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Review Article

Review Paper on Approaches in Developing Inbred Lines in Cross-Pollinated Crops

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Abstract: Plant breeding aims to constantly develop crop cultivars with improved yields and quality and tolerant to droughts, diseases and pests. Use of genetically improved crop cultivars and better management practices are among the best strategies to increase food production and meet a projected doubling of food demand. Inbred lines are homozygous genotypes produced by repeated selfing with selection over several generations. It is developed and maintained by repeated selfing of selected plants. In cross-pollinated species with strongly expressed self-incompatibility, various techniques are used to overcome the incompatibility. The technique of doubled haploids may be used to produce complete homozygous diploid lines in just 1 year (versus more than 4 years in conventional breeding) by doubling the chromosome complement of haploid cells. Doubled haploidy is and will continue to be a very efficient tool for the production of completely homozygous lines from heterozygous donor plants in a single step. Haploids contain half the chromosome number of somatic cells. Anthers/stigma Contain immature microspores or pollen grains with the haploid (n) chromosome number. If successfully cultured (anther culture), the plantlets resulting will have a haploid genotype. To have maximum genetic variability in the plantlets, breeders usually use anthers from F1 or F2 plants. Usually, the haploid plant is not the goal of anther culture. Rather, the plantlets are diplodized (to produce diploid plants) by using colchicine for chromosome doubling. This strategy yields a highly inbred line that is homozygous at all loci, after just one generation.

Keywords: Doubled, Haploid, Homozygous, Loci, Inbreeding

1. Introduction

Plant breeding has made remarkable progress in crop improvement and play a key role in increasing food production. It aimed at developing genetically improved crop cultivars with economic benefits for farmers. Population growth, declining agricultural land and global climate change presents increasing risks to crop production. Consequently, plant breeding aims to constantly develop crop cultivars with improved yields and quality and tolerant to droughts, diseases and pests. Use of genetically improved crop cultivars and better management practices are among the best strategies to increase food production and meet a projected doubling of food demand in the next 40 years [1]; [2]; [3] and [4]. Fundamental discoveries of Mendel established the scientific basis for plant breeding and genetics at the turn of the 19th century. Similarly, the recent integration of advances in biotechnology, genomic research, and molecular marker applications with conventional plant breeding practices has created the foundation for molecular plant breeding, an interdisciplinary science that is revolutionizing 21st century crop improvement. Though the methods of molecular plant breeding continue to evolve and are a topic of intense interest among plant breeders and crop scientists [5]; [6] and [7].

Molecular breeding is now considered an essential component of current crop improvement efforts for major crops by large companies, the broad applicability of modern molecular approaches to conventional plant breeding remains a source of debate among some practicing plant breeders in the public sector, particularly for minor crops [8] and [9]. Inbred lines are homozygous genotypes produced by repeated selfing with selection over several generations. The technique of

doubled haploids may be used to produce complete homozygous diploid lines in just 1 year (versus more than 4 years in conventional breeding) by doubling the chromosome complement of haploid cells. Such doubling may be accomplished *in vivo* naturally or through crossing of appropriate parents, or *invitro* through the use of colchicine. The success of doubled haploids as a breeding technique depends on the availability of a reliable and efficient system for generating haploids and doubling them in the species [2].

Haploids have two primary uses in plant breeding. The first is the accelerated production of homozygous lines and pure cultivars. For cross-pollinated crops, haploids are used primarily for the production of homozygous lines, which are in themselves utilized in the production of hybrid seed. At the present time, more than 200 varieties have been developed by utilizing a doubled haploid approach [10]. The second main use is that haploids provide a possibility of screening breeding material for the presence of advantageous genes. In both, haploids and doubled haploids, all alleles are expressed. This facilitates the selection of genotypes that are important for breeders. Selected haploids can be used for the improvement of any breeding material, including increasing the frequency of favorable genes in populations [11].

