THEORETICAL BIOLOGY FORUM

TBF: 365–376 (2023)
Review Article

Reverse Breeding and Genetics for Targeted Heterosis and Functional Plant Genomics

Shivam Sharma[1*], Sonia Sood[2], D.R. Chaudhary[3], Viveka Katoch[4], Harish B.M.[5], Neha Sharma[6], Shubham Thakur[7], Anuj Choudhary[8*]

[1,5,6]Ph.D. Scholar, Department of Vegetable Science and Floriculture, CSK HPKV Palampur (H.P)-176062
[2]Professor, Department of Vegetable Science and Floriculture, CSK HPKV Palampur (H.P) - 176062
[3]Professor and Head, Department of Vegetable Science and Floriculture, CSK HPKV Palampur (H.P)-176062
[4]Principal Vegetable Breeder, Department of Seed Science and Technology, CSK HPKV Palampur (H.P)-176062
[7]Department of Genetics and Plant Breeding, CSK HPKV Palampur (H.P)-176062
[8]Department of Biology and Environmental Sciences, CSK HPKV Palampur (H.P)-176062

ABSTRACT

Hunger and malnutrition are major issues that plague much of the world's population of almost 7.5 billion and are estimated to be up to 9 billion by 2050. So, needs new strategies to feed the world. The most expected outcome of the heterosis breeding program is to get a hybrid superior in traits of interest i.e., yield, quality, and growth characteristics. But this statement is not true for always. In addition, they can be inferior and the underlying mechanism behind this is still unknown and understood to date limiting hybrid stability. In this review, we focussed on a novel plant breeding strategy using engineered meiosis i.e., gene silencing to create 100% true homozygous parental lines instead of nearly homozygous inbred lines of conventional plant breeding programs along with genetic tools of reverse genetics i.e., insertional mutagenesis, ectopic expression, and Target Induced Local Lesions in Genome (TILLING) for functional plant genomics.

Keywords- *RNAi, VIGS, TILLING, Reverse Breeding, Reverse Genetics*

ARTICLE HISTORY
03 November 2022: Received
22 February 2023: Revised
10 May 2023: Accepted
27 July 2023: Available Online

DOI:
https://doi.org/10.61739/TBF.2023.12.2.365

CORRESPONDING AUTHOR: **Shivam Sharma & Anuj Choudhary**

E-MAIL ID: **ajchoudhary784@gmail.com**

1. Introduction

The most expected outcome of the heterosis breeding program is to get a hybrid superior in traits of interest i.e., yield, quality, and growth characteristics. But this statement is not true for always. In addition, they can be inferior and the underlying mechanism behind this is still unknown and understood to date limiting hybrid stability. So, we can consider this approach as an unpredictable type of approach limiting outcomes of heterosis breeding. In this review, our focus is on discussing the efficiency of this novel plant breeding strategy using engineered meiosis i.e., reverse breeding utilizing gene silencing mechanism to suppress meiotic recombination and focus on the trait of interest [1]. This strategy uses 100% true homozygous parental lines instead of nearly homozygous inbred lines of conventional plant breeding programs [2]. The prime objective of any plant breeding program is to get a sufficient amount of variation. The main limitation behind the picture is the maintenance of unknown heterozygotes through seeds. Desired favorable alleles might be lost in the F_2 generation due to segregation. Because of this fact, we can think of a novel plant breeding technique i.e., reverse breeding. Although, the conventional breeding program contributed to various agronomic traits improvements like yield and quality skilled labor, time and capital investment is the limiting factor to date which is the main cause of lagging for further improvement. These breeding techniques require a large time period (4-8 years) to generate the new desirable traits in plants. With the help of reverse breeding, we can produce the ambient number of new desirable hybrids in a shorter time i.e., 1.5-2 years.

Hybrid vigor is a must to generate/produce highly productive and potentially farmer/consumer-preferred crop varieties [3]. Although, the conventional breeding program allows to development of homozygous inbred lines through continuously selfing homozygous inbred lines developed by this strategy are not 100% homozygous i.e., nearly homozygous. In addition, desirable heterozygous accession stability via a hybrid seed multiplication program is not possible at all due to segregation in the F_2 generation [4]. Due to this fact, we have to focus on the genetic improvement of parental lines for enhancing hybrid vigor and thereby, improving outcomes of heterosis breeding. However, the development of apomictic lines can be used to reserve heterozygous phenotypes, but to date, it is neither applied commercially nor contributed to any significant crop improvement program as achievement of genetic gain can be possible through parental lines improvement [5].

From the earliest time to till date breeding strategy for heterosis breeding is still dependent on the plant breeder's experience and outcomes of the progeny test in which individual plant progenies are evaluated. Further for distinguishing different heterotic groups test cross of selected progenies with their tester is done. This pattern makes it so long i.e., taking a minimum period of 6-7 years with the possibility that the heterotic pattern may be changed with the course of time having low fixation frequency of favorable genes and their

combinations in desirable populations.

Production of complementing homozygous parental lines through gene silencing is the leading goal of reverse breeding in which gene silencing is employed to remove meiotic crossing over by targeting specific genes involved in the formation of chiasmata and synaptonemal complex i.e., *MSH5*; *SmDMC1* in *Solanum melongena*, *BoMSH5*; *BoDMC1* in *Brassica oleracea* and *LeDMC1* in *Solanum lycopersicon* [6]. The reverse breeding concept is based on the selection of an "end product" obtained through traditional breeding at the start of the breeding cycle [7].

Hence it cracks the limitation of hybrid instability by producing complementing homozygous parental lines [6]. This is achieved by targeting genes responsible for crossover formation followed by the selection of non-recombinants from homozygous doubled haploid lines. It also facilitates the production of chromosome substitution lines (CSLs) to evaluate epistatic interactions at the individual chromosome level. Unlike conventional breeding programs, it generates 100% true homozygous parental lines instead of nearly homozygous. Hence this technique is going to be useful in planning productive heterosis breeding programs it adds an extra bit of efficacy when compared with conventional breeding programs.

Moreover, reverse genetics deals with the integration of desirable genetic sequences having specific effects on living organisms and helps to study plant functional genomics by which several plant genomes are already sequenced [8]. Being a powerful tool, it forms a direct connection between biochemical gene product function and its application in vivo conditions [9]. This strategy begins with DNA or protein lacking genetic information to end up with a mutant phenotype carrying a mutant gene [10]. Hence both techniques together not only favor stability fixation of unknown heterozygotes but also allow additional applications in terms of plant functional genomics and chromosomal substitution lines providing an advantage over clonal propagation for the future crop improvement program.

2. Mechanism

Achiasmatic meiosis, i.e., meiosis without the chiasmata formation is the main mechanism behind reverse breeding [Fig.1]. There will be no homologous chromosome pairing, no bivalent formation, no crossing over, and hence no recombination, and all this is achieved through gene silencing [11]. In angiosperms, synapsis favors the generation of cross-over in prophase I of meiotic division. The whole process is mediated by a proteinaceous structure known as a synaptonemal complex. During the pachytene stage, crossing over results in the joining of 2 homologous chromosomes to form bivalents. Chiasmata represents the site of crossovers after the formation of the synaptonemal complex and remained the same until anaphase came followed by their movement to opposite poles.

