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For Copies Contact

Director

ICAR-NAARM, Rajendranagar

Hyderabad-500 030, Telangana, India

Tel: 040-24581322/24581285

Fax: 040-24015912

Email: director@naarm.org

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Emerging Tools and Techniques in Crop Improvement for Higher Productivity and Multiple Stress Tolerance

Amar Kant Kushwaha[1], Vishal Dinkar[1], R. Thribhuvan[1], Neethu Mohan[1], Sujatha Mulpuri[2] and Raman Meenakshi Sundaram[3]

[1]ICAR-National Academy of Agricultural Research Management (NAARM), Hyderabad-500030, Telangana
[2]ICAR – Indian Institute of Oilseeds Research (IIOR), Hyderabad-500030, Telangana
[3]ICAR – Indian Institute of Rice Research (IIRR), Hyderabad-500030, Telangana

Abstract

Ensuring food and nutritional security along with sustainable growth in the immediate future is a serious challenge faced by developing countries like India. To meet these demands, strategic research for development of crop varieties with higher production potential along with resilience to changing climate and resistance to new plant pathogens and pests is needed. Recent advancements in biotechnology led to cost effective, accurate, high-throughput genome sequencing, advanced phenomics platforms, CRISPR based gene-editing, molecular breeding, etc. which are complementing the conventional breeding and add precision to crop improvement efforts. Better tools for exploration of germplasm and conservation, understanding pan-genome and its utilization for gene discovery, use of induced mutants and natural variants in genetic dissection of complex traits will elevate our level of knowledge on genotype response in different environment. Availability of better algorithms and software for analysing data from huge phenotyping data under controlled and field conditions, with robust molecular marker technology enhanced the pace of varietal development for higher yield, enhanced nutritional value with tolerance against abiotic and biotic stresses. By addressing few obstacles related to the application of these emerging tools and techniques in agriculture, it will be possible to fulfil the existing needs and potential food demands of the burgeoning population.

Keywords: Pan-genome, Genetic Mapping, Phenomics, Gene editing, CRISPR

1.Introduction

Global food security is a major concern in the light of growing population and dwindling resources. To meet the growing demand, producing food in sufficient quantities, with adequate nutritional attributes and in a sustainable manner is an important priority of agricultural researchers. Due to anthropogenic activities and a rapidly changing climatic pattern, there is loss of biodiversity and different biotic and abiotic stress factors are emerging, limiting the possibility of improvement and inflicting production losses. To address these challenges, identification and pragmatic utilization of genetic resources with the help of modern tools and techniques of crop improvement is the need of hour (Dinkar *et al.,* 2020). Several tools and techniques in the recent past have been developed to identify and modify traits of interest in crop plants. These techniques assist plant breeders to add precision and make the process more rapid as compared to conventional tools and techniques. Genomics has paved the path for accelerating plant breeding by rapid identification of genetic factor(s) underlying various traits of agronomic importance and by providing large marker sets, which increase accuracy of the breeding process. Further, the science of phenomics is gaining importance in recent years as accurate phenotyping decides the accuracy of marker-trait associations unraveled with the help of genomic tools. Disruptive technologies like genome editing have tremendous potential in generation of specific mutants as compared to non-specific mutations which have been a part of plant breeding in the past. In this chapter, some of the key emerging tools and techniques used for crop improvement are discussed.

2. New tools and technology for exploration and utilization of germplasm

Genetic diversity is the key factor for crop improvement. There is enormous quantity of germplasm stored in the global gene banks and utilization of these lines will accelerate plant breeding efforts. Efficient utilization of germplasm depends on understanding the genetic and molecular basis of the diversity. At molecular level, the diversity in the germplasm is represented by single nucleotide polymorphism (SNP), copy number variation (CNV), insertion and deletions (InDels), and other kinds of structural variations (SVs).

2.1 Understanding the usefulness of pan-genome

A pan-genome representing the full complement of genes of a biological clade such as species can be classified into two sets i.e. one set of core genes -shared by all individuals and second set of dispensable genes -can be partially shared or may be individual specific. Use of this pan-genome has increased its potential in plant breeding and evolution studies with the availability of rapid sequencing techniques and cost-effective genotyping platforms. Earlier, the reference sequences of cultivated crop species. are used to tap different types of genotypic variation at genic level, which are represented by single nucleotide variations (SNPs) and insertion-deletions (InDels). But these kind of variations represent only a part of diversity for the trait of interest. To understand and identify the cause of diversity, there is need to zoom into other forms of structural

variation also *viz.* CNVs and large SVs. Pangenome based identified SNP/InDels and variation of other forms are choice of markers as they are suitable for high-throughput analysis and cost effective, as well as they are representing diversity in germplasm too. This approach has tremendous potential in crops where genomic diversity is not fully captured in plant breeding programmes. Though the concept of crop diversity is not new and the potential to be utilized in crop breeding and genetic analysis of wild relatives have been known to whole plant breeding community but the concept of the pan-genome has been put forth by Tettelin *et al.* (2005). Schatz *et al.* (2014) and Wang *et al.* (2018) have reported that rice has largest pan-genome (40,093 genes) as this crop has most of the reported genes or genic variation from the various germplasm accessions that were characterized. Similarly, pan-genome in other crop/plant species has also been reported (Table 1). There is still more to explore in terms of pan-genome development, and it has been suggested to include wild relatives along with cultivated species to tap most of exploitable variability (Montenegro *et al.,* 2017). In creation of pan-genome, it is important to concentrate on the dispensable genes as most of the variation occurs in these genes. These dispensable genes are mostly related to defence against biotic and abiotic stresses, encoding signalling molecules, anti-oxidant, receptors, *etc.* (Golicz *et al.,* 2016). Different kinds of structural variations play very important role in genetic control of agronomic traits.

Table 1. List of Pan-genomes

No.	*Crop*	*Species*	*Reference*
1	Rice	*O. s ativa* L.	Schatz *et al.,* 2014
		O. sativa L.	Yao *et al.,* 2015
		O. sativa L.	Wang *et al.,* 2018
		O. rufipogon Griff.	Zhao *et al.,* 2018
2	Wild soybean	*Glycine soja* Siebold & Zucc.	Li *et al.,* 2014
3	Brassica	*Brassica oleracea* L.	Golicz *et al.,* 2016
		Br assica napus L.	Hurgobin *et al.,* 2018
		Brassica rapa L.	Lin *et al* ., 2014
4	Wheat	*Triticum aestivum* L.	Motenegro *et al.,* 2017
5	Maize	*Zea mays* L.	Hirsch *et al.,* 2014
6	Sesame	*Sesamum indicum* L.	Yu *et al.,* 2019
7	Sunflower	*Helianthus annuus* L.	Hubner *et al.,* 2019
8	Tomato	*Solanum lycopersic um* L.	Gao *et al.,* 2019
9	Poplar	*Populus demolus*	Pinosio *et al.,* 2016
10	Brachypodium	*Brachypodium distachyon* (L.) P. Beauv	Gordon *et al.,* 2017

Further a fine version of pan-genome, where in the development is based on utilization of different accessions from the different species of the crop is known as super-pan-genome. This will ensure that we can tap most of the structural variations of dispensable kind (Khan *et al.,* 2020).

