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Reverse Breeding–A Breakthrough Approach of Modern Plant Breeding

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Abstract—*In conventional plant breeding, a large number of inbred lines are evaluated to identify best combining lines which produce high heterosis in a series of crosses. This approach involves randomness in identification of heterosis in progenies making it sometimes less reliable. While in some cases elite or high performing heterozygous lines that have undefined parentage may be identified in a breeding programme. To overcome these obstacles in hybrid breeding, an alternative strategy based on the reversal of crop selection, the generation of defined populations with high levels of heterozygosity and random variation was proposed. This is reverse breeding. Large population of plants can be screened and well defined plants can be regenerated indefinitely even without prior knowledge of their parentage. This technique enables the breeder to go backwards from F_1 hybrid to directly recreate the homozygous parental lines that created it. Having its own advantage in the fixation of unknown heterozygous genotypes, application of reverse breeding could profoundly change looming plant breeding.*

1. INTRODUCTION

From times immemorial, cultivation of plants for human survival is a time-honoured practise. Domestication of plants has been initiated by the pre-historic humans somewhere about 1000 decades ago. To feed the ever-growing population, breeders started developing high- yielding varieties and hybrids gradually. In the last century, fast development of some of the crops like maize (*Zea mays*) was noticed due to the development of single and double cross hybrids. The diversity in the germplasm available was exploited to increase the yield potential. In the process of development of hybrids, selfing is done to develop homozygous inbred lines. Later on heterosis is achieved through combining lines from different heterotic groups. This method exploits the heterozygosity advantage to develop the vigorous F_1 hybrids. However, a favourable heterozygous genotype cannot be stably propagated through hybrid seeds because parental chromosomes may recombine when they pass on to progeny [1]. As the hybrids possess the limitation of being stable, breeders have to recreate elite hybrids afresh through crossing homozygous parental lines. Thus, one of the way to enhance the hybrid performance, is to boost the genetic improvement of parental lines. The barrier to utilizing the entire genetics content in elite hybrid crops in current plant breeding programs is that uncharacterized heterozygotes are difficult to reproduce by hybrid seed propagation. Favorable allele combinations of the elite heterozygote are lost in the next generation due to segregation of traits. Thus the development of methods for easy preservation or regeneration of heterozygous genotypes is one of the greatest challenges in breeding hybrid varieties. Clonal propagation (or apomixis) allows for the preservation of the parental genotype [2], but its further genetic gain can be only achieved through improvement of parental lines. For many years, breeding procedures started from hybrid materials have been mainly depending on the experience of breeders and the yield testing of their progenies. In some cases, desirable heterozygous plants of undefined parentage may be identified in the population. If these heterozygous plants can be reconstituted, they can be of immense value to the farming community. In such cases, new approaches of plant breeding has to be developed. Reverse breeding is one such approach which can answer this problem. This review contemplates on the use of reverse breeding in plants, procedure and its applications.

2. REVERSE BREEDING:

Reverse breeding is a method in which the homozygous parental lines can be reconstituted from the heterozygous plant by crossing. It provides more flexibility in combining the desirable traits in the heterozygous offspring. Reverse breeding, meets the challenge of fixation of complex heterozygous genomes by constructing complementing homozygous lines [3]. 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 [4].

The procedure of reverse breeding involves two main steps:

i. In the selected desirable heterozygous plant, meiotic recombination is made to suppress

ii. Regeneration of the doubled haploids from the spores containing non-recombinant chromosomes.

The basic idea of reverse breeding is to inactivate the chromosome recombinase, so as to eliminate chromosome recombination in the segregating progenies of the hybrid. It uses a genetic modification step to suppress the recombination of chromosomes, followed by specific tissue culture to create homozygous parent lines. These lines are then used to stably produce the heterozygous elite plants through seed which is depicted in figure 1 (Source : Dirks et al. 2009[4]).