Achiasmatic meiosis occurs during meiosis I, whereas meiosis II proceeds normally. Allele recombination is influenced by crossing over followed by homologous chromosome orientation at the metaphase plate. On the other side, achiasmatic chromosomes do not form recombinants and remain univalent. Unlike univalent, bivalent favors the movement of homologues toward opposite poles instead of the same pole due to a lack of chiasmata in univalent [12].

Here our main motive is to clear that the presence of a single crossing-over does not provide any limitation in the utilization of a successful reverse breeding program. These bivalents segregate towards opposite poles. There are other homologous which remain univalent. These univalents segregate randomly towards the opposite poles. With the end of Telophase II, only 50 % of gametes are desirable i.e., out of 4 gametes, 2 will be recombinant while the rest 2 non-recombinants are used further for running a desirable reverse breeding program. In normal meiosis, the bivalent segregates towards the opposite poles. But in the case of achiasmatic meiosis, there is a non-disjunction of homologous chromosomes, which means univalent segregate randomly towards any pole. Here, both the univalent move towards the same pole which results in the production of unbalanced gametes which are sterile and of no use in reverse breeding programs.

2.1 Probability of getting balanced gametes

Considering a situation where every formed univalent has the same chance of migration to opposite poles, then in that case probability of getting a balanced chromosome complement will be $(1/2^x)$, with, X= haploid set of chromosomes. *Arabidopsis thaliana* (2x=10) is considered as "Drosophila of Plant Kingdom" as multiple genes have been transferred to brassicas, probability of getting a balanced gamete will be $(1/2^5)$, i.e., one out of 32 with a 3% success rate. Hence success rate of reverse breeding gets declines when the basic chromosome number exceeds 12 as a large screening population is required with a known probability of less success rate appears to be a quite risky factor limiting the success of reverse breeding.

2.2 Probability of getting complementing homozygous parent lines

1. 2^x (X= basic chromosome no.) represents the highest DHs in numbers gained from a diploid heterozygous plant in the reverse breeding experiment (Table 1).
2. So, the possibility of getting 2 double haploids (DHs) to form a pair of "complementing homozygous" parental lines will be $(½)^x$.
3. The probability that if any two DHs, when crossed with each other then there will be no possibility of genetic reconstruction of the parent used in the program is = $(1 - 1/2^x)$.
4. Moreover, n (n-1) / 2. (n= no. of double haploids) will be the probability of getting no. of combination with different double haploids (DHs) with the condition that on attempting reciprocal crosses it will generate the same phenotype.
5. The possibility of getting no complementary doubled haploids (DHs) within the screening population will be $[(2^{x-1})/2^{x]n(n-1)/2}]$.
6. Out of 2 DHs, the frequency of getting 1 complementing homozygous parental line combination will be $1 - [(2^x-1) / 2^x]^{n(n-1)/2}]$. Hence huge population is required to be screened to get balanced gametes

3. Reverse breeding and genetics strategies

Reverse breeding and genetics for studying plant functional genomics involve different strategies i.e., gene silencing, ectopic expression, insertional mutagenesis, and TILLING, having their specific benefits and bottlenecks (Table 2). Based on the goal of reverse genetics these approaches must be selected carefully for a successful plant functional genomics program.

3.1 Gene silencing strategies i.e., RNA interference (RNAi), Dominant-negative mutations (DNMs), Virus-Induced Gene Silencing (VIGS), and Graft transmission

3.2 Insertional mutagenesis i.e., Transposon and T-DNA

3.3 Target Induced Local Lesions in Genome (TILLING)

3.1 Gene silencing strategies: Reverse breeding is employed to identify parental lines of unknown heterozygotes i.e., requires gene silencing strategies for suppression of meiotic recombination [28]. First of all, we select the elite heterozygous plant and it is then subjected to the suppression of meiotic recombination so that the desired allelic combinations can be reconstituted. New allelic combinations will not be formed by suppressing the crossing over. As a result, we obtain the spores/gametes containing random combinations of maternally or paternally inherited chromosomes [6].

3.1.1 Suppression of meiotic recombination: It can be done either by producing gametes directly from heterozygotes or, by suppressing of recombination during spore formation. The latter can be achieved by suppressing genes involved in meiotic recombination which further involves four types: a) RNA interference (RNAi)/siRNAs, in which gene silencing works at the post-transcriptional stage,(b) Dominant-negative mutation,(c) Virus-induced gene silencing (VIGS) i.e., used in crops like cotton, tomato, lettuce and soybean where getting stable transformants is a difficult task (d) Graft transmission i.e., silencing molecules from rootstock are transferred to scion at the site of action and (e) Recombination inhibiting chemicals.

a) RNA interference (RNAi)

RNAi in eukaryotes sequence-specific nucleotides regulate expression of a gene either at transcriptional/TGS or post-transcriptional level gene sequencing/PTGS which get activated when RNA becomes double-stranded i.e., in nature, this takes place due to pathogenic attack in response to natural defense system [29], [30], [31]. With the help of RNAi target genes involved in the construction of chiasmata, chromosome pairing, and gene conversion are silenced [32] Table 3). RNAi induces PTGS (Post Transcriptional Gene Silencing). Moreover, the most essential elements of the RNAi pathway are different proteins viz., Argonaute (AGO), and Dicers for their antiviral role at different levels [33], [34], [35]. Andrew Fire and Craig Mello got the "Noble Prize in Physiology or Medicine" in 2006 for the detection of RNAi mechanism in nematodes i.e., *Caenorhabditis elegans*. In addition, "Science Magazine" 2002 named it "Technology/Breakthrough of the Year" owing to its importance. Moreover, it can be used for biotic and abiotic disease resistance i.e., [36] developed potato virus X (PVX) varieties namely "Katahdin, Russet & Burbank" by targeting/silencing the VbMS gene. Moreover, the intensity of RNAi gene silencing can be altered by the presence of biochemicals (enhancer/retarder) present within the plant system. [37] in their study concluded that the vast majority of chemicals were enhancers namely, ortin1 andisoxazolone providing the most significant and consistent result in *Arabidopsis thaliana* making it "Drosophila of Plant Kingdom" and the most suitable for successful reverse breeding, and genetics program.

b) Dominant-negative mutations

Antimorphs (dominant-negative mutations) inhibit the target gene expression when interacting with the target gene [38]. Overexpression of these antimorphs leads to the formation of malfunctioned subunits. These malfunctioned subunits then interact with the target gene leading to the formation of a poisoned dimer (which is an otherwise normal subunit that leads to the formation of a dimer). The poisoned dimer then leads to the inhibition of target gene expression [39]. Here, genes encoding oligomeric protein are targeted leading to truncated polypeptide formation. These are deleterious and lead to specific loss of function. [40] also found a reduced amount of water loss as compared to control on overexpression of dominant-negative mutant gene *OsKAT2* (*T235R*) reflecting the importance of dominant-negative mutation in plant functional genomics.

c) Virus-induced gene silencing (VIGS)

