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Functional Markers in Zea mays L

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Functional Markers in *Zea mays* L.

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Introduction

Great advancements have been made in the area of plant breeding and genetics since the turn of the century. Many of these advancements stem from research in maize, which is an important global crop. This paper seeks to answer the question: How has the study and mapping of genetic variation in maize germplasm at a genome-wide level facilitated the development of markers for maize breeding? Since the first types of molecular markers were identified, their utility and functionality has increased greatly to the plant breeding community. Functional Markers (FMs) are polymorphic markers located within a gene whose function has been proven to regulate a trait of interest. Maize breeders are gaining more access to published FMs, which are being created based on an increased understanding of gene function coming from gene cloning and mutant analyses. They can use these markers to increase the efficiency of their breeding programs through marker-assisted selection (MAS), which has revolutionized traditional breeding methods. Current research and literature have been reviewed surrounding sequencing technologies, the maize genome, and specific gene sequences and their functions. Also reviewed are examples of how these technologies have aided the development of Functional Markers in maize.

Maize: A World Crop and Model Organism

We have come a long way from the domestication of ancient Teosinte, which modern genetic studies have shown is the most likely plant species that created what we know today as corn (van Heerwaarden et al., 2011). Corn, or maize (*Zea mays*), is one of the most important crops in the world today. As one of the main cereal grains, along with rice and wheat, maize is the primary source of food and feed for many countries around the globe. Its popularity as a crop

is largely due to its many diverse functions as a food source for both humans and animals. The kernels of the maize plant can be consumed straight from the cob, parched, boiled, fried, roasted, ground, and even fermented for use in breads, porridges, gruel, cakes, snacks and alcoholic beverages. Maize can be further processed to use as food thickeners, sweeteners, oils, and non-consumables such as thermoplastics (Nuss and Tanumihardjo, 2010). According to a recent release of the USDA's World Agriculture Supply and Demand Estimates report, during 2019-2020 the United States produced 345.96 million metric tons of corn (USDA, 2021). The National Corn Growers Association estimates the value of the 2019-2020 crop to be $48.49 billion (https://www.worldofcorn.com/#us-corn-crop-value). See Table 1.

Maize is not only an important crop for feed, food, and other domestic uses but it has also been proven to be a model plant for genetics research since the early 20th century. Maize has several characteristics that enhance its attractiveness as a genetic system (Strable and Scanlon 2009). It can be cultured on any scale, whether that be a few plants in pots or many acres in fields. It can be grown successfully year-round in greenhouses and growth chambers with proper lighting; it is also quite hardy, successfully growing outdoors under a wide range of environmental conditions ranging from tropical to temperate climates (Shaw 1988). Maize naturally acts as an outcrossing species, with the ability to receive pollen from plants in adjacent fields and even similar plant species. This makes the genetic architecture (diversity, linkage, recombination, etc.) of maize more similar to other outcrossing organisms such as humans, rather than self-pollinating plants (Rafalski and Morgante 2004; Wallace et al. 2013). While it is an outcrossing species, maize retains a major strength for plant genetics: the ability to self pollinate and quickly produce homozygotes or F_2 populations. Maize is a monoecious plant, allowing for crosses that are easily controlled, which helps make it the premier system for studying the

genetic basis of heterosis (hybrid vigor) and exploiting heterosis. In fact, heterosis is the key to the success of maize as an agricultural crop. It produces many large seeds held in rows, often hundreds per cob, making it easy to score rare recombination events in kernel composition or color and quickly identify segregating ratios (Nannas and Dawe, 2015). The strength of maize as a model organism has led to years of breeding trials and genetic experiments providing a clear history of its evolution and creating a global community of researchers continuously working on its genetic improvement.

Functional Markers: Description and Development

Polymorphic markers that are created for a mutation which occurs within a gene and are causally linked to phenotypic variation in that gene are defined as Functional Markers (FMs) (Andersen, 2003). One of the main benefits of using FMs instead of traditional molecular markers such as morphological markers or random DNA markers, like amplified fragment length polymorphisms (AFLPs), is that their predictive ability is not diminished by recombination unless that recombination happened to occur within the gene, which could result in a complete loss of function of both alleles of that gene. Functional markers can be observed as simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs), insertion-deletions (INDELS), and other marker types, though these types of markers are only considered FMs if they are located within the gene of interest. The predictive value of traditional markers is heavily reliant on the strength of linkages with the trait of interest. Therefore, it requires extensive high density and high-resolution genetic and quantitative trait loci (QTL) mapping. Extensive mapping is essential because QTL subsets can be polymorphic within populations leading to linkage phase discrepancies even in closely related genotypes (Lübberstedt et al., 2005).

