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NEW TECHNOLOGIES FOR IMPROVING MAIZE BREEDING

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Abstract

Limited sources of germplasm used in maize breeding programs, increased the importance of prebreeding activities. Advances in technologies have allowed other methods to add efficiencies to modern commercial maize breeding. These include the use of Doubled haploids, genomics, molecular markers and transformation. Doubled haploids method creates completely homozygous inbreds in 1-2 years versus 7 in traditional breeding. Molecular markers serve as a starting point for genes and have a significant role in selection. MAS is the use of markers to identify the presence of a specific gene or combination of genes that carry a desirable trait, which allows direct use of the inbred to create specific combinations and more rapid trait improvements can be made. Genomics helps scientists to identify which genes determine important traits, and how genes interact with each other. The complete DNA sequence of the maize genome, along with more comprehensive transcriptome, proteome and metabolome information, help to further unravel the complexities of how genes and gene networks function to produce productive maize plants. Genetic improvement is resulting in improved agronomic, disease, and/or end use traits. Application of all new technologies will help breeders to achieve greater harvestable yield and product development systems.

Key words: maize, prebreeding, molecular breeding, genomics

Introduction

Maize is an important crop for food, feed, forage, and fuel across tropical and temperate areas of the world. It is also a classical genetic model for plant research. It has a number of characteristics that are favorable for an experimental model for crop plants: a multiple purpose crop with worldwide cultivation;<59 000 and 42 000–56 000 genes with moderate genome size (~2400Mb of DNA per haploid nucleus in the B73 inbred), which is approximately six times larger than rice and six times smaller than wheat, although a large proportion of the genome represented by repetitive elements; outbreeding reproduction system with tolerance of inbreeding: existence of multiple breeding products (inbreds, hybrids, synthetic cultivars, open-pollinated varieties improved landraces), and wide adaptability including good sources of resistance to environment stresses. The maize genome harbors tremendous molecular diversity that mirrors its substantial phenotypic variability. When considering nucleotide polymorphism in genes, two maize lines are on average as diverged from one another as humans are from chimpanzees (Buckler et al, 2006).

The conventional breeding is the mainstay of inbred and hybrid development. It is based on the theory of heterosis. Advances in technologies have allowed other methods to add efficiencies to modern commercial maize breeding (Babic et al 2011). These include the use of doubled haploids, genomics, molecular markers and transformation. These technologies have allowed scientists to make more informed decisions around specific genetic combinations to improve genetic gain, and allowed for more rapid identification of lines carrying a particular trait of interest. Utilization of conventional breeding coupled with new techlologies has led to the rapid increase in yield gain in maize.

Doubled Haploid (DH) lines are routinely applied in much commercial hybrid maize breeding programs. Doubled haploids are created by a special genetic process and have one set of chromosomes. They undergo chromosome doubling through a chemical process that produces a completely homozygous, fertile doubled haploid plant. Conventional inbred development, it takes seven generations to do this, and the plants are still not 100 percent pure. The purity and genetic uniformity of doubled haploid lines make it easier to measure characteristics and reduce product development time.

Considering the progress in the various "omics'' areas and the integration of different disciplinary applications facilitated by bioinformatics, as well as high-throughput genotyping approaches combined with automation, MAS will gradually evolve into more holistic "genomics-assisted" breeding strategies. Since genomics resources in maize are among the best of the major crop species, the role of genomics is set to become more and more important in maize breeding. However, conventional selection will remain a vital element of the process to finally confirm the best candidate genotypes for progression into the advanced stages of crop improvement and cultivar selection. MAS will no replace conventional breeding programs, but rather will increase reliance on genomics data alongside other technology interventions in an ever evolving and refining breeding system. Genomics-assisted breeding systems will be evaluated in terms of their ability to increase the scope of breeding goals, to provide new added-value traits, to decrease the cost of breeding programs, and to improve the pace of developing new cultivars, and finally to enhance impact of resultant products to command increasing areas of production.

