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Cross-Resistance to Herbicides of Five ALS-Inhibiting Groups and Sequencing of the ALS Gene in *Cyperus difformis* L.

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Resistance to ALS-inhibiting herbicides in *Cyperus difformis* has evolved rapidly in many rice areas worldwide. This study identified the mechanism of resistance, assessed cross-resistance patterns to all five chemical groups of ALS-inhibiting herbicides in four *C. difformis* biotypes, and attempted to sequence the ALS gene. Whole-plant and ALS enzyme activity dose−response assays indicated that the WA biotype was resistant to all ALS-inhibiting herbicides evaluated. The IR biotype was resistant to bensulfuron-methyl, orthosulfamuron, imazethapyr, and propoxycarbazone-sodium and less resistant to bispyribac-sodium and halosulfuron-methyl, and susceptible to penoxsulam. ALS enzyme activity assays indicated that resistance is due to an altered target site yet mutations previously found to endow target-site resistance in weeds were not detected in the sequences obtained. The inability to detect resistance mutations in *C. difformis* may result from the presence of additional ALS genes, which were not amplified by the primers used. This study reports the first ALS gene sequence from *Cyperus difformis*. Certain ALS-inhibiting herbicides can still be used to control some resistant *C. difformis* biotypes. However, because cross-resistance to all five classes of ALS-inhibitors was detected in other resistant biotypes, these herbicides should only be used within an integrated weed management program designed to delay the evolution of herbicide resistance.

KEYWORDS: Acetolactate synthase; herbicide resistance; rice; smallflower umbrella sedge; sulfonylurea target site

INTRODUCTION

Since first reported in a wild carrot (*Daucus carota* L.) biotype resistant to 2,4-D (*1*), the increasing incidence of herbicide resistance worldwide has seriously worsened weed problems in agriculture. Today, at least 320 herbicide-resistant biotypes have been found in 185 weed species worldwide (*2*). Resistance to herbicides that inhibit the ALS enzyme (acetolactate synthase; also known as acetohydroxy acid synthase, AHAS; EC 2.2.1.6 formerly EC 4.1.3.18) has evolved rapidly and occurs more frequently than resistance to any other herbicide group (*2*−*4*).

The ALS enzyme is the primary target site of five classes of herbicides, namely sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinylthiobenzoates (PTB), and sulfonylamino-carbonyl-triazolinones (SCT). This enzyme catalyzes the first committed step in the branched-chain amino acid (valine, isoleucine, and leucine) biosynthetic pathway. Although enhanced metabolism can confer resistance to ALS-inhibiting herbicides (*5*), most cases of ALS herbicide resistance are due to one of several known nonsynonymous point mutations in the ALS gene, which result in target site insensitivity (*4, 6*). Six naturally occurring ALS gene mutations causing amino acid substitutions resulting in target site insensitivity have been found in weeds: Ala_{122} to Thr, Pro_{197} to several amino acids, Ala_{205} to Val, Asp_{376} to Glu, Trp_{574} to Leu, and Ser_{653} to Thr or Asn (*6, 7*).

The different classes of ALS-inhibiting herbicides have partially overlapping binding sites on the ALS enzyme (*8*). Therefore, point mutations at those sites can cause resistance to ALS-inhibiting herbicides in one or more herbicide classes and different cross-resistance patterns are determined depending on the mutation present (*6, 7*). In most plants studied, naturally occurring target site ALS herbicide resistance was reported to be controlled by a single nuclearly encoded gene (*3, 4*) that is dominant (*6*) or incompletely dominant (*9*). However, more than one ALS gene has been found in certain species (*3, 10, 11*). Complete determination of the differential expression of these genes and assessment of the implications for resistance have

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not been conducted. However, naturally occurring ALS target site resistance in weeds has been shown to be conferred by nonsynonymous point mutations in a single gene in all studies carried out to date (*3*). Most studies do not report the presence of introns in the ALS gene of higher plants (*6*), except for one study with sulfonylurea-resistant *Lindernia* spp. (*12*).

Cyperus difformis L. (smallflower umbrella sedge) is a highly self-pollinated C3-type annual aquatic species (*13*) and is one of rice's major weeds worldwide (*14*). It is usually present at high densities in rice fields where grain yields can be reduced by 22−43% (*14*). Resistance in *C. difformis* is a classical example of rapid adaptive evolution to ALS inhibitors. In 1992, after only four consecutive seasons of bensulfuron-methyl use in California rice, resistant biotypes of *C. difformis* and *Sagittaria montevidensis* Cham. & Schltdl. were found in two rice fields of the Sacramento Valley (*15*). Currently, only about 5% of the rice area is treated with bensulfuron-methyl due to resistance in the main targeted weeds, including *C. difformis* (California Rice Commission, unpublished). However, ALS-inhibiting herbicides are important herbicides in all crop systems due to their efficiency at low rates, flexibility of use, and mainly because of their favorable environmental profile and low mammalian toxicity (*16*) in comparison with other herbicide alternatives.

Cross-resistance to SU, IMI, and PTB herbicides has been found in *C. difformis* biotypes from California (*17*), Korea (*18*), Spain, and Italy (*19*, *20*). However, information on cross-resistance to all five chemical classes of ALS inhibitors at the whole-plant and target-site levels is not available for this species. In addition, the ALS gene sequence and nonsynonymous point mutations causing target site insensitivity have not been described. DNA sequences can be obtained using degenerate oligonucleotide primers designed from heterologous sequences when the gene of interest has never been sequenced. This approach can result in failures to amplify the desired target gene (*21*). However, if primer selection is based on sequences from closely related species, amplification conditions can often be optimized for the target species. Relationships among species can then be inferred using genetic distance and/or phylogenetic analyses (*22*).

In addition to bensulfuron-methyl, other ALS-inhibiting herbicides of the same or different chemical group have recently been introduced or are currently being investigated for use in rice production systems in California and worldwide. The impact of bensulfuron-methyl resistance on *C. difformis*'s response to these other herbicides is not known. Assessing cross-resistance patterns to ALS inhibitors in *C. difformis* biotypes is essential to determine the usefulness of other herbicides with the same mode of action for controlling this weed and to define rational herbicide use programs to delay or slow the evolution of resistance to ALS inhibitors. Associating causal mutations with observed cross-resistance patterns would help predict what specific ALS-inhibiting herbicides may or may not be successfully used and would also help elucidate the evolution and spread of resistance to ALS-inhibitors in *C. difformis* throughout California rice fields. In this study, we characterized resistance patterns using whole-plant dose−response assays and ALS-enzyme activity assays to establish if resistance was due to target-site insensitivity. We also sequenced the ALS gene to investigate possible target site mutations corresponding to the observed resistance patterns; the sequence obtained and the process of primer design led to inferences concerning genetic relationships among *C. difformis* and other species.

MATERIALS AND METHODS

Plant Material. Four *C. difformis* biotypes were used in the determination of cross-resistance patterns to ALS-inhibitors. Seeds from the IR biotype were collected in a rice farm in the Po Valley (Northern Italy), and seeds from the WA, BI, and AS biotypes were collected in rice fields of California's Northern Sacramento Valley. The California fields were separated by at least 100 km. Lack of control with bensulfuron-methyl was noted in the fields from where the IR, BI, and WA biotypes originated, while AS was a known susceptible accession (*17*). Five plants from each population were placed in individual greenhouses, covered with a plastic bag for isolation, and selfed twice.

