Reverse breeding: A modern plant breeding approach for hybrid recreation

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Abstract
Reverse breeding is a modern plant breeding method for producing complementing parental lines for any heterozygous plant through achiasmatic meiosis (meiosis without crossovers). The achiasmatic meiosis leads to univalent segregation at meiotic metaphase-I and the generation of aneuploid gametes. These gametes are then regenerlated as doubled-haploid (DH) plants. Each DH carries combinations of its parental chromosomes, and complementing DH pairs can be crossed to reconstitute the initial hybrid. In reverse breeding, the suppression of meiotic crossovers in a hybrid ensures the transmission of non-recombiant chromosomes to haploid gametes. The PAIR2 gene is required for homologous chromosome synapsis at meiosis-I in plants. An insertional mutation in the rice PAIR2 gene, the ortholog of Arabidopsis thaliana ASY1, results in a defect in homologous chromosome pairing during meiosis, display univalents at metaphase-I. Essentially, reverse breeding follows an approach akin to the generation of a DH population from an F1 hybrid, carrying a dominant-acting transgene that down-regulates the expression of Disrupted Meiotic cDNA1 (DMC1), resulting in inhibition of crossover recombination and thereby enabling intact-chromosome inheritance. In earlier reports on reverse breeding in A. thaliana, a hybrid was constructed of using two of its natural ecotypes (Col-0 and Laer-0), carrying an RNAi transgene targeting the meiotic recombinase (RecA homolog) DMC1 that prevented the formation of meiotic crossover recombination. This method mainly included steps: (i) the generation and selection of RNAi: DMC1 transformed lines; (ii) the generation of achiasmatic hybrids; (iii) the crossing of achiasmatic hybrids to GFP-tailswap to generate haploid chromosome substitution lines (CSLs); (iv) the generation of DHs by spontaneous doubling of haploid CSLs; and (v) the crossing of complementing CSLs to regenerate the initial hybrid. The scope of reverse breeding could be envisioned for the improvement of agricultural crops, as it may enable the generation of parental breeding lines for the recreation of hybrid.

Keywords: Achiasmatic meiosis, double haploids, RNAi, DMC1 gene

1. Introduction
The term “Reverse breeding” was originally introduced to describe a technique in plant cell cultures, where homoygous lines are produced from heterozygous parent lines (Dirks et al., 2009; Wijnker et al. 2012) [4, 16]. Here, the term “reverse breeding” includes the earlier proposed usage but goes beyond the original definition by widening the methods used to produce homoygous lines (Palmgreen et al. 2014). Homoygous parental lines are crossed to recreate elite hybrids afresh as the hybrids are not stable. The uncharacterized heterozygotes cannot be reproduced by hybrid seed production because it leads to loss of favorable alleles combinations due to segregation in the next generation (Yi-Xin et al. 2015) [17]. Reverse breeding (RB) is a novel plant breeding method designed to produce parental lines for any heterozygous plant. It generates perfectly homoygous parents, through engineered meiosis, that when mated together produce the same heterozygote. This method eliminates the phenomena of meiotic crossover by silencing the gene responsible for the formation of chiasmata between the non-sister chromatids of homologous chromosome. RB can be executed in plants, fungus, animals but not in humans (Dirks et al. 2008). In some genetic modifications, the residues of shuttles such as bacteria and fungus are left in the host plant but with new breeding technique like RB which makes it possible to develop homoygous lines without introduced DNA sequence (Dirks et al. 2009) [4]. Neither of the authorities like ACRE
and COEGM see any justification that the resultant product produced by RB is GMOs (ACRE, 2013) \(^{[1]}\) and transgenesis is just an intermediate step to pave path for breeding and selection (Kuligowska et al. 2013) \(^{[7]}\) and with knockdown constructs such as GFP-Tailswap, on different chromosomes, multiple transgenic lines can be used to generate a full array of complementary DHs not having transgenes (Wijnker and de Jong, 2008) \(^{[13]}\) but the question arises whether the product obtained, should be considered GMO even in the absence of insert (Parisì. 2013) \(^{[10]}\). However, according to the European legislations, the progeny of GMO should be considered genetically modified whether the concerned gene is present is the succeeding generations (Hartung and Schiemann. 2014) \(^{[6]}\). RB is a new breeding technique which allows for production of new hybrid plant varieties in a much shorter time frame and ambient numbers compared to conventional plant breeding. Another reconstruction technique has been proposed called Near Reverse Breeding, in polyploids or species with high chromosome numbers that is based on the omission of the second meiotic division, which give way to unreduced second division restitution (SDR) spores. These SDR spores facilitate the near reconstruction of desired phenotypes, and also provide the possibility of obtaining CSLs (Van Dun and Dirks, 2006) \(^{[12]}\).

**Following goals could be achieved through RB:**
- To establish breeding lines for uncharacterized hybrid
- To enhance hybrid performance by genetic improvement of parental lines
- To maintain the stability of hybrid
- To maintain a highly heterozygous plant from a homozygous parental line

**Applications of RB**
- As RB can construct homozygous parental lines, that, when mated perfectly constitute the selected heterozygous hybrid plant afterwards.
- These homozygous parents can be propagated indefinitely by breeders
- The technical feasibility in A. thaliana suggests that it might be possible to apply this technique in crop improvement.
- Backcrossing in CMS background.