Prebreding

Prebreeding concept represent a link between genetic resources and breeding activities, aimed to develop germplasm directly or indirectly usable for creation of new cultivars (Nass and Paterniani, 2000). Prebreeding programs can develop new base populations and also help in heterotic patterns identification, necessary for maize breeding. An additional advantage is the establishment of core collections. Maize Research Institute (MRI) Zemun Polje has a genbank which is among the ten largest in the world (FAO, 2010). It conserves collection of 2217 landraces from the former Yugoslav territories and 3258 introduced genotypes from 40 countries (Andjelković and Ignjatović-Micić, 2012). Local landraces are not used directly in breeding, rather for the creation of synthetic populations or for the formation of core collections. In the last decade different research programs had aim to identify superior genotypes within accessions at MRI gene bank. In order to identify cytoplasm types sources of CMS were screened using PCR assay with specific primers for C, T and S cytoplasm (Vančetović et al., 2010). A set of 54 landraces representing 18 agroecological groups from MRI collection was analysed for grain quality components (Mladenovic Drinic et al., 2011). Project of identification of new sources of drought tolerance among more than 6000 samples (gene bank accessions and elite inbred lines) started in 2008. All samples were grown under controlled drought stress in Egypt (Babic et al., 2011). A total of 672 accessions were chosen for further testing in temperate climate. After testing of general combining ability and heterotic pattern 41 genotype is selected for core collection formation. Among them, six accessions produced superior crosses with three lines representing different heterotic groups (Andjelković and Ignjatović-Micić, 2012). Within current four year project the whole collection will be analysed for grain quality. Genotypes with the highest macro and micro nutrient content will be tested in the field conditions. Identified genotypes with high grain quality and/or drought tolerance will be genotyped using SNPs in order to identify alleles for specific target traits which would be used in breeding programs and improve their efficiency.

Double haploids

The most common method of inbred line development in conventional maize breeding is based on pedigree method, but recently a new method of breeding that creates inbred lines, doubled haploids was developed. Two key differences between the Pedigree and Doubled haploid (DH) methods are that DH inbreds are completely homozygous and developed in 1-2 years versus 6-8 generation to derive lines with 99% homozygosity by convetnional breeding. Use of doubled haploid (DH) lines produced by in vivo induction of maternal haploids are routinely used in maize breeding. To induce maternal haploids, the donor plant is pollinated by a specific maize stock (line, single cross, or population) called inducer. The first recognized inducer line was the genetic strain Stock 6, with an haploid induction rate of up to 2.3% (Coe, 1959), which was subsequently improved by hybridization and further selection. Today, modern haploid inducing lines display high induction rates of 8 to 10% (Geiger & Gordillo, 2009). Beside regular F1 kernels, the pollination results in a certain proportion of kernels with a haploid maternal embryo and a regular triploid endosperm. Such kernels display a normal germination rate and lead to viable haploid seedlings. After artificial chromosome doubling the successfully treated seedlings are selfed leading to completely homozygous and homogeneous progenies (DH lines).

A key issue to apply the in vivo haploid induction approach on a commercial scale is an efficient screening system allowing the breeder to differentiate between kernels or seedlings generated by haploid induction and those resulting from regular fertilization. Haploid embryos can be selected based on morphological and physiological markers. The most efficient haploid identification marker is the ‘red crown’ or ‘navajo’ kernel trait encoded by the dominant mutant allele R1-nj of the ‘red color’ gene R1, which causes deep pigmentation of the aleurone layer in the crown region and scutellum (Geiger & Gordillo, 2009). In a haploid inducing cross, the marker should be homozygous recessive in the female parent and homozygous dominant in the pollinator inducer line. After pollination, kernels with a red aleurone crown containing a nonpigmented scutellum are visually selected from the hybrid kernel of regular fertilization with both aleurone and scutellum pigmented. Another cheap and fast haploid identification method was suggested by Rotarenko *et al* (2007), who observed that kernels with a haploid embryo have a significantly lower oil concentration than those with a diploid F1 embryo. This is due to the reduced size of haploid embryos when compared with diploid embryos. Inducers with an above-average oil concentration should be best suited for this approach. Major advantages of DH lines in hybrid breeding are: maximum genetic variance between lines for per se and testcross performance from the first generation; complete homozygosity; acceleration of inbred line development; perfect fulfillment of DUS criteria reduced expenses for selfing and maintenance breeding; greater efficiency and precision of selection especially when used in combination with molecular markers, and increased efficiency in MAS, gene introgression, and stacking genes in lines. Because DH technology offers a faster way to obtain completely homozygous lines, it can save significant time and resources for implementing genetic studies: establishment of DH mapping populations, improve the precision of genetic and mapping studies, analysis of linkage disequilibrium, analysis of haplotype/trait associations, accelerate gene pyramiding, evaluation, and conservation of genetic resources, extraction of individual gametes from heterozygous materials transforming them into DH lines.

