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## Reverse breeding: Accelerating innovation in Plant breeding

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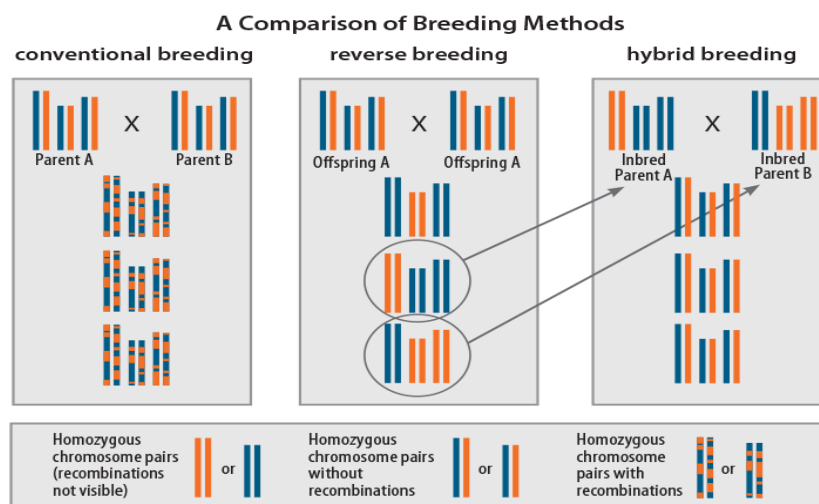
### Abstract

Reverse breeding (RB) is a novel plant breeding technique designed to achieve one of the most sought goal of plant breeding by directly producing parental lines for any heterozygous plant. RB generates perfectly complementing homozygous parental lines through engineered meiosis. The main objective of this review article is to understand the method of producing homozygous parental lines which is based on reducing genetic recombination in the selected heterozygote by eliminating meiotic crossing over. Male or female spores obtained from such plants contain combinations of non-recombinant parental chromosomes which can be cultured in-vitro to generate homozygous doubled haploid plants (DHs). From these DHs, complementary parents are selected and used to reconstitute the heterozygote. The main advantage of this breeding method is that it facilitates the selection of superior hybrid plants. Large population of plants can be generated and screened and superior performing plants can be regenerated indefinitely without prior knowledge of their genetic constitution. The technique will be particularly important for crops lacking large existing collections of breeding lines.

**Keywords:** Plant breeding, engineered meiosis, DHs.

### Introduction

Hybrid plants are often superior in size, growth and yield compared to the parent plants. This phenomenon called heterosis has been exploited in plant breeding since the early 20<sup>th</sup> century. However, hybrid breeding has always been more or less based on a trial and error approach, since it is difficult to predict which parental lines will give the best progeny. Many pairs of parents then need to be crossed and their progeny tested. Reverse breeding puts this century-long endeavor up-side-down by starting with superior hybrid selection followed by recovery of parental lines. This is an excellent tool in the plant breeder's toolbox, as it allows for a much more efficient and targeted hybrid plant production.



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A barrier to achieving high levels of variation in current plant breeding programs is that uncharacterized heterozygotes are difficult if not impossible to reproduce by seeds. Favorable allele combinations of the elite heterozygote are lost in the next generation due to segregation of traits. Because of this difficulty, the development of methods for easy preservation of heterozygous genotypes is one of the greatest challenges in plant breeding. Reverse breeding as a novel plant breeding technique to directly produce homozygous parental lines from any heterozygous plant was proposed by Dirks *et al.* in 2009.

Reverse breeding, meets the challenge of fixation of complex heterozygous genomes by constructing complementing homozygous lines. This is accomplished by the knockdown of meiotic crossovers and the subsequent fixation of non-recombinant chromosomes in homozygous doubled haploid lines (DHs). The approach not only allows fixation of uncharacterized germplasm but provides breeders with a breeding tool that, when applied to plants of known backgrounds, allows the rapid generation of chromosome substitutions that will facilitate breeding on an individual chromosome level.

In reverse breeding, an individual plant is chosen for its elite quality. By suppressing normal genetic recombination, homozygous parental lines are derived from this plant. Upon crossing, these parental lines can reconstitute the original genetic composition of the selected elite plant from which the lines were derived. During Reverse breeding, a genetic constitution step is employed to suppress genetic recombination and thus yielding intermediate plants which fall under GMO-legislation. However, the final selected variety and their parental lines do not contain this genetic modification and thus fall outside the scope of the GMO-legislation.

#### **Need of Reverse Breeding:**

1. To maintain the hybrid stability
2. Genetic improvement of parental lines to enhance the hybrid performance
3. To establish the breeding lines for uncharacterized heterozygotes
4. To multiply a highly heterozygous plant from a homozygous parental lines.

