Molecular Techniques of Crop Breeding

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Molecular approaches are considered as a versatile way to scan out diverse genomic resources which could facilitate to widen up the genetic pool of crop species and further adopted to develop superior cultivars with a wide range of economically demanding traits. In this chapter the tools like RNA interference, CRISPR, Marker assisted selection and genomic selection tools are discussed. The advanced molecular tools has accelerated the probability to manipulate and speed up the crop breeding programme.

Keywords: *Crop breeding, RNA interference, CRISPR, Marker Assisted Selection, Genomic Selection*

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Introduction

Agriculture, heart of global food security faces unprecedented challenges in this modernized world. A rapidly expanding population, projected to exceed 9 billion by 2050, continues to escalate the demand for food, feed and raw materials. At the same time, climate change, loss of arable land, water scarcity and the emergence of new pests and pathogens are intensifying the strain on food production systems. Conventional breeding strategies have long played a key role in crop improvement, but these methods often require decades to develop new varieties and are constrained by limited genetic resources. Traditional plant breeding relies on crossing and selection to incorporate desirable traits, but this process is time-consuming, imprecise and dependent on naturally available genetic variation. The modern molecular tools like RNAi, CRISPR technology, Marker assisted technology and Genomic selection fills this gap by enabling direct and targeted changes in the plant genome (Watanabe et al., 2012; Kumari et al., 2024 and Kaur & Talekar, 2024).

RNA interference for target genes in plants

RNA interference technology refers to a complex interplay of enzymes and proteins that render a particular mRNA inactive, thereby silencing the expression of a target gene in the organism. It is a naturally occurring

process first reported in *Caenorhabditis elegans* (Fire et al., 1998) where degradation of *par -1* gene was observed upon introduction of sense and anti-sense RNA. Though, it was initially believed that the ds RNA involved in the RNA interference were exogenous in origin, a number of studies have also found the endogenous RNAs (Ghildiyal et al., 2008; Tam et al., 2008; Song et al., 2011).

Mechanism of RNA Interference

The double stranded RNA, either injected exogenously or arising with in the cell is cleaved by DICER (an RNase III family enzyme) into exact 21nt long segment called small interfering RNA (si RNA), with a characteristic 2 nucleotide overhang at each end. The si RNA associates with a multiprotein complex called RISC (RNA Induced Silencing Complex) which comprises of a number of agronaute proteins (AGO), helicases, exonucleases and endonucleases. Inside RISC the si RNA unwinds in to the guide strand and the passenger strand, where the later is degraded subsequently. The remaining guide strand in association with PAZ domain of AGO protein helps to find the complementary mRNA to be degraded by PIWI domain of AGO. In some cases, the passenger strand is not degraded rather it is copied by RNA dependent RNA polymerase (RDRP) to form more si RNAs which increases the precision of target gene silencing (Kaur & Talekar, 2024).

Applications of RNAi in plants

The advances of biotechnological research in understanding and manipulating the cellular processes at molecular levels has opened up new vistas for crop improvement. Exploration of non-coding RNAs (nc RNA), siRNA, miRNA, CRISPER-cas etc. have increased the understanding of the control that RNAs exercise over gene expression post-transcriptionally. RNA interference is seen as a tool for improving the plant traits in context of increasing yield, improved stress tolerance and disease resistance, enhanced shelf life, high nutritional value, altered phenotype and more.