Applications of molecular markers in maize breeding

Molecular markers have a significant role in selection. They serve as a starting point for genes that are responsible for the phenotypic response. Different kinds of molecular markers exist, such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs) markers, amplified fragment length polymorphisms (AFLPs), microsatellites and single nucleotide polymorphisms (SNPs). They may differ in a variety of ways; such as their technical acquirements; the amount of time, money and labor needed; the number of genetic markers that can be detected throughout the genome; and the amount of genetic variation found at each marker in a given population. The information provided to the breeder by the markers varies depending on the type of marker system used. Each has its advantages and disadvantages and, in the future, other systems are likely to be developed.

In the past 25 years, maize breeders have tried different ways of using molecular markers to improve grain yield and other agronomic traits. Molecular markers have been extensively used in maize genetic diversity studies for: analysis of genotype frequencies for identification of deviations at individual loci and for characterization of molecular variation within or between populations, construction of “phylogenetic” trees and determination of heterotic groups (Drinic et al., 2011) and, analysis of correlation between genetic distance and hybrid performance, heterosis and specific combining ability (Srdic et al 2011, Drinic et al, 2012).

The development of single nucleotide polymorphism (SNP) markers in maize offers the opportunity to utilize DNA markers in many new areas of population genetics, gene discovery, plant breeding and germplasm identification. Among all types of markers, SNP markers are increasingly the marker-of-choice for all genomics applications in maize breeding. SNP markers use in various areas of molecular genetics and plant breeding, including gene/QTL mapping, linkage-disequilibrium-based association mapping, map-based gene/QTL cloning, germplasm characterization, genetic diagnostics, event characterization, marker-assisted trait introgression, and finally marker-assisted selection (MAS). In order to conduct most of the above-mentioned SNP applications, researcher must know the order of the markers on chromosomes, which can be obtained by constructing recombination-based genetic linkage maps. SNP discovery has been performed on over 3,000 genes, with genetic mapping data on over 1,100 SNP markers being collected on Nested Association Maps using diverse maize inbred lines (Yu et al. 2008). Compared to other types of molecular markers, SNP markers have several advantages, including high abundance and even distribution through the genome. In addition, SNP markers provide highly reproducible codominant information, and there is an increasing range of cost effective high-throughput SNP genotyping systems. Most of the public SNP markers in maize are developed from B73 and Mo17 cultivars. Taking into account massive intraspecific variations among maize inbred lines (Springer et al., 2009), SNPs developed from a few lines will capture only a small portion of all allelic variations happening between parents of a cross designed for a QTL study. Over 2000 SNP markers that were developed for SNP chip-based genotyping are being mapped using three RIL populations.

Molecular marker-facilitated mapping of genes underlying specific traits in maize was first reported by Stuber et al. (1987) followed by Edwards et al. (1992). Since then, more than 2000 QTL related to various traits of agronomic importance in maize, including yield, yield components, plant morphology and physiology, and biotic and abiotic stress responses have been reported (http://www.maizegdb.org). Genetic mapping has been developed through conventional linkage mapping and more recently through linkage disequilibrium-based association analyses. Genetic mapping in maize was first carried out using morphological markers generating a genetic map consisting of 62 morphological trait loci. The first generation of molecular marker maps in maize was constructed using RFLP, which were later

saturated with SSR and other types of PCR-based markers. Most recently, linkage mapping is being raised to a new level as maps are being developed with large numbers of SNP markers and/or candidate gene-based markers. Genetic mapping is carried out using segregating populations, including F2, backcross, recombinant inbred lines (RILs) or doubled haploids. In order to improve the resolution and extend the total map distance, the Maize Mapping Project developed RILs through several generations of intermating an F2 population derived from the single cross of the inbred lines B73 and Mo17. As a result, the resolution of the genetic map was improved significantly, consisting of abot 1000 RFLP and 1000 SSR markers.

