REVIEW

Breeding and biotechnological interventions for trait improvement: status and prospects

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Abstract
Main conclusion **Present review describes the molecular tools and strategies deployed in the trait discovery and improvement of major crops. The prospects and challenges associated with these approaches are discussed.**

Abstract Crop improvement relies on modulating the genes and genomic regions underlying key traits, either directly or indirectly. Direct approaches include overexpression, RNA interference, genome editing, etc., while breeding majorly constitutes the indirect approach. With the advent of latest tools and technologies, these strategies could hasten the improvement of crop species. Next-generation sequencing, high-throughput genotyping, precision editing, use of space technology for accelerated growth, etc. had provided a new dimension to crop improvement programmes that work towards delivering better varieties to cope up with the challenges. Also, studies have widened from understanding the response of plants to single stress to combined stress, which provides insights into the molecular mechanisms regulating tolerance to more than one stress at a given point of time. Altogether, next-generation genetics and genomics had made tremendous progress in delivering improved varieties; however, the scope still exists to expand its horizon to other species that remain underutilized. In this context, the present review systematically analyses the different genomics approaches that are deployed for trait discovery and improvement in major species that could serve as a roadmap for executing similar strategies in other crop species. The application, pros, and cons, and scope for improvement of each approach have been discussed with examples, and altogether, the review provides comprehensive coverage on the advances in genomics to meet the ever-growing demands for agricultural produce.

Keywords Molecular markers · Genomics-assisted breeding · Speed breeding · Transgenics · RNA interference · Gene editing

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Introduction

The human population is growing at a tremendous rate, and it is projected to reach 10 billion by 2050. This necessitates the need to produce enough grains to meet the food and nutritional security. Thus, agriculture holds the key to meet the needs of the ever-growing population. However, various factors, including challenges associated with the availability of water and irrigation systems, deteriorating soil fertility, erroneous rainfall, rise in atmospheric temperature and heatwaves, insect pests and pathogens, and their evolution to form highly virulent strains, leaves a heavy toll on agricultural productivity. For example, extreme heat and frequent droughts have resulted in about a 10% reduction in yield of cereal crops throughout the world from 1964 to 2007. The damage had been more in developed countries with an 8–11% higher loss than developing countries (Lesk et al.

2016). On the other hand, intensification of crop improvement programmes using biotechnological and breeding interventions had resulted in the trait discovery and release of improved varieties that could mitigate the adverse effects (Fig. 1). Conventional breeding transformed into molecular breeding, which then took the shape of genomics-assisted breeding to meet the challenges in agriculture. Similarly, gene cloning and overexpression or knockout/down diversified to different approaches, including RNAi, VIGS, gene/genome editing, to develop lines with enhanced agronomic as well as climate-resilient traits. Advances in high-throughput genomics strategies at a whole-genome level, including genetic association mapping, map-based cloning, genomic selection, and speed breeding, are also proven useful in improvising genetic gains for expediting the crop improvement processes.

In one or the other way, these approaches contributed to the increase in the yield of staple crops like maize, rice, and wheat (Bailey-Serres et al. 2019). However, there are some drawbacks, mainly due to the asymmetric support in sub-Saharan and other impoverished areas. Also, the lack of investment in underutilized crops and the replacement of fruits and vegetables with grain crops led to a dietary shift from foods rich in macro- and micronutrients to a calorie-rich diet (Pingali 2012). Achieving nutritional and food security by increasing the production of nutrient-rich fruits, vegetables, cereals, etc. is the call of the hour and a significant challenge for agricultural scientists (Muthamilarasan et al. 2016; Bailey-Serres et al. 2019). While the technologies and approaches for crop improvement get advanced on the one hand, the problems in agriculture and productivity increase on the other, which is majorly due to the changing climate. FAO predicts that crop yields will decline by 25% by 2050 if we do not address climate change. Also, studies on the effect of combined environmental stresses are limited as compared to studies on individual stresses, which is again a limiting factor. Though it has been repeatedly underlined that the response of plants towards combined stress is unique

as compared to individual biotic or abiotic stress, studies in this direction to delineate the molecular machinery underlying such tolerance and extrapolating the information for crop improvement remain largely elusive. While studies in this direction are need of the hour, synergistic application of two or more integrated modern genomic approaches for developing better varieties also gains momentum. Given this, the review enumerates the different genomics approaches being deployed for trait improvement with examples and provides the roadmap for studying the genomic regions regulating any trait and harnessing the information for an expedited improvement and release of elite varieties.

Genomic resources for trait discovery and crop improvement

Whole-genome sequencing, resequencing and pangenome analysis

Genome sequencing has provided direct access to the structural and functional aspects of protein-coding genes organized within the chromosomes of any species. Also, genome sequencing provides information about the noncoding elements, including transposons and promoters, that are crucial for understanding evolution and diversification. Identification of upstream regulatory elements of each gene through genome sequencing has also enabled fine-tuning the expression of target genes, and all these are possible due to the advent of NGS approaches. Started with *Arabidopsis thaliana* (Arabidopsis Genome Initiative 2000), genome sequencing expanded its horizon by including several crops, plants, and tree species. Rice was the first crop to be sequenced (Yu et al. 2002; Goff et al. 2002; International Rice Genome Sequencing Project 2005), followed by maize (Schnable et al. 2009), sorghum (Paterson et al. 2009), and Soybean (Schmutz et al. 2010). Recently, an annotation-grade whole-genome sequence data of wheat
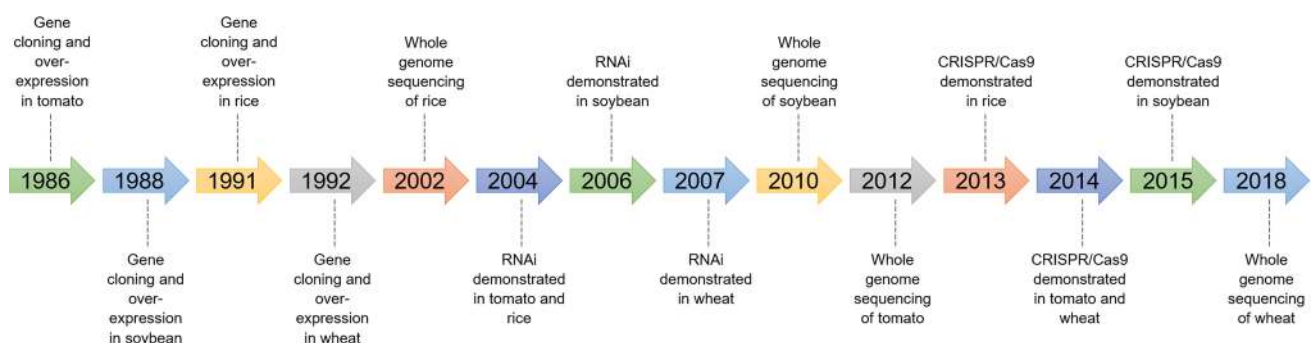

