

Engineering herbicide-resistant maize using chimeric RNA/DNA oligonucleotides

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Maize plants resistant to imidazolinone herbicides were engineered through targeted modification of endogenous genes using chimeric RNA/DNA oligonucleotides. A precise single-point mutation was introduced into genes encoding acetohydroxyacid synthase (AHAS), at a position known to confer imidazolinone resistance. Phenotypically normal plants from the converted events (C₀) were regenerated from resistant calli and grown to maturity. Herbicide leaf painting confirmed the resistance phenotype in C₀ plants and demonstrated the anticipated segregation pattern in C₁ progeny. DNA cloning and sequencing of the targeted region in resistant calli and derived C₀ and C₁ plants confirmed the expected mutation. These results demonstrate that oligonucleotide-mediated gene manipulation can be applied to crop improvement. This approach does not involve genomic integration of transgenes. Since the new trait is obtained through modifying a gene within its normal chromosomal context, position effects, transgene silencing, or other concerns that arise as part of developing transgenic events are avoided.

Keywords: herbicide resistance, chimeraplasty, gene modification, genetic inheritance, acetohydroxyacid synthase

Imidazolinones are nonselective, broad-spectrum herbicides for effective control of a majority of grass and broadleaf weeds. They competitively inhibit acetohydroxyacid synthase (AHAS; EC 4.1.3.18), the first enzyme in the biosynthetic pathway of branched-chain amino acids¹. However, the effectiveness of these herbicides can be significantly reduced by mutations in AHAS that reduce the affinity between the chemical and protein². One such mutation is an amino acid substitution from serine (AGT) to asparagine (AAT) near the C-terminal end of the mature *Arabidopsis* AHAS protein^{3,4}. In maize, the corresponding region flanking the Ser621 position was identified⁵. These mutations can be used to genetically engineer crops resistant to imidazolinone herbicides, and improve selectivity of the herbicide.

Currently, the most effective way to engineer a herbicide-resistant plant is to introduce an in vitro-manipulated resistance gene into plant cells through transformation. However, it can be difficult to control the genomic integration and expression of the transgenic herbicide-resistant gene^{6,7}, because of the random nature of transgene integration and variation of expression level and stability.

Recently, a new strategy for targeted manipulation of endogenous genes by DNA repair using chimeric DNA/RNA oligonucleotides was developed^{8,9}. It has been applied to gene repair in mammalian cells¹⁰⁻¹³ and gene activation and modification in plant cells^{5,14}. In these examples, genes of interest were precisely modified, using sequence-specific chimeric oligonucleotides as DNA repair templates, to introduce single-point mutations in critical positions. We have shown that, in plants, the introduced chimeric oligonucleotides is degraded within the cells several hours after delivery⁵. Based on the unique designed structure and the predicted mode of action of the chimeric oligonucleotides¹⁵, genomic integration is not expected. This method thus provides a valuable alternative for genetic engineering of plants, not requiring the integration of foreign gene or regulatory sequences.

To use this approach for crop improvement, it is important that the modified genes are transmissible through mitosis and meiosis, with a predictable segregation pattern (mendelian inheritance) in the progeny. In this study, we demonstrate the successful generation

of herbicide-resistant mutants created by chimeric chimeric oligonucleotides and the resulting stable inheritance and expression in progeny of the converted AHAS allele. The results described here demonstrate the practical application of this strategy to engineering commercially useful traits in important crop species.