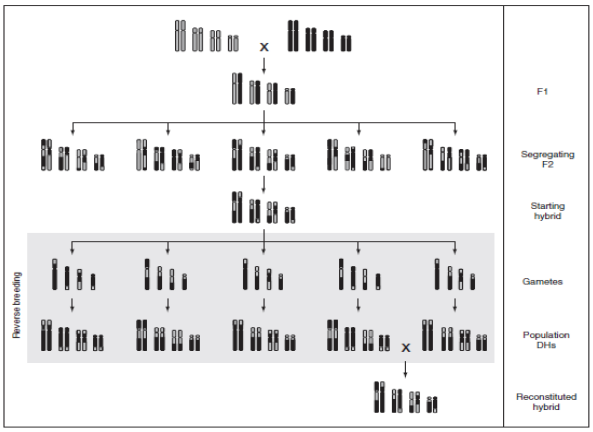

Figure 1: Reverse breeding can be used to fix unknown heterozygotes. Crossing two homozygous parents (grey and black bars) creates a heterozygous F1. When selfed, the F1 produces a segregating F2 population. A starting hybrid of unknown genetic constitution is selected for its desirable characteristics, and subjected to the two steps of reverse breeding (grey box) (Source: Dirks et al. 2009 [4])

2.1. Suppression of meiotic recombination:

Reverse breeding is based on suppression of crossover formation by RNAi or comparable gene silencing techniques. Using the knowledge of the molecular mechanisms underlying unreduced gamete formation and the genes involved in the model plant Arabidopsis, it is now possible to develop strategies to induce unreduced gamete formation in desirable crop species through the targeted knockdown of specific proteins. Techniques that involve knockdown of RNA levels, such as RNA interference (RNAi), virusinduced gene silencing (VIGS; [13,14,15,16]), or mutagenesis of the encoding gene by techniques such as site-directed mutagenesis through zinc finger nucleases [17,18] or tilling [19,20], could be used to knock down the level of the selected protein. Reverse breeding relies on the effective suppression of meiotic crossovers [4]. Studies have shown that RNAi silencing of essential early meiotic genes, such as DISRUPTED MEIOTIC CDNA1 (DMC1), can lead to (almost) complete suppression of crossover formation [5,6]. Consequently, homologues are not joined by chiasmata (the physical manifestation of crossing over) during meiotic prophase I and remain as univalents at anaphase I. These univalents (non-recombinant parental chromosomes) then segregate randomly to daughter cells during the first meiotic division [7]. Most resulting spores will be unbalanced, containing either none, one or two copies of a given chromosome. However, balanced spores, containing one copy of each chromosome, will be formed at a probability of $(1/2)^x$, where x equals the basic chromosome number. Consequently, the chance of obtaining balanced spores decreases exponentially with the chromosome number and seems feasible for species in which the chromosome number equals 12 or less [3]. In reverse breeding, any given elite heterozygote is transformed using an RNAi construct targeting a gene that encodes a protein that mediates the formation of crossovers. The resulting plant is expected to produce low numbers of viable balanced haploid spores that are then regenerated into doubled haploid, perfectly homozygous plants. Other spores with an unbalanced chromosome number will, if they are still viable, produce aneuploid individuals with poor regeneration rate and vigour. Among the doubled haploids, parents with complementary genotypes can be recruited that, upon crossing, will reconstitute the exact genotype of the elite hybrid again. In Arabidopsis, various mutants that lack crossovers (almost) exclusively produce univalents [8,9], although their chromosome behaviour during meiotic prophase can be different. It was recently shown that in univalent-producing mutants (desynaptic1 [dsy1], meiotic prophase amonipeptidase1 [mpa1]) in which chromosomes pair normally during prophase, univalents segregate preferentially to opposite poles during metaphase I [10]. This suggests that pairing, even without chiasma formation, to some extent orients homologues to opposite poles. Targeting such genes for reverse breeding might be fruitful because the chance of recovering balanced gametes increases substantially. As such, genes such as PARTING DANCERS (PTD), for which the mutant shows complete pairing and few residual crossovers next to high levels of univalents [11], might also be of interest to reverse breeding. The benefit of its regular segregation might well outweigh the downside of few remaining crossovers[12].