Fig. 1 Timeline of significant achievements in the deployment of approaches, including overexpression of candidate genes, genome sequencing, use of RNAi and genome editing for trait improvement in major species, namely rice, wheat, tomato and soybean

has been released (International Wheat Genome Sequencing Consortium 2018). In case of foxtail millet, Zhang et al. (2012) had sequenced the genome and reported that around 1500 genes are unique to this crop, where ~580 are annotated as 'response to water'. Reannotation of these genes provided insights into their classification, wherein a few stress-responsive genes were characterized as genome-wide level (Muthamilarasan and Prasad 2015, 2017a, b). Singh et al. (2019) characterized aquaporin-encoding genes of foxtail millet, and showed that overexpressing SiPIP3;1 and SiSIP1;1 in heterologous yeast system provides tolerance to dehydration and salt stress. Recently, Sood et al. (2020) had optimized the transformation procedure of foxtail millet using *Agrobacterium tumefaciens* as a medium, and this would enable the overexpression of candidate genes in the crop per se, which would contribute to trait improvement. The decoding of genome sequences of diverse major food crops provides useful genomic information related to structural, functional and comparative genomics for novel trait discovery and genetic enhancement of these crops. Resequencing of germplasms enables the integrated genomics approach of sequence information to identify novel quantitative trait loci (QTLs), genes as well as SNPs that regulate any specific agronomic trait. Significant efforts in this regard have been made in case of rice by sequencing of its 3000 diverse accessions belonging to different populations (Wang et al. 2018). Resequencing of 429 accessions of chickpea is one of the large-scale exercises conducted to determine the candidate genes underlying thirteen traits (Varshney et al. 2019). In this context, the concept of pangenome has gained significant attention, which includes the complete genetic information available within the accessions of the species (Hurgobin and Edwards 2017). Reference genome, together with the resequencing data of all available accessions of a given species, have the considerable potential to expedite molecular breeding as it deploys the entire genetic diversity existing in the species. Development of pangenomes for crop species has been carried out in several plants, such as rice (Schatz et al. 2014; Zhao et al. 2018; Zhou et al. 2020), maize (Hirsch et al. 2014; Xu et al. 2014), soybean (Lam et al. 2010; Li et al. 2014; Liu et al. 2020a, b) and Brassica (Golicz et al. 2016a; Bayer et al. 2019). The quality of the reference assembly determines the appliance of plant pangenomics in terms of size, completeness and annotation, selection, and dense phenotyping of appropriate genotypes (Golicz et al. 2016b). Weckwerth et al. (2020) had underlined the future of pangenomics in enhancing the accuracy for marker-dependent trait performance, increasing the resolution of markers, and combining this approach with other next-generation strategies for accelerating the crop improvement programmes. The application of pangenome instead of single genome sequence as a reference would allow us to determine the retention or loss of valuable genes due to breeding and artificial selection in cultivars. It will further expedite the exploitation of crop wild relatives as well as cultivated accessions/species to improve abiotic and biotic stress tolerance, nutritional properties, architecture, and other economically significant traits in popularly grown cultivars.

Genomics includes the analysis of gene function, their regulation, and inter-networking for association with biological traits. In the field of crop science, comparative functional genomics and transcriptomics are primarily focused on the identification of allelic variations responsible for the improved phenotype. Condition-specific transcriptional activation of a large number of functional and regulatory genes is measured through micro- and macroarrays, quantitative PCR (qPCR), massively parallel signature sequencing (MPSS), serial analysis of gene expression (SAGE) and NGS-based RNA sequencing (RNA-seq) techniques. Global transcriptome profiling in diverse tissues and developmental stages of different crop accessions has led to the generation of Atlas rich in differentially regulated transcripts/genes indicating complex developmental intricacies in crops (Ref). Global transcriptomic resources may be utilized for an integrative genomics evaluation to delineate the function of genes (Muthamilarasan et al. 2014; Singh and Prasad 2017; Azodi et al. 2020). For example, genome-wide RNA-seq analysis of two contrasting sorghum genotypes: IS20351 (drought-sensitive) and IS22330 (drought tolerant) explored the correlation between physiological response to drought stress and differential gene expression (Fracasso et al. 2016). The abundance of drought-related transcripts was more in the drought-sensitive genotype. A total of 1036 up- and 809 down-regulated genes were identified under drought conditions, among which, 428 and 393 were exclusively up- and down-regulated in the tolerant genotype, respectively. The study revealed the different strategies adopted by both the genotypes in coping with drought conditions. The tolerant IS22330 genotype initiated the synthesis of secondary metabolites, including glycinbetaine and glutathione, whereas sensitive IS20351 genotype hydrolyzed carbohydrates and sugars. Therefore, the extent of drought imposition and perception was more in susceptible genotype than the tolerant. The tolerant genotype could be used as a genetic donor in sorghum germplasm improvement related to drought tolerance traits. Similarly, global transcriptome analysis in response to high temperature and drought has identified 29,395 differentially expressed genes (DEGs) in wheat (Liu et al. 2015), 696 and 808 DEGs under low- and high-nitrogen conditions in rice (Xin et al. 2019), 267 and 1421 DEGs under short- and long-term hypoxia in tomato roots (Safavi-Rizi et al. 2020) and various other crops under different conditions. The DEGs are being functionally characterized and utilized in crop improvement through breeding, genetic engineering, or genome editing.

Development and deployment of molecular markers for breeding and allied research

Molecular markers form the backbone of classical genetics and plant breeding as it enables selection and breeding for any given trait. Development of molecular markers, construction of genetic linkage maps, mapping of QTLs, saturation of maps, and fine mapping of the precise gene were once time-consuming and labour-intensive processes. However, the advent of next-generation sequencing (NGS) has enabled the development of large-scale molecular markers, including microsatellite or simple sequence repeat (SSR), insertion-deletions (InDels), and single nucleotide polymorphisms (SNP). These markers enabled the development of high-density genetic maps useful for mapping of target genes and utilize them in crop breeding. Molecular markers are also employed for the detection of genetic variation associated with valuable agronomic traits among cultivars in a species and facilitate the identification of appropriate parents for molecular breeding. They further ease the selection of desirable offspring resulted from the parental cross at the early stage of their development. Genome-wide marker analysis in Chinese spring wheat has led to the identification of 364,347 SSR markers from 10,603,760 sequences of the genome with 36.68 SSR markers/Mb density (Han et al. 2015). A total of 488 forms of SSR motifs were detected with a maximum proportion of dinucleotide repeats (42.52%), followed by a trinucleotide (24.94%), hexanucleotide (24.65%), tetranucleotide (4.62%) and pentanucleotide (3.25%) in the genome. AG/CT, AAG/CTT, AGAT/ATCT, AAAAG/CTTTT, and AAAATT/AATTTT remained the most abundant repeats of di- to hexanucleotide SSR motifs. Similarly, in foxtail millet, genome-wide 28,324 microsatellite-repeat motifs have been identified covering 405.3 Mb of the whole-genome sequence (Pandey et al. 2013). The abundance of trinucleotide repeats (48%) was more in this case than the dinucleotide repeats (46%). In barley, Zhou et al. (2015) have identified 436,640 InDels throughout the genome after aligning the DNA sequences from two different accessions—Morex and Barke. Among these 1140 InDel markers were integrated with 383 SSRs, 1544 DarT (diversity arrays technology), and 3909 gene-based SNP markers into a single barley genetic map. In addition to these, the development of other classes of markers such as transposable element-based and miRNA-based markers was demonstrated in different crops, including foxtail millet (Muthamilarasan and Prasad 2015). These markers were proven useful in large-scale genotyping applications in millets, cereals, and bioenergy grass species. Their application has also been extended to evolutionary studies and phylogenetic relationships, genetic diversity

analysis, map-based cloning, and DNA-fingerprinting. Garrido-Cardenas et al. (2018) has exquisitely described the applications and trends of molecular markers research in the field of plant science.

