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Molecular markers and their role in crop Improvement

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Abstract
Molecular markers are unique DNA fragments that can be identified inside the entire genome and are effective tools used to 'flag' the location of a specific gene or the inheritance of a definite trait. The development of molecular markers combined with high throughput technologies have paved the way for achieving the desirable traits as well as induced biotic and abiotic stress tolerance in plant, which enhanced the crop breeding. Highly polymorphic molecular markers are developed for gene mapping, estimation of genetic Diversity, finding out the evolution and phylogeny of crop, analysis of heterosis, assessment of diploid/haploid crops and genotyping of cultivars along with Marker Assisted Selection (MAS). This review reveals about the role of various recently developed molecular markers in the improvement of crop.

Keywords: Genetic diversity, gene mapping, evolution crop, marker assisted selection, molecular markers

Introduction
There has been a quick growth in the discipline of Plant Biotechnology since past days decades and the different techniques used in it. Biotechnological methods produce an innovative, effective and profitable output or products. Biotechnology is a branch of biology that manipulates living organisms or any part of particular living organism to develop animals, plants and microorganisms for any specific purpose. The techniques used in biotechnology decreases the use of chemicals in agriculture, increases productivity of food, decreases dismissive environmental effects of conventional methods and reduces the cost of unprocessed material. These subjects have implementation in the use of biological approach for the people as well as manage studies for proper knowledge of basic life activities.
Conventional Plant Breeding method is an effective field of plant sciences depends on selection of genotypes and genetic variation which are used to develop the attributes demanded or needed by the farmers and the people. Introduction of new resistance genes for abiotic and biotic resistance from different sources like related plant varieties or gene bank has also developed the traits of crops. However, conventional breeding techniques have been fortunate in producing improved genotypes of different crops, but the new advancement in molecular breeding enhances the development of genotypes in a very less duration of time. The field of Plant Breeding can be improved by new technologies involved in molecular breeding or Plant Biotechnology and increases our interest of genetics. The genetic composition of organisms was easily understood by the discoveries of Polymerase Chain Reaction (PCR) by Kary Mullis in 1983 (Mullis, 1990) [40] and restriction enzyme by Smith and Wilcox (Smith and Wilcox., 1970) [60]. These discoveries achieved presumed genetic fingerprint. These experiments are done by the dissociation of DNA fragments on a gel electrophoresis that is obtained through a selective amplification of DNA utilizing Polymerase Chain Reaction (PCR) and by the segregation of DNA with enzymes. DNA fragments

Molecular Markers/DNA Markers
DNA markers also called as molecular markers comprise of particular molecules, which manifest simply detectable dissimilarities between various strains of a species or between different species. These markers may be contingent on proteins for instance DNA or isozymes. There are several markers dependent on DNA sequence, and is highly adaptable. A molecular marker is described as a DNA fragment or gene with a familiar site on a chromosome and correlated with a specific gene or character. It can be reported as a variation, which may be created by mutation in the loci of genome that can be perceived.

- Molecular markers are basically classified into two groups, *viz*, based on hybridization and based on Polymerase Chain Reaction (PCR). Types of molecular markers based on hybridization are: RFLP, microsatellite and minisatellite. Various molecular markers based on PCR are: Arbitrarily Primed PCR (AP-PCR), Start Codon Targeted Marker (SC_OT), Random Amplified Polymorphic DNA (RAPD), Inter simple sequence repeat (ISSR), Single nucleotide polymorphism (SNP), Simple sequence repeat (SSR), Amplified Fragment Length Polymorphism (AFLP), Cleaved Amplified Polymorphic Sequence (CAPS) and Expressed Sequence Tag (EST). And markers based on DNA sequencing like Single Nucleotide Polymorphism (SNP) marker. These markers are categorized based on their expressions; they are: -
  A. Dominant markers: only one form of the character which is selected to be marked is linked with the marker, in contrast the other form of the character is not linked with any marker. (Datta *et al*., 2011) [10] for e.g.: RAPD
  B. Co-dominant marker: both forms of the character which is selected to be marked are linked with the marker. (Datta *et al*., 2011) [10] for e.g.: RFLP, AFLP, SSR, SNP, EST, etc.