- **RNAi for enhanced disease resistance**

Plants are infected by a range of parasites such as viruses, bacteria, fungi, insects, nematodes etc. that cause a number of diseases in plants. The annual global losses due to plant disease is estimated to be about US$220 billion by the Food and Agriculture Organization. Pathogen derived resistance (PDR) is the main strategy that has been exploited by many researchers for inducing virus resistance in plants. The coat protein gene of viruses has been targeted by RNAi in a number of transgenic plants such as *Solanum tuberosum* (Potato virus Y), *Nicotiana benthamiana* (Plum pox virus), - common beans (Gemini virus resistant) etc. (Missiou et al., 2004; Bonfim et al., 2007; Hily et al., 2007). The genetic resistance against the bacterial infection seems to be a viable approach for controlling bacterial infections which are otherwise hard to contain. Dunoyer et al., 2006 reported that silencing of two genes (iaaM, ipt) of *Agrobacterium tumefaciens* stopped the formation of crown gall in Arabidopsis. Such strategy can prove beneficial in many fruit trees where crown gall is a major disease. Fungi being eukaryotes entail for a different strategy as both plant and plant fungal pathogen have same RNAi machinery which on one hand is used by host immune system and on the other hand by pathogen to infect, multiply and for pathogenicity (Muhammad et al., 2019). Resistance to *Phytophthora infestans* and *Blumeria graminis* f. sp. *Tritici*, was achieved by targeted silencing of SYR1 gene in potato and MLO gene in wheat respectively (Riechen, 2007 and Eschen et al., 2012). A number of reports are available where RNAi has been used successfully to induce resistance to nematodes in plants like *Arabidopsis thaliana* (Joshi et al., 2020), *N. benthamiana* (Li et al., 2017), *Glycine max* (Hu et al., 2019, Tian et al., 2019).

- **RNAi for qualitative traits**

Plants are the fundamental resource for alleviating hunger and fulfilling the nutritional need of ever-growing global population. A sea of literature is available where the efforts have been made to increase the yield and nutritional index of the plants. Starch accumulation in the leaves of *Arabidopsis thialana* and *Zea Mays* as a result of down regulation of phosphoglucan phosphatse (SEX4) and glucan water dikinase (GWD) has been achieved by RNA interference (Weise et al., 2012). Similarly, a significant decrease in the quantity of α-linoleic acid (1-3 %) as compared to non-transgenic lines in the soybean, as the presence of later decreases the stability of soybean oil (Florres et al., 2008). The enhanced shelf life of fruits, vegetables, cut-flowers and ornamental plants can reduce the economic loss due to spoilage during the transportation to the markets. Insertion of dsRNA inhibited α-mannosidase (α-Man) and β-d-N-acetyl hexosaminidase (β-Hex), two key enzymes of ethylene production pathway in tomatoes (Gautam et al. 2022). New flower colour, decorative foliage, overall robustness is sought after characters in the global flower market. RNAi mediated reduction in the polyacylated anthocyanins can cause modulation of flower colour (Seitz et al., 2007; Nakatsuka et al., 2010). Likewise, the area of pharmaceutical sciences dwells upon developing the new plant metabolic products or enhancing the production and efficacy of existing plant based secondary metabolites. Metabolic engineering offers the ways of improving the nutraceutical value of plants by enhancing the content of useful amino acids, fatty acids, secondary metabolites, fibers quality, reduction of toxins (Sourabh et al., 2014). This is an age of exploration of biotechnological solutions to the global food challenges and RNAi has emerged as a highly promising functional genomic tool that can meet the global demand of better performing plants.

CRISPR Technology in Modern Agriculture for Sustainable Crop Improvement

CRISPR-Cas systems were originally discovered as part of the immune defense mechanism of bacteria, enabling them to recognize and destroy viral DNA. This mechanism has been adapted into a gene-editing tool that allows researchers to target specific genes with remarkable accuracy. In practice, a guide RNA is designed to recognize a precise sequence within the plant genome, and the Cas nuclease enzyme, usually Cas9, introduces a cut at that location (Barrangou et al., 2007). The plant's natural DNA repair pathways then make changes at the site, which can be exploited to either disable undesirable genes or incorporate beneficial traits. What makes CRISPR particularly attractive is its ability to perform these edits with high specificity, efficiency and relatively low cost compared to earlier technologies (Mulepati et al., 2014). The potential applications of CRISPR in crop science are wide-ranging. One of the most pressing needs is to enhance crop yield to meet the demands of a growing population. By targeting genes that regulate plant architecture, flowering time or seed development, CRISPR can produce varieties with increased productivity. For instance, gene edits in rice and wheat have already shown promise in improving grain number and size, thereby contributing to higher yields (Thiruppathi et al., 2024). Beyond productivity, CRISPR is also being used to improve the nutritional value of crops. Biofortification through genome editing has enabled enhancements such as increased vitamin A in rice, higher protein content in maize and improved oil composition in soybean (Bindu et al., 2024).

