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Acetohydroxyacid synthase mutations conferring resistance to imidazolinone or sulfonylurea herbicides in sunflower

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Abstract Wild biotypes of cultivated sunflower (*Helianthus annuus* L.) are weeds in corn (*Zea mays* L.), soybean (*Glycine max* L.), and other crops in North America, and are commonly controlled by applying acetohydroxyacid synthase (AHAS)-inhibiting herbicides. Biotypes resistant to two classes of AHAS-inhibiting herbicides—imidazolinones (IMIs) or sulfonylureas (SUs)—have been discovered in wild sunflower populations (ANN-PUR and ANN-KAN) treated with imazethapyr or chlorsulfuron, respectively. The goals of the present study were to isolate *AHAS* genes from sunflower, identify mutations in *AHAS* genes conferring herbicide resistance in ANN-PUR and ANN-KAN, and develop tools for marker-assisted selection (MAS) of herbicide resistance genes in sunflower. Three *AHAS* genes (*AHAS1*, *AHAS2*, and *AHAS3*) were identified, cloned, and sequenced from herbicide-resistant (mutant) and -susceptible (wild type) genotypes. We identified 48 single-nucleotide polymorphisms (SNPs) in *AHAS1*, a single six-base pair insertion-deletion in *AHAS2*, and a single SNP in *AHAS3*. No DNA polymorphisms were found in *AHAS2* among elite inbred lines. *AHAS1* from imazethapyr-resistant inbreds harbored a C-to-T mutation in codon 205 (*Arabidopsis thaliana* codon nomenclature), conferring resistance to IMI herbicides, whereas *AHAS1* from chlorsulfuron-resistant inbreds harbored a C-to-T mutation in codon 197, conferring resistance to SU herbicides. SNP and single-strand conformational polymorphism markers for *AHAS1*, *AHAS2*, and *AHAS3* were developed and genetically mapped. *AHAS1*, *AHAS2*, and *AHAS3* mapped to linkage groups 2 (*AHAS3*), 6 (*AHAS2*), and 9 (*AHAS1*). The C/T SNP in codon 205 of *AHAS1* cosegregated with a partially dominant gene for resistance to IMI herbicides in two mutant × wild-type populations. The molecular breeding tools described herein create the basis for rapidly identifying new mutations in *AHAS* and performing MAS for herbicide resistance genes in sunflower.

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Introduction

Wild biotypes of cultivated sunflower (*Helianthus annuus* L.), a species native to North America (Rogers et al. 1982), are weeds in corn (*Zea mays* L.), soybean (*Glycine max* L.), and other crops (Schweizer and Bridge 1982; Geier et al. 1996). Controlling sunflowers in corn and soybean was difficult before sulfonylurea (SU) and imidazolinone (IMI) herbicides were introduced (Al-Khatib et al. 1998). Such herbicides have since been widely used to control sunflowers in corn, soybean, and other crop rotations and have selected for herbicide resistance in wild sunflowers (Al-Khatib et al. 1998, 1999; White et al. 2002, 2003; Heap 2003).

SU and IMI herbicides are specific and potent inhibitors of acetohydroxyacid synthase (AHAS, EC 2.2.1.6), also known as acetolactate synthase (ALS), the enzyme that catalyzes the first step in branched-chain amino acid biosynthesis (Umbarger 1978; Duggleby and Pang 2000).

AHAS-inhibiting herbicides impair the synthesis of branched-chain amino acids, thereby severely or fatally disrupting metabolism in herbicide-susceptible genotypes (Shaner 1991; Tranel and Wright 2002; Pang et al. 2003). Species differ in herbicide susceptibility and can develop resistance to different classes of AHAS inhibitors. With few exceptions (Christopher et al. 1992), resistances to AHAS-inhibiting herbicides, in otherwise susceptible species, are caused by point mutations in genes encoding AHAS that reduce the sensitivity of the enzyme to herbicide inhibition (Umbarger 1978; Saari et al. 1989; Subramanian et al. 1990; Jander et al. 2003). In plants, five highly conserved amino acids (Ala_{122}, Pro_{197}, Ala_{205}, Trp_{574}, and Ser_{653}) have been identified that, when mutated, confer resistance or cross-resistance to one or more AHAS-inhibiting herbicides (Tranel and Wright 2002; Jander et al. 2003). Other than Ser_{653} (Bernasconi et al. 1995; Patzoldt et al. 2001), the five amino acids are conserved among wild types (herbicide-susceptible genotypes) across genera.

Spontaneous mutations conferring resistance to AHAS-inhibiting herbicides can rapidly increase in frequency in wild populations under strong herbicide selection (Tranel and Wright 2002). The first weed biotype resistant to AHAS-inhibiting herbicides was discovered in prickly lettuce (*Lactuca serriola* L.) after five generations of chlorsulfuron treatment (Mallory-Smith et al. 1990). Weed biotypes resistant to AHAS-inhibiting herbicides have since been identified in more than 80 species, more than any other herbicide group (Gressel and Segel 1978; Tranel and Wright 2002; Heap 2003;). Typically, resistant biotypes are selected in populations chronically exposed to specific AHAS-inhibiting herbicides (Mallory-Smith et al. 1990; Primiani et al. 1990). Common sunflower populations with cross-resistance to both SU and IMI herbicides were first discovered in 1996 in Kansas (Al-Khatib et al. 1998) and South Dakota (White et al. 2002) in fields that had been repetitiously treated with herbicides for 7–8 years. Presently, AHAS resistance has been confirmed in common sunflower from Kansas, South Dakota, Missouri, and Iowa (Heap 2003). White et al. (2003) recently described an Ala205Val mutation in an *AHAS* gene from common sunflower R biotypes from South Dakota.

Genes for resistance to AHAS-inhibiting herbicides in sunflower have been introgressed from resistant wild populations (ANN-PUR and ANN-KAN) into elite inbred lines for the purpose of developing and deploying herbicide resistant cultivars and hybrids (Al-Khatib and Miller 2000; Miller and Al-Khatib 2002, 2004). Traditionally, sunflower producers have had few herbicides for controlling broadleaf weeds. Resistance to AHAS-inhibiting herbicides has greatly increased the spectrum of herbicides for controlling broadleaf weeds in sunflower. While herbicide resistances in the ANN-PUR and ANN-KAN populations are probably caused by mutations in *AHAS*, the specific mutations have not been identified.

Typically, R genes for AHAS-inhibiting herbicides show partial or complete dominance (Sebastian et al. 1989; Newhouse et al. 1991; Hart et al. 1993; Wright and Penner 1998; Foes et al. 1999). The R gene identified by Bruniard and Miller (2001) from ANN-PUR showed partial dominance. The degree of resistance was affected by a second gene in some genetic backgrounds. While resistance to AHAS-inhibiting herbicides is highly heritable, the phenotypic effects of *AHAS* mutations are often affected by other factors, e.g., herbicide absorption, metabolism, and the rate of translocation of the herbicide to the active site (Newhouse et al. 1991, 1992). The goals of the present study were to identify genes for resistance to SU and IMI herbicides in sunflower and develop tools for marker-assisted breeding of herbicide resistance genes in cultivated sunflower by: (1) characterizing the *AHAS* gene family, (2) identifying single-nucleotide polymorphisms (SNPs) and other DNA polymorphisms in *AHAS* genes in wild-type and mutant lines, (3) identifying *AHAS* mutations that confer resistance to SU and IMI herbicides in ANN-PUR and ANN-KAN; (4) developing SNP and other high-throughput sequence-tagged-site (STS) markers for genotyping *AHAS* genes and distinguishing mutant from wild-type *AHAS* alleles, and (5) genetically mapping members of the *AHAS* gene family and phenotypic loci for herbicide resistance.