Results

Analysis of target AHAS genes in maize. It has been reported that maize, like tobacco, has two families of AHAS genes, AHAS108, and AHAS109¹⁶. For chimeric chimeric oligonucleotides targeting and mutation detection, the copy number for each AHAS gene was estimated by Southern blot analysis. This was done by comparing the intensity of hybridization signals present in restricted genomic DNA and reference plasmid DNA containing AHAS genes. The Hill¹⁷ line used in this study contains approximately two copies of AHAS108 and five copies of AHAS109 (Fig. 1). In addition to copy number, Southern analysis also revealed a high level of polymorphism between these two genes, as well as among different tissues and lines. In order to accurately determine the sequence of the target region, amplified fragments from BMS (Black Mexican Sweet)¹⁸ and Hill cells were cloned. We selected 40 clones from each cell line for sequence analysis. In a 300 bp sequence within the Hill line we detected 23 single-nucleotide polymorphisms (SNPs), and 10 additional SNPs were identified between BMS and Hill cells (data not shown). However, no polymorphism was observed in the region spanning the sequence to which the chimeric oligonucleotides was designed to target.

Based on the sequence and Southern data described, the chimeric oligonucleotides PHPC621A was designed to be able to target both AHAS108 and AHAS109 (ref. 5). To ensure the effectiveness of its targeting, sequences showing homology to PHPC621A were determined by searching the Pioneer/DuPont maize expressed sequence tag (EST) database (having a complexity of >400,000 ESTs). Among 30 high-score hits, four were non target hits. These non target hits have a significantly lower score compared to the target hits, as a result of less homology and higher mismatch in strand direction. Thus the

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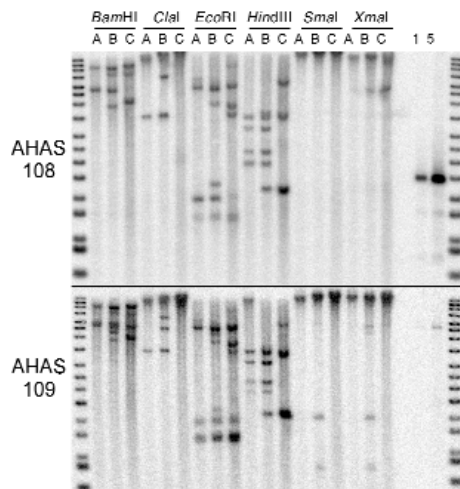


Figure 1. Southern analysis of maize AHAS genes. Restricted maize genomic DNA was hybridized with probes for AHAS108 or AHAS109. Multiple copies of AHAS108 and AHAS109 were revealed by comparing with one- or five-copy controls of plasmids containing a corresponding AHAS gene (1 or 5, respectively, in figure). Restriction fragment length polymorphism exists between the two gene classes (AHAS108 and AHAS109), between the maize BMS (C) and Hill (A and B) lines, as well as between leaf (A) tissue from a Hill plant and callus (B) tissue established from immature embryos from an independent Hill (A188×B73) plant. Molecular weight markers on right range from 26.3 to 0.53 kb.

PHPC621A chimeric oligonucleotide was predicted to bind the AHAS target with very high specificity, with no expected binding to other maize coding sequences. In order to detect chimeric oligonucleotides-introduced mutations in both AHAS108 and AHAS109 simultaneously, a pair of DNA chimeric oligonucleotides common to both gene sequences was identified and used as PCR amplification primers.

Engineering and selection of herbicide-resistant mutants. PHPC621A was delivered into 29 plates of maize Hill cells in two microprojectile bombardment experiments. An additional eight plates were treated as various negative controls as described⁵. Resistant calli from the treatment plates containing selection medium were identified three to four weeks after delivery. No resistant calli were identified from the negative control plates. The putative events were

initially screened by sequence analysis of the PCR-amplified target fragments. We selected 12 of the putative events showing apparent mutations for regeneration and further study. The regenerated C₀ plants were transferred to soil and cultivated in the greenhouse.

Expression of herbicide resistance genes in C₀ plants. C₀ seedlings from each event were selected for the herbicide resistance test. Although symptom development was monitored weekly, data were collected two and four weeks after herbicide application. Among nine events examined, two events, along with the wild-type control, were sensitive to 1× field dose of Lightning, evidenced by extensive discoloration and tissue necrosis. Four events showed no symptoms at 1× field dosage, and showed only localized symptoms at 4× field dose and were classified as tolerant. The other three events showed no symptoms at either 1× or 4× levels of the recommended field application dose and were classified as resistant (Fig. 2A–D). Three additional C₀ plants were not tested for herbicide resistance but were used for subsequent molecular analyses. These results are summarized in Table 1.