Using the marker map, genes affecting traits of interest can then be detected by testing for statistical associations between marker variants and any trait of interest. These traits might be genetically controlled by one or a few genes. Alternatively, they could be genetically complex quantitative traits, involving many genes (i.e. so-called quantitative trait loci [QTL]) and environmental effects. Association mapping is important tool to identify genes responsible for quantitative variation of complex traits by examining the marker-trait associations that can be attributed to the strength of linkage disequilibrium between markers and functional polymorphisms across a set of diverse germplasm (Zhu et al. 2008). Association mapping in plants can be based on candidate genes or whole genome scanning. The latter has become increasingly applicable in maize due to the recent development of large numbers of SNP markers. As a new alternative to traditional linkage analysis, association mapping offers three advantages, increased mapping resolution, reduced research time, and greater allele number (Yu and Buckler, 2006).

Marker-assisted selection (MAS) as a process refers to the selection of superior genotypes using molecular markers. In contrast to phenotypic selection, MAS does not rely on environmental conditions because it detects the structural polymorphisms at molecular level, requires leaf tissue collected at seedling stage, which is very useful for traits that are expressed at later stages of development and which also helps to avoid adverse weather conditions that could kill the plant at adult stage, could be cheaper and less labor intensive, allows selection in off-season nurseries and has a potential to accelerate breeding process. The essential requirements for MAS in breeding are: marker(s) should co-segregate or be closely linked (1 cM or less is probably sufficient for MAS) with the desired trait; an efficient means of screening large populations for the molecular marker(s) should be available. At present, this means, relatively easy analysis based on PCR technology; the screening technique should have high reproducibility across laboratories, be economical to use and be user friendly. According to Collard and Mackill (2008) applications of MAS in plant breeding were grouped into four broad categories: (1) marker-assisted germplasm evaluation including pedigree verification, purity assessment, evaluation of genetic diversity, identification of heterotic patterns and event characterization; (2) marker-assisted trait introgression, (3) marker-assisted pyramiding of genes and (4) genomic selection (GS). Current MAS strategies fit the breeding programs for traits with high heritability and are governed by a single gene or one major QTL that explains large portion of the phenotypic variability. However, the application of MAS for breeding traits with complex genetics based on the interaction of multiple QTL with minor effects has been inefficient. In classical MAS projects researchers use molecular markers that show statistically significant association with a phenotype and are linked to major QTL. Because minor QTL have small effects on phenotype, they have not been applicable in MAS. In comparison to conventional marker-assisted selection, which utilizes only a subset of genetic markers associated with a trait to predict breeding values (BVs), genome-wide selection (GWS) improves prediction accuracies by incorporating all markers into a model simultaneously. This strategy avoids risks of missing quantitative trait loci (QTL) with small effects. Bernardo and Yu (2007) compared

genome wide selection with marker-assisted recurrent selection by evaluating doubled haploids for testcross performance in Cycle 0, followed by two cycles of selection based on markers. They found that across different numbers of quantitative trait loci (20, 40, and 100) and levels of heritability, the response to genome wide selection was 18 to 43% larger than the response to MARS. Guo et al (2013) evaluated the accuracy of prediction for three corn flowering traits days to silking, days to anthesis, and anthesis-silking interval with GWS based on cross-validation experiments using a large data set of 25 nested association mapping populations in maize. GWS via ridge regression-best linear unbiased prediction (RR-BLUP) gave significantly higher predictions compared to MAS utilizing composite interval mapping.