Materials and methods

Plant materials and DNA isolation

Fully expanded leaves were harvested from herbicide-resistant (IMISUN-1, IMISUN-2, HA425, 29023, SURES-1, and SURES-2) and -susceptible (HA89, RHA409, HA370, HA372, RHA280, RHA266, RHA801, NMS373, ZENB9, ZENB13, ZENR1, ZENR7, ZENR13, ZENR16, ZENR17, 24311, 32450, CAS3, and ANN1811) genotypes of cultivated sunflower for DNA isolation. The herbicide R gene donor for IMISUN-1, IMISUN-2, 29023, and HA425 was ANN-PUR (Al-Khatib et al. 1998). IMISUN-1 and IMISUN-2 are IMI-resistant F_2 bulks isolated from HA89*3/ANN-PUR and RHA409//RHA376*2/ANN-PUR, respectively (Al-Khatib and Miller 2000). IMISUN-1 and IMISUN-2 are homozygous for resistance. HA425 is an F_6 oilseed maintainer isolated from HA89*3/ANN-PUR (Miller and Al-Khatib 2002). 29023 is a BC_5F_3 proprietary inbred line obtained by the introgression of resistance from IMISUN-1 into the susceptible ZENB9 line, using marker-assisted selection (MAS). The herbicide R gene donor for SURES-1 and SURES-2 was ANN-KAN (Al-Khatib et al. 1999). SURES-1 is an SU-resistant, F_3-derived F_4 oilseed maintainer isolated from HA424/3/HA406//HA89/ANN-KAN and SURES-2 is an SU-resistant, F_3-derived F_4 oilseed restorer isolated from RHA377/3/RHA392//RHA376/ANN-KAN (Miller and Al-Khatib 2004). Besides screening parents in the pedigrees of IMISUN-1 and IMISUN-2, HA425, and SURES-1 and SURES-2, the parents of three genetic mapping populations were screened to identify DNA polymorphisms in *AHAS*

genes (HA370 and HA372, RHA280 and RHA801, and NMS373 and ANN1811, Tang et al. 2002; Yu et al. 2003; unpublished data). Seed of ANN1811, a wild *H. annuus* population (PI 494567), was acquired from the USDA–ARS National Plant Germplasm System, North Central Plant Introduction Station, Ames, Iowa. ZENB9, ZENB13, ZENR1, ZENR7, ZENR13, ZENR16, ZENR17, 24311, and 32450 are proprietary inbred lines. The other genetic stocks or inbred lines have been publicly released (Fick et al. 1974; Roath et al. 1981; Miller 1992; Miller and Gulya 1999). Leaf tissue was frozen at −70°C, lyophilized, and ground to fine powder. Total genomic DNA was isolated from the powdered samples as described by Tang et al. (2002).

AHAS gene discovery

DNA sequences from several sources were used to design oligonucleotide primers for amplifying *AHAS* gene fragments and isolating *AHAS* genes from sunflower. First, a cDNA probe (ZVG437) for a restriction fragment length polymorphism (RFLP) marker isolated by Berry et al. (1995) was sequenced and, through BlastN and BlastX analyses, was found to be homologous to the 3′ end of *AHAS* genes isolated from common cocklebur (*Xanthium stromarium* L., U16279 and U16280, Bernasconi et al. 1995). Three forward primers (p-AHAS1, p-AHAS2, and p-AHAS3) from the 5′ end of the cocklebur *AHAS* cDNA and two reverse primers (p-AHAS4 and p-AHAS5) complementary to the sunflower ZVG437 cDNA probe were designed (Fig. 1; Table 1). In a second strategy, the nucleotide sequences of the cocklebur and lettuce (*Lactuca* sp.) *AHAS* genes (Mallory-Smith et al. 1990) were aligned. The lettuce DNA sequences were kindly supplied by Dr. Carol Mallory-Smith (Oregon State University, Corvallis, Ore., USA). We designed moderately degenerate forward (p-AHAS6, p-AHAS7) and reverse primers (p-AHAS8, p-AHAS9) based on conserved sequences in the cocklebur-lettuce *AHA*S alignment.

Unless otherwise noted, PCR reaction conditions were the following: 1× buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM each primer, 1.25 U *Taq* DNA polymerase, and 2 ng genomic sunflower DNA in a total volume of 25 μl. After an initial denaturation step at 94°C for 1 min, a program of 40 cycles was used, consisting of 10 cycles of touch-down PCR (94°C for 30 s, 67 to 58°C for 30 s, 72°C for 30 s), followed by 30 cycles of the same cycling regime, but with a fixed annealing temperature of 58°C, and a final elongation step of 72°C for 10 min. PCR products were purified using the Concert Rapid PCR Purification System (Invitrogen Life Technologies, Carlsbad, Calif., USA) or the QiaQuick PCR Purification System (Qiagen, Valencia, Calif., USA), and directly sequenced on an ABI 3700 DNA Analyzer (Applied Biosystems, Foster City, Calif., USA) or cloned prior to sequencing using the PCR-Script Amp Cloning Kit (Stratagene, La Jolla, Calif., USA) or Topo-TA Cloning Kit (Invitrogen Life Technologies). When cloned prior to sequencing, PCR products were produced using a proof-reading polymerase (Platinum *Taq* DNA Polymerase, High Fidelity, Invitrogen Life Technologies).

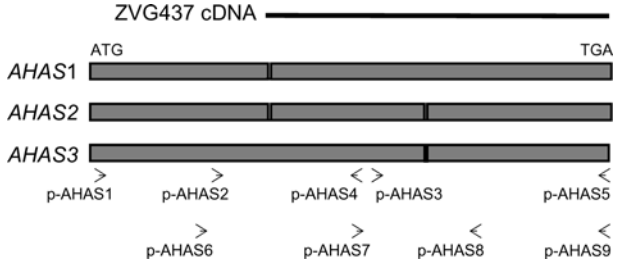


Fig. 1 *AHAS1*, *AHAS2*, and *AHAS3* coding regions in sunflower (1,959–1,977 nucleotides in *AHAS1*, 1,941–1,947 nucleotides in *AHAS2*, 1,941 nucleotides in *AHAS3*). The positions of insertion-deletions (INDELS) that distinguish the three genes are indicated by *gaps*. The positions of primers used in gene discovery are indicated below the *aligned genes*. The *AHAS1* cDNA ZVG437 is aligned above *AHAS1*

AHAS gene fragments were amplified using various combinations of the aforementioned primers. Internal coding sequences were completed by primer walking, using gene-specific primers. The 5′ and 3′ ends of the coding sequences were completed by genome walking (Universal Genomewalker kit, BD Biosciences, Palo Alto, Calf., USA). Briefly, genomic DNA was digested with six restriction enzymes (*Dra*I, *Eco*RV, *Msc*I, *Pvu*II, *Sca*I, *Ssp*, and *Stu*I) and ligated to adaptors to create six fragment libraries according to the manufacturer's instructions. The 5′ ends of *AHAS1* and *AHAS2* were completed using *Dra*I and *Sca*I libraries; the 5′ end of *AHAS3* was completed using *Eco*RV, *Stu*I, and *Ssp*I libraries; the 3′ end of *AHAS2* was completed using *Dra*I, *Pvu*I, and *Sca*I libraries; and the 3′ end of *AHAS3* was completed using *Stu*I and *Msc*I libraries. Nucleotide and amino acid multiple sequence alignments were generated using ClustalW (http://www.ebi.ac.uk/clustalw), and the output was edited and annotated using GeneDoc software (http://www.psc.edu/biomed/genedoc). Numbering of amino acids followed that of the precursor *AHAS* from *Arabidopsis thaliana* (GenBank accession no. X51514, Sathasivan et al. 1990). Gene sequences reported herein have been deposited in GenBank, with accession numbers AY541451–AY541458.

DNA marker development and genetic mapping

AHAS alleles were sequenced from herbicide-resistant and -susceptible lines for polymorphism discovery, DNA marker development, and genetic mapping of *AHAS* genes. Homologous DNA sequences were aligned and searched for SNPs and insertion-deletions (INDELs). SNP or INDEL markers for the three *AHAS* genes were developed and genotyped in one of two segregating populations for which dense reference genetic linkage maps have been developed, RHA280 × RHA801 (Tang et al. 2002; Yu et al. 2003) and NMS373 × ANN1811 (unpublished data). Genetic mapping analyses were