Single nucleotide polymorphism is another critical class of molecular marker abundantly distributed in the genome and detected through a comparative study of whole-genome sequence or transcriptome data of different accessions or genotypes (Habash et al. 2009). Advancements in NGS technologies with simultaneous reduction of their cost have prompt the detection and utilization of large-scale SNP marker for crop improvement. Approximately 20 million SNPs from rice were identified after aligning the reads from 3000 genome sequences with the Nipponbare genome as reference (Alexandrov et al. 2015). In wheat, 46,977 gene-associated SNPs from 90,000 high-density SNP array were genetically mapped using the combination of eight mapping population (Wang et al. 2014). In total, 6,385,011 SNP with high density were identified from 15 different drought-responsive inbred maize lines and B73 reference genome (Xu et al. 2014). The abundance of SNPs was more in the intergenic region (approximately 85%) and intronic (7.67%) region followed by upstream, exon, UTR, and splice sites. 271 non-synonymous SNP (nsSNPs)-associated candidate genes responsible for drought tolerance were also revealed (Xu et al. 2014). Resequencing of 588 *Brassica napus* accessions from 21 countries has generated about 5,294,158 SNPs and 1,307,151 indels. Through genome-wide association study (GWAS), 60 loci significantly associated with agronomic traits such as oil content, seed quality, stress tolerance were identified, which may be proven as a valuable resource for genetic improvement (Lu et al. 2019). The study also revealed the origin of *B. napus* from the hybridization between domesticated *Brassica rapa* and *Brassica oleracea* approximately 1910–7180 years back. These genetic resources have enormous significance in diversity studies and understanding the genetic basis of trait variation throughout the population. The genome and transcript sequences available for diverse crops have led to the generation of numerous genomic and genetic sequence-based markers like SSRs, SNPs, and InDels for their further use in genomics-assisted crop improvement. These markers are found efficient in rapid large-scale genotyping among natural germplasm accessions and bi-parental mapping populations through association and QTL mapping for trait discovery in crops.

QTLs associated with agronomic traits

The analysis of quantitative trait locus (QTL) is a statistical approach that correlates the phenotypic measurements with the genotypic data to evaluate the genetic basis of variations among complex traits. The pipeline for QTL mapping

requires a mapping population segregating for understudied agricultural traits, precise phenotyping, development of large scale high throughput genomic markers, construction of genetic map through genotyping of mapping population with polymorphic genomic markers and finally mapping of QTL utilizing both phenotypic and genotypic data (Mir et al. 2012). The biparental mapping population used for QTL mining is either of F_2, double haploids, backcrosses, near-isogenic lines (NILs), or recombinant inbred lines (RILs). This approach of linkage analysis-based QTL mapping was used thoroughly during the last decade for various crops. However, this process encompasses several limitations, as illustrated by Myles et al. (2009); therefore, to overcome the constraints, linkage disequilibrium (LD)-based association mapping was introduced to map QTLs for dissecting complex agronomically significant traits (Singh et al. 2017). The advantages of association mapping over bi-parental linkage mapping are (1) superior mapping resolution through the exploration and utilization of each recombination event that happened in the evolutionary history of the species, (2) use of natural germplasm collection rather than the development of specialized mapping population, (3) less time consuming and cost-effective, (4) use of same association mapping panel and genotyping data for mapping other traits and (5) larger number of alleles can be mined compared to the linkage analysis-based QTL mapping where only two alleles are usually sampled (Mir et al. 2012). With the availability of crop genetic and genomic resources, genome-wide association study (GWAS), candidate gene-based association mapping, QTL mapping, fine mapping, and map-based cloning are becoming popular to discover novel QTLs, genes, and alleles associated with traits of agronomic importance in major food crops. More recently, the Department of Biotechnology (DBT), Government of India, has initiated the genotypic and phenotypic characterization of more than 65,000 germplasm accessions of rice, wheat, minor pulses, and minor oilseeds conserved at National Genebank through GWAS for trait discovery and genetic improvement of these crops.

Multi-parent advanced generation inter-crosses (MAGIC) and nested association mapping (NAM) are specially designed population structures for multi-parent association studies (Ladejobi et al. 2016). For genotyping, restriction-site associated sequencing (RAD-Seq), genotyping-by-sequencing (GBS), skim-sequencing, and whole-genome resequencing approach are being exploited for mid to high-density trait mapping through QTL-based analysis (Roorkiwal et al. 2020). Molecular markers linked with various agronomic traits derived from association mapping are reported in crops including soybean (Hu et al. 2014), brassica (Qu et al. 2017; Zhu et al. 2019), rice (Feng et al. 2016; Rao et al. 2018), chickpea (Bajaj et al. 2016; Li et al. 2018), foxtail millet (Jaiswal et al. 2019a, b) and various others

plants (reviewed by Muthamilarasan et al. 2019). In plants, QTLs were mostly identified for a variety of agronomic traits, including abiotic and biotic stress tolerance, yield and yield contributing factors, flowering time, root architecture and nutrient uptake, and nitrogen fixation (in case of soybean). A few landmark QTLs associated with nutritional traits in major cereal crops (rice, wheat, and maize) are listed in Table 1. In addition to these genomic QTLs, several other types of QTLs, namely expression QTL (eQTL), proteomic QTL (pQTL), metabolic (mQTL), and phenomic QTL (phQTL) are seeing their dawn in breeding for crop improvement. An eQTL illustrates the genetic variance of a gene expression phenotype (Nica and Dermitzakis 2013), while in pQTL, protein abundance is correlated with genetic polymorphism (Rodziewicz et al. 2019). On the other hand, chromosomal regions that encompass loci that contribute to genetic variation in phenotypic traits are called phQTL. The targeted metabolome profiling of wheat kernel through LC–MS/MS followed by linkage analysis has resulted in the identification of 1005 mQTLs distributed unevenly in the genome. Twenty-two candidate genes underlying these mQTLs regulating the level of different metabolites were functionally annotated (Shi et al. 2020). Comprehensive information about these QTLs is essential for facilitating the effective use of genes and genomic regions that regulate key traits. Various modern NGS-driven QTL mapping strategies like bulk population resequencing: QTL-seq, individual population resequencing, and MutMap utilizing bi-parental mapping and mutant populations are found expedient for identification of major QTLs modulating agronomic traits in crop plants. All these advanced genomics, including novel QTL strategies, enabled to detect both major as well as minor QTLs governing gene regulatory networks underlying vital agronomic traits for quantitative dissection of complex traits and further genetic improvement of crops.

Genomics-assisted breeding for trait improvement

Genomics-assisted breeding (GAB) is initiated with the identification of genomic markers associated with QTL or gene(s) related to the agronomic trait of interest and then their application in the breeding platform (Fig. 2). Molecular markers assist in an assortment of desired offspring in the breeding cycle at the early growth stage utilizing NGS-based high throughput genotyping platforms (Singh et al. 2017; Crossa et al. 2017). Numerous GAB strategies have been deployed for crop improvement, including marker-assisted backcrossing, marker-assisted recurrent selection, and genomic selection. Recently, speed breeding is added to the list to expedite breeding processes. Countries like India predominantly rely on breeding for crop improvement and

Table 1 Summary of major QTLs identified for nutrition-related traits in major cereal crops

