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A Look at Product Development with Genetically Modified Crops: Examples from Maize

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ABSTRACT: Plant breeding for crop genetic improvement involves the cycle of creating genetic diversity and exploiting that diversity to derive an improved cultivar with outstanding performance for specific traits of interest. Genetic modification through transformation essentially expands the genepool to facilitate access to genes otherwise not available through crossing. Transgenic events are defined by the DNA sequence that has been incorporated into the target genome and the specific point(s) of insertion. In the development of a new transgenic trait, typically many events are generated and evaluated with the aim of identifying one exhibiting consistent trait expression at or above specified thresholds, stable inheritance, and the absence of any negative effects. With transgenic traits for maize, once commercial candidates have been identified, these events are introgressed into elite lines, often through the use of molecular markers that can accelerate the breeding process and aid in producing a quality conversion. Converted elite lines are yield-tested to ensure performance equivalency with their unconverted counterparts. Finally, before commercial sale of seed, quality control monitoring is conducted to ensure event identity and purity and the absence of any unintended events. This monitoring complements other quality control measures to confirm seed viability and line/hybrid purity and uniformity in seed treatments, all in an effort to ensure customer satisfaction and to comply with governmental regulations. Thus, genetically modified (GM) cultivars are subject to significant testing and auditing prior to seed sale and distribution to farmers, more testing and auditing than with non-GM cultivars.

KEYWORDS: *biotech trait development, genetically modified, plant breeding, maize, transgenic event, trait introgression*

INTRODUCTION

Plant breeding is the science of applying genetic principles to improve plants for human use. It affects the life of every individual on the planet because it involves the creation and manipulation of economically important traits in plants used for food, animal feed, fuel, fiber for clothing and wood products, and landscape aesthetics. Plant breeding has been enormously successful. Case in point: in the United States in the 1930s and the decades prior, the average yield of maize was approximately 30 bushels per acre. By 2004, average maize yields in the United States exceeded 150 bushels per acre,[1] representing a 5-fold increase in the course of about 70 years! Whereas a proportion of this increase is attributable to use of fertilizers, herbicides, mechanization, and improved agricultural practices, the majority of the increase is due to plant breeding.

The plant breeding community as a whole has been working toward doubling crop yields by 2050[2] to feed a growing global population expected to be at 9 billion by then. Although the world population grows by approximately 73 million people per year and demand for grain for livestock feed escalates as meat consumption in developing countries increases, no appreciable change in available land for agricultural production is anticipated, water tables around the world continue to fall,[3] and reductions in crop inputs, especially chemical fertilizers, are sought to minimize greenhouse gas emissions and reduce the carbon footprint of agriculture.[4] In addition, with climate change resulting in more extreme weather patterns, there is greater risk of crop failure on a regional basis.[5] Certainly, plant breeders face a number of formidable challenges in reaching this ambitious goal.

How does biotechnology fit into the development of new improved crop cultivars through plant breeding? The principles of plant genetic improvement are simple: cross the "best" parents and identify outstanding progeny that outperform the parents for traits of interest. Today, the development of new crop cultivars involves an integrated approach to genetic improvement that includes use of biotechnology (i.e., genetically modified (GM) traits) and genomics-based applications (e.g., molecular markers or DNA sequence information) along with conventional breeding practices[6] (Figure 1).

Plant breeders create useful genetic variation by crossing lines with favorable genes as parents to produce populations of progeny with new gene combinations. Biotechnology comes into play as it facilitates access to novel genes (and traits) through transformation, which otherwise might be unavailable through crossing. In addition, genes from the target species can be engineered to generate useful novel forms of expression such as tissue-specific, growth-stage-specific, higher/lower threshold, or silenced expression. Genomics-based technologies can aid in identifying 'best' lines to use as parents; for example, breeding values can be estimated for candidate parents on the basis of the molecular marker profile of the lines[7] and compared.

