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Targeted manipulation of maize genes *in vivo* using chimeric RNA/DNA oligonucleotides

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ABSTRACT **Site-specific heritable mutations in maize genes were engineered by introducing chimeric RNA/DNA oligonucleotides. Two independent targets within the endogenous maize acetohydroxyacid synthase gene sequence were modified in a site-specific fashion, thereby conferring resistance to either imidazolinone or sulfonylurea herbicides. Similarly, an engineered green fluorescence protein transgene was site-specifically modified *in vivo*. Expression of the introduced inactive green fluorescence protein was restored, and plants containing the modified transgene were regenerated. Progeny analysis indicated Mendelian transmission of the converted transgene. The efficiency of gene conversion mediated by chimeric oligonucleotides in maize was estimated as 10^{-4}, which is 1–3 orders of magnitude higher than frequencies reported for gene targeting by homologous recombination in plants. The heritable changes in maize genes engineered by this approach create opportunities for basic studies of plant gene function and agricultural trait manipulation and also provide a system for studying mismatch repair mechanisms in maize.**

Site-directed manipulation of chromosomal genes has become the method of choice for determining gene function in bacteria, yeast, and mammalian cells. The primary methods used in site-directed gene manipulation rely on gene replacement via homologous recombination using an appropriately designed gene targeting vector (1). In plant cells, gene targeting has been limited by the low frequency of homologous recombination (1, 2). Homologous DNA fragments are randomly integrated into the genome at a much higher frequency (2–7). Even with recent improvements in transformation and selection conditions, the reported frequency of gene targeting in plant cells is still about one event in 10^5–10^7 targeted cells (8–10).

Procedures being developed for mammalian gene therapy provide potential alternatives for gene targeting in plants. One such example is an approach using chimeric RNA/DNA oligonucleotides (ONDs) (11, 12). In mammalian cells, chimeric ONDs that contain both DNA/DNA and RNA/DNA duplex regions with homology to a target locus are capable of specifically correcting mutations at a high frequency in both episomal and chromosomal target genes (11, 12). Gene conversion requires RNA/DNA duplex regions in the targeting ONDs and can occur in 30–40% of recipient cells (12–14), a frequency that is several orders of magnitude higher than gene targeting via homologous recombination. Targeting in mammalian cells with chimeric ONDs is also highly specific, because base alteration was found not to occur in related genes with mismatches in the sequence spanned by the RNA region of the ONDs (12). To date, however, only a few examples of chimeric OND-based gene targeting experiments have been reported in plant systems, and no significant data have been published. Furthermore, the heritability of targeting events reported in mammalian cells has not been vigorously investigated (11–16). For practical utility in plants, stable transmission of modified genetic traits to progeny is a requirement.

Mutations induced by chimeric RNA/DNA ONDs generally have involved alteration of 1–2 bp in the target site, which is adequate for many applications such as site-specific mutagenesis, gene knockouts, and allelic replacements. We report here targeted modification of an endogenous gene and an engineered transgene in maize by using chimeric RNA/DNA ONDs. Our results demonstrate that maize genes can be modified specifically and efficiently by chimeric ONDs and suggest that reverse genetics and engineering of endogenous genes in commercially important crops will be feasible by using this approach.

MATERIALS AND METHODS

Transformation Vectors. Transformation vectors were constructed as controls by using standard gene cloning methods. The plasmid pPHP10247 contains the *in vitro* mutagenized maize acetohydroxyacid synthase (AHAS) 108 gene encoding the Ser-621–Asn mutant form (17). The gene is flanked by the maize ubiquitin-1 promoter (18) and the nopaline synthase polyadenylation signal (19). The plasmid pPHP12322 contains the Pro-165–Ala mutant form of maize AHAS108 (20). Both plasmids have pUC-derived backbones. The plasmid pPHP3528 contains the *Streptomyces hygroscopicus bar* gene (21) driven by the maize ubiquitin-1 promoter.