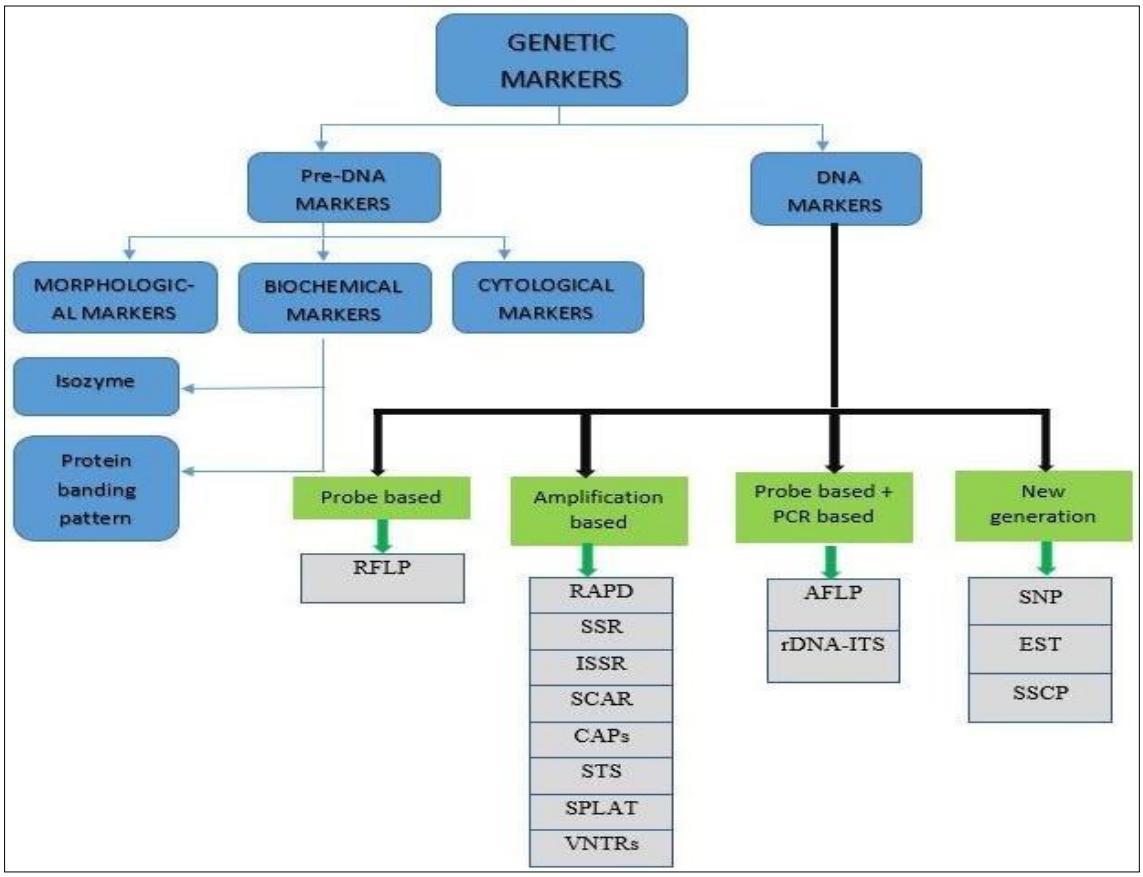

Fig 1: Types of molecular markers used in crop breeding (Gouda *et al*., 2020) [22]