When trying to develop FMs there are a series of steps that must be completed. The first of these is: 1) identification of the gene of interest. Once the candidate gene for a FM is identified: 2) the alleles must then be sequenced. After sequencing the alleles: 3) any polymorphic, functional motifs that affect plant phenotype must be identified. Finally, 4) the associations between the polymorphisms and the trait variations they cause must be validated. Maize is a strong candidate crop for developing FMs because there are currently over 50,000 gene locations and their descriptions are available in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/gene/?term=%22Zea+mays%22). There are also now more than 2 million *Zea mays* expressed sequence tags (ESTs) available to the public domain through NCBI. (https://www.ncbi.nlm.nih.gov/). The creation of these databases helps to facilitate the completion of steps one and two of functional marker development. As genetic research continues, plant breeders can only expect to have more access to genes of interest and their allelic sequences.

Current Tools for Developing Functional Markers

The Maize Genetics and Genomics Database (MaizeGDB; https://www.maizegdb.org/) is the most beneficial database available to the maize research community (Portwood et al., 2019). The MaizeGDB supports genome sequences of several maize inbred lines with a genome browser application that is user-friendly. It also contains, among other features, large numbers of datasets covering seed stocks, polymorphism information, mutants and phenotypes, genetic maps, QTL information, gene annotations, and transcriptomic and proteomic data. Furthermore, it provides tools used for querying and visualizing datasets, such as SNPversity, which allows the SNP diversity of a selected genomic region to be visualized, and qTeller (https://qteller.maizegdb.org/), which can be used to explore the expression of genes of interest.

Finally, it provides additional services that benefit the maize community such as the coordination of the annual Maize Genetics Meeting, recommendation of current publications by the MaizeGDB Editorial Board, and the distribution of germplasm with the handling of stock requests through the Maize Genetics Cooperation Stock Center (https://www.maizegdb.org/data_center/stock) (Liu et al., 2020). The development of the MaizeGDB in the early 2000s and the complete sequencing of the genome of the B73 variety to be used as a reference genome was groundbreaking in the plant breeding and genetics communities. Since the release of the B73 reference genome sequence in 2009 and subsequent release in 2017, MaizeGDB has been extensively used for maize functional genomics research (Liu et al., 2020). In 2020 there was a fifth version update of the entire B73 genome released through MaizeGDB. New sequencing technologies and the continued decrease in cost has led to the release of whole genome sequences for several other maize varieties since 2016. The maize varieties that have currently been sequenced can be found below in Table 2. The high-quality assemblies enabled by new single molecule sequencing technologies, which are currently available for B73, Mo17, and SK, have greater advantages in annotation of genome features. These high-quality assemblies also aid in the identification of important elements, such as promoter regions and transposable elements (TEs) and variations, especially structural variations, compared with the assemblies that were based on short-read sequencing (Jiao et al., 2017; Sun et al., 2018; Yang et al., 2019).

Identifying Polymorphisms and Functional Motifs

As molecular markers have been applied to construct linkage maps and map single genes and QTL for quantitative traits, many types of mapping populations have been generated including the classic F_2, backcross, recombinant inbred line (RIL), and multi-parent advanced

generation intercross populations. Maize researchers have constructed high-resolution mapping populations that have been used extensively for genetic mapping (Liu et al., 2020). One such population is the intermated B73-Mo17 (IBM) population, a type of intermated RIL population. To create the IBM population the F_2 progenies of crosses between B73 and Mo17 were intermated for four generations before repeated selfing by single-seed descent method to generate RILs. Due to the extra generations of intermating, there was a 2.7-fold increase in recombination leading to a 3.86-fold increase in map length as compared to that developed using no extra intermating (Lee et al., 2002). Thus, such mapping population provides enhanced resolution in single gene and QTL mapping. Another approach used in developing mapping populations for high-resolution mapping is the nested association mapping (NAM) population consisting of 25 RIL populations with a common parent line (B73). Both the IBM and NAM populations have been used for accurate linkage mapping and association mapping to help determine the genetic basis of complex traits, such as flowering time and disease resistance (Balint-Kurti et al., 2007; Buckler et al., 2009; Kump et al., 2011). At the turn of the century, (Thornsberry et al., 2001) tested associations between 123 polymorphic sites covering dwarf8 and flowering time genes in an association mapping population consisting of 92 maize inbred lines and using 141 SSR loci to control for population structure. Since then, many natural maize populations have been used for candidate-gene-association studies (reviewed in Yan et al., 2011; Xiao et al., 2017; Liu and Yan, 2019). In maize, association tests were subsequently extended to genome-wide association studies (GWASs) in both mutant populations and natural variants. The ‘‘Goodman’’ maize diversity panel is one of these types of populations; it contains 302 inbred lines that captures the diversity present in public breeding programs (Flint-Garcia et al., 2005). This panel has been genotyped using the Illumina MaizeSNP50 Array (Olukolu et al., 2013) and

genotyping-by-sequencing (GBS; Romay et al., 2013), and is now covered under the maize HapMap 3 project (Bukowski et al., 2018).