Cross-Resistance Experiments. *Whole-Plant Assay.* Dose−response experiments were conducted in the greenhouse where average temperatures ranged from 24 to 30 °C, and natural sunlight was supplemented by 900 μmol m^{-2} s^{-1} photosynthetic photon-flux density from high-pressure sodium lamps to expose plants to 16 h daylight. Seeds of each of the four *C. difformis* biotypes were planted in individual 40 cm^2 pots filled with steam-sterilized Stockton clay adobe soil (fine, montmorillonite, thermic, Typic Pelloxert). Five days after emergence, plants were thinned to two uniform plants per pot. Pots were irrigated daily with a soluble fertilizer solution containing 6 g L^{-1} N, 27 g L^{-1} P_2O_5, 57 g L^{-1} K_2O (GrowMore No Boron, National Research and Chemical Co. Gardena, CA), 0.15 kg L^{-1} $MgSO_4$, and 0.3 kg L^{-1} $Ca(NO_3)_2$.

The herbicides were applied at 0, 0.125, 0.25, 0.5, 1, 2, and 4 times the recommended field rate (active ingredient). The herbicides applied and their recommended field rates (indicated in parentheses) were: bensulfuron-methyl (70 g ha^{-1}), halosulfuron-methyl (70 g ha^{-1}), orthosulfamuron (75 g ha^{-1}), imazethapyr (60 g ha^{-1}), bispyribac-sodium (30 g ha^{-1}), penoxsulam (30 g ha^{-1}), and propoxycarbazone-sodium (60 g ha^{-1}). Commercial formulations of the herbicides were used. The silicon-based adjuvant Kinetic (Helena Chemical Company, Collierville, TN) (0.125%, v/v) was added to bispyribac-sodium and 2.5% and 0.125% (v/v) crop oil concentrate (RNA Corporation, Fresno, CA) was added to the penoxsulam and orthosulfamuron, respectively. The herbicides were sprayed using a cabinet sprayer with an 8001-VS flat-fan nozzle calibrated to deliver 140 L ha^{-1} at a pressure of 275 kPa. Plants were sprayed at the four-leaf stage of growth, and 23 days after spraying, they were clipped at soil level to measure aboveground fresh weight. The experiment was conducted as a completely randomized design with six replicates and was repeated once.

ALS Activity Assay. C. difformis seeds were planted in 20 cm × 40 cm × 5 cm trays filled with soil. *C. difformis* biotypes, soil type, and growing conditions were the same as described earlier. ALS enzyme activity was assayed "in vitro" using a modification of Ray's (*23*) technique. This assay detects the acetoin produced after decarboxylation of the ALS enzyme product acetolactate; detailed procedures are described elsewhere (*5*, *17*). Herbicide concentrations used for the ALS activity assay were $0-1 \times 10^6$ nM for imazethapyr and $0-1 \times 10^5$ nM for bensulfuron-methyl, halosulfuron-methyl, orthosulfamuron, bispyribac-sodium, penoxsulam, and propoxycarbazone-sodium. The possible production of acetoin from other sources (*24*) was not considered. The experiment had three replicates and was conducted twice.

Statistical Analyses. Plant fresh weight and absorbance data were expressed as percentage of the mean untreated control value and were square root transformed. Both experiments were subject to ANOVA using the JMP IN software (Version 4.0.3 Academic, SAS Institute, Inc., Cary, NC) with experiments, herbicides, and herbicide doses as sources of variation. There were no significant ($P > 0.01$) experiment effects, thus data from repeated experiments were pooled. The interaction of herbicide and dose was significant ($P < 0.01$). The following four-parameter log−logistic model (*25*) was fit to the data for each herbicide and biotype:

$$Y = c + [d - c/1 + \exp[b(\log(x) - \log(e))]] \qquad (1)$$

where Y is the response expressed as percentage of the untreated control, c and d are asymptotic values of Y at the lower and upper limits, respectively, b is the slope of the curve around the point of inflection, and e is the herbicide rate giving response halfway between d and c

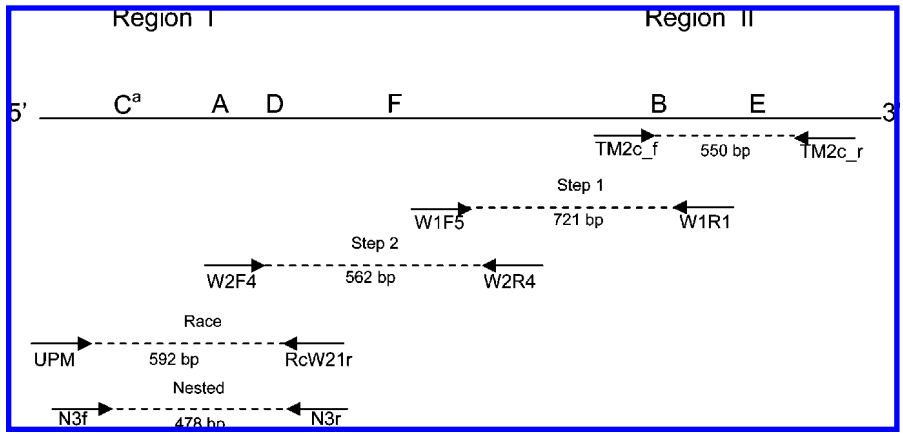

Figure 1. Schematic representation of the *Cyperus difformis* ALS gene amplification procedure. Primer names are as described in **Table 1**. Letters C, A, D, F, B, and E represent the conserved regions known as domains where mutations endowing herbicide-resistance have previously been found. Domain C: amino acids 115 to 133, mutation at Ala_{122}; domain A: amino acids 191 to 203, mutation at Pro_{197}; domain D: amino acids 205 to 219, mutation at Ala_{205}; domain F: amino acids 365 to 380, mutation at Asp_{376}; domain B: amino acids 573 to 576, mutation at Trp_{574}; domain E: amino acids 651 to 655, mutation at Ser_{653}; of the standard *Arabidopsis thaliana* ALS gene X51514 sequence (*35*).

Table 1. Nucleotide Sequences of the Oligonucleotide Primers Used for Amplification of the *Cyperus difformis* ALS Gene

primer name	sequence (5′ − 3′)	sequences used for primer design[a]	amplicon name[b]
M2cf	GTYGGRCARCAYCARATGTGGG	1, 2, and 8	region II
M2cr	TCCKGCCATCWCCWTCCRKK		
W1f5	NATGYTNGGNATGCAYGG	1, 3, 4, 5, 6, 7, and 8	step 1
W1r1	CATCAGGAAGGAACCATCACCGTC	region II	
W2f4	ACNGAYGCNTTYCARGARAC	1, 3, 4, 5, 6, 7, and 8	step 2
W2r4	TGCTTTGCTAACTGCATAAT	step 1	
RC2W21r	TTCAGGTTGCTTTGGCAGGCGAGAGGT CTAATACGACTCACTATAGGGCAAGCA	step 2	
UPM	GTGGTATCAACGCAGAGT CTAATACGACTCACTATAGGGC	provided with the race kit	race/region I
N3f	GATGTCCTCGTCTGAGGCTCT	race/region A	region I/nest
N3r	ATGATGCGAGGTATGTCATCTACGTC	race/region A	

[a] Numbers refer to the following species and GenBank nucleotide accession numbers: (1) *Amaranthus retroflexus* L. (AF363369); (2) *Bromus tectorum* L. (AF488771); (3) *Brassica napus* L. (Z11526); (4) *Camelina microcarpa* Andrz. ex DC. (AY428880); (5) *Helianthus annuus* L. (AY541451); (6) *Gossypium hirsutum* L. (Z46960); (7) *Nicotiana tabacum* L. (X07645); (8) *Oryza sativa* L. (AY885675). [b] Amplicon name as described in **Figure 1**.

