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Molecular Markers in a Commercial Breeding Program

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In the 1980s, DNA-based molecular markers were identified as having the potential to enhance corn (*Zea mays* L.) breeding. Research has demonstrated the advantage of using molecular markers for selection of simply inherited traits, however only a few studies have evaluated the potential to enhance genetic gain for quantitative traits. In the late 1990s, Monsanto decided to implement marker assisted selection for quantitative traits in our global plant breeding programs. We built genotyping systems and information tools and developed marker assisted methodologies that increased the mean performance in elite breeding populations.

DNA-based molecular markers were identified as having potential utility in corn breeding in the 1980s (Helentjaris et al., 1985; Paterson et al., 1988). The identification of restriction fragment length polymorphisms (RFLPs) (Botstein et al., 1980) created a new research discipline typically referred to as molecular breeding. The central dogma of molecular breeding involves the utilization of molecular marker fingerprints to improve selection efficiency in plant breeding programs.

Scientists have researched applications such as the characterization of genetic variation, molecular marker assisted backcrossing, quantitative trait mapping, and molecular marker assisted selection (Charcosset and Gallais, 2003; de Vienne and Causse, 2003; Hoisington and Melchinger, 2004; Frisch, 2004; Mohler and Singrun, 2004). Numerous quantitative trait loci (QTL) mapping studies have been published on a wide range of phenotypic traits (Lawrence et al., 2004, 2005, 2007). However, after 20 years of research there are a limited number of publications demonstrating results in plant breeding programs.

This article focuses on the application of molecular marker technologies to Monsanto's plant breeding programs with emphasis on selecting for quantitative traits.

The goals of this article are to

- define the major components needed to implement large-scale molecular marker assisted breeding methodologies;
- outline the structural changes in Monsanto's breeding programs to accommodate each of these components;
- provide empirical results from molecular marker assisted breeding methodologies for quantitative traits;
- outline one of Monsanto's methods to improve the precision in estimating QTL genetic locations and account for population structure in association mapping.

Key Components

As Monsanto moved from experimentation to commercial application of molecular marker assisted breeding methodologies, five major components needed modification to enable successful implementation. These components were

- breeding program structure
- molecular markers
- genotyping platform
- phenotypic information
- information technology (IT) systems

Abbreviations: IT, information technology; MARS, marker assisted recurrent selection; MTI, multiple trait index; PCR, polymerase chain reaction; QC, quality control; QTL, quantitative trait loci; RFLP, restriction fragment length polymorphisms; SNP, single nucleotide polymorphism; SSR, simple sequence repeat; TDT, transmission disequilibrium test.

Breeding Program Structure

Molecular marker information increases the complexity of a breeding program. In addition to the standard plant breeding procedures (such as seed processing, planting of summer nursery and yield trials, pollinating, collecting phenotypic data, harvesting, and data analysis), molecular marker assisted breeding methodologies require the analysis and interpretation of genotypic data, joint analysis of genotypic and phenotypic data, and decision making using molecular marker information all within the same limited timeframe that North America corn breeders deal with each fall. Marker assisted breeding programs have an approximate sevenfold increase in the amount of data and analysis that must be completed compared to conventional breeding programs. With accelerated marker assisted recurrent selection (MARS) schemes (Fig. 1), breeders make selection decisions three to four times per year instead of the typical one to two times per year. More complex decision-making on a larger information base combined with more frequent selection decisions requires a plant breeder to spend additional time on the front-end of the breeding process.

Monsanto's North America corn breeders also develop and help deploy unique and high performing products to multiple commercial channels. Commercial channels are different mechanisms to provide products to customers. These could include multiple national brands, regional brands, and genetic licensing models. The complexity associated with running a large-scale yield testing program combined with deployment of commercial products to multiple channels requires a plant breeder to spend additional time on the back-end of the breeding process.