A. van Kammen was the first person to use "VIGS" to explain the event of recovery from virus infection [41]. Although RNAi is highly efficient and commercially utilized in the majority of crops cotton, lettuce, and tomato, getting stable transformants for the meaningful reverse breeding program is still lacking. In that case, Virus-induced gene silencing (VIGS) proves an effective and efficient tool for functional plant genomics in which at least 50 VIGS vectors are already designed to date which majority of them were developed for dicotyledonous plants i.e., RNA-based (**Table 4**; [1], [57]). Upon plant infection with the virus, defense response gets activated cleaving viral RNA with type III endonucleases i.e., DICER-like enzyme yield give rise to multiple copies of siRNA which act on plant endogenous mRNA with RNA interfering silence complex i.e., RISC [58], [59].

d) Transmission by grafting

Suppression of target gene expression occurs in the scion. siRNA and miRNA move from GM rootstock to non-GM scion and then lead to gene silencing in the scion. Grafted tobacco rootstock reflecting RNA-directed DNA methylation (RdDM) against cauliflower mosaic virus (CaMV) on non-transgenic scion revealed a low level of DNA methylation for *CaMV 35S* promoter in the scion [60]. On the contrary, [61] concluded that variation in vascular cambium with cytoskeleton from cell to cell via plasmodesmata determines the frequency and success rate of gene silencing varying from crop to crop. A grafting experiment by [48] reflected that the presence of DICER-LIKE2 (*DCL2*) is a must in scion to receive the signal from the transgenic rootstock of *Nicotiana benthamiana* to carry out successful post-transcriptional gene silencing (PTGS). ACC oxidase 1 (*ACO1*) overexpression lines of tomato produced siRNA when grafted on transgenic rootstock enhancing gene silencing in pre-existed gene silencing plants [62]. Continuing the same result, [63] evaluated the *ACO1* antisense (AS) line with an upgraded level of antisense *ACO1* transgene mRNA while the downgraded level of siRNAs to strong *ACO1* gene silenced rootstock revealed a decreased level of AS mRNA i.e., 2 weeks after grafting.

3.1.2 Production of Doubled Haploids

Spores/gametes give rise to haploid plants which are sterile and these haploid plants are then subjected to the formation of doubled haploids [Fig.2; 64]. These doubled haploids are the lines containing random combinations of maternally or paternally inherited chromosomes [65]. According to well-established protocols various in-vitro and in-vivo methods can be utilized [66]. In-vitro methods included (i) Androgenesis: Anther-Pollen culture [67], **(**ii) Gynogenesis: Ovary-Ovule culture, and (iii) Embryogenesis while in-vivo methods include haploidization by wide and intra-specific crosses. Colchicine treatment is given to the obtained haploid plantlets and embryos to obtain doubled haploid plants [68].

3.1.3 Screening homozygous complimentary parental lines through marker-assisted selection

The various Doubled Haploid Lines (DHLs) are obtained. Among these, we have to find out the complimentary lines that when combined, result in the production of a desirable F_1 hybrid [69]. The rest of the DH lines which do not complement each other are discarded due to GMO legislation imposed by the European Union (EU).

3.2 Insertional mutagenesis

Transposon and T-DNA insertional mutants are released to create alternation in the target gene of interest through PCR screening to study gene function [70], [71]. Insertional mutagenesis favors specific mutation in which a low frequency of recombination for homologous chromosomes is there [72]. The highly effective and efficient strategy for insertional mutagenesis is gene disruption [73]. The efficiency of this technique depends upon transformants generated along with PCR screening to identify introduced mutations utilizing lines within desirable genes [8]. In comparison, transposons possess multiple advantages over T-DNA with the production of multiple lines from initial lines and via recombination [74].

3.3 Target Induced Local Lesions in Genome (TILLING)

Target Induced Local Lesions in Genome (TILLING) methodology is utilized for screening and mapping of induced point mutations. Credit for developing this technique goes to the Henikoff laboratory aimed to screen EMS (Ethyl Methane Sulfonate) treated *Arabidopsis thaliana* desirable mutant alleles [9]. To date, many TILLING projects are running across the globe (Table 5). TILLING integrates traditionally used mutagenesis along with genome screening for a point mutation in the gene of interest [75]. The reasons behind its wide applicability in reverse genetics programs are high efficiency and running at a long cost making it more affordable [76]. Beyond laboratory-designed model plants, TILLING technique is implicated in agronomically important crops i.e., tomato, wheat, rapeseed, rice, maize, and soybean [24]. In addition, this technique does not require any transformation protocols making it most efficient and feasible for recalcitrant species and due to non-GMO technology also avoids controversies [77] In addition, large-scale applicability is making it a potential genetic tool for future plant genomics [23]. Moreover, undoubtedly most recent advancement in agriculture technology is the development of Next Generation Sequencing (NGS) which significantly lower down economy of whole genome, transcriptome sequencing, and plant functional genomics [78].

4. Perspectives and future directions

4.1 Benefits, applications and requirement of reverse breeding [Fig.3]

1) Reconstruction of heterozygous germplasm

2) Breeding on the single chromosome level i.e., to study epistatic interactions

3) Advantage over clonal propagation/apomixis

4) Providing seed-propagated varieties in vegetatively propagated crops to create diversity

4.2 Bottlenecks of reverse breeding

1) Necessity of Double Haploid Lines (DHLs) constrained the outcomes of reverse breeding in which double haploidy is not common i.e., lettuce, tomato, and cotton

2) Limited host range i.e., crops having haploid chromosome number of 12 or less.

3) Uncertain present scenario regarding novel plant breeding techniques such as reverse breeding, CRISPR/Cas9, and TALENs has to be kept under European Union (EU) GMO legislation or not.

4.3 Organizations

The following organizations are currently working in the reverse breeding program:

1. Rijk Zwaan Breeding B.V. R&D EersteKruisweg 9, 4793 RS Fijnaart, The Netherlands
2. CHIC Project (www.chicproject.eu)
3. NBT Platform (www.nbtplatform.org)
4. European Plant Science Organization (http://www.epsoweb.org/)

4.4 Future Thrust area

1. RNAi-mediated reverse breeding and genetics is a young work and requires extensive study to overcome technical problems.
2. Extensive research is required to improve the potential for the production of doubled haploids in reverse breeding programs.
3. Emphasis should be given to the production of hybrids in crops like cucumber, onion, broccoli, and cauliflower where seed production is problematic.

5. Conclusion

Hybrid stability/fixation is one of the most difficult tasks in heterosis breeding due to segregation in the F_2 generation. The conventional breeding program allows the development of nearly homozygous lines limiting hybrid vigor and usually taking 7-8 years. Reverse breeding appears to be the need for the hour and an emerging solution to crack this limitation. Rather to date, it applies to crops having haploid chromosome number <12 constraining efficiency, success, and wider adaptability of reverse breeding for other crops. So, a further extensive investigation must be taken into consideration. In addition, genetic tools of reverse genetics can be utilized for functional plant genomics studies. Collaboration of both novel plant breeding strategies can be an emerging solution for the heterosis revolution and functional plant genomics.

Declarations

Acknowledgment: All the authors would like to thank Mata Naina Ugratara, Dev Payindal Rishi, Mata Saraswati, and last but not least Sree Radhika Rani for their continuous support during the preparation and execution of the manuscript.