#### **Benefits of Reverse breeding**

Reverse breeding accelerates the breeding process considerably and increases the number of available genetic combinations which allows breeders to respond much quicker to the needs of farmers and growers with better varieties. Other main advantage of reverse breeding is that it facilitates selection of superior hybrid plants. Large populations of plants can be generated and screened and well performing plants can be regenerated indefinitely without prior knowledge of their genetic constitution. This essentially removes the randomness in earlier hybrid breeding. Reverse breeding is currently limited to crops with a relatively small, diploid genome.

#### **Mechanism of Reverse breeding**

Reverse breeding comprises two essential steps: the suppression of crossover recombination in a selected plant followed by the regeneration of DHs from spores containing non-recombinant chromosomes. The DH lines can then be used to recapitulate the elite heterozygote on a commercial scale.

Reverse breeding relies on achiasmatic meiosis: Achiasmatic chromosomes (chromosomes that did not form crossovers) remain as univalent. Chiasmata that in bivalents promote segregation of homologues to opposite poles (regular disjunction), are absent in univalent and the homologues may segregate to the same pole instead (non-disjunction). This leads to unbalanced chromosome numbers (aneuploidy) in the spores. Consequently, achiasmatic plants are highly sterile.

#### **Effective suppression of recombination**

Reverse breeding relies on the effective suppression of

meiotic crossovers. Therefore, genes that are essential in crossover formation but leave the chromosome structure intact are particularly useful. The knockdown of gene expression, essential for RB, can be achieved by targeting genes using RNA interference (RNAi) or siRNAs, which will result in predominantly post-transcriptional gene silencing (PTGS). Alternatively, dominant-negative mutations of the target gene can be used. The human meiotic recombination protein DMC1 forms octomeric rings but is fully defective in both ssDNA and dsDNA binding activities, when an amino terminal deletion lacking 81 amino acids is made. In crops in which stable transformation is difficult or impossible to achieve, other techniques i.e. Virus-induced gene silencing (VIGS) can be an effectively use for induction of PTGS. Exogenous application of compounds that cause inhibition or omission of recombination during meiosis would speed up the application of RB enormously. Crossover suppression need not be complete to be useful for RB. A single residual crossover may still occur in any chromosome pair(s). A single crossover causes regular segregation of the homologues involved (thereby increasing the chance of obtaining a balanced gamete twofold). A crossover also generates two recombinant chromatids, which are not useful for RB. But since a crossover affects only half of the chromatids of the bivalent pair, the other two chromatids are non-recombinant, and useful. Consequently, half of the resulting spores are potentially useful for RB. Residual crossovers (provided there is only one per bivalent) increase the incidence of DHs carrying recombinant chromosomes, but still produce 50% of spores carrying non-recombinant chromosomes. These non-recombinant spores can be selected for by using molecular markers.

#### **Doubled haploids**

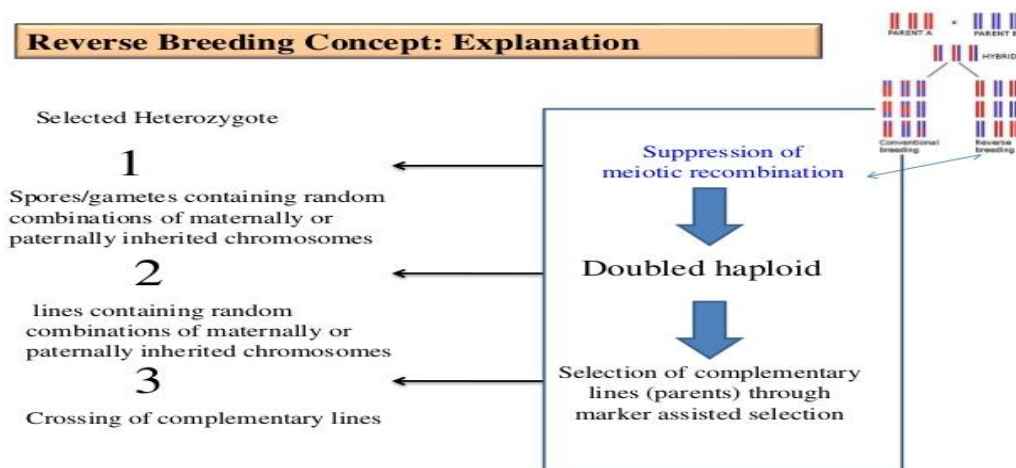
Doubled haploid plants resulting from achiasmatic meiosis can be obtained from unfertilized ovules (gynogenesis) or from microspore and anther cultures (androgenesis). The efficiency of DH formation from haploid spores is species dependent. Development of RB is limited to those crops where DH technology is common practice. For the great majority of crop species this technology is well established and professional breeding companies routinely use such techniques in their breeding programs. There are, however, some notorious exceptions such as soybean, cotton, lettuce and tomato where doubled haploid plants are rarely formed or not available at all. Genotyping of DHs by molecular markers is routine practice in contemporary plant breeding and is also indispensable for RB. In the complete absence of meiotic recombination one polymorphic molecular marker per chromosome would suffice to genotype every DH since the entire chromosome would behave as a single linkage block. In the presence of any residual crossovers, two markers (as distally located as possible) are required per chromosome.