Monsanto decided to restructure its North America corn breeding program to allow plant breeders to focus on either the front-end or back-end of the breeding process. Two groups were developed in the North America corn breeding program (Fig. 2). The front-end of the breeding process, called the Line Development Breeding group, has responsibility for developing new inbred lines using all available technologies and breeding methodologies. Line development breeders manage the process from developing new breeding populations through placement of hybrids in the first year company-wide yield trials. The back-end of the breeding process called the Commercial Breeding group has responsibility for development and commercialization of new commercial hybrids and management of all yield trials. Commercial breeders manage the process of advancing hybrids beginning with the first year company wide yield trials through commercial products. Commercial breeders also develop new hybrid combinations of elite performing inbred lines. The division of the breeding process allows a line development breeder to focus on implementation of new technologies that improve the plant breeding process, while the commercial breeders

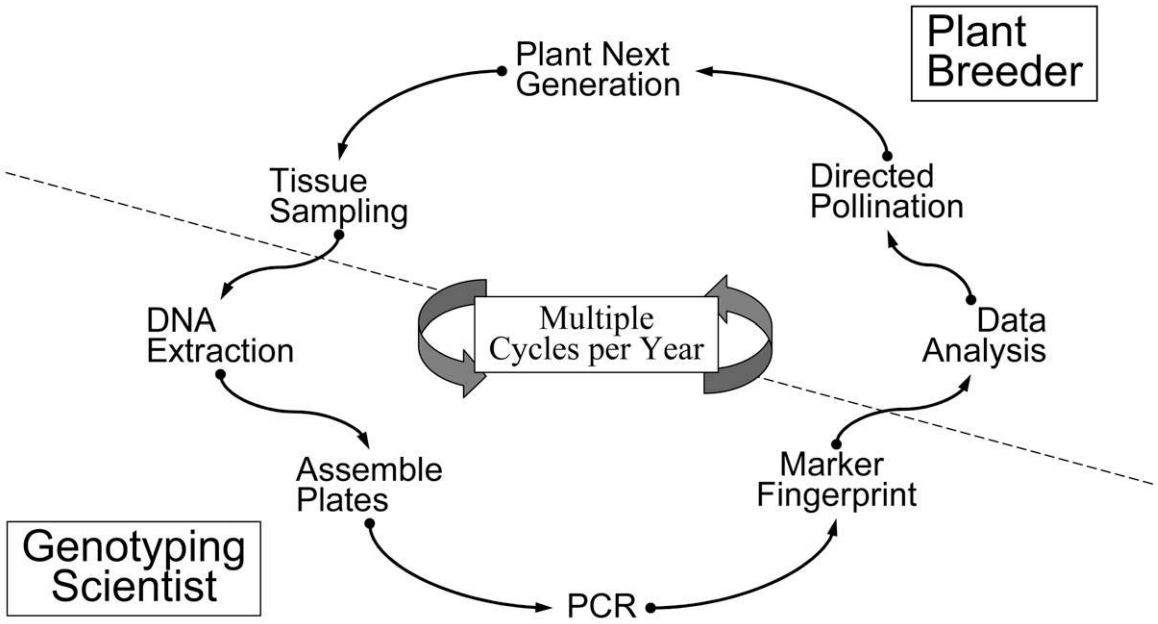

Figure 1. Marker assisted recurrent selection scheme.

can focus on running high quality yield trials, hybrid advancement, and product deployment.

Besides the line development and commercial breeding groups, two other key groups were defined. Upstream of the line development group is the breeding technology organization. This organization encompasses a number of teams that are responsible for generating molecular marker fingerprints, evaluating new technologies for plant breeding, statistical support, biotech trait integration, management of multiseason nursery programs, and plant pathology support. Technologies that pass proof-of-concept experiments flow from the breeding technology organization into the line development breeding group for large-scale implementation and optimization. On the back-end of the process a product deployment group works with the commercial breeders and the Monsanto commercial channels to optimally place products into each channel.

