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Chimeric RNA/DNA oligonucleotide-directed gene targeting in rice

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Abstract Site-specific mutagenesis in a rice genome was obtained by introducing chimeric RNA/DNA oligonucleotides (COs) by means of particle bombardment. Three COs were designed to target the independent codons for Pro-171, Trp-548 and Ser-627 of the endogenous rice acetolactate synthase (*ALS*) gene so it would confer resistance to *ALS*-inhibiting herbicides. Sequencing of the *ALS* gene of herbicide-resistant plants demonstrated that the *ALS* sequence was modified in a site-specific fashion. The efficiency of gene conversion mediated by COs was estimated to be 1×10^{-4} . These results demonstrate that CO-directed gene targeting is feasible in rice.

Keywords Acetolactate synthase · Chimeric RNA/DNA oligonucleotide · Gene targeting · Herbicide resistance · *Oryza sativa* L.

Abbreviations *ALS*: Acetolactate synthase · *BS*: Bispyribac-sodium · *Cf*: Chlorsulfuron · *CO*: Chimeric RNA/DNA oligonucleotide

Introduction

Chimeric RNA/DNA oligonucleotides (COs) have been used to cause site-specific base changes in chromosomal targets in mammalian and plant cells (for review: Hohn and Puchta 1999; Oh and May 2001; Graham and Dickson 2002). COs consist of 68 synthesized oligonucleotides which have a DNA ‘mutator’ region of five nucleotides complementary to the target site flanked by 2'-*O*-methyl RNA bridges of eight to twelve nucleotides

each. This DNA ‘mutator’ region includes a mutation that is introduced into the endogenous gene.

The first use of COs to cause site-specific base changes in plant cells was reported in maize (Beetham et al. 1999) and tobacco (Zhu et al. 1999). In both cases, gene targeting was directed towards generating base changes that resulted in a chemically selectable phenotype. The targeted gene was an acetohydroxyacid synthase (*AHAS*) gene in maize (Zhu et al. 1999, 2000) and an acetolactate synthase (*ALS*) gene in tobacco (Beetham et al. 1999). Both *ALS* and *AHAS* are the first common enzymes in the biosynthetic pathway of branched-chain amino acids, leucine, isoleucine and valine, and mutations of specific amino acids in these proteins have been known to confer resistance to sulfonylurea and imidazolinone herbicides (Shimizu et al. 2002). The efficiency of gene conversion mediated by COs in maize was estimated to be 10^{-4} . CO-directed gene conversions have been demonstrated to follow Mendelian patterns of segregation and are stable in subsequent generations (Zhu et al. 2000). Further studies in other species have not been reported to date.

In the investigation reported here, the feasibility of CO-directed gene targeting was examined in rice. We designed three COs to introduce single-point mutations in the rice *ALS* gene. Five plants resistant to *ALS*-inhibiting herbicide were selected, and the nucleotide sequence of the *ALS* gene of each was determined. Specific conversions were detected at the targeted bases.

Materials and methods

Plant material

A *japonica* rice variety *Taichung 65* (*Oryza sativa* L.) was used in the present study. A 6- to 8-day-old callus derived from scutellum of mature seeds (Nakagawa et al. 2001) and a 6- to 7-week-old callus derived from anther culture (Toriyama and Hinata 1985) were used as target cells. The callus was cultured on an N6 medium (Chu et al. 1975) supplemented with 30 g l⁻¹ sucrose, 2 mg l⁻¹ 2,4-dichlorophenoxyacetic acid, 2.88 g l⁻¹ L(-)-proline, 0.3 g l⁻¹ casamino acids and 3 g l⁻¹ Gelrite. The calli were placed on a plate in a circle 2.5 cm in diameter for delivery of the COs.