Table 1 Primers for sunflower *AHAS*

Primer name	Purpose	Sequence (5′–3′)	Sequence source
p-AHAS1	Gene discovery	TTCCATCACCACCAAACCAC	*Xanthium AHAS*
p-AHAS2	Gene discovery	GGAGCTACGAACCTAGTAAG	*Xanthium AHAS*
p-AHAS3	Gene discovery	TTTGATGATCGTGTGACGGG	*Xanthium AHAS*
p-AHAS4	Gene discovery	CCATGCATCCCAAGCATATG	*Helianthus* cDNA
p-AHAS5	Gene discovery	CGGTGATCACATCCGAGAAA	*Helianthus* cDNA
p-AHAS6	Gene discovery	CTGGTCTTCCCGGCGTMTGT	*Xanthium/Lactuca AHAS*
p-AHAS7	Gene discovery	GGRACNGTTTATGCGAATTATGC	*Xanthium/Lactuca AHAS*
p-AHAS8	Gene discovery	TGAGCAGCCCACATCTGATG	*Xanthium/Lactuca AHAS*
p-AHAS9	Gene discovery	AATATTTMATTCTGCCRTCGCC	*Xanthium/Lactuca AHAS*
p-AHAS10	Gene expression, *AHAS1* RT-PCR	TTGGAGGAAAAGAATTCGGTGACT	*Helianthus AHAS1*
p-AHAS11	Gene expression, *AHAS1* RT-PCR	CGCCCGTTAACTCATCAAGAACT	*Helianthus AHAS1*
p-AHAS12	Gene expression, *AHAS2* RT-PCR	CAAGATTTTGGAGGAGAAAAATTCTC	*Helianthus AHAS2*
p-AHAS13	Gene expression, *AHAS2* RT-PCR	CCCGTTAACTCATCGAGCACA	*Helianthus AHAS2*
p-AHAS14	Gene expression, *AHAS3* RT-PCR	TGCGATCCAAGTGCTGAATGAAG	*Helianthus AHAS3*
p-AHAS15	Gene expression, *AHAS3* RT-PCR	CGATGTCAACCACGACTGCATCT	*Helianthus AHAS3*
p-AHAS16	Genotyping, *AHAS1* alleles	CCCCGTTTCGCATTACCCATCACT	*Helianthus AHAS1*
p-AHAS17	Genotyping, *AHAS1* alleles	ACCAACACGTCTGCGCCTTTTCTC	*Helianthus AHAS1*
p-AHAS18	Genotyping, *AHAS1* alleles	TTCCTCCCCCGTTTCGCATTAC	*Helianthus AHAS1*
p-AHAS19	Genotyping, *AHAS1* alleles	CGCCGCCCTGTTCGTGAC	*Helianthus AHAS1*
p-AHAS1c205F	Mapping, *AHAS1* target F primer	CAAGTTCCCCGGAGAATGAT	*Helianthus AHAS1*
p-AHAS1c205R	Mapping, *AHAS1* target R primer	CGAAAAATCAAGATTAGTCACCGAAT	*Helianthus AHAS1*
p-AHAS1c205SNP	*AHAS1* codon 205 SNP primer (rev)	CCTCAACAATTGGGGTTTCTTGAAAC	*Helianthus AHAS1*
p-AHAS1c281SNP	*AHAS1* codon 281 SNP primer (fwd)	CGGGTTATTTGTCTAGAATGCC	*Helianthus AHAS1*
p-AHAS2indelF	Mapping, *AHAS2* target F primer	CTTCCATCACCGCCAAACCAC	*Helianthus AHAS2*
p-AHAS2indelR	Mapping, *AHAS2* target R primer	GTACCGGGAGACGAATGGC	*Helianthus AHAS2*
p-AHAS3c581F	Mapping, *AHAS3* target F primer	CTTCCTGTTAAAATGATGGTGCTT	*Helianthus AHAS3*
p-AHAS3c581R	Mapping, *AHAS3* target R primer	CAACATATTTGGGAATATACCCGAT	*Helianthus AHAS3*
p-AHAS3c581SNP	*AHAS3* codon 581 SNP primer (rev)	AAGTAGGTGTGCGCGCGGTT	*Helianthus AHAS3*

performed using MAPMAKER (Lander et al. 1987), essentially as described by Tang et al. (2002).

Genotyping assays were developed for SNPs in codons 205 and 281 of *AHAS1* (*AHAS1*-c205C/T and *AHAS1*-c281G/A, respectively) and in codon 581 of *AHAS3* (*AHAS3*-c581G/A). SNPs were scored using the fluorescence polarization-template-directed incorporation assay (Chen et al. 1999; Kwok and Chen 2003) and commercial kits (AcycloPrime-FP SNP Detection Kit, PerkinElmer Life Sciences, Boston, Mass., USA). Target amplification and terminator incorporation reactions were performed as recommended by the kit manufacturer. Briefly, a region of the genome containing the SNP was amplified using standard PCR. The *AHAS1* target fragment was 732 bp in length and encompassed codons 205 and 281. The *AHAS3* target fragment was 141 bp in length. Excess primers and dNTPs were removed by addition of shrimp alkaline phosphatase and *Escherichia coli* exonuclease (ExoSAP-IT, USB, Cleveland, Ohio, USA) supplied with the AcycloPrime kit. The single-base extension reaction was performed using SNP detection primers that terminated adjacent to the SNP sites and acylclo-dideoxynucleotide triphosphate terminators supplied in the kit. SNP genotypes were read on a Wallac 1420 VICTOR3 fluorescence polarization plate reader (PerkinElmer), and alleles were called using an EXCEL macro supplied by PerkinElmer for AcycloPrime SNP genotyping. Sequences of the target amplification and SNP detection primers are provided in Table 1.

AHAS1 and *AHAS3* were integrated into the genetic linkage map of sunflower by genotyping the *AHAS1*-c281G/A and *AHAS3*-c581G/A markers on 94 RHA280 × RHA801 recombinant inbred lines (RILs). *AHAS2* was integrated into the genetic linkage map of sunflower by genotyping a single-strand conformational polymorphism (SSCP) marker for *AHAS2* on 94 [(NMS373 × ANN1811) × NMS373] BC_1 progeny. The positions of *AHAS2* and DNA markers linked to *AHAS2* on the NMS373 × ANN1811 genetic linkage map are reported in the present paper. The complete NMS373 × ANN1811 genetic linkage map is to be reported elsewhere. DNA markers from previously published genetic linkage maps (Tang et al. 2002; Yu et al. 2003) were used to group and order *AHAS2* on the NMS373 × ANN1811 genetic linkage map. The *AHAS2* SSCP primer pair (p-AHAS3indelF and p-AHAS3indelR) flanked a 6-bp insertional polymorphism in ANN1811. The DNA fragment amplified by these primers was 185 bp long in NMS373 and 191 bp long in ANN1811. SSCP analyses was performed on a 50-cm wide by 20-cm high polyacrylamide gel apparatus (CBS

Scientific Products, Del Mar, Calif., USA), essentially as described by Slabaugh et al. (1997), except that one of the glass plates was treated with γ-methacryloxypropyltrimethoxysilane (Sigma Chemical, St. Louis, Mo., USA) so that the gel remained attached during silver staining.

To facilitate breeding and MAS, a PCR-based assay was developed to detect allelic length variants in the simple sequence repeat (SSR) in *AHAS1*. Primers p-AHAS16 and p-AHAS18 are *AHAS1*-specific primers upstream of the SSR, and p-AHAS17 and p-AHAS19 are downstream primers (Table 1). Primers p-AHAS16 and p-AHAS17 produced 176–191-bp fragments, and primers p-AHAS18 and p-AHAS19 produced 313–328-bp fragments from genomic DNA. PCR products were amplified in a 10-μl reaction containing 10× buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM primers, 2 ng genomic DNA, and 0.5 U Platinum *Taq* DNA polymerase (5 U/μl, Invitrogen Life Technologies). The PCR program utilized an annealing temperature of 60°C. Products were analyzed on agarose or acrylamide gels.