Molecular Markers and Genotyping Platform

With the advent of polymerase chain reaction (PCR), public institutions and commercial organizations switched to PCR-based molecular markers like simple sequence repeats (SSRs) (Akkaya et al., 1992). PCR-based molecular markers along with advancement in automation of molecular genotyping dramatically reduced the cost per molecular marker data point (defined as the genotype of one genetic sample revealed by one molecular marker). As genomic technologies improved, genotyping moved to single nucleotide polymorphism (SNP) markers. Unlike SSRs, SNP detection is not limited to gel- or capillary-based fragment size separation. With gel-free genotype detection systems like MALDI-TOF MS (Sequenom, San Diego, CA), TaqMan (Applied Biosystems, Foster City, CA), Invader (Third Wave Technologies, Madison, WI), SNPStream (Beckman-Coulter, Fullerton, CA), Pyrosequencing (Uppsala, Sweden), and Illumina (La Jolla, CA) additional automation of the process of molecular fingerprinting was possible (Jenkins and Gibson, 2002). Fully automated molecular marker fingerprinting systems from DNA extraction through allele calling of fluorescent DNA reads are possible.

To facilitate our large-scale implementation of molecular marker assisted breeding methodologies, we identified and developed assays for thousands of corn SNPs. A large percentage of these SNPs are in putative genes and all SNPs are integrated into a consensus linkage map based on multiple biparental mapping populations. Our consensus linkage map utilizes the intermated B73 × Mo17 population genetic material (Lee et al., 2002) to provide better genetic precision of individual SNP locations.

In 2000, Monsanto switched to SNP-based genotyping at our Ankeny, IA, facility with gel-free detection systems and a fully automated genotyping process. From 2000 to 2006, total molecular marker data point production grew over 40-fold, while cost per data point decreased over sixfold.

Phenotypic Information

The quality of marker phenotype associations is dependent on the quality of the phenotypic information. By focusing on the front-end of the breeding process, line development breeders are able to spend more time collecting high quality phenotypic information on their breeding populations. This information provides better phenotypic