For the transgene target we created a translational fusion between phosphinothricin-*N*-acetyltransferase (PAT), a gene product conferring resistance to bialophos (22), and the green fluorescence protein (GFP) (23). The fusion was created by cloning a 3′ *Bgl*II site in PAT to a 5′ flanking *Bam*HI site on GFP. By site-directed mutagenesis (MORPH kit, 5 Prime→3 Prime), the start codon (ATG) from GFP was removed, and a native PAT termination codon (TGA) was inserted in the junction of PAT/GFP. The plasmid PHP11129 contains the coding sequence for the PAT/TGA/GFP fusion target sequence, the maize ubiquitin-1 promoter, and the pinII terminator in a superbinary vector pSB1 suitable for *Agrobacterium*-mediated transformation (24). The plasmid PHP10699 is a positive control of the fusion without the TGA codon.

Cell Culture, Transformation, and Selection. Cultured maize HiII (25) or Black Mexican Sweet (BMS) (26) cells, as well as immature embryos from GS-3 (HiII equivalent), were used for transformation experiments. Transformation medi-

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Abbreviations: AHAS, acetohydroxyacid synthase; OND, oligonucleotide; PAT, phosphinothricin-*N*-acetyltransferase; GFP, green fluorescence protein; BMS, Black Mexican Sweet.

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ated by particle bombardment was performed according to Tomes *et al.* (27). *Agrobacterium*-mediated transformation was performed according to Ishida *et al.* (24).

Transformed cells were plated on solid culture medium containing either 0.7 μM imazethapyr (AC263, 499, or Pursuit, technical grade, American Cyanamid) for AHAS621 or 20 ppb chlorsulfuron (Glean, technical grade, DuPont) for AHAS165. Putative events were identified 4–6 weeks after bombardment and subsequently selected on fresh media containing 1.0–2.0 μM imazethapyr or 50 ppb chlorsulfuron.

The transgenic positive control lines were established by particle bombardment-mediated transformation of HiII cells with either pPHP10247 (AHAS621) or pPHP12322 (AHAS165) together with pPHP3528. Transformants expressing the *bar* gene were selected on media containing 3 mg/liter of bialaphos (Meiji Seika, Tokyo), and further selected on imazethapyr or chlorsulfuron. These transgenic events served solely as positive controls for imazethapyr or chlorsulfuron selection testing in culture and were not advanced for plant regeneration.

Stable lines with the PAT/GFP transgene were established via *Agrobacterium*-mediated transformation. Transformed immature embryos were selected on media containing 3 mg/liter of bialaphos. Plants were regenerated from HiII embyogenic callus containing verified converted PAT/GFP transgene according to Register *et al.* (28). Developing T_0 plantlets were transferred to soil and grown to maturity in the greenhouse. After pollination with HiII pollen, the T_1 seeds were collected. Forty seeds were germinated for progeny segregation analysis.

OND Synthesis, Labeling, and Plant Nuclease Resistance. Chimeric RNA/DNA ONDs were synthesized and purified according to ref. 11. Chimeric OND SC2 (12) was 3′ end-labeled with tetramethylrhodamine-6-dUTP (Boehringer Mannheim) by using terminal transferase according to the manufacturer's instructions.

Whole-cell extract was prepared from maize BMS cells by using a Bionebulizer (Glas-Col, Terre Haute, IN). Double-strand DNA, 2′-*O*-methyl-RNA, DNA/RNA hybrid, and RNA/DNA chimera, with similar length and secondary structure, were labeled with ^{32}P by using T4 polynucleotide kinase. Samples with the same amount of radioactivity were incubated with whole-cell extract at 17°C for 90 min. Controls included incubation in nuclease-free water and whole-cell extract inactivated at 65°C for 5–10 min. Results were examined by 12% PAGE and autoradiography. The percentages of intact OND in each sample were quantified from the autoradiogram by using ALPHAEASE software (Alpha Innotech, San Leandro, CA).