Generally mating is a way by which plant breeders impact the gene frequencies in a population. Inbred lines are homozygous genotypes produced by repeated selfing with selection over several generations. The technique of doubled haploidsis used to produce complete homozygous diploid lines in just 1 year (versus more than 4 years in conventional breeding) by doubling the chromosome complement of haploid cells. Therefore, the objective of this paper was:-to understand the fastest method of developing inbred line in cross pollinated crops

2. Review of Literature

2.1. Inbred Line Development in Cross Pollinated Crops

Inbred lines are homozygous genotypes produced by repeated selfing with selection over several generations. It is a breeding material that is homozygous. An inbred line consists of individuals with the same genotype. It is developed and maintained by repeated selfing of selected plants. In principle, developing inbred lines from cross-pollinated species is not different from developing pure lines in self-pollinated species. The pedigree method of breeding is the most widely used method to develop inbred lines. The pedigree method of inbred development is referred to as “standard method” when an open pollinated population is sampled [12].

Because of the mode of reproduction, breeding lines from cross-pollinated species are more challenging to develop and maintain. Inbred lines may be developed from heterozygous materials obtained from a natural population, or from F2 selected genotypes. Depending on the breeding procedure, parents for hybrid production may be developed in the conventional fashion, or non-conventional fashion. Normal inbreds are developed by repeatedly self-pollinating selected plants, from S0–S*n*(for materials drawn from natural populations) or from F1–F*n*(for materials obtained from crossing). The goal is to attain a level of homozygosity at which the inbred lines are uniform in characteristics and will remain so under continued selfing, with no further loss of vigor. At this stage, the inbred line may be maintained by self-pollination. Hybrid varieties are commonly developed by crossing two unrelated, homozygous inbred lines. Traditionally, the maize plants’ cross-breeding nature required recurrent self-pollinations for 6–10 generations, i.e., 3–5 years when two seasons per year can be accomplished, to obtain sufficiently homozygous inbred lines [13].

Inbred lines are genotypes that are developed to be used as parents in the production of hybrid cultivars and synthetic cultivars in the breeding of cross-pollinated species. They are not meant for direct release for use by farmers. They are homogenous and homozygous, just like pure lines. However, unlike pure lines, they need to be artificially maintained because they are produced by forced selfing (not natural selfing) of naturally cross-pollinated species. The success of a crop breeding program relies on choice of the best parents possessing complementary and desired traits. Thus, breeders continuously select potential parent populations from diverse sources including landraces, modern cultivars, obsolete or primitive cultivars, wild or semi-wild species. Parents with high specific or general combining abilities are selected via progeny testing through well-designed recombination. The progenies are evaluated to determine the genetic potential of parents for subsequent breeding and to discern the type of cultivar to be developed, i.e., pure line, hybrid, or open-pollinated. Progeny testing is performed in a set of target and representative environments with half-sibs, full-sibs, testcrosses or recombinant inbreds [2] and [3].

Development of inbred parents can follow different breeding methods such as pedigree breeding, backcrossing, bulking, single seed descent, double haploids, etc. Inbred lines are developed by selfing of heterozygous population and doubling of haploids. Various population viz. open pollinated varieties, synthetic varieties or any other heterozygous population can be used for selfing. Superior plants on the basis of vigour, disease resistant and yield are selected and selfed. Progeny of selected plants are grown separately from the selfed seed in the next season. Again selection is made for the superior progeny and selfed. This processes is continue to get superior homozygous inbred.

Pedigree breeding is the most widely used breeding system to develop maize inbreds. Typically, specific crosses are made between inbred lines, and then self-pollination is applied to the F1 and subsequent generations to develop inbred lines that are superior to either parent (transgressive segregants) through genetic segregation and recombination. Selection is applied among progeny rows and among plants within S1 families. It is common to have replicated nurseries for the S1 families exposed to different Disease, insect, or abiotic stresses. This process of selfing and selection is repeated in successive generations (S2, S3, S4, S5...S*n*) until homozygous elite inbreds are developed. Effective phenotypic selection and