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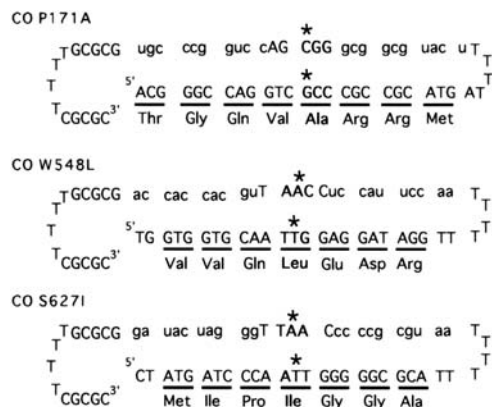


Fig. 1 Chimeric RNA/DNA oligonucleotide. Uppercase letters DNA residues, lowercase letters 2'-O-methyl-RNA residues, bold letters each target codon, * the nucleotide that should be introduced into the target sequence

COs design and delivery

Three COs were designed based on the sequence of the rice *ALS* gene (accession numbers AB049822 and AB049823 in the DDBJ databases). CO P171A was designed with the Pro-171 codon CCC altered to contain a mismatch codon GCC, which encodes alanine. CO W548L was designed to substitute TTG (a leucine codon) at codon 548 for TGG (a tryptophan codon). In CO S627I, AGT for serine was replaced by ATT for isoleucine (Fig. 1). COs were delivered to the callus cells by particle bombardment. They were co-precipitated with 150 μ l 2.5 mM CaCl₂ and 4 μ l 0.1 M spermidine onto 6 mg 1.6- μ m gold particles (Bio-Rad, Hercules, Calif.) that were suspended in 100 μ l 100% ethanol. An aliquot (10 μ l) of the resuspended gold particles was bombarded twice to each plate. Bombardment was carried out at 1,100 psi with a target distance of 10 cm using a Bio-Rad PDS-000 He delivery system. CO P171A (2 μ g) was delivered to callus derived from anther culture, and calli were selected using chlorsulfuron (Cf, Wako Chemicals, Tokyo, Japan). A mixture of CO W548L and CO S627I (0.1–0.5 μ g each) was delivered to callus derived from scutellum, and calli were selected using bispyribac-sodium (BS, Kumiai Chemical Industry Co, Shizuoka, Japan). Some calli were placed on a high osmotic N6 medium supplemented with 0.25 M each of mannitol and sorbitol 6 h pre- and 16–20 h post-bombardment in order to increase the efficiency of gene delivery (Kemper et al. 1996; Chen et al. 1998).

A plasmid containing the cauliflower mosaic virus 35S promoter and green fluorescent protein (35S-GFP, Chiu et al. 1996) was also delivered in order to be able to estimate the number of cells receiving gold particles.

Selection of herbicide-resistant callus

Two days after bombardment, calli were transferred onto an N6 medium supplemented with 1.4 μ M Cf or 0.5 μ M BS. They were subcultured every 2 weeks. Actively growing calli were transferred to a regeneration medium—MS (Murashige and Skoog 1962) medium supplemented with 2.1 μ M Cf or 0.5 μ M BS, 2 μ g l⁻¹ 6-benzylaminopurine, 1 μ g l⁻¹ α -naphthaleneacetic acid, 30 g l⁻¹ sucrose, 30 g l⁻¹ sorbitol, 2 g l⁻¹ casamino acids, and 4 g l⁻¹ Gelrite (pH 5.8). Regenerated plants were transferred to petri plates containing a rooting medium consisting of MS medium, 2.1 μ M Cf or 0.5 μ M BS, 30 g l⁻¹ sucrose and 8 g l⁻¹ agar and incubated under light at 28°C.

Molecular characterization of herbicide-resistant plants

Genomic DNA was extracted from leaves of the regenerated plants. Target sequences were amplified from the DNA using Ex *Taq* polymerase (Takara, Shiga, Japan) and the following primers—ALS primer 12 (5'-GCAACCAACCTCGTGTCCGC-3') and ALS primer 22 (5'-GCGTCATACAGGAAGCCTTC-3') for the experiment of CO P171A; ALS primer 13 (5'-GAATTGCGCTG-TTTTGTGA-3') and ALS primer R2 (5'-GGATCTGT-CGTCCTTCGATT-3') for CO W548L and CO S627I. Cycling conditions were 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 90 s, ending with a 3-min extension at 72°C. The amplified fragments were gel-purified using the Min Elute Gel Extraction kit (QIAGEN, Valencia, Calif.). Purified PCR products were either directly sequenced using a ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif.) or sequenced after cloning into a pCR 2.1 TOPO vector using TOPO TA cloning kits (Invitrogen, Carlsbad, Calif.).