These advances not only support food security but also address hidden hunger caused by micro nutrient deficiencies. Another critical area where CRISPR is proving invaluable is in developing resistance to diseases and pests, which account for major agricultural losses globally. By knocking out susceptibility genes or strengthening plant immune responses, scientists have created crop varieties that can withstand bacterial, fungal and viral infections. For example, tomatoes edited using CRISPR have shown resistance to powdery

mildew, while other studies have focused on making rice more resilient against blast disease (Tyagi et al., 2021). Alongside biotic stress resistance, CRISPR has been applied to improve tolerance to abiotic stresses such as drought, salinity, and extreme temperatures. As climate change continues to intensify these challenges, the ability to rapidly develop stress-resilient crops will be critical for ensuring stable agricultural production. Sustainability is another major dimension where CRISPR can make a difference. By creating crops that require fewer chemical inputs, such as fertilizers and pesticides, CRISPR contributes to environmentally friendly farming practices. For example, pest-resistant varieties reduce the need for pesticide applications, thereby lowering environmental pollution and production costs for farmers (Woo et al., 2015). Similarly, crops with enhanced nutrient-use efficiency minimize fertilizer dependency, reducing soil degradation and water contamination. These contributions align with the broader goals of sustainable agriculture, which seek to balance productivity with ecological conservation and social well-being.

Marker assisted selection for crop trait improvement

The foundation of plant breeding rests on the selection of the crops with desirable traits such as increased yield, higher tolerance to biotic/abiotic stresses, improved performance, robustness to climatic variation and more. A molecular marker is essentially an inheritable DNA sequence which is present at a particular locus and shows silent variations among the related genomes. Restriction fragment length polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Single Sequence Repeat (SSR) or microsatellites, Single Nucleotide Polymorphism (SNP), Inter simple sequence repeat (ISSR), Cleaved Amplified Polymorphic Sequences (CAPD), CAAT box derived Polymorphism (CBDB), Sequence Characterised Amplified Region (SCAR) etc. are some of extensively used markers for estimating crop genetic diversity (Table-1) (Nair and Pandey., 2021). Area of selection-breeding has seen unprecedented progress with not only with the precision marking, tracking and characterization of germplasm, clustering of promising genes and release of superior plant varieties in a matter of few years but a simultaneous development of Plant Genetic Resource (PGR) information system is attributed to the immense potential of molecular markers. Recent arrival of functional molecular markers (FMM) that obviate the limitations of random DNA markers is seen as a promising tool for plant breeders to achieve the targeted selection of the selected genes for overall crop improvement.

Table 1. Molecular markers linked to disease resistance and qualitative traits

Markers Used	Crop	Pathogen	Reference
Disease Resistance			
RAPD	*Phaseolus vulgaris* L., *Allium cepa L.*, *Cicer arietinum*, *Oryzae sativa*	*Collectotrichum lindemuthianum*, *Botrytis cinerea*, *Ascochyta rabiei*, *Magnaporthe oryzae*	Balardin & Kelly, 1997; Kim et al., 2021; Baite et al., 2020; Ashkani et al., 2011
SSR	*Pisum sativum*, *Zea mays*, *Malus* spp,	*Uromyces fabae*, *Exserohilum turcicu*, *Colletotrichum gloesporioides*	Singh et al., 2016; Kumar et al., 2022; Gustavo et al., 2016
ISSR	*Triticum* sp.,	*Puccinia graminis* f.	Khan et al., 2005;