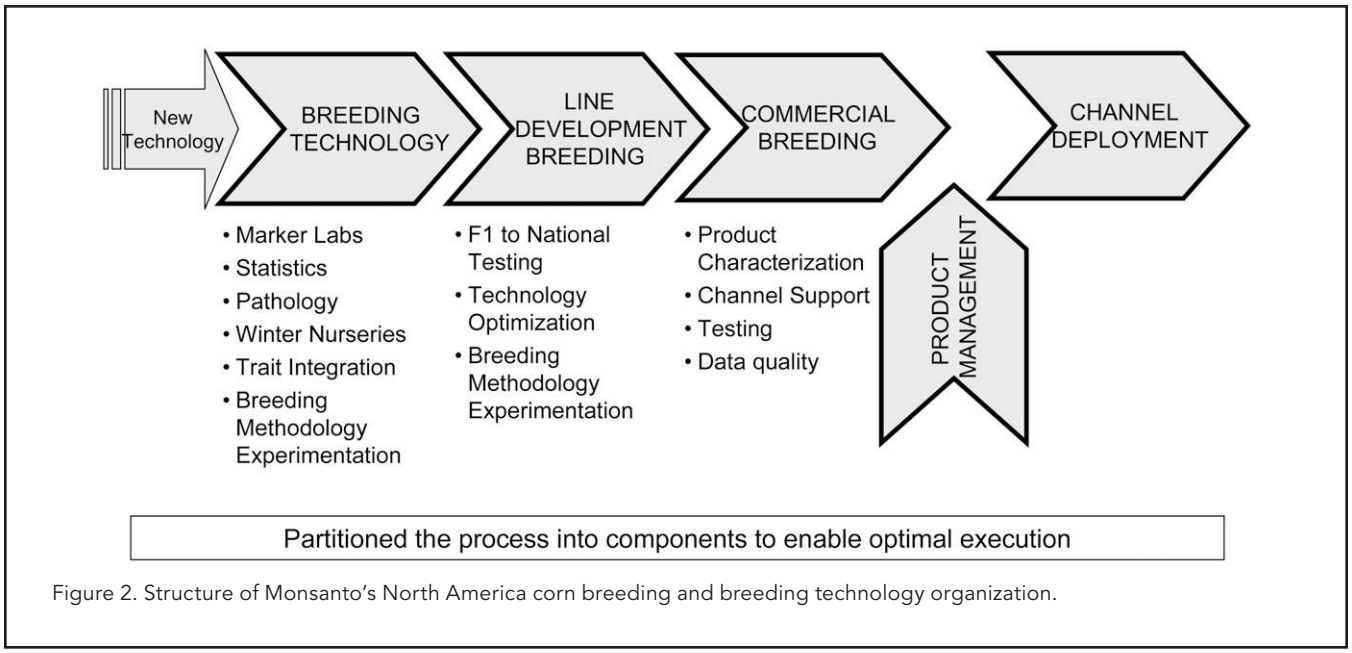

Figure 2. Structure of Monsanto's North America corn breeding and breeding technology organization.

characterization of breeding lines and is used in subsequent molecular marker assisted breeding methodologies. The commercial breeders are focused on the back-end of the breeding process and are able to handle the planting and harvesting of additional yield trial plots. Monsanto invested in the development of improved equipment for efficient yield testing programs. The combination of specialized breeders and equipment enabled an 80% increase in yield trial plot capacity in the last four to five years. These additional yield trial plots provide better characterization of the breeding pipeline and provide more phenotypic data for development of marker phenotype associations.

Information Technology Systems and Algorithms

A North American corn breeder deals with a lot of information (Crosbie et al., 2006). Breeders must manage their entire breeding pipeline including operations such as tracking pedigrees, designing and planting nursery and yield trials, collecting phenotypic information, analyzing data, and making selection decisions. The Monsanto family of seed companies had multiple plant breeding software systems. To replace these, we built a global all crop plant breeding system to handle all of Monsanto's plant breeding programs. This centralized database system allows the breeders to manage all aspects of their programs. It also enables access to genetic material inventory, pedigree information, and phenotypic data for all crops in all of our global breeding programs. The system enforces key requirements such as uniform pedigree nomenclature, trait definitions and rating scales, and data quality control (QC). Every plant in this system is uniquely identified and can be tracked from the day it was created.

A similar information system was developed for the molecular genotyping laboratories. This system tracks tissue samples throughout the genotyping process and assures that genotypic information is correctly linked to genetic material. Scientists can manage all aspects of the genotyping process including activities such as developing marker lists, scheduling projects for genotyping, managing molecular marker inventory, tracking the genotyping progress, running genotypic QC routines, and making marker scoring decisions. Every tissue sample is uniquely identified and is linked to our field breeding system.

After building phenotypic and genotypic transactional systems, we built an integrated molecular marker decision-making system. This system is Web based, which enables rapid methodology enhancement and access from any company computer system. This data analysis system enables breeders to submit populations for molecular marker assisted selection, track project status, develop genetic molecular marker models for selection, and make selection decisions. Breeder decisions are transferred back into the field IT system and/or the laboratory IT system to provide a seamless flow to the next stage of the breeding process.

Selection for Complex Traits

Quantitative traits such as grain yield are a major driver for the success of commercial products (Crosbie et al., 2006). Customers want products that combine favorable characteristics for a number of complex traits such as grain yield, grain moisture at harvest, standability, and test weight. These traits are primary breeding targets for corn breeding programs; therefore, molecular marker assisted breeding methodologies capable of improving selection efficiency for complex traits are desired. The core principle of molecular marker assisted selection follows the concept of correlated traits selection (Falconer, 1960). A methodology that combines both phenotypic and genotypic information

was described by Lande and Thompson (1990). The ability of molecular marker information to enhance selection relative to phenotypic selection was demonstrated in a few studies (Stuber and Edwards, 1986; Edwards and Johnson, 1994; Eathington et al., 1997; Johnson, 2001, 2003).

