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REVERSE BREEDING: ADVANCING BREEDING PARADIGMS

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ABSTRACT

Reverse breeding is an innovative plant breeding technique designed to produce complementary parental lines for any heterozygous plant through a mechanism called achiasmatic meiosis, which occurs without crossovers. This process leads to univalent segregation during meiotic metaphase-I and the production of aneuploid gametes, which are then regenerated as doubled-haploid (DH) plants. Each DH plant carries combinations of parental chromosomes, and complementary DH pairs can be crossed to reconstruct the original hybrid. In reverse breeding, the inhibition of meiotic crossovers in a hybrid ensures the transmission of non-recombinant chromosomes to haploid gametes. The PAIR2 gene, necessary for homologous chromosome synapsis during meiosis-I in plants, plays a crucial role in this process. Mutations in the PAIR2 gene, such as an insertional mutation in rice or orthologous mutations in other species like Arabidopsis thaliana (e.g., ASY1), lead to defects in chromosome pairing during meiosis, resulting in the formation of univalents at metaphase-I. Reverse breeding essentially follows a strategy similar to generating a DH population from an F1 hybrid, incorporating a dominant-acting transgene that down-regulates the expression of Disrupted Meiotic cDNA1 (DMC1), thereby inhibiting crossover recombination and facilitating intact-chromosome inheritance. In previous studies on reverse breeding in A. thaliana, a hybrid was created using two natural ecotypes (Col-0 and Laer-0), with an RNAi transgene targeting the meiotic recombinase DMC1 to prevent meiotic crossover recombination. This method involved several steps: (i) generating and selecting RNAi: DMC1 transformed lines; (ii) producing achiasmatic hybrids; (iii) crossing achiasmatic hybrids with GFP-tailswap to generate haploid chromosome substitution lines (CSLs); (iv) generating DHs by spontaneously doubling haploid CSLs; and (v) crossing complementary CSLs to recreate the original hybrid.The potential of reverse breeding extends to the improvement of agricultural crops by enabling the generation of parental breeding lines for hybrid reconstruction.

***Keywords*:** Reverse breeding, homozygous lines, achiasmatic meiosis.

Introduction

In this work, we demonstrate how complementing homozygous lines are created using reverse breeding, a novel method that addresses the difficulty of fixing complex heterozygous genomes (Dirks *et al.*, 2003). Although the earlier suggested usage of the word "reverse breeding" is included, it expands on its original meaning by referring to a wider range of techniques used to create homozygous lines (Palmgreen *et al.*, 2014). This is achieved through suppressing meiotic crossovers and stabilizing non-recombinant chromosomes within homozygous doubled haploid lines (DHs). This method not only enables the fixation of uncharacterized genetic material but also furnishes breeders with a valuable technique. When applied to plants with known backgrounds, it

facilitates the swift creation of chromosome substitutions, thereby streamlining breeding at the individual chromosome level. Following a concise introduction to the RB breeding scheme, we delve into its foundation: the distinctive feature of achiasmatic meiosis. According to the theory, reverse breeding allows for the rearrangement of chromosomes between two homozygous plants. Some genetic modifications can leave residues, such as bacteria and fungus, in the host plant. However, innovative breeding methods like reverse breeding enable the creation of homozygous lines without introducing new DNA sequences. Despite the involvement of recombinant DNA in the reverse breeding process, the selected homozygous parental lines and their offspring are not considered transgenic. Studies on Arabidopsis thaliana using reverse breeding suggest its potential applicability in crop development. Because elite hybrids are unstable, homozygous parental lines are crossed to make new hybrids. Hybrid seed production cannot reproduce uncharacterized heterozygotes because it results in the segregation of the following generation, which eliminates advantageous allele combinations (Yi-Xin *et al*., 2015).

However, implementing this approach might be challenging for crops with a higher number of chromosomes. Commercial CMS lines, commonly used in modern agriculture, are well-suited for reverse breeding. This strategy is effective only in crops capable of renewing doubled haploids from spores and possessing a haploid chromosomal count of 12 or below. For polyploid animals or those with a high chromosome count, an alternative reconstruction technique has been proposed. In the reverse breeding process, a genetic constitution step is employed to prevent genetic recombination, resulting in intermediate plants subject to GMO regulations. However, the final selected variety and its ancestors do not carry this genetic modification, making them exempt from GMO regulations. According to both ACRE (2013) and COEGM (April 1), there is no evidence to support the claim that the product generated by RB is genetically modified and transgenesis is only a stopgap measure to clear the way for selection and breeding. (Kuligowska *et al*., 2013)