Table 1: Commonly used molecular markers are as below

Molecular Markers	Type	Amplification of marker/ technique used for identification	References
Restriction fragment length polymorphism (RFLP)	Co-dominant	Depends on point mutation in a restriction site	Botstein *et al*. (1980) [3]
Random amplified polymorphic DNA (RAPD)	Dominant	Point mutation at primer annealing site in the specific region of a DNA strand	Williams *et al*., 1990, Welsh and McClelland.,1990 [65, 63]
Sequence Tagged Sites (STS)	Co-dominant	Depends on mutation at primer annealing site in the specific region of a DNA strand.	Olson *et al*., 1989 [46]
Sequence characterized amplified region (SCAR)	Co-dominant	Depends on mutation at primer annealing site in the specific region of a DNA strand.	Paran and Michelmore., 1993 [48]
Amplified fragment length polymorphism (AFLP)	Dominant	Depends on mutation at primer annealing site in the target DNA and change in restriction site in the target DNA.	Vos *et al*., 1995 [62]
Cleaved amplified polymorphic sequence (CAPS)	Co-dominant	Depends on: - 1. Mutation at primer annealing site in the target DNA. 2. Change in restriction site in the target DNA.	Konieczny and Ausubel., 1993 [31]
Simple Sequences Repeats (SSRs)/microsatellites	Co-dominant	Differences in the number of repeats of motif	Litt and Luty., 1989, Akkaya *et al*. 1992 [35, 1]
Diversity Arrays Technology (DArT)	Dominant	Microarray hybridization, DArT arrays are produced from genomic libraries through amplification of candidate or random clones	Wenzl *et al*., 2004 [64]
Single nucleotide polymorphism (SNP)	Co-dominant	Point mutation along with sequence information	Rafalski. 2002 [52]

Applications of molecular markers in Crop Improvement

Gene tagging

The development of huge number of new molecular markers provided a deep insight about the construction of high-resolution map and molecular breeding procedures in plants (Singh *et al*., 2017, Griffiths *et al*., 2000, Mishra and Tomar., 2014) [58, 23, 38]. Molecular markers are highly used for construction of genetic linkage mapping. It is also used in recognition of useful alleles in the variety or wild species. A molecular marker acts as a site of heterozygosity for some set of silent DNA differentiation, which are not associated with any quantifiable phenotypic variation. Such a locus of DNA, when in heterozygous form, can be utilized in gene mapping analysis like a conventional pair of heterozygous alleles can be used (Griffiths *et al*., 2000) [23]. Hence, the use of molecular markers, such as SSR, SNP, DArT, Microsatellite, ISSR, RFLP, RAPD, etc. along with high- throughput technologies has facilitated mapping of gene, QTL gene discovery and various other advancements in plant breeding. Mapping of Quantitative Trait Loci QTLs for grain protein content (PC) in rice useful for biofortification was studied using 98 molecular markers. Seven markers out of ninety-eight markers were strongly linked QTLs for grain protein content in rice. The protein content of grain was controlled by three novel QTLs, *viz*, qPC3.1, qPC5.1 and qPC9.1 and qPC6, qPC6.1, qPC6.2 also reported for protein content in rice. Four QTLs, qPC3, qPC8, qPC6.1 and qPC12.1 were detected for use in plant breeding programme. RM 407, marker was detected close to protein controlling QTL, *viz*, qProt8 and qPC8. The strongly linked markers with protein content of rice were; qPC3, qPC3.1, qPC5.1, qPC6.1, qPC8, qPC9.1 and qPC12.1, which will be applicable for their pyramiding for producing protein rich high yielding rice varieties (Pradhan *et al*., 2019) [49].