Fragments containing sequences spanning the targeted region in the AHAS gene were amplified from both the resistant callus and the leaf tissue of the resulting C₀ plants. Because the mutation to be introduced into AHAS genes was associated with loss of a *Bfa*I site⁵, *Bfa*I restriction fragment length polymorphism (RFLP) was used to enrich the potential mutant allele. Unrestricted fragments were reamplified and cloned. Sequence analysis of the cloned fragments confirmed the expected mutation in these events. A guanine to adenine nucleotide substitution leading to a change of serine to asparagine at amino acid position 621 was observed (Fig. 3A–C). Plants from the confirmed events were pollinated with wild-type Hill pollen, and grown to maturity. C₁ seeds were collected for the progeny analysis.

Heritable transmission of herbicide-resistant gene through meiosis. A total of 270 C₁ seeds from seven events (approximately 40 from each event) were planted for the analysis. Lightning herbicide was applied to leaves as described above, and symptoms were monitored weekly. Because of physical damage to some of the tested leaves during greenhouse transfers, data were collected from only 209 of the plants at four weeks after herbicide application. Overall, approximately half of the progeny seedlings showed the herbicide-tolerant phenotype whereas the other half were herbicide-sensitive (Fig. 2E–H, and Table 1).

Leaf samples from 26 C₁ plants representing the seven events were collected. Sequences from the targeted regions of the AHAS genes were amplified and cloned from the genomic DNA of the leaf

Table 1. Sequence analyses of cloned AHAS621 alleles and herbicide tolerance phenotype assessment in C₀ events and segregating C₁ progeny following chimeroplasty

Event	Herbicide-selected callus and C ₀ plants				C ₁ progeny seedlings					
	Plant phenotype	Cloned sequence analyzed ^a	No. with sequence mutation ^b	No. with predicted conversion ^c	Plants analyzed	Cloned genes analyzed	No. with sequence mutation ^b	No. with predicted conversion ^c	Progeny plants scored	Herbicide-tolerant frequency
123-1A1	Not tested	–	–	–	8	27	9	4	39	0.62
123-1A2	Tolerant	10	1	0	4	9	6	4	40	0.58
123-2A5	Resistant	8	1	1	4	13	3	1	40	0.49
123-2A8	Tolerant	5	0	0	–	–	–	–	–	–
206-12D1	Sensitive	4	0	0	–	–	–	–	–	–
206-12D4	Tolerant	–	–	–	3	8	2	1	32	0.71
206-18B6	Resistant	14	2	1	3	7	2	1	40	0.59
206-19B8	Tolerant	2	2	0	3	16	4	1	39	0.39
206-19B10	Resistant	13	4	1	1	1	1	0	40	0.52
206-3112	Not tested	2	0	0	–	–	–	–	–	–
206-3114	Not tested	9	8	1	–	–	–	–	–	–
206-3115	Sensitive	7	1	0	–	–	–	–	–	–

Total = 270 Mean = 0.55

^aCloned sequences represent individual PCR products amplified through the segment spanning the targeted region from total plant or callus DNA.

^bGenerally, only one of the multiple copies of the endogenous AHAS gene was converted by the chimeric oligonucleotide, hence the small number of sequences with mutations relative to the number analyzed.

^cIn all cases, mutations that were not the predicted conversion were either alternate nucleotides at the predicted position or a base change one (and in one case two) nucleotide(s) upstream of the predicted site.