Candidate gene analyses

Candidate gene analyses were performed on [(HA425×-HA89)×HA89] BC_1 (Bruniard and Miller 2001) and (IMISUN-2×ZENB9) F_2 progeny segregating for the incompletely dominant herbicide resistance gene from ANN-PUR (Ar_{PUR}). The AHAS-inhibiting herbicide resistance (*Ar*) locus was scored in both segregating populations by inferring *Ar* genotypes from herbicide resistance phenotypes. Eighty [(HA425 × HA89)×HA89] BC_1 progeny that were previously phenotyped for resistance to imazamox (Raptor, BASF, Mt. Olive, N.J., USA; Bruniard and Miller 2001) were genotyped for the *AHAS1*-c205 SNP. Imazamox was applied at a rate of 33.2 g ai/ha to greenhouse-grown plants at the six-leaf stage, and plants were visually phenotyped for herbicide injury and scored as susceptible (*arar*) or moderately resistant ($Ar_{PUR}ar$) 1 week after herbicide treatment. In a separate experiment, 200 (IMISUN2 × ZENB9) F_2 progeny were phenotyped for resistance to imazamox (Sweeper, BASF). Imazamox was applied at a rate of 100 g ai/ha to field grown plants (Balcarce, Argentina) at the four to six true-leaf stage. Plants were rated visually on a scale of 1–4 for herbicide injury 2 weeks after treatment. Plants in which the apex had died were rated 1, plants with severe injury (yellowing and leaf deformation) were rated 2, plants with one or two leaves showing a mosaic of yellowing were rated 3, and plants with no apparent herbicide injury were rated 4. Eighty-three randomly chosen plants from this F_2 population were genotyped by sequencing the codon 205 region of *AHAS1*. The fit of observed to expected segregation ratios for the *Ar* and *AHAS1* loci were tested using χ^2-statistics (1:1 for *Ar* and *AHAS1* in the BC_1 and 3:1 for *Ar* and 1:2:1 for *AHAS1* in the F_2). The effect of the *AHAS1* locus on herbicide tolerance was estimated in the IMISUN-2×ZENB9 F_2 population by performing an analysis of variance on *AHAS1*-c205 SNP marker genotypes, using SAS PROC GLM (Littel et al. 1996). The additive (*a*) and dominance (*d*) effects and degree of dominance (*d*/*a*) of the *AHAS1* locus were estimated as described by Falconer and Mackay (1996). The proportion of the phenotypic variance explained by the *AHAS1* locus was estimated by SS_M/SS_T, where SS_M is the sum of squares for the SNP marker, and SS_T is the total sum of squares.

AHAS gene expression analyses

The presence of *AHAS1*, *AHAS2*, and *AHAS3* transcripts was determined in 28-day-old IMISUN-1, IMISUN-2, HA89, and RHA409 seedlings, using RT-PCR. Harvested tissue was immediately placed in liquid nitrogen, ground to a powder with a mortar and pestle, and total RNA was extracted using Trizol reagent (Invitrogen Life Technologies) as recommended by the manufacturer. RNA was quantified with a spectrophotometer and RNA integrity was assessed on a 1% denaturing agarose gel (Sambrook et al. 1989). Reverse transcription was carried out using MMLV reverse transcriptase (Invitrogen Life Technologies) with an oligo $(dT)_{12\text{-}18}$ primer according to the protocol of the manufacturer. One-tenth of the reverse transcription mix was used as template for cDNA amplification. *AHAS1* cDNA was amplified using primers p-AHAS10 and p-AHAS11; *AHAS2* cDNA was amplified using primers p-AHAS12 and p-AHAS13; and *AHAS3* cDNA was amplified using primers p-AHAS14 and p-AHAS15 (Table 1). The PCR products were sequenced to confirm gene-specific amplification.

Results

The *AHAS* gene family in cultivated sunflower

BlastN analysis on a proprietary cDNA sequence database (Advanta Seeds) identified a cDNA clone (ZVG437) as highly homologous to the catalytic subunit of *AHAS* genes cloned from other plant genera. The highest homologies were to cDNAs isolated from wild-type and herbicide-resistant cocklebur (*X. stromarium* L., GenBank accession nos. U16279 and U16280). The cocklebur *AHAS* cDNAs are 2,156 bp long and encode proteins 648 amino acid residues long. The first 77 amino acids have been tentatively identified as chloroplast targeting signals (Bernasconi et al. 1995). By aligning the ZVG437 and cocklebur cDNA sequences, the former was identified to be a 1,262-bp fragment from the 3′ end of *AHAS*. Subsequently, a sunflower EST database was searched (http://compgenomics.ucdavis.edu; Kozik et al. 2002), and 11 sunflower *AHAS* ESTs were identified (QHF14PO1, QHA8G11, QHB10A23, QHB23I24, QHI1F03, QHE14F03, QHI10G24, QHE20B02, QHI4B16, QHE20P09, and QHI15 N13).

Genomic sequences of sunflower *AHAS* genes were isolated by designing forward primers complementary to

conserved nucleotide sequences in the 5′ ends of the cocklebur cDNAs (p-AHAS1, 2, and 3) and reverse primers complementary to ZVG437 (p-AHAS4 and 5), in addition to moderately degenerate forward and reverse primers complementary to highly conserved sequences between cocklebur and lettuce *AHAS* cDNAs (p-AHAS6, 7, 8, and 9, Table 1; Fig. 1). Various combinations of forward and reverse primers were used to amplify genomic DNA from IMI-resistant (IMISUN-1, IMISUN-2, HA425) and -susceptible (RHA409, HA89, RHA280, and RHA801) inbred lines, the former originating from ANN-PUR, an imazethapyr-resistant wild biotype (Al-Khatib et al. 1998; Al-Khatib and Miller 2000; Miller and Al-Khatib 2002). When the DNA sequences of various amplicons were aligned, we discovered that three paralogous *AHAS* genes, designated *AHAS1*, *AHAS2*, and *AHAS3*, had been amplified from resistant and susceptible genotypes. Whereas both *AHAS1* and *AHAS2* were amplified by most primer pairs, *AHAS3* was only amplified by degenerate primers p-AHAS7 and p-AHAS9. The discovery of multiple *AHAS* genes was expected because ZVG437 hybridized to several fragments when used as an RFLP probe on Southern blots (unpublished data).

Complete coding sequences were obtained for the three genes by using paralogue-specific primers and genome walking to sequence the 5′ and 3′ ends. No introns were found in any of the sunflower genes, as is the case in other plant *AHAS* genes (Tranel and Wright 2002). The deduced amino acid sequences of the sunflower *AHAS* genes were aligned with catalytic subunits of *Arabidopsis* and cocklebur *AHAS* genes (Fig. 2) and numbered in reference to the *Arabidopsis* sequence (Sathasivan et al. 1990).

Two of the three sunflower *AHAS* genes were highly homologous. The nucleotide sequences of *AHAS1* and *AHAS2* were 92% identical, whereas *AHAS3* was 72% identical to *AHAS1* and 73% identical to *AHAS2*. Excluding multiple differences in the putative chloroplast targeting sequence, *AHAS2* was distinguished from *AHAS1* by a nine-base pair deletion in frame of codons 435–437. Similarly, *AHAS3* was distinguished from *AHAS1* and *AHAS2* by a three base pair deletion in the same location, in addition to a nine-base pair in-frame insertion between codons 268–269 (Fig. 2).