Indirect Functional Markers (IFMs) can be identified with reasonable effort using association studies that allow for assigning function to short sequence motifs in large germplasm collections. This approach may be limited by linkage disequilibrium, i.e., extended haplotype structures (Lübberstedt et al., 2005). In heterogeneous genotype collections, associations that identified specific sites for IFMs could be confounded with effects from other genome regions, especially in the case of population stratification (Pritchard et al., 2000). Only comparisons of isogenic lines will confirm the phenotypic effects of short sequence motifs, revealing direct FMs. Isogenic lines for genes of interest can be generated in different ways (Andersen and Lübberstedt, 2003). One approach is to create mutations for the target IFMs using transposons. Maize researchers mainly use transposon mutagenesis approaches to tag genes for cloning. Several research programs have tagged maize genes with active *Mu*, including the Trait Utility System for Corn, Maize Targeted Mutagenesis database, *Mu* array, *RescueMu*, Photosynthetic Mutant Screen, and UniformMu (reviewed in Brutnell, 2002). UniformMu is currently the most widely used among these programs (McCarty et al., 2005). Apart from transposon mutagenesis, targeting induced local lesions in genomes (TILLING) has also been used to construct maize mutant libraries (Till et al., 2004; Lu et al., 2018). In this approach, point mutations that alter protein function, induced by exposure to ethyl methanesulfonate, are detected with high-throughput methods or next generation sequencing (NGS). The conventional approach of selfing RILs that are heterozygous for individual IFMs could produce near-isogenic lines for the validations of the IFMs.

Application of Functional Markers in Maize

While increasing yield has always been the central goal in breeding programs there are now many new kinds of maize lines developed for specific purposes. Silage corn, waxy corn, high-amylose corn, high-oil corn, high-quality-protein corn, sweet corn, and popcorn are some examples of the so-called "specialty corns" (Liu et al., 2020).

Case study 1: Functional Markers for Sweet Corn

A significant portion of the global demand for maize comes in the form of sweet corn. The demand for sweet corn has increased tremendously in the last few years, partially due to urbanization, increased consumption, and the availability of organized food-processing industries (Mehta et al. 2017). The recessive sugary1 (*su1*) gene has been extensively used for development of sweet corn cultivars worldwide. In addition to *Su1*, the recessive *sh*2 allele has also been used to develop commercial sweet corn cultivars (Lertrat and Pulam 2007). Traditionally, SSRs linked to the *su1* allele are used as markers for the molecular breeding of sweet corn (Hossain et al. 2015). However, this style of linked markers may not always be polymorphic between donor and recipient parents. There is a chance that individuals selected were recombinants and would show a false positive because of crossing over between the gene and marker (Gupta et al. 2013). In order to enhance the efficiency of marker-assisted breeding for kernel sweetness in maize, Chhabra et al. (2019) developed (*i*) su1-based functional marker(s) for the *sugary1* gene that encodes starch debranching enzyme, which affects kernel sweetness and (*ii*) validated the markers' effectiveness by studying segregating populations and among diverse inbreds. In their study, they used five diverse wild-type maize inbreds (*Su1Su1*) and six sugary-type maize inbreds (*su1su1*) to study nucleotide variations in the *su1* gene. They also studied seeds of five F_2

populations carrying 100-120 individuals for segregation of alleles at the *su1* locus. A set of 230 inbreds, including 47 sugary (*su1su1/Sh2Sh2*), 85 shrunken *(sh2sh2/Su1Su1*) and 98 normal maize lines (*Sh2Sh2/Su1Su1*), was used to validate the developed FMs for the sugary trait in corn.

Chhabra et al. (2019) studied the 11.7kb *Su1* gene for single nucleotide polymorphisms (SNPs) and insertion and deletion markers (INDELs). Twenty-seven overlapping primers were designed using Primer3 online software (http://bioinfo.ut.ee/primer3-0.4.0/) covering the 11.7-kb *Su1* gene (GenBank accession number AF030882) to amplify polymerase chain termination reaction (PCR) products of 500–900 bp. Overlapping primers were used to amplify PCR products from selected inbreds: *(i)* five wild type and *(ii)* six sugary type. SNP- and INDEL-specific primer pairs were also designed after studying the sequence variation in the *Su1* alleles of selected genotypes. They applied a novel PCR approach to amplify both *Su1* alleles simultaneously using primers specific to indel and SNP loci located within the *Su*1 locus. (Table 3).

They aligned sequences of the *su1* gene in the five wild- (*Su1*-) and six sugary- (*su1su1*) type inbreds with the reference *Su1* gene sequence available in the public domain (GenBank accession number AF030882). In total, 12 indels and 96 SNPs were identified that clearly differentiated wild-type and sugary inbreds. Of the 108 markers that differentiated wild and sugary inbreds, only two indels and one SNP were found to be associated with the functional variation among the *Su1* alleles. Deletion of 36 bp in the promoter was found to be associated with a TATA box sequence, which is necessary for the formation of an active transcription-initiation complex (Greenblatt, 1991). A 6-bp indel in intron-10 was localized to the exon-splicing enhancer site leading to aberrant su1 transcripts and lack of a functional starch

debranching enzyme (Wu and Maniatis 1993). A point mutation in exon-2 with a C to G transversion mutation in the *su*1 mutant results in the nonsynonymous mutation from phenylalanine (UUC) in wild type to leucine (UUG) in the mutant at amino acid position 163. No association of other SNPs and INDEL mutations of the *Su1* locus with Su1 function was observed.