3. Genetic mapping strategies

To unravel the genetics behind the performance of economically important agronomic traits and their utilization in varietal development, marker-trait relations are ought to be established. Linkage and association mapping are the major mapping strategies used in genetic mapping. For this purpose, there are several kind of mapping populations available *viz.* families-based mapping populations, introgression lines, libraries, germplasm collections, complex mapping populations, *viz*. Multiparent Advanced Generation Inter-crossed (MAGIC) populations, Nested Association Mapping (NAM) populations, *etc*. The choice of mapping population depends on the crop, reproduction behaviour and the targeted trait(s) including its inheritance.

3.1 Linkage mapping of Oligogenes

Genetic maps can be of three types, (i) linkage maps, (ii) cytogenetic maps, and (iii) physical maps. A linkage map is constructed to represent various genetic markers positioned on chromosome based on frequencies of recombination between marker pairs. The recombinations frequency is calculated from observations made on an appropriate mapping population and are translated to map distances. Depending upon the genetic distance, the molecular or genetic markers are clustered into linkage groups, and their sequence in the linkage group is represented as the linkage map (Singh and Singh, 2015).

3.1.1 Generalized protocol for construction of linkage map

1. Construction of suitable mapping population by crossing genetically divergent parents
2. Parental polymorphism survey using large number of markers
3. All individuals in the population used for mapping are genotyped with polymorphic markers
4. Estimation of recombination frequencies between markers using appropriate software
5. Phenotyping of mapping population and classification of various phenotypic groups
6. The phenotypic and genotype (using marker) data are analysed with an appropriate software for the identification of markers linked with oligogene(s) controlling the desired trait and to determine recombination frequency between the gene and markers

It is not necessary to genotype all the individuals in the mapping population for all the polymorphic markers to predict the gene associated markers (Dinkar *et al.,* 2020). One can use strategies like Bulked Segregant Analysis (BSA) and its several other variants *viz.* Bulked Segregant RNA seq (BSRseq), MutMap, MutMap^{+} and MutMap Gap to identify a small set of polymorphic markers linked to the target gene.

3.1.2 Bulked Segregant Analysis (BSA)

BSA is a great technique for identification of diagnostically linked markers to the gene of interest with least effort and outlay (Michelmore *et al.,* 1991). BSA, involves selected and pooled individuals, has been widely used in gene mapping with biparental populations and mapping by sequencing with oligogenic mutants. Extreme phenotypes are pooled for genome wide association study (Zou *et al.,* 2017). The extreme phenotypes would be from any population with two different types of genetic backgrounds: (i) variants from segregating populations and, (ii) variants from any populations of a species with diverse genetic backgrounds (Zou *et al.,* 2017). With the advancements in genome sequencing and molecular breeding technologies, BSA has also witnessed many improvements.

3.2 QTL Mapping

QTL analysis relies on the idea of finding linkage disequilibrium (LD) between the trait and the marker. Markers help to classify the mapping population into distinct genotypic groups based on the presence/absence type of polymorphism of a specific marker locus and to decide whether substantial differences exist between groups with regard to the trait of interest (Collard and Mackill 2008). There are several methods available for QTL mapping with their own advantages and limitations (Table 2).

Table 2: Statistical methods of QTL mapping

QTL mapping method	*Advantage*	*Limitations*
Single Marker Analysis (SMA)	Simplest and easiest, Performed using simple softwares, No linkage map is required	No information about recombination rate between marker and QTL QTL position is unknown Low QTL detection power
Simple Interval Mapping (SIM)	Statistically more powerful, QTL position is provided in support interval, Provides more reliable QTL effect estimates	Chance to detect ghost QTL More computational time Unable to detect interacting QTLs
Composite Interval Mapping (CIM)	Statistically more powerful than SIM	Unable to detect interacting QTL Randomness in cofactors selection for analysis
Multiple interval mapping (MIM)	Synchronous QTL mapping in several marker intervals	Computationally challenging
Bayesian Approaches	Detection of multiple QTL	Complexity in computation Lack of user-friendly software

3.3 Association mapping

Association mapping depends on exploring linkage disequilibrium that is non-random association of alleles between different loci within genome. It depends on the initial LD decay present in a population whose rate is determined by the genetic distance between loci and the number of generations since it arose. In addition to the historical recombination events that might have occurred during establishment of association panel, this non-random allelic association is ascribable to several evolutionary factors such as mutation, genetic bottlenecks, genetic drift, and migration *etc*. Association mapping and knowledge of LD has several favourable consequences to a plant breeder. As opposed to family-based QTL mapping, AM does not require any segregating population or a linkage map. AM exploits the genetic diversity existing among the naturally occurring diverse genotypes and saves the time for mapping population development. Historical data on phenotypic information recorded on diverse genotypes over the years can also be used. Moreover, AM assess entire range of allelic diversity in the AM panel and all identifies more alleles than biparental mapping, making it more realistic for QTL discovery (Bohra, 2013). Before taking up association studies for any crop species, prior knowledge on the genetics of the crop, like ploidy level, and the breeding behaviour of the crop is mandatory (Zhu *et al.,* 2008). General procedure for carrying out association mapping is as follows:

i. Constitution of the Association mapping (AM) population

ii. Phenotyping

iii. Genotyping for population structure analysis

iv. Structure and kinship analysis

v. Genotyping for LD analysis

vi. AM and LD analysis

Pang *et al.* (2020) performed a large-scale genome-wide association study on 768 wheat cultivars using genotyping-by-sequencing approach (GBS) and detected 395 quantitative trait loci (QTLs) for 12 traits under 7 environmental conditions. Total 273 QTLs were mapped to ≤1.0 Mb intervals. They detected eight candidate genes for three QTLs that were enhancing spike seed setting and grain size with the assistance of gene expression data and subsequently validated them in three bi-parental populations. The study indicated that by increasing genome-wide association study (GWAS) population size and marker density, QTLs underlying a quantitative trait can be detected which in turn facilitates fine mapping, candidate gene identification and their validation and in development of functional markers in wheat.