Crop	Trait	No. of QTLs identified	Linkage group	Phenotypic variation explained (%)	References
Rice	Zn, Se and Cd accumulation in grains	5	4, 5, 6 and 9	13.8–16.4	Liu et al. (2020a)
	Fe and Zn concentration in grain	23	All accept 4, 8 and 11	7.18–22.03	Calayugan et al. (2020)
	Cooking and eating quality of grain	14	1, 2, 3, 4, 6, 9 and 10	5.33–37.72	Park et al. (2019)
	Nutrient content and yield	72	All except 5 and 10	2.3–75.6	Kinoshita et al. (2017)
	Sugar-related trait in grain	17	1, 3, 4, 5, 6 and 8	7.5–18	Yang et al. (2014a, b)
	Amylose, protein and lipid content in grain	8	1, 2, 3, 6, 8, 9 and 10	30.0–40.0	Yun et al. (2014)
	Macro- and microelements	139	All chromosomes	3.1–12.9	Zhang et al. (2014)
	Amylose, lipid and protein content of grain	8	2, 3, 6, 7, 9 and 11	3.9–19.3	Lee et al. (2014)
	Lipid content	1	5	20.0	Kim et al. (2013)
	Lipid metabolism	29	All except 9 and 10	7.16–37.93	Ying et al. (2012)
	Fat content	2	7	10.0–18.6	Shen et al. (2012)
	Grain chalkiness	3	5, 8 and 10	9.6–25.0	Liu et al. (2012)
	Chalkiness, amylose and protein content and viscosity	132	All chromosomes	2.0–68.2	Liu et al. (2011)
	Mineral content in grain	31	All except 7	5.0–19.0	Garcia-Oliveira et al. (2009)
	Viscosity and food quality of grain	26	All except 10	1.0–88.0	Wang et al. (2007)
	Phytate and micronutrient content	11	1, 2, 5, 7, 8 and 12	12.8–26.5	Stangoulis et al. (2007)
	Starch synthesis and cooking quality	7	5 and 6	11.3–72.8	He et al. (2006)
	Cooking and eating quality of rice grain	12	1, 2, 3, 6 and 11	8.0–80.3	Tian et al. (2005)
	Grain quality	27	All except 9	3.0–73.7	Aluko et al. (2004)
	Protein and fat content	5	1, 2, 4, 5, 6 and 7	5.1–23.0	Hu et al. (2004)
Wheat	Gluten strength	5	1A, 1B and 3A	3.4–40.1	Ruan et al. (2020)
	Gran protein content and thousand kernel weight	23	1A, 1B, 2A, 3A, 4A, 4B, 5A, 6A, 6B and 7B	0.6–24.4	Fatiukha et al. (2020)
	Grain protein content and yield	22	1B, 2A, 2B, 3A, 4A, 4B, 4B, 5B, 7A and 7B	8.0–23.0	Giancaspro et al. (2019)
	Grain protein content and protein deviation	17	2B, 3A, 3B, 4A, 4B, 5A, 5B, 6B, 7A and 7B	4.1–8.7	Nigro et al. (2019)
	Grain Fe, Zn and protein content and thousand kernel weight	16	1A, 2A, 2B, 3A, 4A, 5A, 5B, 7A, and 7B	2.3–6.8	Krishnappa et al. (2017)
	β-Glucans and protein content and grain yield/spike	19	1B, 2A, 2B, 3A, 3B, 4A, 5A, 6B, 7A and 7B	–	Marcotuli et al. (2017)
	Fe and Zn content	8	1A, 2A, 3D, 4A, 4D, 7B and 7D	29.1–51.45	Roshanzamir et al. (2013)
	Starch granule size	3	1D, 4A and 7B	3.8–5.6	Feng et al. (2013)
	Protein content	25	1D, 2A, 2B, 3B, 4 A, 5B, 5D, 6B and 7A	4.11–10.90	Wang et al. (2012)
	Grain water-soluble oligosaccharide	10	1B, 1D, 2B, 2D, 3B, 4A, 5A, 5D, and 6B	6.98–38.30	Fu et al. (2011)
	Quantity of protein fraction in grain	55	1A, 1B, 1D, 3A, 3B, 4A, 5D and 7A	2.1–73.2	Zhang et al. (2011)
	Grain protein concentration	9	1A, 1B, 2A, 2B, 5B, 6B, 7A and 7B	11.1–17.6	Suprayogi et al. (2009)
	Protein and mineral concentration	82	All chromosomes	1.0–23.0	Peleg et al. (2009)
	Trait related to protein and starch in grain	35	1D, 2A, 2D, 3B, 3D, 5A, 6A, 6B, 6D and 7B	7.99–40.52	Sun et al. (2008)
	Grain protein content	13	2A, 2B, 2D, 3D, 4A, 6B, 7A and 7D	2.95–32.44	Prasad et al. (2003)
	Grain protein content	7	4B, 5A, 6A, 6B, 7 A and 7B	17.0–31.7	Blanco et al. (2002)

Table 1 (continued)

Crop	Trait	No. of QTLs identified	Linkage group	Phenotypic variation explained (%)	References
Maize	Starch content	8	1, 2, 3, 7, and 9	5.45–6.84	Lin et al. (2019)
	Starch granule size	7	3, 6 and 7	–	Liu et al. (2018)
	Kernel oil and protein content	21	All except 9	4.6–11.4	Yang et al. (2016)
	Mineral content, concentration and grain yield	74	All chromosomes	5.84–38.14	Gu et al. (2015)
	Grain protein content	16	3, 5, 6, 7, 8 and 9	4.4–13.4	Yang et al. (2014a, b)
	Oil, protein and starch content in grain	22	All chromosomes	2.4–20.6	Guo et al. (2013)
	Grain starch and grain-oil content	37	1, 3, 4, 5, 8, 9 and 10	3.24–12.35	Yang et al. (2013)
	Grain protein concentration	5	3, 6, 8 and 10	6.2–13.4	Li et al. (2009)
	Fatty acid composition in kernel oil	18	All except 4 and 5	15.4–59.6	Wassom et al. (2008)

variety release. In such cases, strengthening the breeding strategies and modernizing the approaches are required to meet the challenges faced by agriculture, on time.

Marker-assisted backcrossing and recurrent selection

Marker-assisted backcrossing (MABC) is the introgression of a genomic region (QTL or locus or gene) contributing the desired trait from a donor genotype into a breeding line or elite cultivar without linkage drag through backcrossing after multiple generations. The resultant product of MABC contains the whole genome of an elite parent with the genetic loci or QTL or gene(s) contributing to the desired phenotype from the donor parent (Gupta et al. 2010). Quantity of molecular marker used, the strength of marker association with the phenotype, undesirable linkage drags, and size of the population used for each generation of backcrossing determines the efficiency of MABC. This method has been used extensively to generate superior lines of varieties for biotic and abiotic stress tolerance. For instance, NILs of drought-tolerant rice have been developed through introgression of root QTLs derived from a drought-tolerant japonica upland cultivar CT9993 into a lowland susceptible indica cultivar IR20 (Suji et al. 2012). Yang et al. (2019) had introgressed the blast resistance gene, *Pi2*, into an elite line by coupling MABC with genomics-based background selection. Kang et al. (2019) exploited the rapid generation advance system for expedited development of near-isogenic lines (NIL) from rice stripe virus resistant and susceptible parents, followed by MABC to develop a virus-resistant line in a short duration. In peanut, MABC assisted in developing elite lines with a high oleic/linoleic fatty acid ratio (Bera et al. 2019). In chickpea, Mannur et al. (2019) had developed Fusarium wilt resistant high yielding desi chickpea cultivars, Super Annigeri 1, and improved JG 74 lines in a

record time of 10 years. Significant efforts have also been made to introgress, especially the genes and major QTLs regulating submergence, drought, and salinity tolerance as well as bacterial blight, sheath blight and blast resistance to develop stress-tolerant improved cultivars with enhanced yield and productivity in rice (Singh et al. 2019).

The marker-assisted recurrent selection (MARS) was introduced to counter the inefficiency of MABC in transferring multiple QTLs regulating complex traits like yield or broad-spectrum disease resistance. MARS involves the detection and selection of large QTLs or multiple genomic regions controlling complex agronomic traits within a single or across the populations and their pyramiding in a single genotype (Ribaut et al. 2010; Kulwal et al. 2011). This approach makes use of the F_2 population and is most effective for cross-pollinating species. In disparity with MABC, favourable alleles may be contributed by both the parents, and the selected improved genotype becomes the chimera of their parents. The superior allele enrichment involves the phenotypic and marker effect for desired traits in the F_2 population, followed by two or multiple cycles of marker-assisted selection (Eathington et al. 2007). In the past few years, the Hyderabad situated International Maize and Wheat Improvement Center (CIMMYT) has made significant headway in the development of drought-tolerant maize inbred lines through MARS approach in their Asia Maize Drought Tolerance (AMDROUT) project. Other applications of this method have also been reported from rice, wheat, barley, soybean, cotton, pea, and sunflower improvement, particularly for evolving durable resistance.

