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Evolution in crop improvement approaches and future prospects of molecular markers to CRISPR/Cas9 system

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ABSTRACT

The advent of genetic selection and genome modification method assure about a real novel reformation in biotechnology and genetic engineering. With the extensive capabilities of molecular markers of them being stable, cost-effective and easy to use, they ultimately become a potent tool for variety of applications such a gene targeting, selection, editing, functional genomics; mainly for the improvisation of commercially important crops. Three main benefits of molecular marker in the field of agriculture and crop improvement programmes first, reduction of the duration of breeding programmes, second, they allow creation of new genetic variation and genetic diversity of plants and third most promising benefit is help in production of engineered plant for disease resistance, or resistance from pathogen and herbicides. This review is anticipated to present an outline how the techniques have been evolved from the simple conventional applications of DNA based molecular markers to highly throughput CRISPR technology and geared the crop yield. Techniques like using Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9) systems have revolutionised in the field of genome editing. These have been promptly accepted in both the research and commercial industry. On the whole, the widespread use of molecular markers with their types, their appliance in plant breeding along with the advances in genetic selection and genome editing together being a novel strategy to boost crop yield has been reviewed.

1. Introduction

With the global development throughout, still today, considerable disparities exist between the crop produces and worldwide consumption of food. Crops, majorly wheat, rice, sugarcane, maize, etc. being the staple food world wide is the chief sources of nutrition. Many plant diseases, pests, insects, a number of stresses caused by biotic and abiotic factors, leads to very low crop productivity and serious yield losses thereby resulting in food shortages. Therefore, for sustainable crop development and its improvement, breeders require to produce cultivars continuously, which have disease and pest resistance, stress tolerance, high nutritional value crop and high yield, etc. (Leng et al., 2017).

Past few decades had witnessed great progress in plant breeding (Zamir, 2001). Earlier methods of conventional breeding were based upon phenotypic characters and moreover with the breeders experiences many improved crop varieties were produced. Conventional methods of breeding were a vibrant section in research based upon applied sciences. It basically relied upon genetic traits and selection method in order to produce cultivars that are of breeder's and the consumer's interest (Datta et al., 2011). Another important way is to introduce genetic material (genes) from differing sources; for instance, gene bank or other related plant species. Undoubtedly through these methods many hybrid varieties were produced. But the recent advances in molecular marker technology have a more powerful impact.

Advances molecular marker technology has been seen during 1980s, and this has brought about many changes in the fate of plant breeding techniques and crop improvement (Mao et al., 2013). A thorough

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perception of molecular markers and its technology has perceived in the fields of genetics and molecular breeding, along with genetic selection and editing, which thereby comprehends into the diversity which is accessible for crops and breeding schemes (Nadeem et al., 2018). Many large scale marker trait association analysis have been enabled by High throughput genotyping technology and phenotyping platforms, such as genome wide association studies (GWAS), to specifically explore the genetic constitution of plant traits (Leng et al., 2017).

Besides molecular markers technology, genome editing technology using engineered endonucleases are way much popular in present day. These include genome editing technologies using Zinc Finger Nucleases (ZFNs) (Carroll, 2011) and Transcription Activator-Like Effector Nucleases (TALENs) (Mahfouz et al., 2011; Li et al., 2012) which are capable of generating genomic modifications, along with being easily accessible, reasonable and trouble free engineered (Shan et al., 2013). With more advances in these technologies and prokaryotic immune system, a new strategy for genome editing has been introduced, popularly known as CRISPR associated Cas9 systems (Cong et al., 2013). CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats. Availability of the genomic sequences, has led to advances in the studies of discovering new technologies for crop improvement which ultimately has resulted in making it a possible way to breed for any kind of genomic trait (Jaganathan et al., 2018).

In both animal and plant systems, last decade has remarked precise genomic editing by the extensive use of site specific nucleases (SSNs). These create double stranded breaks (DSBs) in the targeted DNA. Thereafter starts a repair mechanism through Non-Homologous End Joining (NHEJ) or the other one Homology-Directed Recombination (HDR) pathways (Jinek et al., 2012). These ultimately result in creating Insertion/Deletions (INDELs) and mutations in the region(s) which is targeted. Unlike the pathways of creation of transgenic organisms, the genome editing pathways are way more defined as they produce only the desired mutations, thereby becoming the most potent tool in improvisation of crop genetics and its functional genomics. Also, the plants which are genetically engineered possess added benefits over those transgenic plants as they contain their modified DNA for the desired trait (Malzahn et al., 2017).

2. Genetic markers

Genetic markers regarded as molecular markers, are beneficial for the analysis carried out for plant genomes and hence becoming the significant tool in crop breeding and improvement techniques in present scenario. These markers are basically a small sequence of DNA (or gene) that characterize a specific gene or trait. They are tightly linked and related to the desired targeted gene and act as a sign or flag (Collard and Mackill, 2008). By following a simple pattern of Mendelian's inheritance, these molecular markers depict the heritable dissimilarities in the nucleotide sequences which lie at a corresponding position on the homologous chromosome (Kesawat and Das, 2009). DNA based molecular markers are a resourceful tool in the branches of plant breeding and economic botany such as embryology, plant taxonomy and its physiology, and genetic engineering, etc. (Schlotterer, 2004). A careful consideration is required to choose one or the other methods of the various kinds of molecular markers with their respective different principles, methodologies and applications (Kesawat and Das, 2009). The parameters that describe the term genetic marker includes its capability of being easily phenotyped, be locus specific and depict polymorphism in the desired population which is studied. The attributes of a molecular marker are typically determined, either by its Heterozygosity or Polymorphism Information Content (PIC) in the studied population.

2.1. How to select a desirable marker system

Various kinds of molecular markers were identified during the past 20th century (Farooq, 2001; Gillet, 1999). There are many places still were the biochemical markers such as isozymes are being used in place of DNA Markers because the former ones are inexpensive as compared to latter. For numerous tree species, we have well established set of protocols, the former are products of structural genes whose metabolic role and level of variation is well known which makes it a suitable marker (Farooq and Sayyed, 1999a; Farooq and Sayyed, 1999b). The selection is totally objective dependent. For instance, if distinction was to be made between two consents of a species then DNA based molecular markers such as hybridisation markers RFLP, RAPD, AFLP, etc. were to be used (Farooq et al., 1996). Conversely, if only the distinction was to be made between two closely related species that differ only in few characters environmentally then the use of non-neutral DNA based markers such as microarrays or isozymes are considered to be a better option. Yet there are several cases, where isozymes could not resolve the issue. For instance, it would be difficult to distinguish between two dwarf mutant cultivars of commercial rice Basmati 370 due to very low levels of polymorphism. So instead of using biochemical markers in this case, RAPD or AFLP markers could be used (Farooq, 2001).

