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Alien Gene Introgression in Crop Improvement – New Insights

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ABSTRACT

Alien genes have contributed several traits in the crop plants which are not available in the cultivated background. These have helped plant breeders in creating newer genetic diversity, thereby providing additional avenues of selection of better plant types. Vertical and horizontal transfer of alien genes has changed the fate of several crops by imparting resistance to diseases and insect-pests, tolerance to abiotic stresses[22]. Several challenges such as pre- and post fertilization barriers in distant crosses, problems in normal chromosome pairing, linkage drag, pleiotropic effects and role of recipient genome background on the expression of introgressed alien gene(s) and in HGT, erratic regeneration protocols, difficulty in isolation of genes from wild species and their expression in recipient plants, and possibilities of gene flow pose significant challenges to make alien gene transfer a routine process across all crop species[7]. However, recent advances in sequencing and genotyping technologies have made it possible to develop molecular markers as well as undertake genotyping at large scale in both major as well as minor (or so called orphan crop species) that can be used not only for developing high density genetic and physical maps but also for generating transcriptome or sequence data. In parallel, the high-throughput sequencing and genotyping approaches can be used to detect genetic variation existed in germplasm collection not only in cultivated gene pool but also in landraces and wild species. Furthermore, the QTLs or genes or superior alleles for the trait of interest identified through linkage mapping, association mapping, AB-QTL approach can be introgressed or pyramided in elite varieties or genotype of interest by using MAGIC, MABC, MARS or GWS approaches.

Key words: *Introgression, Genotyping, Fertilization, QTL*

INTRODUCTION

The twentieth century has witnessed tremendous improvement in global crop production. Besides several factors such as increase in cultivated area, improved agronomic practices, increased use of plant protection measures and better crop management, improved varieties of crop plants have played a dramatic role in improving the productivity of different crops. The genetically improved crop cultivars have been developed through modern plant breeding by introducing improved alleles at existing loci through conventional hybridization, of late, aided by molecular marker technology and genetic transformation.

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The aim of all these techniques has been either exchange of genes between sympatric or neighbouring populations of crops and related taxa or transfer of genes from related taxa into the cultivated gene pool of a crop. This led to the development of numerous improved cultivars with high yield, stress resistance and superior agronomic performance. In nature, gene transfer from one population to another is slow as compared to man-made systems, whereas it is faster and often mediated by hybridization followed by a number of backcrossings and rigorous phenotypic selections.

The influence of alien gene transfer on the way the fate of a crop can be changed was first demonstrated as early as in 1956 when a small segment from *Aegilops umbellulata* Zhuk. carrying a gene for resistance to leaf rust was translocated onto the wheat chromosome 6B[40]. This revolutionary work ushered an era of utilization of wild genetic resources for the improvement of crop plants. Consequently, interspecific and intergeneric hybridization have been widely adopted by plant breeders across different crops and used to develop improved cultivars with enhanced agronomic performance, resistance to biotic and abiotic stresses, and quality improvement. However, the success in utilizing wild genetic resources for transfer of desirable genes has not been uniform across all crop species since alien gene transfer largely depends upon availability of such resources, ease of hybridization and expression of the trait of interest in the progeny. Owing to these requirements, routine alien gene introgression from wild species is still a challenge before scientists for harnessing the desirable genes of wild species across all species. While evaluation of wild relatives and identification of genes conferring traits of interest themselves pose greatest challenges to breeders, effecting successful hybridization and obtaining a viable progeny with the gene(s) of interest transferred into them is further a difficult task. For involving wild species in hybridization, these need to be available in the vicinity of the recipient parent and their flowering must synchronize with that of the cultivated species. Raising wild species and exotic germplasm in field condition is often not easy and development of controlled conditions such as glass houses and plant growth chambers requires huge investments on time and money.

Alien gene introgression is sometimes associated with several other difficulties such as linkage drag and pleiotropic effects; while in some instances this is associated with some unforeseen advantages also such as development of chromosome elimination technique for doubled haploidy breeding in wheat and barley. Advancements of in vitro techniques, in vivo and in vitro hormonal manipulations, techniques such as somatic hybridization and protoplast fusion and the most recent development of cisgenesis and intragenesis have offered commendable opportunities towards alien gene introgression.

Need for alien gene transfer

Genetic variation is essential for developing new plant varieties, and this can be created by introducing genes from a related species, sometimes from a relatively distant species or even an unrelated species. The need for gene transfer in a crop species depends upon the extant genetic variability in that crop as well as availability of a trait of interest in the donor in intense form. In most of the cultivated crop species, limited popular and high yielding varieties are grown over wide areas and these are often derived from a relatively narrow representation of gene pool, mostly from the primary gene pool, and therefore these have a narrow genetic base and limited genetic buffer. Making selections for desired traits such as non-shattering habit, uniform maturity, improved seed fertility, seed dormancy, increased seed number, increase in seed and fruit size, modified plant architecture, and conversion from perennial to annual forms during the process of crop domestication led to a gradual loss in genetic diversity[46]. This reduction or loss in genetic diversity during crop domestication could be attributed to (i) selection by human beings for desirable "domestication related traits", (ii) genetic drift