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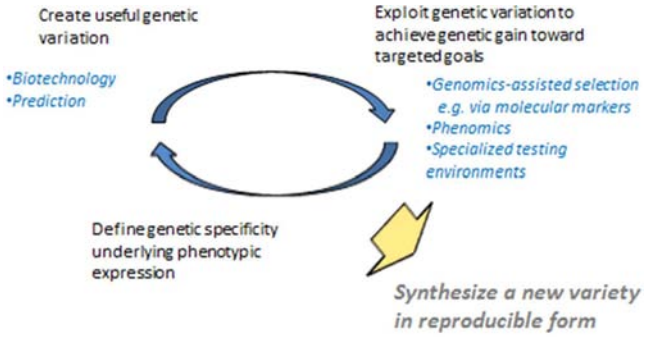

Figure 1. Cycle of creating and exploiting genetic variation in cultivar improvement, featuring the role of biotechnology and other 21st century tools.

Then, once created, plant breeders exploit the genetic variation in a breeding population to achieve genetic gain toward specific breeding objectives (Figure 1). Performance is evaluated to identify outstanding progeny. Generally, this takes the form of evaluation for yield and other important attributes at numerous locations within the target market region. Molecular markers and other genomics-based technologies can help to spotlight individuals that have particular genes or gene combinations shown to be associated with top performance.[8,9] In addition, identifying outstanding progeny can be facilitated through the use of specialized testing environments that enable highly controlled evaluation of particular performance attributes, for example, drought tolerance[10] or nitrogen use efficiency. Automated systems for data collection and analysis enhance efficiency and facilitate high throughput. Secondary traits, known to be correlated with desired performance attributes, may be measured and analyzed to further improve throughput and accuracy in selection.[11] "Phenomics" has been coined to describe the study of plant growth, performance, and composition, with the goal to bridge the gap between genomics, plant function, and agricultural traits.[12] It is focused on expression of the genome as traits in a given environment.

Once identified, outstanding progeny then become the basis for synthesizing an improved variety or hybrid (Figure 1). Furthermore, these genetic materials can become the basis for repeated cycles of selection; that is, top performers may be recombined to create new populations focused on continued improvement. As knowledge of the genetic architecture of key performance attributes is accumulated (e.g., location, numbers, and expression of genes), this information can be used to identify parents with more accuracy to exercise greater efficiency and greater genetic gain in the next cycle directed to cultivar improvement.

Since their debut in the mid 1990s, GM traits have been rapidly adopted in the United States and other countries around the world including Brazil, Argentina, India, Canada, China, and South Africa.[13] In 2012, 93% of all soybeans and 88% of all corn grown in the United States was GM.[14] The trend toward GM traits has steadily increased the number of U.S. corn hybrids with >1 GM trait (i.e., "stacked" trait hybrids) to 52% in 2012.[14] The appetite for GM cultivars has been fueled among U.S. farmers on the basis of increased profit margin and decreased environmental impact associated with pesticide use and greenhouse gas emission from agriculture.[15]

The range of GM traits continues to expand.[16] Some categories of traits protect genetic potential, for example, herbicide tolerance, insect resistance, and other abiotic and biotic stress tolerance of factors (e.g., drought tolerance and disease resistance, respectively). Other categories stretch genetic potential: to enhance productivity such as grain yield or to improve nutritional quality, for example, high-lysine corn and altered oil profile to support human health. Still other categories are designed to lower crop production inputs, for