3. Major types of genetic markers

There are three major types of genetic markers: (a) morphological markers (also called "classical" or "visible" markers) which are phenotypic traits, (b) biochemical markers, which are called isozymes, including allelic variants of enzymes, and (c) DNA markers (or molecular markers), which reveal sites of variation in DNA (Jones et al., 1997; Winter and Kahl, 1995). Many markers such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs), single nucleotide polymorphism (SNP) and diversity arrays technology (DArT) markers are developed and characterised under DNA Markers (Sharma et al., 2014; Jiang, 2013).

4. Classical markers

4.1. Morphological markers

From long back, markers have been used as an assisting tool in plant breeding techniques in order to select plant with the desired traits. Some observable features such as leaf shape or size, colour of pod and flower or seed, shape of seed, awn type in cereals, colour and shape of rind (exocarp), length of the stem, etc. lay the basis of utilization of molecular markers in the breeding technologies (Jiang, 2013; Khlestkina, 2014).

Genetic polymorphism is exhibited by these markers which is easily identified and manipulated. A successful use of these markers was at the time of green revolution when semi dwarfism varieties of wheat and rice contributed to high yields cultivar production. The dwarfism in wheat crop breeding, governed by gene Rht10 was interrogated in Nuclear male sterile cultivar of wheat, through the process of backcrossing. By result, a linkage was produced in the gene Rht10 along with the male sterility gene Ta1. With these results, the trait dwarfism was considered to be a molecular marker for the detection of male sterile plants in the studied population (Liu et al., 1999).

4.2. Biochemical markers

Isozymes are nothing but alternative structural variants of enzymes which have catalytic activity similar whereby differing in molecular weights and electrophoretic mobility. By amino acid substitution, a point mutation creates dissimilarities in electrophoretic mobility, through which isozymes reflect the products of different alleles (Xu, 2010). Thereby these isozymes are mapped over chromosomes in order to map other genes. Despite of being trouble free to use, all these biochemical or morphological markers, does not depict so much of

Table 1
List of Molecular Markers (Rautela et al., 2019).

Name of Molecular Marker	Acronym	Name of Molecular Marker	Acronym
Amplified Fragment Length Polymorphism	AFLP	Sequence Characterised Amplified DNA	SCAR
Anchored Simple Sequence Repeats	ASSR	Short Tandem Repeats	STR
Cleaved Amplified Polymorphic Sequence	CAPS	Sequence Tagged Sites	STS
Diversity Arrays Technology	DArT	Simple Sequence Length Polymorphism	SSLP
Expressed Sequence Tags	EST	Simple Sequence Repeats	SSR
Inter-Simple Sequence Repeats	ISSR	Single Nucleotide Polymorphism	SNP
Inter-Retro Transposon Amplified Polymorphism	IRAP	Single Stranded Conformational Polymorphism	SSCP
Retro Transposon- Microsatellite Amplified Polymorphism	REMAP	Variable Number Tandem Repeats	VNTR
Random Amplified Polymorphic DNA	RAPD		

Table 2
Important Characteristics of commonly used molecular markers (Nadeem et al., 2018).

Characteristics	RFLP	AFLP	RAPD	SSR	SNP	DArT
Co-dominant/Dominant	Co-dominant	Dominant	Dominant	Co-dominant	Co-dominant	Dominant
Reproducibility	High	Intermediate	High	High	High	High
Polymorphism level	Medium	High	very high	High	High	High
Required DNA quality	High	High	High	Low	High	High
Required DNA quantity	High	Low	Medium	Low	Low	Low
Marker index	Low	Medium	High	Medium	High	High
Genome abundance	High	Very high	Very high	Medium	Very high	Very high
Cost	High	High	Less	High	Variable	Cheapest
Sequencing	Yes	No	No	Yes	Yes	Yes
Status	Past	Past	Past	Present	Present	Present
PCR requirement	No	Yes	Yes	Yes	Yes	No
Visualization	Radioactive	Agarose gel	Agarose gel	Agarose gel	SNP-VISTA	Microarray
Required DNA (ng)	10,000	500–1000	20	50	50	50–100

accuracy and is also time consuming and expensive. Hence, these evolved to molecular markers which are more consistent and cost effective (Sharma et al., 2014).

4.3. DNA markers

There are different types of markers based upon the technique which will be used for detection and amplification of the particular segment of DNA. For instance, based on modifications of restriction site in the target DNA and consequent hybridization with probe DNA, Restriction Fragment Length Polymorphism (RFLP) Marker will be applicable. Whereby others such as Random Amplified Polymorphic DNA (RAPD), Sequence characterized amplified region (SCAR) and Sequence tagged sites (STS) are based upon mutation in the primer annealing site at the target DNA. Furthermore, Simple sequence repeat (SSR), Inter simple sequence repeat (ISSR) and Single nucleotide polymorphism (SNP) markers are also included (Table 1) (Datta et al., 2011). A standard DNA marker is the one which is able to detect higher levels of polymorphism, which depicts co-dominance and high level of reproducibility and is present consistently throughout genome (Mondini et al., 2009). Numerous kinds of DNA markers are developed and is practical into many agronomically improved crop varieties and cultural practices. These molecular markers can be organized into the following:

1. Co-dominant or dominant markers (which are based upon the gene action of the marker).
2. Hybridisation or Polymerase Chain Reaction (PCR) Markers (which are solely dependent on the method of detection.
3. The others are considered under the category where the markers are based upon the method of transmission (Semagn et al., 2006).

4.4. Hybridization marker (RFLP)

The ever first and the only marker technology was based upon hybridisation which was Restriction Fragment Length Polymorphism (RFLP). The technique was initially discovered and applied to detect DNA sequence polymorphisms especially for the genetic mapping of a temperature sensitive mutation of adenovirus stereotypes. Earlier this was applied only to human genome mapping, but later it was brought to plant genomics too (Botstein et al., 1980). Nucleotide base substitutions, insertions, deletions, duplications, and inversions within the whole genome removing or creating new restriction sites are the molecular bases of RFLP technique. These INDELs (Insertions-Deletions) lead to polymorphism in the target DNA.