4. Recent techniques for rapid identification of gene/QTLs governing important traits in crops

Creation of mutation and their utilization in mapping of factor(s) responsible has been progressed very rapidly in the last ten years with the advent of sequencing techniques and cost-effective genotyping protocols. Techniques like MutMap, MutMap Gap, MutMap^{+}, MutChromSeq and MutRenSeq are based on development of population using mutants created by different mutagens. QTL-seq, RNA-seq, NIKS (needle in the k-stack), Exome Capture, NGM (Next Generation Mapping) and Associated genetics R gene

enrichment sequencing (AgRenSeq) have been utilized for analysing mapping populations derived from natural variants or germplasm (Table 3 and 4).

4.1 Mapping using mutations

4.1.1 MutMap

The F_2 or other advance populations generated from crossing with mutants and wild types are used for MutMap. High quality reference genome is needed for this technique. Alignment of short reads generated from whole genome re-sequencing of parental as well as high trait value bulk and low trait value bulk from the population is done to generate SNP index and based on delta SNP value causal SNPs for the mutant are identified (Abe *et al.,* 2012).

Table 3. Technique utilizing mutants

No.	*Technique*	*Crop*	*Gene*	*References*
1	MutMap	Rice	*OsRR22*	Takagi *et al.,* 2015
			LOC_Os06g29380	Deng *et al.,* 2017
			OsCADT1	Chen *et al.,* 2020
			OsNRAMP5	Cao *et al.,* 2019
		Wheat	*MS1*	Wang *et al.,* 2017
		Oilseed rape	*Bna.IAA7.C05*	Cheng *et al.,* 2019
		Maize	*ZmCLE7*	Tran *et al.,* 2020
		Soybean	*Glyma.04g242300*	Al Amin *et al.,* 2019a
2	MutMap Gap	Rice	*Pii-1*	Takagi et al, 2013b
			Pii-2	Takagi *et al.,* 2013b
3	MutMap$^+$	Rice	*BEIIb*	Nakata *et al.,* 2018
			OsLAP6/OsPKS1	Zou *et al.,* 2017
		Chenopodium	*CqCYP76AD1 -1*	Imamura *et al.,* 2018
4	MutChromSeq	Barley	*Eceriferum -q*	Sánchez -Martin *et al.,* 2016
			Rph1	Dracatos *et al.,* 2019
		Wheat	*Pm2*	Sanchez-Martin *et al.,* 2016
5	MutRenSeq	Wheat	*Sr22, Sr45*	Steuernagel *et al.,* 2016
			Yr7, Yr5 and YrSP	Marchal *et al.,* 2018
6	NIKS	Rice	*OsCAO1*	Nordström *et al.,* 2013

Table 4. Techniques utilizing natural variants

No.	Technique	Crop	Gene	Reference
1	QTL-seq	Rice	*Nortai qPi -nor1(t) qPHS3 -2*	Takagi *et al.,* 2013a
			qTGW5.3	Yaobin *et al.,* 2018
		Chickpea	*SW QTL (CaqSW1.1)*	Das *et al.,* 2015
		Cucumber	*Ef1.1*	Lu *et al.,* 2014
		Soybean	*Glyma.13 g249400*	Zhang *et al.,* 2018
		Squash	*QtlPC -C04*	Ramos *et al.,* 2020
2	BSR-seq	Wheat	*Stem Rust res. QTL*	Edae and Rouse, 2019
		Maize	*Glossy13*	Li *et al.,* 2013
3	Exome capture	Wheat	*Rht-B1*	Mo *et al.,* 2018
			Lr21	Hussain *et al.,* 2018
4	AgRenSeq	Wheat	*Sr33, Sr45, Sr46 and SrTA1662*	Arora *et al.,* 2019

4.1.2 MutMap Gap

It is the extension of MutMap where in there is a gap between the sequence data generated out of the parental as well as high trait value bulk and low trait value bulk and the available reference sequence of the species (Takagi *et al.,* 2013b).

4.1.3 MutMap^{+}

With respect to traits related to fertility, lethality, *etc*. the use of MutMap technique is very difficult with respect to the development of mapping populations. To map such gene(s), MutMap^{+} method was developed by Fekih *et al.,* (2013). In this method, only one bulk which has no defect can be utilized to identify the location of the causative gene associated with the defect.

4.1.4 MutChromSeq

This technique has potential to be utilized in polyploid crops such as wheat, where a number of chromosomal cytogenetic stocks are available. In such species, chromosome number is high and sequencing of each chromosome is additional cost and time taking. In this, the chromosome from the mutant individuals in the population generated using mutant stocks harbouring the mutation is isolated using flow cytometry and then subjected to alignment over reference sequence and site is identified (Sánchez-Martin *et al.,* 2016).

4.1.5 MutRenSeq

A novel technique which is a 3-step method (mutagenesis, exome capturing and cloning of gene(s)), has reduced the time required to identify the gene from the wild germplasm and making it amenable to incorporate in cultivated lines in a single breeding cycle. Steuernagel *et al.* (2016) have introduced and shown the potential of this technique by identifying stem rust resistance gene *Sr33* and *Sr22* in wheat from mutagenized source. In this

technique, information on signature of disease resistance gene is utilized as most of the resistance genes are having nucleotide binding domain leucine rich repeats (NLRs). Availability of reference genome sequence and low-cost sequencing platform is essential to this technology.

4.1.6 NIKS (Needle in the k-stack)

This is a reference free method on the basis of comparison of k-mers in the whole genome sequence of mutants. In this technique, there is no requirement of segregating population and genetics maps. Nordstrom *et al.* (2013) developed this technique to map mutations in rice.

4.2 Mapping using natural variants

4.2.1 QTL-Seq

This method is based on the re-sequencing and is widely utilized in mapping quantitative trait loci (QTLs). Biparental mapping population generated from diverse parents for the trait of interest is utilized and extreme bulks as well as parental lines were subjected to re-sequencing. The reads were then aligned with reference to calculate the delta SNP index and causal SNP for the trait of interest identified (Takagi *et al.,* 2013a).

4.2.2 BSR-Seq

RNA based bulk segregant analysis is done through RNA isolated from mutants and non-mutants from segregating population to form separate pools and are subjected to RNA-seq, reads so obtained are aligned over reference sequence to identify the causal mutant allele (Liu *et al.,* 2012).