Hybrid necrosis genes, namely, Ne1 and Ne2 were mapped through microsatellite markers in wheat. Ne1 and Ne2 are two dominant complementary genes present on 5BL and 2BS chromosome arm, respectively. Interaction between these genes causes hybrid necrosis. Hybrid necrosis was visible in the F1 hybrid produced from the cross between Bread wheat genotype, 'Alsen' and 4 synthetic hexaploid wheat (SHW) lines, *viz*, TA4152-19, TA4152-37, TA4152-44, and TA4152-60 developed at CIMMYT. Genetic analysis showed that Alsen variety had ne1ne1Ne2Ne2 genotype and SHW had Ne1Ne1 ne2ne2 genotype. Xbarc74 and Xbarc55, microsatellite markers were used to map the genes in backcross populations. Xbarc74 was linked to Ne1 genes on chromosome arm 5BL at a genetic distance of 2.0cm whereas Xbarc55 was linked to Ne2 genes on chromosome 2BS at a genetic distance of 3.2cm (Chu *et al.*, 2006) [7]. High resolution mapping of Barley Leaf Rust (*Puccinia hordei*) resistance gene, $Rph_{MBR1012}$ was studied using 56 molecular markers (Fazlikhani *et al*., 2019) [17]. For the construction of a high-resolution mapping population of $Rph_{MBR1012}$, 537 segmental homozygous recombinant inbred lines obtained from 4775 F_2-plants were manipulated. To develop 56 molecular markers; six SSRs (Simple Sequence Repeats), seven size polymorphism and twenty-four SNPs (single nucleotide polymorphisms) marker obtained from the Barley Genome Zipper (GZ), the 9k iSelect Bead Chip (Dragan *et al*., 2013) [15], the Illumina 50K infinium chip and Genotyping By Sequencing (GBS) (Mascher *et al*., 2017) [36] were used for gene mapping. Quantitative trait loci (QTL) identification was performed across different environment for yield and its component and fiber quality characters in cotton (Ramesh *et al*., 2019; Diouf *et al*., 2018; Shang *et al*., 2016) [55, 12, 57]. CottonSNP63k Illumina infinium SNP array is widely used for genotyping different RILs and the parents (Ramesh *et al*., 2019; Li *et al*., 2016) [55, 34]. The genetic map revealed a total length of 3,149.8 cM with an average marker interval size of 2.2 cM (Ramesh *et al*., 2019) [55]. Phenotypic variance from five different environments were analysed using composite interval mapping which showed a total fifty-six QTL justifying phenotypic variances in the range of 8.18%–28.91% and 34 QTL out of 64 QTL resulted in QTL X Environment interactions, which were identified through Multi-Environment Trials Analysis (Ramesh *et al*., 2019) [55]. QTL x Environment interactions are detected for fiber quality through composite interval mapping which identified 62 new stable QTL across different environments (Shang *et al*., 2016) [57]. Yield traits and morphological features were identified using molecular markers inn maize. 346 SNPs and 623 SilicoDArT (969 markers) out of 49,911 identified polymorphic molecular markers were associated with yield traits and analysed morphological traits (Tomkowiak *et al*., 2019) [61]. DArTseq is a combination of Next Generation Sequencing (NGS) and DArT (diversity array technology) complexity reduction methods (Tomkowiak *et al*., 2019, Kilian *et al*., 2012, Cruz *et al*., 2013, Sansaloni *et al*., 2011) [61, 30, 8, 56]. The DArTseq method is used between others to identify SNPs and gives a large population of so-called silicoDArTs. SilicoDArTs have a dominant trait as the variability is found by the single point mutation (Sansaloni *et al*., 2011) [56]. Hence, silicoDArT and SNP markers can be used to group lines with incomplete data of origin and group lines in terms of origin of a species as well as in determining the applications in the selection of parents for crossing in heterosis and for predicting the hybrid formula in maize (Tomkowiak *et al*., 2019) [61]. An ameliorated genetic map with 1205 loci, having an average space of 2.2cM between loci and spanning 2598.3cM was developed in the recombinant inbred line (RILs) (TAG 24 × ICGV 86031) varieties of groundnut using a marker of high-density 58K SNP "Axiom_Arachis" array. Analysis of QTL was performed from the phenotypic data made for twenty drought tolerance and two iron deficiency tolerance related parameters at two locations in India and from 8 seasons, which resulted in nineteen majors main- effect QTLs 10.0 to 33.9% phenotypic variation for iron deficiency tolerance and drought tolerance related parameters (Pandey *et al*., 2021) [47].

Assessment of Genetic Diversity

Recent progress in the technology of molecular markers act as an important tool for profundity of genetic diversity and increased the knowledge abound breeding strategies (Ramesh *et al*., 2020) [54]. Markers are used for estimation or assessment of genetic diversity in germplasm collection, advanced breeding material and other cultivars which helps in the characterization of germplasm, developing PGR information system and for evolving varietal information structure. SSR, SCoT, CBDP, DArT, ISSR, SNP, CAPS, SCAR, RFLP, AFLP, RAPD, etc. are some of the different markers, which are extensively used for the estimation of crop genetic diversity (Hossain *et al*., 2020, Gaballah *et al*., 2021, Ghobadi *et al*., 2021, Kasoma *et al*., 2020) [26, 18, 20, 28].