At Monsanto, we utilize both phenotypic and genotypic information through a proprietary methodology to develop a framework of knowledge that breeders use as a basis for genetic modeling in a breeding population. The breeder combines germplasm knowledge and breeding population objectives with molecular marker phenotypic trait association information to develop a molecular marker assisted multiple trait selection model for each breeding population. This selection model is utilized to rapidly increase the frequency of the molecular marker alleles associated with favorable phenotypic traits within the breeding population. Breeders may decide to drop a breeding population based on observed or predicted population metrics or can choose to run multiple selection models on an individual population.

After a breeder develops a selection model for a breeding population, the population is enhanced via marker assisted recurrent selection. During this process progeny from a given breeding population are fingerprinted with specific molecular markers to enable the calculation of a genotypic value for each progeny. Controlled pollinations are made within the pool of selected progeny to provide offspring for the next cycle of molecular marker assisted selection. With the use of continuous nursery programs and preflowering genotypic information, multiple cycles (three to four) of molecular marker assisted selection and controlled pollinations can be completed within one year. This scheme of MARS rapidly accumulates favorable molecular marker alleles linked to desired QTLs in the breeding population. The breeder can select different MARS schemes depending on the selection model and the desired genetic structure (inbreeding level, genetic drift, and favorable allele frequency accumulation) of the population after MARS. The MARS schemes are optimized for field and laboratory resource utilization, execution of the process, and accumulation of favorable allele frequency while minimizing genetic drift. By increasing the frequency of favorable alleles in a breeding population, the probability of recovering a genotype with the combination of desired alleles is increased. By changing the favorable allele frequency from 0.5 to 0.96 the probability of recovering the ideal genotype for 20 independent regions moves from one in a trillion to one in five. This change in allele frequency should result in a change in the mean performance of the population for the selected trait, which is typically a multiple trait index (MTI) that combines the values of multiple traits into a single index with weights on individual traits.

Data Summaries

The molecular marker assisted breeding methodology described in the previous section was applied to breeding populations by plant breeders. After one year of MARS, a set of lines were derived from the MARS population and evaluated against lines selected through conventional breeding schemes from the same population. The breeder made all decisions on the selection model, selection of lines, and derivation of the MARS lines. All seed was produced in a common nursery and yield tested in the same experiment to minimize confounding effects associated with seed source and testing environments. Mean performance of the conventionally selected lines was compared to the mean performance of the MARS lines. A MTI value was calculated for each of the MARS and conventionally selected lines using the MTI parameters (phenotypic traits and their respective weights) defined in the selection model that was built for the specific breeding population.

For North America and European corn breeding programs, the results were computed in each of 248 breeding populations and then averaged within the testing year (Table 1). The MTI value was adjusted to a parental mean value of zero. Three key points are apparent in the results. First, Monsanto breeding programs are making genetic gain in the early generations of selection. Second, the MARS-derived lines are higher performing compared to the conventionally selected lines. Finally, the amount of gain for both breeding methods varies across years.

The results of MARS in 43 soybean [*Glycine max* (L.) Merrill] breeding populations are presented in Table 2. Various selection schemes were used in the soybean breeding populations so results are presented as the average performance of the MARS lines minus the average performance of the conventionally selected lines for the key traits grain yield and relative maturity. The MARS lines showed a 37.6 kg ha^{-1} advantage with a slight delay in relative maturity.

Table 1. Comparison of multiple trait index (MTI) values following one year of marker assisted recurrent selection (MARS) (three cycles) and conventional selection (two cycles) in corn.

Year	No. of unique breeding populations	Multiple trait index†	
		Conventional selection	MARS
2002	79	0.63	1.10
2003	97	0.25	0.97
2004	72	0.76	1.62
All years	248	0.50	1.18

†Multiple trait index is scaled to the have the parental lines equal to zero. This index includes traits like grain yield, grain moisture, test weight, standability, etc.