2.2. Regeneration of the doubled haploids from the spores containing non-recombinant chromosomes:

Doubled haploid plants resulting from achiasmatic meiosis can be obtained by gynogenesis (from unfertilized ovules) or by androgenesis (from microspore and anther cultures), according to well established protocols that have been developed for a variety of plant species including crops [21]. The efficiency of DH formation from haploid spores is species dependent [22]. The unique characteristic of DHs made from spores produced through achiasmatic meiosis is explained in Figure 1: they contain non recombinant parental chromosomes[4]. Note

however that aneuploid unfertile spores, which are in fact most prevalent, were not depicted. Selection of the required euploidspores is in part automatically achieved since only spores containing at least one copy of all chromosomes can pass through all developmental stages, from cell division and embryogenesis to plant regeneration. Hyperploid offspring could be selected against using co-dominant markers or flow cytometry. 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 [23,22]. 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 [24,25,26]. Genotyping of DHs by molecular markers is routine practice in contemporary plant breeding [27] 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. The final step of reverse breeding is crossing appropriate DH lines on the basis of matching molecular markers to develop desirable superior hybrids. The general procedure of RNAi mediated reverse breeding is depicted schematically in figure 2.

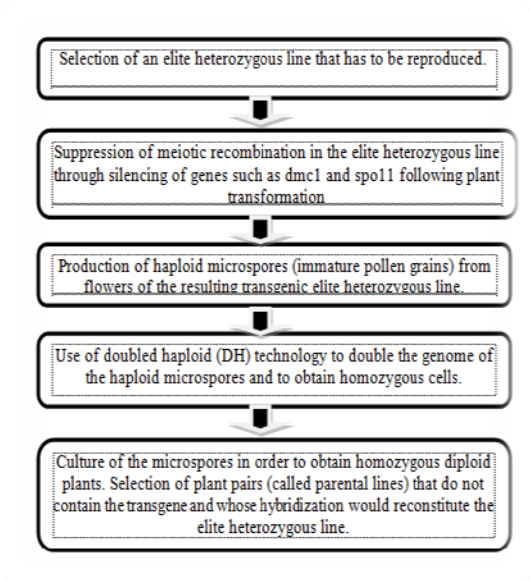

Figure 2: General procedure of RNAi mediated reverse breeding.

3. GENERAL PROTOCOL OF MARKER-ASSISTED REVER SEBREEDING [28]:

(1) Extract DNA from seed embryo and pericarp of a selected elite hybrid separately.

(2) Select genotyping platform and molecular markers that provide high density of genome coverage with high throughput genotyping available.

(3) Genotype the seed embryo and pericarp DNA samples to derive the parental genotypes. Since all the pericarp DNA of a hybrid is from the maternal parent, whereas one half of the embryo DNA is from the maternal parent and the other half from the paternal parent, we can derive the high-density genotypes of the two parents based on the embryo and pericarp genotypes of the hybrid.

(4) Select a subset of markers that are polymorphic between the parental genotypes for the following marker-assisted selection.

(5) Self the hybrid F_1 to generate F_2seeds and genotype the F_2 seeds or plants with the subset markers to identify the progeny with the highest levels of similarity to their maternal and paternal genotypes, respectively.

(6) Self the selected F_2 plants to get F_2-derived F_3families, and continue with selection among F_3 seeds or plants to identify the progeny with the highest levels of similarity to their maternal and paternal genotypes, respectively.

(7) Self the selected F_3 plants to get F_3-derived F_4families, and continue with selection among F_4seeds or plants to identify the progeny with the highest levels of similarity to their maternal and paternal genotypes, respectively.

(8) Move to the next stage or continue with marker-assisted selection until the selected progeny reach a desirable level of similarity to the parental lines.

(9) Use DH technology or continue with selfing to obtain fixed genotypes.

4. APPLICATIONS OF REVERSE BREEDING:

4.1. 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 (also see Figure 1). The number of DHs that is required is surprisingly low. For instance in maize (x = 10) just 98 DHs are expected to contain a set of two reciprocal genotypes (P = 99%)[4].

4.2. Generation of chromosome-substitution lines:

The procedure for the generation of chromosome-substitution lines is depicted in the figure 3 [4].

