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Resistance to acetohydroxyacid synthase (AHAS)-inhibiting herbicides has been reported in over 90 weedy species, including wild sunflower biotypes, since the herbicides were developed in 1982. The *AHAS* gene family in sunflower, consisting of three paralogs *AHAS1*, *AHAS2*, and *AHAS3*, has been targeted for inducing herbicide resistance. A polymorphism identified in an elite sunflower line bred for resistance to the class of AHAS-inhibiting herbicides - sulfonylureas (SU) allowed development of a genotyping assay to be used in marker assisted selection (MAS). The expression level of the *AHAS* gene family was assayed in eight selected sunflower tissues. Diversity of the *AHAS* gene family was assessed among wild and domesticated sunflowers. A single nucleotide polymorphism (SNP) was discovered in the SU resistant sunflower. Development of a SNP assay, using fluorescently probed acyclo-dNTPs, facilitated genotypic determination. Non-quantitative reverse transcription-PCR (RT-PCR) and real-time RT-PCR were used to observe the expression level of each gene in the AHAS family. Diversity within the AHAS gene family among wild germplasm and domesticated germplasm was assessed using sequence alignment of 46 different accessions. The discovery of the SNP at codon

197 in *AHAS1* of the resistant line was predicted to confer resistance to the SU class of AHAS-inhibiting herbicides. The mutation at codon 197 has been observed in other plant species that confer resistance to the SU herbicides. Identification of the presence or absence of herbicide resistance genes using the SNP assay yields reliable genotyping that can be used to compliment phenotyping data. The *AHAS1* paralog was shown to be the most highly expressed gene in the *AHAS* gene family. All three genes are expressed in sunflower with the highest expression of *AHAS1* exhibited in leaf tissue. Overall gene diversity was greatest in *AHAS1*. Gene diversity was particularly high in the wild sunflower accessions for all three genes. The *AHAS1* gene is likely to be the target of mutations conferring herbicide resistance because it is the most highly expressed gene in the family. Ultimately, MAS for herbicide resistance genes in sunflower was improved by the development of a reliable SNP assay for codon 197 in *AHAS1*.

The Acetohydroxyacid Synthase Gene Family its Role in Herbicide Resistant Sunflowers

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To my grandmother
Myrna Hons

INTRODUCTION

Acetohydroxyacid synthase (AHAS, EC 2.2.1.6) is the first enzyme in the pathway synthesizing the branched-chain amino acids valine, leucine and isoleucine (Duggleby and Pang, 2000). It functions in a common pathway among bacteria, fungi, algae and higher plants and aligned sequences of the *AHAS* gene have indicated that it is a highly conserved gene (Duggleby and Pang, 2000). The enzyme is essential for a balanced supply of the three amino acids utilized by developing plants. AHAS catalyzes two reactions: (1) the condensation of two molecules of pyruvate to form acetolactate in the biosynthesis of valine and leucine, and (2) the condensation of pyruvate and 2-ketobutyrate (2-KB) to form acetohydroxybutyrate in the biosynthesis of isoleucine (Chang AK, 1997; Duggleby and Pang, 2000; McCourt and Duggleby, 2005). The absence of these amino acids in developing plants results in rapid inhibition of root and shoot growth, eventually leading to plant death (White et al., 2003). For this reason, many AHAS-inhibiting herbicides have been developed.

Herbicides inhibiting AHAS were introduced in 1982 and have become important tools for weed management in a variety of crop species (Saari et al., 1994). There are several families of AHAS inhibiting herbicides, including sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines, pyrimidinyl oxybenzoates, N-Phthalylvaline anilide and sulfonylcarboxamide (Duggleby and Pang, 2000; Tranel and Wright, 2002). The primary mechanism by which these herbicides are effective in causing plant death is through starvation of the plant for valine, leucine and isoleucine. Secondary effects of these herbicides include buildup of 2-KB, disruption of protein

