them through reproduction is known as Transgenic organism. The inserted gene(s) are known as the transgene and method involved in gene transfer is called genetic transformation. This manipulation or alteration in genetic makeup of a plant genome by biotechnological approaches is scientifically termed as genetic engineering, also often called genetic modification. A genetically modified organism (GMO) is any organism whose genetic material has been altered using genetic engineering techniques (i.e. genetically engineered organism). Genes for desired traits may come from wide range of living organisms, either from a related species or from a completely different organism. It is very important to detect the presence of a transgene in a plant so as to confirm whether it is transgenic. It is possible to confirm the presence of any gene in a transgenic by the simple method of polymerase chain reaction. PCR (polymerase chain reaction): PCR (polymerase chain reaction) is a revolutionary technique in molecular biology that permits the detection of any short sequence of DNA (or RNA) even in samples containing only minute quantities of DNA or RNA. PCR is used to reproduce (amplify) selected sections of DNA or RNA for analysis. Kary Mullis was awarded the Nobel Prize in 1993 for inventing the polymerase chain reaction. PCR has now become a routine analytical tool for quickly analysing plant transformants for the presence of a transgene.

Principle of PCR

PCR is used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The PCR technique of amplification involves basic three steps:-

1. **Denaturation** of the double stranded DNA template at high temperature.
2. **Annealing** of the oligonucleotide primers to the DNA template at low temperature
3. **Extension** of annealed primer using *Taq* DNA polymerase.

These three steps form one cycle. Repeated cycles of heat Denaturation of template DNA, annealing of primers and extension of the annealed primers with polymerase results in the amplification of the template DNA defined by the 5' end of the two primers. The extension product of one primer serves as the template for the other primer and each cycle doubles the amount of DNA produced in the previous cycle and results in the exponential accumulation of the DNA template in several million folds in a short time. Finally the reaction product is visualized in agarose/Polyacrylamide gel after electrophoresis.

Components of PCR

There are various components required for PCR which are as follow:-

1. DNA Template: The segment of DNA to be amplified is called as template. When using DNA as the starting template, nanogram to microgram amounts of genomic DNA, or up to 20,000 target copies are chosen to start optimization trials. However, even very low levels of sample (i.e., mRNA, DNA from single cells or individual viral genomes) may be sufficient for PCR amplification.

2. DNA polymerase enzyme: A type of enzyme that synthesizes new strands of DNA complementary to the target sequence. DNA polymerase contains a polymerization dependent 5'-3' exonuclease activity. The first and most commonly used of these enzymes is *Taq* DNA polymerase (from *Thermis aquaticus*), whereas *Pfu* DNA polymerase (from *Pyrococcus furiosus*) is used widely because of its higher fidelity when copying DNA. DNA polymerase have two advantages, First it has a good activity rate around 75°C. Second, it should be able to withstand temperatures of 95-100°C so that more enzyme does not have to be added at the beginning of each new cycle. Apart from the thermostability, there are two aspects of a DNA polymerase that should be considered.

1. Processivity refers to the rate at which that polymerase enzyme makes the complementary copy of the template. The standard here is *Taq* polymerase, which has a processivity of 50-60 nucleotides (nt) per second at 72°C.

2. Fidelity is the accuracy of the complementary copy being made. *Taq* DNA polymerase has among the highest error rates of the thermophilic polymerases at 285 x 10^{-6} errors per template nucleotide because of absent of 3'-5' exonuclease (proof reading).

3. dNTPs - The four different deoxyribonucleotide triphosphates (dNTPs), adenine (A), guanine (G), cytosine (C), and thymine (T) are needed to provide the building blocks for DNA replication. DNA polymerase will add each complementary base to the new growing

DNA strand according to the original strand's sequence following normal A-T and C-G pairings.

4. Bivalent cations: Bivalent cations act as cofactor fr enzyme activity**.** Magnesium or manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be used for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis.