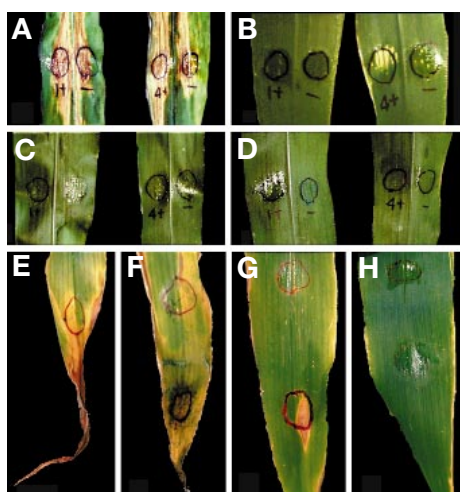


Figure 2. Results from herbicide application on C_0 and C_1 plants following conversion. (A–D) Two leaves from each C_0 plant were painted with either 1× (1+ on leaves) or 4× (4+ on leaves) of the recommended field dose of herbicide in a lanolin paste as described in Experimental protocol. Lanolin paste without herbicide was applied to the same leaves (- on leaves) for direct comparison. Plants were scored as sensitive, tolerant, or resistant on the basis of phenotype following herbicide application. (A) Wild-type control; (B) tolerant event; (C, D) resistant events. (E–H) Herbicide paste with 1× (upper marker circle) and 4× (lower marker circle) field dose of herbicide was applied to a single leaf from each C_1 seedling. The four bottom panels show the different segregating phenotypes in C_1 seedlings from one of the events: (E, F) sensitive; (G) tolerant; (H) resistant.

samples (Table 1). Sequence analysis of the cloned targeted fragments again confirmed the predicted mutations generated by chimeric oligonucleotides (Fig. 3D, E).

For those events that showed no symptoms on a 4× level of herbicide application, at least one cloned AHAS gene sequence containing the predicted conversion was recovered from the C_0 events (Table 1). The sequence analysis data in Table 1 represent results from only a subset of individual cloned genes from some of the events studied. Since there are multiple copies of AHAS genes in maize, the observation that some herbicide-tolerant events did not yield clones with correct mutations could be because the specific AHAS gene carrying the introduced mutation was not among the recovered and sequenced gene clones from those events. This is substantiated by the observation that for some of the C_0 events, for which a cloned gene with the correct mutation was not originally recovered (e.g., events 206-19B8 and 123-1A2), we were able to clone out a gene with the correct mutation from the derived C_1 progeny plants. Additionally, since more than one copy of the gene could be converted within one event, albeit at low frequency, this could contribute to variation in level of tolerance among different events.

Discussion

As an alternative to transgenic approaches, endogenous genes can be modified by homologous recombination, however its use in plants is limited by low frequencies^{19–21}. Previously, we presented an alternative approach for manipulation of the plant genome and improvement of plant traits using chimeric oligonucleotides⁵. Here we have further investigated the herbicide resistance phenotype and the integrity of the AHAS gene sequence in the progeny derived from the converted events, demonstrating that chimeric oligonucleotide-modified AHAS genes are transmitted through both mitosis and meiosis, in a predictable mendelian fashion. The modified genes behave the same as any other normal endogenous maize gene, and so the plants derived from these modified genes can be used in breeding of herbicide-resistant maize. An advantage in modifying the target sequence in its normal chromosomal context may be that many of