(GR_{50} and I_{50} for plant fresh weight and enzyme activity, respectively). The four-parameter logistic model was fit to the data using the statistical freeware program R and the software package *drc* (*26*). Goodness of fit and coefficient significance were evaluated using lack-of-fit *F*-tests (*25*) and *t*-tests, respectively. For a given herbicide, lack of differences ($P > 0.05$) in *c* and *d* across a set of regressions (one for each biotype) allowed estimation of parameters common to all curves using the *collapse* procedure of the *drc* package (*26*). The magnitude of biotype resistance was quantified by a resistance index (RI = GR_{50} or I_{50} of the resistant biotype/GR_{50} or I_{50} of the susceptible biotype). Confidence intervals (95%) were calculated for RI, GR_{50} and I_{50} values.

ALS Gene Sequencing. *Genomic DNA Extraction.* Leaves from the plants and biotypes used for the ALS activity assay were individually sampled at the four-leaf stage for DNA extraction. Genomic DNA was extracted from frozen leaf tissue of five plants per biotype using the CTAB (cetyltrimethylammonium bromide) DNA protocol (*27*). The resulting DNA concentration was determined using 1.5% agarose gel electrophoresis under ethidium bromide staining and a Low DNA Mass Ladder (Invitrogen Corporation, Carlsbad, CA) as reference.

ALS Gene Isolation: Regions I and II. Because no information regarding the ALS gene sequence of any species of *Cyperaceae* was available, 18 regular and degenerate oligonucleotide primers for polymerase chain reaction (PCR) intended to amplify regions I and II of the ALS gene (**Figure 1**) were designed based on the nucleotide and amino acid sequences of ALS enzymes from the following species, and the respective GenBank (National Center for Biotechnology Information, Bethesda, MD, http://www.ncbi.nlm.nih.gov/) nucleotide accession numbers: *Amaranthus retroflexus* L. (AF363369), *Arabidopsis thaliana* (L) Heynh (X51514), *Bromus tectorum* L. (AF488771), *Brassica napus* L. (Z11526), *Camelina microcarpa* Andrz. ex DC. (AY428880), *Helianthus annuus* L. (AY541451), *Gossypium hirsutum* L. (Z46960), *Lolium multiflorum* Lam. (AF310684), *Nicotiana tabacum* L. (X07645), *Oryza sativa* L. (AY885675), *Raphanus raphanistrum* L. (AJ344986), *Triticum aestivum* L. (AY210408), *Zea mays* L. (X63553) and *Xanthium strumarium* L. (U16279). These species represent several plant families where the ALS gene has been sequenced. The ClustalW program (EMBL-EBI, European Bioinformatics Institute, Cambridge, UK, http://www.ebi.ac.uk/clustalw/) was used for sequence alignment and primers were designed using Primo Pro and Primo Degenerate (Chang Bioscience, Inc., Castro Valley, CA, http://www.changbioscience.com) software. Primers were synthesized by MWG-Biotech Inc. (MWG-Biotech Inc., High Point, NC). PCR amplification was conducted in a 15 μL mixture of 1× *Taq* buffer, $MgCl_2$ (2.0 mM), each forward and reverse primer (0.5 μM), dNTPs (200 μM), one unit of *Taq* DNA polymerase (QIAGEN Inc., Valencia, CA) and 20 ng genomic DNA. Amplification was conducted in an MJ Research PTC-200 thermocycler (Bio-Rad Laboratories Inc., South San Francisco, CA) using the following cycles: DNA denaturation for 3 min at 94 °C, and 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, and 1.5 min elongation at 72 °C. Finally, the samples were subjected to a 10 min elongation at 72 °C. PCR products were analyzed in 1.5% agarose gels stained with ethidium bromide using a 100 bp DNA ladder (Promega Corporation, Madison, WI) for reference.

The degenerate oligonucleotide primers M2cf and M2cr (**Table 1**) produced a clear 551 bp amplification product corresponding to the expected size. These oligonucleotide primers amplified region II of the ALS gene corresponding to domains B and E (**Figure 1**). The amplified fragment was purified using ExoSAP-IT (USB Corporation, Cleveland, OH) following the manufacturer's instructions, and sequenced. The PCR sequencing reaction was performed using 15 ng of the amplicon,

forward or reverse primers (1.7 μM), BigDye 3.1 sequencing reaction mix (Applied Biosystems, Foster City, CA) (1 μL), and 0.5X BigDye 3.1 sequencing buffer (Applied Biosystems, Foster City, CA) in 5 μL total volume. The PCR sequencing product was precipitated using ethanol/sodium acetate. The resulting DNA fragment was resuspended in 10 μL formamide and denatured for 5 min at 95 °C, after which samples were maintained on ice for 5 min. Sequencing products were loaded on an automated capillary sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA). Both strands of the DNA amplicon were sequenced using M2c forward and reverse primers individually. Sequences were edited with the Chromas 1.3 software (Conor McCarthy, Griffith University, Brisbane, Queensland, Australia), and nucleotide sequences were manipulated and aligned using BioEdit v7.0.5 (Ibis Therapeutics, Carlsbad, CA). The nucleotide sequence obtained was queried using Blastn (National Center for Biotechnology Information, Bethesda, MD, http://www.ncbi.nlm.nih.gov/) analysis resulting in 86% identity with the standard ALS gene sequence from *Arabidopsis thaliana* gene (X51514) and indicating that the sequence obtained corresponds to the ALS gene.

Attempts to amplify region I of the ALS gene (**Figure 1**) did not result in any ALS gene sequence. Different PCR reagent concentrations (*28*), PCR cycles (*29*), PCR additives (*30*), primer characteristics (*21*), and DNA *Taq* polymerases were tested without successful amplification of region I. Therefore, a "targeted gene walking" procedure was used to amplify this region starting from region II of the ALS gene. This procedure is used to amplify unknown DNA sequences adjacent to a short known sequence of the gene of interest. It consists of designing a regular primer based on the known sequence and a second "walking" primer is designed according to putative homologous sequences.

Targeted Gene Walking PCR. In the first step of this procedure (step 1), the amplification started in region II of the ALS gene and proceeded toward the 5′ end (**Figure 1**). Several reverse regular oligonucleotide primers were derived from the sequence of region II obtained as described in the preceding section and several degenerate forward oligonucleotide primers were designed based on ALS amino acid sequences available in the GenBank database described earlier. The forward walking primer W1F5 and the reverse targeted primer W1R1 (**Table 1**) produced an amplicon with the expected size of 721 bp. This fragment was excised from the gel and purified using a modification of the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA) procedure whereby two independent dry spin steps were added after the ethanol rinsing procedure in order to remove ethanol traces from the final DNA extract. The PCR procedure and the purification and sequencing of amplification products was conducted as described earlier. For the second amplification step (step 2) shown in **Figure 1**, several reverse targeted primers were designed based on the sequence obtained in step 1 and several degenerate forward walking primers were designed based on the ALS amino acid sequences available in the GenBank database. The primer pair W2F4 and W2R4 amplified a 562 bp fragment corresponding to a section of the ALS gene that included domains D and F (**Figure 1**). A similar approach was used to attempt to obtain sequences for the remaining domains C and A. However, the ALS gene sequence from this latter region could not be obtained.