Disclosure statement: The authors report there are no competing interests to declare

Data availability statement: The data that support the findings of this study are openly available in doi: 10.1111/j.1467-7652.2009.00450.x

Funding: Not applicable

Figure Legends

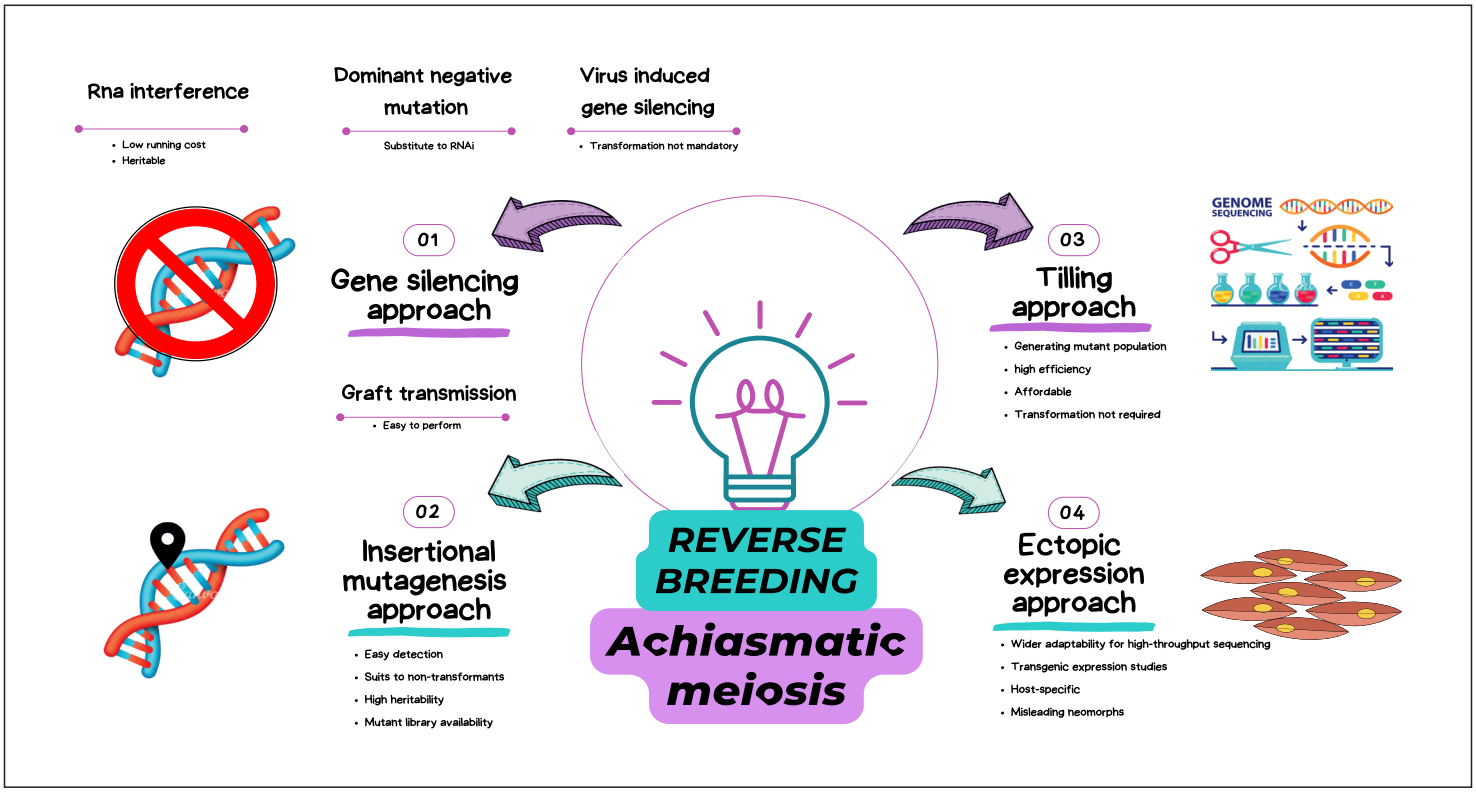

Fig.1. Multiple approaches of reverse breeding relying on achiasmatic meiosis

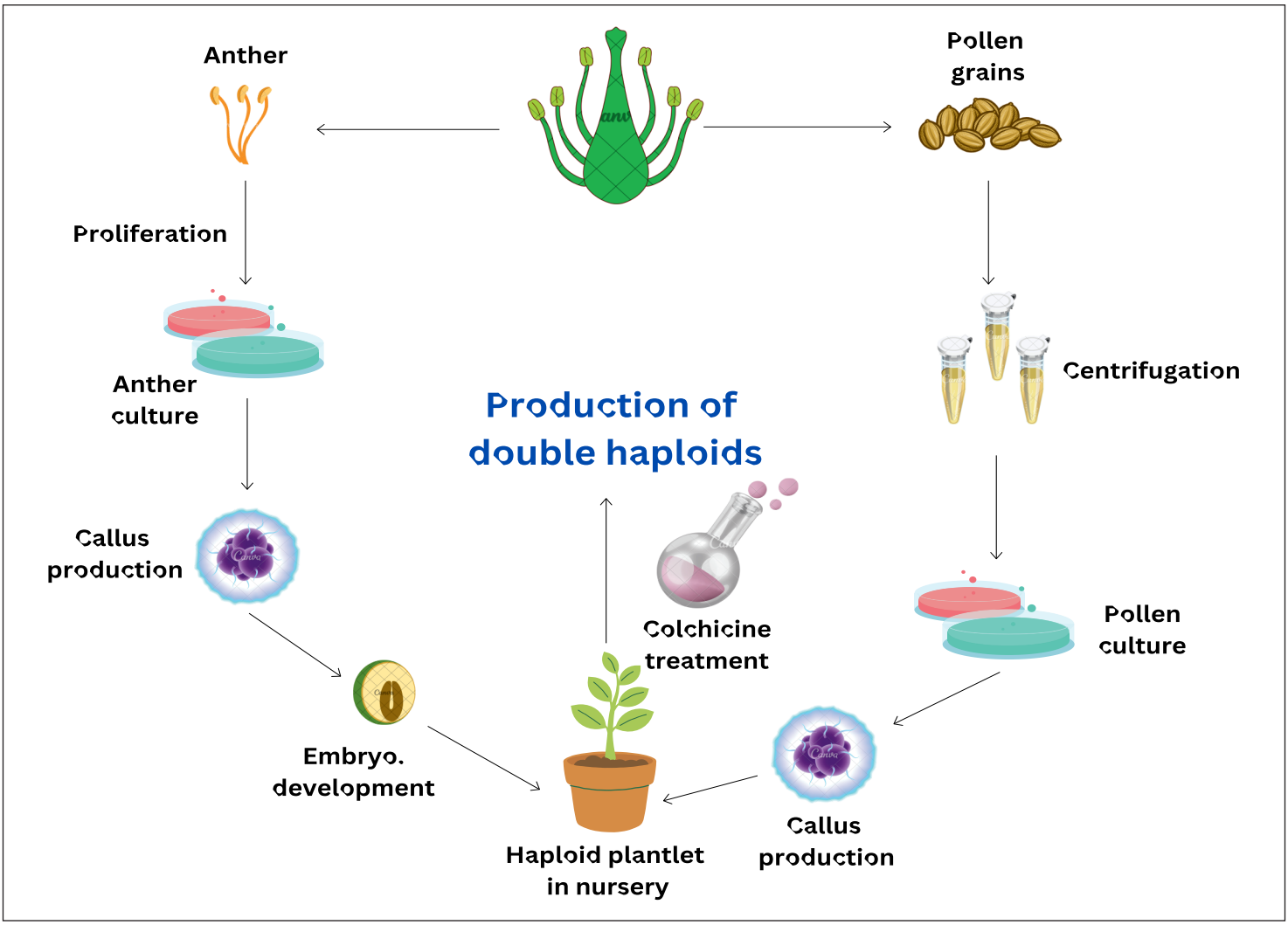

Fig.2. Schematic view for production of double haploids

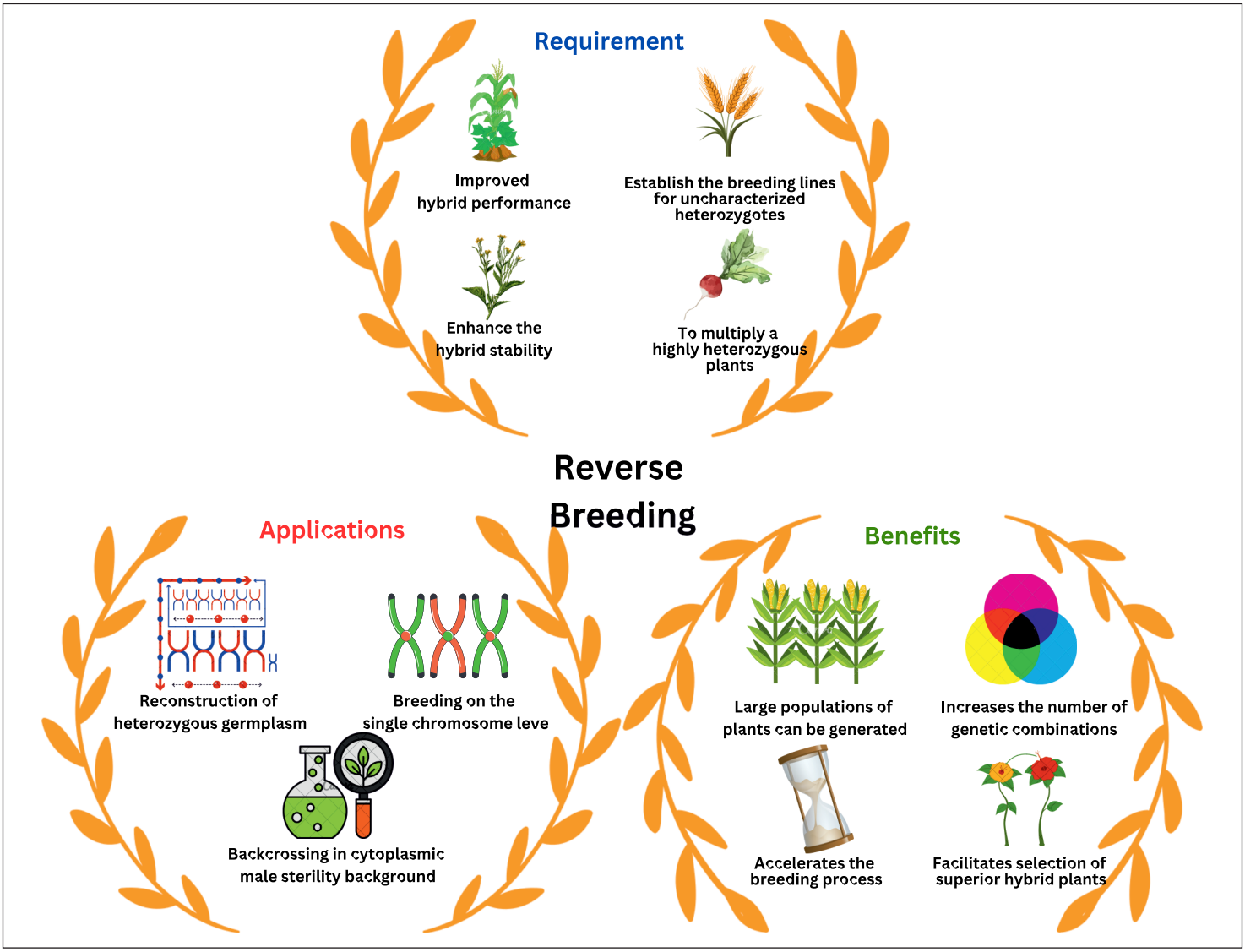

Fig.3. Applications, benefits and requirements of reverse breeding

Table 1. Non-recombinant DHs required to reconstitute desirable hybrid plant

S.No.	Crop	Haploid chromosome number (n)	Probability level			
			0.90	0.95	0.99	1.00
1.	*Arabidopsis thaliana*	5	13	14	18	47
2	Spinach, maize	6	18	20	25	67
3	Cucumber	7	25	28	35	94
4	Onion	8	35	40	49	133
5	Barley, carrot, beetroot, brassica vegetables, and lettuce	9	49	56	69	188
6	Maize, sorghum, asparagus, and cocoa	10	69	79	98	266
7	Banana, watermelon, celery, fennel, and common bean	11	98	111	138	377
8	Tomato, pepper, and brinjal	12	138	157	195	532

Table 2. Reverse breeding and genetics techniques for plant functional genomics

Method	Benefits	Bottlenecks	References
RNAi	1. Low running cost 2. Gene silencing of multiple homologous genes at the same time 3. Heritable and can be tissue-specific or temporal	1. Unstable frequency of gene silencing 2. Range of application limited to some species 3. Effect on non-desirable/non-target genes	[10], [13], [14]