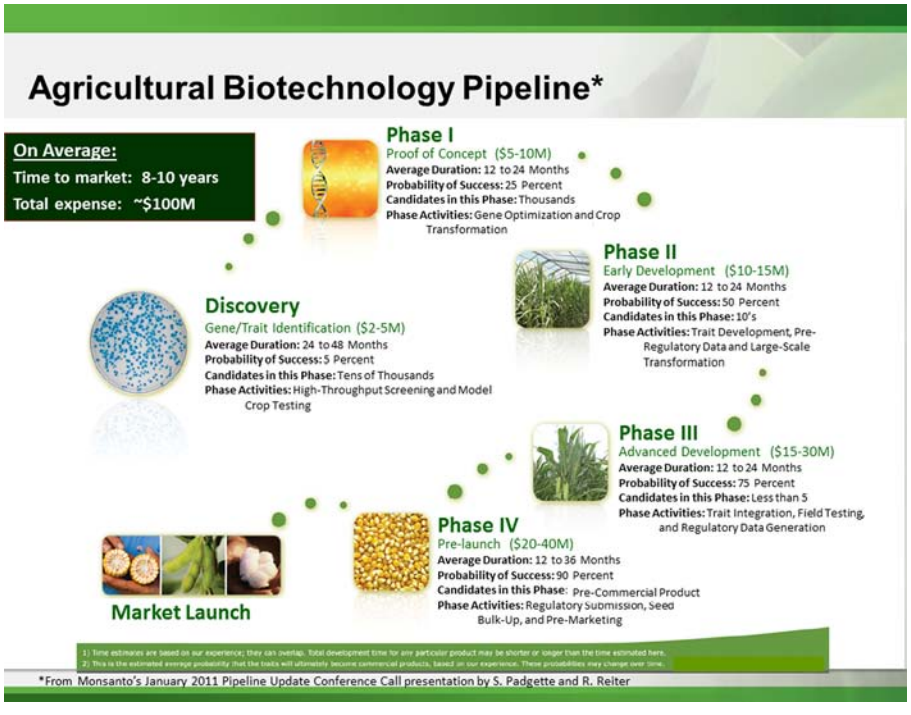

Figure 2. Example of a commercial pipeline process to develop transgenic events, which includes several phases to market launch.

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example, nitrogen use efficiency and reduced water requirement.

The purpose of this paper is to describe the processes by which GM crops are developed for commercialization and the data produced by analysis and testing to support advancement through the product pipelines. Because processes may differ somewhat by crop due to a variety of factors including plant biology (e.g., ease of crossing, average number of seed produced per cross), typical uniformity within a cultivar (e.g., varieties may be mixtures of genotypes), and diversity of commercial and pre-commercial transgenic events, hybrid maize product development is highlighted as the primary example throughout. Considering the safety of GM crops, this paper provides an inside view of GM crop product development, specifically highlighting two product pipelines: the pipeline for development of biotech traits, and the trait introgression pipeline for conversion of improved cultivars that will include the biotech traits as a key component. Note that other evaluations may be conducted in support of data packages submitted to governmental authorities to demonstrate safety, for example, earthworm feeding studies, compositional analysis, and livestock feeding studies. Although there is significant overlap in data assembled for product development decisions and for regulatory approvals, the latter are outside the scope of this work and are addressed in the paper by Laura Privalle (also included in this special issue of *Journal of Agricultural and Food Chemistry*), which considers deregulation of GM events.

BIOTECH TRAIT DEVELOPMENT PIPELINE

The development of biotech traits involves a pipeline process (Figure 2) upstream of GM seed product development. Transgenic events are created through transformation, which can take the form of *Agrobacterium* transfer or bombardment (Figure 3). In either case, a vector with core elements comprising the gene(s) of interest, a promoter, and a terminator sequence is assembled. In addition, other elements such as enhancers to expression (e.g., transit peptides which can direct the transgenic protein to appropriate cell organelles) may be included. With *Agrobacterium* transformation, the vector is inserted into the *ti* plasmid, which has effectively been "disarmed" pathogenically so that the target DNA is transferred to the intended host but disease is not. With bombardment, the vector is affixed to a gold or tungsten particle and propelled with force into plant tissue of the intended host. In either case, through transformation, the DNA becomes incorporated into the host genome in one or more cells, which are screened and induced to regenerate into fertile plants. The screening for cells that have incorporated the introduced DNA is often facilitated through use of a so-called "selectable marker" (Figure 3), which conveys a property or attribute that enables easy identification and recovery of transformed cells. Typically, selectable markers are introduced in transformation using a separate vector as this enables selection against the marker attribute after plant regeneration to eliminate the selectable marker DNA from the resulting event prior to commercialization.