in the form of "domestication bottlenecks" (Eyre-Walker *et al*. 1998), and (iii) modern plant breeding practices that resulted in the development of high yielding and uniform crop varieties. This reduction in diversity has been more prominent in self-pollinated crops like wheat, where the level of genetic variation in cultivated pool has often been reported to drops below 5 % of that available in nature. It makes crops more vulnerable to biotic and abiotic stresses. This may also result in huge losses in yield and quality as observed previously by the attack of shoot fl y and Karnal bunt in India and the Southern corn leaf blight in the United States[46]. Moreover, it reduces chances to identify new and useful gene combinations for crop improvement. In this way, modern plant breeding although increased crop productivity worldwide, it also eroded the genetic variability of the crops. Consequently, our major crop species represent the relatively few species that were selected by our ancestors from a multitude of extant species, and the resulting narrow germplasm forms the basis of modern monoculture in many areas of the world[22]. This makes them fragile to global climate change and vulnerable to new races of pathogens and insect pests. Due to narrow genetic variability, options to execute selection for desirable plant types also become limited.

To overcome these concerns and for further genetic improvement in crops plants, the natural variation available in wild relatives, landraces, and primitive cultivars of the crop species is required to be harnessed for a rapid and sustainable improvement of crop species for many years[46]. Wild species are a rich reservoir of useful alien genes which are no longer available within the cultivated gene pool[46]. Since these species have had much longer time and increased opportunities to evolve and adapt to natural environments, therefore, these often have genes for resistance to diseases and insect pests and for tolerance to drought, temperature stress, salinity and other extreme environmental conditions. Further, they have wide genetic buffers to withstand unexpected adversities.

3. Sources of alien genes and their characterization

The genes present in distant relatives (i.e., wild species) are usually known as alien genes. Therefore, these species are important source of such alien genes in crop plants. Among these species, phylogenetic relationships have been established on the basis of crossability of cultivated species with the wild species and other cytological and molecular analysis. This has led to characterization of wild species into primary, secondary, and tertiary gene pools according to the gene pool concept of Harlan and De Wet[24]. This gene pool concept provides the knowledge regarding the possibilities of transferring alien genes controlling desirable traits from wild species either through conventional crossing or by using the advanced modern technologies. In general, species within the primary gene pool are easily crossable with each other and hence have been used easily for transfer of alien genes. Although wild species belonging to secondary gene pool may also cross readily with cultivated species, some post-zygotic barriers restrict their use in alien gene transfer. However, recent advances in tissue culture techniques have made it feasible to use the species of this group. Wild species of tertiary gene pool are not found cross compatible with cultivated species. A large proportion of wild species belongs to this group and consequently is of no use for crop improvement through sexual manipulations.

4. Importance of alien gene introgression

Genetic variation of crop plants is continuously decreasing due to domestication and modern plant breeding practices. This has although resulted in development of high yielding, uniform crop varieties, but it has happened largely at the cost of extinction of primitive ancestors. Consequently, these uniform and high yielding varieties become more vulnerable to attacks by diseases and insect-pests leading to heavy losses and in some cases, to near extinction of a crop.

4.1 Grassy Stunt Virus (GSV) epidemics in Rice

During the early 1970s, before the release of resistant rice cultivars in 1974, GSV epidemics destroyed more than 116,000 ha (287,000 acres) of rice in Indonesia, India, Sri-Lanka, Vietnam, and Philippines. After this, screening of ~17,000 cultivated and wild rice lines for resistance to GSV for 4 years led to the identification of a population of *Oryza nivara* growing wild near Gonda in Uttar Pradesh, India and showing resistant to GSV. This resistance in *O. nivara* for " *grassy-stunt virus strain 1* " was governed by a single resistance gene. This gene was transferred from *O. nivara* into cultivated rice and it is believed that GSV resistant hybrids containing the wild Indian gene are grown across 110,000 km^2 of Asian rice fields. This is one of the most important examples showing how wild relatives of crop plants came to the rescue of cultivated crops and thus prevented massive crop failure and famine.