RACE PCR. The RACE (Rapid Amplification of cDNA Ends) (Clontech, Mountain View, CA) procedure consists of a gene sequence isolation strategy to overcome problems with DNA amplification through regular PCR. The RACE procedure was used to obtain the remaining sequences for domains C and A toward the 5′ end of the ALS gene (**Figure 1**). RNA was extracted from approximately 100 mg of fresh leaf tissue from four-leaf stage plants of the AS biotype using the RNeasy Plant mini kit (QIAGEN Inc., Valencia, CA). RNA quality and quantity were evaluated by measuring absorbance at 260 nm and through electrophoresis using 1.5% agarose gels stained with ethidium bromide and a Low DNA Mass Ladder (Invitrogen Corporation, Carlsbad, CA). Synthesis of cDNA for the 5′ RACE procedure was conducted using the Smart Race cDNA amplification kit starting with 1.0 μg of total RNA. The gene-specific regular reverse primer Rc2W21r (**Table 1**) for the RACE amplification was based on the step 2 sequence obtained earlier in the targeted gene walking procedure (**Figure 1**). The RACE PCR cycling program used followed the manufacturer's instructions (Clontech, Mountain View, CA). The RACE PCR fragment was excised from the gel, purified as described earlier, cloned into a plasmid vector and transformed into competent *Escherichia coli* using the Topo-TA Cloning (Topo-TA Cloning) procedure. Five colonies were chosen and suspended individually in 20 μL of water. PCR was conducted using one μL of this suspension as template and the universal oligonucleotide M13 reverse and M13 forward (−20) primers. PCR products were checked for the expected size and the amplicon excised and purified as described earlier. DNA sequencing was conducted in both directions using the M13 reverse and M13 forward (−20) primers. Nucleotide sequence manipulation and alignment were conducted as previously described. A single amplicon of about 592 bp was obtained and queried in the GenBank database using Blastn analysis and was found to have high similarity with the ALS gene.

Nested PCR of Region I. The RACE procedure enabled the sequencing of domains C and A. However, this procedure is laborious for large numbers of samples. Therefore, a nested PCR for sequencing all biotypes studied was conducted using genomic DNA as template and several regular forward and reverse primers designed from the RACE sequence. The primers N3f and N3r produced a single 478 bp amplicon (**Figure 1**) that was cloned and sequenced using the methods described earlier. The sequence obtained was similar to the 5′ end of the ALS gene according to Blastn analysis and corresponds to the region where domains C, A, and D are located (**Figure 1**).

In summary, by using multiple approaches including amplification of region II, targeted gene walking steps 1 and 2, and the nested PCR procedure, we sequenced the full ALS gene for all four biotypes. The partial sequences obtained were arranged in a unified 1709 bp contig that has been deposited in the GenBank database with the accession number EF061294 (**Figure 2**).

Comparison of ALS Gene Sequences. The ALS-gene sequence obtained in this study is the first for *C. difformis* in the GenBank database. We compared this sequence to those of the other species in GenBank in order to provide information that could be useful for primer design for other species with unknown ALS-gene sequences. The analysis was based on a 350 bp fragment of ALS region I where domains C, A, and D are located (**Figure 1**). Sequences were aligned using ClustalW software. Genetic distances between species were estimated using Kimura's two-parameter model (*31*):

$$d_{AB} = -1/2 \ln[(1 - 2P - Q)\sqrt{1 - 2Q}] \qquad (2)$$

where P and Q are the observed rates of transitions and transversions, respectively, between the two sequences. Transitions are changes within the purine (A and G) and pyrimidine (C and T) groups and transversions are changes between the groups.

RESULTS AND DISCUSSION

Herbicide Cross-Resistance. The *C. difformis* biotypes studied differed in their aboveground biomass responses to the herbicides used (**Table 2**). Comparing biotype responses to that of the susceptible (S) control biotype AS indicated the WA biotype was resistant (R) to all herbicides (RI values ranged from 5.9 to 21.1). The IR biotype was R to bensulfuron-methyl, orthosulfamuron, imazethapyr, and propoxycarbazone-sodium (RI $\geq$ 6.9), but less resistant to bispyribac-sodium (RI = 4.7). Biotypes with RI values $\leq$2.0 in whole-plant assays are generally considered to indicate susceptibility (*32*). Thus, IR was S to halosulfuron-methyl, as observed in a previous study (*20*), and to penoxsulam, while BI was S to all herbicides (RI values ranged from 0.6 to 1.9). The susceptibility of biotypes with RI $\leq$ 2 (*32*) can indicate variability related to natural herbicide tolerance among biotypes regardless the evolution of any mechanism of herbicide resistance.

Biotype responses in the ALS-activity assays (**Table 3**) reflected those of the whole plant assays (**Table 2**), suggesting all cases of resistance involved a target-site alteration. Furthermore, the enzyme assay results suggested that the lower whole-plant resistance to bispyribac-sodium (RI = 4.7) observed in