4.2.3 AgRenSeq

Resistance genes in plants are characterized by the presence of peculiar signature sequences such as Nucleotide Binding Domain Leucine Rich Repeat (NLRs) which is most prevalent. Wild relatives of crop plants are reservoirs of such genes as they have been exposed to different variants of the pathogen as a result of trench-warfare. Green revolution resulted in narrowing down of genetic base and the varieties or advanced lines of today are devoid of such NLR genes and hence, there is need to utilize wild resources. However, utilization of such wild genetic resources is restricted due to many genetic factors like crossing barriers, ploidy differences and linkage to undesirable genes. To overcome these limitations, a method of rapid identification and cloning of such genes has been developed utilizing germplasm panel, known as AgRenSeq (Arora *et al.,* 2019). AgRenSeq combines enrichment of R genes using k-mer based association genetics without the use of sequence information. Arora *et al.,* (2019) identified and cloned three genes *viz., Sr33*, *Sr46* and *SrTA1662* using AgRenSeq from 195 diploid accessions of wheat species *Aegilopes tauschii* ssp. *strangulata* and *Ae. tauschii* ssp. *tauschii.* They also established that the panel size required for mapping does not need to be large as they could be able to map all the three genes using only 140 accessions. Also, it can identify multiple genes having epistatic effects and the time taken to map these genes is very less. AgRenSeq technique is able to tap disease resistant genes from the pan-genome variation representing a wide range of genetic resources. The technique has shown its potential in wheat and can be further extended to crops where there is no limitation germplasm

availability. This technique gives importance to utilization of wild germplasms in regular breeding programme consistently.

5. Marker Assisted Selection (MAS)

The pre-genomic era of plant breeding was predominated by visual phenotypic selection and crossing the best phenotypes to bring out a new cultivar. DNA marker technology has shown great potential in plant breeding (Singh *et al.,* 2011; Sundaram *et al.,* 2011). Based on genetic linkage maps, DNA markers help to detect the allelic variation in the genes governing various agronomic traits. DNA markers help to accelerate plant breeding by enhancing the efficiency and precision of plant selection. Several activities can be performed using information on marker-trait associations such as, precise assessment of genetic diversity, marker assisted backcross breeding, marker assisted recurrent selection, marker assisted pedigree selection, genomic selection, *etc*.

5.1 Marker Assisted Backcross Breeding (MABB)

Backcrossing is most common technique to integrate one or a few genes into an agronomically superior variety which is deficient in one or two major traits. DNA markers in backcross breeding increase the selection efficiency through indirect selection of target genes (foreground selection), rapid recovery of recipient parent background (background selection), and minimization of linkage-drag (recombinant selection). Moreover, MABB facilitates trait, genotype, heritability, growth stage, and environment independent selection which were very difficult with conventional methods (Xu and Crouch, 2008). MAS has been successfully used in several breeding programmes such as development of Improved Pusa Basmati-1 resistant to bacterial blight (Gopalakrishnan *et al.*, 2008), improvement of KMR-3R, a stable restorer line and subsequently its derived rice hybrid KRH2 for bacterial blight resistance and grain quality by marker-aided introgression of three genes (Hari *et al.,* 2011), JG-11 chickpea variety developed by introgression of a QTL-hotspot genomic segment for better root system and drought tolerance (Varshney *et al.,* 2013), improvement of widely grown but rust susceptible wheat variety HD2932 by pyramiding three rust resistance genes *Lr19*, *Sr26* and *Yr10* (Mallick *et al*., 2015), 'Pusa Basmati 1121' improved for resistance against blast and bacterial blight diseases by adding *Pi2*, *Pi54, xa13 and Xa21 genes* (Ellur *et al.,* 2016), development of biofortified pro-vitamin A rich QPM variety 'Pusa Vivek QPM-9 Improved' by combining *crtRB1, lcyE*, and *o2* genes (Gupta *et al.,* 2019), enhancement of β-carotene concentration in an improved version of CO6 maize by introgression of *crtRB1* gene (Natesan *et al.,* 2020), *etc*.

5.2 Marker Assisted Recurrent Selection (MARS)

Recurrent selection aims to accumulate all desirable alleles of several genes/QTLs in a segregating population based on specific sub-set of markers showing significant association with agronomically important traits in a population (Bankole *et al.,* 2017). For each individual in population, additive effects of all alleles for all the marker loci are added to calculate the net marker score (m). A cumulative selection index (I)

can be computed based on both molecular marker data and phenotype data by formula given as

$I = ¼ b_z z + b_m m$

where b_z is the weightage for trait phenotype (z)

b_m is the marker score (m).

For a given selection intensity, the efficiency of marker score-based selection relative to that of phenotypic selection can be estimated by the formula (Singh and Singh, 2015)

$$\sqrt{p/h^2}$$

where p is the fraction of additive genetic variance (V_A) of the trait contributed by all the marker loci included in the marker score calculation

h^2 is heritability of the trait

A number of researchers have utilized the potential of MARS in crop improvement, such as genetic gain for yield attributing traits in maize under drought stress (Bankole *et al.,* 2017), improvement of wheat yield under drought condition during multi-location trials after each cycle of recombination based on higher marker score (Harikrishna, 2017), successful combination of several QTLs of small effect for crown rot resistance in wheat (Rahman *et al.,* 2020) *etc*.

5.3 Genomic Selection (GS)

Genomic selection (GS) was put forwarded by Meuwissen *et al.* (2007) to deal with the lacunas associated with MAS and MARS. The GS makes use of the information of genome-wide marker-trait associations irrespective of the fact that the associations with the trait(s) of interest are significant or not.GS focuses to predict breeding values. It integrates marker and phenotypic data in a training population (TRN) to derive genomic estimated breeding values (GEBVs') of individual genotyped but not phenotyped line in breeding population (Crossa *et al.,* 2017) (Figure 1).

Recently, several researchers (Rutkoski *et al.,* 2017; Wang *et al.,* 2018; Robertsen *et al.,* 2019; Voss-Fels *et al.,* 2019; Xu *et al.,* 2020) have reviewed and highlighted the power of GS in crop improvement. Li *et al.* (2018b) used the whole-genome re-sequencing approach to sequence the 132 chickpeas under drought-hit environments and found number of SNPs from auxin-related genes were highly correlated with yield and yield associated traits. They inferred that instead of using all SNPs, use of SNP subsets significantly correlated with the traits of interest resulted in increased prediction accuracies. Gaikpa *et al.* (2020) detected favourable alleles in two European maize landraces related to Gibberella ear rot using GS. Similarly, Sertse *et al.* (2020) evaluated a mini-core collection of flax for 11 drought related traits and identified a number of quantitative trait nucleotides (QTNs) linked with drought-related traits, including grain yield as well as with heat and salt stress.

6. Breeding by design

Breeding by Design is a novel plant breeding concept that has been designed to accumulate all allelic variations for all genes of agronomic importance into a single ideal cultivar. It can be achieved by combining precise genetic mapping, high-resolution chromosome haplotyping and extensive phenotyping through the steps given below (Peleman and Van der Voort, 2003).