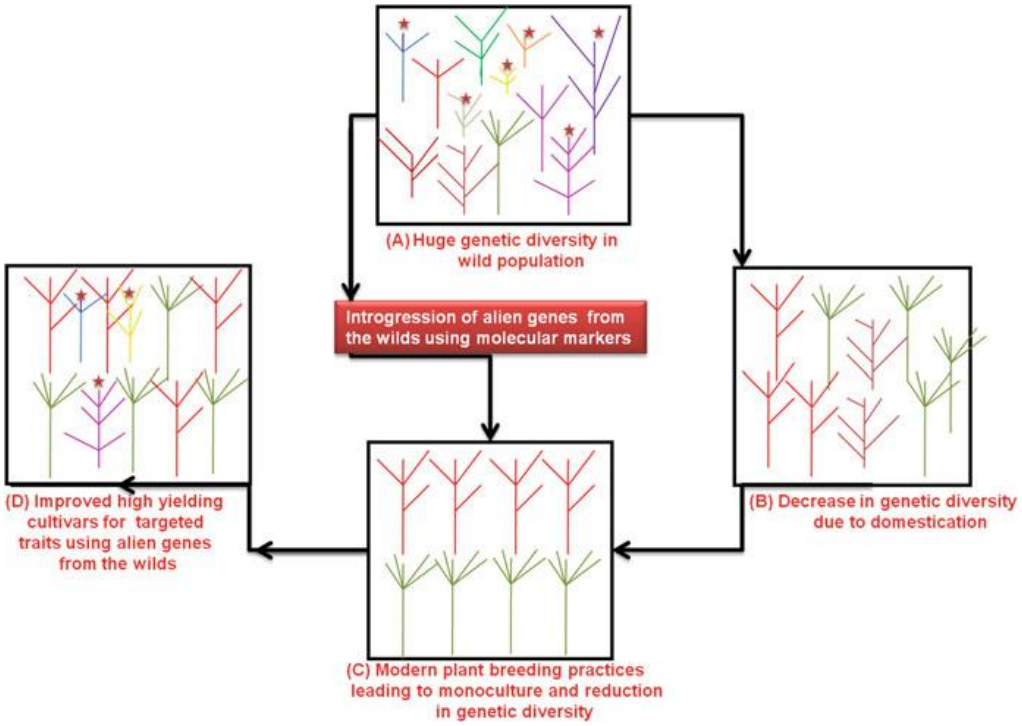

Fig 1. Diagrammatic representation of loss of genetic diversity in crop species (A) due to domestication, (B) modern plant breeding practices, (C) introgression of alien genes from the wild species to improve modern uniform crop varieties (D) for a variety of traits. The Symbol (*asterisk*) indicates the novel genetic variation that has not been selected during domestication and due to modern plants breeding practices, and these novel alleles are now being introgressed from the wild species into elite cultivars with the help of molecular markers to improve them for biotic and abiotic stresses.

Challenges in alien gene transfer

Alien gene introgression has opened new ways and opportunities in creating additional genetic variability and providing newer avenues of useful selection in crop plants besides helping in evolution of the crop species. While developments in hybridization strategies and advancement of in vitro techniques have made alien gene introgression in cultivated species easier, certain challenges are still there which make alien gene introgression a routine practice a bit difficult in plant breeding. There are two ways to transfer the alien gene(s) into cultivated species: transferring alien gene from cross-compatible wild species through hybridization (vertical gene transfer)

and transfer of gene(s) from other sources as well as cross-incompatible wild species through genetic transformation and somatic hybridization (horizontal gene transfer).

5.1. Vertical Gene Transfer

5.1.1 Crossability barriers

Wild species are an important reservoir of useful genes. However their use, especially of those species belonging to the tertiary gene pool, has been limited for transferring the useful genes due to crossability inhibition and limited recombination between chromosomes of wild and cultivated species[6,27,43]. These crossability barriers developed during the process of speciation frustrate breeders' efforts in successful hybridization between species of different gene pools. The pre-fertilization cross incompatibility between parent species arises when pollen grains do not germinate, the pollen tube does not reach ovary, or the male gametes do not fuse with female gametes[13,42].

5.1.2 Chromosome Pairing

Pairing of chromosomes of wild species with the cultivated species in their hybrids is the key to transfer of gene(s) across species. Genetic control of pairing of chromosomes derived from two different genomes has been identified in wheat, where this gene is known as *Ph1*. The suppressing of the *Ph1* pairing regulation of polyploidy wheats and oat has resulted in desired chromosome pairing and hence alien gene transfers into these crop species[25]. Such cytogenetic manipulations, including the suppression of the *Ph1* system, for recombining desirable alien chromatin into wheat were termed as chromosome engineering[41]. Essentially similar cytogenetic manipulations affecting gene transfer can also be done in hexaploid oats[26]. In rice, very limited chromosome pairing has been observed at metaphase I in F 1 hybrids of cultivated and wild species. Therefore, it has been difficult to transfer the alien genes from wild species to cultivated species[7].

5.1.3 Linkage Drag

One of biggest challenges in using wild species for introgression of alien genes into cultivated background is the association of undesirable genes with the useful alien genes, known as linkage drag. Its effect is more severe in crops with diploid genetic systems because their genomes are more sensitive to genetic imbalance compared to relatively more buffered polyploid genomes. This has resulted in exploitation of only a few exotic genes in alien germplasm in agriculture[17]. In wheat, for example, genes other than the targeted gene (e.g., *Sr39* transferred from wild species ' *Aegilops speltoides Tauschii'*) was carried on the alien chromatin[49] during introgression, which had a deleterious effect on yield and quality[47,32,31]. Therefore, it became important to eliminate the excess *Aegilopes speltoides* chromatin surrounding *Sr39* in order to make this gene useful for fighting against *Ug99*[37].

5.1.4 Background effects

It has been observed that there is a problem of variable expression of introgressed alien genes in cultivated backgrounds. In wheat, Chinese spring and *Leymus racemosus* translocated chromosomes carrying genes for resistance to *Fusarium* Head blight have been transferred to different common wheat backgrounds. However, expression of resistant gene was observed to be uniform among the resultant lines. These results demonstrated that effects of genetic backgrounds on the expression of alien resistance genes in wheat were due to epistatic interactions[10]. Therefore, it is evident that efficient manipulation of alien chromatin and selection of proper recipient genotypes play a crucial role in the success of alien introgression for Fusarium head blight resistance[44,18].

5.1.5 Pleiotropic effects of Alien Genes

Sometimes introgressed alien genes affect more than one trait. If such effects are positively associated with desirable traits, introgression of alien genes with pleiotropic effects can be useful. However, if these are associated with undesirable traits, it becomes a challenge for breeders to use them in crop improvement. For example in wheat, introgression of leaf rust resistance gene *Lr47* (from *Triticum speltoides*) led to an overall reduction of 3.8 % in grain yield; nevertheless it varied significantly across genotypes and environments. At the same time, lines with the alien *Lr47*segment showed consistent increase in grain and fl our protein concentration, while there was significant decrease in fl our yield and an increase in fl our ash[8]. Similarly, the

slow rusting genes in wheat often have pleiotropic effects on multiple rust diseases. Cloning of the well-characterized pleiotropic resistance gene *Lr34/Yr18/Sr57/Pm38/Sb1/Bdv1/Ltn1* showed that it belonged to the ABC transporter group and was distinct from cloned race-specific resistance genes[29].