**2. Mechanism of RB**

**2.1 Selection of heterozygote**
A highly heterozygous plant with favorable trait combination is chosen whether its parentage is known or not. Gamete from the heterozygote is produced.

**2.2 Suppression of meiotic recombination during spore formation**
This is best achieved by dominant suppression of one of the several genes required for meiotic recombination. Recombination can be prevented or repressed by several ways, particularly through dominant transgenic accesses, dominant negative mutation or chemical treatment. RNA interference which is a post transcriptional gene silencing (PTGS) tool, is used for silencing of genes responsible for recombination. DMC1 gene which encodes the meiotic recombination protein DISRUPTED MEIOTIC cDNA1 in hybrids of A.thaliana, so that non-recombined parental chromosomes segregate during meiosis. RNA silencing being genetically dominant approach, it makes easy to obtain progeny devoid of the RNA cassette. *Brassica carinata DMC1* is 91.1 percent identical to A. thaliana DMC1. Genes required for the happening of meiotic recombination are following:
1. DMC1 gene: Disrupted Meiotic cDNA
2. SPO1 gene: Sporulation Specific gene
3. RecA gene: Recombinase A gene

Suppression of meiotic recombination is also achieved by chemical compounds like MIRIN, an inhibitor of Mre11-Rad50-Nbs1 complex. It arrests G2 stage and inhibits phosphorylation of ATM i.e. Ataxia Telangiectasia Mutated=serine/threonine protein kinase (Dupree et al., 2008).

**2.3 Generation of Double haploids**
DH technique was included for the selection of fertile selfing lines which can produce the same hybrid genotype as produced by the original parents (Wijnker et al. 2012, 2014) \(^{[15]}\). Using pollen culture technique, the resulting achaïmatic gametes are grown on suitable media to develop into adult haploid plants and the seeds harvested from these haploid plants are crossed to Cenh3-1 GFP-Tailswap resulting into homozygous diploids shown in Fig:1. (Wijnker et al. 2014) \(^{[15]}\).

**2.4 Crossing of complementary parents**
Using Marker Assisted Selection (MAS), the complementary parents are detected and they are crossed to regenerate the initial hybrid. In the condition of complete deprivation of meiotic recombination one polymorphic molecular marker per chromosome would be sufficient to genotype every DH as the entire chromosome would behave as a single linkage block and if there is a presence of any residual crossovers, two markers are required per chromosome (Dirks et al. 2009) \(^{[4]}\).

The hybrid obtained through RB does not carry the transgene and hence they should not be considered as GM.

**2.5 Marker Assisted Reverse Breeding in Maize**
In maize, chip-based SNP genotyping was done for the selection of homozygous plants similar to the two parents, so it is named as marker-assisted reverse breeding (MARB). This breeding procedure took four crop seasons each with a cycle of marker-assisted selection completed in a year. The maternal and paternal inbreds developed look phenotypically similar to those from two standard US heterotic groups, Lancaster and Reid, respectively. RMRB and MARB take same span of time due to population development and chip screening. Both RB methods are more efficient than that of conventional breeding which take six–ten years to produce homozygous parental lines (Yi-Xin et al. 2015) \(^{[17]}\).
2.6 Difference of end product of conventional and reverse bred crops

- The end products of reverse bred crops are as similar as parental lines obtained by conventional breeding.
- RNAi silencing is confined to only meiotic crossover; there will be no change in the DNA sequence. The products are safe to use.
- Reverse bred crops are non-genetically modified so, there is no bioethical issue.

3. Limitations of RB

- This technique is confined to those crops only where double haploid technology is common practice.
- There are some exceptions such as soybean, cotton, lettuce and tomato where DHs is barely formed (Croser et al. 2006) [2].
- It is confined to crops having haploid chromosome no. of 12 or less than it or in which spores can be regenerated into DHs. In the plants having higher number of chromosomes, the number of non-recombinant double haploids needed for searching the complementary pair that reconstitute the original heterozygous plant would be extremely high and practically not feasible (Lusser et al. 2011) [9].
- Due to the complete homozygosity of the received plants there is no room for further selections which limits the genetic variation wanted in plant breeding (Van Dun and Dirks, 2008).

4. Future prospects

- New possibilities for the selection and improvement of favorable genotypes by RB may contribute to increasing future crop production.
- The scope of RB could be envisioned for the improvement of agricultural crops, as it may enable the generation of parental lines for the recreation of hybrids.

5. Conclusions

Though, RB is used as an intermediate step of the breeding process, but it has huge implication in crop breeding as it generates homozoygous parental lines from complex genotypes. Transgenesis and marker-assisted selection techniques behind many commercial varieties of agricultural crops produced in the last two decades are now have new tools derived from modern biotechnology. Now-days it is believed that the extent of the adoption and the application of the techniques will depend on factors such as the need to increase the technical efficiency of some processes and the decisions on related-regulatory status.

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