PCR primers for four marker loci were used against the five created segregating populations to identify wild-type homozygotes (*Su1Su1*), heterozygotes (*Su1su1*) and recessive sugary homozygotes (*su1su1*) that segregate in the expected 1:2:1 ratio using the primers listed in Table 3. When validated against the panel of 230 inbred lines consisting of *su1*, and *sh2* mutant and wild-type inbreds as controls, Su36Del-FR and both SNP-specific markers (SNP-2703 and SNP2703-CG-85/89) amplified PCR products only in *su1* inbreds and not in the control *sh2* mutant and wild-type inbreds, whereas wild-type-specific primers amplified only in *sh2* and normal *Su1* inbreds, but not in *su1* inbreds. However, the primers for the SuDel6-FR locus were not successfully validated with the inbred panel. This suggests that the 6-bp indel is not the causal agent for the *su1* mutation, which illustrates the importance of validating molecular markers in a diverse set of inbreds in order to demonstrate their relationships with the loss of function mutation.

Although microsatellite markers linked to *su1* genes are available in the public domain (Hossain et al., 2015), so far there is no report on universal functional markers for the *su1* mutations. Three SNPs for *su1* alleles have been previously reported (Shin et al., 2006); however, those mutations were not observed in the study conducted by Chhabra et al. (2019), and thus could not be validated. Tracy et al. (2006) also identified three SNPs and one transposon-mediated indel among various recessive *su1* alleles present in 57 sweet corn

cultivars. However, the polymorphisms identified by Tracy et al. (2006) could not differentiate the wild-type and mutant alleles of *Su1* in the germplasm of the Chhabra study (2019).

The study by Chhabra et al. (2019) is the first report of development and validation of universal functional markers for selection of the *su1* allele. Two markers, SNP2703-CG-85/89 and SuDel36-FR, were confirmed as functional markers by studying F2 populations and a panel of diverse inbreds. These markers behave as codominant and thereby separate the homozygotes from the heterozygotes. These markers can be used for precise selection of desirable plants in the segregating generations, for improving the selection efficiency of kernel sweetness.

Case study 2: Functional Markers for Increased Amylose Content

Normal maize starch consists of 20 to 30% amylose. One of the recessive mutants that has been identified in maize is the *amylose-extender* (*ae*) mutant. This *ae* mutant lacks the major form of starch branching enzyme gene (SBEIIb) and produces starch with amylose content from 50 to 90% (Garwood et al., 1976; Hedman and Boyer, 1982). The starch from this type of high amylose corn is commonly used in textiles, gumcandies, cosmetics, pharmaceuticals, biodegradable plastic products, numerous industrial applications, and as an additive to increase the level of dietary fiber and lower the rate of energy release without changing the taste or texture. Two recent advances have created an increasing interest in the use of high amylose starches and have seen its production increase over the past decade. The first has been in the development of biodegradable thermoplastics which are starch based. The second comes from food companies who would like to use amylose from maize as a source of resistant starch (RS), a type of starch that is not digested as readily as normal starch (Chen et al., 2013). Consumers could benefit from RS as a food additive, where it has been shown to lower the risk of colon cancer (Nugent, 2005; Regina et al., 2006).

Campbell et al. (2007) released a maize inbred line, GEMS-0067 (registration number GP-550 [PI 643420]), to the public without restriction of any kind. This line possesses a higher amylose modifier gene which raises the amylose content in starch to over 70% when accompanied by the recessive *amylose-extender* (*ae*) gene. Since GEMS-0067 is the first public germplasm possessing the recessive gene and a modifier gene or genes that raises amylose level to over 70%, Chen et al., (2013) sought to develop and validate functional codominant molecular markers of *Sbe*I and *Sbe*IIb for GEMS-0067 that were able to distinguish *AeAe*, *Aeae*, and *aeae* genotypes.

Seventeen maize inbred lines, including seven *ae* mutants and 10 conventional inbreds, were used in this study. The seven *ae* mutants included GEMS-0067, and HAM-1-6; and the 10 conventional lines included B73, Mo17, and SN01-9. Primers were designed for full-length cDNA cloning of *Sbe*I, *Sbe*IIa, *Sbe*IIb, and *waxy1* genes (Table 4). There are 15 SNPs between the *Sbe*Is of B73 and GEMS-0067, all of which could be used as markers. The SNP on the sixth exon (C→A) happened to be the restriction site for *Alu*I, which cuts the palindrome sequence AGCT and was chosen for FM design. A primer pair for *Sbe*I FM (ae-SbeISNP) [F (5′-gtacacatttaagcatcctc-3′) and R (5′-atcatggctctcagcatatg-3′) Table 4] were designed from the sixth exon that amplifies a PCR product of 906 bp. The amplified PCR products for the *ae-Sbe*ISNP locus were 906 bp from all 17 inbred lines used in this study. When completely digested with *Alu*I, only the 906-bp product from GEMS-0067 was not digested by the enzyme, while the products from all other inbred lines were cut into two smaller fragments (617 plus 289 bp), confirming that the *Sbe*I sequence of GEMS-0067 was mutated in the sixth exon. The codominant nature of *ae-Sbe*ISNP was confirmed by digesting the PCR product amplified from the F1 heterozygous *Aeae* genotype, which yielded three bands. In all seven *ae* mutants, the ninth