synthesis, and disruption of photosynthate transport (Shaner, 1991). These herbicides are not competitive inhibitors but bind to the enzyme at a site distinct from the active site (Pang et al., 2003). The binding of the herbicide blocks the substrate access channel, therefore preventing the interaction of AHAS with the substrates essential for the production of the amino acids. These herbicides were rapidly and widely adopted because of their many production advantages. These herbicides are effective at low rates, grams per hectare of active ingredient as opposed to kilograms per hectare typical of many other herbicides, have low mammalian toxicity, exhibit broad-spectrum weed control, and have flexible application timing in a variety of crops (Mazur and Falco, 1989; Patzoldt and Tranel 2002). In fields of corn (*Zea mays* L.) and soybean (*Glycine max* L.), the common sunflower (*Helianthus annuus* L.) has been a troublesome weed. With the introduction of the AHAS inhibiting herbicides good control was achieved using the SU and IMI herbicides (Al-Khatib et al., 1998).

Although these herbicides are very effective, over-reliance on them has resulted in weed populations that are resistant to the AHAS inhibiting herbicides. The appearance of resistant populations is the result of intense selective pressure exerted by a lack of diversity in weed management practices (Gressel and Segel, 1978). In 1987 the first report of resistance was observed in prickly lettuce (*Lactuca serriola* L.), just five years after the initial use of the herbicides (Mallory-Smith, 1990). Since then there have been 92 species added to the list of weeds resistant to AHAS inhibiting herbicides to date (Heap, 2005). Included on the list are wild biotypes of the common sunflower that have developed resistance to IMI herbicides (ANN-PUR) and to SU herbicides (ANN-KAN) (Al-Khatib et al., 1999).

There are two mechanisms that account for the resistance of plants to acetohydroxyacid synthase (AHAS)-inhibiting herbicides: (1) a change in the regulatory subunit of the enzyme, or (2) a change in the ability of the plant to metabolize the herbicide. Predominately, the mechanism underlying AHAS resistance is an altered amino acid in the regulatory subunit of AHAS that changes its interaction with the herbicides (Lee and Duggleby, 2001; Fabie and Miller, 2002). The mutations typically affect herbicide binding to AHAS and do not impact the active site of the enzyme where the substrates bind (Pang 2003). Thus, the enzyme continues to function despite the mutation. Substitutions at five different amino acid positions, as the result of a single nucleotide polymorphism (SNP), have been shown to confer resistance or cross-resistance to one or more AHAS-inhibiting herbicides in several weed species including pigweed (*Amaranthus blitoides, Amarnthus hybridus, Amaranthus quitensis*), common cocklebur (*Xanthium strumarium*), eastern black nightshade (*Solanum ptycanthum*), prickly lettuce (*Lactuca serriola*), kochia (*Kochia scoparia*), wild turnip (*Brassica tournefortii*), Indian hedge mustard (*Sisymbrium orientale*), ragweed (*Ambrosia arteisiifolia, Ambrosia trifida*), and sunflower (White et al., 2003; Kolkman et al., 2004; http://www.weedscience.org/in.asp). These substitutions occur in the highly conserved amino acids Ala122, Pro197, Ala205, Trp574 and Ser653 (Guttieri et al., 1992; Guttieri et al., 1995; Boutsalis et al., 1999; Sibony et al., 2001; White et al., 2003; Kolkman et al., 2004).

In sunflower, three genes that encode AHAS have been identified (Kolkman et al., 2004). Multiple *AHAS* genes have been identified in several other plant species as well: six in cotton (*Gossypium hirsutum*; Grula JW, 1995), two in tobacco (*Nicotiana*

tabacum, Keeler et al., 1993), and five in *Brassica napus* (Ouellet et al., 1992). In sunflower, a Pro197Leu mutation in *AHAS1* has been shown to confer resistance to SU herbicides in the wild biotype ANN-KAN, and an Ala205Val mutation, also in *AHAS1*, has been shown to confer resistance to IMI herbicides in the wild biotype ANN-PUR (Al-Khatib et al., 1999; Kolkman et al., 2004). In at least six plant species a mutation at Pro197 has conferred resistance to SU herbicides (Guttieri et al., 1992; Guttieri et al., 1995; Boutsalis et al., 1999; Sibony et al., 2001; Kolkman et al., 2004).