#### **Chances of finding complementing parents**

The maximum number of different DHs obtained from a heterozygous diploid in a RB experiment equals  $2^x$ , with  $x$  being the basic chromosome number. The probability that two DHs form a pair of complementary parents equals  $2^x / (2^x)^2 = (1/2)^x$ , and the probability that they, upon crossing, do not reconstruct the original genotype is  $1 - (1/2)^x = (2^x - 1) / 2^x$ . The number of combinations between different DHs, presuming that reciprocal crosses result in the same phenotype, is  $n(n-1) / 2$ . In the case of  $n$  DHs, the probability of not finding a complementary pair of lines is therefore  $[(2^x - 1) / 2^x]^{n(n-1)/2}$  and

the probability of at least one complementary combination of two DHs is given by the formula  $[(2^x-1)/2^x]^{n(n-1)/2} = 0.01$  ( $P = 99\%$ ). This equation can be solved for different values of  $x$ .

The number ( $n$ ) of DHs that must be generated for finding a complementary match is highly dependent on the haploid chromosome number ( $x$ ).



### Applications of Reverse breeding

Reverse breeding as a new concept proposed in 2009 has not yet reached commercial application, though it has already been adopted at a research stage by many breeders. The potential is enormous and so is also the growing interest in this technique-as breeders for many decades have sought after a reliable method to maintain superior hybrid crop plants independent of access to the parental lines. It is expected to benefit breeding of important agricultural crops such as cucumber, onion, broccoli, cauliflower, watermelon, tomato, eggplant and many crops. Other important applications are as follows:

- 1. Reconstruction of heterozygous germplasm:** For crops where an extensive collection of breeding lines is still lacking, RB can accelerate the development of varieties. In these crops, superior heterozygous plants can be propagated without prior knowledge of their genetic constitution.
- 2. Breeding on the single chromosome level:** Many interesting characteristics in crops are based on polygenic gene interactions, very often located on different chromosomes. These quantitative traits are therefore not easy to breed on. RB is applied to an F1 hybrid of known parents. These homozygous chromosome substitution lines provide novel tools for the study of gene interactions. When crossed with one of the original parents, hybrids can be formed in which one of the chromosomes is homozygous whereas it is also possible to produce hybrids in which just one chromosome is heterozygous.
- 3. Reverse breeding and marker assisted breeding:** Especially in combination with (high throughput) genotyping, reverse breeding becomes a versatile tool. Evidently, high throughput genotyping speeds up the process of identification of complementing parents in populations of DHs in early stages. Also use in the study of gene interactions of the various heterozygous inbred families (HIFs) that can be produced by crossing and backcrossing the products of RB. The screenings of populations that segregate for traits on a single chromosome allow the quick identification of QTLs, when genotyping is combined with for example-transcriptome or metabolome profiling. Such HIFs further aid the generation of chromosome specific linkage maps and the fine mapping of genes and alleles. RB can

as such provide highly valuable insights into the nature of heterotic effects.

- 4. Backcrossing in CMS back ground:** In several vegetable crops such as cabbages and carrots, breeders make use of cytoplasmic male sterility (CMS). In these systems, the presence of male sterility presents a special challenge to RB. In these cases, gynogenesis rather than androgenesis can be used to obtain DH plants. This is perfectly compatible with RB in the sense that the chromosomes from the maintainer line can be recovered directly in the cytoplasm of the sterile line in one step. Gynogenesis has been described in several crops such as Brassica, maize, sugar beet, cucumber, melon, rice, onion, sunflower, and barley. In cases where the efficiency of gynogenesis is too low, it is possible to cross the male sterile (A) lines with maintainer lines (B) that carry one copy of a restorer gene. The AB combination will be fertile and RB can be performed. It should therefore be possible to use a restorer gene and a gene for crossover suppression in the same vector (both transgenes) and perform RB in a double suppressed (CMS and cross-over) background.

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