The process initiates with the isolation of the DNA segment which are then mixed with the restriction enzymes leading into a huge number of copy DNA of varying length. These DNA fragments are then separated using agarose or polyacrylamide gel electrophoresis in the form of different bands. The main reasons due to which the variation occurs in the RFLP pattern are mutations, base pair (bp) deletions, insertions, trans positions or locations (Nadeem et al., 2018). RFLP technique, also known as genetic fingerprinting, profiling or testing was one of the first methods used for genomic typing and editing (Al-Samarai et al., 2013).

4.5. Polymerase chain reaction (PCR) based markers

The next description of markers (Table 2) is based upon polymerase chain reaction, these includes Random Amplified Polymorphic DNA (RAPD) (Kumar and Gurusubramanian, 2011). The others are Amplified Fragment Length Polymorphism (AFLP), Single Nucleotide Polymorphism (SNPs) and Single Sequence Repeats (SSRs).

RAPD: The technique of RAPD, since the last decade has been increased as it is one of the most common technique used. It was first discovered and analysed in 1990, that RAPD markers are potent to detect and determine the different traits among strains of specific species (Williams et al., 1990; Welsh and McClelland, 1990; Govarthanan et al., 2011). The genomic DNA is amplified through the extensive use of random and short primers of usually around 10 nucleotides. These sequences act both as forward and reverse primers as they are able to amplify fragments from 1 to 10 sites simultaneously. The amplification of fragments shows dependency upon the genomic DNA and the primer's length and size (Nadeem et al., 2018). The amplified fragments of

basically 0.5–5 Kbs in length and are later separated by the agarose gel electrophoresis. Later on, by ethidium bromide staining the polymorphisms is identified.

Over several other markers these RAPD Markers have many advantages in terms of time consumption, cost effectiveness, stable and reliable to environmental variation, with very small amount of DNA nucleotides needed, but main disadvantage of RAPD marker is low reproducibility rate (Kesawat and Das, 2009; Datta et al., 2011).

AFLP: This is basically the combination of the power of hybridisation marker RFLP along with the PCR based technique of RAPD which ligates the primer recognition sequences to the restricted DNA (Kesawat and Das, 2009). According to (Saal and Wricke, 2002), high genomic abundance, wide range of applications, significant reproducibility, production of several bands which are the result of variations in the restriction sites or in the intervening regions along with the fact that there is no requirement of any sort of prior database for the primer construction. Visualization of these banding profiles are done by agarose gel electrophoresis or polyacrylamide gel which are stained with $AgNO_3$ or autoradiography (Mishra et al., 2014). The detection of sequence variants is similar in both RFLP and AFLP but the number of polymorphisms found is higher in the latter one.

Microsatellites or SSRs: Microsatellites are short tandem repeats of DNA sequences of only a few (1–6 bp) in length. These were named microsatellites also known as short tandem repeats (STRs) (Edwards et al., 1991) or simple sequence repeats (SSRs) (Jacob et al., 1991), differing from VNTRs (minisatellite shaving sequence repeats ranging from 11 to 60 bp) (Nakamura et al., 1987). The term microsatellite was denoted in order to characterise these simple sequences which are ultimately amplified by polymerase chain reaction (Litt and Luty, 1989).

Single Nucleotide Polymorphism (SNP): There comes another class of molecular markers which are based on nucleotide sequences. Single nucleotide polymorphism (SNPs) is basically polymorphisms (substitutions, insertions and deletions) which are specific to different single nucleotides and occur frequently (Grover and Sharma, 2016; Sobrino et al., 2005). They serve as a desirable tool for carrying out several different works including mapping, marker assisted breeding and map based cloning (Kesawat and Das, 2009). In both plants and animals, SNPs are found in abundance as their frequency ranges between 1 SNP in every 100–300 bp (Xu, 2010). In an experiment it was observed that there lies a single nucleotide polymorphism (SNP) for every 170 bp, when the sequences from japonica rice cultivar were compared with indica cultivar (Yu et al., 2002). SNPs are observed widely in a genomic sequence and can be retrieved in the coding or non coding region(s) or even within intergenic region having different frequencies (Xu, 2010).

Depending upon diverse systems of allelic distinction and detecting pathways, a huge number of SNP genotyping methods have been devised which include SNP being the simplest and trouble free method. Also, the CAPS marker system can be directed in the SNP detection (Nadeem et al., 2018). Numerous high throughput genotyping methods including Next Generation Sequencing (NGS), Genotype Based Sequencing (GBS) and chip based NGS, Allele specific PCR have been devised which eventually makes SNPs as the most desirable markers for genotyping (Agarwal et al., 2008).

4.6. Microarray based markers

Diversity Array Technology (DArT): Another is DArT sequencing technique which is highly reproducible microarray technique (Wenzl et al., 2004). By this advanced technique genotyping of several hundred polymorphic loci, which are present over the genome, can be done simultaneously (Jaccoud et al., 2001). Since past few years, it is been observed that DArT has undertook the status of favourably reliable, and beneficial marker based assay, along with the genetic mapping by the use of linkage or association studies both in a variety of model and non model crops including Eucalyptus (Sansaloni et al., 2010), Wheat (Orabi et al., 2014; Terracciano et al., 2013), Barley (Lex et al., 2014), Sugarcane (Aitken et al., 2014), Brassica (Raman et al., 2012), Pearl Millet (Supriya et al., 2011), and Carrot (Grzebelus et al., 2014).

Conversely, this technique lacks behind in some applications and have limitations as being a micro array based technique require the desired software (DArTsoft and DArTdb), along with laboratory facilities, huge investment, and skilled manpower. A distinctive DNA fragment is represented by them. Therefore, being primarily dominant (whether present or absent or different) in their intensity, DArT markers limit their value in some applications (Kesawat and Das, 2009).

4.7. Uses of molecular markers in plant breeding and genome analysis

4.7.1. Genetic mapping

The wide use of DNA based markers is giving way to rise of new technologies such as genetic mapping in which the locus of the gene can be identified along with the determination of the distance between the two genes. The markers applications are making it possible for researchers to govern the gene sequence in the chromosomes and also the distances between them (Datta et al., 2011). The chromosomal recombination during the process of meiosis which results in the segregation of genes is the main principle underlining genetic mapping (Nadeem et al., 2018). These linkage maps set basis and delivers an outline for detection of marker trait associations and also, they help in determining the marker which are to be used in marker assisted breeding (MAB). Henceforth, a genetic linkage map is very essential in marker assisted breeding (Jiang, 2013). The factors on which the construction of genetic maps depends are:

- Completeness of detection of recombinational events.
- Linkage distance between loci.
- Number of individuals analysed.