RB (Reverse Breeding) could be employed to achieve the following objectives:

1. **Create breeding groups for unidentified hybrids:** RB can be utilized to establish breeding groups specifically for hybrids whose genetic composition is not well-characterized.
2. **Genetically advance parental lines to enhance hybrid performance:** RB provides a means to genetically advance parental lines, leading to improved performance in hybrid plants.
3. **Retain a homozygous parental line that has produced a highly heterozygous plant:** RB allows for the preservation of a homozygous parental line that has given rise to a highly heterozygous plant.
4. **Study gene interactions using homozygous chromosomal replacement lines:** Homozygous chromosomal replacement lines generated through RB offer exceptional resources for studying gene interactions, providing valuable insights into genetic mechanisms.

Mechanism of Reverse Breeding

1. Heterozygote Selection

In this process, a highly heterozygous plant exhibiting a desirable combination of characteristics is chosen, regardless of whether information about its parents is available. The selected heterozygote then undergoes development into a gamete.

2. Meiotic Recombination Suppression at the Time of Spore Production

Effective suppression of meiotic crossovers is a crucial requirement for the success of reverse breeding. Therefore, genes that play a role in crossover formation while preserving the chromosome structure are particularly advantageous. Targeting these genes using RNA interference (RNAi) is a preferred method, as it predominantly leads to post-transcriptional gene silencing. This reduction in gene expression is essential for reverse breeding to achieve the desired outcome.

The following genes are necessary for meiotic recombination to occur:

1. Disrupted Meiotic cDNA: DMC1 gene
2. Sporulation-specific gene SPO1
3. Recombinase gene RecA A gene

In hybrids of A. thaliana, the DMC1 gene, responsible for encoding the meiotic recombination protein Disrupted Meiotic cDNA1, plays a crucial role in preventing non-recombined paternal chromosomes from undergoing recombination.

An alternative approach to expedite the reverse breeding process involves the use of chemical agents that halt recombination and accelerate meiosis. Compounds such as MIRIN and inhibitors of the Mre11-Rad50-Nbs1 complex can interrupt the G2 stage, preventing the phosphorylation of ATM (Ataxia Telangiectasia Mutated), as demonstrated by Dupree *et al.* in 2008. The absence of transgenes in Reverse

Breeding (RB) products, specifically Doubled Haploids (DHs) produced with crossover-suppressing chemicals, is a noteworthy advantage.

In situations where conventional transformation methods are challenging or impossible, alternative approaches like Virus-Induced Gene Silencing (VIGS) may be employed. VIGS has proven to be a useful method for inducing Post-Transcriptional Gene Silencing (PTGS).

For RB to be effective, complete suppression of crossover events should be avoided. It is preferable to allow some residual crossing in any chromosomal pair. This residual crossing leads to the regular segregation of the implicated homologues, doubling the probability of generating a balanced gamete. While a crossing results in two recombinant chromatids, they are ineffective for RB. The other two chromatids in the bivalent pair, which remain non-recombinant due to the crossover affecting only half of them, are valuable for the RB process.

3. Creation of Double Haploids

Doubled haploid plants can be generated through achiasmatic meiosis, and there are also alternative methods, such as anther culture and isolated microspore culture. Fig. 1 shows the homozygous diploids that resulted from crossing these haploid plants to Cenh3-1 GFP-Tailswap. Wijnker *et al.* (2012, 2014). In anther culture, immature pollen grains develop into cell colonies through tissue culture methods, while isolated microspore culture involves the development of cell colonies from microspores. These colonies are then transferred to media containing plant growth regulators and carbohydrates to stimulate shoot and root growth. The success of doubled haploid (DH) production from haploid spores varies among species. (Dupree *et al*., 2008).

4. Selection of Complementary Lines through Marker-Assisted Selection:

Complementary parents are identified and crossed to regenerate the original hybrid using Marker-Assisted Selection (MAS). Utilizing one polymorphic molecular marker per chromosome would be adequate for genotyping every Doubled Haploid (DH) under the condition of complete absence of meiotic recombination, as the entire chromosome functions as a single linkage block. However, in the presence of any residual crossovers, two markers per chromosome are necessary. Importantly, since the hybrid generated through Reverse Breeding (RB) does not incorporate a transgene, it should not be classified as genetically modified (GM).