5. Buffer solution: maintains pH and ionic strength of the reaction solution and also contains monovalant (K^{+}or Na^{+}ion) or divalent (Mg^{2+}, Mn^{2+}) in it. It also contains additives such as detergent, PEG, glycerol, gelatine, DMSO etc.

6. **Primers:** The specificity of PCR depends upon the primers. The following factors are important in choosing effective primer

- Should be 17 to 30 bp in length.
- GC content is about 50% is ideal. For low GC content to choose a long primer.
- No complementary between two primers and should not be form primer-dimer.

Stages of PCR

Initialization step- For DNA polymerases that require heat activation by hot-start PCR. This step consists of heating the reaction to a temperature of 94–96 °C for 1–10 minutes.

Denaturation step- This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 30 sec–2 minutes. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Annealing step- The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3–5 °C below the Tm of the primers used. Stable DNA–DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.

Extension/elongation step- The temperature at this step depends on the DNA polymerase used; *Taq* polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to amplify.

Final elongation- This single step is occasionally performed at a temperature of 70–74 °C (this is the temperature needed for optimal activity for most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final hold- This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

Protocol for screening of transgenic using PCR

- Adjust the concentration of sample DNA to approx. 50 to 100ng/µl.

Component	Concentration	Final concentration	Volume per reaction (µl)
DNA sample		50-100ng	-
dNTP mix	25mM each	200µM	0.5
$MgCl_2$	25mM	1.5mM	1.5
10X buffer	10X	1X	2.5
Taq polymerase	5U/µl	1U	0.5
Primer (Forward)	100µM	20µM	0.5
Primer (Reverse)	100µM	20µM	0.5
Sterile water			-
Total volume			25µl

- Mix the reagents thoroughly and spin for few seconds to bring down the mix to the bottom of the PCR tube.
- Place the tubes on a Thermo cycler block for standard programme-

i. Step 1 : 94^{o}C for 5 min
ii. Step 2 : 94^{o}C for 1 min
iii. Step 3 : 58^{o}C for 30 sec
iv. Step 4 : 72^{o}C for 1 min

30 Cycles

v. Step 5 : 72^{o}C for 10 min
vi. Step 6 : 4^{o}C for ever

- After the completion of PCR programme the samples are mixed with 6X loading dye and checked in agarose gel for the desired gene product.

Different approaches of PCR application in Transformant analysis

PCR is a very useful technique which is used for various kinds of analysis in transformants. Some of these are mentioned below-

1. Confirmation and Screening of transgenic plants through PCR

Gene specific primers can be designed for detecting the presence of foreign gene in the DNA of transgenic plant. However, in case of endogenous gene (where the gene is already present in the non transgenic plant) the primers to be designed from marker/ reporter gene. However, one gene specific primer and other one from the marker/ reporter gene is also preferred in certain cases.

2. Expression of transgene in transgenic through PCR

The expression of the transgene can be done through RT-PCR (Reverse transcription Polymerase chain Reaction). For this RNA can be isolated from desired tissue at desired point of time.

3. PCR analysis for identification of transgenic events

Another important aspect of transgenic plants is the **EVENT**. Same gene can be integrated at different site of the genome, and each site of integration is known as an event. Transgenic plant can behave drastically different way from one event to another and hence identifying event of a transgenic plant is important. Inverse PCR and TAIL PCR (Thermal asymmetric interlaced PCR) is commonly used to know the insertion site of the T-DNA.

4. Transgene copy number estimation by PCR

Transgene copy number can greatly affect the expression level and genetic stability of the target gene, making estimation of transgene copy numbers an important area of genetically modified plant research. Molecular biological analysis of transgenic plants, like real time PCR is also used to determining the copy number in transformed plants.