As soon as one or few markers are observed in association with the desired trait in a given population, a dense molecular map will generate in reference to a standard population to provide with the identified of markers that are flank the targeted gene. Later, fine mapping can also be done to classify the markers that are closely linked to the gene which controls the trait. Also, to accurately locate the desired QTLs/genes, the constructive genetic map should possess a sufficient number of evenly spaced polymorphic markers (Babu et al., 2004).

4.7.2. Construction of linkage and QTL mapping

One of the most needed application of DNA markers in the field of plant biology and breeding technology are the construction of linkage maps for different plant population. These maps are eventually used for the identification of single gene trait or quantitative trait gene by QTL analysis (Mohan et al., 1997). QTL stands for Quantitative Trait Loci which is basically a DNA segment (locus) which play a role in phenotypic value of quantitative traits. A single gene or group of genes is present in the loci is inherited together with those tight linkages (Datta et al., 2011).

The detection of QTLs controlling traits is only made possible because of the construction of genetic linkage maps which is principally based upon genetic recombination in the meiosis, thus allowing their analysis in progeny (Tanksley and Nelson, 1996; Paterson, 1996). This technique was a major break through in the determination of quantitative trait loci (Paterson et al., 1988). Specifically talking about rice cultivars (Weng et al., 2008), more than 22 Quantitative trait loci of rice grain have been reported (Aluko et al., 2004; Redoña and Mackill, 1998; Huang et al., 1997; Lin et al., 1995; Tan et al., 2000; Xu et al., 2002; Li et al., 2004). The presence/absence of alleles in markers and the associated traits are mapped onto a linkage map which is hereby analysed in QTL Mapping. Phenotyping and genotyping are the two main phases that come in the methodology of QTL Mapping (Fig. 1 A).

QTL mapping is a technique, in which by the extensive use of

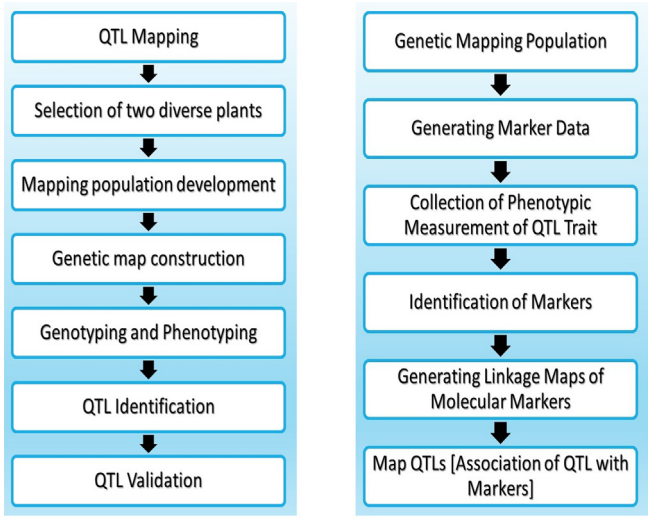


Fig. 1. (A) QTL Mapping procedure and its validation (B) Significant steps of QTL mapping.

molecular markers, genes that affect the desired traits are located and identified (Nadeem et al., 2018). These traits are further classified into two and are identified upon the variations they show. Such as discontinuous variations are depicted by qualitative traits and continuous variations by the quantitative ones. Molecular (DNA) markers are of great application in both QTL analysis and Molecular Assisted Breeding (MAS) as well (Angaji, 2009). Important steps in QTL analysis include the following (Fig. 1 B).

For mapping, the two of the parents which possess a desirable level of polymorphism are selected. DHs, Backcrosses (BCs), Near isogenic lines (NILs), Recombinant Inbred Lines (RILs), and F_2 populations are used (Paterson, 1996). Markers including RFLP, AFLP, ISSR, SSR, SNPs, DArTs have been eventually used in the creation and determination of linkage maps (Semagn et al., 2006). Yet, with the introduction of next generation sequencing, several thousand DNA markers are put in use for high throughput genetic mapping (Dhingani et al., 2015; Bernardo et al., 2015). The detection of QTL genes is most importantly done by methods such as single marker analysis (Tanksley and Nelson, 1996), simple interval analysis (Lander and Botstein, 1989), composite interval analysis (Silva et al., 2012) and multiple interval analysis (Datta et al., 2011). Few significant statistical programs and software that are widely used in QTL Mapping are R (Broman et al., 2003), QGENE (Nelson, 1997), PLABQTL (Utz and Melchinger, 1996), QTLNetwork (Yang et al., 2008), MAPMANAGER, QTLSTAT, MAPQTL, Map Manager QT (Datta et al., 2011), MapChart (Voorrips, 2002).

4.7.3. Merits and limitations of QTL mapping

This technique of QTL mapping is extensively used for the detection of genes that control the desired or targeted traits (Mohan et al., 1997), useful in the genome wide scan or genomic wide association study (GWAS) in the plants. Pests and diseases are the major issue in the development and breeding in agricultural practices and through QTL mapping, the genes which are responsible for diseases can be easily detected (Young et al., 1992). The major drawbacks of this technique include less allelic diversity, more time consumption in case of mapping population development (Neale and Savolainen, 2004), lower number of recombination events (Price, 2006), and specificity in terms of the detected QTLs for a given population (Lübberstedt et al., 2005).

4.7.4. Marker assisted selection

Molecular or DNA markers serve as potent research tools making a way in determination of genetic composition of plants, along with being a reference points to evaluate the dissimilarities in the DNA sequences and thus, the allele arrangements in plant biology (Ibitoye and Akin-Idowu, 2010). Marker assisted selection is a breeding program in which the detection and selection of DNA marker are integrated (Li et al., 2012; Kordrostami and Rahimi, 2015). This technique opens ways and help to preclude the difficulties that are concerned with traditional methods of breeding. This has totally transformed the standards of selection (Mohan et al., 1997; Tabor et al., 2002). This approach of MAS in current breeding technologies permits the selection by genotype using DNA markers being closely linked with the desired gene (Khlestkina, 2014). The MAS program has proven itself in the development of gene pyramids, and also at backcrossing and linear selections (Moose and Mumm, 2008).

In the MAS breeding program (Fig. 2), the linkages are obtained between molecular markers and the desired traits as in biotic stress tolerance, pathogen and pest resistance, resistance to nematodes, some quality and quantity parameters (Jonah et al., 2011). As compared to conventional phenotypic selection, MAS is a much simpler method and the selection is carried at breeding stage only and the single plants are selected with high reliability (Collard and Mackill, 2008). The technique of MAS is ordinarily used by plant breeders to identify appropriate dominant or recessive alleles across a generation along with the most favoured traits in an individual within a progeny (Francia et al., 2005). In MAS, the molecular markers are first validated, and the selection of parent is done to check out the relationship with the marker and the trait, which is followed by mapping of the population. The procedure takes place in few major steps as depicted in Fig. 3.