Results

Introduction of CO P171A and selection of Cf-resistant plants

CO P171A was introduced to four plates of calli derived from anther culture. In the period 7–12 weeks following the bombardment, nine calli grew on the selective medium containing 1.4 μ M Cf, which were subsequently transferred onto the regeneration medium containing 2.1 μ M Cf. Two calli regenerated plants, although they were albino. One of the albino plants showed active growth on the rooting medium containing 2.1 μ M Cf; this was named Cfr#1 (Fig. 2A). In contrast, a wild-type plant did not show any root growth and eventually died in the same rooting medium (Fig. 2A).

Genomic DNA of Cfr#1 was extracted from leaves, and the target region of *ALS* was amplified by PCR for sequence analysis. Direct sequencing of the PCR products showed two overlapping peaks of C and G and, consequently, the N nucleotide designation in the chromatograms at the target nucleotide in codon 171 (Fig. 2B, panel 1). Sequencing of the cloned PCR products demonstrated that 7 of the 14 clones contained the predicted conversion sequence, G, at the target nucleotide (Fig. 2B, panel 3) and that the other seven clones exhibited a nucleotide C (Fig. 2B, panel 2), which was the same as that of the wild-type sequence (Fig. 2B, panel WT). These results indicated that both unconverted wild-type and converted mutant alleles were present in this plant. Sequencing of the whole coding region of *ALS* gene indicated that there were no other mutations (data not shown).

Introduction of CO W548L and CO S627I and selection of BS-resistant plants

A mixture of CO W548L and CO S627I was delivered into calli derived from scutellum of mature seeds. Cells with herbicide resistance were selected using BS, which is known to be a stricter selection agent than Cf for rice.

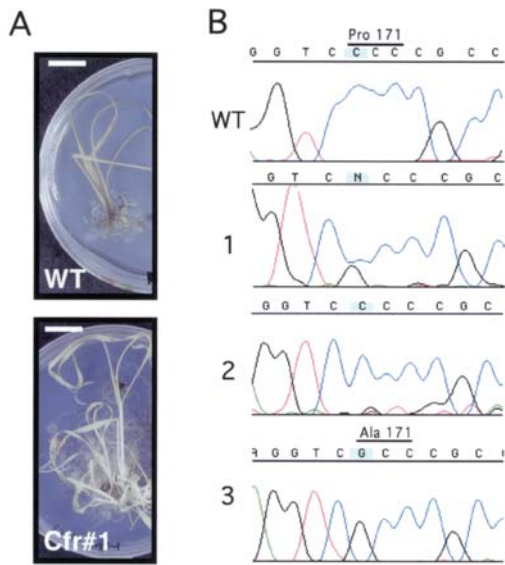


Fig. 2 Plants obtained after delivery of CO P171A show resistance to Cf (A) and nucleotide conversion in the target codon 171 (B). A A Cf-resistant albino plant actively growing in rooting medium containing $2.1 \mu\text{M}$ Cf (*Cfr#1*) in contrast to a wild-type plant (WT) showing withered brown leaves and no root growth after 3 weeks of culture. B Sequencing chromatograms of the *ALS* target region around codon 171. Direct sequencing indicated two overlapping peaks of C and G (1). Either unconverted nucleotide C (2), which is identical to that of the wild type (WT), or converted nucleotide G (3) was observed in the cloned PCR products

Two independent calli were selected from 32 plates of calli, and another two independent calli were obtained from ten plates of calli treated with the high osmotic medium pre- and post- bombardment. BS-resistant plants subsequently regenerated from these and were named BSr#1 to BSr#4 (Fig. 3A), but no BS-resistant calli were obtained from the 20 plates of non-bombarded calli that had been placed on the N6 medium containing $0.25 \mu\text{M}$ BS. Genomic DNA was isolated from the regenerated plants and used for PCR amplification of the target sequences. Direct sequencing of the PCR products showed two overlapping peaks of T and G and, consequently, the N nucleotide designation in the chromatograms at the target nucleotide in codon 548 (Fig. 3B, panel 1). Sequencing of the cloned PCR products demonstrated that five out of eight clones from BSr#1, two out of eight clones from BSr#2, three out of seven clones from BSr#3 and two out of six clones from BSr#4 contained the predicted conversion sequence T at the target nucleotide position (Fig. 3B, panel 3). The other clones exhibited the nucleotide G (Fig. 3B, panel 2), which was identical to that of the wild-type sequence (Fig. 3B, panel WT). However, no base changes were observed in the whole coding region of the *ALS* gene, including another target codon 627. These results indicated that both unconverted wild-type and converted mutant alleles were present in codon 548 in all four plants, BSr#1 to BSr#4.