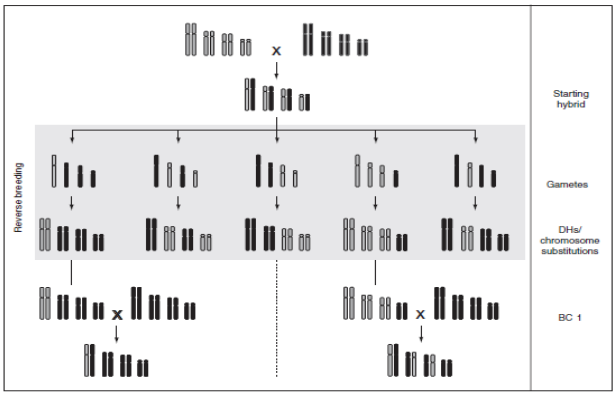

Figure 3: Schematic diagram depicting the procedure for developing chromosome-substitution lines using reverse breeding [4].

4.3. 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 [4]. The success in application of MARB in maize suggests that this technology is applicable to any hybrid crop to breed new inbredswith improved hybrid performance but the same heteroticmode[28].

5. LIMITATIONS OF REVERSE BREEDING:

- Mostly suited for the vegetatively propagated crops where elite heterozygous varieties exist without inbred parents
- It is applicable for the plants with a chromosome number less than 12.
- When a species with a large number of chromosomes is used, many of the gametes will be unbalanced and either unviable or producing aneuploidy individuals with poor vigor.
- Requires molecular markers for all of the chromosomes.
- Development of RB is limited to those crops where DH technology is common practice eg. Cucumber, onion, broccoli, sugarbeet, maize, pea, sorghum.
- There are, some exceptions such as soybean, cotton, lettuce and tomato where doubled haploid plants are rarely formed or not available at all.

6. CONCLUSION

Plant breeding is progressing towards a new path. In such cases, advanced breeding approaches and tools have to be followed. One such technique which can provide a solution for the hit-or-miss nature of production of hybrids by conventional breeding is reverse breeding. One important application is the production of complementary homozygous lines that can be used to generate specific F_1 hybrids. Additionally, when RB is applied to F_1 heterozygotes, it is possible to generate chromosome substitution lines that allow targeted breeding on the single chromosome scale. RB is fully compatible with commercial CMS lines that are frequently used in modern agriculture[4].Further progress in the field of RNAi silencing and DH technology can provide the solution for the limitations of reverse breeding.

REFERENCES

[1] Wijnker E, van Dun K, de Snoo C B, Lelivelt C L C, Keurentjes J J B, Naharudin N S, Ravi M, Chan S W L, de Jong H, Dirks R. Reverse breeding in *Arabidopsisthaliana* generates homozygous parental lines from a heterozygous plant. *NatureGenetics 2012*, 44: 467-470.

[2] Van Dijk P and Van Damme J. Apomixis technology and the paradox of sex. *Trends in Plant Science,2000*.**5**: 81-84.

[3] Dirks, R.H.G., van Dun, C.M.P. and Reinink, K. RijkZwaanZaadteelt en Zaadhandel B.V. Reverse Breeding,WO⁄ 2003⁄017753. 2003.

[4] Dirks, R. *et al.* Reverse breeding: a novel breeding approach based on engineered meiosis. *Plant Biotechnol. J.* 2009.**7**, 837–845 .

[5] Siaud, N. et al. Brca2 is involved in meiosis in Arabidopsis thaliana as suggested by its interaction with Dmc1.EMBOJ. 2004.23, 1392–1401.

[6] Higgins, J.D. et al. The Arabidopsis MutS homolog AtMSH4 functions at an early step in recombination: evidence for two classes of recombination in Arabidopsis. Genes Dev.2004. 18, 2557–2570.

[7] Couteau, F. et al. Random chromosome segregation without meiotic arrest in both male and female meiocytes of a dmc1 mutant of Arabidopsis. Plant Cell.1999. 11, 1623–1634.

[8] Ma, H. A molecular portrait of Arabidopsis meiosis. In The Arabidopsis Book (Somerville, C.R. and Meyerowitz, E.M., eds), pp. 1–39, American Society of Plant Biologists,2006.