greater selection intensity can be applied in Initial inbreeding stages for traits with high heritability such as pest resistance, maturity, morphological traits, etc. The backcross breeding method is used widely in maize breeding to transfer one or a few traits/genes from the donor parent to the recurrent and most desirable parent. With the advent of genetically modified organisms, major emphasis is devoted to accelerate Backcrosses to transfer the transgenes to elite inbreds. The use of DNA molecular markers has facilitated both the speed and accurate recovery of the recurrent parent, and the reduction of linkage drag. The bulk method, where the seeds for each selfing generation are harvested in bulk, and single-seed descent, where one or a few seeds from each genotype are advanced each generation until approximate fixation is reached, are also used because of their simplicity and low space requirements. In cross-pollinated species with strongly expressed self-incompatibility, various techniques are used to overcome the incompatibility reaction. For instance in *Brassicas*, bud pollination is enhanced by treatment in a CO_2 enriched atmosphere [14] or by application of gibberelic acid, sodium chloride, urea or ammonium sulphate on stigmas.

2.1.1. Doubled Haploid Breeding Methods

Haploids produced from diploid species (2n=2x), known as monoploids, contain only one set of chromosomes in the sporophytic phase (2n=x). The production of pure lines using doubled haploids has several advantages over conventional methods. Using DH production systems, homozygosity is achieved in one generation, eliminating the need for several generations of self-pollination. In vitro production of haploid plants followed by doubling of somatic chromosomes is the quickest means to produce pure breeding doubled haploids (DHs) [15] and [16].

Cross-pollinated species are known to possess numerous deleterious recessive alleles that are not expressed in heterozygous states. They are gradually fixed during self pollination, causing inbreeding depression and difficulties in producing homozygous lines during conventional breeding. Doubled haploid technology helps to overcome these problems through the rapid fixation of genes in one generation and early elimination of deleterious alleles from populations. Their complete homozygosity enables true breeding and stable field performance over generations of progeny, although the complete lack of heterozygosity and heterogeneity in varieties is thought to be more vulnerable to environmental changes and altered cropping systems.

Hence, the key to increased genetic gains and accelerated development of improved varieties is reducing the time needed for inbred development. This can be most effectively achieved by application of the doubled haploid (DH) technology. The doubled haploid method has several advantages in crop breeding programs. Firstly, production of doubled haploids leads to homozygosity in a single generation after recombination of selected parents. This is unlike the conventional selection method that requires six to seven selfing generations to achieve a practical level of homozygosity. Secondly, selection is more efficient for oligogenic or polygenic traits in DHs because the genes are fixed in a homozygous background, limiting dominance genetic variation and segregation [15]. Thirdly, the DH method prevents losses of valuable genetic variations better than the conventional selection method. Traditionally, early generation segregating populations are selected in a single environment where certain genotypes perform poorly are discarded. These genotypes may have carried useful genes that would be expressed in other target environments. Owing to the gains in speeded up cultivar development and the creation of desirable genetic backgrounds, doubled haploids are widely utilized in breeding as well as in genetic studies of various crops and traits [16]; [17]; [18] and [19]. Overall, the DH technology allows for the creation of stable haploids after recombination of parents with broad genetic variation. Thus, DH derivatives can be selected for improved traits such as yield, earliness, plant height, nutritional quality and pest and disease resistance, in a fully homozygous state. Selected genotypes can be used as homogenous varieties or as breeding parents in the ensuing crosses and selection cycles.

2.1.2. Marker Assisted Selection (MAS)

In order to select those phenotypes which display the desired trait, a selection procedure is indispensable following creation of variability (via hybridization or mutagenesis) in or following genetic transformation of a target organism. Traditionally in plant breeding morphological markers are being used (e.g. pigmentation, dwarfism, leaf shape) in the selection of desired individuals. With the introduction of in vitro techniques (e.g. cell and tissue cultures) marker systems which allow for selection early in plant development before the final phenotype has been developed are needed. Advances in biotechnology enabled the development of more efficient selection systems (e.g. biochemical or molecular marker systems) replacing traditional phenotype-based selection systems.