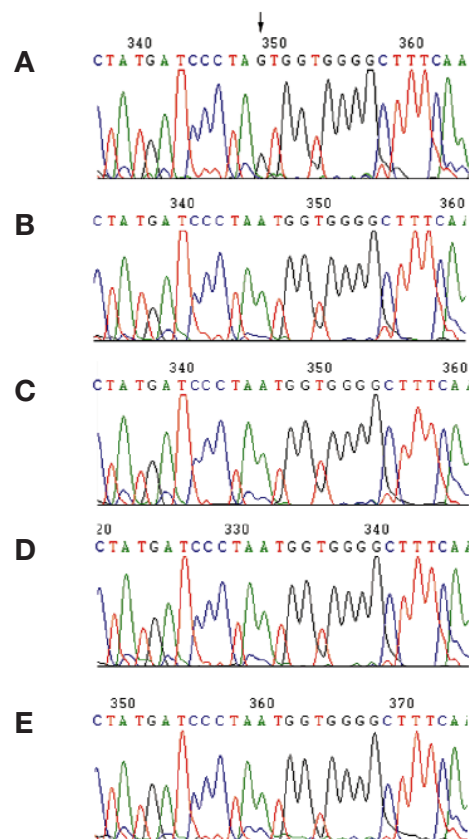


Figure 3. Sequencing chromatograms of cloned fragments from conversion events. DNA sequences spanning the target region were cloned and sequenced. Results are from (A, B) C_0 callus (C) a C_0 seedling, and (D, E) C_1 seedlings, showing (A) wild-type sequence and (B–E) converted sequences in the AHAS621 gene (nucleotide targeted for conversion indicated by arrow).

the expression variation concerns associated with transgenic plants are not an issue, since presumably the genomic environment is not markedly perturbed as with transgene integration.

In this paper we propose and use the designations C_0 , C_1 , and so on, to describe the different generations of events from conversions using chimeric oligonucleotides, to distinguish them from the T_0 , T_1 , etc., designations used commonly in the literature to describe different generations of transgenic events. Although we used the latter nomenclature in our earlier report⁵, we believe the proposed new designations would be more appropriate. The two processes, introduction of new transgenes by transformation and modification of existing endogenous sequences by chimeraplasty, lead to distinct end products, and it would be important to distinguish them.

Manipulation of plant traits using chimeric oligonucleotides has some advantages compared to conventional transformation methodologies. The first is target site specificity. Manipulation of genes using chimeric oligonucleotides is sequence-dependent. Therefore, compared to random integration, where multiple insertions can occur, or where integration can happen in undesirable locations in the genome, the converted event is easy to manage in breeding programs. A second advantage is that no foreign gene or other vector sequences are integrated. Chimeric oligonucleotides are site-specific mutagens that are rapidly degraded *in vivo*. The expression of the manipulated gene is driven from its own endogenous promoter in its native chromosomal context. With transgenes there may be competition among native and introduced promoters for endogenous transcription factors; this is not an issue with the chimeric oligonucleotides approach.

Interestingly, according to a recent study in *Arabidopsis thaliana*, the outcrossing rate of naturally occurring AHAS mutants is signifi-

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cantly lower than the rate of a transgenic mutant expressing the same mutant allele²². Whether similar results might be expected for the chimeric oligonucleotides-induced single-point mutations versus transgenic approaches to generate the same traits in maize or other plants would merit further investigation.

The normal inheritance of the modified herbicide resistance trait establishes a foundation for applying this approach to crop improvements in general. Although the low frequency (10^{-4}) observed to date with this method in plants^{5,14} has not precluded the generation in our hands of new herbicide-resistant lines potentially suitable for commercial development, many other important traits cannot be readily selected at the single-cell level in culture following chimeraplasty. Also, studies to date have utilized dominant genes as targets for conversion. Equally important will be the ability to modify and select for the many recessive traits that contribute to critical biological processes or agronomic performance in crop plants. Thus, increases in overall efficiencies of nucleotide conversion or alternative screening strategies will be required for application of this approach to recessive traits, or those without scorable or selectable phenotypes. However, considering the success of chimeraplasty to date in introducing targeted modifications in several different genes in both monocots⁵ and dicots¹⁴, this approach is expected to have a broad range of potential applications in crop improvement.

Experimental protocol

Chimeric oligonucleotides design, synthesis, and purification. Chimeric oligonucleotides PHPC621A (ref. 5) was synthesized and purified (Kimeragen, Inc., Newtown, PA) according to published methods^{8,15}. The crude oligonucleotides were purified by PAGE and analyzed by HPLC. DNA ONDs were synthesized in a similar fashion using DNA phosphoramidites.