	Hordeum vulgare, *Solanum tuberosum*	*Blumeri agraminis* f. *Synchytrium endobioticum*	Ahmad et al., 2017
SNP	*Prunus cerasus*	*Blumeriella jaapii*	Prodhomme et al., 2020
Shelf life	*Solanum lycopersicum,* *Mangifera* sp.	*alc gen,* ACC synthase gene *pgACS1*	Osei et al., 2019; Eltaher, 2025; Mathiazhagan, 2021
Qualitative traits			
SNPs SSR	*Zea Mays, Citrullus lanatus* *Oryzae sativa, Vitis vinifera, Pisum sativum*	Carotenoids, chlorophyll biosynthesis Heat resistance, high productivity	Gao et al., 2024; Zhang et al., 2023 Gebremeskel et al., 2023; Panday et al, 2021

Genomic Selection

The plant breeding programmes are time consuming and costly method to develop new varieties as it requires extensive testing of the new variety at different location and different time points before the release of new variety. The adoption of breeding tool which speed up the entire process in effective way out is genomic selection. The genomic selection layout comprises of training population, breeding population, genome wide molecular marker (Kumari et al., 2024: Kaur & Talekar, 2024). In Genomic selection, the effect of all the markers were estimated individually and genomic estimated breeding values (GEBV) was calculated by combining the the effect of all the markers. The genomic selection starts by developing a training population. The genotypic and phenotypic information was restored from each individual of training population. This information is further used to model construction. In this model the genotype is act as predictor and phenotype as response. The information gathered from this model could be used to calculate the GEBV with respect to breeding population. The breeding population constitute individuals have only genotypic information. The plants with maximum GEBV could be used as a parent in a breeding programme for desirable trait improvement. The stages of genomic selection is presented in Figure 1.

The main preference to foster genomic selection for crop breeding is reduction of time of breeding cycle and cost utilized for extensive phenotyping. To gain success in breeding programme through genomic selection there are certain key consideration which influence the genomic prediction accuracy, includes the size of both training and breeding population, broad genetic pool of breeding population, effect of interaction between genotype with an environment, frequency of the marker on genome coverage, number of QTLs, linkage disequilibrium, genetic relatedness between the training and breeding population and most important model used.

It has been analyzed that most of the models used for the estimation of GEBV does not includes the effect the GX E interaction, which has great impact on economic traits of crop. Juliana et al., in 2020 optimized the model include GXE interaction to enhance the prediction accuracy (Perez-Rodríguez et al., 2017). Genomic predictions were executed by using genomic-best linear unbiased prediction model by using ‘R’ package BGLR ((Habier et al., 2013 and Perez and Campos, 2014)