Chimeric OND Delivery. Chimeric ONDs were delivered to plant cells by particle bombardment. Briefly, onion epidermis was freshly prepared before bombardment. Cultured maize HiII or BMS cells were suspended in liquid N6 medium and then plated on a VWR Scientific glass fiber filter. Chimeric ONDs (0.4 μg) were coprecipitated with 15 μl of 2.5 mM $CaCl_2$ and 5 μl of 0.1 M spermidine onto 25 μg of 1.0-μm gold particles. Microprojectile bombardment was performed by using a Bio-Rad PDS-1000 He particle delivery system.

Fluorescent Microscopy. The *in vivo* fate of the rhodamine-labeled chimeric ONDs was monitored by using a Leica DM RB microscope with filter 41002b (Chroma Technology, Brattleboro, VT). Images were recorded by a CH350 charge-coupled device camera (Photometrics, Tucson, AZ). Superimposed images were processed by using Adobe Photoshop 4.0 (Mountain View, CA). Green fluorescence from GFP-expressing cells was surveyed by using a Leica MD-10 epifluorescence microscope with a Leica GFP filter set (10446093) 4 days after transformation. Images were recorded on Fujichrome Sensia film (ASA400).

PCR Amplification and Sequence Analysis. Target sequences were amplified from the extracted genomic DNA of putative events by *Pwo* polymerase (Boehringer Mannheim), with 30 cycles of 35 s at 95°C, 35 s at 60°C, and 35 s at 72°C. For the AHAS621 target, primers common to both AHAS108 and AHAS109 were designed as 5′-GCAGTGGGACAGGTTCTAT (PHN21971) and 5′-AGTCCTGCCATCACCATCCA (PHN21972). For the AHAS165 target, the following primers were used: 5′-ACCCGCTCCCCCGTCAT (PHN21973) and 5′-ATCTGCTGCTGGATGTCCTTGG (PHN21974). For the PAT/GFP target, primers used were: 5′-CGCAACGCCTACGACTGGA (PHN21976) and 5′-TGATGCCGTTCTTCTGCTTGTC (PHN21978). PCR fragments were purified and either cloned or directly sequenced in both directions on an Applied Biosystems ABI377 automated sequencer.

Restriction Fragment Length Polymorphism Analysis and Cloning. PCR fragments were digested with excess *Bfa*I (New England BioLabs) and analyzed by electrophoresis on gels containing 2% metaphor and 1.5% Seakem LE agarose (FMC) by using 1× Tris-borate EDTA. Undigested fragments were extracted and purified from gel slices by using a QIAquick gel extraction kit (Qiagen, Chatsworth, CA) and subcloned into the cloning vector pCR2.1-TOPO or pCR-Blunt (Invitrogen). Vectors containing subcloned fragment were transformed into Invitrogen's competent *Escherichia coli* One-Shot Top10 cells. Cloned fragments were sequenced by using M13 forward and reverse primers.

RESULTS

Nuclease Resistance and *in Vivo* Fate of Chimeric ONDs. First, we examined the stability of the radioactively labeled chimeric RNA/DNA ONDs in maize whole-cell extract. Quantitative analysis of the autoradiogram indicated that approximately 40–50% of chimeric ONDs remained intact after 90 min of incubation. To examine their fate *in vivo*, a rhodamine-labeled chimeric OND SC2 was bombarded into onion epidermis and BMS maize suspension culture cells. After bombardment, cells were rinsed with liquid culture medium and examined by fluorescence microscopy over time. Other than a diffuse signal in the cytoplasm, rhodamine fluorescence was localized mainly in nuclei and occasionally associated with gold