Genetic diversity was estimated for 16 rice varieties under waters stress condition using SSR markers for drought tolerance. The varieties Giza 178, Giza179 and GZ1368- S-5-

4 were found to be drought tolerant (Gaballah *et al*., 2021) [18]. Start codon targeted (SCoT**)** polymorphisms and CBDP CAAT box–derived polymorphism (CBDP) are effective markers for the estimation of genetic diversity in hexaploid wheat, two Aegilops species (*Aegilops crassa* and *Aegilops cylindrica*) of wheat because 15 SCoT producing 262 fragments and 15 CBDP primers produced 298 fragments, all were polymorphic (Ghobadi *et al*., 2021) [20]. Genetic diversity was successfully estimated in 48 accessions of barley (introduced from ICARDA, Lebanon) using 150 SSR markers, out of which 51 SSR marker showed polymorphism that were utilized for final analysis. These 51 SSR markers generated 158 polymorphic loci in the form of genotypic data with a mean of 3.275 alleles per SSR locus. Hence, the existence of favourable allelic diversity was confirmed which is important for estimation of genetic diversity (Kumar *et al*., 2020) [32]. DArT Sequencing-derived single nucleotide polymorphism markers revealed the genetic diversity of 59 genotype of maize for economic characters and resistance to the fall armyworm pest. A moderate level of genetic diversity among the genotypes were generated when evaluated using SNP markers as the mean gene diversity were 0.29 and Polymorphic information content (PIC) were 0.23. ZM 7114, ZM 4236 and Pool 16 were the maize genotypes with favourable agronomic traits and fall armyworm resistant (Kasoma *et al*., 2020) [28]. Thus, recently developed microarray-based molecular marker technology shows high efficiency in determining the genetic diversity in maize (Kasoma *et al*., 2020 [28], Badu-Apraku *et al*., 2021 [2], Zebire, 2020 [67]). A large genetic variation in data set using CottonSNP63K array provided an opportunity to differentiate *G. hirsutum* from other species of Gossypium and even distinctly separated wild types from cultivated types of *G.hirsutum*, and additionally, SNPmarker identified the loci associated with seed nutritional characters (Hinze *et al*., 2017) [25]. Thirteen SSR markers were used to estimate genetic diversity. 119 genotypes were studied across two locations to evaluate agronomic characters and leaf spot and rust disease susceptibility. Moderate level of genetic diversity with 0.34 and 0.63 mean polymorphic information content and gene diversity, respectively which proved genotypes ICG 12725, ICGV-SM 16608, ICGV-SM 16575 and ICGV-SM 06737 are useful for the improvement of groundnut (Daudi *et al*., 2021) [11].

Phylogenic relationship of crop

Advancement in the technologies of molecular markers have enhanced facts related to the genetic structure of a crop. Earlier, the evolution was studied on the basis of morphological or geographical changes between the crops, but, nowadays molecular markers are largely used to reconstruct a genetic map to reveal information about phylogeny and evolution of a crop (Nadeem *et al*., 2018) [41]. Chloroplast markers are considered to be ideal for the evaluation of plant phylogeny, because of their stable and simple genetic construct. (Dong *et al*., 2012) [14]. Phylogenetic analysis is the organization of various species in a group based on their genetic relationship, which indicates the degree of genetic variation. Pattern of evolution of various species can be from this groups using molecular markers (Singh and Singh, 2015) [59]. A map of genome variation in rice proved that *Oryza sativa L* (Japonica rice) to be the first domesticated rice species from a particular population of *O. rufipogon* and a cross made between *japonica rice* and wild local rice eventually developed the Oryza sativa (Indica rice) (Oka 1988, Cheng *et al*., 2003, Huang *et al*., 2012) [45, 6, 27]. Phylogenetic study revealed that SNPs are fixed by ancestors of *indica–japonica*, which resulted that 45% of the ancestral alleles of SNPs are fixed in *japonica* and 55% in *indica* (Huang *et al*., 2012) [27]. Zhang *et al*., 2021 [70] studied genome distribution patterns in Triticum aestivum and its diploid and tetraploid progenitors comparing the numbers of coding sequences i.e., AAC, AAG, AGC and AG. SSRs sequence evolution carried out for the identification of different chromosomes which in result indicated more sensitivity of B genome expansion and elimination during wheat evolution. Phylogenetic study of 14 cereals (4 wheat, 3 barley, 3 rice, 2 maize and 2 sorghum) using 10 ISSR-PCR marker, 15RAPD-PCR marker and NTSYS-pc program data of both sets evinced wheat is more closely related to barley than rice followed by maize and sorghum (Rabey *et al*., 2015) [16]