Table 2. Comparison of phenotypic trait values following one year of marker assisted recurrent selection (MARS) (three cycles) and conventional selection (two cycles) in soybean, sunflower, and corn.

Crop	Geography	MARS minus conventional selection				
		Selection index	Relative maturity	Grain yield	Grain moisture	Kernel oil
			d	kg ha^{-1}	g kg^{-1}	%
Soybean	North America	–	0.06	37.6	–	–
Sunflower	Europe	–	–	10.0	-11.0	0.5
Corn	Brazil	1.47	–	287.2	0.10	–

The results of MARS in one European sunflower (*Helianthus annuus* L.) breeding population demonstrated improvement in grain yield, grain moisture at harvest, and percent oil in the MARS lines compared to conventionally selected lines (Table 2). Finally, in Monsanto's Brazilian corn breeding program, MARS lines outperformed conventional selected lines for selection index, grain yield, and grain moisture at harvest (Table 2).

To evaluate the impact of using different genetic models, 23 corn breeding populations from eight different breeding programs were selected for two different selection models (Table 3). Each population was selected using an MTI model, which averaged 3.5 traits and a grain yield model, which averaged 1.9 traits and had 62% more weight on grain yield compared to the MTI model. The populations went through MARS and a random sample of progeny from each selection model was evaluated. On average, the progeny selected with the grain yield model had higher grain yield levels compared to the progeny selected with the MTI model. However, correlated traits like grain moisture and test weight were controlled better in the MTI model compared to the grain yield model.

Information Database

By implementing the process of genetic mapping and MARS in our commercial plant breeding programs, we have assembled a very large database of marker phenotype associations. Since 2000, our association database has grown 50-fold. This database of information represents the core of the next wave of plant breeding methodologies. It will be possible to utilize this database of information in predictive breeding methodologies. With the development of these new methodologies, the enhanced selection efficiency that molecular markers have enabled for backcrossing, selection for simply inherited traits, and selection for complex traits can be applied to all stages of a plant breeding program.

One application of this association database is the prediction of progeny performance before phenotypic evaluation of these progeny. We evaluated this concept for hybrid grain moisture at harvest in four breeding populations. For each population, a selection model was built using information in the association database combined with the molecular marker fingerprints of the parental inbreds. Each parental inbred contributed genomic regions for both higher and lower hybrid grain moisture at harvest. A divergent MARS scheme was applied to the progeny of each population with selection for higher and lower hybrid grain moisture at harvest. A random set of 20 to 30 lines was derived from each of the divergent populations, crossed to one tester of the opposite heterotic pattern, and evaluated at multiple locations. All four populations showed response to selection. All populations selected to have lower grain moisture at harvest had lower grain moisture at harvest compared to the populations selected for higher grain moisture at harvest. The lines per se also showed a directional change in grain moisture at harvest that matched the hybrid response. Overall the hybrids had a change in grain moisture at harvest of 2.5 percentage points, while the lines changed 3.9 percentage points. The divergent populations had changes in phenotypic traits such as growing degree units to flowering and silking and husk characteristics.

Key Learnings

Molecular marker information represents another tool in the plant breeding toolbox. This tool is most effective when it is combined with the breeder's germplasm knowledge and breeding population objectives. It is important for breeders to perform phenotypic selection on the lines per se that are going to be utilized in a MARS scheme. In addition, breeders need to continue phenotypic evaluation and selection among and within derived lines after MARS.

While building genetic models for MARS schemes, breeders have to switch from selecting on observed phenotypic information to selecting toward a desired phenotype. Understanding how to interpret marker based phenotypic predictors and correlated trait response is important in determining the potential success in each breeding population.