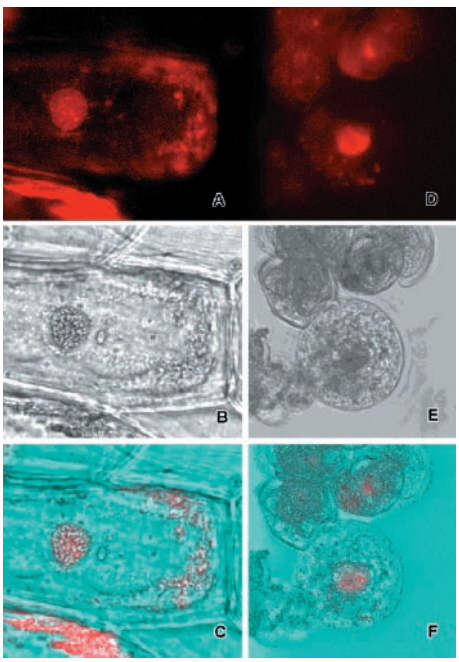

FIG. 1. Nuclear localization of rhodamine-labeled chimeric ONDs in onion epidermal cells (*A–C*) and maize BMS cells (*D–F*) 1 hr after bombardment, illustrated by rhodamine signal (*Top*), cellular organization (*Middle*), and their superimposed images (*Bottom*).

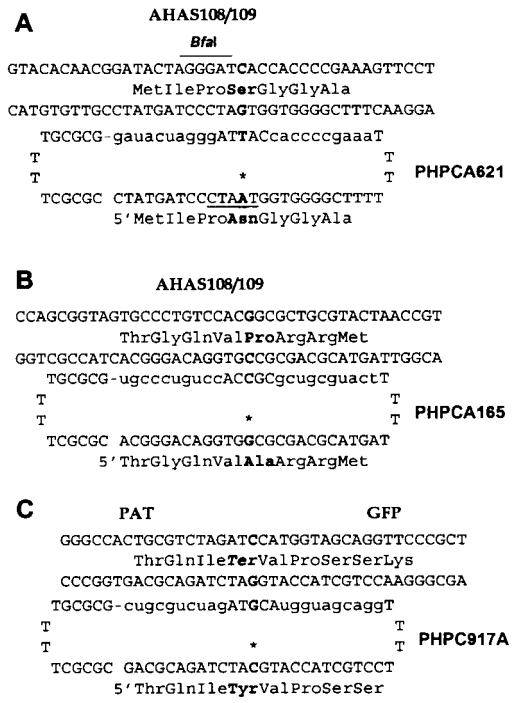

FIG. 2. Chimeric ONDs and target sequences of (*A*) AHAS Ser-621–Asn, (*B*) AHAS Pro-165–Ala, and (*C*) Ubi::PAT/GFP fusion Ter-996–Tyr. DNA residues are indicated in uppercase, and the modified RNA residues are shown in lowercase. Nucleotides in bold differ between the target sequence and chimeric OND. * indicates the nucleotide that should be introduced into the target sequence. The overscored sequence highlights the *Bfa*I restriction site and the underlined sequence indicates the site after sequence modification.

particles within cells (Fig. 1). We found that chimeric ONDs accumulate preferentially in the nuclei of these plant cells within 1 hr after bombardment. At 24 hr after bombardment, the rhodamine fluorescence was either very weak or no longer visible.

Conversion of Maize Endogenous *AHAS*. The endogenous gene target we chose for modification encodes AHAS (E.C. 4.1.3.18), the first enzyme in the biosynthetic pathway of branched chain amino acids. It is the target of imidazolinone and sulfonylurea herbicides (29, 30), and several mutations are known that confer resistance to these chemicals.

Maize AHAS Ser-621 corresponds to AHAS Ser-653 in *Arabidopsis* (17). A dominant single point mutation results in an amino acid substitution from Ser (AGT) to Asn (AAT) at the carboxyl terminal end of the mature AHAS, thus conferring resistance to the imidazolinone herbicide family. Two AHAS genes, *AHAS108* and *AHAS109*, previously have been reported in maize (31). In the maize HiII cell line, there are two copies of *AHAS108* and five copies of *AHAS109*, both of which are identical in nucleotide sequence at the target site (T.Z., M. Rudert, and C.L.B., unpublished data). Chimeric OND PHPCA621 was designed to modify Ser-621 in both AHAS108 and AHAS109, while simultaneously removing a *Bfa*I site (Fig. 2).

Maize AHAS Pro-165 corresponds to the AHAS Pro-196 site in tobacco (20). Various dominant mutations at this position lead to sulfonylurea herbicide resistance in many species (32). Chimeric OND PHPCA165 was designed to introduce a Pro-165–Ala mutation through a single nucleotide substitution from a CCG to a GCG in either *AHAS108* and *AHAS109*, because the two sequences are identical at this target site. No alteration of restriction sites was associated with this change (Fig. 2).