Genomic selection and speed breeding

Genomic selection (GS) or genome-wide selection (GWS) utilizes the large-scale DNA markers dispersed throughout the genome to develop superior germplasm lines. Thus, the

Fig. 2 Mapping of quantitative trait loci associated with complex agronomic traits and their application in genomics-assisted breeding. Linkage analysis in mapping population segregating for desired phenotype conquer QTL identification which generally employs in MAS
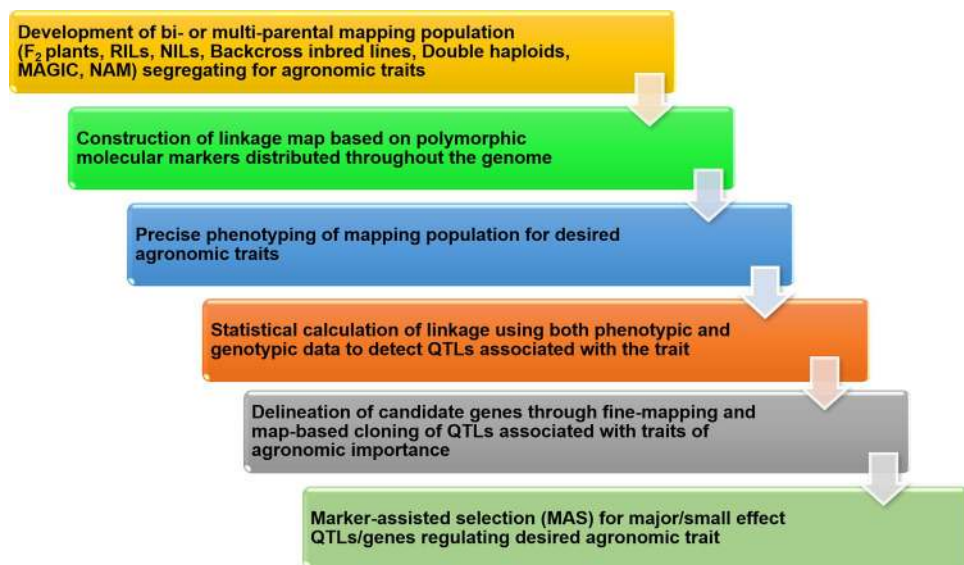
genomic selection approach has the potential to capture multiple QTLs/genes widely distributed with minor additive effects. Vigorous phenotyping is not mandatory for a breeding population, and subsequent offspring selection primarily focused on genotypic predictions, which combines the genomic and pedigree data for several generations of the breeding cycle (Nakaya and Isobe 2012). Genomic estimated breeding value (GEBV), the sum of the information index with a combined effect of genome-wide molecular markers, is the basis of recurrent selection (Singh et al. 2017). High-density molecular markers where each QTLs are in linkage disequilibrium with a minimum of single genomic markers are prerequisites for precise GEBV, and thus, for GWS (Habier et al. 2007). The success of GS also depends on the quantity and diversity of the training population (breeding lines selected for the GWS programme). The reduced number of selection events has decreased the time and cost of breeding. This approach can be equally applicable for both cross- and self-pollinated species with slight alterations (Bernardo 2010). Few examples of crop improvement through this approach are the development of wheat lines resistant to stem rust caused by *Puccinia graminis* f. sp. *tritici* (Rutkoski et al. 2010), drought-tolerant high-yielding lines in maize (Ziyomo and Bernardo 2013), improved yield and related traits under drought in chickpea (Li et al. 2018), and improved productivity in superior hybrids of rice (Cui et al. 2020). GS has also expanded its horizon towards underutilized or less studied crops for their improvement. de C. Lara et al. (2019) have demonstrated the use of GS in an autotetraploid forage grass, *Panicum maximum*. Similarly, in miscanthus, Slavov et al. (2019) have combined index selection and genomic prediction to achieve multiple breeding targets. These include increased biomass, delayed flowering, reduced lignin, and increased cellulose contents. In cassava,

Torres et al. (2019) have deployed GS for early selection and breeding for agronomic traits such as fresh root yield, dry matter content, dry yield, fresh shoot yield, and harvest index. These reports suggest the popularity and applicability of GS in enhancing the traits at a quicker phase that might lead to the early release of improved genotypes for agricultural production.

In case of breeding, time is an important factor that decides the release of genotypes to the farmers. Conventional breeding takes 3–7 years for crossing experiments, followed by 4–5 years for testing the yield, diseases and quality, and another 1–3 years for the release of varieties. Altogether, considerable time is invested in improving a single genotype or variety. Given this, the approach of modulating day-light and duration for accelerating the life cycle, termed 'speed breeding', has been introduced (Fig. 3). This recently developed speed breeding technology shortens the breeding cycle by accelerating crop generation by providing controlled rapid growth-promoting conditions in glasshouses and growth chambers (Watson et al. 2018; Ghosh et al. 2018). By modulating lighting, photoperiod, humidity, temperature, and other factors, the approach can achieve six generations per year for crops like wheat, barley, chickpea, and canola (Hickey et al. 2019). In contrast, in the glasshouse, these crops can undergo only three generations a year (Hickey et al. 2019). Early anthesis was reported from plants grown under speed breeding setup with fully viable mature seeds. Seed produced (g per plant) was unaffected between speed breeding and normal photoperiod conditions in almost all crops (Watson et al. 2018). The adaptation of technique in the breeding programme will accelerate the generation of mapping populations, reduce the duration of MABC/MARS/GWS, and expedite the progression towards homozygosity. Apart from major crops that are mostly annual or

biannual, the method also has immense potential to hasten the improvement of woody shrub or perennial plants. The optimization of methods which headed towards the reduction of juvenile phase from 5 years to 10 months in apple and 7 to 2 years in chestnut are some example of the application of accelerated breeding cycle in perennial crops (Baier et al. 2012; van Nocker and Gardiner 2014). Rana et al. (2019) had coupled marker-assisted selection with speed breeding for developing salt-tolerant rice lines. Similarly, Bauerle (2019) has shown that the generation per year of hops could be enhanced from one (under field conditions) to four (through speed breeding) that could accelerate selection for flower yield and quality in this crop. Jighly et al. (2019) have combined GS with speed breeding to enhance genetic gains in allogamous plants like tall fescue. The approach named SpeedGS is gaining popularity among the breeders for achieving higher genetic gain per cycle, especially for traits with low heritability.