Genetic Resolution

A biparental F_2 population has the maximum amount of linkage disequilibrium. This genetic structure was important in the initial QTL mapping studies since the cost of molecular marker fingerprinting was relatively high. Therefore, a limited number of molecular markers could be used in the mapping study. However, a disadvantage of a biparental F_2 population structure is the inability to localize the position of a detected QTL (Kearsey and Farquhar, 1998). This lack of precision impacts molecular marker assisted selection and hinders the ability to resolve tightly linked QTLs from pleiotropic effects. Fine mapping of QTL position can be categorized into mathematical,

recombinational, and substitution mapping approaches (Paterson, 1998). The recombinational method can be subcategorized into procedures that generate recombinations for the purpose of fine mapping and procedures that try to utilize historical recombinations (Darvasi and Soller, 1995; Xiong and Guo, 1997).

Random mating is an effective method of generating genome wide recombinations. Random mating of the Illinois high oil (C70) and Illinois low oil (C70) was done for 10 generations followed by derivation of random S2 lines to create a mapping population with a relatively low level of linkage disequilibrium (Laurie et al., 2004). The genetic resolution was estimated to be on the order of 2 to 3 cM based on marker to marker linkage disequilibrium estimates. This resolution combined with high density genotyping, which is now possible with thousands of SNP assays and automated genotyping procedures, enabled a detailed mapping of QTLs for percent grain oil. Increased genetic resolution helps narrow the list of possible candidate genes in a region associated with phenotypic variation.

There is a lot of interest in utilizing historical recombinations for fine mapping. Researchers might sample historical recombinations that are present in germplasm collections or utilize genetic material that is derived in a plant breeding program. Monsanto has a large collection of inbred lines that were derived in our plant breeding programs that could be used for an association study with improved genetic resolution.

It is important to understand the nature of the linkage disequilibrium in the set of genetic material that may be used for an association study. Linkage disequilibrium, which more appropriately is called gametic disequilibrium, can be caused by factors other than linkage. Spurious associations in a population of germplasm can be due to linkage disequilibrium between unlinked genomic regions and between genomic regions on different chromosomes. This concept is demonstrated in an example using elite Monsanto soybean lines.

Table 3. Effect of one year (three cycles) of marker assisted recurrent selection in 23 corn populations for two different selection models.

Type of selection model	MARS minus original lines (C0)†			
	MTI‡	Grain yield	Grain moisture	Test weight
		kg ha^{-1}	g kg^{-1}	kg m^{-3}
MTI	0.73	105.4	0.10	1.03
Grain yield	–0.15	264.0	3.90	–3.86

†MARS, marker assisted recurrent selection.

‡Multiple trait index (MTI) model was applied to both MARS populations.

A total of 750 soybean lines were genotyped with hundreds of SNPs. Approximately, half of these lines were Roundup Ready® soybeans, which are resistant to glyphosate herbicides such as Roundup® agricultural herbicides. The lines were classified into resistant and susceptible categories based on their phenotypic reaction to glyphosate herbicide. Using a standard association analysis, 49 molecular markers were significantly ($P < 1 \times 10^{-9}$) associated with the phenotypic reaction to the application of glyphosate herbicide (Fig. 3). A significant association was identified on 15 different chromosomes. Through de novo genetic mapping studies, segregation analysis, and sequence analysis, the location of the transgenic event 40-3-2 (Padgette et al., 1995) is known to be on linkage group D1b (U19) of the USDA genetic map of soybean (Cregan et al., 1999) and is a single insert. Therefore, nearly all of these 49 molecular marker associations are false positives due to the linkage disequilibrium structure in this set of