4.7.5. Drawbacks of marker assisted selection technique

- The technique has a major limitation of the cost factor.
- Major need of technical skills.
- The technique being automated.
- Validation of the maker is to be ensured before each breeding population. Any sort of presumption in this case could be hazardous.
- Unlike the DNA markers, traits may have an affect by the environment and depict G × E interactions. Henceforth, in the development of markers, the phenotypingshould be done in different environments and consequences of G × E interactions should be well

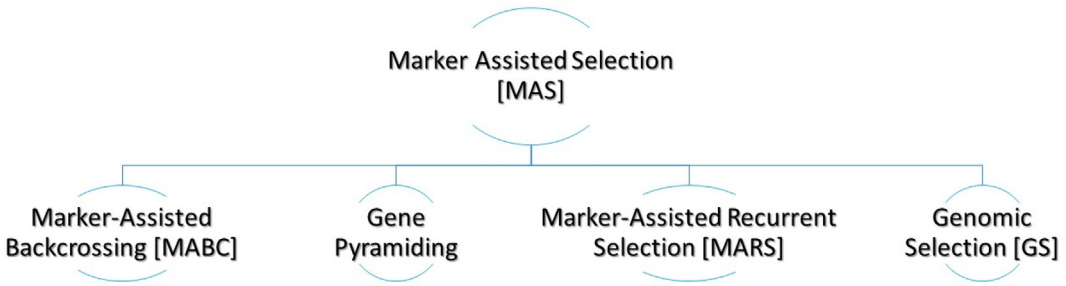


Fig. 2. Major schemes of molecular assisted breeding (MAS) (Nadeem et al., 2018).

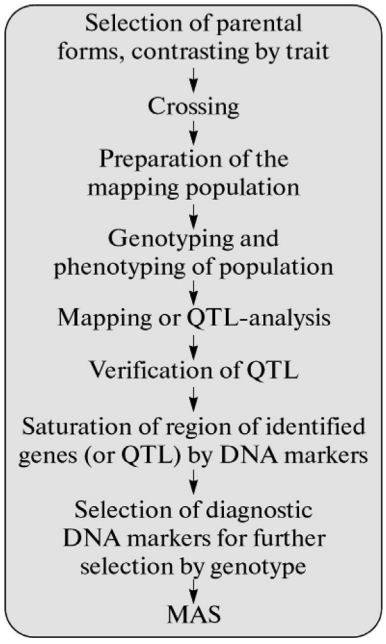


Fig. 3. Stepwise procedure of marker assisted selection (Khlestkina, 2014).

considered (Datta et al., 2011).

4.8. Genomic selection

Genomic Selection (GS) (Fig. 4) is a kind of marker assisted selection technique which evaluates all the loci, effects of markers and haplotypes throughout the genome concurrently in order to determine genomic estimated breeding values (Meuwissen et al., 2001; Dekkers 2007). This selection is wholly dependent upon these GEB (Genomic Estimated Breeding) values (Nakaya and Isobe, 2012). These values are basically a prediction model, in which the pedigree data is taken along with the phenotypic ratios along with markers for a better accuracy. In contrast to the MAS technique, GEBV is all marker dependent (Newell and Jannink, 2014).

In the genomic selection genotypic data, markers are used to calculate the traits with accuracy within the whole genome, so that the selection could be made on the same basis (Jiang, 2013). This technique of genomic selection is based upon the extensive utilisation of high density markers. The scheme summary of genomic selection is as follows in Fig. 5.

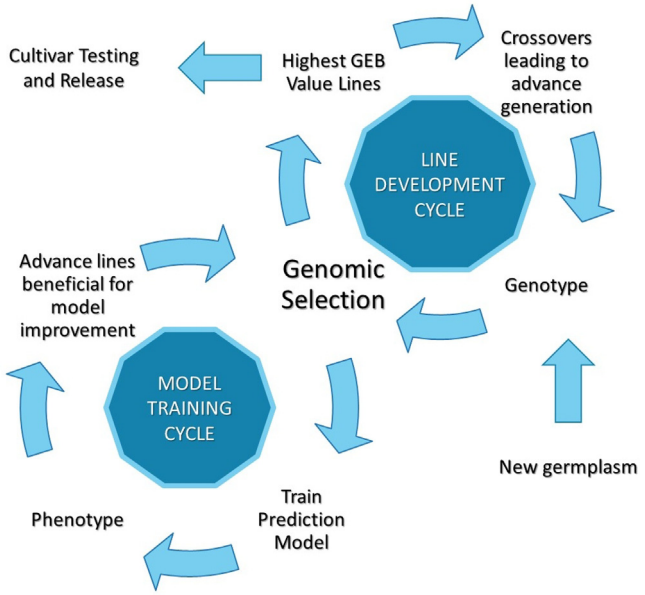


Fig. 5. Schematic representation of genomic selection (Heffner et al., 2009).

4.9. Genomic editing: a new way to improve plant breeding

Genomic editing is described as an effective technique in genetic engineering, which to a great extent, is used by engineers to edit the genome such as for insertions, substitution, removal or disruption of DNA sequences. This is done by tool such as molecular scissors or artificially engineered nuclease enzymes and eventually making them more useful for the basic and applied science fields (Punwar et al., 2014). In 1985 and 1986, first research work on genetic manipulation was published (Smithies et al., 1985; Thomas et al., 1986). They did insert a gene into the mammalian genome by homologous recombination. These genome editing technologies have evolved as potent tools in the creation of new allelic alternatives in the genomes of cultivated varieties. It can be performed via several pathways such as ZFNs, TALENs, or the novel CRISPR systems, Genome Editing (Fig. 6). In biotechnology and agriculture to extensively create genetically modified organisms, such as crops with higher yield and resistance to pathogen and pests and cattle genetically modified that don't have horns.