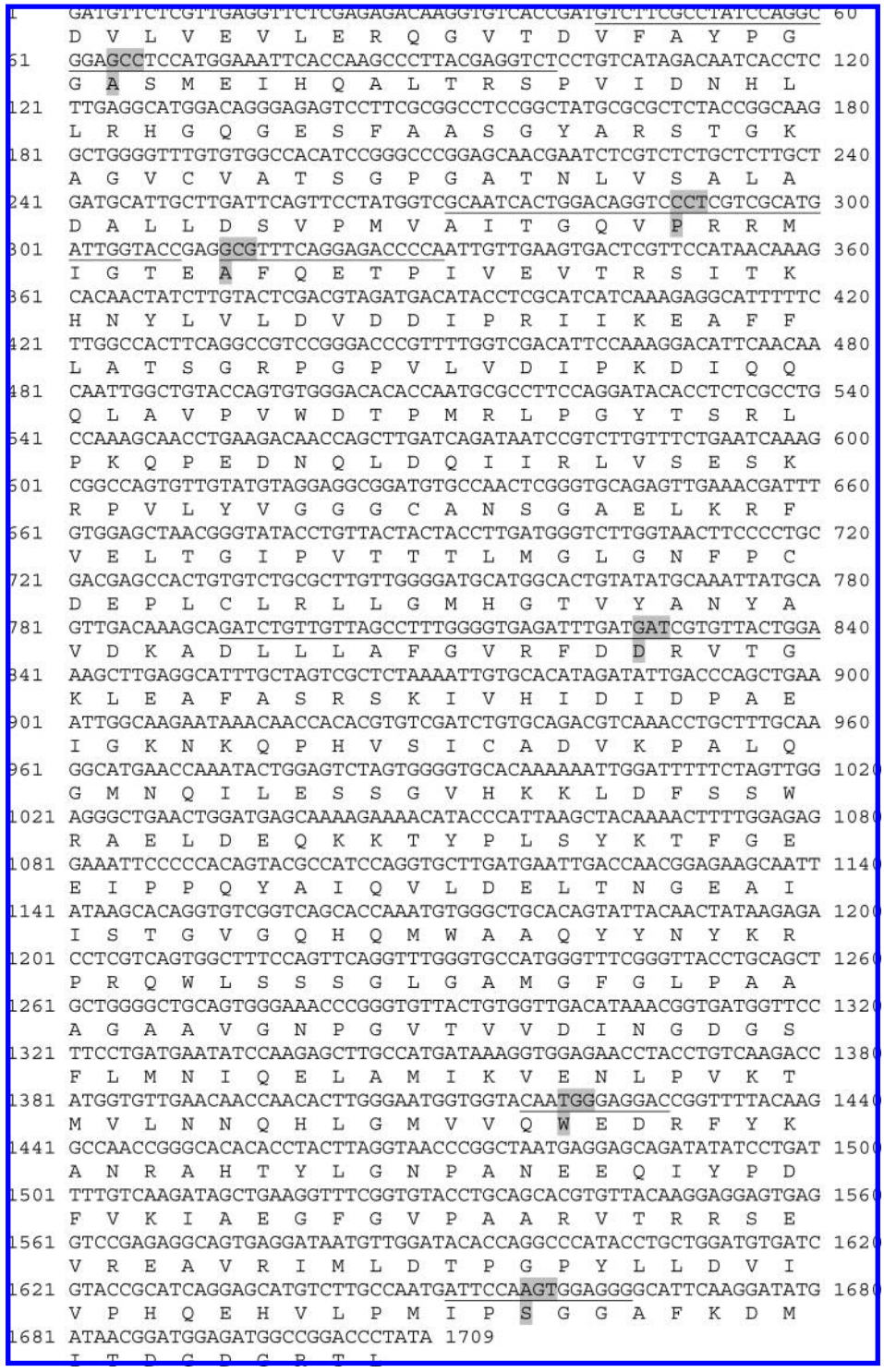

Figure 2. Consensus nucleotide and deduced amino acid sequence of the *Cyperus difformis* ALS gene obtained in this study for four biotypes (GenBank accession number EF061294). Positions of the highly conserved amino acid domains are underlined and show the mutations (shaded) identified in other studies to cause field-evolved resistance to ALS inhibiting herbicides. In the order they are shown, the domains and amino acid substitutions found to confer resistance in other studies are: domain C (amino acid 115 to 133, Ala_{122}), domain A (amino acid 191 to 203, Pro_{197}), domain D (amino acid 205 to 219, Ala_{205}), domain F (amino acid 365 to 380, Asp_{376}), domain B (amino acid 573 to 576, Trp_{574}), and domain E (amino acid 651 to 655, Ser_{653}), of the standard *Arabidopsis thaliana* ALS gene X51514 sequence (*35*). See **Figure 1** for positions of the domains within regions I and II.

the biotype IR (**Table 2**) relates to a lower sensitivity at the target site (RI = 5.7 in **Table 3**). Plant processes such as absorption, transport, and metabolic detoxification modulate herbicide action in whole-plants (*5*, *33*). Most of these nontarget-site factors are removed in the enzymatic assays where extracted proteins are isolated from cell components and differences in target site sensitivities among biotypes are best resolved. In this study, the GR_{50} values of the IR and WA biotypes for propoxycarbazone-sodium were much closer than their respective I_{50} values thus indicating that the ALS of WA is an order of magnitude more resistant than that of IR (**Tables 2** and **3**). Differences in levels of target-site resistance to a certain herbicide have been previously observed among biotypes, even when resistance is endowed by the same mutation (*33*). These

Table 2. Parameters of the log−logistic Equations[a] Used to Calculate the Herbicide Concentrations Required for 50% Reduction of Aboveground Fresh Weight (GR_{50}) of *Cyperus difformis* Biotypes in Whole-Plant Dose−Response Studies, and Ratios of Resistant to Susceptible GR_{50} Values (Resistance Index, RI)[a]

				e (GR_{50})		RI[b]	
herbicide/biotype	*b*	*c*	*d*	g · ha^{-1}	95% CI[c]	ratio	95% CI[c]
				Bensulfuron-methyl (SU)[d]			
IR	3.4	4.3	97.8	191.1**[e]	183.6−198.6	14.8**	14.1−15.5
WA	1.6			123.5**	116.3−130.7	9.6**	9.0−10.2
BI	1.9			22.3**	21.1−23.5	1.7**	1.6−1.8
AS	3.5			12.9**	12.5−13.4		
				Halosulfuron-methyl (SU)			
IR	1.5	1.6	97.1	10.6**	9.8−11.4	1.3**	1.2−1.4
WA	5.7			124.1**	119.0−129.2	15.2**	14.1−16.3
BI	1.1			4.6**	4.0−5.2	0.6**	0.5−0.7
AS	1.8			8.2**	7.6−8.8		
				Orthosulfamuron (SU)			
IR	4.3	3.3	96.3	177.4**	171.3−183.5	15.9**	15.0−16.8
WA	3.0			238.9**	229.0−248.8	21.1**	19.8−22.4
BI	1.8			10.5**	9.8−11.2	0.9	0.8−1.0
AS	2.3			11.1**	10.6−11.6		
				Imazethapyr (IMI)			
IR	3.5	2.1	96.5	169.1**	163.3−174.9	6.9**	6.4−7.3
WA	2.3			143.9**	137.6−150.2	5.9**	5.5−6.3
BI	1.7			39.1**	37.1−41.1	1.9**	1.8−2.0
AS	1.2			24.3**	22.8−25.8		
				Bispyribac-sodium (PTB)			
IR	1.4	3.9	96.8	24.1**	22.5−25.7	4.7**	4.4−5.0
WA	3.7			79.1**	76.2−82.0	15.3**	14.6−16.0
BI	1.6			4.3**	4.0−4.6	0.8*	0.7−0.9
AS	3.0			5.1**	4.9−5.3		
				Penoxsulam (TP)			
IR	2.9	5.2	96.9	18.5**	17.8−19.2	2.0**	1.0−2.1
WA	3.8			89.2**	85.9−92.5	9.7**	9.1−10.3
BI	1.9			9.1**	8.7−9.5	1.0	0.9−1.1
AS	1.3			9.1**	8.6−9.6		
				Propoxycarbazone-sodium (SCT)			
IR	2.0	2.1	97.6	159.3**	151.3−167.3	16.0**	15.1−16.9
WA	3.8			183.6**	176.7−190.5	18.4**	17.4−19.4
BI	2.8			11.1**	10.7−11.5	1.1*	1.0−1.2
AS	2.1			9.9**	9.5−10.3		

[a] $Y = c + [d - c/1 + \exp[b(\log(x) - \log(e))]]$, where *Y* is the response expressed as percentage of the untreated control, *c* and *d* are asymptotic values of *Y* at the lower and upper limits, respectively, *b* is the slope of the curve around the point of inflection, *e* is the herbicide rate giving response halfway between *d* and *c* (made common for all four curves from each herbicide); $P < 0.001$ for all log−logistic equations. [b] Resistance index: GR_{50} ratio between the correspondent biotype and the susceptible AS biotype. [c] Values in this column are 95% confidence intervals. [d] ALS-inhibiting herbicide class: SU, sulfonylurea; IMI, imidazolinone; PTB, pyrimidinylbenzoate; TP, triazolopyrimidine; SCT, sulfonylaminocarbonyltriazolinone. [e] * and **represent $P \leq 0.05$ and $P \leq 0.01$, respectively, according to *t*-tests performed on coefficients of the four-parameter logistic model.

differences can result from posttranslational alterations that modify the level of expression of the ALS gene or be due to differences in the small subunit of the ALS enzyme among biotypes (*33*).

In this study, evolved resistance to bensulfuron-methyl did not result in broad cross-resistance among SU herbicides. Thus the IR biotype exhibited resistance to bensulfuron-methyl and orthosulfamuron but was very sensitive to halosulfuron-methyl. A single amino acid substitution in the channel of access to the active site of the ALS can result in insensitivity to an herbicide that normally binds to the amino acid residue (*18*), but the same mutation may not affect as much the binding of other herbicides from the same chemical group that do not interact with this amino acid residue. Differences among biotypes in cross-resistance patterns within an herbicide chemical group have also been found for IMI herbicides (*18*). An increase in ALS gene expression is unlikely to cause this cross-resistance pattern in the IR biotype because an increase in gene expression would not be specific for a certain ALS herbicide but not others.

Target-site cross-resistance in *C. difformis* to SU, PTB, and IMI herbicides has been previously reported (*17, 18*) and similar patterns have been observed in whole-plant studies but with no indication of the mechanisms involved (*19, 20*). Responses to TP and to the new SCT group of ALS-inhibiting herbicides were not evaluated in those studies. This is thus the first time cross-resistance among all five classes of ALS inhibitors has been evaluated in *C. difformis*. Although SCT herbicides are not currently used in rice, knowledge on full-spectrum cross-resistance is essential for comprehensive ALS-resistance management in *C. difformis*.