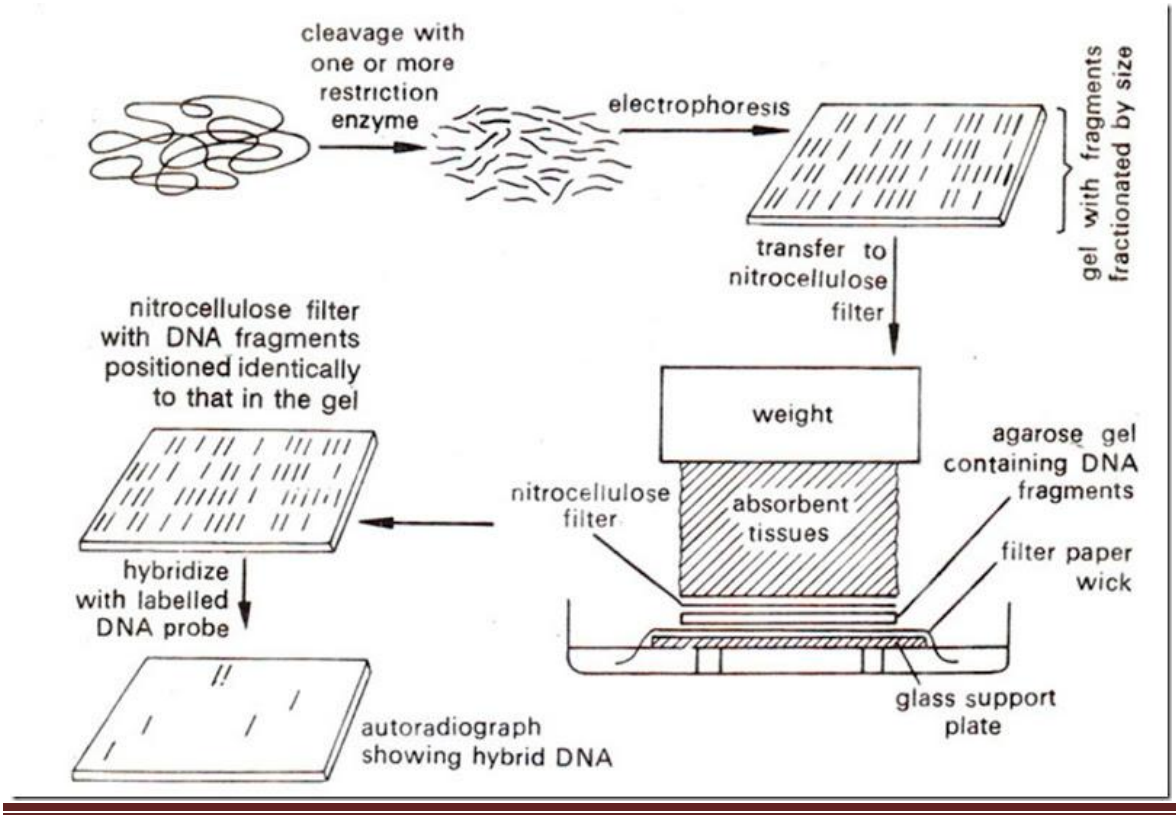
Southern and Northern Blotting

Rekha Kansal

ICAR-National Research Centre on Plant Biotechnology, New Delhi

Southern Blotting

Southern blotting is one of the central techniques in molecular biology and was developed by E.M.Southern in 1975. It is the capillary transfer of DNA fragments,from an electrophoresis gel to a nitrocellulose or nylon sheet, in such a way that theDNAbanding pattern present in the gel is reproduced on the membrane. During transfer or as a result of subsequent treatment,the DNA becomes immobilized on the membrane and can be used asa substrate for hybridization analysis with labeled DNA or RNA probes that specifically target individual restriction fragments in the blotted DNA. The ability to detect rare sequences in a complex population of restriction fragments paved the way for the cloning of eukaryotic genes, reverse genetics and modern biology. In essence,Southern blotting is therefore a method for 'detection of a specific restriction fragment against a background of many other restriction fragments.