The basic principle concept of genome editing lies on the perception of DNA Double Stranded Breaks known as DSBs and its repair mechanisms. Non homologous end joining (NHEJ) and Homology directed repair (HDR) are the two pathways which are aligned to DSB mechanism (Fig. 7). The difference between the homologous

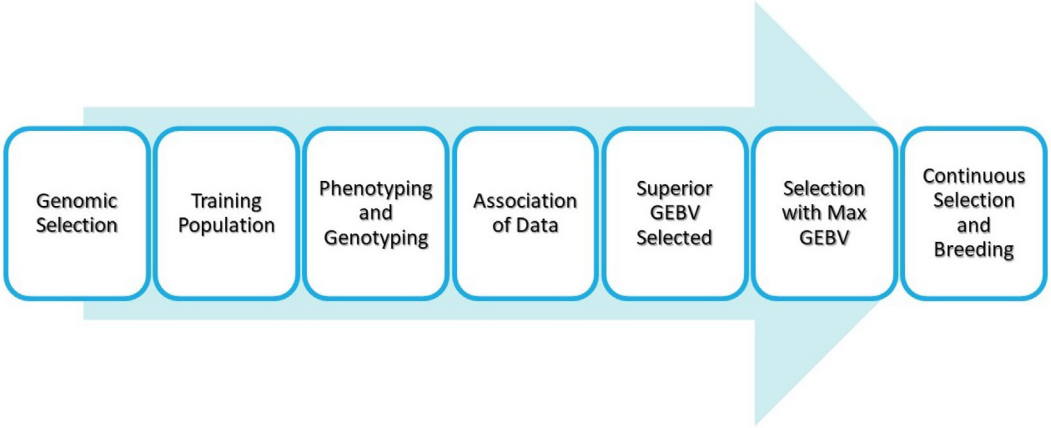


Fig. 4. Major steps in Genomic Selection Technique (Nadeem, 2018).

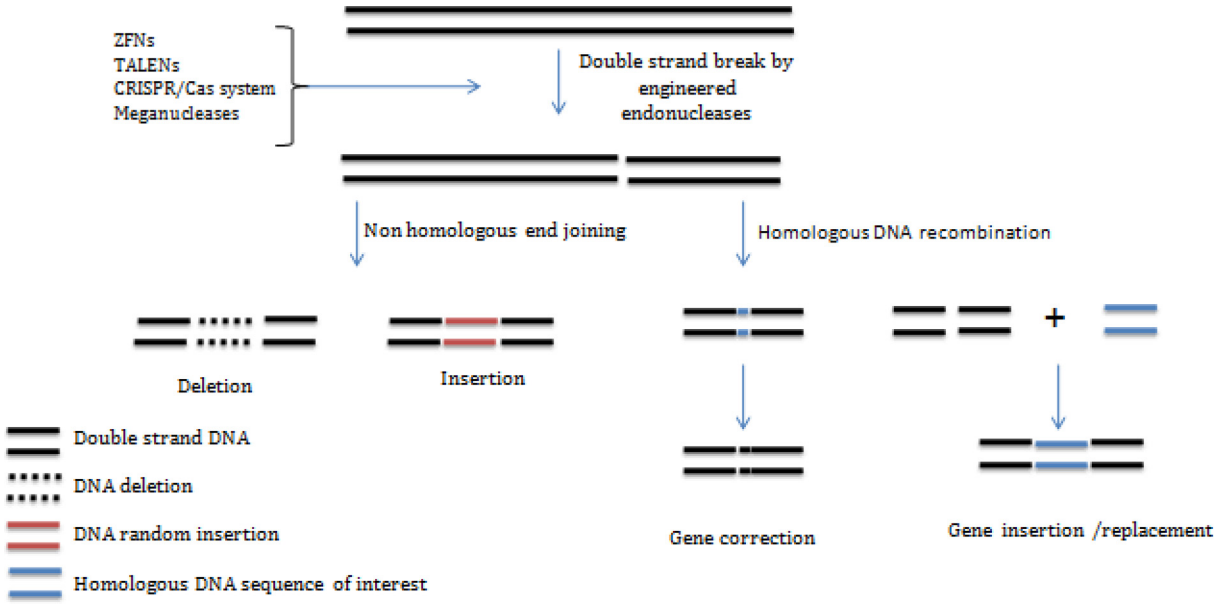


Fig. 6. Genomic Editing by artificial engineered nucleases.

recombination and NHEJ is that the former use sister chromatids as template for DNA repair and the latter directly ligates the DSB (Punwar et al., 2014). These are only completed by DSBs which are produced through the ionizing radiation which leads to sister chromatid cross-overs (Latt, 1981).

4.10. Mechanisms of genome editing

The method of genomic editing brings along a new modification in the conventional breeding practices that were based solely on recombination and to a small extent, to genetic recombination. For the first time in 1996, the protein domains were found to be working as site specific nucleases such as zinc fingers which were coupled with FokI endonuclease (Kim et al., 1996). These were named Zinc Finger Nucleases (ZFNs) with further advancements tools such as Transcription Activator-Like Effector Nucleases (TALENs), and more recent Clustered Regularly Interspaced Short Palindromic Repeats/ Associated Protein-9 Nuclease (CRISPR/Cas9) System have been discovered. Few unique alterations produced by TALEN has led to a 6.7% haploid induction rate which is only triggered by a mutation in the pollen specific phospholipase (Kelliher et al., 2017). With the most recent system, CRISPR, plant genome editing has accelerated to higher levels because of the possible generation of trans gene free wheat mutants (Zhang et al., 2016; Zhang et al., 2014; Noman and He, 2016).

Zinc Finger Nucleases (ZFNs): ZFN recognises target sites that consist of two zinc finger binding sites that flank upto 5–7 bp spacer sequence recognised by FokI endonuclease cleavage domain. Three to four zinc finger domains; Cys_2-His_2 zinc finger (ZF) domains, together constitute to form a ZFN, In which each individual domain has a composition of approximately 30 amino acids residues that are organised in a ββα motifs (Gaj et al., 2013; Palpant and Dudzinski, 2013; Pabo et al., 2001; Petolino, 2015). The ever first sequence specific transcription factor in eukaryotic cell had zinc binding repeats in its DNA binding domain (Miller et al., 1985; Cathomen and Joung, 2008). These zinc fingers as the name suggests are involved in DNA binding.

Zinc Finger Nucleases has evolved as an extensively used genome editing tool by making its ability evident to manipulate the desired genomic sites and being applied in both basic and applied science fields. In terms of efficiency, minimal non target effects, high specificity and current attempts which are duly based on improvisation of design and delivery makes it more strategic in developing diverse crops of interest (Kamburova et al., 2017).