These chimeric ONDs were introduced independently into maize HiII and BMS cells by microprojectile bombardment. Resistant calli were selected on imazethapyr or chlorsulfuron, and the results are summarized in Table 1. In various negative controls (see below), five spontaneous mutants resistant to imazethapyr were identified from 86 plates, each plate containing approximately 10^6 cells, and one spontaneous mutant resistant to chlorsulfuron was selected from 50 plates. Thus the frequencies of spontaneous mutations conferring imazethapyr or chlorsulfuron resistance were 10^{-7}–10^{-8}.

Fragments containing the targeted region of *AHAS* from herbicide-resistant calli were amplified by PCR for sequence analysis. For AHAS621, mutant alleles first were identified by restriction fragment length polymorphism using *Bfa*I. Fragments containing the wild-type allele produced restriction fragments of 244 bp and 44 bp after digestion, whereas fragments with the mutant allele remained unrestricted by *Bfa*I (Fig. 3 *A* and *B*). In the positive control callus lines, where multiple copies of mutant *AHAS* were introduced by bombardment (data not shown), there are approximately equal amounts of restricted and unrestricted fragments, indicating multiple copies of endogenous wild-type *AHAS* genes. Among the fragments amplified from two herbicide-resistant calli obtained after chimeric OND treatment, a band corresponding to the unrestricted *Bfa*I fragment was clearly present (Fig. 3*B*). However, the restricted fragments were still prevalent, indicating that only a proportion of endogenous target sites were converted. The unrestricted fragments from both events were isolated and cloned. Sequence analysis of these clones indicated that 34 of 40 clones contain the change predicted by the specific chimeric OND (G to A, Fig. 3*C*). Unexpectedly, the remaining six clones contain three alternative mutations in adjacent bases (Table 2). Each of the three alternative mutations also resulted in loss of the *Bfa*I restriction site in the target sequence.

Sequence alterations from 16 additional herbicide-resistant calli and three controls were examined by direct sequencing of fragments amplified by PCR. Mutated target sequences were observed in fragments amplified from 11 of 16 chimeric OND-derived events and two positive control calli, but not in

Table 1. Summary of gene conversion experiments

Target	Cell type	Plates bombarded	Total cells receiving oligos*	Putative events selected	Events analyzed†	Confirmed mutation‡	Predicted conversion frequency§
AHAS621	HiII	130	2×10^5	40	18	13	1.4×10^{-4}
AHAS165	BMS	86	9×10^4	29	11	9	1.0×10^{-4}
PAT/GFP (T_0)	HiII	48	5×10^4	11	1	1	1.5×10^{-4}
PAT/GFP (T_1)	HiII	89	9×10^4	139	N/D	N/D	1.1×10^{-3}

*Total cells receiving chimeric ONDs were estimated by transient expression of GFP using bombardment of pPHP10699.
†Events were selected by their herbicide resistance or GFP phenotypes and analyzed by direct sequencing of PCR products or, where applicable, by restriction fragment length polymorphism and cloning.
‡Mutations induced by chimeric ONDs include those with the desired base change at the target site, as well as base changes at positions adjacent to the target sites (see Table 2).
§Predicted conversion frequency = (putative events selected × molecular confirmation rate)/total cells receiving chimeric ONDs.