[9] Mercier, R. and Grelon, M. Meiosis in plants: ten years of gene discovery. Cytogenet. Genome Res. 2008. 120, 281–290.

[10] Pradillo, M. et al. An analysis of univalent segregation in meiotic mutants of Arabidopsis thaliana: a possible role forsynaptonemal complex. Genetics. 2007. 175, 505–511.

[11] Wijeratne, A.J. et al. The Arabidopsis thaliana PARTING DANCERS gene encoding a novel protein is required for normal meiotic homologous recombination. Mol. Biol. Cell. 2006. 17, 1331–1343.

[12] T.G. (Erik) Wijnker. Meiotic recombination and its implications for plant breeding. Wageningen University. Wageningen, NL. 2013. ISBN 978-94-6173-440-2.

[13] Kusaba M. RNA interference in crop plants. Current Opinion in Biotechnology. 2004.15, 139–143.

[14] Robertson D. VIGS vectors for gene silencing: many targets, many tools. Annual Review of Plant Biology, 2004.55, 495–519.

[15] Galun E. RNA silencing in plants. In Vitro Cellular and Developmental Biology–Plant. 2005.41, 113–123.

[16] Angaji SA, Hedayati SS, Hoseinpoor R, Samadpoor SS, Shiravi S, Madani S. Application of RNA interference in plants. Plant Omics Journal. 2010.3, 77–84.

[17] Townsend JA, Wright DA, Winfrey RJ, Fu F, Maeder ML, Joung JK, Voytas DF. High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature. 2009.459, 442–445.
[18] Shukla VK, Doyon Y, Miller JC, et al. Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. Nature. 2009.459, 437–441.
[19] Stemple DL. TILLING—a high-throughput harvest for functional genomics. Nature Reviews Genetics. 2004.5, 145–150.
[20] Barkley NA, Wang ML. Application of TILLING and EcoTILLING as reverse genetic approaches to elucidate the function of genes in plants and animals. Current Genomics. 2008. 9, 212–226.
[21] Jain, S., Sopory, S.K. and Veilleux, R.E. In vitro haploid production in higher plantsIn Current Plant Science and Biotechnology in Agriculture, Dordrecht, Boston, London:Kluwer Academic Publishers.1996.
[22] Forster, B.P., Heberle-Bors, E., Kasha, K.J. and Touraev, A. The resurgence of haploids in higher plants. Trends Plant Sci.2007.12, 368–375.
[23] Maluszynski, M., Kasha, K.J. and Szarejko, I. Published protocols for other crop plant species. In Doubled Haploid Production in Crop Plants: A Manual (Maluszynski, M., Kasha, K.J., Forster, B.P. and Szarejko, I., eds), pp. 309–336, Dordrecht, the Netherlands: Kluwer Academic Publishers.2003.
[24] Croser, J., Lu¨ lsdorf, M., Davies, P., Clarke, H., Bayliss, K., Mallikarjuna, N. and Siddique, K. Toward doubled haploid production in the Fabaceae: progress, constraints, and opportunities. Crit. Rev. Plant Sci.2006. 25, 139–157.
[25] Segui-Simarro, J.M. and Nuez, F. Embryogenesis induction, callogenesis, and plant regeneration by in vitro culture of tomato isolated microspores and whole anthers. J. Exp. Bot. 2007. 58,1119–1132.
[26] Zhang, X, Jin, S, Liu, D, Lin, Z, Zeng, F, Zhu, L, Tu, L and Guo, X. Cotton biotechnology: challenge the future for cotton improvement. In Advances in Plant Biotechnology (Rao, G.P., Zhao, Y., Radchuk, V.V. and Bhatnagar, S.K., eds), pp. 241-301. Houston, Texas: Studium Press LLC. 2008.
[27] De Vienne, D. Molecular Markers in Plant Genetics and Biotechnology. Enfield, NH, USA: Science Publishers, Inc. 2003.
[28] Yi-Xin, G., Bao-hua, W., Yan F., Ping L. Development and application of marker-assisted reverse breeding using hybrid maize germplasm. *J Integr Agri.*2015.14(12): 2538-2546.