Transcription Activator-Like Effector Nucleases (TALENs): These nucleases are artificial enzymes that are composed of TALE domain along with FokI DNA cleavage domain. TALE binding domain consists of a series of repeat domains which has approximately 32 residues. Each repeat connects to the DNA through the amino acid residues at 12 and 13 position and are called as Repeat Variable di-

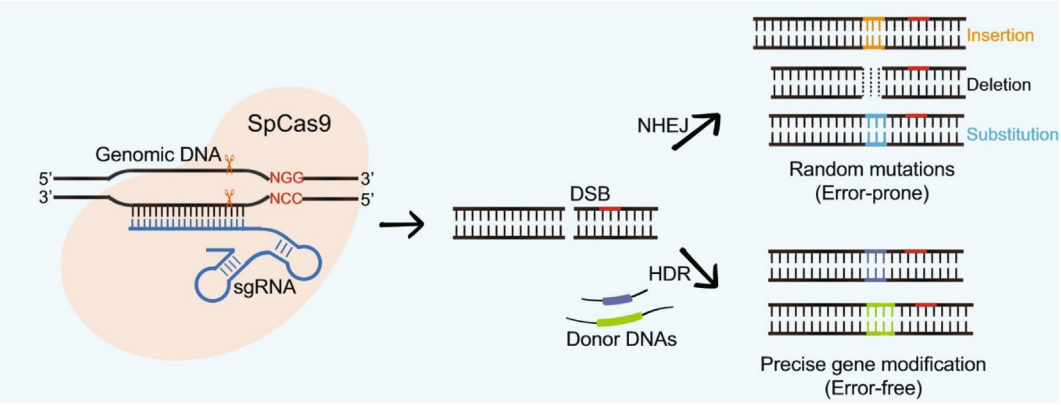
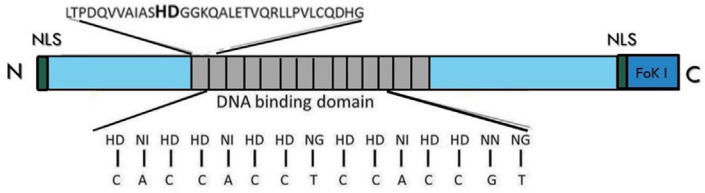
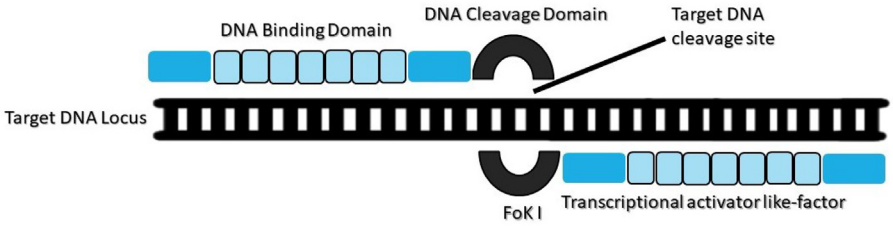


Fig. 7. DSB repair mechanism via NHEJ and HDR in genome engineering (Wang et al., 2019).



Di-Amino Acid	Nucleotide Bound
NI	A
NG	T
HD	C
NN	A/G

(A)



(B)

Fig. 8. (A) Structure of TALEN along with RVDs (Novodvorsky, 2014), (B) Diagrammatic representation of TALEN (Punwar et al., 2014).

residues (RVDs) (Fig. 8 A). The most common RVDs used for assembling the artificial TALE arrays include NI for Adenine (A), NG for Thymine (T), and HD for cytosine, and NN or HN for Guanine or Adenine. These specific regions define the nucleotide specificity. The TALE proteins identify and stimulate the specific promoters present in plant with the help of tandem repeats, forming the basis to the construction of novel genome editing system comprising of TALENs or Chimeric Nucleases (Jankele and Svoboda, 2014).

Like ZFNs, dimerization of TALEN protein is mediated by the FokI cleavage, which separates the TALE binding sites by a cut at 12–19 bp spacer sequence (Fig. 8 B). Conversely to zinc fingers which identify DNA triplet, the TALE protein only identifies a single bp, with little to no target site overlap from adjacent domains. In 2007, the functional activity of TALE proteins as DNA binding protein was discovered (Romer et al., 2007) and later the recognition code of target DNA sequence was decoded by TALE proteins (Boch et al., 2009). With advances in the experimentation and the wide applications of TALE Nuclease System, within three years of decoding their function, this system was considerably used to alter the genes in both animal and plant species (Xiong et al., 2015; Kamburova et al., 2017). The animal species include rat (Tesson et al., 2011), zebrafish (Sander et al., 2011; Huang et al., 2011), human cells (Miller et al., 2011; Hockemeyer et al., 2011) and the plant species specifically cereals and horticultural crops include wheat (Wang et al., 2014), rice (Li et al., 2012), Arabidopsis (Christian et al., 2010; Cermak et al., 2011; Li et al., 2012), tomato (Lor et al., 2014), and potato (Sawai et al., 2014).

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR): This latest genome editing technology has created a buzz in the science world and is applied to revolutionise to crop plant improvement techniques. This gives access to researchers to demonstrate DNA free insertions, gene knock-ins or insertions, and gene knout-outs. In general, it is called CRISPR which is a short hand for CRISPR/Cas9 (Fig. 9). CRISPR is basically specialised stretches of DNA and Cas9 is an enzyme working as molecular scissors to cleave the DNA. CRISPR was formerly identified in bacteria and archaea, where they function as acquired immunity mechanism against viral DNA and RNA, the usage of CRISPR/Cas9 to applied areas of research has developed genetic engineering, and opens ways for the novel therapeutic appliance and plant breeding and crop improvement (Sander and Joung, 2014; Liang et al., 2015; Maeder and Gersbach, 2016; Makarova et al., 2011; Kim et al., 2017; Newton et al., 2019).

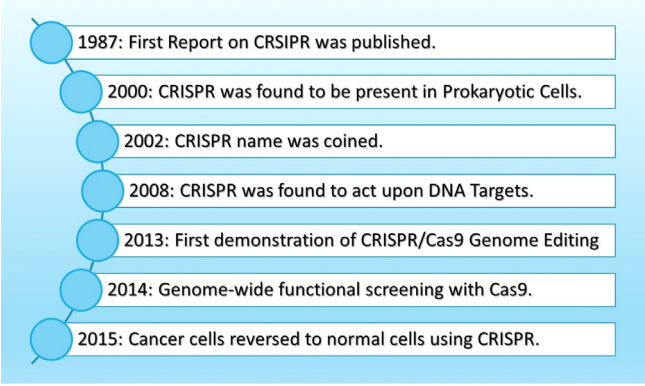


Fig. 9. CRISPR Timeline.