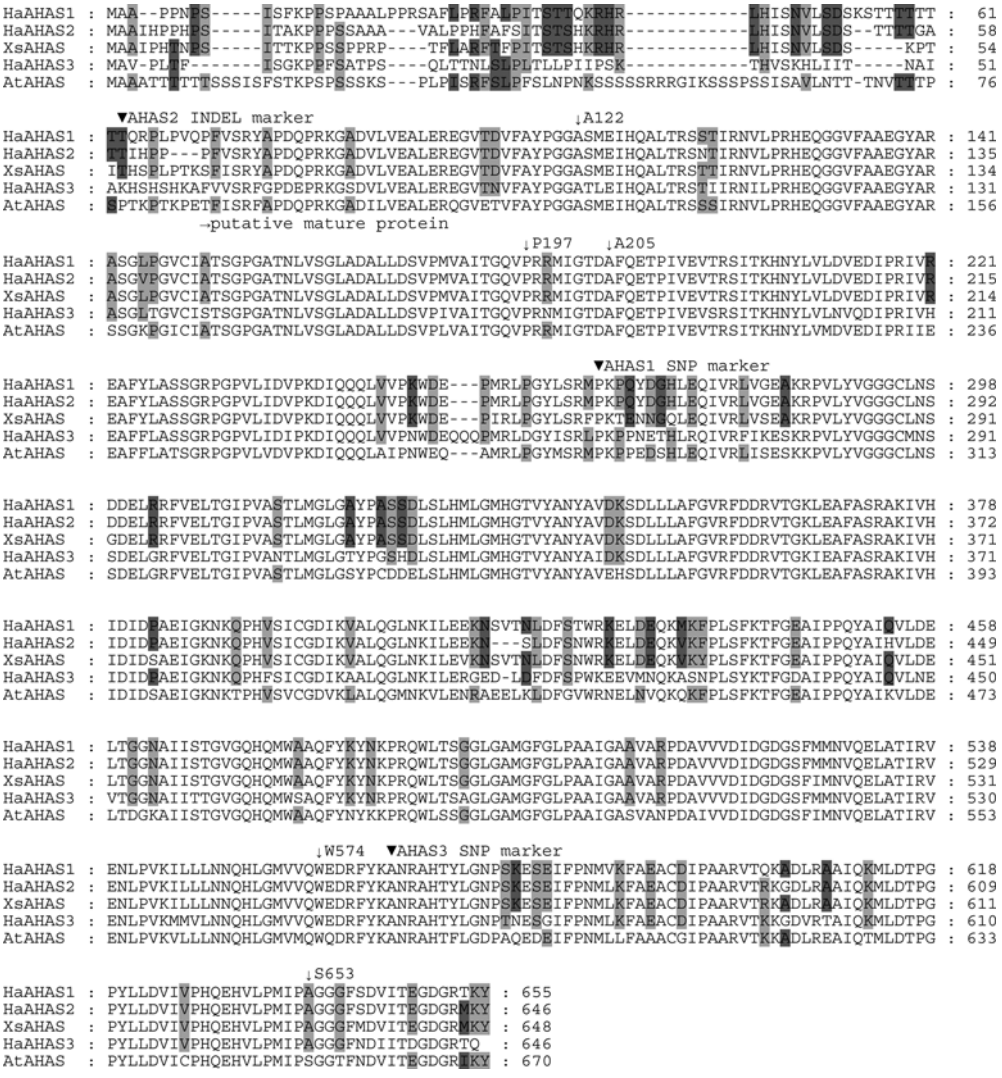


Fig. 2 Deduced amino acid sequences of AHAS proteins from sunflower (HaAHAS1, HaAHAS2, and HaAHAS3) aligned with the amino acid sequences of *Arabidopsis thaliana* (AtAHAS) and *Xanthium stromarium* (XsAHAS). The mutations in AHAS that have been shown to confer herbicide resistance in plants are indicated by *arrows* and are numbered according to *A. thaliana* (Sathasivan et al. 1990). The positions of markers for genes encoding HaAHAS1, HaAHAS2, and HaAHAS3 are indicated by *solid triangles*

DNA polymorphisms in *AHAS* genes

Subsequent to identifying the three *AHAS* paralogues, *AHAS1*, *AHAS2*, and *AHAS3* alleles were sequenced from additional herbicide-resistant and susceptible sunflower genotypes. The resistant genotypes included SURES-1 and SURES-2, SU-resistant lines developed from ANN-KAN, an herbicide resistant population of common sunflower (Al-Khatib et al. 1999; Miller and Al-Khatib 2004; Table 2). Among *AHAS1* allele sequences from 23 elite inbred lines, we identified 48 SNPs and an $[ACC]_n$ repeat comprising five haplotypes (Fig. 3). In contrast, no DNA polymorphisms were identified among *AHAS2* allele sequences from a nearly identical set of elite inbred lines (Table 2). We subsequently sequenced *AHAS2* from ANN1811, the wild parent in an elite × wild (NMS373 × ANN1811) mapping population, and identified a six-base pair insertion in the transit peptide-encoding region of the *AHAS2* allele (Fig. 3). DNA polymorphisms were also rare in *AHAS3*. We identified a single DNA polymorphism, a synonymous G-to-A SNP in codon 581, among *AHAS3* allele sequences from 12 elite inbred lines (Fig. 3). The probability of observing a SNP or INDEL among elite inbred lines, calculated from haplotype frequencies reported in Table 2, was 0.77 for *AHAS1*, 0.00 for *AHAS2*, and 0.44 for *AHAS3*.

Herbicide resistance is correlated with mutations in *AHAS1*

Resistance to IMI herbicides in IMISUN-1, IMISUN-2, and HA425 (originating from ANN-PUR) correlated with *AHAS1* haplotype 5, whereas resistance to SU herbicides in SURES-1 and SURES-2 (originating from ANN-KAN) correlated with *AHAS1* haplotype 3 (Table 2). Susceptible genotypes had *AHAS1* haplotypes 1, 2, or 4. Thus, *AHAS1* was identified as the prime candidate gene for herbicide-resistant phenotypes originating in the two wild populations (ANN-PUR and ANN-KAN, Al-Khatib et al. 1998, 1999). Only six of the 48 SNPs discovered in *AHAS1* would cause amino acid substitutions. Two of these, Pro_{197} and Ala_{205}, have previously been shown to confer resistance to AHAS-inhibiting herbicides in other plant genera (Tranel and Wright 2002). The ANN-PUR *AHAS1* allele, as identified from IMISUN-1, IMISUN-2, and HA425 *AHAS1* alleles, harbored an alanine (GCG)-to-valine (GTG) mutation in codon 205, whereas the ANN-KAN *AHAS1* allele, as identified from SURES-1 and SURES-2 *AHAS1* alleles, harbored a proline (CCC) to leucine (CTC) mutation in codon 197. Recently, White et al. (2003) reported an independent AHAS Ala205Val mutation in a South Dakota common sunflower population that was cross-resistant to imazethapyr and chlorimuron ethyl.

Marker development and genetic mapping of sunflower *AHAS* genes

SNP markers were developed for the SNPs in codon 205 of *AHAS1* (*AHAS1*-c205C/T), codon 281 of *AHAS1* (*AHAS1*-c281G/A), and codon 581 of *AHAS3* (*AHAS3*-c581G/A, Fig. 4). SSCP markers were developed for the six-base pair INDEL in *AHAS2* (*AHAS2*-INDEL) and the G/A SNP in *AHAS3* (*AHAS3*-INDEL, Fig. 5). To facilitate MAS based on *AHAS1* haplotypes, we developed an SSR marker based on the poly-Thr ($[ACC]_n$) repeat (Fig. 2) in the putative transit peptide of AHAS1 (not shown). *AHAS1* and *AHAS3* were genotyped and genetically mapped in RHA280 × RHA801, using the *AHAS1*-c281 and *AHAS3*-c581 SNP markers, respectively (Fig. 4). *AHAS2* was genotyped and genetically mapped in NMS373 × ANN1811, using the *AHAS2* SSCP marker (Fig. 5). The three loci mapped to linkage groups 2 (*AHAS3*), 6 (*AHAS2*), and 9 (*AHAS1*) of the public sunflower map and were flanked by previously mapped SSR or INDEL markers (Tang et al. 2002; Yu et al. 2003; Fig. 6).

Table 2 *AHAS1*, *AHAS2*, and *AHAS3* haplotypes of cultivated and wild sunflower germplasm

Gene	Haplotype	Germplasm	Herbicide resistance
AHAS1	1	HA89, RHA409, RHA801, HA370, ZENB9, ZENR1	S
AHAS1	2	HA372, ZENB13, ZENR13, ZENR16, ZENR17, 32450, 24311	S
AHAS1	3	SURES-1, SURES-2	R[a]
AHAS1	4	RHA280, RHA266, CAS3, ZENR7	S
AHAS1	5	IMISUN-1, IMISUN-2, HA425, 29023	R[b]
AHAS2	1	HA89, RHA409, RHA801, RHA280, RHA266, NMS373, HA370, HA372, SURES-1, SURES-2, IMISUN-1, IMISUN-2, ZENB9, ZENR1, ZENB13, ZENR13, ZENR16, ZENR17, ZENR7, CAS3, 32450, 24311, 29023	S and R[a,b]
AHAS2	2	ANN1811	Unknown
AHAS3	1	HA89, RHA409, RHA801, SURES-2, IMISUN-1, IMISUN-2	S and R[a,b]
AHAS3	2	RHA280, RHA266, SURES-1	S and R[a]