5.2 Horizontal Gene Transfer (HGT)

5.2.1 Regeneration Protocol

Horizontal transfer of alien genes across genera requires suitable regeneration system for the development of transgenic plants. This is one of the major challenges, especially in recalcitrant species such as food legumes that restrict transfer of alien genes through genetic transformation (i.e., transgenics). Tissue culture techniques are part of a large group of strategies and range from molecular genetics to recombinant DNA studies, genome characterization, gene-transfer and in vitro regeneration of plants. All these tools require totipotent tissues that readily respond to tissue culture procedures. In most of the species, in vitro regeneration is highly genotype specific and cultivated varieties are rarely amenable to regeneration. Additionally, morphogenesis is generally very slow and very often there are problems like development of albinos and vitreous tissues, and no response in dedifferentiated calli. Therefore, successful and reliable plant regeneration in many crop species still remains an aspiration that requires considerable refinement in technology and training of the human resources to develop the skills that are needed to generate green plants.

5.2.2 Isolation of genes from wild species

Desirable genes present in the background of wild species belonging to primary gene pool have been exploited in development of improved varieties in several crops. These can be isolated through map-based cloning. However introgressions are accompanied by linkage drag where recombination is suppressed in the target gene region, and standard recombination-based approaches cannot be used in the molecular dissection of the target genes[22]. In addition to this isolation of desirable genes from species belonging to secondary and tertiary gene pools is still difficult through map-based cloning. Although the wild species are good genetic resources for genes controlling resistance to biotic and abiotic stresses as well as quality traits, these could not be used extensively in breeding programs in either way.

5.2.3 Expression of alien genes

HGT trough genetic transformation is one of the most exciting approaches that opened practical opportunities for the improvement of crop plant without any limitation of genome boundaries. However, the unpredictable silencing or variable expression of transgenes is a ubiquitous phenomenon and it is an important challenge for genetic engineering of crops. This has been observed invariably in all plant species studied[12]. There is not yet a reliable way to prevent silencing, although the converse affects consistent gene silencing has been reported[3]. Silencing resulting from interactions among multiple copies of transgenes and related endogenous genes involves homology based mechanisms that act at either the transcriptional or post-transcriptional level[35]. It has been shown that high level expression of foreign proteins in plants often leads to gene silencing[39].

5.2.4 Gene Flow

Flow of alien transgenes from transgenic plants to their weedy and wild relatives through sexual reproduction and/or vegetative propagation is one of major concerns with potential ecological risks. Gene flow from the transgenic plants having resistance to diseases and insect-pests, drought and salt tolerance, and herbicide resistance can significantly enhance the ecological fitness of weedy and wild populations. As results, they can become aggressive weeds that can have unpredictable consequences to local ecosystems. This gene flow can also change the original wild populations and better ecological fitness could even lead to the extinction of endangered wild species populations locally[28]. Therefore, alien gene transfer through genetic transformation has a challenge of its negative impact of present ecological system.

6. New genetic approaches for harnessing the natural variation

The domestication of the plant species for food, fodder or any commercial purpose for mankind is one of the very ancient practices. However, while carrying out domestication or breeding of any crop species, the gene pool has been narrowed with number of alleles (46). Therefore, in general, breeders work with a limited number of alleles available in the cultivated gene pool and are unable to utilize the natural variation present in the germplasm collection of a particular species. In this context, wild species can serve as a reservoir of useful alleles to use them in breeding programme. Conventional methods of breeding, however, have limited scope as they render the transfer of only a fraction of the genetic variation from wild to cultivated species. Some selected approaches have been described below that have potential to utilize the alleles from wild species to breeding lines.

6.1 Introgression of exotic germplasm

Wild species together with landraces represent natural variation within the species. Domestication of these landraces which are highly heterogeneous in nature is the first step to produce cultivars. Extensive studies have been done on the natural variation in crop species to study both evolutionary and ecological potential of the genes. It has been demonstrated that quantitative trait modification which includes phenotypic and compositional changes cannot be achieved by mutagenesis or transgenic but can be introgressed through wide genetic variation studies using molecular marker assisted breeding[1]. Several other strategies have been used for introgression of favourable gene/QTL/chromosomal segment by developing isogenic lines using wild species and the variety /genotype of interest[23]. Based on the protocol used for the development, the generated lines are referred as introgression lines (ILs), back-cross recombinant inbred lines (BCRIL)[36], recombinant chromosome substitution lines (RCSLs)[33], chromosome segment substitution lines (CSSLs)[48]. Introgression/ exotic libraries are constructed using introgression lines each of which carries a fragment of defined homozygous chromosomal segment form donor exotic parent with a homozygous genetic background of elite parent. These exotic libraries have been used for identification of QTLs controlling tomato aroma, fruit nutrition and antioxidant content. In rice, a large set of CSSL libraries were constructed which resulted in the transfer of brown plant hopper (BPH) and the white-backed plant-hopper (WBPH) resistance in the line. Hence, this approach can be employed to enrich the genetic variation which was lost during the domestication of crop plant.