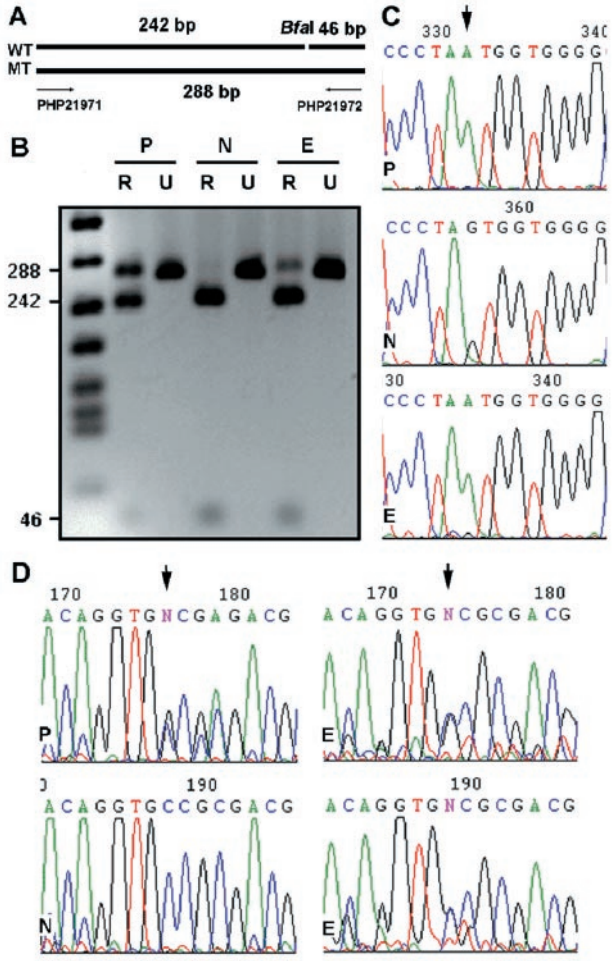

FIG. 3. AHAS621 conversion examined by restriction fragment length polymorphism and sequence analysis. (*A*) A map of the amplified AHAS621 target sequence from both wild-type and mutant alleles indicates the positions of PCR primers and the *Bfa*I restriction site. (*B*) Polymorphism of wild-type and mutant alleles in target PCR fragments from positive control (P), negative control (N), and a representative event (E) before (U) and after *Bfa*I restriction (R). (*C*) Sequence comparison of cloned AHAS621 alleles from the above samples. (*D*) Sequence comparison of cloned AHAS165 alleles from a positive control event (P), a negative control event (N), and two events with the predicted nucleotide conversion (E). Sequences were generated directly from PCR-amplified DNA from maize tissues. Because multiple AHAS genes exist in maize, as expected, both unconverted wild-type and converted mutant alleles are present in the events, as represented by the two overlapping peaks and the N nucleotide designation in the chromatograms.

wild-type callus. Thus, through direct sequencing of PCR products, 69% (11/16) of herbicide-resistant calli resulting from PHPCA621 treatment exhibited mutations at the target site.

For AHAS165, PCR fragments from target regions of resistant calli and a transgenic positive control line were sequenced directly. In the positive control callus expressing a Pro-165–Ala mutant form of maize AHAS from the ubiquitin promoter, the predicted change was detected from the chromatograms. The predicted conversion (Pro-165–Ala) also was detected from two of the chlorsulfuron-resistant calli analyzed (Fig. 3*D*). However, in a large proportion of the resistant calli, a T rather than the expected G was introduced at the predicted position, which resulted in a Pro→Ser conversion (Table 2). Nevertheless, because a substitution of Pro-165 with various amino acids will confer chlorsulfuron resistance, the mutations induced by PHPCA165 resulted in the desired phenotype.

No mutations were detected at the target position in herbicide-resistant calli arising by spontaneous mutation or in various negative controls, and no other mutations within 800 bp of surrounding sequence were found in any of the clones derived from the manipulated cells. The negative controls included: (*i*) unbombarded cells, (*ii*) cells bombarded with gold particles only, (*iii*) cells bombarded with PHPC917A chimeric OND that contains no homologous sequence to the AHAS target sites, and (*iv*) cells bombarded with a DNA-only version of PHPCA621.

Conversion of a *PAT/GFP* Transgene. The engineered transgene we used in this study is a stably integrated *PAT/GFP* fusion with a termination codon between the two genes, which prevents translation of the GFP protein. A chimeric OND (PHPC917A) was designed to replace G with C at nucleotide position 2990 (Fig. 3*B*), thereby eliminating the termination codon and allowing for expression of GFP as part of the PAT/GFP fusion protein. By using this scheme, we expected that modified cells should be identifiable by GFP fluorescence without chemical selection.