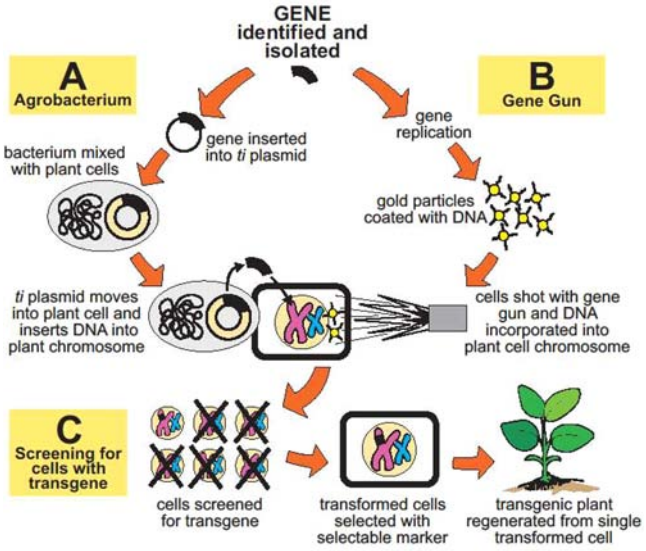

Figure 3. Gene of interest can be engineered for desired expression and introduced to a target species through transformation via *Agrobacterium* or bombardment. Each event originates as a fertile plant regenerated from a single cell incorporating the introduced DNA. Graphic courtesy of North Dakota State Univeristy, Michael D. Peel, http://www.ag.ndsu.edu/pubs/plantsci/crops/a1219w.htm.

Thus, each event originates as a single plant (referred to as a T0 plant) regenerated from a single transformed cell. An event is defined by the specific DNA sequence that has been inserted into the host genome through transformation as well as the particular site(s) of insertion. Because of the high cost of development and requirements for government approvals to support commercial release (reportedly in the $50−100 million range), biotech trait developers aim to create and identify a sole event that optimally meets the biotech trait product goal in terms of expression, stability, safety, and utility. Thus, even with the use of elite lines for transformation, a single event chosen for commercialization originating from a single source (e.g., T0 plant) will eventually be integrated into numerous cultivars to maximize market penetration of the GM trait; this has important implications for the trait introgression pipeline (discussed in the next section).

The biotechnology pipeline involves several phases to create and select the single "best" event for government authorization and commercial release (Figure 2). The process begins with discovery of a gene(s) that elicits a desired response, for example, production of a given insecticidal protein active against a prominent crop pest. Phase I focuses on identifying elements of a commercial vector to optimize expression of that gene. In phase II, many events are created and evaluation begins. In phase III, events are tested with the goal of narrowing to ≤5 events. As the number of candidate events decreases, the number of elite lines converted for the candidate events is increased. Phase IV activities focus on a single event. Data packages are submitted to regulatory authorities to show safety and efficacy in an effort to win commercialization and/or grain import approvals for target markets, full-scale trait integration is implemented, and performance testing of potential seed products is conducted as well as seed bulk-up in preparation for market launch. Thus, the process involves intense selection for an optimal event with dependable expression for the trait of interest in keeping with the trait development target, with no adverse phenotypic effects.

Throughout the biotech trait development pipeline, transgenic events are subject to intense evaluation and stringent selection prior to choosing the one event for commercialization.[17] Criteria for event selection emphasize several factors: (a) single copy insertion and structural fidelity of the DNA introduced through transformation relative to the transformation vector; (b) phenotypic expression according to

desired threshold levels, tissue specificity, and timing in the plant life cycle; (c) consistent and reliable expression regardless of environmental conditions and stresses, genetic background, and presence of other events; (d) stable inheritance through numerous generations; and (e) absence of negative or detrimental effects. Potential problems may arise when the inserted DNA represents a rearrangement (compared to vector DNA) because complex integration patterns have been associated with unstable expression and silencing.[18] Silencing of transgenic event expression has also been attributed to stress and to the presence of duplicate DNA sequences, for example, as with stacked events having the same promoter. If the event interacts with "native" genes in the genome, expression of other traits could be affected, either positively or negatively. Furthermore, with a given event, performance for a key trait could be negatively affected due to disruption from the DNA insertion into the genome during transformation; for example, the insertion site for a particular event could alter regulatory expression elements controlling gene(s) for yield, leading to an unintended negative effect such as yield loss.