Methodology:

1. Place the gel in a tray containing 0.2N HCl. Rock the tray gently for 20-30 minutes.

(Take enough of the acid solution to cover the gel completely and the tray should be rocked continuously.The acid treatment allows depurination to occur-the depurinated sites are cleaved during the alkali treatment and the smaller DNA fragments transfer more efficiently)

2. Decant the acid solution and rinse the gel with sterile water. Pour off water.

(The BromophenolBlue tracking dye changes colour from blue to yellow, indicating that the acid treatment is complete)

3. Add denaturation solution to the tray to cover the gel completely. Rock the tray gently for 20 minutes.

(The Bromophenol Blue tracking dye should once again turn blue, indicating that the deanaturation step is complete)

4. Decant the denaturation solution. Add neutralization solution and rock the tray gently for 30 minutes.

5. Accurately measure the length and width of the gel and cut one piece of membrane filter (nylon or nitrocellulose) such that it fits the gel nicely.

(Always use gloves when handling membranes and filters. Oils present on ungloved hands can get on the membranes and cause transfer artifacts)

6. Wet the cut filter in a tray of water for 1 min, then place filter into a tray of 20XSSC buffer.

7. Cut 3-5 sheets of Whatman 3MM paper of the size of the gel.

(This will result in 1-2 cm stack of cut paper)

8. Prepare a wick by cutting one piece of Whatman 3MM paper which is 2 cm bigger than the width and length of the gel.

9. Place about 700-800 ml of 20XSSC in a large tray. Wet the wick thoroughly in the 20XSSC. Put a glass tray over the rubber corks and place the wick on plate with the ends of the wick hanging over plate into the 20XSSC buffer.

(Remove air bubbles trapped between the wick and glass plate by rolling a pencil back and forth over the wick)

10. Lift the gel out of neutralization solution and allow most of the liquid to drip off the gel and place on top of the Whatman 3MM wick. Remoe air bubbles trapped between the gel and the wick as above.

11. Remove the membrane filter from the tray of 20XSSC and lay it on the top of the gel. Remove trapped air bubbles as above.

(Make sure that the membrane does not overhang the gel. Also it is important to lay the filterdown precisely the first time, as detectable transfer can take place almost immediately)

12. Wet one piece of the cut Whatman 3MM paper in 20XSSC and place it on the top of membrane filter. Place the stack of cut Whatman 3MM papers on top of the first piece of 3MM paper. Put a 3-5 cm stack of filter papers or paper towelson top of the 3MM paper.

13. Keep the entire pyramid pressed together. Place a glass plate on top and weight of 500gm on the glass plate.

14. Cover up the ends of the tray with plastic wrap to minimize evaporation during the transfer. Allow the transfer to proceed for overnight or 16-18 hrs.

15. After the transfer is complete, take apart the pyramid so that the membrane is still lying on the gel. With a lead pencil mark on the filter the location of the slot.

16. Using blunt forceps remove the membrane and place it in atray of 2XSSC for 5 min to wash away excess salts. Blot the membrane dry on a piece of 3MM paper and bake at 80°C for 2 hrs.

(The transfer can be checked and documented by restaining and photographing the gel. Little or no DNA should be retained on the gel, if the transfer is performed properly.

Immobilization of the DNA transferred to membrane can also be done by cross linking for 1 min at 990 Jls in the UV cross linker)

Prehybridizing, hybridizing and washing the filter

1. Transfer the membrane with the help of forceps into the hybridization bottles.

2. Add 15 ml pre-hybridization buffer (Church and Gilbert buffer) pre-warmed at 65^0C into the hybridization bottle and wet the membrane thoroughly

3. Incubate the membrane with prehybridization buffer at slow speed for 2hrsat 65^0C.

Composition of CHURCH and GILBERT BUFFER

Phosphate buffer (pH – 7.2) : 0.5 M

SDS : 7% (w/v)

EDTA :10mM

Preparation of probe

4. The DNA of the gene of interest is used to make the probe

5. Following components are mixed in a microcentrifuge tube:

DNA Template (100 ng) : x µl

dNTPS in 5X reaction buffer : 10 µl

Nuclease free water : up to 40 µl

6. Vortex the tube,spin down the components,incubate the tube in a boiling water bath for 5-10 min and cool on ice. Spin down quickly.