Two HiII transformants containing the *PAT/GFP* fusion gene were established by selection on bialaphos after *Agrobacterium*-mediated transformation. No GFP expressing cells were observed in either cell line (data not shown). Four days after introducing PHPC917A, GFP-expressing cells were identified in each cell line by fluorescence microscopy. In initial experiments using recipient cell lines that had been maintained in culture for 10 months, 11 GFP-positive events were detected in 48 bombardment plates. Subsequent experiments used freshly initiated cell lines derived from T_1 embryos of plants regenerated from the initial transformants; with introduction of PHPC917A, the frequency of GFP-positive cells was approximately 10-fold higher (Table 1). No GFP-positive cells were observed from various negative controls including: (*i*) unbombarded cells, (*ii*) cells bombarded with gold particles only, (*iii*) cells bombarded with a DNA version of PHPC917A, (*iv*) cells bombarded with nonspecific chimeric OND PHPCA621, and (*v*) wild-type cells bombarded with PHPC917A.

Table 2. Summary of mutations induced by chimeric ONDs

	Predicted change		Observed change		Frequency of sequence observed	
Target	Nucleotide	Amino acid	Nucleotide	Amino acid	Clones*	PCR fragments
AHAS621	AGT → AAT	Ser-621–Asn	AGT → AAT	Ser-621–Asn	34/40	13/16
			AGT → GGT	Ser-621–Gly	3/40†	0/16
			CCT → CCC	Pro-620–Pro	2/40†	0/16
			CCT → CAT	Pro-620–His	1/40†	0/16
AHAS165	CCG → GCG	Pro-165–Ala	CCG → GCG	Pro-165–Ala	–	2/12
			CCG → TCG	Pro-165–Ser	–	7/12
			CCG → ACG	Pro-165–Thr	–	1/12
PAT/GFP	TAG → TAC	Ter-996–Tyr	TAG → TAT	Ter-996–Tyr	–	1/1

*Clones were analyzed from two independent events, as described in text.
†Frequency enriched by *Bfa*I restriction.

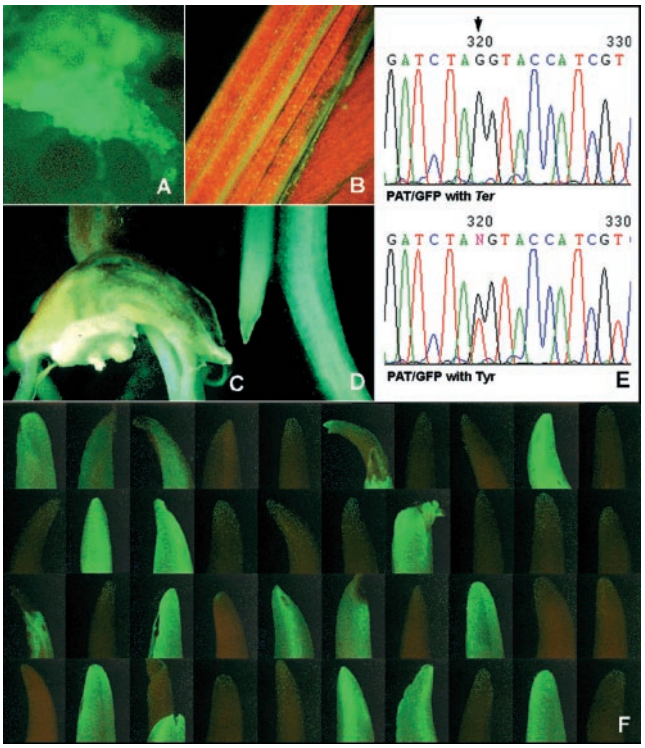

FIG. 4. GFP expression in T_0 callus (*A*), leaves (*B*), germinating embryo (*C*), and roots (*D*) after PAT/GFP conversion. (*E*) Chromatogram of the junction region of the targeted PAT/GFP showing sequence obtained directly from PCR amplification. (*F*) T_1 progeny segregation. In seedlings with GFP expression, strong green fluorescence was detected in the coleoptiles that lack chlorophyll. In contrast, coleoptiles in seedlings without GFP expression were semitransparent under the excitation and emission spectra used, and leaf chlorophyll autofluorescence could be seen through coleoptile tissue.