“Omica” tehnologies

Genomics is a relatively new field of study, and may be described as the science of the genetic material of a chromosome set. This tool helps scientists identify which genes determine important traits in maize, and how genes interact with each other. Functional genomics utilizes the vast wealth of data produced by genome sequencing projects to understand the gene functions, and their interactions. It is often referred to the study of the genes, their functions, interactions, and regulation to provide a biological function in an organism. There are four major types of high-throughput measurements that are commonly performed: genomic SNP analysis (i.e., the large-scale genotyping of single nucleotide polymorphisms), transcriptomic measurements (i.e., the measurement of all gene expression values in a cell or tissue type simultaneously), proteomic measurements (i.e., the identification of all proteins present in a cell or tissue type), and metabolomic measurements (i.e., the identification and quantification of all metabolites present in a cell or tissue type). Each of these four is distinct and offers a different perspective on the processes underlying disease initiation and progression as well as on ways of predicting, preventing, or treating disease. Transcriptomic measurements (often referred to as gene expression microarrays or "gene chips") are the oldest and most established of the high-throughput methodologies. The most common are commercially produced "oligonucleotide arrays", which have hundreds of thousands of small (25 bases) probes, between 11 and 20 per gene. RNA that has been extracted from cells is then hybridized to the chip, and the expression level of ~30,000 different mRNAs can be assessed simultaneously.

Maize genome sequencing

Gene sequencing technology is used to determine the nucleotide sequence of genes or regions of the genome important for trait improvement. This technology has also been used to create a dense genetic map that helps to locate a specific gene or trait, detect the amount of genetic diversity that exists for that specific gene or trait, and to develop DNA-based diagnostic markers for the trait for use in the breeding programs. Gene sequencing makes trait selection much more accurate, and help breeders to improve plant breeding and product development systems. This allows more rapid creation and testing of superior products by selecting the best possible traits for yield and disease resistance. The application of gene sequencing technology to native trait discovery and molecular breeding is in active area of use.

Sanger sequencing method dominated the industry for almost two decades and still considered the gold standard for sequencing, but its limitations, especially with respect to throughput and cost, necessitated high demand for new and improved technologies for sequencing genomes. These alternative technologies collectively termed as Next generation sequencing (NGS) technologies (Varshney et al., 2009).

A wide range of sequence level variation exists in maize including single nucleotide polymorphisms (SNPs), small insertions/deletions, presence/absence variation (PAV), and

copy number variation (CNV). The US-based consortium of researchers decoded the genome of an inbred line of maize B73, an important commercial crop variety. The 2.3-billion-base sequence includes more than 32,000 protein-coding genes spread across maize's 10 chromosomes. Transposable elements are the most abundant parts of the sequence, spanning almost 85% of the genome (Schnable et al. 2009). A maize variety from the Mexican highlands called Palomero were sequenced (Viella-Calzada,2009) and the Palomero genome is around 400 million nucleotides smaller and contains about 20% less repetitive DNA than B73. More than a dozen genes related to heavy-metal detoxification and environmental-stress tolerance that were conserved in B73 and Palomero were found, but that were absent from teosinte, suggesting that these genes were involved in the domestication process. Another team, from Cornell University in Ithaca, sequenced part of the gene-rich region of 27 maize varieties to map haplotypes. This 'HapMap' revealed thousands of genes around the centres of the chromosomes, where they were unlikely to be shuffled around during recombination. Recombination is necessary for plant breeders to unite favourable genes from different crop varieties in a single plant, so this could explain why farmers often need to cross-breed different inbred lines to produce the superior maize varieties. Schnable et al (2009) compared the genome structures of B73 with inbred line Mo17. They found hundreds of genes that appeared only once in one or other of the two genomes. This suggests that crossing the two varieties could produce hybrids containing a higher number of beneficial genes.

Most of the public SNP markers in maize are developed from B73 and Mo17 cultivars. Consequently, there is a big chance that majority of allelic variations, including the causative mutation between parents of this cross will be missing. In order to avoid this situation, resequencing of genomes of both parents and discovery of allelic variations in low and single copy regions could be implemented using NGS technologies coupled with genome complexity reduction techniques. Discovered cross-specific polymorphisms can later be converted into any modern SNP genotyping assay (Mammadov et al., 2010).

RNA based sequencing (RNA-seq) is a powerful approach for transcriptional analysis, assessing sequence variation, and identifying novel transcript sequences, particularly in large, complex, repetitive genomes such as maize. Hansey et al (2012) sequenced RNA from whole seedlings of 21 maize inbred lines representing diverse North American and exotic germplasm. *De novo* assembly of RNA-seq reads that did not map to the reference B73 genome sequence revealed 1,321 high confidence novel transcripts, of which, 564 loci were present in all 21 lines, including B73, and 757 loci were restricted to a subset of the lines. 145 of the novel *de novo* assembled loci were present in lines from only one of the two heterotic groups consistent with the hypothesis that, in addition to sequence polymorphisms and transcript abundance, transcript presence/absence variation is present and, thereby, may be a mechanism contributing to the genetic basis of heterosis.