Researchers are now exploiting the AHAS targeted site resistance and developing crops resistant to AHAS-inhibiting herbicides. Resistance has been established in canola (*Brassica napus*), corn (*Zea mays*), rice (*Oryza sativa*), sugarbeet (*Beta vulgaris*), and sunflower (Anderson and Georgeson, 1989; Croughan, 1996; D'Halluin et al., 1992; Al-Khatib and Miller, 2000; Miller and Al-Khatib, 2004). These crops have been developed through a variety of approaches, including somatic cell selection, mutation breeding, plant transformation, and interspecific and intraspecific crossing (Tranel and Wright, 2002). In sunflower, two lines have been developed with resistance to SU herbicides and two to IMI herbicides by introgressing mutations from wild isolates ANN-KAN and ANN-PUR, respectively, into elite breeding lines (Miller and Al-Khatib, 2000). SURES-1 and SURES-2 (Miller and Al-Khatib, 2004) carry SU resistance at Pro197, while IMISUN-1 and IMISUN-2 (Al-Khatib and Miller, 2000) carry IMI resistance at Ala205 (Kolkman et al., 2004). In *Arabidopsis,* resistance to SU herbicides conferred by a single nucleotide polymorphism at Pro197 in *AHAS* was also seen following ethylmethanesulfonate (EMS) mutagenesis (Jander et al., 2003).

Expression patterns of the *AHAS* gene family vary depending on the complexity of the gene family. In *Arabidopsis*, the single *AHAS* gene is constitutively expressed, cotton, in comparison, has a six-gene family where four genes are constitutively expressed and the other two are tissue-specific paralogs (Grula JW, 1995; Chang AK, 1997). In *B. napus* expression of one of the five *AHAS* genes is restricted to reproductive tissues (Ouellet et al., 1992). In sunflower, expression of the three *AHAS* genes was detected in apical meristems and leaves (Kolkman et al., 2004). In developing plants, one *AHAS* gene must be constitutively expressed (Ouellet et al., 1992; Keeler et al., 1993; Grula, 1995; Chang, 1997). A mutation that confers herbicide resistance may occur in this gene but it will still continue to function.

Thus far, mutations conferring resistance to AHAS-inhibiting herbicides in sunflower have been identified exclusively in the *AHAS1* gene. Determination of the expression pattern of *AHAS* gene family members in sunflower may provide information as to why *AHAS1* is the targeted paralog in this species. This information may be relevant to the production of new lines of herbicide resistant crops, including sunflowers, for example, when designing site directed mutagenesis to produce herbicide resistance in AHAS. Additionally, such knowledge will facilitate ease in development of AHAS markers for marker assisted selection (MAS) in crops.

A greater understanding of the *AHAS* gene family may be elucidated from a study in the diversity of the alleles within the family. *Helianthus annuus* is geographically the most widespread wild sunflower in North America. It can survive in a variety of habitats and has many morphological variations (Arias and Rieseberg, 1995). The wild sunflowers tend to be weedy and highly branched. They have small

flowering inflorescences and relatively small achenes (single-seeded fruits) that are released at maturation (Arias and Rieseberg, 1995; Burke et al., 2005). Domesticated germplasm incorporates both elite breeding lines and Native American land races, either of which could be unbranched with a single large head or branched with a larger primary head, all of which tend to have larger achenes that are held until maturation (Arias and Rieseberg, 1995; Tang and Knapp, 2003; Burke et al., 2005). Domestication has not occurred in a single lineage as variable sunflower germplasm has been used to develop modern cultivars (Arias and Rieseberg, 1995). The species has been domesticated several times with parallel development of domesticated lines, and breeding bottlenecks narrowing the genetic diversity from wild to domesticated lines (Tang and Knapp, 2003).