Virus-induced gene silencing (VIGS)	1. Transformation: not mandatory 2. Rapid outcomes and easy to use 3. Potential of inducing partial loss of gene function 4. Alternative technology where RNAi is not applicable i.e., lettuce and tomato	1. Highly transient 2. Limited host range 3. Lack of missense mutations 4. Silencing variability	[15], [8], [16], [17]
Insertional mutagenesis	1. High throughput potential and heritable 2. Obtain stable desirable with less unwanted mutation 3. Well adapted for studies of loss/gain function 4. Generation of overexpression lines/mutants 5. Availability of mutant libraries 6. Easy detection of mutation site by PCR 7. Suitable for non-transformants	1. Being T-DNA/transposon-based, the probability is always there that the desired mutation might never be found 2. T-DNA mutagenesis cannot be studied 3. Less efficient in providing desirable information about genes of interest	[18], [19], [20]
TILLING	1. Suitable for non-transformable species having stable mutations 2. Having multiple alleles per locus 3. Can obtain missense mutation with complete loss of function 4. Highly efficient	1. Having low-medium throughput 2. High running cost and comparatively expensive 3. Screening of large mutant population required 4. Low probability of getting desirable mutation	[21], [22], [23], [24]
Ectopic expression	1. Wider adaptability for high-throughput screening 2. Right choice for studying transgene expression or gain of function	1. Limited range of hosts i.e., not suitable for non-transformants 2. Generation of misleading neomorphs 3. Exogenous regulatory sequence can alternate levels of expression	[25], [26], [27]