Table 3. Parameters of the log−logistic Equations[a] Used to Calculate the Herbicide Concentrations Required for 50% Reduction of the ALS Activity (I_{50}) of Susceptible and Resistant Biotypes of *Cyperus difformis* from ALS Enzyme Activity Studies, and Ratios of the I_{50} Values (Resistance Index, RI)[a]

				e (I_{50})		RI[b]	
herbicide/ biotype	*b*	*c*	*d*	nM	95% CI[c]	ratio	95% CI[c]
Bensulfuron-methyl (SU)[d]							
IR	0.8	2.5	95.3	2066.9**[e]	1838.9−2294.9	333.1**	287.1−379.1
WA	0.5			1890.3**	1608.3−2172.3	304.6**	255.6−353.6
BI	0.5			14.9**	12.7−17.1	2.4**	2.0−2.8
AS	0.7			6.2**	5.5−6.9		
Halosulfuron-methyl (SU)							
IR	0.7	8.6	92.6	5.9**	5.1−6.7	2.9**	2.4−3.4
WA	0.9			492.9**	426.9−558.9	238.1**	199.1−277.1
BI	0.6			3.6**	3.0−4.2	1.7*	1.4−2.0
AS	1.0			2.0**	1.7−2.3		
Orthosulfamuron (SU)							
IR	0.6	2.2	95.0	3280.0**	2148.9−4412.9	644.4*	353.4−935.4
WA	0.7			6544.3*	3829.3−9259.3	1285.4*	667.4−1903.4
BI	0.3			14.3*	8.3−20.3	2.8	1.4−4.2
AS	0.4			5.1*	3.1−7.1		
Imazethapyr (IMI)							
IR	0.5	6.6	95.3	26368.0**	25115.0−27621.0	46.4**	36.4−56.4
WA	0.6			8879.3**	7376.3−10382.3	15.6**	12.6−18.6
BI	0.6			430.3**	355.3−505.3	0.7	0.6−0.8
AS	0.5			567.1**	460.1−674.1		
Bispyribac-sodium (PTB)							
IR	0.8	0.3	90.1	70.2**	62.1−78.3	5.7**	4.9−6.5
WA	0.7			467.3**	408.3−526.3	38.5**	32.5−44.5
BI	0.6			21.3**	18.3−24.3	1.7**	1.5−1.9
AS	0.6			12.1**	10.5−13.7		
Penoxsulam (TP)							
IR	0.6	5.5	105.5	1.9**	1.5−2.3	0.7	0.6−0.8
WA	0.4			84.0**	61.0−107.0	32.0**	24.0−40.0
BI	0.5			2.6**	2.0−3.2	0.9	0.7−1.1
AS	0.3			2.6**	1.9−3.3		
Propoxycarbazone-sodium (SCT)							
IR	0.6	9.5	99.7	2189.9**	1934.7−2345.8	32.0*	29.2−34.7
WA	0.8			11522.0**	3111.6−2183.5	167.4*	12.5−322.2
BI	0.5			75.1[ns]	55.0−95.4	1.0	−10.3−12.3
AS	0.5			68.8[ns]	52.0−86.9		

[a] $Y = c + [d - c/1 + \exp[b(\log(x) - \log(e))]]$, where Y is the response expressed as percentage of the untreated control, c and d are asymptotic values of Y at the lower and upper limits, respectively, b is the slope of the curve around the point of inflection, e is the herbicide rate giving response halfway between d and c (made common for all four curves from each herbicide); $P < 0.001$ for all log−logistic equations. [b] Resistance index: GR_{50} ratio between the correspondent biotype and the susceptible AS biotype. [c] Values in this column are 95% confidence intervals. [d] ALS-inhibiting herbicide class: SU, sulfonylurea; IMI, imidazolinone; PTB, pyrimidinylbenzoate; TP, triazolopyrimidine; SCT, sulfonylaminocarbonyltriazolinone. [e] * and ** represent $P \leq 0.05$ and $P \leq 0.01$, respectively, according to *t*-tests performed on coefficients of the four-parameter logistic model.

ALS Gene Sequencing and Comparisons. Because the ALS "in vitro" assay suggested that a target site alteration could be the cause of resistance in the WA and IR biotypes, we attempted to isolate and sequence the ALS gene in order to search for the ALS gene mutations previously observed to confer resistance to ALS inhibitors in weeds. The procedure was successful with amplification of region II using the M2cf and M2cr degenerate primer set (**Table 1**) designed based on the ALS gene sequences from *Amaranthus retroflexus* L., *Arabidopsis thaliana* L., *Bromus tectorum* L., and *Oryza sativa* L. A similar approach and several PCR adjustments did not result in amplification of region I of the ALS gene. Therefore, a targeted gene walking approach toward the 5′ end of the ALS gene was used by defining the starting point on the DNA sequence obtained for region II. Thus, two amplicons corresponding to the sequence between regions I and II were obtained (**Figure 1**). However, even using this technique, we still could not amplify region I of the ALS gene. The RACE approach was then attempted successfully. GC-rich DNA regions are generally difficult to amplify using regular *Taq* DNA polymerases and standard PCR reagents and amplification conditions (*34*). Analysis of the region I sequence (**Figure 2**) shows a GC content of 71%, suggesting that this could be the cause of the amplification difficulty in this region.

Using the multiple approaches, we obtained ALS gene sequences for five individuals of each of the four *C. difformis* biotypes. The sequences correspond to a 1709 bp coding region (**Figure 2**), equivalent to nucleotide positions 609 to 2317 of the standard *Arabidopsis* ALS gene sequence X51514 (*35*). We did not detect any variation in nucleotide and amino acid sequences among individuals of a biotype and also did not observe any variation between the S and R biotypes analyzed.

Table 4. Genetic Distances between the ALS Gene Sequence (Region I Where the Domains C, A, and D are located, **Figure 1**) Obtained for *Cyperus difformis* and the ALS Sequences of the Species Used for Primer Design

	CMAMI[a]	ARBTH	BRSNN	RAPRA	AMARE	HELAN	XANSI	NIOTA	GOSHI	BROTE	ORYSA	TRIAE	LOLMU	ZEAMX	CYPDI
CMAMI	0.000														
ARBTH	0.102	0.000													
BRSNN	0.201	0.181	0.000												
RAPRA	0.212	0.197	0.069	0.000											
AMARE	0.414	0.367	0.398	0.422	0.000										
HELAN	0.308	0.310	0.317	0.321	0.326	0.000									
XANSI	0.289	0.303	0.320	0.309	0.299	0.140	0.000								
NIOTA	0.368	0.380	0.348	0.326	0.322	0.252	0.256	0.000							
GOSHI	0.380	0.356	0.306	0.279	0.324	0.285	0.292	0.249	0.000						
BROTE	0.504	0.518	0.384	0.355	0.456	0.395	0.434	0.416	0.475	0.000					
ORYSA	0.538	0.521	0.398	0.389	0.454	0.364	0.444	0.420	0.433	0.093	0.000				
TRIAE	0.508	0.507	0.394	0.381	0.455	0.390	0.440	0.417	0.468	0.045	0.080	0.000			
LOLMU	0.549	0.552	0.422	0.418	0.477	0.424	0.470	0.436	0.472	0.084	0.087	0.077	0.000		
ZEAMX	0.473	0.479	0.360	0.324	0.445	0.382	0.441	0.405	0.430	0.097	0.111	0.0121	0.121	0.000	
CYPDI	0.465	0.459	0.426	0.410	0.360	0.418	0.443	0.469	0.450	0.330	0.343	0.365	0.354	0.328	0.000

[a] CMAMI (*Camelina microcarpa* Andrz. ex DC.), ARBTH (*Arabidopsis thaliana* (L.) Heynh), BRSNN (*Brassica napus* L.), RAPRA (*Raphanus raphanistrum* L.), AMARE (*Amaranthus retroflexus* L.), HELAN (*Helianthus annuus* L.), XANSI (*Xanthium strumarium* L.), NIOTA (*Nicotiana tabacum* L.), GOSHI (*Gossypium hirsutum* L.), BROTE (*Bromus tectorum* L.) ORYSA (*Oryza sativa* L.), TRIAE (*Triticum aestivum* L.), LOLMU (*Lolium multiflorum* Lam.), ZEAMX (*Zea mays* L.) and CYPDI (*Cyperus difformis* L.).