Interest in the frequency and nature of SNPs in plants has recently increased. Nucleotide variation, especially SNPs, are the most abundant polymorphisms in the genome of any species (Schneider et al., 2001). They are highly informative markers in the study of within-species diversity for nucleotide diversity estimates and have been used to identify evolutionary constrictions and selection pressures in the domestication of many plant species including maize, soybeans, sunflowers, and conifers (Kado et al., 2003; Tang and Knapp, 2003; Zhu et al., 2003; Tenaillon et al., 2004; Kolkman, unpublished data). By sequencing gene fragments, it is possible to detect SNPs between alleles at a single locus (Schneider et al., 2001).

The sunflower *AHAS1*, *AHAS2* and *AHAS3* genes were mapped to linkage groups 9, 6, and 2, respectively (Kolkman et al., 2004). Haplotypes were determined for each gene by sequencing 23 *AHAS1* alleles, 24 *AHAS2* alleles, and nine *AHAS3*

alleles (Kolkman et al., 2004). This analysis primarily looked at elite inbreds although SURES-1 and -2 and IMISUN-1 and -2, as well as a wild *H. annuus*, ANN1811, were included. Very little nucleotide diversity was detected in *AHAS2*. Twenty-three of 24 lines carried identical *AHAS2* coding sequences, whereas the wild accession, ANN1811, was distinguished by a single in-frame 6 bp insertion (Kolkman et al. 2004). Linkage group 6, the location of the *AHAS2* gene, historically lacks in polymorphism (Yu et al., 2003; Kolkman et al., 2004; Burke et al., 2005). For *AHAS3,* a single SNP at codon 581 differentiated two haplotypes for the gene. Both *AHAS3* haplotypes were observed among elite inbreds. In contrast, sequencing of *AHAS1* alleles revealed five haplotypes distinguished by 48 SNPs. Three haplotypes were observed among herbicide-susceptible inbreds and two haplotypes were accociated with sulfonylurea and imidazolinone resistance. A marker that mapped to within 5cM of *AHAS1*, ZVG41, was found to be hyperpolymorphic, supporting the notion that this is a highly variable region of the sunflower genome (Kolkman, unpublished data).

The goal of this study was to gain a greater understanding of the highly conserved acetohydroxyacid synthase gene family in sunflower. Our specific objectives were to: (i) identify a mutation in a herbicide resistant Pioneer Hi-Bred International sunflower line that confers resistance to SU herbicides; (ii) develop a DNA-based marker for the mutation; (iii) assess the reliability and utility of the marker; (iv) determine the expression pattern of the sunflower *AHAS* genes in eight tissues; (v) assess the diversity of the *AHAS* genes in wild and domesticated sunflower lines.

MATERIALS AND METHODS

Identification of a Polymorphism and Development of a SNP Genotyping Assay

Plant materials and DNA isolation

Pioneer Hi-Bred International (Woodland, CA, USA) supplied lyophilized leaf tissue from a known herbicide-resistant (PH-SUR) and herbicide-susceptible (PH-SUS) sunflower. Total genomic DNA was isolated from the dried leaf samples using a modified CTAB method (Webb and Knapp, 1990). Pioneer Hi-Bred International supplied sunflower seed for use as a blind test of the *AHAS* marker. Leaf disks were harvested from fully expanded leaves from 184 sunflowers grown from the seed supplied by Pioneer. Total genomic DNA was isolated from the leaf samples using the Wizard Magnetic 96 DNA Plant System (Promega, Madison, WI, USA).

DNA marker development

Nucleotides 255 to 685 of the *AHAS1* gene (GenBank accession no. AY541451) were amplified from known herbicide-resistant and –susceptible sunflower lines provided by Pioneer were, sequenced and used for polymorphism discovery and DNA marker development. The PCR conditions were as follows: 1X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each gene specific primer (ALS-5'F, ALS-5'R, Table 1), 1U/rxn *Taq* DNA polymerase, and 30 ng genomic sunflower DNA in a total volume of 30 μl. After an initial denaturation step at 95°C for 4 min, a program of 39 cycles was used, consisting of 6 cycles of touch-down PCR (95°C for

Table 1. Oligonucleotide primer names, sequences, purpose and the source of the sequence used for the SNP detection.