[a]Resistant to sulfonylurea herbicides
[b]Resistant to imidazolinone herbicides

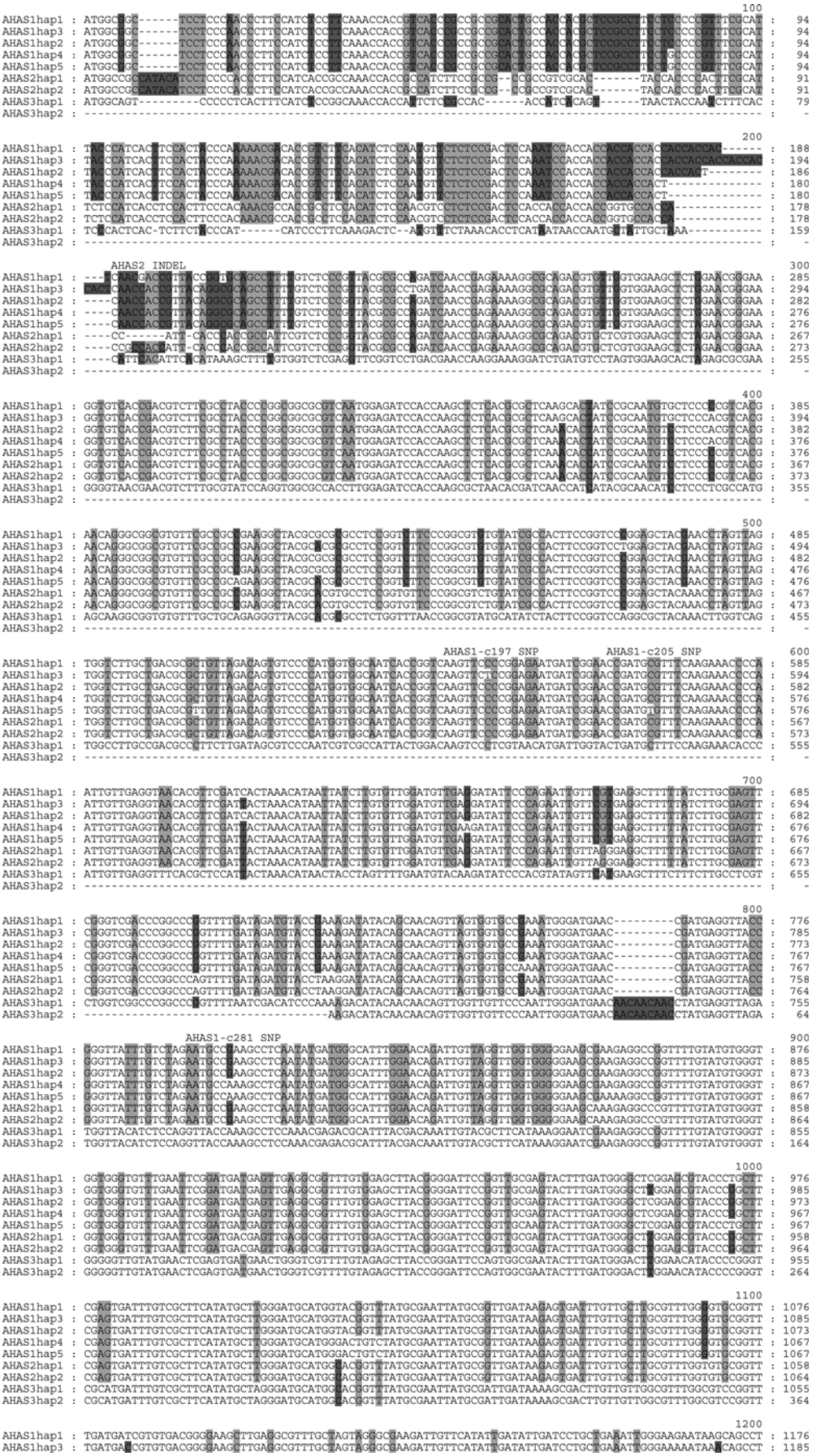
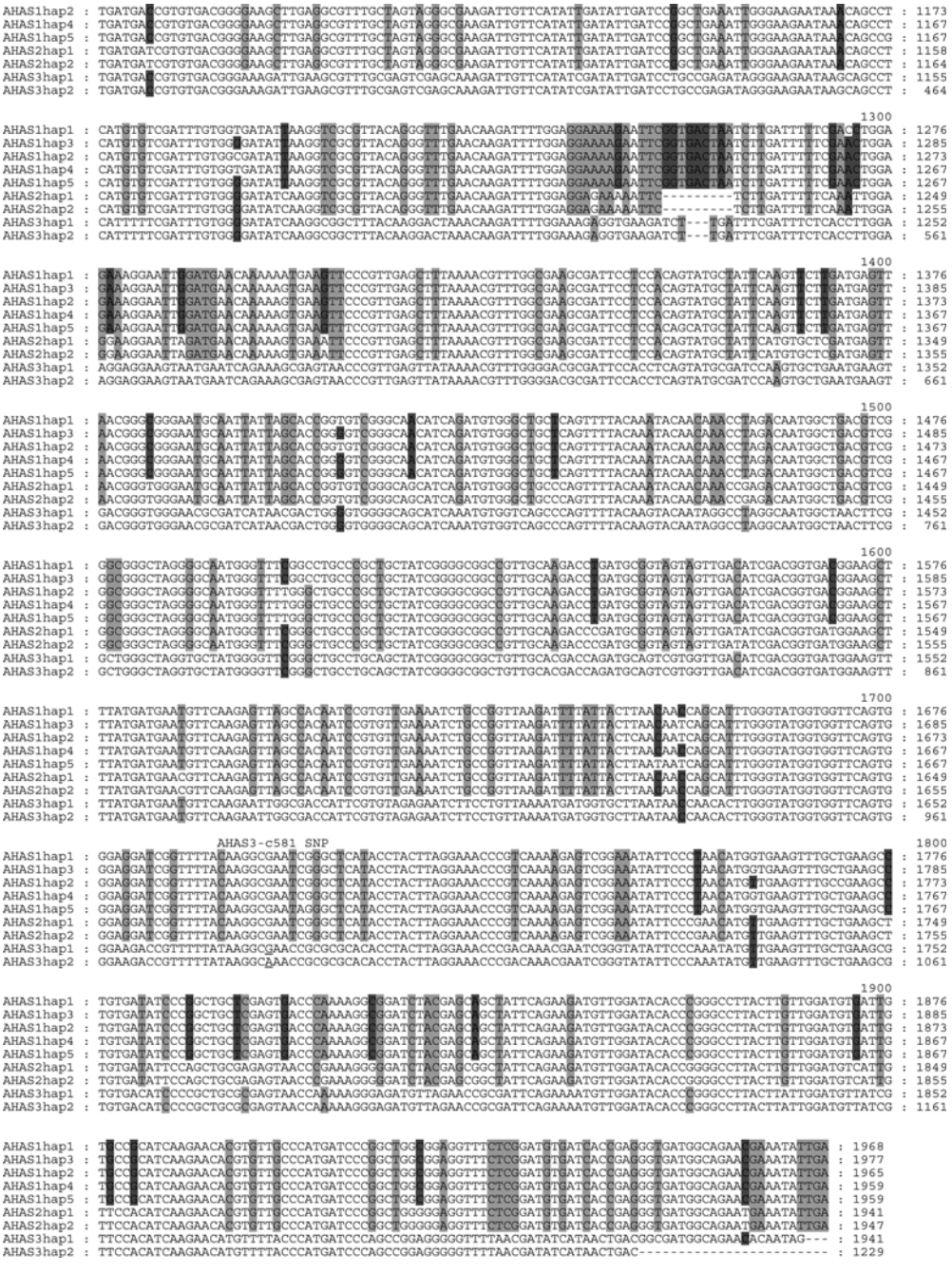


Fig. 3 Nucleotide alignment of five *AHAS1* haplotypes, two *AHAS2* haplotypes, and two *AHAS3* haplotypes from sunflower. INDELs and single-nucleotide polymorphisms (SNPs) used to map the three *AHAS* genes and *AHAS1* mutations putatively conferring herbicide resistance (codons 197 and 205) are *underlined* and *labeled*

Fig. 3 (continued)



The *AHAS1* mutation in codon 205 cosegregated with resistance to IMI herbicides

The *AHAS1*-c205 SNP marker was genotyped on 80 [(HA425 × HA89) × HA89] BC_1 progeny segregating for the ANN-PUR IMI resistance gene (Ar_{PUR}), previously phenotyped for resistance to imazamox (33.2 g ai/ha, Bruniard and Miller 2001). The observed segregation ratio for the *Ar* locus was not significantly different ($P<0.73$) from the expected segregation ratio for a single partially dominant gene segregating in a BC_1 (1 $Ar_{PUR}ar$:1 *arar*). *AHAS1*-c205 SNP genotypes (Fig. 4) completely cosegregated with herbicide resistance phenotypes, 41 C/C–39 C/T. Susceptible progeny (*arar*) were homozygous for the wild-type *AHAS1* allele (C/C), whereas moderately resistant progeny ($Ar_{PUR}ar$) were heterozygous for wild-type and mutant *AHAS1* alleles (C/T) in [(HA425 × HA89) × HA89] BC_1.