Table 3. Target genes involved in chiasmata formation, chromosome pairing and gene conversion

S. No.	Target genes for chiasmata formation	Target genes involved in chromosome pairing	Target genes required for gene conversion
1.	*SPO11*	*RAD51, RAD52, RAD54, RAD55 and RAD57*	*SGS1*
2.	*MER1, MER2*	*MER3, ZIP1, ZIP2, ME15, MEI218, SSC1*	*MSH4*
3.	*MEI4*	*DMC1* i.e., the most commonly targeted gene among all (*Brassica carinata*, *Brassica oleracea, Arabiopsis thaliana, Solanum melongena, Solanum Lycopersicon, Nicotiana tabacum*	*MSH5* (*Solanum melongena*)
4.	*RAD50*	*SMC3*	*ZIP1, ZIP2*
5.	*MRE2, MRE4, MRE11*	*MND1, MSH2, MSH3, MSH6, PMS1*	*MLH1, MLH3*
6.	*RED1*	*SAE2, SAE3*	*MEC1*
7.	*REC102, REC104, REC114*	*REC8, RPA, SCP3*	-
8.	*HOP1*	*HOP2*	-
9.	*XRS2*	*HIM6, RED1, RAD57,*	-

Table 4. List of recent VIGS vectors available with host crop plants

VIGS vector	Form	Virus genus	Crop	Viral response	Reference
Tomato zonate spot virus	RNA	Orthotospovirus	Tomato	Severe	[42]
Maize rayado fino virus	RNA	Marafivirus	Maize	Mild	[43]
Tobacco Rattle Virus (TRV)	RNA	Tobravirus	Tobacco (*Nicotiana attenuate*)	Mild	[44]
Papaya leaf distortion mosaic virus (PLMV)	RNA	Potyvirus	Papaya	Mild	[45]
Cucumber green mottle mosaic virus (CGMV)	RNA	Tobamovirus	Cucurbits	Mild	[46]
Apple latent spherical virus (ALSV)	RNA	Cheravirus	*Silene latifolia*	Severe	[47]
Tobacco Mosaic Virus (TMV)	RNA	Tobamovirus	Tomato, tobacco, chili	Severe	[48]
Cucumber Mosaic Virus (CMV)	RNA	Cucumovirus	Tomato, cucumber, *Arabidopsis thaliana*, maize	None	[16], [49]
Barley strip Mosaic Virus (BSMV)	RNA	Hordeivirus	Maize, wheat & barley	Moderate	[50]
Turnip Yellow Mosaic Virus	RNA	Tymovirus	Brassicas	Mild	[51]

African Cassava Mosaic Virus	DNA	Begomovirus	Cassava	Variable	[52]
Tobacco Rattle Virus (TRV)	RNA	Tobravirus	Spinach & potato	Mild	[53]
Potato Virus X (PVX)	RNA	Potexvirus	Potato	Moderate	[54]
Cabbage Leaf Curl Virus (CbLCV)	DNA	Begomovirus	Cabbage, cauliflower, and broccoli	Moderate	[55]
Tomato Yellow Leaf Curl Virus (TYLCV)	DNA	Begomovirus	Tomato	No	[56]

Table 5. List of TILLING projects running across the globe

Crop	List of TILLING Project	Organization
Arabidopsis	CAN-TILL	University of British, Columbia, Canada
Rice	Rice TILLING platform	National Institute of Genetics, Japan
	Davis TILLING Project	University of California, USA
Wheat	Arcadia Bioscience TILLING	Arcadia Bioscience, USA
Barley	Barley TILLING	SCRI, Scotland
Beans	USDA Bean TILLING Project	USDA
Cabbage, mustard, turnip	MBGP TILLING	Multinational Consortia
Drosophila	Seattle TILLING Project	Seattle, USA
Pea, rapeseed, tomato	URGY TILLING Project	URGY, France

References

1. Zhou T, Dong L, Jiang T, Fan Z. Silencing specific genes in plants using virus-induced gene silencing (VIGS) vectors.. Methods Mol Biol. 2022;2400:149-161.
2. Kumar J, Kumar A, Sen Gupta D, Kumar S, De Pauw RM. Reverse genetic approaches for breeding nutrient-rich and climate-resilient cereal and food legume crops. Hered. 2022;1-24.
3. Chen ZJ. Molecular mechanisms of polyploidy and hybrid vigor. Tr. Plant Sci. 2010;15: 57-71.
4. Wijnker E, Van KD, De CBS, Lelivelt CL, Keurentjes JJ, Naharudin NS, Ravi M, Chan SW, Jong H, Dirks R. Reverse breeding in *Arabidopsis thaliana* generates homozygous parental lines from a heterozygous plant. Nat genet. 2012;44:467-470.
5. Perotti E, Grimanelli D, John P, Hoisington D, Leblanc O. Why is transferring apomixis to crops still a dream? In: Fischer T, Turner N, Angus J, McIntyre L, Robertson M, Borrell A, Lloyd D, editors. New Directions for a Diverse Planet: Proceedings for the 4th International Crop Science Congress. Brisbane, Australia; 2004.
6. Dirks R, Van DK, De SCB, Van DBM, Lelivelt CL, Voermans W, Woudenberg L, De WJP, Reinink K, Schut JW, Van DZE. Reverse breeding: a novel breeding approach based on engineered meiosis. Plant Bio J. 2009;7:837-845.
7. Stupar R, Gardiner J, Oldre A, Haun W, Chandler V, Springer N. Gene expression analyses in maize inbreds and hybrids with varying levels of heterosis. BMC Plant Biol. 2008;8: 33.
8. Amar AB, Daloul S, Reustle GM, Krczal G, Mlikia A. 2016. Reverse genetics and high throughput sequencing methodologies for plant functional genomics. Curr Genom. 2008;17: 460-475.
9. Gilchrist E, Haughn G. Reverse genetics techniques: engineering loss and gain of gene function in plants. Brie. Funct.Geno. 2010;9(2):103-110.
10. Aklilu E. Review on forward and reverse genetics in plant breeding, All Life. 2021;14:1,127-135.
11. Jiang L, Du Z, Zhang G, Wang T, Jin G. Advances in RNA-silencing related resistance against viruses in Potato. Genes. 2022;13:731.
12. Hartung F, Wurz-Wildersinn R, Fuchs J, Schubert I, Suer S, Puchta H. The catalytically active tyrosine residues of both SPO11-1 and SPO11-2 are required for meiotic double-strand break induction in Arabidopsis. Plant Cell. 2007;19:3090-3099.
13. Mezzetti B, Smagghe G, Arpaia S, et al. RNAi: What is its position in agriculture. 2020. J Pest Sci. 2020;93:1125-1130.

14. Kumar S. RNAi (RNA interference) vectors for functional genomics study in plants. Natl Acad Sci Lett. 2014;37(3):289-294.