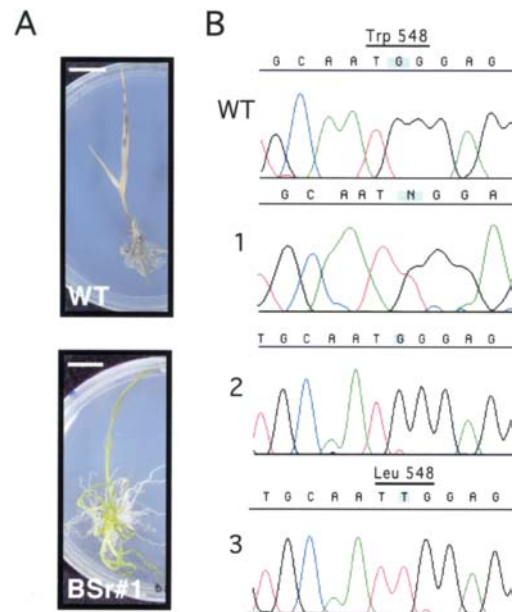


Fig. 3 Plants obtained after delivery of the mixture of CO W548L and CO S627I show resistance to $0.5 \mu\text{M}$ BS (A) and nucleotide conversion in the target codon 548 (B). A A BS-resistant green plant actively growing in rooting medium containing $0.5 \mu\text{M}$ BS (*BSr#1*) and a wild-type plant (WT) showing withered leaves and no root growth after 2 weeks of culture. B Sequencing chromatograms of *ALS* target region around codon 548. Direct sequencing indicated two overlapping peaks of G and T (1). Either unconverted nucleotide G (2), which is identical to that of the wild type (WT), or converted nucleotide T (3) was observed in the cloned PCR products

Efficiency of gene conversion

The number of cells receiving COs was estimated using the efficiency of the bombardment of the plasmid of 35S-GFP into scutellum-derived calli as an indicator. The number of cells exhibiting transient expression of GFP was 550 cells per plate when calli were plated on the N6 medium and 1,800 cells per plate when calli were treated with the high osmotic medium. In the experiment using a mixture of CO W548L and CO S627I, the efficiency of gene conversion per number of cells receiving COs was estimated to be 1.1×10^{-4} ($2/32/550$) for cells plated on the N6 medium and 1.1×10^{-4} ($2/10/1,800$) for cells plated on the high osmotic medium.

Discussion

We chose the *ALS* gene of rice as a target gene. The *ALS* gene is shown to exist as a single-copy gene in rice based on Southern blot analysis (unpublished data) and a BLAST search in DNA databases. CO-directed gene targeting is thought to be less complicated in rice than in tobacco and maize because tobacco and maize have an allotetraploid genome, and thus two copies of *ALS* genes.

The target position of rice Pro-171 corresponds to the tobacco Pro-196 site and the maize AHAS Pro-165 site. The mutation at this position is known to confer resistance to sulfonylurea herbicides, such as Cf, and has been used in model experiments of CO-directed gene targeting in tobacco and maize (Beetham et al. 1999; Zhu et al. 1999). Recently, mutations at both Trp-548 and Ser-627 have been reported to confer resistance to the novel pyrimidinylcarboxy herbicide BS (Shimizu et al. 2002). A single mutation at Trp-548 alone also provides BS resistance, although the degree of resistance is weaker than that of the double mutants (Shimizu et al. 2002). In the current study, we obtained plants in which only the nucleotide in the 548th codon for tryptophan was changed, although we used a mixture of CO W548L and CO S627I expecting the creation of double mutations. The co-delivery of two distinct COs that direct conversions at separate sites is the next subject to be achieved in order to direct base changes for non-selectable genes.

In previous studies of CO-directed gene targeting in maize and tobacco, nonspecific conversions were reported at one nucleotide 5' upstream of the targeted nucleotide, in addition to the precise expected alteration (Beetham et al. 1999; Zhu et al. 1999). In the current study, precisely the expected alterations were detected in all five plants examined. The efficiency of gene conversion was estimated to be approximately 1×10^{-4} , which is almost the same as that reported by Zhu et al. (1999) in their study of maize. Our results indicate that CO-directed gene targeting is feasible in rice and creates opportunities for basic studies of functional genomics and the manipulation of agricultural traits in rice.

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