exon (84 bp in length) of the *Sbe*IIb gene was deleted, resulting in a 28 amino acid deletion in SBEIIb compared to that of AF072725 (*AeAe*). Since all seven *ae* mutants had the ninth exon deleted an *ae*-indel was chosen to generate a marker, with primers designed from the sequences flanking the 84-bp deletion specific for *Sbe*IIb. The forward primer from the eighth exon (5′-ctggatcaagtactcagtgc-3′) and the reverse primer from the 10th exon (5′-tgcactgcattgtatccaagt-3′) were designed to create the FM *ae*-indel, which gave rise to a 700-bp fragment from all seven *ae* mutants and a 1576-bp fragment from 10 normal maize inbreds in PCR amplification. Sequence comparison between the 700 bp fragment from GEMS-0067 and 1576 bp fragment from SN-01 indicated a 116 bp deletion in the intron between exons 8 and 9 and a 675 bp deletion in the intron between exons 9 and 10. The 84 bp ninth exon, together with these two flanking introns, were deleted in GEMS-0067.

To answer the question whether *Sbe*I is directly involved in increasing amylose content, a population of 94 F_2 individuals were derived from the hybridization between HAM-1 and GEMS-0067. The two parental lines HAM-1 and GEMS-0067 have the same *ae* background with a nonfunctional SBEIIb. HAM-1 has a normal functional *Sbe*I allele; however, the *Sbe*I allele of GEMS-0067 has 15 SNPs resulting in a six amino acid change compared to that of HAM-1. The F_2 individuals were genotyped using the *Sbe*I-specifc *ae*-*Sbe*ISNP molecular marker; and amylose content of each individual was assayed. The results showed that there were significant differences among the group means of amylose content of the F_2 population. The amylose content of the aa group was significantly higher than that of AA and Aa groups; where AA represents the genotype homozygous SbeI allele of HAM-1, aa represents the genotype homozygous SbeI allele of GEMS-0067, and Aa is the genotype of their F_1. There was no significant difference found between AA and Aa groups. These results provided direct evidence

that a mutated SBEI could increase amylose content in *amylose extender* background. *The sbe*I explained 49% of the total amylose content variation in the study by Chen et al. (2013) and therefore this mutation in SBEI enhances the levels of amylose content. The role of mutation in an SBE in increasing amylose content was established earlier in pea (Bhattacharyya et al., 1990). The r locus in pea studied by Gregor Mendal governed the wrinkled seed shape. Mutation of a starch branching enzyme SBEI gene located in the r locus by a transposon insertion results in increased levels amylose and sucrose (sweet pea) contents and reduced levels of the starch, amylopectin (Bhattacharyya et al., 1990).

Discussion

Chen et al. (2013) described a set of molecular markers that could be considered as functional markers for increasing amylose contents. They identified a gene of interest, determined the allele sequence, and identified polymorphic motifs within the gene that were shown to be functional in increasing amylose content. Finally, Chen et al. (2013) validated the markers created from the observed polymorphisms of the GEMS-0067 line for variations in amylose content within an F_2 population made up of the cross between GEMS-0067 and another proven high-amylose line, HAM-1. The *ae-Sbe*ISNP and *ae*-indel markers distinguish differences in amylose content based on genotype within this population. The *ae*-indel marker in the *Sbe*II is a functional marker for enhancing amylose content in maize because the indel marker is based on the deletion that causes the loss of *SBEII* function. However,

*ae-Sbe*ISNP requires additional validation to consider it as a functional marker. The *ae-Sbe*ISNP was selected from 15 SNPs and it is unknown if this SNP is a causal mutation for loss of the *SBE*I function.

The study by Chhabra et al. (2019) provides an excellent example of Functional Marker development. The researchers identified a gene candidate for kernel sweetness, determined the sequence, identified polymorphic motifs shown to be responsible for increasing kernel sweetness, and validated the association of the polymorphism and variation in kernel sweetness across differing maize germplasms. The panel of 230 inbreds consisting of different sweet corn and normal maize varieties used to validate the SNP2703-CG-85/89 and SuDel36-FR markers clearly demonstrate their use as a Functional Marker. These markers should have a strong utility in sweet corn breeding programs for use in marker assisted selection (MAS). The importance of using a diverse set of germplasms was proven essential, when the inbred panel failed to validate a candidate marker that had been successful in the experimental lines. This study also provides a novel approach for Functional Marker development. Using a PCR mix with two different forward primers and a common reverse primer allowed Chhabra et al. (2019) to detect the presence of both *Su*1 and *su*1 alleles simultaneously, which allowed the assigning of genotypes for individual plants. The efficacy of this approach should be applicable in developing FMs for other traits.