4.11. How does CRISPR/Cas9 works

The working of CRISPR/Cas9 begins with the formation of editing complex. The subsequent step involves the pairing of Cas9 enzyme with the guide RNA (gRNA), it carries the complementary sequences and helps in the delivery of Cas9 to the genome. Following this process, the complex (Cas9, gRNA and the complementary sequence) pairs with the targeted gene in the genome (Jinek et al., 2013; Qi et al., 2013). The target gene on the genome is then allowed to be cut by the Cas9 enzyme. Attempts are made by the cell to repair the DNA but it creates a mutation thus disabling its function permanently. Now, the insertion of short DNA fragment or the desired gene with specific function occurs for filling the gap and the original gene is finally replaced (Carroll, 2017; Barrangou et al., 2007; Barrangou and Marraffini, 2014). This finally leads to the production of desired protein (Fig. 10). Some recent instances of genetically modified crops on the basis of CRIPSR includes wheat variety that is resistant to diseases (Zhang et al., 2017), soy crop that yield much healthier soya oil (Demorest et al., 2016), and production of potatoes that don't sweeten on storage (Clasen et al., 2016). Genome editing is favourable over other breeding strategies and methods of production of GMO's as the single desired trait can be inserted in a generation without disturbing the background. Similar kind of alteration can be introduced in different varieties or cultivars produced in different environment without being resulted into

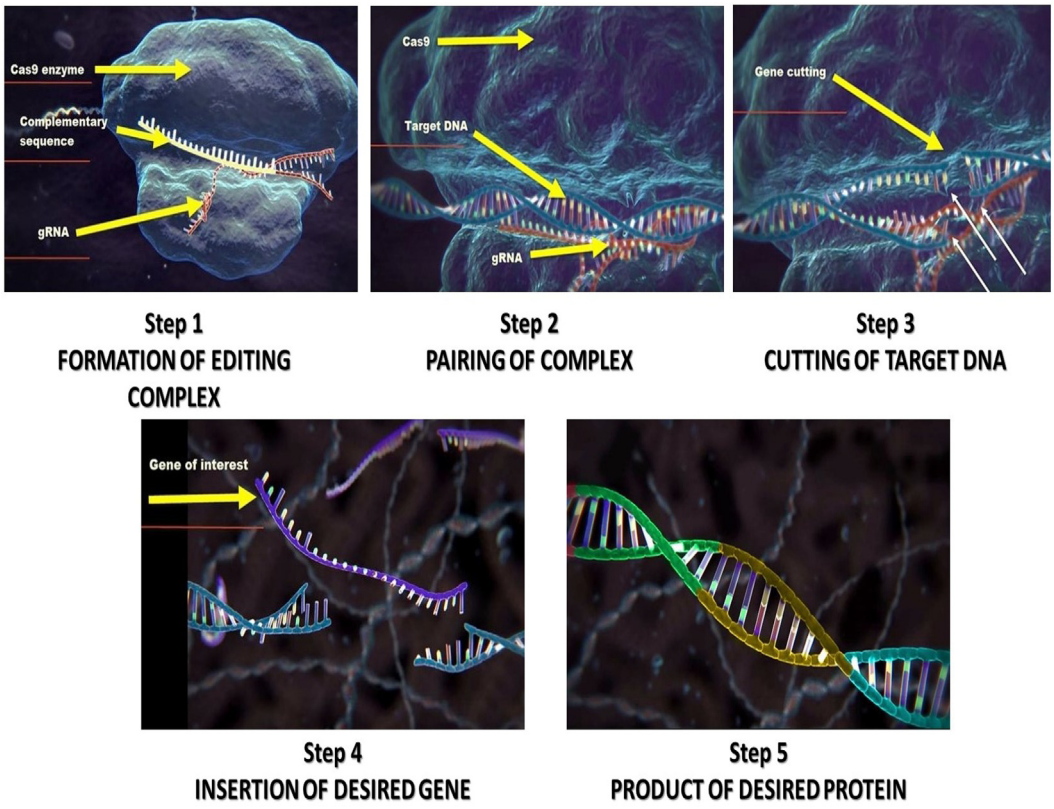


Fig. 10. Mechanism of CRISPR/Cas9.

Table 3
Application of CRISPR system in crops (Jaganathan et al., 2018).

Crop	System	Source	Gene of interest	Trait	References
Rice	Fncpf1	*Francisellanovicida*	*OsDL, OsALS*	Leaf morphology	Endo et al., 2016
Tobacco	Fncpf1	*Francisellanovicida*	NtPDS, NtSTF1	Pigmentation, Leaf morphology	Endo et al., 2016
Rice	Lbcpf1	*Lachnospiraceae bacterium*	OsEPFL9	Stomatal development	Yin et al., 2017

monoculture.

The applications of genomic editing that take in consideration the use of homology directed repair (HDR) mechanism of CRISPR/Cas are also of magnificent interest in the plant research industry (Table 3). The HDR tool functions to insert or knock-in the DNA fragment, like tags or new domains, as well as allele replacements and recoded genes (Belhaj et al., 2015). Despite of novel desirable molecular breeding techniques for DNA free editing, CRISPR/Cas9 being a revolutionary editing system promises for better complex genomic relocation (Ricaño-Rodríguez et al., 2018).

5. Conclusion

With the advances in the molecular marker technology and basically the molecular genetics, the last 3–4 decades have seen a great development as novel varieties of techniques have come into sight. These new techniques are highly functional in the determination of genetic variation, functional genomics and especially in the development of crop improvement and breeding technologies. The era has changed from the conventional use of molecular marker to the latest genome editing system CRISPR/Cas9. The conventional methods of genomic selection and genome editing by the use of cytological or biological markers were solely based on the breeders' experience of raising the crop; while talking about the progress of molecular markers in this area, the DNA markers have been put into systems such as CRISPR/Cas9 which ultimately works on site specific nucleases such as ZFNs or TALENs which are low cost being high throughput for the crop improvement. These genome editing systems are setting the new standards for functional genomics and raising the economically important crops through genetic selection. Presently, several examples endure proving these systems to be exceptionally unique in the field of understanding of plant biology and improvising the yield of crop with the help of mutagenesis, marker assisted breeding and genetic selection along with genomic editing. Channelling homologous recombination for the gene add-on still remains a challenge in the editing process of the plant genome. With the available bioinformatics tools for selecting optimal CRISPR/Cas9 target sites and predicting off targets, the technique has positioned itself at the forefront of genome editing methods. Being an easy and affordable tool, CRISPR technology has reformed so well in the field of plant breeding and genetics that assists the researchers for directing on genome editing of all the economically significant crops.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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