The cosegregation of herbicide resistance phenotypes and *AHAS1* genotypes was further assessed among 83 herbicide-resistant × susceptible (IMISUN-2 × ZENB9) F_2 progeny phenotyped for resistance to a higher rate of imazamox (100 g ai/ha) and genotyped for *AHAS1* polymorphisms by allele sequencing (Table 3). IMISUN-2 × ZENB9 F_2 progeny homozygous for the *AHAS1*-c205 mutation (T/T) were either uninjured or partially injured, whereas wild-type homozygotes (C/C) were killed by herbicide treatment (Table 3). The heterozygous class (T/

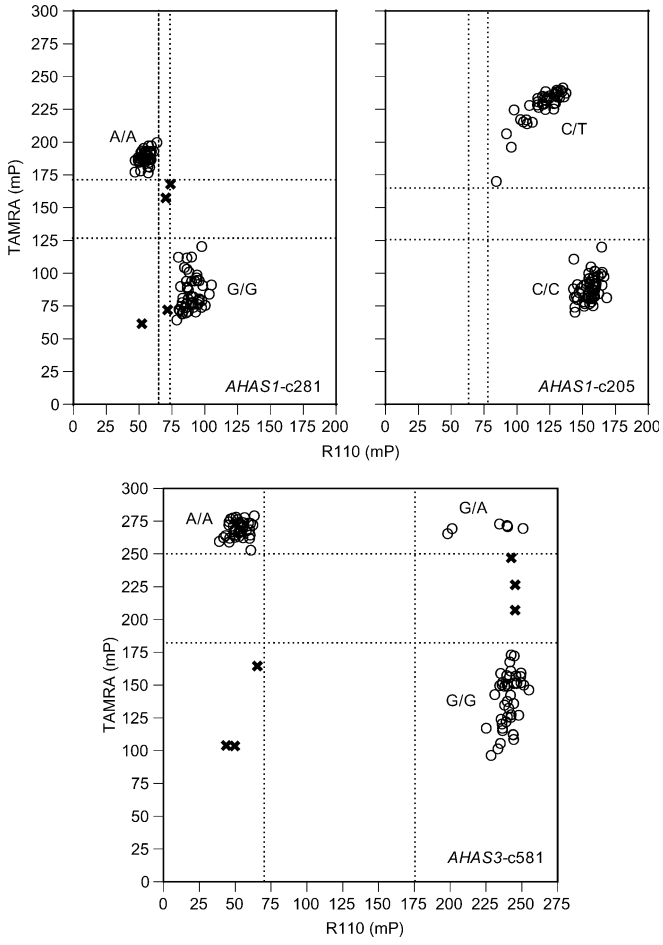


Fig. 4 SNP genotyping assays for a G/A SNP in codon 281 of *AHAS1* (*upper left*), a C/T SNP in codon 205 of *AHAS1* (*upper right*), and a G/A SNP in codon 581 of *AHAS3* (*bottom*) in sunflower. The *AHAS1*-c281 and *AHAS3*-c581 SNP markers were genotyped on 96 RHA280 × RHA801 F_7 recombinant inbred lines and the *AHAS1*-c205 SNP marker was genotyped on 80 [(HA425 × HA89) × HA89] BC_1 progeny. Data points marked × were not assigned a genotype

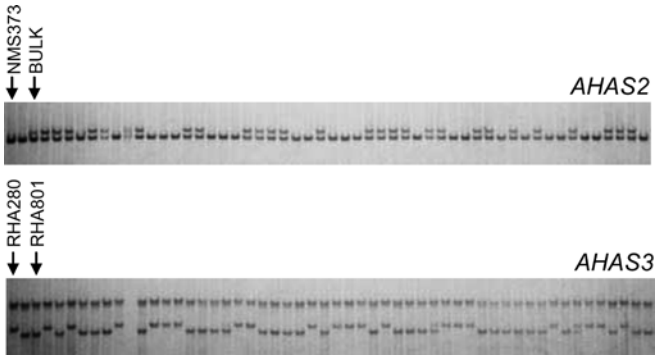


Fig. 5 Single-strand conformational polymorphism markers for *AHAS2* and *AHAS3* genotyped on 94 [(NMS373 × ANN1811) × NMS373] BC_1 progeny and 96 RHA280 × RHA801 F_7 recombinant inbred lines, respectively. *Upper panel lanes* containing markers produced from NMS373 and a bulk of 20 BC_1 progeny are labeled, and *remaining lanes* show genotyping data from a subset of the segregating BC_1 population. *Lower panel lanes* containing markers produced from RHA280 and RHA801 are labeled, and *remaining lanes* show genotyping data from a subset of the segregating recombinant inbred line population. NMS383 and ANN1811 *AHAS2* fragments were 185 bp and 191 bp in length, respectively. RHA280 and RHA801 *AHAS3* fragments were 141 bp in length, and the alleles differed at a single G/A SNP site

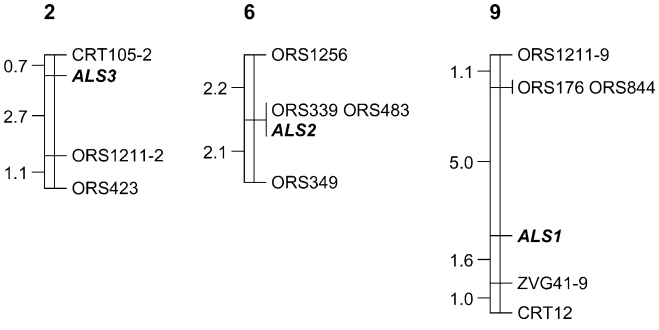


Fig. 6 Location of the *AHAS3*, *AHAS2*, and *AHAS1* genes on the public map of the sunflower genome relative to simple sequence repeat (*ORS* and *CRT*) and INDEL (*ZVG*) marker loci on linkage groups 2, 6, and 9, respectively

C) was more variable. Three heterozygotes were killed by the high rate of herbicide treatment and, solely on the basis of phenotypic analysis, were classified as *arar*. These individuals, however, were found to be heterozygous for the C/T SNP and inferred to be heterozygous for the resistance gene ($Ar_{PUR}ar$). The additive effect of the C/T SNP (a=1.41) was highly significant (P<0.0001), whereas the dominant effect (d=0.25) effect was nonsignificant (P<0.11). The degree of dominance (d/a) was 0.18; hence, the effect of Ar_{PUR} was nearly additive in IMISUN-2 × ZENB9 (Table 3). The C/T SNP (Ar_{PUR} locus) explained 66.5% of the phenotypic variance for herbicide resistance.

Table 3 Herbicide resistance phenotypes and ratings for IMISUN-2 × ZENB9 F_2 progeny segregating for the *AHAS1*-c205C/T SNP and Ar_{PUR} resistance gene

Phenotype	Phenotypic rating	Number of F_2 progeny		
		T/T	T/C	C/C
Dead	1		3	23
Severely damaged	2		20	
Mildly damaged	3	3	10	
Undamaged	4	13	11	
Mean		3.81	2.66	1.00

Expression analysis of *AHAS* genes

ESTs encoding *AHAS1* and *AHAS2* were identified in the sunflower EST database; however, no ESTs encoding *AHAS3* were identified (Kozik et al. 2002). Ten *AHAS1* ESTs (QHA8G11, QHB10A23, QHB23I24, QHI1F03, QHE14F03, QHI10G24, QHE20B02, QHI4B16, QHE20P09, and QHI15 N13) and one *AHAS2* EST (QHF14PO1) were identified from 44,061 ESTs (0.03%) isolated from diverse tissues and development stages; hence, *AHAS1* seems to be more strongly expressed than *AHAS2*. However, using RT-PCR analysis with gene-specific primers, we detected mRNA transcripts from all

three genes in seedling apical meristems and leaves. Transcript levels of the three genes did not significantly differ between herbicide-resistant sunflower lines and their recurrent parents (data not shown). Because the three sets of gene-specific primers supported unequal amplification efficiencies, we were unable to accurately quantify relative transcript levels of the three *AHAS* genes.

Discussion

This study identified two mutations in the sunflower *AHAS1* gene that likely confer resistance to AHAS-inhibiting herbicides. We discovered an Ala205Val mutation in sunflower lines developed by introgressing Ar_{PUR} (Al-Khatib et al. 1998) into elite inbred lines (Al-Khatib and Miller 2000; Miller and Al-Khatib 2002) and a Pro197Leu mutation in sunflower lines developed by introgressing Ar_{KAN} into elite inbred lines (Al-Khatib et al. 1999; Miller and Al-Khatib 2004).