7. Based on choice of labeled triphosphate (dATP or dCTP), use mix A or mix C respectively

Add the following components in the same tube:

Mix C :3µl

[α^{-32P}]-dCTP : 2µl

Klenow fragment : 1µl

Nuclease Free Water : 4 µl

8. Shake the tube and spin down the contents for 3-5 sec.

9. Incubate for 5 min. at 37 ^{0}C

10. Add 4 µl of dNTP mix and incubate for 5 min. at 37^{0}C

11. Incubate the tube in boiling water bath for 5 min.

Mixing of probe with hybridization buffer

12. Take the microcentrifuge tube behind the shield.

13. Change the pre-hybridization buffer of hybridization bottles.

(Add only the pre warmed buffer)

14. Add probe directly into the solution with pipette. Mix it well and incubate overnight at slow speed at 65^{0}C.

Washing of probe:

15. The next morning discard the hybridization buffer and probe solution.

16. Add 2X SSC + 0.1 % SDS to the hybridizations bottles. Incubate at medium speed for 5 min. at 65 ^{0}C.

17. Repeat the above step with 2X SSC + 0.1 % SDS. Discard the solution.

18. Add 1 X SSC + 0.1 % SDS to the hybridizations bottles and incubate at medium speed for 10 min at 65^{0}C. Repeat this step once more.

19. Add 0.1 X SSC + 0.1 % SDS to the hybridizations vials and incubate at medium speed for 15 min at 65^{0}C.

20. Take out membrane on a clean glass plate and check the radioactive count. The count should be between 40-100 cpm (counts per min.)

21. If count is greater than 100 cpm, then, the membrane is again washed with 0.1 X SSC + 0.1 % SDS

Wrapping of membrane and x-ray film exposure

22. Cut sufficient cling film so as to wrap a membrane tightly. Put some of the solution 0.1 X SSC + 0.1 % SDS on the cling film and place the membrane onto the film. Fold the edges of film tightly so as to avoid leaking of the internal solution.

23. Place the membrane in a cassette and expose an X-ray sheet in the dark room

24. Keep it overnight at -70^{0}C.

25. Next morning, thaw the cassette for atleast 2 hours. Open the cassette and remove the x-ray film in dark room

26. Put the X-ray film in developer till bands are just visible and quickly wash it water for 6-7 sec and immediately put in fixer for 5-10 min.

27. Wash in sufficient running water for atleast 10 min

28. Hang the X-ray film and let it dry

Materials required for southern blotting

1) **Tris - cl (1M, pH - 8.0):100 ml**

   Weigh 12.11 g of Tris base and dissolve in 80 ml of autoclaved double distilled water. Adjust the pH to 8.0 with concentrated HCL and make up the volume to 100 ml with autoclaved double distilled water.

2) **EDTA (0.5 M, pH – 8.0): 100 ml**

   Weigh 18.61 g of Disodium dihydrate EDTA and add 80 ml of autoclaved double distilled water. Adjust the pH to 8.0 with NaOH pellets. EDTA salt will not dissolve until the pH of the solution is 8.0. Make up the volume to 100 ml with autoclaved double distilled water.

3) **NaCl (5.0 M) : 100 ml**

Weigh NaCl as per requirement and dissolve in 80 ml of autoclaved double distilled water. Make up the volume to 100 ml with autoclaved double distilled water

4) **Depurination solution:**

Add 11 ml HCl to 989 ml of autoclaved distilled water. Store at room temperature

5) **Denaturation solution:**

Add 87.66 g NaCl and 20 g NaOH to 800 ml autoclaved distilled water. Mix to dissolve and make up the volume to 1000ml.