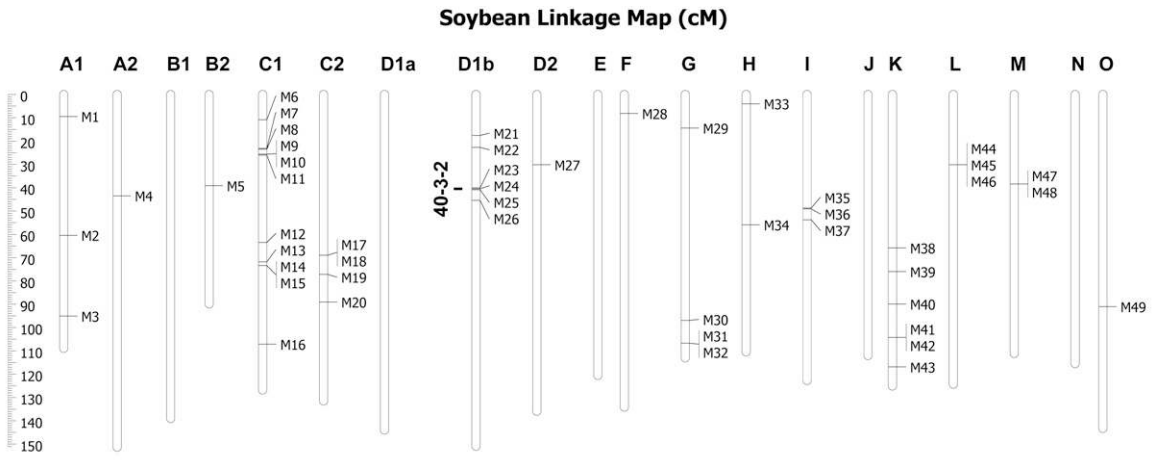

Figure 3. Association analysis for the soybean transgene 40-3-2 that provides resistance to glyphosate herbicide. Markers on the right side of each chromosome are single nucleotide polymorphism (SNP) markers significantly associated ($P < 1 \times 10^{-9}$) with the herbicide resistance trait.

elite soybean lines. A proprietary data analysis method to account for this population structure was applied to this data set, which resulted in identification of the only significant ($P < 1 \times 10^{-9}$) genomic region containing the 40-3-2 transgene on linkage group D1b.

Population structure in an association study can be handled in a number of ways. A method developed by Pritchard et al, (2000a) and Thornsberry et al. (2001) utilizes molecular marker information to define the population structure and account for this structure in the analysis. A publicly available program called *structure* (Falush et al., 2003; Pritchard et al., 2000b) was developed to analyze association studies (Thornsberry et al., 2001). Another method is to remove the linkage disequilibrium at unlinked loci by one generation of meiosis. The transmission disequilibrium test (TDT) is a family-based methodology to remove linkage disequilibrium at unlinked loci (Spielman et al., 1993). In a TDT, only progeny derived from a heterozygous individual are used in the association analysis.

After evaluation of the linkage disequilibrium structure in Monsanto's elite corn germplasm, we decided to utilize a TDT scheme to remove significant linkage disequilibrium among unlinked loci. A TDT scheme can be applied to a collection of inbred lines by generating random F_1s among a set of selected inbred lines and deriving random progeny from each F_1. The parental lines or the F_1 generation along with the progeny are genotyped at a set of molecular markers. Phenotypic information is collected on the random progeny. The TDT analysis is performed with each molecular marker using only progeny derived from a heterozygous F_1.

Summary

The first DNA-based molecular markers were identified in corn more than 20 years ago. Since then researchers identified numerous applications and demonstrated the utility of these applications. At Monsanto, we implemented large-scale molecular marker assisted breeding methodologies in our plant breeding programs. Today, molecular marker assisted breeding is becoming our conventional breeding process. We built the necessary systems including the organization of our breeding program to facilitate implementation of these new breeding methodologies. Controlled experimentation was conducted on hundreds of breeding populations across crops, years, world regions, and many individual plant breeding programs. These experiments showed that molecular marker assisted breeding methodologies increased the mean performance of progeny compared to our conventional breeding methodologies. As a final confirmation, Monsanto has commercial products derived from MARS methodologies in multiple crops.

In the past, plant breeding information databases contained knowledge of pedigrees, phenotypic performance, and general and specific combining ability. Today, plant breeding information databases also contain knowledge of molecular marker fingerprints and marker phenotype associations that will drive the next wave of predictive breeding methodologies.

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