Biotechnological interventions for crop improvement

Transgenic or genetically modified (GM) crops have modified genomes at gene level achieved through several genetic engineering techniques. While breeding is time-consuming and allows only the transfer of genetic information from closely related species, genetic engineering or transgene-based research facilitates the transfer of genes from any source into the plants. However, an established protocol for introducing the gene into host species and rigorous selection is required to achieve greater success. *Agrobacterium tumefaciens*-mediated genetic transformation is one of the reliable approaches being used to achieve stable transgenic lines. In contrast, other techniques, including particle bombardment (biolistics), sonication, and electroporation, are used for transient expression of the foreign DNA. Singh and Prasad (2016) had comprehensively discussed the merits and demerits of *A. tumefaciens*-mediated genetic transformation in cereals. The prime bottleneck in this approach is the lack of optimized protocol for several important species, and the optimization is a time consuming and labour-intensive
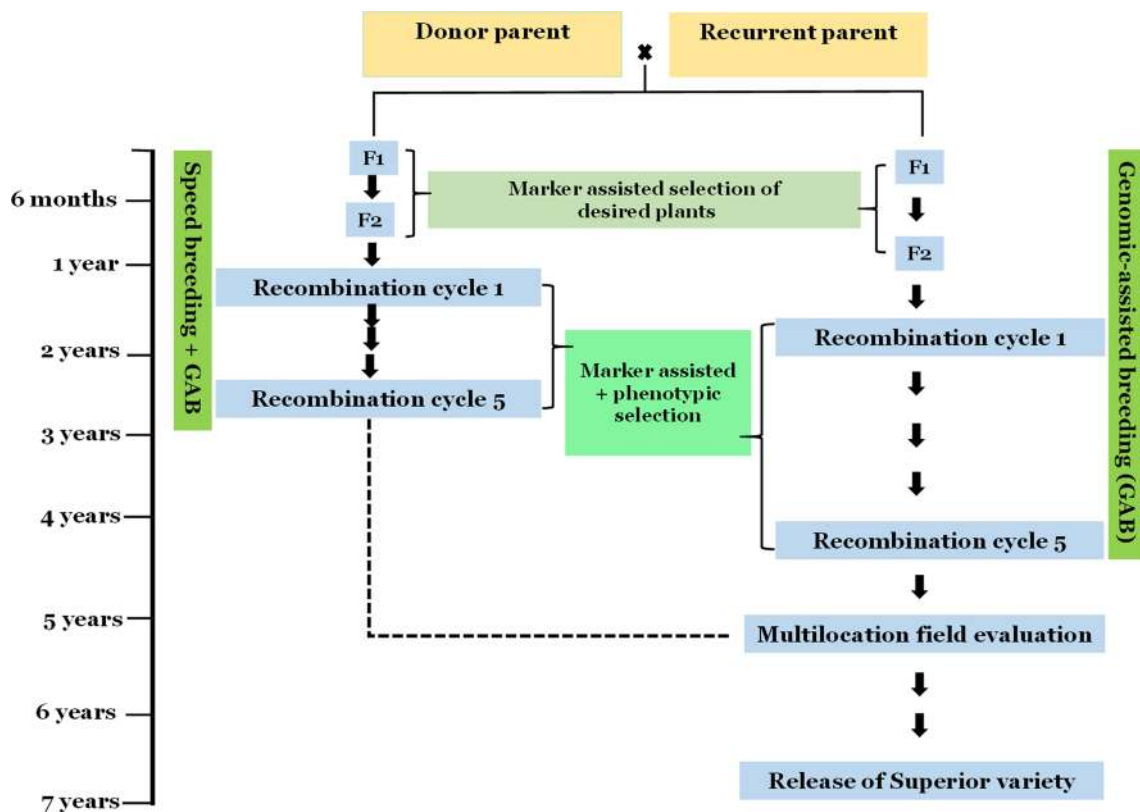

Fig. 3 Diagrammatic representation of the application of speed breeding in genomic-assisted breeding. Speed breeding significantly reduces the length of breeding cycle and accelerates the process of crop improvement. Conventional marker-assisted breeding (MAB) approximately takes 7–8 years to release an improved cereal variety while speed-breeding-assisted MAB would be completed within 3–4 years

process. Achieving higher transformation efficiency is another issue that requires the fine-tuning of the experimental parameters. Once established, the protocol will serve as a key to introduce several genes into the target genome to attain better performance and phenotype. So far, 525 transgenics in 32 crops have been commercialized, of which *Zea mays* accounts for the highest number. Cultivation of transgenic crops has boosted agricultural productivity to about 22% leading to a 68% increase in profits (Kumar et al. 2020). Several genetic engineering technologies have been utilized for crop improvement, which is briefly discussed in the following sections (Fig. 4). Bt cotton has contributed to the Indian economy for a while, and in low-income countries like Bangladesh, Bt brinjal has secured its economy and livelihood of farmers. Despite these advantages, the public acceptance of transgenic crops has been quite low, and concerns have been raised regarding ecological hazards and safety-related issues in the context of human consumption. However, there is no scientific evidence that shows transgenic crops to cause health hazards (Tsatsakis et al. 2017; de Vos and Swanenburg 2018). Irrespective of these, biotechnology holds the key to the future of agriculture as the challenges faced by the farming sector are on a steady rise. At present, intensive agriculture is securing the life and livelihood of farmers and contributes to the global stock of grains and vegetables; however, issues like the spread of new diseases, insect/pest attack, erroneous rainfall, lack of soil fertility due to overuse of synthetic fertilizers, monotonous cropping, etc. could soon pose a serious threat to the ongoing agriculture. Plants need to withstand multiple stresses rather than single stress in their environment. Modulation of genes that regulate multiple stress responses through biotechnological interventions will help in the development of plants with enhanced efficiency under such conditions (Pandey et al. 2020). Several crops have been modified using biotechnology and have either been released or have the potential to be released. Examples of such crop species have been provided in Table 2. While addressing climate change and introducing good farming practices receives importance on one hand, it is also imperative to release varieties that could be climate-resilient or sustain in abnormal conditions, thus securing the food security of global population.

Expression and overexpression of candidate genes for desired phenotype

Gene cloning and isolation have facilitated the pulling out of a target gene from any genome that can then be transformed into any other genome for its expression. Expression of 'Cry' gene of *Bacillus thuringiensis* in plants is a typical example of this approach, and it is still popular since there is a rise in pests and insects that attacks plants. Expression of genes from other plant species for enhancing the

agronomic or stress tolerance traits of target crops is also being practiced for a while. DREB (dehydration responsive element binding) protein-encoding genes are one such class of genes that were frequently isolated from one species and expressed in another for enhancing the tolerance to different abiotic and biotic stresses. Further, these expression and overexpression strategies also assist in elucidating the function of genes, which is an important task considering a large number of genes are at our disposal due to the advancement of NGS technologies. Their functional characterization becomes essential not only for basic research but also for application purposes. The T-DNA insertion lines of *Arabidopsis thaliana* have served as a vital resource for elucidating gene function. The next step after functional characterization is to utilize the gene for crop improvement programmes, and one of the most widely used methods is the overexpression of candidate genes. Several success stories depict the immense potential of gene overexpression in crops. Overexpression of *ARGOS* genes in *Zea mays* leads to a reduction in sensitivity to ethylene, and transgenic plants show enhanced drought resistance as well as higher grain yield in well-watered as well as drought conditions (Shi et al. 2015). Similarly, transgenic *Glycine max* plants overexpressing *GmWRI1b* show higher oil content and improved plant architecture under field conditions (Guo et al. 2020). The list for crop improvement for biotic and abiotic stresses, nutritional enhancement, increase in yield, biofuel production, herbicide resistance, etc., through overexpression approach is quite long with new additions at an ever-increasing rate. Some recent examples have been presented in Supplementary Table 1. The functional validation of new candidates is also increasing at a rapid pace generating novel resources for crop improvement programmes (Lata and Prasad 2011; Puranik et al. 2012; Singh et al. 2020). The availability of genome sequence information in public domains had facilitated the large-scale analysis of genes and gene families, and characterizing those genes for their physiochemical properties, genomic composition, promoter elements, and expression profiling in response to stress, hormonal treatments and developmental stages had pinpointed several candidate genes that could be subjected to overexpression in target organisms for enhancing the trait-of-interests.