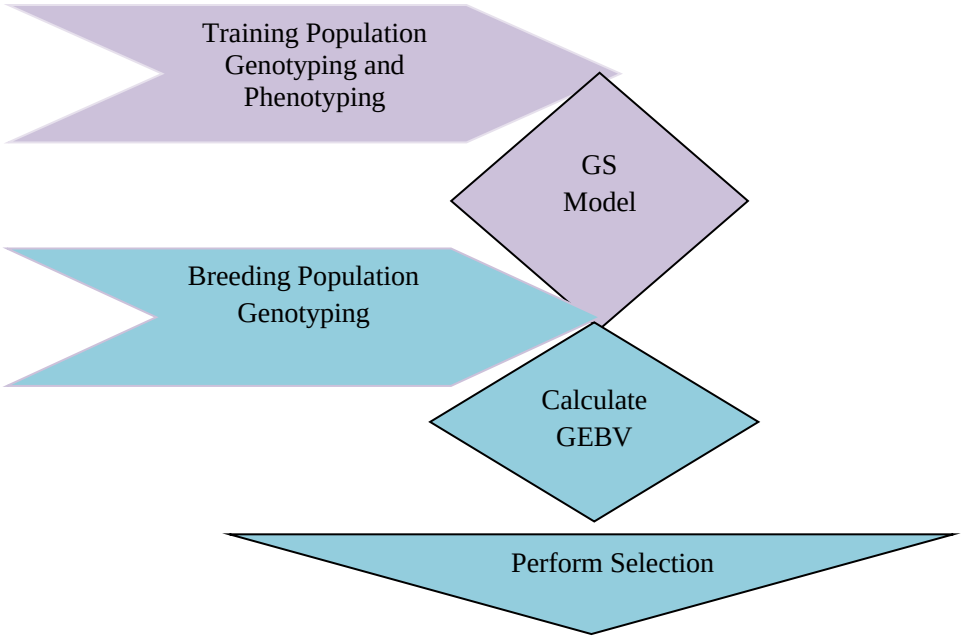

Figure 1. Stages of Genomic selection

Statistical models

The most commonly used method in crop improvements is Ridge Regression Best Linear Unbiased Prediction (RR-BLUP), Genomic Best Linear Unbiased Prediction. (G-BLUP) and Bayesian models. RR - BLUP analysis mainly relies on the effect of markers and variance. Each marker contributed for unique effect despite of similarity in variance (Bernardo et al. 2007). this is the best method for analysis of prediction accuracy, in case of the trait controlled by polygenes (Burgueno et al. 2012). Genomic best linear unbiased predictor (G-BLUP) method estimates prediction accuracy based upon genetic similarity between individual. In this method the matrix is built on the basis of data analysis of marker information. This matrix provides information regarding observed similarity at genotypic level rather than expected similarly on the basis of pedigree analysis. The matrix contains marker information of individuals of training and breeding population. Most of the Bayesian models viz., Bayesian Ridge Regression, Bayes A, Bayes B, Bayes C and Bayesian LASSO are used to analysed the contribution of different markers for different effects and variances (Habier et al., 2011). These models are preferably used to predict the GEBV for complex traits. In Bayesian models most of the markers have null effect on desired trait, due to which markers are excluded from prediction model. This model is used in the breeding for improvement of trait encoded by major effect QTL which contributes to genetic diversity. These models minimize prediction accuracy with respect to increase in number of loci. However, among all models the prediction accuracy of G-BLUP is not influenced by number of QTLs and remains nearly constant. Thus, it is more commonly used model for estimating prediction accuracy for traits controlled by minor genes.

Bioinformatics tools for GS

The complexity of statistical model used for genomic selection calls up a need to explore web-based user-friendly analytical tools and flexible databases for the genomic selection (Kumari et al., 2024). Different analytical tools used for genomic selection are SolGS (http://cassavabase.org/Solgs), G Selection (http://CRAN.R-project.org/package=GSelection), Breed Wheat Genomic Selection (BWGS), Bayesian

generalizedlinearregression(https;//CRAN.Rproject.org/package=BGLR),STGS(https;//CRAN.Rproject.org/package=STGS), MTGS used for multiple traits genomic selection and available freely on website https;//CRAN.R-project.org/package=MTGS.

Conclusion

The prospect of plant breeding offers innovative breeding strategies to tackle worldwide food security. The tools like RNAi and CRISPR-Cas9 enable the precise alteration of particular genes to incorporate desirable characteristics in crop. Molecular markers and genomic selection tools felicitate the selection of superior genomic resources with respect to resistance against biotic and abiotic stress. Together, these tools play a crucial role in cultivating crops with precisely modified traits, accelerate breeding cycle and enhanced crop productivity. Compared to traditional crop breeding these tools reduces reliance on synthetic insecticides and pesticides. Thereby, offers higher accuracy, speed and cost efficiency in developing crop breeding programme that could meet out the goal of global food productivity.

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