6.2 Advanced-backcross (AB-QTL) analysis :

AB-QTL analysis is an approach for simultaneous discovery and transfer of QTLs from a wild species to a crop variety which was proposed earlier by Tanksley and Nelson. In this approach, a wild species is backcrossed to a superior cultivar, and during backcrosses, the transfer of desirable gene/QTL is monitored by employing molecular markers. The segregating BC2F2 or BC2F3 population is then used not only for recording data on the trait of interest, but also for genotyping using polymorphic molecular markers. These data are then used for QTL analysis, leading to simultaneous discovery of QTLs, while transferring these QTLs by conventional backcrossing. Many AB-QTL studies concluded that wild species contain favourable alleles for enhancement of quantitative traits for cereal crops.

6.3 Association genetics :

Association genetics is an approach that utilizes natural variation and linkage disequilibrium (LD) existed in natural population to identify the gene(s) / genomic regions associated with trait. In general, conventional linkage analysis using a bi-parental mapping population such as F2 lines, back cross (BC) population and recombinant inbred lines (RILs) is a commonly used method for trait mapping. However, such mapping populations are derived from a few cycles of recombination events, hence limit the

resolution of genetic maps and localize QTLs from 10 to 20 cM intervals and also do not essentially use the germplasm that is being actively used in breeding programs. In contrast, association mapping, based on LD measures the degree of non random association between alleles at different loci. It does not require a segregating population and in some cases more powerful than linkage analysis for identifying the genes responsible for the variation in a quantitative trait[9].

Association mapping offers three advantages over traditional linkage analysis–
(i) increased mapping resolution, (ii) greater allele number, and (iii) reduced research time. Mapping based on LD allows for large scale assessment of allele/trait relationship when combined with a correction for population structure. Under this approach, association between marker and trait is only expected when a QTL is tightly linked to the marker because the accumulated recombination events occurring during the development of the lines will prevent the detection of any marker/trait association in any situation where the QTL is not tightly linked to a molecular marker.
Based on the scale and focus of a particular study, association mapping employs one of following two approaches:
(i) candidate-gene association mapping, which relates polymorphisms in selected candidate genes that have purported roles in controlling phenotypic variation for specific traits.
(ii) genome-wide association mapping, or genome scan, which surveys genetic variation in the whole genome to find signals of association for various complex traits.

Although candidate gene sequencing across several hundreds of genotypes for selected genes using Sanger sequencing was an expensive task in past, use of pools of amplicons for a range of genotypes and/or genes through NGS technologies is expected to
reduce the costs significantly.

6.4 Multi-parent advanced generation inter-cross (MAGIC) population

MAGIC population is a second generation mapping resources for crop improvement which is constructed using multiple parents. This mapping population strategy involves linkage and association methodology for mapping genetic variation in population segregating for multiple QTLs. The MAGIC approach can be considered superior to association and linkage mapping as it allows both coarse and fine mapping of RILs developed from multiple parents by sampling seed of any generation with greater genetic variation. It has been demonstrated that if a large set of RILs are produced, the complex architecture of many traits which are associated with crop yield and quality can be studied using epistatic interactions[11]. Furthermore, the MAGIC lines may show extensive segregation for plant developmental traits like plant height, maturity that may limit the use of MAGIC population in dissection of complex traits.

7. Next Generation Sequencing (NGS) Technologies

Detection and utilization of genetic variation has been a major task for plant breeders. Though, classical molecular markers such as RFLPs (restriction fragment length polymorphisms), RAPDs (random amplified polymorphic DNA), AFLPs (amplified fragment length polymorphisms) and SSRs have been used extensively for this purpose, the SNP marker system that has capability to detect the variation at single base level, however have not been used in many crop species. One of the major limiting factor in this direction has been the higher costs involved in sequencing the genes/ transcriptomes/ part of genomes of related individuals for SNP discovery. Three major sequencing platforms that are currently being used in plant species include Genome sequencer FLX Roche/454 , Applied Biosystems SOLiD and Illumina Genome Analyzer.

These three platforms provide thousands of million sequence reads in a single run in reduced time and less costs as compared to conventional Sanger sequencing technology[30]. Among these three approaches, FLX/454 platform is superior in terms of read

length (about 400 bp) but is rather expensive in terms of cost when compared with the Solexa and AB SOLiD. Yet another approach based on single molecule synthesis is gaining attention and is termed as 3rd generation sequencing. Apart from this many new sequencing technologies are emerging and/or are at their infant stages to facilitate genome wide marker discovery in both model/major and orphan crop species.

Sequence data generated for parental genotypes of the mapping populations by using NGS technologies can be used for mining the SNPs at large scale. While in case of model plant species or major crop species, it is easier to align the NGS data from individuals to the reference genome sequence data, if available or the transcript sequence data available through EST sequencing projects. In case of under-resourced crop species where appropriate or adequate sequence data are not available, the best possible strategy is to sequence the cDNAs with NGS technologies and then align with the transcript data of the species, if available or of the related major/ model crop species. In case, these SNPs have been derived from genes or genic regions, the corresponding markers are also referred as functional markers/FMs[4] or genic molecular markers/GMM.

Apart from developing SNP markers, NGS technologies can be and are being used for other applications such as de novo sequencing, association mapping, alien introgression, transcriptome expression and polymorphism, population genetics, evolutionary biology and genome-wide assembly in several crop species. With the development of rapid and inexpensive sequence technologies, the efficiency and accuracy in sequencing have interpreted the genomic information of many plant species.