6) **Neutralization solution:**

Add 87.66g NaCl and 60.5g Tris base to 800 ml autoclaved distilled water. Mix to dissolve. Adjust the pH to 7.5 with concentrated HCl and make up the volume to 1000ml.

7) **Nucleic acid transfer buffer (20X SSC):**

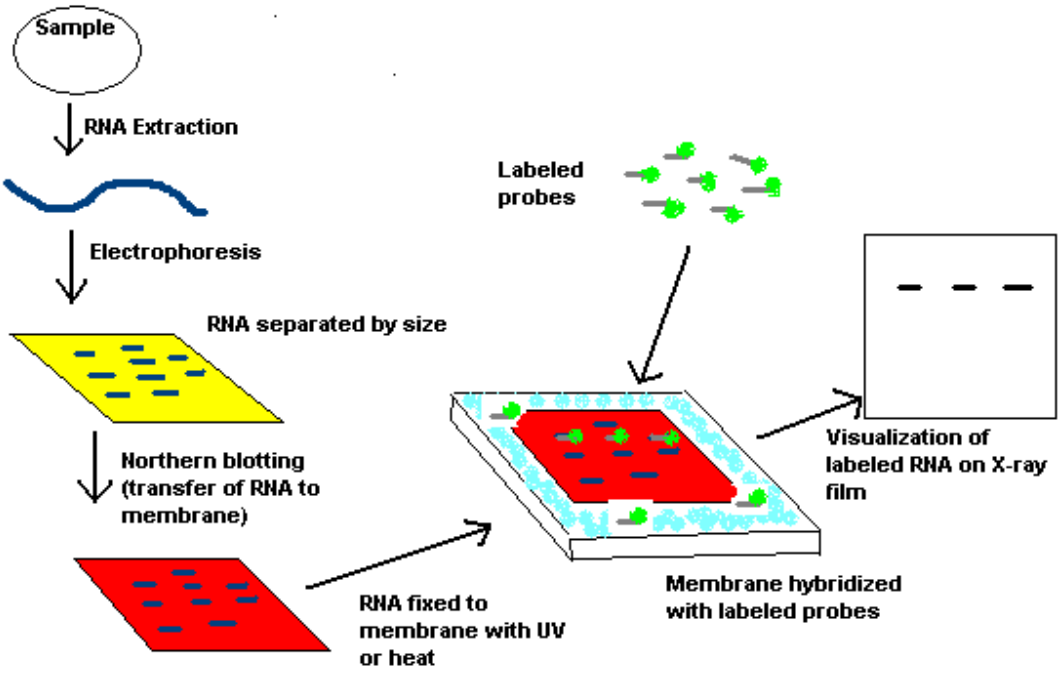
Add 88.23g Tri-Sodium Citrate and 175.32g NaCl to 800 ml autoclaved distilled water. Mix to dissolve. Adjust the pH to 7-8 and make up the volume to 1000ml.

Northern Blotting

A northern blot is a laboratory method used to detect specific RNA molecules among a mixture of RNA. The technique was developed by Alwine and his colleagues in 1979. It is used to analyze a sample of RNA from a particular tissue or cell type in order to measure the RNA expression of particular genes. This method was named for its similarity to the technique known as a Southern blot. The hybridization of RNA is the process of forming a double stranded DNA-RNA hybrid molecule between a single stranded DNA molecule and single stranded target RNA.

The first step in a northern blot is to denature or separate, the RNA within the sample into single strands. This step ensures that the strands are unfolded and that there is no bonding between strands. The RNA molecules are then separated according to their sizes using agarose gel electrophoresis. Following separation, the RNA is transferred from the gel onto a blotting membrane. Once the transfer is complete, the blotting membrane carries all of the RNA bands originally on the gel. Next, the membrane is treated with a small piece of DNA or RNA called a probe, which has been designed to have a sequence that is complementary to a particular RNA sequence in the sample; this allows the probe to hybridize, or bind, to a

specific RNA fragment on the membrane. In addition, the probe has a label, which is typically a radioactive atom or a fluorescent dye. Thus, following hybridization, the probe permits the RNA molecule of interest to be detected from among the many different RNA molecules on the membrane.