Thus, the mutations causing the target site insensitivity that was detected by the ALS activity assay were not found. In studies of other weed species, amino acid substitutions at Ala_{205}, Pro_{197}, Asp_{376}, and Trp_{574} were found to result in broad cross-resistance to SU, IMI, TP, and PTB herbicides (*6*, *7*, *36*), which is the cross-resistance pattern we observed in the WA biotype. It is difficult to predict the mutations that would account for the complex resistance pattern exhibited by the IR biotype. Mutations at Pro_{197} can result in resistance to SU herbicides and to variable levels of resistance and even susceptibility to other groups (*36*). Mutations at this site can also endow simultaneous resistance and susceptibility among SU herbicides (*37*). However, a mutation endowing strong resistance to the SU and IMI herbicides, combined with low resistance or susceptibility to other groups, as detected in this study, has not been previously reported (*36*). Establishing which mutations underlie the observed target site responses would require sequencing all the expressed copies of the ALS gene in *C. difformis*.

The ALS gene sequence obtained in this study does not correspond to the phenotypic expression of the ALS enzyme in response to treatment of plants with the various herbicides used in the enzyme assay experiment. Thus, our results suggest that more than one ALS gene may be present in *C. difformis*, as has been observed in other species. Two and three ALS genes have been found in the diploid common sunflower (*Helianthus annuus* L.) in two different studies (*38*, *11*). In sunflower, 48 single nucleotide polymorphisms (SNPs) were found in the AHAS1 gene, a single six-base insertion-deletion in the AHAS2 gene, and a single SNP in the AHAS3 gene (*11*). No polymorphism in AHAS2 was found among the 25 inbred lines analyzed (*11*). Mutations present in AHAS1 corresponded to cross-resistance patterns to the ALS-inhibiting herbicides, suggesting that this is the gene related to the ALS enzyme expression. Similarly, in another study (*10*), five ALS genes were present in the allotetraploid *Brassica napus*, where two of them encoded the ALS enzyme and the other three appeared to be defective or were not expressed. The difficulties with isolation and sequencing of the ALS gene in *C. difformis* in this study may be related to the existence of multiple copies of ALS genes in this species also. Evidence for variable ploidy levels (diploidy and tetraploidy) and chromosome deletions in *C. difformis* can be found in the literature (*39*−*41*). Further studies, such as Southern blotting and genetic mapping, are required to verify this hypothesis and identify the specific ALS genes conferring target-site resistance to the ALS-inhibiting herbicides in *C. difformis*.

Comparison of the genetic distances between the ALS gene sequence obtained for *C. difformis* in this study and the reference sequences used for primer development indicated substantial differences. As expected, genetic distances between species within the same plant family were smaller than those observed between species from different plant families. For instance, within the Poaceae, genetic distances varied from 0.012 between *Triticum aestivum* and *Zea mays* to 0.121 between *L. multiflorum* and *Zea mays* (**Table 4**). Within the dicots, genetic distances varied from 0.069 between two species in the Brassicaceae, namely, *Brassica napus* and *Raphanus raphanistrum*, to 0.422 between *Amaranthus retroflexus* and *R. raphanistrum*, species in the Amaranthaceae and Brassicaceae, respectively (**Table 4**). Not surprisingly, the ALS gene sequence observed for the monocot species *C. difformis* is more similar to the ALS sequences found in grasses, with genetic distances ranging from 0.328 to 0.365 than that of dicots, with genetic distances ranging from 0.360 to 0.469 (**Table 4**). Despite the greater similarity to the grasses, the ALS sequence obtained for *C. difformis* is still quite different from the grasses, which likely accounts for the difficulties encountered with amplification of the ALS gene based on primers designed from grass and dicot species.

In summary, ALS enzyme activity assays indicated that target-site herbicide-resistance to all groups of ALS-inhibitors exists in *C. difformis*. However, cross-resistance patterns varied among the biotypes studied, suggesting that certain ALS-inhibiting herbicides can still be used to control some resistant biotypes. For instance, susceptibility to halosulfuron-methyl and to penoxsulam in the biotype IR suggests these ALS-inhibitors commonly used in rice can still succeed to control *C. difformis* in rice fields infested with this biotype. The choice of herbicide can only be made once extant cross-resistance patterns in a given field have been determined. However, because the potential to select for resistance to any of these herbicides exists in *C. difformis*, ALS-inhibiting herbicides should only be used within an integrated weed management program. The different cross-resistance patterns possibly result from different mutations of the ALS gene or

even from the possible combined effects of different mutations in different copies of the gene, although no resistance mutations previously observed to confer resistance in other weeds could be detected. This study reports the first nucleotide and deduced amino-acid sequence of an ALS gene from *Cyperus difformis* (GenBank accession number EF061294).