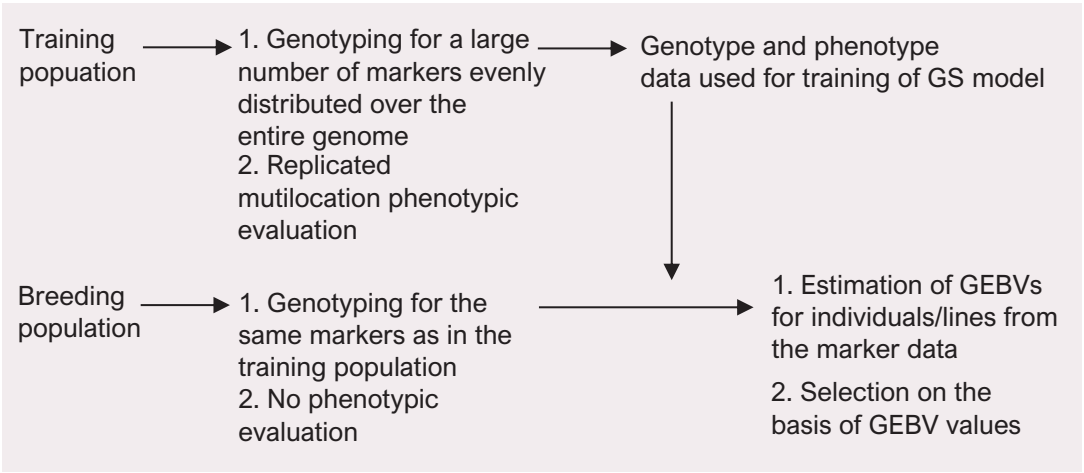

Fig. 1. A diagrammatic view of genomic selection (GS) *(Singh and Singh, 2015)*

1. Mapping of loci govern all agronomically important traits.
2. Analysis of the allelic variation at those loci.
3. Breeding by Design by choosing the parental combinations and the desired recombinants.

This *insilico* breeding approach leads to fast-tracking of desired traits into target genotypes resulting in faster variety development and release. Markers are used as a substitute for phenotyping, which helps to do selection in off-season nurseries which in turn makes the programme cost effective.

7. Advances in phenomics technology

Breeding for higher productivity and mitigation of biotic and abiotic stresses requires good understanding of genotype X environment interactions. This necessitates a well-established and robust protocol for phenotyping of several aspects of plant development and their reaction to changing environment. Plant physiological traits such as water and nutrient uptake and their portioning, photosynthate partitioning, canopy temperature and moisture content, rate of transpiration, heat dissipation, stomatal conductance, *etc*. are key for development of selection criteria for high yielding and climate resilient genotypes.

Phenomics is a trans-disciplinary field that requires expertise from cell biology, system biology, genetics, mathematics, statistics, and information sciences and involves accumulation of total phenotypic

information right from cellular, tissue, organ, whole organism and up to population level. High-throughput phenotyping is essential to have a better understanding of causal link between the genotype and environmental variations and the phenotype. High-throughput phenotyping is necessary to capture phenotypical data in larger and detailed quantities.

7.1 High-throughput phenotyping in controlled environmental conditions

Demand for automated greenhouses equipped with high-throughput phenotyping platforms (HTPPs) are now increasing for automated and rapid phenotypic data acquisition and analysis. Manual or automated high-resolution image capture technologies have delivered HTPPs. HTTPs systems may include stationary camera such as LemnaTec system, designed for barley plants grown in pots or systems that use moving imaging devices over the plants such as PHENOPSIS (Granier *et al.,* 2006) and GROWSCREEN (Walter *et al.,* 2007). The imaging technologies used in different HTTPs are listed in Table 5.

HTTPs assure salient advantages such as non-destructive and non-invasive detection, high-resolution images, ease of phenotyping, multi-trait recording and helps in development of database and predictive models. However, several such HTPPs have high cost of construction, operation, and maintenance, and most of the researchers lack access to these technologies, all traits cannot be measured, storage and maintenance and analyses of huge data require appropriate computer and software facility.

7.2 High-throughput phenotyping in field conditions

Real value of a genotype can only be determined in field conditions. Field based high-throughput platforms (FBPPs) can be either a grounded or an aerial vehicle equipped with high-resolution imaging device, sensors and a computing device that captures phenotypic data, manage and analyse large datasets throughout crop duration in field conditions (Table 6). White *et al.* (2012) had emphasized the inclusion of supportive phenotyping systems in controlled environments or platforms for rapid screening of shoot or root traits.

7.2.1 Ground based fixed (on tower) FBPPs

These are low-cost, easy to place and maintain, but at the same time generate limited crop information. For example, Fukatsu *et al.* (2012) developed Crop Phenology Recording System (CPRS) and used digital imaging for monitoring rice bug. Zhou *et al.* (2017) came up with a FBPP 'CropQuant' supported with web-based control system *viz.* 'CropMonitor' and successfully demonstrated real-time monitoring of dynamic crop growth.

7.2.2 Ground based mobile FBPPs

Single or a combination of sensing devices such as ultrasonic sensor, RGB camera, thermal infrared thermometer or a spectrometer mounted on manually operated vehicles such as a cart can be used to collect data from a relatively larger area and 3D information can be generated. Bai *et al.* (2016) developed a cart mounted multiple sensors based FBPP and used it in soybean and wheat field for phenotyping of

Table 5. List of different imaging techniques used in delivered high-throughput plant phenotyping platforms (HTPPs) (based on Zhao et al., 2019)

No.	*Phenomics Technologies*	*Applications*	*Measurement parameters*
1	RGB (Red, Blue, Green) Imaging	Plant morphology, Digital biomass, height, colour, texture etc. Nutrient deficiencies, Disease symptoms, plant growth and senescence analysis	Whole plant or any plant part, growth dynamics
2	Infrared (IR) Imaging and Near Infrared (NIR) Imaging	Measurements of transpiration, heat dissipation, stomatal conductance	LAI, Canopy temperature and moisture status, seed composition, growth dynamics
3	Fluorescence imaging	Photosynthetic status/quantum yield/seedling structure/leaf disease, etc	Multiple chlorophyll fluorescence parameters and multi-spectral fluorescence parameters
4	Hyperspectral camera	Disease severity assessment, leaf and canopy growth	Water content, seed composition, etc. indoor time series experiment
5	3D imaging	Shoot structure, leaf angle, canopy structure etc.	Plant or organ morphology, structure, and colour parameters, time series at various resolutions
6	Magnetic Resonance Imaging (MRI)	Morphometric parameters/water content.	Water content, morphology parameters (200–500 mm), 1–600 s
7	X-ray tomography (Computer Tomography)	Tissue density, tiller number, seed quality, and tissue 3D reconstruction.	Morphometric parameters in 3D (1–100 mm), minutes- hours
8	Positron emission tomography (PET)	Visualize the metabolic distribution and transport of radionuclides	Transport partitioning, sectorality, flow velocity, 1–2 mm, 10 sec– 20 min
9	Fluorescent protein (FP)-Green, Yellow, Red	Pathogen movement monitoring, Study root colonization using GFP tagged bacteria (Chaerle and Van Der Straeten, 2001), Monitoring cellular functions and signal transduction (Chaerle *et al.,* 2009)	Image based detection and quantification of differences in fluorescence intensity resulted from differential GFP expression.