The maize sequencing project and the constant progress in maize functional genomics are providing new genes and functional genomic DNA sequence information that are increasingly being integrated into the maize genetic map. A total of 25 908 markers have now been integrated into the fingerprinted BAC contig (FPC) map. This includes 1902 genetically mapped markers (SSRs, RFLPs, SNPs, and InDels) and 24 006 sequence-based markers (ESTs, BAC ends, and 40-bp overlapping oligonucleotide overgo probes) (Cone et al. 2009).

Maize transformation

In maize transformation has been extensively used for the development of new commercial pest and herbicide resistant cultivars but more recently also including more complex traits such as grain quality and drought tolerance. Transgenic maize has been cultivated commercially in the United States since 1996. Since then, GM maize production has

expanded to more than 51 million hectares (32%) worldwide. Two traits are expressed by today's GM maize cultivars: insect resistance and herbicide tolerance. More and more, cultivars are being grown that express both of these traits simultaneously (stacked genes). The commercial sector has made substantial progress with pest resistant maize through transformation with genes encoding for insecticidal crystal (Cry) proteins from Bacillus thuringiensis (Bt), which have been particularly successful in providing protection against several corn borers. *Bacillus thuringiensis* is a species of bacteria that produces proteins that are toxic to certain insects. There are a number of Cry toxins that are categorized by their spectrum of activity. For maize pests, primary Cry proteins are Cry1 and Cry2 for Lepidoptera and Cry3 proteins for Coleoptera. Maize can be genetically engineered to produce these specific Cry toxins. IMI (IR/IT) or clearfield (CL) maize was developed by tolerance selection to be resistant/tolerant to imidazolinone herbicides. LibertyLink maize is genetically engineered to allow over-the-top applications of glufosinate herbicide and Roundup Ready maize allows postemergence applications of Roundup and some other glyphosate-type products directly to maize. Naqvi et al. (2009) created elite inbred South African transgenic corn plants in which the levels of three vitamins were increased specifically in the endosperm through simultaneous modification of three separate metabolic pathways. The kernels of the transgenic white corn (Cv. M37W) were found to contain 169-fold the normal amount of carotene, 6-fold the normal amount of ascorbate, and double the normal amount of folate. SmartStax is genetically modified (GM) maize that has eight GM traits combined or 'stacked' together, six for insect resistance (Bt) and two for herbicide tolerance. The traits are combined together using crosses between existing transgenic corn lines rather than using genetic transformation of a single maize strain. While the adoption of first generation traits in maize has been rapid, the next generation, currently in development, holds even more promise. These traits are designed to help maize continue to grow under drought conditions, more efficiently use nitrogen, produce even higher yields, enhance protection against insects and other pests, and improve grain quality for food, animal feed and biofuels.

Conclusion

New molecular technology including molecular markers, genomics, gene sequencing, and transformation has been widely used in maize breeding. It covers many different applications that influence the understanding of gene function. Application of these technologies does not occur independently of conventional breeding, but in association with it. A number of breeding programs have in the past two decades to varying degrees started using markers to increase the effectiveness and to significantly shorten the development time of varieties and therefore maize geneticist consider molecular marker assisted selection a useful additional tool in breeding programs to make selection more efficient. The most significant breakthrough in agricultural biotechnology is coming from research into the structure of genomes and the genetic mechanisms behind economically important traits. The genomics provide information on the identity, location, impact and function of genes affecting important traits, cataloging and mapping single gene markers. The complete DNA sequence of the maize genome, along with more comprehensive transcriptome, proteome and metabolome information, will continue to drive innovations in molecular breeding and biotechnology. These additional layers of information help to further unravel the complexities of how genes and gene networks function to produce productive maize plants. This knowledge will lead to improved predictions and capabilities to assemble native gene variation through molecular breeding as well as more optimal gene selection and regulation in the development of future biotechnology products.

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