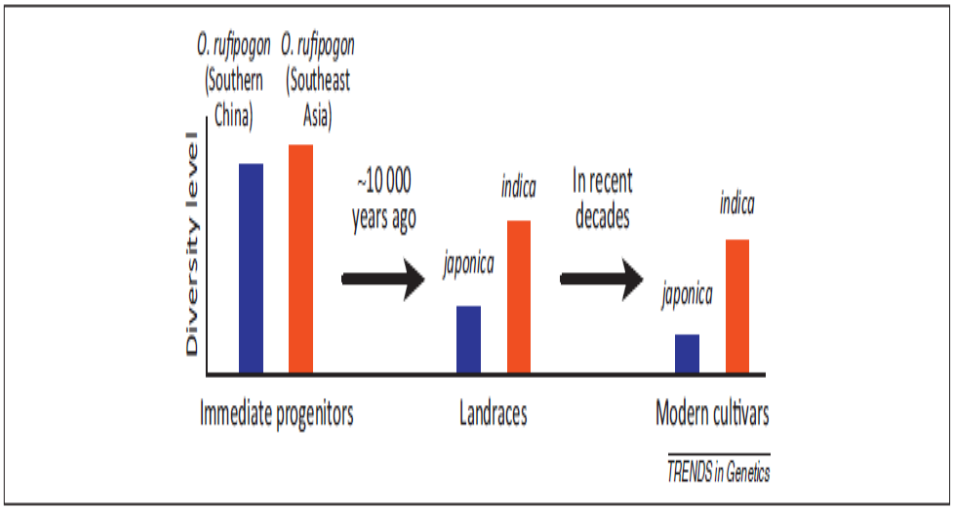

Fig 2: Depicts the genetic diversity lost over the years.

7.2 TLLING :

The emphasis on genomics has been changing from complete sequenced genomes to the study of the functional genomics. To understand the function of gene(s) many approaches like RNAi, gene knockout, site-directed mutagenesis, transposon tagging etc. have been exploited for many years. All these techniques demand the use of transgenic material which is not always possible in many commercially important crops. So it is not only impeding the functional analysis of gene(s) but also retards the improvement of existing as well as the development of improved cultivars. In this review, a non transgenic technique called Targeting Induced Local Lesions IN Genomes (TILLING) was emphasized that determines the allelic sequence of induced point mutations in gene (s) of concern. It allocates the rapid and cost-effective detection of induced point mutations in populations of physical/chemically mutagenized individuals.

Ethyl methanesulfonate (EMS) is mostly used and produce G/C to A/T transition by alkylating the G residues and the alkylated G resides to base pair with T instead of pairing with C.

TILLING (Targeting induced local lesions in genomes) a newly developed general reverse genetic strategy helps to locate an allelic series of induced point mutations in genes of interest. It allows the rapid and inexpensive detection of induced point mutations in populations of physically/chemically mutagenized individuals. In addition to allowing efficient detection of mutations by TILLING approach, EcoTILLING technology is also ideal for examining natural variation. Endonuclease CEL I cut effectively the multiple mismatches in a DNA duplex. Therefore, heteroduplex DNA of unknown sequence with to that of a known sequence reveals the positions of polymorphic sites. Both nucleotide changes and small insertions/deletions are identified. It can be performed more inexpensive than full sequencing the methods currently used for the most single nucleotide polymorphism (SNP) discovery. SNP variation can provide clues to the adaptive strategies and population history that undoubtedly played roles in specie's evolution. It is also used for screening and detection of plants with desired traits by knockdown and knockout mutations in specific genes. This makes TILLING and EcoTILLING an attractive strategy for a wide range of applications from the basic functional genomic study to practical crop breeding.

TILLING antiquity

It was first explored in the late 1990's by the efforts of Claire McCallum and his collaborators (Fred Hutchinson Cancer Research Center and Howard Hughes Medical Institute) who was experimenting on Arabidopsis[5]. He used T-DNA lines and antisense RNA as reverse genetic approaches to illustrate the function of two chromomethylase genes. He was impotent to successfully apply these methodologies to describe the function of CMT2 gene. This technique was developed by pooling chemically mutagenized plants together, creating hetero duplexes among the pooled DNA, intensify the region of concern and using dHPLC (denaturing high performance liquid chromatography) to identify the mutants by chromatographic variations. A less expensive and faster modification of the TILLING protocol was published later, which uses a mismatch specific celery nuclease (CEL I) and LI-COR gel analyzer system[2,38].

In 2001, the standard procedure was developed with practical software that makes the TILLING technique a more routine method to detect mutations to get satisfactory results[14]. Since from its origin, it has been automated and exploited in many plant taxa. As a reverse genetic high throughput method, it purposes to detect SNPs (single nucleotide polymorphisms) and/or INDELS (insertions/deletions) in gene/genes of interest created from a mismatch in a mutagenized populace.

The application of TILLING to crop improvement may also help with another constraint in domesticated species genomes having limited genetic variation. During domestication and subsequent selection, much of the genetic variation available in the wild crop progenitors has been lost[19]. Thus, plant breeders have at times used wild relatives or land races to introduce useful genetic variation. This practice has been successful in wheat for developing disease resistant and higher yielding varieties[50] and a land race was also used for the development of the first full waxy line because it carried a rare deletion allele of one of the waxy loci[20]. As an alternative to the use of wild varieties, TILLING can be a means to introduce genetic variation in an elite germplasm without the need to acquire variation from exotic cultivars, consequently, avoiding the introduction of agriculturally undesirable traits.