traits such as height, canopy temperature, reflectance and 3D pictures. Individual plant architecture can be studied with stereo camera, time-of-flight depth sensor and infrared (IR) camera based FBPP as described by Young *et al.* (2019).

Gantry mounted sensors have been developed such as Field Scanalyzer phenotyping platform (LemnaTec GmbH; Virlet *et al*., 2017) and Crop 3D (Guo *et al.,* 2018). The Field Scanalyzer is a fully-automated, carries out non-invasive phenotyping of plant morphology, physiology and health. Crop 3D uses light detection and ranging (LiDAR) technology integrated with a high-resolution camera, thermal camera and a hyper-spectral imager to acquire three-dimensional (3D) data on plant height, width, leaf area, leaf angle, *etc*.

7.2.3 Unmanned aerial vehicle (UAV) based FBPPs

The sensors that use UAV based remote sensing platforms (UAV-RSPs) typically include digital cameras, IR thermal imagers, LiDAR, multi-spectral cameras, and hyper-spectral sensors, which capture data for crop biomass estimation (Tilly *et al.,* 2015), crop fine-scale geometric traits analysis and canopy surface modelling based on images (Gonzalez-Dugo *et al.,* 2014; 2015), monitoring of crop physiology via chlorophyll fluorescence, monitoring of plant nitrogen levels (Yang *et al.* 2019); detection of plant water (Gómez-Candón *et al.,* 2016; Roitsch *et al.,* 2019). Recently, White *et al.* (2012), Zhao *et al.* (2019) and Yang *et al.* (2020) have discussed the pros and cons associated with the use of UAV based FBPPs based on a number of studies. UAVs are highly portable with great efficiency in data collection and monitoring, low cost and highly suited in field conditions are the major advantages of UAV based FBPPs. But some serious limitations are also associated with UAVs such as lack of algorithms and software for rapid data processing and modelling, UAVs usage is weather dependent and legal regulations on airspace use.

7.3 Future prospects of phenomics

High-throughput phenomics platforms can serve both extensive and intensive phenotyping. Extensive phenomics means recording phenotype of multiple traits but their evaluation in a limited number of contexts. But intensive phenomics involves characterization of one or few traits phenotypes in detail. Plant traits need to be prioritized for investigation under controlled or field condition (Houle *et al.,* 2010). High-throughput phenotyping data can be used to construct a database which ultimately helps in finding association between DNA sequences with structure and function of crop plants. Data acquired under clearly defined environments, digitally stored, with fast and easy retrieval system and objectively described in mathematical terms will for the base for such database (Singh and Singh, 2015). This will ultimately help in linking of genomic information with agricultural traits.

The current phenomics studies are mostly extensive and thus an intensive approach must be included. To develop better field-based phenomics facilities research priority should be on (i) development of effective management of acquired field data, (ii) integration of data collected from several sensors and global positioning system (GPS), (iii) establishment of suitable protocols for trail of newly developed instruments for phenotyping with respect to their calibration, ease of integration in setup and for testing their

Table 6. List of promising applications of Field-based high-throughput phenomics platforms

No.	*Targeted trait*	*Indices measured*	*Applications*	*Reference*
1	Chlorophyll	Canopy chlorophyll content index (CCCI), Normalized difference vegetation index (NDVI) for live green vegetation	Chlorophyll content	Barnes *et al.,* 2000
2	Carotenoids content	Green atmospherically resistant vegetation index (GARI)	Chlorophyll content, photosynthetic rate	Gitelson *et al.,* 2006
3	Cellulose	Cellulose absorption index (CAI)	Bioenergy potential	Daughtry, 2001; Kokaly *et al.,* 2009
4	Nitrogen content	NDVI, CCCI	Plant nitrogen status under stress	Tilling *et al.,* 2007; Bronson *et al.,* 2011
5	Lignin content	Cellulose absorption bands	Stress response, Bioenergy potential	Kokaly *et al.,* 2009
6	Photosystem II activity	Photochemical reflectance index (PRI), Chlorophyll fluorescence	Diurnal radiation use efficiency, Effect of stresses on photosynthesis	Baker and Rosenqvist, 2004
7	Canopy conductance	Canopy temperature (CT), Crop water stress index (CWSI), Normalized water index (NWI)	Transpiration (Crop water status)	Jackson *et al.,* 1981; Chaudhuri *et al.,* 1986
8	Canopy water content	Normalized difference water index (NDWI)	Plant water status	Babar *et al.,* 2006b; Gutierrez *et al.,* 2010
9	Leaf area index and Plant biomass	NDVI, NWI	Overall growth	Babar *et al.,* 2006a
10	Canopy height	Close range photogrammetry, Ultrasonic depth camera	Light interception, Growth, Lodging resistance, Canopy height and width; leaf orientation and size	Bronson *et al.,* 2011
11	Flower number	Image analysis	Plant development	Thorp and Dierig, 2011
12	Maturity	Time series of index, Time series of fluorescence	Seedling emergence, onset of grain-filling, senescence	Ghozlen *et al.,* 2010

reliability over a range of temperature and, (iv) development of strong algorithms for data analysis (Houle *et al.,* 2010). Growing tendencies to get IP protections has been observed as detrimental for innovations in phenomics platforms and delays improvement in software required for field-based phenotyping and thereby increases the installation, operational and maintenance cost of such phenotyping platforms. These are ethical issues needed to be addressed for the betterment of plant breeding research.

8. Breeding by editing

8.1 Genome editing tools in crop improvement

Gene-editing involves the changes made in DNA of any organism, either by replacing, adding, or modifying nucleotides in DNA. It is an appropriate, flexible and preferred tool for crop improvement. The fundamental principle of gene-editing is based on double stranded breaks (DSBs) and their repair mechanisms in DNA. These DSBs are repaired by either of the two pathways *viz.* homology directed repair (HDR) or non-homologous end joining (NHEJ). The difference between these repair-mechanism is that the HDR uses sister chromatids as a DNA repair template whereas the NHEJ causes direct ligation of DSBs (Punwar *et al.,* 2014).