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LITERATURE CITED

(1) Switzer, C. M. The existence of 2,4-D-resistant strains of wild carrot. *Proc. Northeast Weed Control Conf.* **1957**, *11*, 315–318.
(2) Heap, I. International Survey of Herbicide Resistant Weeds (Online), 2008; www.weedscience.com (Accessed 01 September 2008).
(3) Warwick, S. I.; Sauder, C.; Beckie, H. J. Resistance in Canadian biotypes of wild mustard (*Sinapsis arvensis*) to acetolactate synthase inhibiting herbicides. *Weed Sci*. **2005**, *53*, 631–639.
(4) Corbett, C. A. L.; Tardif, F. J. Detection of resistance to acetolactate synthase-inhibiting in weeds with emphasis on DNA-based techniques: a review. *Pest Manage. Sci*. **2006**, *62*, 584–597.
(5) Fischer, A. J.; Bayer, D. E.; Carriere, M. D.; Ateh, C. M.; Yim, K. O. Mechanisms of resistance to bispyribac-sodium in an *Echinochloa phyllopogon* accession. *Pestic. Biochem. Physiol*. **2000**, *68*, 156–165.
(6) Tranel, P. J.; Wright, T. R. Resistance of weeds to ALS-inhibiting herbicides: What have we learned? *Weed Sci*. **2002**, *50*, 700–712.
(7) Whaley, C. M.; Wilson, H. P.; Westwood, J. H. A new mutation in plant ALS confers resistance to five classes of ALS-inhibiting herbicides. *Weed Sci*. **2007**, *55*, 83–90.
(8) McCourt, J. A.; Pang, S. S.; King-Scott, J.; Guddat, L. W.; Duggleby, R. G. Herbicide-binding sites revealed in the structure of plant acetohydroxyacid synthase. *Proc. Natl. Acad. Sci. U.S.A*. **2006**, *103*, 569–573.
(9) Volenberg, D. S.; Stoltenberg, D. E. Inheritance of resistance in eastern black nightshade (*Solanum nigrum*) to acetolactate synthase-inhibiting herbicides. *Weed Sci*. **2002**, *50*, 731–736.
(10) Rutledge, R. G.; Ouellet, T.; Hattori, J.; Miki, B. L. Molecular characterization and genetic-origin of the brassica-napus acetohydroxyacid synthase multigene family. *Mol. Gel. Genet*. **1991**, *229*, 31–40.
(11) Kolkman, J. M.; Slabaugh, M. B.; Bruniard, J. M.; Berry, S.; Bushman, B. S.; Olungu, C.; Maes, N.; Abratti, G.; Zambelli, A.; Miller, J. F.; Leon, A.; Knapp, S. J. Acetohydroxyacid synthase mutation conferring resistance to imidazolinone or sulfonylurea herbicides in sunflower. *Theor. Appl. Genet*. **2004**, *109*, 1147–1159.
(12) Uchino, A.; Watanabe, H. Mutations in the acetolactate synthase genes of sulfonylurea-resistant biotypes of *Lindernia* spp. *Weed Biol. Manage*. **2002**, *2*, 104–109.
(13) Merotto, A., Jr.; Jasieniuk, M.; Fischer, A. J. Estimating the outcrossing rate of smallflower umbrella sedge (*Cyperus difformis* L.) using resistance to ALS-inhibiting herbicides and SRAP molecular markers. *Weed Res*. **2008**, *49*, 29−36.
(14) Swain, D. J.; Nott, M. J.; Trounce, R. B. Competition between *Cyperus difformis* and rice: the effect of time of weed removal. *Weed Res*. **1975**, *15*, 149–152.
(15) Pappas-Fader, T.; Turner, R. G.; Cook, J. F.; Butler, T. D.; Lana, P. J.; Carriere, M. D. Resistance monitoring program for aquatic weeds to sulfonylurea herbicides in California rice fields. *Rice Technol. Work Group* **1993**, *25*, 165.
(16) Shaner, D. L. Resistance to Acetolactate Synthase (ALS)-inhibiting in the United States: history, occurrence, detection, and management. *J. Weed Sci. Technol*. **1999**, *44*, 405–411.
(17) Osuna, M. D.; Vidotto, F.; Fischer, A. J.; Bayer, D. E.; De Prado, R.; Ferrero, A. Cross-resistance to bispyribac-sodium and bensulfuron-methyl in *Echinochloa phyllopogon* and *Cyperus difformis*. *Pestic. Biochem. Physiol*. **2002**, *73*, 9–17.
(18) Kuk, Y. I.; Kim, K. H.; Kwon, O. D.; Lee, D. J.; Burgos, N. R.; Jung, S.; Guh, J. O. Cross-resistance pattern and alternative herbicides for *Cyperus difformis* resistant to sulfonylurea herbicides in Korea. *Pest Manag3. Sci*. **2004**, *60*, 85–94.
(19) Ruiz-Santaella, J. P.; Bakkali, Y.; Osuna, M. D.; de Prado, R. Evaluation of resistance in *Cyperus difformis* populations to ALS inhibiting herbicides. *Comm. Agric. Appl. Biol. Sci*. **2004**, *69*, 91–96.
(20) Busi, R.; Vidotto, F.; Fischer, A. J.; Osuna, M. D.; De Prado, R.; Ferrero, A. Patterns of resistance to ALS herbicides in smallflower umbrella sedge (*Cyperus difformis*) and ricefield bulrush (*Schoenoplectus mucronatus*). *Weed Technol*. **2006**, *20*, 1004–1014.
(21) Rose, T. M.; Schultz, E. R.; Henikoff, J. G.; Pietrokovski, S.; McCallum, C. M.; Henikoff, S. Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. *Nucleic Acids Res*. **1998**, *26*, 1628–1635.
(22) Gaut, B. S.; Morton, B. R.; McCaig, B. C.; Clegg, M. Substitution rate comparisons between grasses and palms: Synonymous rate differences at the nuclear gene *Adh* parallel rate differences at the plastid gene *rbcL*. *Proc. Natl. Acad. Sci. U.S.A*. **1996**, *93*, 10274–10279.
(23) Ray, T. B. Site of action of chlorsulfuron. *Plant Physiol*. **1984**, *75*, 827.
(24) Forlani, G.; Mantelli, M.; Nielsen, E. Biochemical evidence for multiple acetoin-forming enzymes in cultured plant cells. *Phytochemistry* **1999**, *50*, 255–262.
(25) Seefeldt, S. S.; Jensen, J. E.; Fuerst, E. P. Log−logistic analysis of dose−response relationships. *Weed Technol.* **1995**, *9*, 218–227.
(26) Ritz, C.; Streibig, J. C. Bioassay analysis using R. *J. Stat. Software* **2005**, *12* (5), 1−22, http:\\www.bioassay.dk.
(27) Doyle, J. J.; Doyle, J. L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull.* **1987**, *19*, 11–15.
(28) Don, R. H.; Wainwright, B. J.; Mattick, J. S. Touchdown PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res*. **1991**, *19*, 4008.
(29) Levano-Garcia, J.; Verjovski-Almeida, S.; Silva, A. C. R. Mapping transposon insertion sites by touchdown PCR and hybrid degenerate primers. *BioTechniques* **2005**, *38*, 225–229.
(30) Chakrabarti, R.; Schutt, C. E. The enhancement of PCR amplification by low molecular weight amides. *Nucleic Acids Res*. **2001**, *29*, 2377–2381.
(31) Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol*. **1980**, *16*, 111–120.
(32) Valverde, B. E.; Riches, C. R.; Caseley, J. C. *Prevention and Management of Herbicide-Resistant Weeds in Rice: Experiences from Central America with Echinochloa colona*. Cámara de Insumos Agropecuarios: San Jose, Costa Rica, 2000; p 123.
(33) Milliman, L. D.; Riechers, D. E.; Wax, L. M.; Simmons, F. W. Characterization of two biotypes of imidazolinone-resistant eastern black nightshade (*Solanum ptycantum*). *Weed Sci*. **2003**, *51*, 139–144.
(34) Henke, W.; Herdel, K.; Jung, K.; Schnorr, D.; Loening, S. A. Betaine improves the PCR amplification of GC-rich DNA sequences. *Nucleic Acids Res*. **1997**, *25*, 3957–3958.
(35) Sathasivan, K. G.; Haughn, G. W.; Murai, N. Nucleotide sequence of a mutant acetohydroxyacidsynthase gene from and imizadolinone-resistant *Arabidopsis thaliana* var. Columbia. *Nucleic Acids Res*. **1990**, *18*, 2188.
(36) Tranel, P. J.; Wright, T. R.; Heap, I. M. ALS mutations from herbicide-resistant weeds (Online). 2008; www.weedscience.com (accessed 01 September 2008).

(37) Roux, F.; Matejicek, A.; Reboud, X. Response of *Arabidopsis thaliana* to 22 ALS inhibitors: baseline toxicity and cross-resisitane of *csr*1−1 and *csr*1−2 resistant mutants. *Weed Res.* **2005**, *45*, 220–227.

(38) White, A. D.; Graham, M. A.; Owen, M. D. K. Isolation of acetolactate synthase homologs in common sunflower. *Weed Sci.* **2003**, *51*, 845–853.

(39) Bir, S. S.; Cheema, P.; Sidhu, M. SOCGI plant chromosome number reports: VII. *J. Cytol. Genet.* **1988**, *23*, 219–228.

(40) Tejavathi, D. H.; Nijalingappa, B. H. M. Cytological studies in some members of Cyperaceae. *Cytologia* **1990**, *55*, 363–372.

(41) Cheema, P.; Bir, S. S. Cytomorphological studies in *Cyperus* Linn. V. Section *Cyperus* Linn. from Punjab, NW India. *Proc. Indian Sci. Congr. Assoc.* **1995**, *82* (3:VIII), 82–83.

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