RNA interference for in vivo knockdown of target genes

The discovery of RNAi was a breakthrough in the history of biology, and since its finding, it has been widely utilized in functional genomics, reverse genetics and crop improvement (Rosa et al. 2018). RNAi pathway involves the generation of small RNAs (sRNA), which include short interfering RNA (siRNA), microRNA (miRNA), transacting siRNA (ta-siRNA) and natural-antisense siRNA
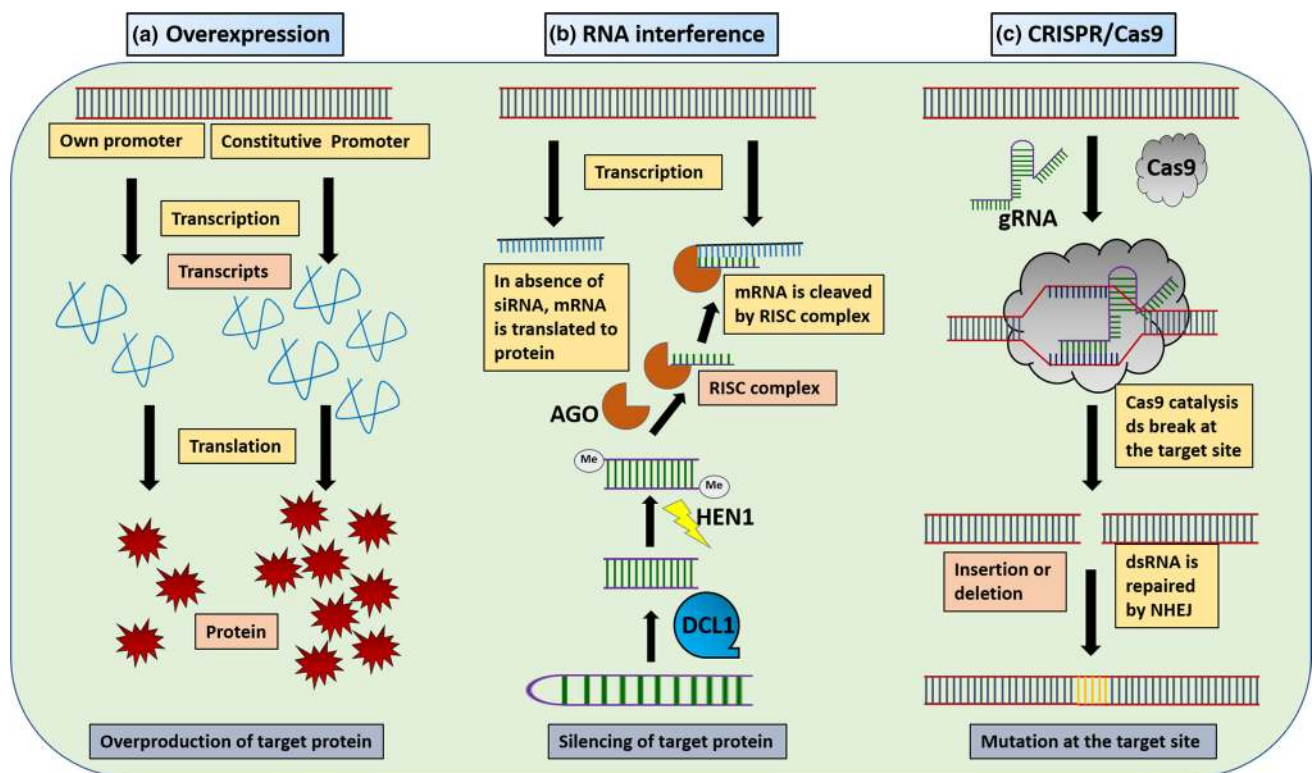
Fig. 4 Strategies for crop improvement through biotechnological approaches. **a** Overexpression leads to greater transcription of target gene which can be translated into protein; **b** RNA interference leads to downregulation of target gene; **c** Gene editing through CRISPR/Cas9 leads to insertions or deletions at target site which gives rise to mutations

Table 2 Genetically engineered crops either released (*) or having the potential to be released (+)

Crop species	Gene	Technology	Trait improved	References
Rice*	Phytoene synthase, phytoene desaturase, lycopene-β-cyclase	Overexpression	Golden rice-provitamin A-rich rice	Ye et al. (2000)
Rice+	Phytoene synthase, phytoene desaturase, β-carotene ketolase, and β-carotene hydroxylase	Overexpression	aSTARice-astaxanthin-rich biofortified rice	Zhu et al. (2018)
Tomato+	Self-pruning, ovate, fasciated, fruit weight 2.2, multiflora and lycopene-β-cyclase	Gene editing	Improved size, number and lycopene content of fruit	Zsögön et al. (2018)
Cotton*	Crystalline endotoxin	Overexpression	Insect-resistant cotton	Umbeck (1992)
Tomato*	Polygalacturonase	RNAi	Flavr Savr tomato reduction in polygalacturonase activity leading to delayed fruit ripening	Sheehy et al. (1988)
Canola*	Tryptophan decarboxylase	Overexpression	Low indole glucosinolate canola	Chavadej et al. (1994)
Potato+	Amaranth albumin 1	Overexpression	High-protein-content potato	Chakraborty et al. (2010)
Maize+	Waxy	Gene editing	High-amylopectin-content corn	Waltz (2016)

(NAT-siRNA) which mediate silencing or epigenetic regulation of their target genes (Prasad et al. 2019). RNAi can be utilized by both transformative and non-transformative strategies. Transformative RNAi has been used in several modified forms like artificial miRNA (amiRNA), artificial ta-siRNA (ata-siRNA), hairpin RNA (hpRNA), intrinsic direct repeat, 3′-untranslated region (UTR) direct repeat, terminator-less, single-stranded promoter antisense and intron delivered promoter hpRNA (Guo et al. 2016). Numerous examples can be cited where RNAi has been
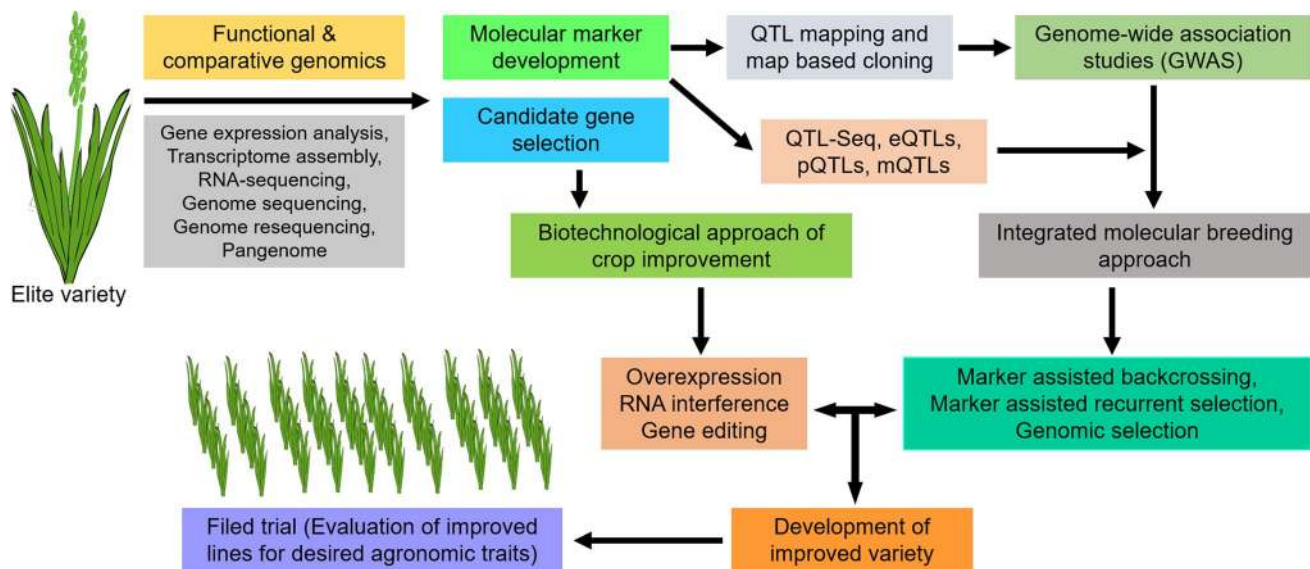
Fig. 5 Application of functional and comparative genomics in marker-assisted breeding and biotechnological approaches for crop improvement. The candidate gene(s) identified from functional genomic studies can be introduced through genetic engineering or tar- geted modify through genome editing technology in crop species for improved agronomic traits. The other approach is through molecular breeding which employ molecular markers to identify genomic region associated with desired traits during breeding programme