Gene-editing technology has witnessed enormous advancement in less than four decades. Currently there are three potent classes of nucleases that can be designed to deliver double stranded breaks at virtually any desired target: Zinc-Finger Nucleases (ZFNs); transcription activator-like effector nucleases (TALENs); and CRISPR-Cas, (Carroll, 2014). While the CRISPR-Cas platform currently dominates research laboratories worldwide, the other two have also been in use in diverse agricultural and medical research.

8.1.1 Zinc-Finger Nucleases (ZFNs)

These targets sites consist of two zinc-finger recognition and binding sites flanking up to 5 - 7 base pair spacer sequences recognised by the Fok-1 endonuclease cleavage domain. Cys2 - His2 zinc finger domains form a ZFN, in which each domain comprises approximately 30 residues of amino acids which are arranged in ββα motifs. (Gaj *et al.,* 2013; Palpant and Dudzinski, 2013; Petolino, 2015).

8.1.2 Transcription Activator-Like Effector Nucleases (TALENs)

These are engineered enzymes having Fok1-endonuclease containing TALE domains. The TALE binding domain comprises of a run of repeat domains of roughly thirty-two residue in which each repeat binds to DNA through the residues of amino acids at positions 12 and 13 referred to as RVDs (Repeat Variable Di-residues). Generally, RVDs which are used to assemble artificial TALE arrays include HN or NN for Adenine or Guanine respectively, NI for Adenine (A), HD for Cytosine (C) and NG for Thymine (T). These regions determine the specificity of the nucleotide base. The TALE proteins recognize and provides stimulus to unique promoters in the plant cell acting through tandem repeats, hence forming the basis of developing this innovative gene editing system (Jankele and Svoboda, 2014; Dheer *et al.,* 2020). Similar to ZFNs, the dimer formation in the TALEN protein is facilitated by the Fok-1 cleavage, which subsequently, splits the TALE binding sites by cutting the 12 to 19 base pair spacer

sequence. In contrary to the zinc fingers that recognize the DNA triplet, only a single base pair is recognized by TALE protein, with minute or no variation between the target sites and the neighbouring domains.

8.1.3 Clustered regularly interspaced short palindromic repeat (CRISPR)

This is an RNA-programmed DNA cleavage mechanism that have been discovered first in bacteria and archaea. CRISPR–Cas9 and CRISPR–Cas12a are the most studied and most commonly deployed CRISPR models (Jinek *et al.,* 2012, Zetsche *et al.,* 2015). Each system has two components: a DNA endonuclease (Cas9 or Cas12a) and an RNA molecule called as single guide RNA (sgRNA) or a CRISPR RNA (crRNA). The only requirement for applying CRISPR to a specified target is the presence of a protospacer adjacent motif (PAM) sequence close to the site of interest. Using CRISPR for different targets thereby needs only different spacer sequences; therefore, it is quick, fast, effective, cheap and adaptable.

Organisms created by genome editing are literally genetically-modified, but vary from earlier GMOs in significant ways (Carroll *et al.,* 2016). In certain cases, no genetic material from another species is incorporated, except where it is inserted in a particular genomic position. Changes that are made are most likely those that may have arisen spontaneously, and entire genome sequencing may be conducted on modified organisms to search for off target mutations. Since seed can be rapidly dispersed into the next generation, validated genomes can easily produce large populations of modified plants. Latest examples of modified crops are summarized in Table 7 and 8.

Gene-editing techniques have an enormous capacity to improve the production, productivity, nutrition and quality of food crops and it is anticipated to help in tackling the world's food scarcity problems. However, the main issue at present is that the producer and the consumer acceptance to commercially available genome edited-products (Bartkowski *et al.,* 2018, Wang *et al.,* 2019). Gene-editing supporters contend that the features of the end product, not the procedure involved, should be used for food safety evaluation (Bain *et al.,* 2019). However, differing interpretations of the gene-edited goods will still remain, in particular due to the different regulatory provisions in various countries. Global science agreement and uniform regulatory initiatives across countries could contribute to the utility of gene-editing technologies beyond the research domain.

In a policy brief on accelerating the use of Genetic Engineering (GE) technologies for food and nutrition security and increasing farmers' income from the National Academy of Agricultural Sciences, NAAS Fellows representing scientists from India's National Agricultural Research System recommended-

- To support environmental release of the GE varieties, which have been established to be bio safe, to extend the advantage of growing these varieties to the consumers and farmers.
- The provision of taking NOC from states for the conduct of confined field trials (CFTs) of GE crop varieties is counter-productive to GE research and must be discarded as the GEAC investigates issues of biosafety from a national perspective and there is no provision for such a step in the regulation of GE plant CFTs in India.

Table 7. Application of CRISPR System in Crops

No.	*Crop*	*Research objective/Target trait*	*Reference*
1	Tomato	Apprehend the role of a photoreceptor in seedling development (stress tolerance)	Liu *et al.,* 2020
		Bacterial speck resistance	Ortigosa *et al.,* 2019
		Fusarium wilt tolerance gene function confirmation	Prihatna *et al.,* 2018
		Improve lycopene content	Li *et al.,* 2018a
		Enhance Tomato Yellow Leaf Curl Virus resistance	Tashkandi *et al.,* 2018
		Improved shelf life	Yu *et al.,* 2017
		Powdery mildew resistant plants (transgene-free)	Nekrasov *et al.,* 2017
		Achieve ideotype	Zsögön *et al.,* 2017
2	Capsicum	Apprehend the role of transcription factors in fruit colour and chloroplast development	Borovsky *et al.,* 2019
3	Carrot	Generate haploid plants	Dunemann, 2016
4	Potato	Reduce enzymatic browning	González *et al.,* 2020
		Overcome self-incompatibility	Ye *et al.,* 2018, Enciso Rodriguez *et al.,* 2019
		Reduce steroidal glycoalkaloids	Nakayasu *et al.,* 2018
		Develop amylopectin starch cultivars	Andersson *et al .,* 2018
5	Sweet potato	Improve Fusarium wilt resistance	Zhang *et al.,* 2020a