Transfer of RNA to Membrane filter

1. After the electrophoresis is complete, carefully transfer the gel in a large tray and rinse several times with water to remove formaldehyde.

2. Decant completely and add 500 ml of 20XSSC buffer to the tray. Place the tray on a rocker and shake gently for about 45 min.

3. Accurately measure the length and width of the gel and cut one piece of membrane filter (nylon or nitrocellulose) such that it fits the gel nicely.

4. Rinse the membrane in distilled water for 20 minutes and keep it in 20X SSC at least for 10 minutes before use.

5. The blotting procedure is the same as done for Southern blotting.

Prehybridizing, hybridizing and washing the filter

6. Place the baked filter in a hybridization bottle and add Northern prehybridization solution.

(The prehybridization solution should be added so that the membrane gets completely wet)

7. Prehybridize overnight at the desired temperature.

(The prehybridization and hybridization temperatures will generally fall between 37° and 42°C. The formamide used in the buffer lowers the Tm of the nucleic acids)

8. Prepare the radiolabelled probe by Nick translation method or random primer labelling method. Boil the probe for 5min and add to the Northern hybridization solution.

9. Hybridize overnight at the same temperature.

10. Next day open the bottle and wash the filter with the following solutions in the given order till background counts are reduced to 2-5 counts per second as detected by monitor (like Mini-Monitor GM tube).

1) 5X SSC and 0.1 % SDS for 10 min at room temperature
2) 2 X SSC and 0.1 % SDS for 10 min at room temperature and
3) 0.1X SSC and 0.1 % SDS for 20 minutes at 42 ^{0}C

11. Wrap the blots in plastic wrap and drain the excess solution by rolling a pipette on the blots.

12. Expose the blots to X-ray films (like Kodak Biomax MR) in cassettes with intensifying screen at -80^{0}C. Develop the autoradiogram after 3-7 days depending on the counts.

Solutions/materials for northern blotting:

1. SSC (Saline Sodium Citrate) Buffer

3.0 M NaCl and 0.3 M sodium citrate at pH 7.0. 20X SSC is used as a blotting buffer to facilitate transfer of nucleic acids to membranes in Southern and northern blotting, dot blots, and colony lifts; as a buffer for hybridization; or as a post-hybridization wash buffer.

2. Denhardt's Solution

Denhardt's solution is a mixture of high-molecular weight polymers capable of saturating non-specific binding sites and artificially increasing the concentration of available probe.

Denhardt's solution is 100-fold concentrated and should be diluted to 50 fold with distilled water.

BSA fraction V	20 g/l
Ficoll® 400	20 g/l
Polyvinylpyrrolidone	20 g/l
pH (25°C)	7 ±0.2
RNase, DNase, Protease free	

3. Formamide

A destabilizer, formamide lowers the melting temperature of hybrids thus increasing the stringency of the probe to target binding.

4. Dextran sulfate

Its presence accelerates the rate of hybridization by 10 fold. The addition of Dextran Sulfate favours the formation of probe networks, resulting in greater sensitivity during membrane-bound nucleic acid analysis.

5. Nylon membrane

Nylon membranes bind nucleic acids irreversibly and are far more durable than nitrocellulose filters. Immobilized nucleic acids can therefore be hybridized sequentially to several different probes.

6. Herring Sperm DNA

The herring sperm DNA is buffered in TE, pH 8.0, and then mechanically sheared through a small gauge needle. Add herring sperm DNA to the prehybridization or hybridization cocktail to a final concentration of 250 µg/ml.

7. BioMax MR film

BioMax® MR-1 is a single emulsion film that provides maximum resolution and sensitivity for detection of ^{35}S-, ^{33}P-, and ^{14}C-labelled samples.