GFP-positive cell clusters (Fig. 4*A*) were excised and transferred to appropriate media for plant regeneration. GFP fluorescence was clearly seen in regenerating T_0 seedlings (Fig. 4 *B–D*). Sequence analysis of one of the T_0 convertants indicated replacement of the termination codon by a tyrosine codon. However, a T rather than the predicted C residue was found at the correct nucleotide position 2990 (Fig. 4*E*). The T_0 plants were fertile and produced viable seeds. Strong green fluorescence was observed from various tissues of T_1 seedlings, except in leaves where GFP fluorescence was masked by chlorophyll autofluorescence (data not shown). Analysis of 40 T_1 progeny indicated that 18 seedlings expressed GFP, consistent with a 1:1 Mendelian transmission of the modified PAT/GFP transgene (Fig. 4*F*).

DISCUSSION

Our results demonstrate that genes in maize can be modified at the nucleotide level with a high degree of precision by using chimeric RNA/DNA ONDs. Although chimeric ONDs with sequences identical to the target were not tested in this study, previous work in mammalian cells has shown that such ONDs apparently are not mutagenic (11, 12).

The overall frequencies of site-specific targeting by chimeric ONDs as reported here (10^{-4}, Table 1) are 2–3 orders of magnitude higher than frequencies of spontaneous mutation (10^{-7}–10^{-8}), and gene targeting by homologous recombination (10^{-5}–10^{-7}) in plant cells (2). However, the frequencies observed in plants are up to 3 orders of magnitude lower than the frequencies reported for chimeric OND-mediated nucleotide conversion in mammalian cells, depending on different target cell lines used (12, 14, 16). One explanation is that the frequency we observed might represent a conservative estimate because it is based primarily on events that survived chemical selection. Observed targeting frequencies using the PAT/GFP fusion target were higher, especially when healthy freshly initiated callus was used (Table 2). Although chemical selection provides a useful means for recovery of cells with the desired phenotype after targeting, some targeted cells may undergo cell cycle arrest as a result of DNA damage/repair (33, 34), and thus may not be easily recovered as colonies when additional stress is imposed. Other factors responsible for the different frequencies may involve experimental variables, such as method of delivery (bombardment vs. lipofection), or differences between mammalian and plant cells in the efficiencies of homologous pairing, strand transfer, or mismatch repair.

In addition to the predicted nucleotide conversions obtained in our studies, different mutated nucleotides were recovered in several cases from individual herbicide-resistant calli. However, it is not clear from our analysis whether any of the unexpected sequence changes result in the observed herbicide resistance. Similarly, it is not clear which of the multiple endogenous AHAS genes were mutated or if all cells in each callus contained each of the mutated AHAS forms detected by our analysis. It is possible that the diversity of mutations may result from decreased fidelity of the mismatch repair machinery in maize, as compared with mammalian cells. Error-prone mismatch repair may somehow be activated by specific sequences in the target region or by the affinity of the repair machinery for mismatched heteroduplex involving DNA and 2′-*O*-methyl RNA. At the very least, our data indicate that chimeric RNA/DNA ONDs could be used to assay the poorly characterized mismatch repair pathways in plants.

The proliferation of targeted cells and heritable transmission of targeted genes shown here suggest that chimeric RNA/DNA ONDs will be useful for applications in plants such as reverse genetics and crop improvement. With previous gene therapy applications, altered genes generally were not transmitted even through mitosis, because the targeted cells, such as lymphoblasts and hepatoma cells, were terminally differentiated (12–15), although a recent study demonstrated the mitotic stability of a corrected gene in melanocytes (16). The present system should provide opportunities for studying gene function through the ability to create targeted gene modifications and for the generation of novel traits in plants, without needing to introduce foreign genes.

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