Estimation of heterosis using molecular markers

Heterosis describes the superiority of F_1 (progeny) over both its parents. The use of polymorphic molecular markers for the estimation of heterosis have gradually increased over last few decades. Molecular markers are effectively used in the analysis of heterosis in various population structure (Gonzalez *et al*., 1999) [39]. Heterosis was successfully predicted in 40 F_1 rice hybrids by using 25 EST- SSR and morphological markers Heterosis was analyzed based on correlation between standard heterosis and coefficient of marker polymorphism (CMP), in which CMP value ranged from 0.40-0.80 (Pavani *et al*., 2018) [50].

Nie *et al*., 2019 [43] concluded that SNP (90K SNP chip) is an effective marker to accurately predict heterosis based on genetic distance (GD) and perfectly allocates the parents to heterotic groups in wheat. The experiment performed by Geng *et al*., 2021 [19], also revealed that SSR and SNP marker serves as an effective tool in predicting the heterosis based on parental GD as well as assigns the varieties into heterotic groups and even provides knowledge for selection of parents in hybrid breeding of cotton. A study was conducted to analyze the heterotic group and patterning in quality protein maize (QPM) in 3 inbreds of maize using SSR markers clustered the better hybrids together but in another sub clusters and flint X dent was the superior heterotic pattern (Rajendran *et al*., 2014) [53]. Heterosis in barley was assessed through cDNA- AFLP marker in 48 crosses. 5 TDFs revealed the significance of heterosis of 1000- kernel weight (Zhang *et al*., 2015) [68].

Genotyping of cultivars

Haploids are described as crops having single set of chromosomes, diploids are crops having two copies of homologous chromosomes each and double haploids (DH) are crops obtained from a single pollen grain and artificially doubled to produce homozygous diploids. This DH or haploid crops are utilized as a mapping population for QTL mapping and other genetic studies (Khush and Virmani, 1996). Radi *et al*., 2020 [29, 51] used an integrated protocol for the identification of haploids in a hybrid population of early seedling stage by using *R1-navajo (R1-nj* phenotypic marker or anthocyanin color marker (Chaikam *et al*., 2015, Melchinger *et al*., 2013) [4, 37] associated with genome size determination and SSR marker were used at pre-seedling stage for the assessment of haploid progenies in maize inbred

lines. Total 38 STMS (Sequence-tagged microsatellite) markers identified heterozygotes from 200 DHs, which were derived from 'BS6444G', an elite indica rice hybrid. Out of 200 DHs, 9 DH line yielded higher than hybrid parents (Naik *et al*., 2017) [42].

Inter-retro transposon amplified polymorphism (IRAP) markers proved to be efficient tools for fast genetic fingerprinting and screening of large germplasm of cotton. The marker works efficiently in identifying the diploids from tetraploid cotton as well as differentiated the cultivars. So as to achieve the data on genetic diversity, 17 diploid and tetraploid *Gossypium* accessions were used for the genetic fingerprinting through the manipulation of IRAP markers, which resulted in moderate (0.0–18%) to high (45–80%) genetic variability (Noormohammadi *et al*., 2018) [44].