7.3 EcoTILLING

An extension of TILLING is EcoTILLING, which uncover natural alleles at a locus contrary to induced mutations. Many crop species cannot be exposed to induced mutation, and EcoTILLING can be used to find the natural variants and their putative

gene functions in these crops. This can be done at low cost of SNP/haplotyping methods, and one can screen many samples with a gene of interest[15]. It does not require all the population to be screened to find the polymorphism which can be arduous and time consuming in TILLING.

Moreover, in open pollinated crops, EcoTILLING can be used to find the heterozygosity level within a gene fragment. As the CEL I can digest small proportion of the hetroduplexes, it can be used to find the multiple polymorphisms in a single gene of interest. Furthermore, phylogenetic diversity estimates, selection and linkage disequilibrium can be estimated. It can be used to detect the DNA polymorphism in satellite repeat numbers. It can also be effectively used as an efficient, rapid technique to identify DNA polymorphisms in populations with high genetic similarity and to mine for SNPs in collections of plant germplasm. The EcoTILLING approach first time used on Arabidopsis thaliana in 2004 to mine for natural polymorphisms[15]. From five different genes that were almost 1 Kb in length, 55 haplotypes were identified in the introns. This study showed that CEL I could cut hetroduplexes to detect SNPs, INDELS and microsatellite repeat polymorphism.

Another important application is to find alleles of resistant genes that could provide immunity to various diseases. EcoTILLING in Mla resistance genes of Hordeum vulgare (barley) was used in 2006 to examine the allelic variation[34]. It demonstrates how effectively it can be used for the evaluation of diversity in natural populations[21]. It was employed to identify polymorphisms in mung bean (Vigna radiata (L.)). The majority of the polymorphisms were detected between sublobata and radiata in putative introns[16]. In general, EcoTILLING shows great promise in the process of identifying natural disease resistance alleles, which can be used in crop improvement. Besides, this technique is applicable to any organism even those that are heterozygous and polyploidy in nature.

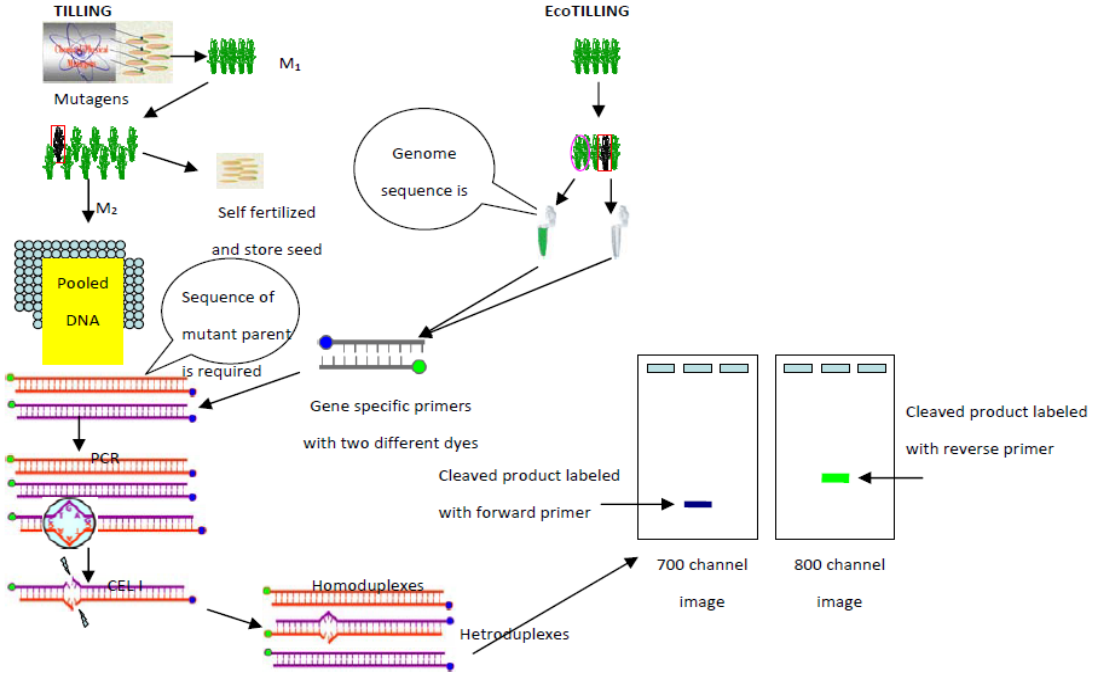

Fig 3. Schematic diagram of the TILLING and EcoTILLING techniques. In TILLING seeds are mutagenized with chemical/physical mutagens to produce M1 plants. M1 plants are self to produce the M2 from which DNA is extracted for analysis. The M2 is allowed to produce seed, which can be easily stored for future analysis. After the DNA is extracted from the mutant population, the DNA concentration of all samples is standardized and pooled together. The number of individuals in a pool depends on the ploidy level of the plant and the amount of naturally occurring SNPs, which may require the number of individuals in the pool to be reduced. The desired gene is amplified using a forward primer with 700 nm dye label and a reverse primer with an 800 nm dye label attached to the 5' ends. The PCR products are heated and cooled to form a mixture of homoduplexes and heteroduplexes among the genotypes in the pool. Any mismatches (SNPs or small INDELS) will be detected by a mismatch endonuclease (CEL I) and cleaved into two separate products, which will be detected in the 700 and 800 dye channel of a LICOR DNA Analyzer.