successfully utilized for improving important traits like modification of plant architecture, improvement in fruit quality in terms of high β-carotene and lycopene content, enhanced shelf life, nutritional enhancement like low gluten content, reduction in toxic terpenoids, biotic stress resistance against viruses, fungi, bacteria and nematodes; and abiotic stress resistance to heat, drought, salinity, and cold (Kamthan et al. 2015).

The non-transformative RNAi technique, spray induced gene silencing (SIGS), has gained widespread attention due to its low cost of application and feasibility of use. It involves spraying plants with double-stranded (ds) RNA/siRNA and has been successfully utilized for controlling insect pests, which are the carriers of several viral pathogens (Cagliari et al. 2019; Worrall et al. 2019). Plants sprayed with dsRNA/ sRNA targeting *DCL1* and *DCL2* of *Botrytis cinerea* showed a significant reduction in grey mold disease symptoms high-lighting the potential of this technology for the generation of next-generation eco-friendly biofungicides (Wang et al. 2016). Transgenic plants are met with criticism in several countries, and widespread acceptance is still lacking. It is estimated that about 130 million dollars are spent to bring a transgenic crop into commercialization (Rosa et al. 2018). However, despite all the efforts and promising features that GMOs have to offer, anti-GMO responses follow. Consider-ing this, SIGS being a non-GMO approach has enormous potential for crop improvement, and it is also crucial that we devise new dsRNA/sRNA delivery strategies for silenc-ing host as well as pathogen genes. Recent crop improve-ments utilizing RNAi as a tool have been summarized in

Supplementary Table 2. In the functional genomics perspec-tive, RNAi was useful in gene characterization studies; how-ever, the recent advent of virus-induced gene silencing has now established its prominence over RNAi.

Gene and genome editing

Precise genome editing has revolutionized genetic engi-neering, and this started in 1996 when for the first time, it was shown that DNA binding zinc finger domains along with Fok1 endonuclease domains could cleave DNA at defined regions and act as site-specific nucleases (SSNs) (Kim et al. 1996). Further research led to the development of transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindrome repeats (CRISPR)/CRISPR-associated protein 9 (Cas9). Meganu-cleases (MegaN) recognize long DNA sequences that are greater than 14 nucleotides (nt) up to 40 nt. Since they have endonuclease activity, they produce double-stranded (ds) breaks at the recognition sites. However, their use in genome editing has been minimal because the variety of MegaNs available is very less and cannot be used for every locus (Silva et al. 2011). However, CRISPR/Cas9 has been more popular because of its ease of use compared to other genome editing technologies (Das et al. 2019). As the name suggests, CRISPR/Cas9 consists of two components: a single-guide

RNA that is customizable and Cas9 endonuclease. Another prime requirement of the system is a Protospacer adjacent motif (PAM) (5′NGG3′), which is required for inducing ds breaks at the targeted sites in the genome. The breaks are repaired through either homology directed repair (HoDR) or non-homologous end joining (NHEJ). Since NHEJ is error-prone, repair leads to insertions or deletions at the target site (Khatodia et al. 2016).

CRISPR/Cas9 has shown immense potential for crop improvement, and several traits ranging from nutritional, biotic, and abiotic stress resistance have been enhanced (Jaganathan et al. 2018; Das et al. 2019) (Supplementary Table 3). However, there are certain limitations like the restrictions due to the requirement of PAM, off-target effects, and low efficacy of HoDR. Another problem is plant viruses that are known to mutate at tremendous rates (Prasad et al. 2020). Reports suggest that the evolution of editing-resistant viruses may lead to viral escapes within a short span of time (Mehta et al. 2019). However, the technology is continually being supplemented with novel innovations overcoming some of the drawbacks.

Single nucleotide polymorphisms (SNPs) are responsible for certain elite traits in crops through genome-wide association studies (GWAS) (Zhao et al. 2019). Base editing is a new approach that can be utilized for editing these SNPs. Cas9 is fused with cytidine deaminase enzyme that has base conversion activity (C → T or G → A), and this modified method does not require dsDNA as a repair template (Komor et al. 2016). The problem of low efficiency of HoDR is also overcome as it is not involved in the base conversion process. Conventional genome editing is associated with the insertion of DNA cassettes at random regions within the genome, and this may lead to other undesirable effects. The regulatory concerns for transgenics are another hindrance. To overcome these problems, DNA free genome editing was developed, which involves the delivery of preassembled CRISPR/Cas9 ribonucleoproteins (RNPs) to protoplasts by particle bombardment (Woo et al. 2015). The only problem with this method is that protoplast regeneration systems are not yet available for a majority of crops, but this can be overcome with a research focus in this direction. CRISPR/Cas9 is also limited due to the requirement of 5'NGG3' PAM, and Cas9 variants are essential to overcome this problem. CRISPR from Prevotella and Francisella 1 (Cpf1) endonuclease recognize T-rich PAMs, thus broadening the target range of the system (Zetsche et al. 2015). Several variants exploiting the CRISPR-based approach to achieve other functions had also been reported. For example, deactivating the nuclease domain and engineering a transcription enhancer to Cas9 could promote the expression of the target gene. Similar engineering of deaminase to the Cas9 protein facilitates the conversion of cytosine residues to thymine. Thus, CRISPR/Cas9-based approach has multiple applications in editing the genes and genomes at base-pair level. Of note, the method is not considered as GM in several countries, which would encourage the use of CRISPR/Cas9 in developing lines that could meet the challenges currently faced by agriculture.

Conclusions and future prospects

The foremost challenge for crop scientists is to increase agricultural productivity to pursue the demand for food supply for a rapidly expanding global population, which is expected to reach approximately 10 billion by the mid of the twenty-first century (United Nations, World Population Prospects 2017). On the one hand, global warming, constrained environmental conditions, and biotic factors are limiting crop yield. On the other hand, fertile farmland is also shrinking due to rapid urbanization and soil erosion. Besides, pandemic situations like the recent COVID-19 prevalence have introduced significant gaps in food and nutritional securities to the global population. Therefore, the rapid release of environmentally sustainable high yielding varieties is required. Molecular breeding and genetic manipulation have emerged as the two most potent technologies which have the potential to attain food and energy security for the coming years (Fig. 5). Advances in NGS technology have enabled the incorporation of genomics with various disciplines of crop breeding. Large-scale genomic markers and high-throughput genotyping are being applied in breeding have accelerated the quantity and cost of cultivar development. Similarly, functional and comparative genomics have provided the platform for gene discovery and their functional characterization. The key gene or genes regulating a molecular pathway are being genetically engineered or edited to develop phenotypically improved crop lines. Whatever be the approach, either molecular breeding or biotechnological tools, the ultimate intention is to ensure food for all through increased productivity per plant and minimize yield loss caused due to external factors. The collaborative research investments of various branches of science are paramount to sustainable crop improvement.

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