15. Alter H, Peer R, Dombrovsky A, Flaishman M, Spitzer-Rimon B. Tobacco Rattle Virus (TRV) as a tool for rapid reverse-genetics screens and analysis of gene function in *Cannabis sativa* L. Plants. 2022;11(3):327.

16. He G, Zhao X, Xu Y, Wang Y, Zhang Z, Xiao L, Hudson M, Hu R, Li S. An efficient virus- induced gene silencing (VIGS) system for gene functional studies in Miscanthus. GCB Bioenergy. 2023;15:805-820.

17. Purkayastha A, Dasgupta I. Virus-induced gene silencing: a versatile tool for discovery of gene functions in plants. Plant Physiol Biochem. 2009;47:967-976.

18. Johnson, Alexander; Mcassey, Edward; Diaz, Stephanie; Reagin, Jacod; Redd, et al. Development of mPing-based activation tags for crop insertional mutagenesis. Agronomy & Horticulture- Faculty Publications. 2021;1433.

19. Ram H, Soni P, Salvi P, Gandass N, Sharma A, Kaur A, Sharma TR. Insertional mutagenesis approaches and their use in Rice for functional genomics. Plants. 2019;8(9):310.

20. Jung KH, An G, Ronald PC. Towards a better bowl of rice: assigning function to tens of thousands of rice genes. Nat Rev Genet. 2008;9, 91-101.

21. Gil J, Andrade-Martínez JS,Duitama J. Accurate, Efficient and User-Friendly Mutation Calling and Sample Identification for TILLING experiments. Front Genet. 2021;12:624513.

22. Bajaj D, Srivastava R, Nath M, Tripathi S, Bharadwaj C, Upadhyaya HD, et al. Eco TILLING-based association mapping efficiently delineates functionally relevant natural allelic variants of candidate genes governing agronomic traits in chickpea. Front Plant Sci. 2022;7:450.

23. Wang N, Shi L. Screening of mutations by TILLING in plants. Methods Mol Biol. 2015;1245:193-203.

24. Dalmais M, Antelme S, Ho-Yue-Kuang S, Wang Y, Darracq O, Bouvier M, et al. A TILLING platform for functional genomics in *Brachypodium distachyon*. PLoS ONE. 2013;8(6);e65503.

25. Chen F, Li Y, Li X, Li W, Xu J, Cao H, Wang Z, Li Y, Soppe WJJ, Liu Y. Ectopic expression of the Arabidopsis florigen gene FLOWERING LOCUS T in seeds enhances seed dormancy via the GA and DOG1 pathways. Plant J. 2021;107(3):909-924.

26. Singh A, Khurana P. Ectopic expression of *Triticum aestivum SERK* genes (TaSERKs) control plant growth and development in *Arabidopsis* . Sci Rep. 2017;12368

27. Ben-Amar A, Cobanov P, Buchholz G, Mliki A, Reustle G. In plant agroinfiltration system for transient gene expression in grapevine. Acta Physiol Plant. 2013;35(11):3147-3156.

28. El-Sappah AH, Yan K, Huang Q, Islam MM, Li Q, Wang Y, Khan MS, Zhao X, Mir RR, Li J, El-Tarabily KA, Abbas M. Comprehensive mechanism of gene silencing and its role in plant growth and development. Front Plant Sci. 2021;12:70524929.

29. Lata H, Sharma A, Chadha S, Kaur M, Kumar P. RNA interference (RNAi) mechanism and application in vegetable crops. J Hort Sci Bio. 2021;97;160-170.

30. Song L, Fang Y, Chen L, Wang J, Chen X. Role of noncoding RNAs in plant immunity. Plant Commun. 2021;2:100180.

31. Guo Z, Li Y, Ding SW. Small RNA-based antimicrobial immunity. Nat Rev Immunol. 2019;19:31-44.

32. Zhao JH, Guo HS. RNA silencing: From discovery and elucidation to application and perspectives. J Integ Plant Bio. 2022;64:476-498.

33. Zheng L, Zhang C, Shi C, Yang Z, Wang Y, Zhou T, Sun F, Wang H, Zhao S, Qin Q, Qiao R, Ding Z, Wei C, Xie L, Wu J, Li, Y. Rice stripe virus NS3 protein regulates primary miRNA processing through association with the miRNA biogenesis factor OsDRB1 and facilitates virus infection in rice. PloSPathog. 2017;13:e1006662.

34. Qin Q, Li G,Jin L, Huang Y, Wang Y, Wei C, Xu Z, Yang Z, Wang H, Li Y. Auxin response factors (ARFs) differentially regulate rice antiviral immune response against rice dwarf virus. PloSPathog. 2020;16:e1009118.

35. Yang Z, HuangY, Yang J, Yao,S, Zhao K, Wang D, Qin Q, Bian Z, Li Y, Lan Y, Zhou T, Wang H, Liu C, Wang W, Qi Y, Xu Z, Li Y. Jasmonate signaling enhances RNA silencing and antiviral defense in rice. Cell H Micro. 2020;28:89-03 e108.

36. Zhao J, Rios CG, Song J. Potato virus X-based microRNA silencing (VBMS) in potato. Jove J Vis Exp. 2020;159:e61067.

37. Jay F, Vitel M, Brioudes F, Louis M, Knobloch T, Voinnet O. Chemical enhancers of post-transcriptional gene silencing in Arabidopsis. RNA. 2019;25:1078-1090.

38. Barajas D, Nagy PD. Expression of dominant-negative mutants to study host factors affecting plant virus infections. Methods Mol Biol. 2012;894:359-76.

39. Zhu X, Dinesh-Kumar SP. Virus-induced gene silencing as a tool to identify host genes affecting viral pathogenicity. Methods Mol Biol. 2008;451:641-8.

40. Moon SJ, Kim HY, Hwang H, Kim JA, Lee Y, Min MK, Yoon IS, Kwon TR, Kim BG. A Dominant Negative OsKAT2 Mutant delays light-induced stomata opening and improves drought tolerance without yield penalty in Rice. Front Plant Sci. 2017;8:772.

41. Gupta B, Saha J, Sengupta A, Gupta K. Recent advances on virus induced gene silencing (VIGS): Plant functional genomics. Plant Biochem& Physio. 2013;1(5).

42. Li J, Chen S, Qiu R-S, Zhang L-Z, Chen Y, Zheng X, et al. Functional analysis of thenon-structural protein NSs of tomato zonate spotvirus. PloS ONE. 2022;17(1):e0262194

43. Sizolwenkosi M, Nitika K, Kristen W, Junhuan X, Jane T, Hong HT, Lucy RS. Coat protein expression strategy of maize rayado fino virus and evidence for requirement of CP1 for leaf hopper transmission, Virology. 2022;10,570:96-106.

44. Rahman J, Baldwin IT, Gase K. California TRV-based VIGS vectors mediate gene silencing at elevated temperatures but with greater growth stunting. BMC Plant Biol. 2021;21:553.

45. Tuo D, Yan P, Zhao G. et al. An efficient papaya leaf distortion mosaic potyvirus vector for virus-induced gene silencing in papaya. Hortic Res. 2021;8:144.

46. Liu M, Liang Z, Aranda MA. Et al. A cucumber green mottle mosaic virus vector for virus-induced gene silencing in cucurbit plants. Plant Met. 2020;16, 9.