Marker Assisted Selection (MAS)

MAS/MAB is the indirect selection of selected or desired plant phenotype depending on the closely linked DNA marker. MAS/MAB is an efficient molecular tool for breeding, in which markers linked with the desired genes are used for indirect selection for that gene in non-segregating or segregating populations. MAS is an important method for the selection of traits that are difficult, like, biotic and abiotic stress tolerance in a crop (Datta *et al*., 2011, Das *et al*., 2017) [10, 9]. Marker assisted selection successfully combines tolerance/resistance to various biotic and abiotic stresses as well as maintains grain quality and higher yield in rice. The experiment was conducted utilizing genes conferring resistance against bacterial leaf blight (Xa4, xa5, xa13, Xa21), blast (Pi9), gall midge (Gm4, Gm8) and drought tolerance QTLs (qDTY1.1 and qDTY3.1). 7 introgression lines (ILs) of rice having 7-10 QTLs combined together developed using MAS in the context of swarna with QTLs of drought. Out of these, 3 ILs *viz*., IL1 consisting of Pi9+ Xa4+ xa5+ Xa21+ Bph17+ Gm8+ qDTY1.1+ qDTY3.1, IL6 which contains Pi9+ Xa4+ xa5+ Xa21+ Bph3+ Bph17+ Gm4+ Gm8+ qDTY1.1+ qDTY3.1 and IL7 having Pi9+ Xa4+ xa5+ Bph3+ Gm4+ qDTY1.1+ qDTY3.1 were resistant\tolerant for multiple abiotic and biotic stresses in the field and glasshouse conditions (gordeeva *et al*., 2020) [21].

MAS methods were used to develop purple-grained cultivars of bread wheat. The breeding scheme initiated from crossing of lines and elite cultivars (recipient) with donor lines which had the complementary genes Pp-D1 and Pp3. Pp genes controls the anthocyanin pigments which results in purple colored grain. The F_2 hybrids followed the 3 step MAS while the dominant genotypes (Pp-D1Pp-D1Pp3Pp3) followed the recurrent backcrossing and morphological markers were used to select the desired $BC1F_{2\text{-}3}$ progenies. The study revealed seventeen lines rich in anthocyanin, purple grains best yield and showed multiple resistance (Gordeeva *et al*., 2020) [21]. Lei *et al*., 2020 [33] newly developed a PCR based co-dominant marker identified the 17-kb deletion of *Hordeum vulgare* ethylene response factor (ERF) gene, that is seen on 7HL chromosome on the *nud* locus which is responsible for naked barley. SNP markers can be successfully used in the MAS of upland cotton because a *SNP_GH1570* was found totally segregated from fruiting branches character and SSR marker BNL3232 was linked to the short fruiting branches was screened by BSA method (Zhang *et al*., 2018) [69]. MAB method was used to pyramid the β-Carotene (*crtRB1*) and Opaque-2 (*O2*) genes in maize. 236 SSR markers equally spread across the maize genome were used for the background selection. *O2* gene from HKI163 (donor) was transferred to *crtRB1*rich inbred lines i.e. UMI1200β+ and UMI1230β+. *CrtRB1 3'TE* and *umc1066* markers were used for *crtRB1and O2*gene, respectively for the foreground selection. The results showed increase in tryptophan (0.073–0.081%), lysine (0.294–0.332%) and β-carotene (6.12–7.38 µg/g) content (Chandran *et al*., 2019) [5].

Conclusion

A desirable molecular marker should have high polymorphism, frequent occurrence, should be easy to use and should be quick, co-dominant inheritance, equally dispersed all over the genome, high transferability and reproducibility, less expensive and phenotypically neutral. Molecular marker plays an important role in plant breeding or crop improvement. The last few years have revolutionized the molecular marker technology from RAPD to DArT markers. Advancement in molecular markers integrated with high throughput technology played a vital role in gene mapping, estimation of Genetic Diversity, finding out the evolution and phylogeny of crop, analysis of heterosis, and assessment of diploid/haploid crops and genotyping of cultivars along with Marker Assisted Breeding (MAB) / Marker Assisted Selection (MAS). Now a days, DArT, SSR, SNP, EST-SSR, ISSR, CAPS, SCAR, etc. with high throughput technologies are very exciting markers, which enhances the crop with desired traits and induces tolerance against biotic and abiotic stresses in a short period of time. Though, technologies have gradually enhanced in this newly developed marker, but all these requirements are not yet fulfilled. Hence, appropriate selection of molecular markers is important, which amalgamates some of these desirable characters to achieve the current demand in crop improvement.

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