CRISPR-Cas9 and the Future of Functional Markers

The clustered regularly interspaced short palindromic repeats (CRISPR) system uses a guide RNA and recruits CRISPR-associated nuclease 9 (Cas9) to mediate mutations in the guide-RNA-specific target sequences (Ran et al. 2013). Compared to protein-based recruitment methods, CRISPR-Cas9 has several advantages including the ease and low cost of synthesizing new guide RNAs, greater specificity of cleavage sites, and the ability to edit multiple loci simultaneously (Ran et al. 2013). CRISPR-Cas9 technology can be used to generate both multiple mutated alleles for a single gene; or mutations of multiple genes in a single maize plant

by integrating multiple guide RNAs into one vector or using just a single guide RNA targeting a conserved region of homologous genes (Doll et al., 2019).

There can be many randomly mutated loci in lines in chemical mutagen-induced mutant libraries derived for TILLING or tagging genes in *Mu* insertion-induced mutant libraries. Mu-induced mutants could be unstable due to high reversion rates (Brutnell, 2002; Lu et al., 2018) exacerbating the difficulty in generating functional markers. In contrast, the CRISPR/Cas9 system introduces stable mutations precisely at specific sites as dictated by the single guide RNA with a much cleaner genetic background (Liu et al., 2020). The ability to target genes for editing with a high degree of specificity makes the CRISPR-Cas9 system ideal for the development of Functional Markers in maize for the foreseeable future. Creating mutant libraries that are genetically similar except for the target genes would aid in the completion of step three for functional marker development; identifying mutations in motifs that affect plant phenotype would be more accurate and efficient because it would limit the interference of randomly mutated loci.

Conclusion

Maize has continued to grow as an important crop and a model plant. There has been a positive feedback loop created with the expansion of the research community around maize; as more research is done and the global databases are updated with new genes and sequences, an increase in the functional understanding of maize is occurring. Sequencing technologies continue to get better and less expensive which should lead to the elucidation of more and more accurate reference genomes. The resequencing of more maize genomes and predicting the polymorphisms

by comparing sequences with the reference genome will make for more accurate predictions of the polymorphisms associated with particular phenotypes, which can be revealed through genome-wide association studies (GWAS). The development of functional markers will become much more efficient with more access to information about gene sequences and their functions. The understanding of gene structure and function necessary for FM development will continue to increase with the new and accurate reference genomes coming from recent advancements in sequencing technology. Functional markers can be extremely beneficial to maize breeders. They can be used to increase the efficiency of breeding programs through marker assisted selection (MAS). The number of functional markers available to maize breeders is currently limiting. There have been many genes identified and cloned for important maize traits that could facilitate the development of additional functional markers (Table 5). Additionally, genes governing traits for many important grain crops have been identified (Table 6). Genes controlling traits in these alternative crops could provide a solid base for identification of orthologous genes in maize, which then can be targeted through CRISPR-Cas9 system to generate possible functional markers. Maize breeders should anticipate the identification of more FMs for important agronomic traits such as germination, vigor, flowering time, kernel size, number, and nutrient composition, disease resistance, and others in future years.

Table 1. U.S. Corn Crop Value (National Corn Growers Association, 2021)