Primer[a]	Purpose	Sequence (5'-3')	Nucleotide position[b]
ALS-5'F	sequencing partial *AHAS1* alleles	AGACGTGTTGGTGGAAGCTCT	255-276
ALS-5'R	sequencing partial *AHAS1* alleles	AACTCGCAAGATAAAAAGCCTCACG	660-685
AHAS1c197targ-F2	SNP assay target forward primer	CCGGTCTTCCCGGCGTG	427-453
AHAS1c197targ-R1	SNP assay target reverse primer	CCACTAACTGTTGCTGTATATCTTTC	719-745
AHAS1c197snp-D	codon 197 downstream SNP primer	ATCGGTTCCGATCATTCTCCGG	546-567

[a] Primers derived from *Helianthus annuus AHAS1* haplotype 1, GenBank accession no. AY541451

[b] Relative to the nucleotide positions of the *AHAS1* haplotype 1 allele in *Helianthus annuus*

20 s, 64°C to 59°C for 45 s, 72°C for 1 min) followed by 33 cycles at a fixed annealing temperature of 59°C, and a final elongation step of 72°C for 10 min. PCR products were purified using the QiaQuick PCR Purification System (Qiagen, Valencia, CA, USA). The samples were sequenced on an ABI Prism 3730 DNA Analyzer at the Nevada Genomics Center (University of Nevada, Reno). DNA sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw) and searched for polymorphisms.

Genotyping assays were developed for the *AHAS1* codon 197 SNP (*AHAS1*-c197 C/T). SNPs were scored using the fluorescence polarization-template directed incorporation assay (Chen et al., 1999; Hsu et al., 2001; Kwok and Chen, 2003) and commercial kits (AcycloPrime-FP SNP Detection Kit, PerkinElmer Life Sciences, Boston, MA, USA). Target amplification and terminator incorporation reactions were performed as recommended by PerkinElmer. A target region of the genome containing the SNP was amplified using the following PCR conditions: 1X buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.1 μM of each gene specific primer (AHAS1c197targ-F2, AHAS1c197targ-R1, Table 1), 3.75U/rxn *Taq* DNA polymerase, and 20 ng genomic sunflower DNA in a total volume of 10 μl. After an initial denaturation step at 95°C for 4 min, a program of 42 cycles was used, consisting of 7 cycles of touch-down PCR (94°C for 20 s, 63°C to 56°C for 45 s, 72°C for 45 s) followed by 35 cycles at a fixed annealing temperature of 56°C, and a final elongation step of 72°C for 15 min. To ensure gene-specific amplification, a hot start reaction was simulated using a pre-heated MJ Research PTC-200 DNA Engine (Waltham, MA, USA) thermocycler. The *AHAS1* target fragment was 318 bp in length. A 5 ul sample from the target

amplification was used to continue the assay. Excess primers and dNTPs were removed by the addition of 2 ul clean-up mix containing exonuclease, alkaline phosphatase, and pyrophosphatase as described by Xiao et al. (2004). The target amplification was incubated with the enzymes at 37°C for 60 min followed by 80°C for 15 min to terminate enzyme reactions (Xiao et al., 2004). A single-base extension reaction was performed using a SNP detection primer that terminated adjacent to the SNP site and acyclo-dideoxynucleotide triphosphate terminators supplied in the kit. The AcycloTerminator mix used in this SNP assay contained an acyclo-dATP fluorescently labeled with TAMRA and an acyclo-dGTP fluorescently labeled with R110. The following conditions were used for the single-base extension reaction: 7 μl target PCR/clean-up mix, 2 μl 10X reaction buffer, 1 μl AcycloTerminator mix (G/A), 0.5 μl of 10 μM SNP detection primer (AHAS1c197snp-D, Table 1), 0.05 μl AcycloPol in a total volume of 20 μl. The AcycloPrime-FP program included an initial denaturation at 95°C for 2 min followed by 25 thermal cycles consisting of 95°C for 15 s and 55°C for 30 s. SNP genotypes were read on a Wallac 1420 VICTOR3 fluorescence polarization plate reader (PerkinElmer), reported in fluorescence units (mP), and alleles were identified using an EXCEL macro supplied by PerkinElmer for AcycloPrime SNP genotyping (PerkinElmer, Boston, MA, USA).