Therefore, in light of these criteria, events are subject to intense evaluation during biotech trait development. Testing can be categorized into four classifications: molecular analysis, efficacy testing, performance testing, and impact evaluation. Molecular analysis concentrates on the genomic location and makeup of each event; for example, single-insert single-copy events encoding the same DNA sequence used in the transformation vector are ideal. Efficacy testing focuses on the expression and phenotypic effect of the event for the trait of interest. It may involve applying intense pressure for the transgenic trait to measure the limits of the event effect. For example, efficacy testing of an event for protection against insect pests may involve artificial infestation to facilitate event expression at extremely high levels of insect pressure. The artificial infestation also fosters uniform pressure across the test plots. Typically, experimental designs that facilitate sharp comparisons between events are utilized (e.g., replicated split plot designs), featuring near-isogenic lines having the same genetic background with and without the event.

Performance testing, on the other hand, involves evaluation at numerous locations across the target market region. It assesses performance with and without trait pressure to ensure that there is not a "penalty" for event expression. As with the example above, in the absence of insect pressure, a yield penalty would be detrimental. Testing is performed across a wide range of environments within the target market region to assess performance with diverse soil types, cultural practices, and stress factors and across multiple years to evaluate performance under different climatic conditions. The performance of the event in a wide range of genetic backgrounds and together in stacks representing desired event combinations is useful to determining whether the event in question is reliable and consistent in its expression and unhampered by other genes. Depending on the trait, other measures may be evaluated to understand the impact of the biotech trait. For example, an event resulting in altered grain composition may be studied to understand the impact of the change in animal feeding diets relative to livestock health and meat quality. Evaluation is aimed at identifying an ideal event for commercialization, one with dependable trait delivery and no "extra baggage", which requires only one copy of the event (e.g., acting in a dominant fashion) in a hybrid cultivar.[19]

Plant materials required for the various classifications and types of testing are produced in accordance with testing goals, test designs, and precision needs.[17] For example, some efficacy testing can be conducted with partial conversions of elite inbreds or hybrids, whereas performance testing requires finished conversions of a number of genetically diverse elite lines for completion. Line conversions are further discussed in the next section.

Another recent trend with biotech trait development is toward multiple modes of action included in the same event or stack of events by assembling multiple genes for a particular trait.[20] For example, SmartStax, which is a stack of four events, represents three genes with different modes of action for Lepidopteran insect control, three genes for control of corn rootworm, and two genes conferring different types of herbicide tolerance.[21] "Quantitative" versus "qualitative" protection is expected to increase both the durability of and the performance for the trait.

TRAIT INTROGRESSION PIPELINE

In corn, backcross (BC) breeding is done to "convert" a target elite hybrid for a particular event of interest. The goal of backcross breeding is to recover the complete and unaltered agronomic package represented by the target elite hybrid plus the desired expression of all events introduced. Event introgression involves repeated crossing to the parent(s) of the hybrid targeted for conversion to recover the vast proportion of the recurrent parent (RP) genome (Table 1).

Table 1. Introgression of a Single Event Involves Repeated Crossing to the Parent(s) of the Hybrid Targeted for Conversion (i.e., Recurrent Parent), Followed by Trait Fixation and Then Performance Testing

generation	activity	seed outcome
1	cross event donor to RP	F1
2	select for event; BC to RP	BC1
3	select for event; BC to RP	BC2
4	select for event; BC to RP	BC3
5	select for event; BC to RP	BC4
6	select for event; BC to RP	BC5
7	select for event; BC to RP	BC6
8	self-pollinate	BC6S1
9	self-pollinate	BC6S2
10	identify line phenotypically similar to RP that stably expresses introduced trait	BC6S3
11	testcross to produce seed to evaluate agronomic performance of converted hybrid	BC6S3 testcross

Starting with 50% in the F1 generation, the proportion of the non-RP germplasm decreases by half with each cross to the RP. If the event acts in a dominant fashion, only one parent of the hybrid need be converted. However, if events act in an additive fashion, conversion of both parents of the hybrid may be required to achieve trait expression targets.