Any selection system that relies on the indirect selection of traits of interest through markers linked to them can be referred to as marker assisted selection (MAS). The most widely known example for marker systems is the use of selectable markers in genetic transformation. They are usually antibiotic or herbicide resistance genes, introduced into the recipient organisms together with the, usually qualitative, trait of interest. Successfully transformed genotypes survive the application of herbicides or antibiotics while those which do not contain the recombinant DNA are eliminated. In addition markers appropriate for screening are being developed, which allow the identification of those genotypes which contain the desired trait without destroying the others. Nowadays MAS refers in particular to selection based on genetic information retrieved through the application of molecular markers [20].

Molecular markers make differences in the DNA sequence visible, which can be related to different phenotypes. So in breeding programs molecular markers are used to select for traits at the DNA level. On the one hand they facilitate the choice for the elite parental lines to be used in cross breeding and on the other hand the decision on which offspring to

continue breeding with or to choose for multiplication (i.e. seed production). So MAS can be very useful to efficiently select for traits that are difficult or expensive to measure or are expressed late in development. This is particularly relevant for crops with long-lasting juvenility (e.g. trees species) as selection is facilitated already at the seedling stage. So MAS is most frequently used to eliminate disease susceptible genotypes or to introgress disease resistance genes into well-adapted elite lines early in the breeding programs [20]. MAS can be particularly useful in pyramiding monogenic resistance gene, which cannot be distinguished by phenotype [21].

However breeding objectives in cross breeding not only involve monogenic (qualitative) traits, but often involve complex traits (e.g. disease or pest resistance). These so called quantitative traits are influenced/specified by various genes (polygenic effect). The respective phenotypes vary in degree and unlike discrete characteristics are measurable on a continuous scale. Thus the challenge is the identification of markers linked to the respective quantitative trait. In other words marker loci need to be identified which lie in close proximity to those loci on the chromosome determining the quantitative trait. As molecular markers are inherited according to Mendelian laws, quantitative trait loci (QTL) analysis can be used for this purpose. QTL analysis comprises the joint study of the segregation of marker genotypes and of phenotypic values of individuals or lines which enables the location and effect-estimation of the genetic elements controlling a trait of interest [20]. Once the relationship between molecular markers and the desired trait is established, MAS can remarkably assist breeding programmes.

2.1.3. DNA Based Molecular Markers and Their Applications in Plant Breeding

Molecular markers reveal genetic differences in the primary structure of DNA between individuals. Compared to protein markers, DNA based polymorphisms are more stable, and can reveal subtle changes in the genomic DNA [22] and [23]. Different DNA based marker techniques have been successfully used such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeats (SSR) and single nucleotide polymorphisms (SNP) [22] and [24].

Molecular markers are 'landmarks' on chromosomes that serve as reference points to the location of other genes when a genetic map becomes available. If genetic maps are constructed, then the plant breeder establishes association between markers and desirable phenotypic traits. The trait of interest is then selected by indirectly selecting for the marker which is readily assayed or observed [25] and [9]. In plant breeding, markers are used to locate the chromosomal positions of candidate genes, to determine genomic organization among different gene pools and to conduct marker-assisted breeding. Identification of DNA markers associated with traits of interest may be facilitated by comparative mapping, i.e., by cross-referencing to the maps of model crop species, owing to gene synteny. These markers may facilitate inter-generic gene transfers and help to minimize linkage drag [25].

2.1.4. Applications of Molecular Markers in Plant Breeding

When molecular markers are available, conveniently co-segregating with candidate genes, marker-assisted selection (MAS) or marker-aided selection may improve the efficiency of selections of simple traits in conventional plant breeding programs [26] and [25]. Broadly, molecular markers are applied in plant breeding in the following areas:

a) To screen for useful single gene traits e.g. disease resistance. This may facilitate the introgression of new genes from a non-adapted parent and in pyramiding desired alleles into enhanced lines of candidate cultivars.
b) To accelerate backcross breeding programs through identification of the gene of interest and to eliminate the undesirable genome of the donor parent. Unlike conventional backcrossing, this method reduces linkage drag and requires few numbers of repeated backcrosses to recover the genotype of the recurrent parent.
c) To characterize diverse germplasm and establish heterotic patterns. Markers are useful to determine the magnitude of genetic diversity for crop improvement and to assign exotic (or non-adapted) germplasm into an appropriate breeding pool. In inbred lines markers assist in establishing heterotic patterns in order to guide the selection of parents for use in a hybrid breeding program. Marker information may be used in combination with phenotypic and pedigree analyses to ascertain genetic differences between lines of different heterotic groups to enable the breeder to predict the performance of hybrids to be developed from different intergroup crosses [27].

2.2. Reverse Breeding

Reverse breeding (RB) is actually a combination of different techniques which are applied in a sequential manner to generate homozygous lines parental lines which recreate a desired heterozygous genotype. The resulting breeding products are in essence identical to the initial elite hybrid crop, which is the starting point for the breeding process [28]. The steps involved in RB can be listed as follows [29]:

a) An elite heterozygous line is selected for its phenotypic characteristics.
b) Meiotic recombination is suppressed (e.g. through RNA interference, RNAi).
c) Gamete cells that do not contain the transgene are regenerated into homozygous, double haploid plants.
d) Parental lines are selected which together will reconstitute the initial heterozygous phenotype – only non-transgenic plants are selected.

A number of techniques may be applied resulting in silencing of meiotic recombination during sexual reproduction. Usually this is achieved by introducing GM modifications, which lead to silencing of genes required to initiate meiotic recombination events [30]. In a proof of concept experiment with Arabidopsis thaliana a GM based dominant RNAi

approach was used to silence the DMC1 gene to suppress crossover recombination [31]. However other techniques were discussed for achieving this objective among them virus induced gene silencing and grafting on GM rootstock to deliver silencing construct, introduction of dominant-negative alleles and use of chemical inhibitors. Application of GM methods is usually associated with propagation of individual cells in cell culture and/or with phases of in vitro tissue culture.

During this step microspore propagation and double-haploid techniques may be applied to convert haploid gametes into diploid plants which are homozygous for chromosomes derived from the initial hybrid [32]. Other approaches resulting in balanced double-haploid offspring may be used depending on the plant species [31].

3. Summary and Conclusions

Plant breeding aims to constantly develop crop cultivars with improved yields and quality and tolerant to droughts, diseases and pests; Use of genetically improved crop cultivars and better management practices are among the best strategies to increase food production and meet a projected doubling of food demand. Inbred lines are homozygous genotypes produced by repeated selfing with selection over several generations. It is developed and maintained by repeated selfing of selected plants. In principle, developing inbred lines from cross-pollinated species is not different from developing pure lines in self-pollinated species. In cross-pollinated species with strongly expressed self-incompatibility, various techniques are used to overcome the incompatibility reaction.

The technique of doubled haploids may be used to produce complete homozygous diploid lines in just 1 year (versus more than 4 years in conventional breeding) by doubling the chromosome complement of haploid cells. Such doubling may be accomplished *in vivo* naturally or through crossing of appropriate parents, or *invitro* through the use of colchicine. The success of doubled haploids as a breeding technique depends on the availability of a reliable and efficient system for generating haploids and doubling them in the species. Doubled haploidy is and will continue to be a very efficient tool for the production of completely homozygous lines from heterozygous donor plants in a single step.

Haploids contain half the chromosome number of somatic cells. Anthers contain immature microsporesor pollen grains with the haploid (*n*) chromosome number. If successfully cultured (anther culture), the plantlets resulting will have a haploid genotype. Haploid plantlets may arise directly from embryos or indirectly via calli, as previously discussed. To have maximum genetic variability in the plantlets, breeders usually use anthers from F1 or F2 plants. Usually, the haploid plant is not the goal of anther culture. Rather, the plantlets are diplodized (to produce diploid plants) by using colchicine for chromosome doubling. This strategyyields a highly inbred line that is homozygous at all loci, after just one generation.

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