Whereas both Ar_{PUR} and Ar_{KAN} originated in common sunflower from Kansas, a presumably independent mutation was recently described in an herbicide-resistant common sunflower population from South Dakota (White et al. 2002, 2003). The South Dakota mutation also occurred in the *AHAS1* gene, based on the deduced amino acid sequence (White et al. 2003).

Prior to these studies, mutation of Ala205 in AHAS inhibitor-resistant plants had only been reported in cocklebur and *Arabidopsis* (Woodworth et al. 1996; Jander et al. 2003). In sunflower and cocklebur, Ala205Val confers moderately high resistance (>tenfold relative to susceptible genotypes) to IMIs and partial resistance (<tenfold relative to susceptible genotypes) to SUs (Woodworth et al. 1996; White et al. 2003).

Mutation of Pro197 is one of the most common mutations found in plants resistant to AHAS-inhibiting herbicides. Substitution of Pro197 with at least eight different amino acids has produced SU resistance in *Lactuca*, *Kochia*, *Brassica*, *Sisymbrium*, *Amaranthus*, and *Arabidopsis* species (Tranel and Wright 2002; Jander et al. 2003). The Pro197Leu mutation was associated with high resistance (>tenfold relative to susceptible biotypes) to both SU and IMI herbicides in *Amaranthus* (Sibony et al. 2001), but high resistance to SU and moderately low resistance to IMI in *Kochia* (Guttieri et al. 1995). The same mutation in sunflower was associated with a resistance pattern similar to that of *Amaranthus* (Fabie and Miller 2002).

Pro197 and Ala205 are conserved amino acids in AHAS enzymes in numerous species (Tranel and Wright 2002). The crystal structure of yeast AHAS in complex with chlorimuron ethyl, an SU herbicide, revealed that both Pro197 and Ala205 make hydrophobic contact with the inhibitor, which binds in the substrate access channel and blocks entry of substrate into the active site of the enzyme (Pang et al. 2003). Indeed, nine of the ten mutations that confer resistance to AHAS-targeted herbicides in yeast involve amino acids that make direct, mainly hydrophobic contacts with bound chlorimuron. These nine amino acids include Pro197, Ala205, and three additional amino acids that when mutated, confer resistance to AHAS-targeted herbicides in plants (Tranel and Wright 2002). Thus, the structurally divergent classes of AHAS-inhibiting herbicides apparently all bind in the same channel, with each herbicide making a unique set of contacts with several amino acid side chains. This model provides a rational basis for the observed variations in cross-resistance to different herbicide classes conferred by *AHAS* mutations in a variety of plant species.

Sequence polymorphisms among different members of the sunflower *AHAS* gene family varied markedly. Within the 23 lines and accessions we sequenced, five *AHAS1* haplotypes were detected. Two haplotypes were associated with introgressed herbicide resistance genes. Overall, *AHAS1* alleles varied at 48 nucleotides within the coding sequence (2.4%), a rate somewhat greater than the overall average (1.6%) observed in exonic sequences in a study of SNPs in a panel of 12 domesticated and wild sunflower germplasms (unpublished data). *AHAS1* alleles were also distinguished by differing lengths of an $[ACC]_n$ repeat (encoding poly-Thr) in the transit peptide-encoding part of the gene. Excluding the two SNPs that created the Pro197Leu and Ala205Val mutations, only four of the 48 SNPs caused amino acid changes. Two SNP sites and the $[ACC]_n$ repeat were exploited to develop robust DNA markers for *AHAS1* genotypes.

In contrast to *AHAS1*, extremely low sequence diversity was found in *AHAS2* and *AHAS3*. Only two alleles were identified for each of these genes, and the polymorphisms detected were a single six-base pair in-frame insertion (*AHAS2*) and a single synchronous SNP (*AHAS3*). *AHAS2* was completely conserved in elite inbred lines; the insertion was found in a wild sunflower, ANN1811. Whether unequal polymorphism rates in the *AHAS* gene family are attributable to selection pressures operating independently on the three genes or to selection events during domestication of sunflower that preserved genomic regions linked to the *AHAS2* and *AHAS3* genes cannot be determined from the present study. However, a marker that maps within 5 cM of *AHAS1* on LG9, ZVG41, was found to be hyperpolymorphic in the aforementioned study (unpublished data), suggesting that this genomic region is highly variable in domesticated sunflower. Conversely, LG6, to which *AHAS2* maps, has historically been one of the sparsest linkage groups in maps produced from crosses between elite inbred lines (Berry et al. 1995; Perez-Vich et al. 2002; Tang et al. 2002; Yu et al. 2003).

The *AHAS1* Ala205Val mutation was partially dominant and did not confer complete resistance to IMI herbicides (Bruniard and Miller 2001; this study). Genes for resistance to AHAS-inhibiting herbicides are partially dominant in several genera (Sebastian et al. 1989; Newhouse et al. 1991; Hart et al. 1993; Wright and Penner 1998; Foes et al. 1999), with mutant homozygotes often being more resistant than heterozygotes. Because *AHAS1*-c205 mutant homozygotes (T/T) were more strongly resistant to herbicides than heterozygotes (C/T) in the IMISUN-2 ×

ZENB9 F_2 family, the introgression of resistant alleles (T) into wild-type (C/C) male and female inbred lines is necessary to produce T/T hybrids and maximize herbicide resistance in hybrids.

Do other genetic factors affect resistance to AHAS-inhibiting herbicides in sunflower populations segregating for the Ala205Val *AHAS1* resistance gene? Line 29023 was developed by introgressing the Ala205Val *AHAS1* mutation from IMISUN-1 line into ZENB9, using MAS to recover both the mutant gene and the ZENB9 genetic background. The resulting line (29023) was less tolerant to IMI herbicides than IMISUN-1 (unpublished data), suggesting that more than one gene may be necessary to achieve high levels of resistance. Bruniard and Miller (2001) studied the inheritance of resistance to imazamox in a sunflower line derived from ANN-PUR carrying the Ala205Val mutation and concluded that resistance was controlled by two genes, Ar_{PUR} and a modifier. Maximum resistance could only be achieved with homozygosity of both genes in inbred lines and hybrids.

In the present study, DNA polymorphisms were not identified between herbicide-susceptible and -resistant inbred lines in the *AHAS2* or *AHAS3* coding sequences, and steady-state levels of *AHAS2* and *AHAS3* mRNAs were not significantly different in resistant and susceptible lines. Thus, *AHAS2* and *AHAS3* seem to be unlikely candidates for quantitative trait loci affecting the degree of resistance in certain genetic backgrounds. Additional genetic determinants such as the efficiency of herbicide uptake, rate of transport, and mode of metabolism are candidates for factors that may affect herbicide resistance phenotypes.

The *AHAS1* gene has provided all of the herbicide-resistant mutations characterized thus far in sunflower. This suggests that either AHAS1 activity is required for branched chain amino acid synthesis in sunflower, or that *AHAS1* is the gene family member that is predominantly expressed in tissues affected by herbicide treatment. The preponderance of *AHAS1* cDNA sequences in the sunflower EST database suggests that the *AHAS1* gene is the most highly expressed member of the family. However, evidence has been presented that isoforms of AHAS are differentially regulated in *Brassica napus* (Ouellet et al. 1992), tobacco (Keeler et al. 1993), and cotton (Grula et al. 1995).

Sunflower has the dual role of a weed and crop in North America. The discovery of resistance to AHAS-inhibiting herbicides in wild sunflowers has created the basis for deploying herbicide resistant hybrids. The *AHAS* allele sequences and DNA markers described herein create tools for monitoring resistance genes in natural populations and commercial production, rapidly developing and deploying herbicide resistant hybrids through MAS, and identifying new *AHAS* point mutations in sunflower. The demonstration of gene flow from IMI-resistant domesticated sunflower to wild relatives (Massinga et al. 2003) and the lack of a competitive penalty associated with the Ala205Val mutation in common sunflower (Marshall et al. 2001) suggests that widespread use of the hybrids could result in emergence of new herbicide-resistant weedy sunflower biotypes by both selection and gene flow. The discovery and careful management of different resistance genes, especially mutations that lack cross-resistance to different classes of AHAS-inhibiting herbicides, is needed for better management of domesticated sunflower and other crops where control of weedy common sunflower is necessary.

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