47. Fujita N, Kazama Y, Yamagishi N, Watanabe K, Ando S, Tsuji H, Kawano S, Yoshikawa N, Komatsu K. Development of the VIGS System in the Dioecious Plant Silene latifolia. Int J Mol Sci. 2019;20(5):1031.

48. Chen W, Zhang X, Fan Y, Li B, Ryabov E, Shi N, Zhao M, Yu Z, Qin C, Zheng Q, Zhang P, Wang H, Jackson S, Cheng Q, Liu Y, Gallusci P, Hong Y. A Genetic Network for Systemic RNA Silencing in Plants. Plant Physiol. 2018;176(4):2700-2719.

49. Rhee S-J, Watt LG, Bravo AC, Murphy AM, Carr JP. Effects of the cucumber mosaic virus 2a protein on aphid–plant interactions in *Arabidopsis thaliana*. Molecular Plant Pathology. 2020;21:1248–1254.

50. Jarugula S, Willie K, Stewart LR. Barley stripe mosaic virus (BSMV) as a virus-induced gene silencing vector in maize seedlings. Virus Genes. 2018;54:616–620.

51. Yu J, Yang XD, Wang Q. et al. Efficient virus-induced gene silencing in *Brassica rapa* using a turnip yellow mosaic virus vector. Biol Plant. 2018;62:826-834

52. Lentz EM, Kuon JE, Alder A *et al.* Cassava geminivirus agroclones for virus-induced gene silencing in cassava leaves and roots. Plant Methods. 2018;14:73

53. Lee J, Cao DV, Kim J. et al. Development of a virus-induced gene silencing (VIGS) system for *Spinacia oleracea* L. in vitro cell. Dev.Biol.-Plant. 2017;53:97-103

54. Bai Y, Han S, Gao Y, Zhang W, Fan G, Qiu C, Shen Y, Zhang S, Shang H, Nie X,Wen J. Pathogenicity of Potato virus Y strains in different potato varieties. Acta Agric. Boreali-occidentalis Sin. 2017;27:1713-1720.

55. Reyes MI, Flores-Vergara MA, Guerra-Peraza O, Rajabu C, Desai J, Hiromoto-Ruiz YH, Ndunguru J, Hanley-Bowdoin L, Kjemtrup S, Jose T. Ascencio JT and Robertson D. A VIGS screen identifies immunity in the *Arabidopsis* Pla-1accession to viruses in two different genera of the Geminiviridae. The Plant Journal. 2017;92:796-807.

56. Xin Huang, Jianming Wei, Dan Wu, Na Mi, Sili Fang, Yao Xiao & Yunzhou Li. Silencing of SlDRB1 gene reduces resistance to tomato yellow leaf curl virus (TYLCV) in tomato (*Solanum lycopersicum*). Plant Signaling & Behavior. 17:1:2149942

57. Bekele D, Tesfaye K, Fikre A. Applications of virus-induced gene silencing (VIGS) in plant functional genomics studies. J Plant Biochem & Physio. 2019;7:1-7.

58. Muhammad T, Zhang F, Zhang Y, Liang Y. RNA Interference: A Natural Immune System of Plants to Counteract Biotic Stressors. Cells. 2019;8(1):38.

59. Nejat N, Mantri N. Emerging roles of long non-coding RNAs in plant response to biotic and abiotic stresses. Crit Rev Biotechnol. 2018;38(1):93-105.

60. Tonosaki K, Fujimoto R, Dennis ES, Raboy V and Osabe K. Will epigenetics be a key player in crop breeding? Front. Plant Sci. 2022;13:958350.

61. Sager R, Lee JY. Plasmodesmata in integrated cell signalling: insights from development and environmental signals and stresses. J Exp Bot. 2014;65(22):6337-58.

62. Shaharuddin NA, Yuanhuai H, Hongying L, Grierson D. The mechanism of graft transmission of sense and antisense gene silencing in tomato plants. FEBS Lett. 2006;580: 6579-6586.

63. Han Y, Grierson D. Enhancement of post-transcriptional gene silencing by grafting. Plant Sig. & Behav. 2008;3:1,30-33.

64. Seguí-Simarro JM. Doubled haploid technology in Methods in Molecular Biology. 2021;Vol. 2287. New York: Springer Science+Business Media.

65. Ren J, Wu P, Trampe B, Tian X, Lübberstedt T, Chen S. Novel technologies in doubled haploid line development. Plant Biotechnol. J. 2017;15(11):1361-1370.

66. Yali Werkissa. Haploids and doubled haploid technology application in modern plant breeding. J of Plant Sci. 2022;10(2), pp.71-75.

67. Hale B, Ferrie AMR, Chellamma S, Samuel JP, Phillips GC. Androgenesis-based doubled haploidy: Past, Present, and future perspectives. Front Plant Sci. 2022;12:751230.

68. Sharma P, Chaudhary HK, Manoj NV et al. New Protocol for Colchicine Induced Efficient Doubled Haploidy in Haploid Regenerants of Tetraploid and Hexaploid Wheats at *In vitro* Level. Cereal Research Communication . 2019;47:356–368

69. Shrestha S, Subedi S, Shrestha J. Marker-assisted selection: A smart biotechnological strategy for modern plant breeding. 2020;10.21704/pja.v4i3.1490.

70. Mathieu M, Winters E, Kong F. Establishment of soybean (*Glycine max*Merr. L) transposon-based mutagenesis repository. Planta. 2009;229:279-89.

71. Thole V, Alves SC, Worland B. A protocol for efficiently retrieving and characterizing flanking sequence tags (FSTs) in *Brachypodium distachyon* T-DNA insertional mutants. Nat Protoc. 2009;4:650–61.

72. Gonzalez-Ballester D, Pootakham W, Mus F, Yang W, Catalanotti C, Magneschi L, et al. Reverse genetics in Chlamydomonas: a platform for isolating insertional mutants. Plant Met. 2011;7:(24).

73. Thole V, Peraldi A, Worland B, Nicholson P, Doonan JH, Vain P. T-DNA mutagenesis in *Brachypodium distachyon*. J Exp Bot. 2012;63(2):567-576.

74. Upadhyaya NM, Zhu QH, Bhat RS. Transposon insertional mutagenesis in rice. Methods Mol Biol. 2010;678:147-177.

75. Chen L, Hao L, Parry MAJ, Phillips AL, Hu YG. Progress in TILLING as a tool for functional genomics and improvement of crops. J Integr Plant Biol. 2014;56:425-443.

76. Tadele Z,Chikelu MBA, Till BJ. TILLING for mutations in model plants and crops. In: Molecular Techniques in Crop Improvement; Jain, S.M.; Brar, D.S., Eds.; Springer; 2010;pp. 307332.

77. Kurowska M, Daszkowska-Golec A, Gruszka D, Marzec M, Szurman M, Szarejko I, Maluszynski M. 2011. TILLING – a shortcut in functional genomics. J Appl Genet. 2011;52, 371-390.

78. Edwards D, Batley J, Snowdon RJ. Accessing complex crop genomes with next-generation sequencing. Theor Appl. Genet. 2013;126:1-11.