8. Role of Molecular Markers in Alien Gene Introgression

8.1 Marker-assisted selection and marker-assisted backcrossing

Once the markers associated with a trait of interest is identified through linkage mapping, association mapping, AB-QTL or transcriptomics approach, etc., the next step is to use these markers in the breeding programme. In this context, the selection of one or a few genes (QTLs) through molecular markers using backcrossing is a highly efficient technique. There are three levels of MABC or MPS:

(i) Foreground selection: which includes screening of target gene or QTL using molecular markers, this step can also be used for selection of recessive allele for backcrossing as recessive alleles require one generation of selfing for its expression.

(ii) Recombinant selection involves selection of the BC progeny containing the target gene and recombination events (between the target locus and linked flanking markers. The purpose of this selection step is to minimize the linkage drag by using markers that flank the target gene. This linkage drag poses a big problem during selection through conventional breeding methods. Furthermore this recombination selection event is usually carried out using two BC generations.

(iii) Background selection: involves use of markers that are unlinked to the target locus for the selection of BC progeny containing highest proportion of recurrent parent (RP). In summary, the MABC employs linked markers to select the target gene/QTL from the donor parent and the unlinked markers to recover RP. Traditional approaches of recovery of RP genome take upto six BC generations but the use of markers enables to achieve the same in even in BC2.

8.2 Marker-assisted recurrent selection :

There are cases where quantitative variation is controlled by many genes (QTLs) with minor effect; in such cases the previous approach (MABC) has certain limitations in the introgression of the target trait. Moreover, the markers identified linked with a trait to be used in MABC are generally identified biparental mapping populations. This limits the study of allelic diversity and genetic background which are very essential in crop breeding program. Limited statistical tools for studying polygenic traits controlled by many small effect loci are yet another drawback of MAS.

Furthermore, minor QTLs show an inconsistent QTL effect. Even though the effect of these minor QTLs is consistent, introgression of these QTLs through MABC approach becomes extremely difficult as a larger number (sometimes unmanageable) of progenies, depending on the number of QTLs, are required to select appropriate lines in MABC. In cases as mentioned, marker assisted recurrent selection (MARS) can be used for pyramiding of several genes/QTLs (of minor effect) in a single genotype. MARS is based on ad hoc significance test which include the identification of trait associated markers and estimation of their effect. The approach involves multiple cycles of marker based selection that includes,

(i) Identification of F2 progeny which contain favourable alleles for most if not all QTLs,

(ii) Recombination of the selected progenies to the selfed ones, and

(iii) Repetition of these cycles.

According to the recent studies, the response of MARS is larger in case of prior knowledge of the QTLs and the response decreases as the knowledge of the number of minor QTL associated with the trait decreases. In sweet corn, MARS was employed to fix six marker loci in two different F2 populations which showed an increase in the frequency of marker allele from 0.50 to 0.80. Similarly in a separate study, enrichment of rust resistance gene (Lr34/Yr18) with the increase in frequency from 0.25 to 0.60 was reported in wheat BC1 through MARS. MARS, becoming a popular approach, can thus be effectively utilized for selection of traits associated with multiple QTLs by increasing the frequency of favourable QTLs or marker alleles.

8.3 Genome-wide selection:

Genome-wide selection is another approach that can be used to pyramid favourable alleles for minor effect QTLs at whole genome level. Unlike MABC or MARS, the GWS calculates the marker effects across the entire genome that explains entire phenotypic variation. The genome wide marker data (marker loci or haplotypes) available or generated on the progeny lines, therefore, are used to calculate genomic estimated breeding values (GEBV). It is important to note that the GEBVs are calculated for individuals based on genotyping data using a model that was 'trained' from individuals having both phenotyping and genotyping data. These GEBVs are then used to select the progeny lines for advancement in the breeding cycle. In summary, the GWS provides a strategy for selection of an individual without phenotypic data by using a model to predict the individual's breeding value.

CONCLUSION

Recent advances in sequencing and genotyping technologies have made it possible to develop molecular markers as well as undertake genotyping at large scale in both major as well as minor (or so called orphan crop species) that can be used not only for developing high density genetic and physical maps but also for generating transcriptome or sequence data. These approaches together with –omics approaches such as transcriptomics, genetical genomics, metabolomics and proteomics can be used to identify the genomic regions or genes involved in expression of trait(s) that are of interest to the breeding community. In parallel, the high-throughput sequencing and genotyping approaches can be used to detect genetic variation existed in germplasm collection not only in cultivated gene pool but also in landraces and wild species. TILLING and EcoTILLING are inexpensive and swift natural polymorphism detection and genotyping methods. They have advantages for determining the range of variation for genetic mapping based on linkage analysis. Such kind of genetic variation (or favourable alleles) can be introgressed in elite variety or genotype of interest by using AB-QTL approach or developing introgression libraries. Furthermore, the QTLs or genes or superior alleles for the trait of interest identified through linkage mapping, association mapping, AB-QTL approach or –omics approach can be introgressed or pyramided in elite varieties or genotype of interest by using MAGIC, MABC, MARS or GWS approaches.

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