Callus initiation, chimeric oligonucleotides delivery, selection, and plant regeneration. Friable embryogenic calli (type II) were initiated from immature embryos of the HiII (A188 × B73) maize germ plasm¹⁷ and maintained on N6 basal medium with 3% (wt/vol) sucrose, 2 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2, 4-D), 0.7 mg L⁻¹ L-proline, 0.85 mg L⁻¹ AgNO₃, and 0.3% (wt/vol) GELRITE (Sigma, St. Louis, MO).

Cultured maize cells were suspended in liquid N6 medium and then plated on a glass fiber filter (VWR, West Chester, PA), and briefly dried down under vacuum. The filter disks were then placed on N6 medium without 2, 4-D and cultured overnight in the dark. Chimeric oligonucleotides (0.4 μg) were co-precipitated with 15 μl of 2.5 M CaCl₂ and 5 μl of 0.1 M spermidine onto 25 μg of 1.0 μm gold particles (Analytical Scientific Instruments, Richmond, CA) and were delivered into the maize cells via biolistic bombardment. Microprojectile bombardment was performed using a PDS-1000 He particle delivery system (BioRad, Hercules, CA) with 650 p.s.i. rupture disks at a 9 cm target distance.

Selection of AHAS mutants was conducted according to Zhu and colleagues⁵. Briefly, following microprojectile bombardment, cells were resuspended in liquid medium and uniformly plated on solid culture medium containing 0.7 μM imazethapyr (AC263,499, or Pursuit; American Cyanamid, Princeton, NJ). Cells were cultured in the darkroom at 25°C and 75% humidity for four to six weeks with subculturing every one to two weeks. Putative events were subsequently transferred to fresh medium containing 1.0–2.0 μM imazethapyr for selection.

Plants containing sequence-verified conversions in the AHAS genes were regenerated from HiII embryogenic callus according to Armstrong and coworkers¹⁷ and Register and colleagues²³. Developing C₀ seedlings were transferred to soil and grown to maturity in the greenhouse. Pollen from control HiII plants was used to pollinate the plants, and C₁ seeds were collected.

Progeny analysis for herbicide resistance. Lightning herbicide (American Cyanamid, Princeton, NJ) was used for application to the C₁ plants in the greenhouse. Based on the per acre recommended application rate of 1.28 ounce in 20 gallons water (1×), we prepared 1× and 4× stock solutions of Lightning mixed with lanolin into a paste. Lanolin mixed with water alone served as a control. Maize seedlings regenerated from the chimeric oligonucleotides conversion experiments were grown in the greenhouse under our standard conditions. The control or herbicide-containing lanolin paste preparations were applied to the third or the fourth leaf of maize seedlings at the V5 stage. Results were scored at two and four weeks after herbicide applications.

Molecular analysis of AHAS wild-type and mutant alleles. Genomic DNA was extracted from maize callus or leaf tissue using a Plant DNAase extrac-

tion kit (Qiagen, Valencia, CA). Copy number of the endogenous AHAS genes was determined by Southern blot analysis following standard procedures. Probes used in the analysis are pPHP10247 (containing the AHAS108 gene) and pPHP2545 (containing the AHAS109 gene).

Fragments including sequences spanning the target sites were amplified from the extracted genomic DNA of putative events, by *Pwo* or *Taq* polymerase (Boehringer Mannheim, Indianapolis, IN) with 30 cycles of 35 s at 95°C, 35 s at 60°C, and 35 s at 72°C, using a MJ thermocycler (MJ Research, Watertown, MA). The primers used are common to both AHAS108 and AHAS109 genes, having sequences 5'-GCAGTGGGACAGGTTCTAT (PHN21971) and 5'-AGTCCTGCCATCACCATCCA (PHN21972). Amplified fragments were digested with excess *Bfal* (New England Biolabs, Beverly, MA), and analyzed by agarose gel electrophoresis. Unrestricted fragments were amplified and subcloned using the TOPO TA cloning system (Invitrogen, San Diego, CA). Cloned fragments were sequenced with an ABI 377 automated sequencer (Perkin-Elmer, Norwalk, CT).

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