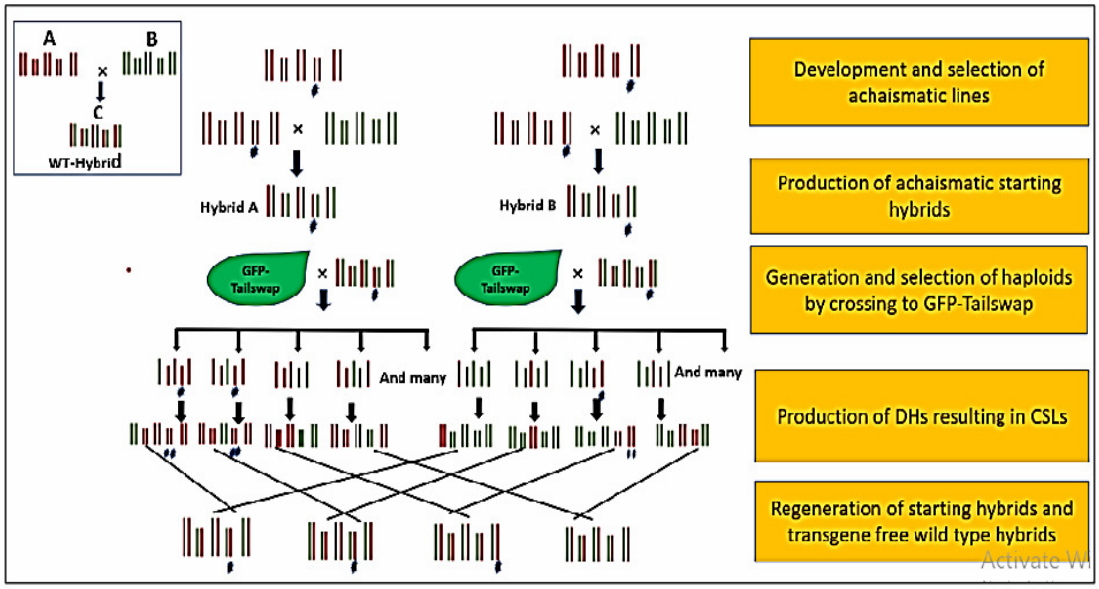

Fig. 1: Flow chart of RB for regeneration of starting hybrids (adopted from Wijnker *et al.,* 2014).

Firstly, two transgenic lines at different chromosomes viz. DMC1: RNAi are developed; crossing of resulting achaismatic lines with second accession to develop starting hybrids; haploids are generated using haploids constructs like GFP-Tailswap; generation of DHs and intercrossing of chromosome substitution lines (CSLs) to regenerate starting hybrids and WT- type hybrid with no transgene.

Applications of reverse breeding:

- RB has the capability to produce homozygous parental lines, which, when appropriately mated, result in the creation of the desired heterozygous hybrid plant.
- Breeders have the ability to perpetuate these homozygous parents indefinitely through propagation.
- The demonstrated technical feasibility in A. thaliana suggests that there is potential for applying this technique in crop improvement.
- Additionally, backcrossing in a CMS (cytoplasmic male sterility) background is an important consideration in utilizing this technique effectively.

Limitations of RB

- This technique is confined to those crops only where double haploid technology is common practice.
- Some crops, like soybean, cotton, lettuce, and tomato, exhibit limited formation of double haploids (DHs) according to Croser *et al.* (2006)
- The application of this technique is restricted to crops with a haploid chromosome count of 12 or less, or those in which spores can be regenerated into double haploids. For plants with a higher chromosome count, the impracticality of obtaining the necessary number of non-recombinant double haploids to find the complementary pair and reconstitute the original heterozygous plant is emphasized by Lusser *et al.* (2011)
- The complete homozygosity of the obtained plants precludes further selections, thereby limiting the desired genetic variation in plant breeding, as highlighted by Van Dun and Dirks (2008).

Future prospects:

- Potential advancements in RB hold promise for enhancing future crop yields by facilitating the selection and enhancement of advantageous genotypes, thus potentially boosting overall crop production.
- RB could be envisaged as a valuable tool for enhancing agricultural crops, as it may facilitate the development of parental lines necessary for hybrid regeneration.

Conclusion

While RB serves as an intermediary stage in the breeding process, its significance in crop breeding is substantial, as it plays a pivotal role in producing homozygous parental lines from intricate genotypes. Modern biotechnology has introduced new tools derived from techniques like transgenesis and marker-assisted selection, which have contributed to the development of numerous commercial varieties of agricultural crops over the last two decades. The widespread adoption and application of these techniques are now contingent on factors such as the imperative to enhance the technical efficiency of certain processes and decisions regarding their regulatory status.

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