No.	*Crop*	*Research objective/Target trait*	*Reference*
6	Watermelon	Vacuolar sugar transporter gene function validation	Ren *et al.,* 2020
		Obtain gynoecious genotypes	Zhang *et al.,* 2020b
		Fusarium oxysporum f. sp. niveum Race 1 resistance	Zhang *et al.,* 2020c
		Fruit flesh sugar accumulation gene functional characterization	Guo *et al.,* 2019
		Herbicide resistance	Tian *et al.,* 2018
7	Banana	Inactivate banana streak virus	Tripathi *et al.,* 2019
8	Soybean	Change in the overall lipid content	Al Amin *et al.*, 2019b
		Delay in flowering time	Cai *et al.*, 2018
9	Rice	Rice blast disease tolerance	Yin *et al.*, 2017
		Stress tolerance and panicle development	Lee *et al.*, 2019
		Bacterial blight resistance (*Os SWEET genes)*	Varshney *et al.*, 2019
		Increasing Amylopectin by amylose ratio	Perez *et al*., 2019
		Increased resistance to Rice tungro Virus (spherical)	Macovei *et al.*, 2018
10	Apple	Early flowering	Charrier *et al.*, 2019
11	Wheat	Grain morphology and yield	Zhang *et al.*, 2019a,b
		Gluten content	Sanchez Leon *et al.*, 2018
		Grain weight and length	Liang *et al.*, 2017
12	Barley	Grain development and germination	Holme *et al.*, 2017
		Drought stress	Curtin *et al.*, 2018
13	Maize	Albino explants	Feng *et al.*, 2018

Table 8. Application of ZFN and TALEN Systems in Crops

No.	*Crop*	*Trait*	*Target gene*	*Reference*
ZFN				
1	Soybean	Herbicide transmission	*DCL*	Curtin *et al.,* 2011
2	Tobacco	Chromosome breaks	*GUS: NPTII*	Wright *et al.,* 2005
3	RICE	Detection of safe harbour loci Herbicide	*OsQQR*	Cantos *et al.,* 2014
TALEN				
1	Wheat	Powdery mildew resistance	*TaMLO*	Wang *et al.,* 2014
2	Potato	Herbicide resistance	*ALS*	Butler *et al.,* 2016
		No reducing sugars and improved food safety	Vacuolar invertase	Clasen *et al.,* 2016
3	Sugarcane	Reduced lignin and improved biofuel production	Caffeic acid O-methyltransfe rase	Jung *et al.,* 2016
4	Rice	Fragrant rice	*OsBADH2*	Shan *et al.,* 2015
5	Soybean	Low polyunsaturated fats	*FAD2 -1A, FAD2 -1B*	Demorest *et al.,* 2016
6	Wheat	Powdery mildew resistance	*TaMLO - (A1, B1, D1)*	Wang *et al.,* 2014

- A clear public awareness campaign on issues relating to GE technologies must be established to restrict the production of false public opinions, based on unsubstantiated knowledge.

9. Novel plant breeding technologies in heterosis breeding

To increase the yield of crop significantly, heterosis breeding has been identified as a potential technology, since several years. There are three requirements for a crop to have successful hybrid *viz.* higher level of heterosis, pollination control mechanism and efficient and cost-effective seed production technology and each of these are indispensable. High level of heterosis has been reported in many of the crops and pollination control mechanisms like cytoplasmic and genetic male sterility occurring in nature have been identified and exploited. Further, many techniques such as transgenesis and CRISPR-mediated gene editing have shown potential to create male sterility trait artificially (Barman *et al.,* 2019). Further, economic viability of seed production system have to be improved in terms of production of parental lines.

9.1 Seed Production Technology (SPT)

In cross-pollinated crops, there is problem of mixture of fertile seeds in the production of male sterile parental line, utilizing genetic male sterility system. Wu *et al.,* (2016) developed a transgenic maintainer that does not affect the composition of produced male sterile line that leads to production of non-transgenic male sterile line and utilizing colour marker linked to fertile allele in maintainer line is amenable to machine colour seed sorting. The maintainer line is designed with following genes (i) non transgenic fertility gene, (ii) pollination inhibitor gene having alpha-amylase domain, and (iii) colour-based marker gene. Though the technology has been identified and being utilized in maize crop but has the potential where genic male sterility gene(s) have been identified and further this technique broadens the scope to utilize any line form the germplasm to be utilized in hybrid seed production.

9.2 Reverse breeding method

Reverse breeding is a method where from a hybrid the parental lines can be isolated. It utilizes the engineered meiosis which supresses the recombination in the hybrid or F_1 (Dirks *et al.,* 2009). In addition, this technique also allows the production of chromosomal substitution lines which serve as useful cytogenetic as well genetic stocks in understanding genetics of different traits. Though the technique has the potential but has its own limitations as the probability of getting parental type reduces by the exponential factor with an increase in the number of chromosomes. This technology has the potential where in natural population, superior heterozygotes have been identified and one is keen to develop parental lines from those. Several meiotic suppressor loci in different crops have been identified *e.g*. *ASY1* gene in Arabidopsis (Caryl *et al.,* 2000), *PAIR2* gene from rice (Nonomura *et al.,* 2004) and similarly *ptd, sds, spo11* and *dmc1* (Couteau *et al.,* 1999; Azumi *et al.,* 2002; Stacey *et al.,* 2006; Wijeratne *et al.,* 2006). Different biotechnological techniques like RNAi, CRISPR can be further used to target these genes for development of mutants to utilize in conventional plant breeding programmes. In combination of the above, genetic suppression of meiosis through chemical induction would expedite the reverse breeding application.

10. Conclusion

Biotic and abiotic stresses are the major limiting factors in crop growth and yield. To assure the food security in future, comprehensive knowledge of modern plant breeding and biotechnological tools and techniques is foremost need. A number of tools and techniques are available to understand the molecular mechanisms of response of crop plants at population as well as cellular level under stress conditions. It is essential to discover stress-responsive genes and elucidation of their functions to understand plant response under various stresses. With the revolution in genomics such as genome sequencing platforms has enabled plant scientists for rapid and low-cost genetic dissection of traits, gene discovery, molecular marker development, comparative genome analysis, better understanding and utilization of pan-genome, characterization of signalling cascade and better insights into coping mechanism of crop plant under stress. For the development of superior multiple stress tolerant crop varieties genomics and transcriptomics tools have been proved to

be most promising and convenient for plant breeders, but research advancements in phenomics are yet lagging behind. The incorporation of innovative modern tools and techniques into the present crop improvement programs will uncover new possibilities for the rational design of superior performing multiple stress-tolerant crop varieties.

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ICAR-National Academy of Agricultural Research Management
(ISO 9001:2015 Certified)
Rajendranagar, Hyderabad-500030, Telangana, India
+91-40-24581300/333, Fax: 091-40-24015912
email: director@naarm.org.in
https://naarm.org.in