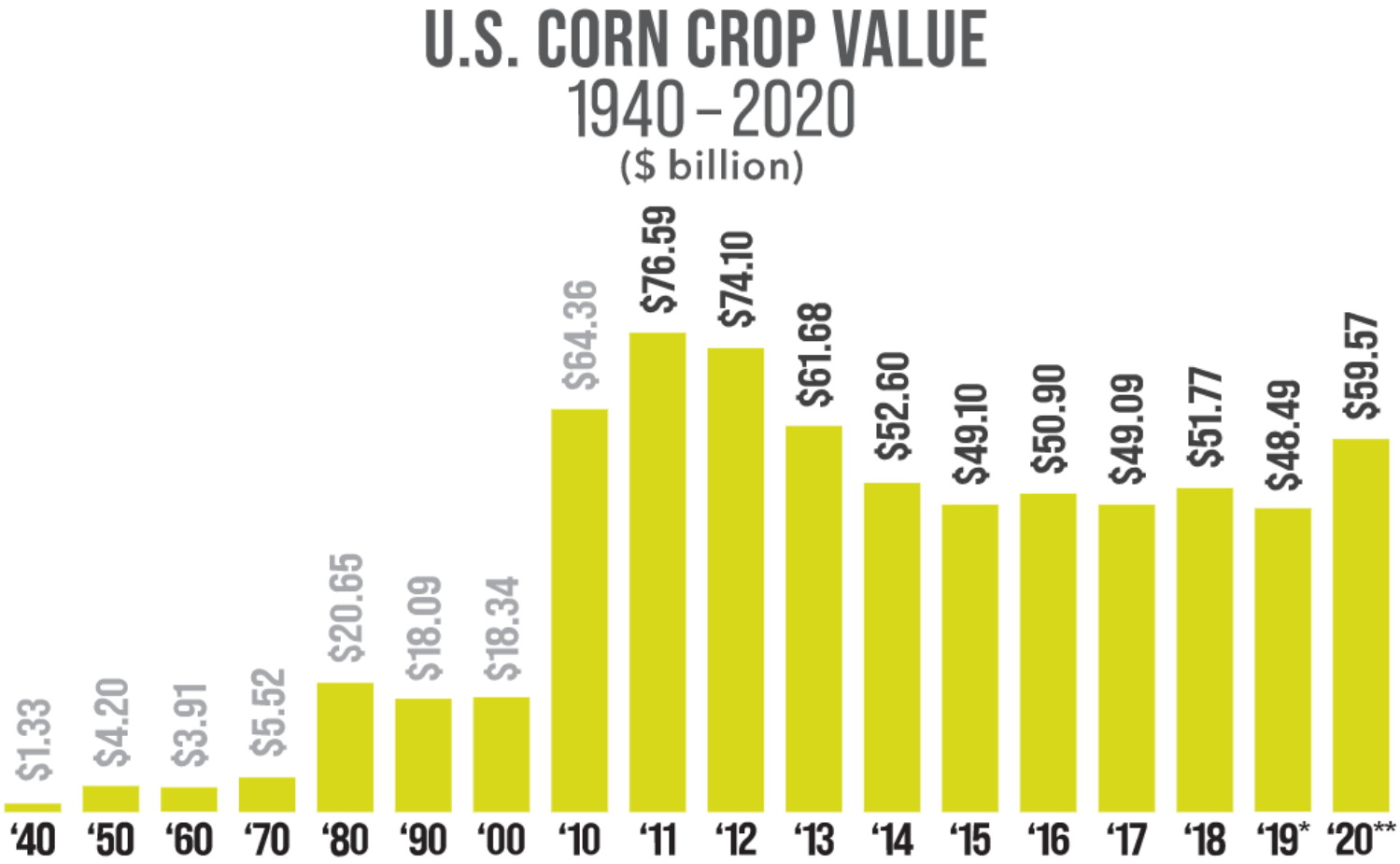

Table 2. Summary of Released Maize Genomes (Liu et al., 2020)

Genome	Assembly size (Mb)	No. of scaffolds	Scaffold N50	Contig N50	No. of gene models
B73	2106	625	9.56 Mb	1.18 Mb	39 324
Mo17 (CAU)	2183	2560	10.2 Mb	1.48 Mb	38 620
W22	2134	306	35.52 Mb	72.4 kb	40 789
SK	2161	708	73.24 Mb	15.78 Mb	43 271
PH207	2102	127 488	654 kb	5.3 kb	37 613
Mo17 (Yan)	2042	48 268	3.00 Mb	60.5 kb	40 003
mexicana	1204	107 418	108 kb	26.6 kb	31 387
HZS	2209	12	223.93 Mb	78.2 kb	40 893

Table 3. SNP/INDEL Markers Developed for the *Su1* locus (Chhabra et al., 2019)

No.	Primer	Sequence (5'-3')	Amplicon size	Annealing temp. (C)
1	SuDel6-FR	F-GCCATTCCCAACCATAGAAA R-GAGCTGGGCTTTTCAATCAA	146 bp in wild type, 140 bp in mutants	60
2	SuDel36-FR	F-ACCACCCACACCGTACAAAT R-GGACGTGGGCTTTTTCTGT	415 bp in wild type, 379 bp in mutants	60
3	SNP-2703	F1-GGTTCGATGGCGTCTT**C** F2-GGTTCGATGGCGTCTT**G** R-CCCGTCACCTTAGCGTAT	85 bp in both wild type and mutants	52
4	SNP2703-CG-85/89	F1-TACAGGTTCGATGGCGTCTT**C** F2-GGTTCGATGGCGTCTT**G** R-CCCGTCACCTTAGCGTAT	89 bp in wild type, 85 bp in mutants	51.1

Table 4. Primers for High Amylose sbeI and sbeII mutations (Chen et al., 2013)

Gene	Primer (5'-3')	NCBI Accession
Sbe I	F-ATGCTGTGCCTCGTGTCGCC R-GGCTTCATTTGGTATCTTGATCG	NM_001111900
Sbe IIa	F-GGACTTGCCGTCGGTGCTCT R-CTATTCGTCCTCTGCACCT	ZMU65948
Sbe IIb	F-ATGGCGTTCCGGGTTTCTGG R-TCACTCCACTGGAGCATAGACG	AF072725
Waxy 1	F-ATGGCGGCTCTGGCCACGTC R-TCAGGGCGCGGCCACGTT	NM_001111531
Sbe I FM	F-GTACACATTTAAGCATCCTC R-ATCATGGCTCTCAGCATATG	
Sbe IIb FM	F-CTGGATCAAGTACTCAGTGC R-TGCACTGCATTGTATCCAAGT	