Molecular markers may be used to speed the conversion process. In such applications, molecular markers essentially act as points along the chromosomes to facilitate comparison of the genomes of different individuals. For trait introgression, use of molecular markers enables identification and selection of individuals with higher proportion of RP germplasm recovery in a given backcross generation,[17] which can reduce the number of generations required for complete conversion. Individual

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backcross progeny differ not only for the proportion of RP germplasm recovered but also for the location of non-RP segments. The most stubborn genomic locations for RP recovery are those in close proximity to the event insertion site because there is reduced opportunity for recombination in these segments of linked DNA. When the event is selected in the conversion process, it also tends to bring along this linked DNA from the donor source, which is especially problematic if the donor source is nonelite or from an opposite heterotic group (e.g., from the female genepool when the inbred targeted for conversion is a male line). Thus, molecular markers can be used to guide elimination of undesirable DNA from the donor source in close proximity to the event insertion site (i.e., linkage drag).[22] Furthermore, molecular markers for the event can be used to identify event homozygotes (i.e., lines with two copies of the event) during trait fixation, saving one to two generations in the process. Overall, use of molecular markers in trait introgression can speed time to market and minimize risk of failure to recover all of the desirable attributes of the elite line by facilitating removal of linkage drag.[23] Thus, accelerated and precise conversion is critical to timely commercial launch of new GM seed products, introduction of value-added traits in elite genetics, and efficient management of seed inventories.

In addition to molecular markers, other resources can be employed to speed the trait introgression process, including use of continuous nurseries, winter nurseries, or greenhouse facilities to cycle multiple generations per year and use of techniques to accelerate the rate of plant development through its life cycle.[17] Thus, the duration of the process may differ across organizations.

Typically, multiple versions of each conversion are produced and performance tested. The probability of success in recovering a hybrid conversion with equivalent performance to the unconverted target hybrid is a function of the amount of residual non-RP germplasm remaining in the finished hybrid conversion and the number of versions of each RP that are produced and tested in hybrid combination.

QUALITY CONTROL OF COMMERCIAL SEED

Seed volumes for commercial release are monitored to ensure event identity and purity.[17] The monitoring is designed to confirm the presence of all intended events at or above specified purity thresholds and uphold label specifications.[24] For example, the label on a unit of seed sold to farmers may specify that ≥98% of that seed contains the event of interest. At the same time, monitoring also confirms the absence of unintended events, that is, no adventitious presence. Likewise, seed used in certain product testing as well as data packages for regulatory approvals is also typically monitored in this manner. Such testing supplements other testing done to confirm line/hybrid purity and uniformity, seed viability, and seed quality. Quality control testing of seed is essential to protecting research investments, maintaining compliance with government regulations, and ensuring farmer satisfaction.

Quality control monitoring is bolstered by quality assurance practices.[17] These include standard operating procedures (SOPs) for pipeline activities involving seed handling and seed management in the product pipeline to facilitate uniform application of best practices throughout the organization, and auditing as a means of proper stewardship verification.[25]

In closing, biotechnology provides a key advantage in crop genetic improvement, allowing the plant breeder to tap into useful genetic variation not otherwise accessible for the target species. Transgenic events are subject to intense evaluation and stringent selection prior to commercialization. Commercial candidate events are introgressed into elite cultivars, often using molecular markers for efficiency, to offer farmers biotech traits in desirable agronomic packages. Final seed products are performance tested and quality inspected to ensure genetic and event identity and purity. Thus, GM cultivars are products of significant testing and auditing prior to seed sale and distribution to farmers, more testing and auditing than with non-GM cultivars.

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Notes

The authors declare the following competing financial interest(s): As an expert in transgenic product development, R.H. Mumm has provided consulting services through GeneMax Services to a number of companies that develop transgenic traits in crops and/or transgenic seed products since 1999.

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