Assaying Expression Levels of Three *AHAS* Genes in Selected Tissues of Sunflower

RNA isolation and first strand cDNA synthesis

From sunflower seeds germinated in plastic boxes roots were harvested approximately 1 week after germination. Pre-anthesis ray flowers were harvested from greenhouse grown sunflowers. Tissues were immediately frozen in liquid N_2 upon harvesting and stored at -80°C. One gram of each of the frozen tissue types was ground in liquid N_2 and total RNA was extracted using 9 ml of the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The samples were immediately vortexed and incubated at room temperature for 15 min. After the addition of 2 ml of chloroform the samples were mixed vigorously for 30 s and centrifuged at 10,000 x g for 20 min at 4°C. The top phase was transferred to a new 15 ml tube and an additional chloroform wash was performed. The top phase was transferred to fresh 15 ml tubes and 5 ml isopropanol was added to each sample. After incubation at room temperature for 10 min, the samples were then centrifuged at 3,000 x g for 10 min at 4°C. The supernatant was discarded and the pellet was washed with 10 ml of 75% ethanol. The pellet was vortexed until it detached from the side of the tube and the sample was centrifuged at 3,000 x g for 5 min. The ethanol wash was repeated. The supernatant was discarded and the pellet was left to dry for 10 min. The RNA was resuspended in 500 µl of diethyl pyrocarbonate (DEPC)-treated water. The extraction was completed using Qiagen RNeasy Midi RNA clean-up kit, following the manufacturer's instructions for ≤0.5 mg RNA Cleanup with an additional elution step (Qiagen, Valencia, CA, USA).

Apex tissue was collected from greenhouse grown plants at the 8 leaf stage. The tissue was immediately frozen in liquid N_2 upon harvesting and stored at -80°C. The frozen tissue was ground in liquid N_2 and 100 mg was used to extract total RNA

using the Qiagen RNeasy Plant Mini kit according to the instructions of the manufacturer (Qiagen, Valencia, CA, USA). Total RNA samples were DNase treated with Promega DNase according to the manufacturer's instructions with a total volume of 100 µl. The extraction was completed using Qiagen RNeasy Midi RNA clean-up kit and following the manufacturer's instructions for ≤0.5 mg RNA Cleanup at ¼ the suggested volumes (Qiagen, Valencia, CA, USA).

Total RNA samples from leaves (3 week old seedlings) and developing kernels at 10, 14, 18 and 22 days after flowering (DAF) were supplied by Dr. G. Felix Schuppert (Schuppert, 2005). A 5 µg aliquot of total RNA from developing kernels at 10, 14, 18 and 22 DAF, leaves, ray flowers, apex and roots was used as template for the first strand cDNA synthesis. The synthesis was carried out using MMLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with an oligo $(dT)_{12-18}$ primer according to the instructions of the manufacturer.

The cDNA was tested for DNA contamination by running a standard PCR using primers, KASII F/R (Schuppert, 2005), known to amplify an intron-containing region of the sunflower beta ketoacyl synthase II gene. The PCR reaction was checked for multiple bands and size specific bands.

AHAS1, AHAS2 and AHAS3 transcript detection in eight different tissues

Gene specific primers were designed for *AHAS1, 2* and *3* to amplify a fragment between 200 and 250 bp (Table 2). Primers were designed for the actin gene that amplified a fragment of 125 bp (Table 2). These primers were optimized by running a temperature gradient PCR and comparing the level of amplification at each

Table 2. Oligonucleotide primers used for RT-PCR, real-time RT-PCR, and sequencing.