Table 5. Positionally Cloned Genes for Quantitative Traits in Maize (Liu et al., 2020)

Gene	Phenotype	Functional annotation	Causal variation
ZmRap2.7	Flowering time	AP2-like transcription factor	*MITE* TE~70 kb upstream of this gene
ZmCCT10	Flowering time Disease resistance	CCT transcription factor	*CACTA*-like TE ~2.5 kb upstream of this gene
ZmCCT9	Flowering time	CCT transcription factor	Harbinger-like TE ~57 kb upstream of this gene
ZmCLA4	Leaf angle	Ortholog of *LAZY1* in rice	–
ZmRAVL1	Leaf angle	B3 domain–containing protein	2-bp InDel ~9.5 kb upstream of this gene
brd1	Leaf angle	Brassinosteroid C-6 oxidase	–
ZmGA3ox2	Plant height	GA3 β-hydroxylase	Two small InDels in the promoter
qph1	Plant height	ABC transporter	A mis-sense SNP
UB3	Kernel row number	SBP-box transcription factor	1.2-kb transposon-containing insertion located ~60 kb downstream of this gene
ids1/Ts6	Kernel row number	AP2 transcription factor	–
ZmBAM1d	Kernel size and weight	CLV1/BAM-related receptor kinase-like protein	–
DGAT1-2	Oil content and composition	Diacylglycerol acyltransferase	Extra phenylalanine insertion
Zmfatb	Palmitic acid	Acyl-ACP thioesterase	11-bp InDel in the sixth exon
ZmPORB2	Tocopherol concentration	Protochlorophyllide oxidoreductase	A small InDel in 5’ UTR
ZmAuxRP1	Disease resistance	Domain of unknown function 966	–
ZmPLA1/MTL /NLD	Haploid induction rate	Phospholipase A	A 4-bp insertion led to a frame shift
ZmDMP	Haploid induction rate	DUF679 domain membrane protein	One single-nucleotide change

Table 6. Candidate Genes for Functional Marker Development in Crop Species (Salgotra and Stewart, 2020)

Trait	Gene(s)	Chromosomal Location	Sequence	Crop
Agronomic traits				
Semi-dwarf stature	*Rht-B1 and Rht-D1*	4B, 4D	F-TCTCCTCCCTCCCCACCCCAAC R-CCATGGCCATCTCGAGCTGC & F-CGCGCAATTATTGGCCAGAGATAG R-CCCCATGGCCATCTCGAGCTGCTA	Wheat
Grain weight	*TaGW2*	6	F-ATGGGGAACAGAATAGGAGGGAGGA R-CGAGTATGCCTAGAATGGAAAGAC	Wheat
Photoperiod response	*Phd-H1*	2	F-ACGCCTCCCACTACACTG R-CACTGGTGGTAGCTGAGATT	Wheat
Plant stature	*tb1*	1	F-CACATGAGCCCATGCCTCTC R-AAAGCGGTAAGTCCATGGGG	Maize
Plant height	*Dwarf8*	1	F-ACACTATCACCGCTCTATTG R-ACTCTTTCCCTGACTTCATT	Maize
Quality Traits				
Forage quality for digestibility	*Bm3*	4	F-TTCAACAAGGCGTACGGGAT R-AGTGGTTCTTCATGCCCTCG	Maize
Sweetness	*sugary1*	4	F-TCCCGACTTCAGAACGGTTG R-ACAACAGAGCAACCCCAACA	Maize
Yellow pigment content	*Psy1*	7A	F-ACATGCCGCTACTCCTATCC R-GTAGAGTGGCCAGACAAGGT	Wheat
Fragrance	*badh2*	8	F-AGTTATGGTCTGGCTGGTGC R-TTGTGTGCTACCCACCCTTC	Rice
Oil content	*DGAT1-2*	6	F-TGGCTCTGCAATCAGGAGAA R-TGAAGCAGCAAACAACGAGC	Maize
Intermediate amylose content	*Wx-in*	6	F-CAGCGTCGACGTAAGCCTAT R-CAGGCCCCTGAAATCCATGT	Rice
β-Carotene & Flesh	*QA/QC*	3	F-AGTGCGGGACAAGATGATCA R-TCCCGAACATCTGAGCAAGT	Sweet potato
Drought tolerance	*MYBE1*	5	F-GGTACCCTGTCAAGGTTCGG R-AATTACTGGCCCCAGGTTCG	Maize
Photoperiod response	*Phd-H1*	2H	F-GTTGAGATCGACAGTCCCCA R-GGGCTCCTATCTCCAACTCC	Barley

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