References:

Ausubel, F.M., Bent, R.Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K.(1987). Current Protocols in Molecular Biology, Green Publishing Associates and Wiley-Interscience.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: A Laboratory Manual, Cold Spring Harbar Laboratory Press, New York

RNA interference and genome editing

Debasis Pattanayak

ICAR-National Research Centre on Plant Biotechnology

Pusa Campus, New Delhi -12

Email: debasispattanayak@yahoo.co.in

Small non-coding RNA-mediated gene silencing pathways, collectively called RNA interference (RNAi), are involved in regulation of endogenous gene expression and plant defense. It is manifested through two broad classes of small non-coding regulatory RNAs, small interfering RNA (siRNA) and micro RNA (miRNA). siRNAs, generated from cleavage of long hairpin RNA by RNase III-class endonuclease, Dicer-like, mediate transcriptional or post-transcriptional gene silencing. At transcriptional level, 24 nt long siRNAs guide an effector complex for DNA methylation, which leads to heterochromatinization of target loci and consequently transcriptional silencing. At post-transcriptional level, a different size class of 21 nt long siRNAs guides a silencing complex, called RISC, for cleavage of target mRNA. *cis*-acting siRNAs are involved in plant defense against viruses and transposons, and *trans*-acting siRNAs regulate endogenous genes involved in plant growth. miRNAs are generated from processing of imperfect stem-loop RNA precursors by Dicer-like. They regulate plant growth and adaptive stress responses by either degradation or translational repression of target mRNAs. The field of RNAi has provided deep insights into the process occurring at the cellular level. Small RNA-mediated gene expression interference technologies herald a new era in medicine and plant biology.

Genome editing or the ability to make specific changes at targeted genomic sites employing programmable DNA binding proteins (PDPs) ushered a new horizon in biology. Designer nuclease, nuclease domain fused with DNA binding domain, creates double-strand breaks (DSBs) at predefined DNA sequences. DSBs are repaired either by homologous recombination (HR), or, in the absence of a homologous repair template, via non-homologous end joining (NHEJ). NHEJ causes small insertions or deletions as the broken ends are pieced back together. This proclivity for indel generation is exploited as a convenient method for knocking genes out. A numbers of tools, like meganuclease, ZFN (Zinc-Finger Nuclease), TALEN (Transcription Activator-Like Effector Nucleases) and CRISPR-Cas (Clustered,

Regularly Interspaced, Short Palindromic Repeats-CRISPR Associated endonuclease) are now available for genome editing. Meganucleases are endodeoxyribonucleases characterized by a large recognition site that can be used to replace, eliminate or modify sequences in a highly targeted way through protein engineering. In case of ZFN and TALEN, engineered ZFs and TALEs, respectively, are fused with nuclease domain of Fok1 edonuclease. CRISPR-Cas is part of an adaptable bacterial immune system that mediates cleavage of foreign viral DNA and plasmids. Short fragments of such foreign sequences, termed protospacers, integrate into the clustered regularly interspaced short palindromic repeat (CRISPR) locus of the bacterial genome. Transcribed CRISPR RNAs (crRNAs) bind to auxiliary trans-activating crRNAs (tracrRNA) and these RNA hybrids direct sequence specificity of the Cas endonuclease. In Cas9-based designer nucleases, the specificity is mediated by a single guide RNA (gRNA) that mimics the natural crRNA–tracrRNA hybrid. In such RNA-guided endonucleases (RGENs), reprogramming of DNA specificity requires no changes in the Cas9 protein, but only in the recombinant gRNA. Genome editing tools, especially CRISPR-Cas9, are increasingly being used in crop improvement.

हर कदम, हर डगर
किसानों का हमसफर
भारतीय कृषि अनुसंधान परिषद

Agrisearch with a human touch

Designed & Printed by:
SANSHU ENTERPRISES: 09810384501