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'
AHAS1	AGACGTGTTGGTGGAAGCTCTG	CGATACACACGCCGGGAAG
AHAS2	GAGTGATTTGTTGCTTGCGTTTGGT	CCTTCCTCCAATTTGAAAAATCAAGAGAATTT
AHAS3	GATTGATAAAAGCGACTTGTTGTTG	GTGAGAAATCGAAATCAAGATCTTCA
Actin	GCAAAAAGCAGCTCGTCTGT	AGCAGCTTCCATTCCAATCA

temperature. To determine the presence or absence of gene expression in each tissue type a standard PCR was performed using cDNA from 8 different tissues: developing kernels at 10, 14, 18 and 22 days after flowering (DAF), leaves, ray flowers, apex and root), a positive control of genomic DNA (RHA 373), and a negative no template control (water). PCR conditions were as follows: 1X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, 0.5 U/rxn Taq DNA Polymerase and template (8 ng of cDNA or 10 ng of genomic DNA or 2 μl water) in a total volume of 15 μl. After an initial denaturation step at 95°C for 4 min, a program of 35 cycles was used, consisting of 95°C for 20 s, 59°C for 30 s, 72°C for 30 s, and a final elongation step of 72°C for 10 min. The samples were electrophoresed on a 1.5% agarose gel and scored for presence or absence of RT-PCR products. To confirm gene specificity of the primers, PCR products were purified using the QiaQuick PCR Purification System (Qiagen, Valencia, CA, USA) and sequenced on an ABI Prism 3730 DNA Analyzer at the Nevada Genomics Center (University of Nevada, Reno).

Real-time PCR Quantification of mRNAs in the AHAS gene family

A two-step real-time PCR procedure was performed in all experiments. The first step was first strand cDNA synthesis; the second step was real-time PCR analysis performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The reaction contained 4 ng of cDNA, 1X SYBR Green PCR Master Mix (ABI SYBR GREEN 2X Master Mix, Applied Biosystems, Foster City, CA, USA) and 1 μM of the forward and reverse primer in a total volume of 25 μl. The actin gene was used as an endogenous control within each tissue type.

The relative standard curve method was used to quantify the relative expression of the tissues; therefore each real-time PCR run included a standard curve (16, 8, 4, 2 and 1 ng of cDNA) for the gene of interest and for *actin*. All reactions were run in triplicate on a single plate containing a gene of interest and the *actin* control. The comparative threshold (Ct) value was set using the ABI Sequence Detection Systems 1.0.1 software (Applied Biosystems, Foster City, CA, USA). Following the guidelines in the ABI Prism 7700 Sequence Detection System User Bulletin #2, the slope (m) and y-intercept (b) of the standard curve for the gene of interest and control (log[input amount] vs. Ct value of input amount) was calculated. The input amount of RNA for the unknown samples was determined by first calculating the log input amount (X) =([Ct value of unknown sample]-b)/m and, 2) and then calculating the input amount (IA)= 10^ (X). To determine the normalized amount of the gene of interest divide the input amount (IA) of the gene of interest (*AHAS1* or *AHAS2*) by the input amount (IA) of the control actin. The highest normalized transcript level was used as the calibrator, which was leaf in this experiment. Then to determine the relative expression level of the other seven in comparison to leaf the normalized leaf expression value was divided by each of the normalized expression values of the other seven tissues. The difference between the tissues was determined by dividing the relative expression of leaf by the relative expression of the other seven tissues.

Calculating Gene Diversity for the *AHAS* Gene Family in Domesticated and Wild Sunflower

Genotyping

Genomic DNAs from single plants representing 12 wild accessions and DNA from 15 domesticated lines (Table 3[a]; Tang and Knapp 2003) were used to investigate sequence diversity in the AHAS gene family. Paralog-specific primers (Table 4) were designed to amplify a ~1,000 bp fragment from each gene. Standard PCR conditions were used for amplification: 1X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM each primer, 1 U/rxn *Taq* DNA polymerase and 20 ng of genomic DNA in a total volume of

30 μl. After an initial denaturing step at 95°C for 4 min a program of 39 cycles was used, consisting of 6 cycles of touch-down PCR (94°C for 20 s, 64°C to 59°C for 45 s, 72°C for 20 s) followed by 33 cycles at a fixed annealing temperature of 59°C, and a final elongation step of 72°C for 15 min. PCR reactions were purified using the Qiaquick Qiagen PCR Purification System (Qiagen, Valencia, CA, USA) and sequenced on an ABI Prism 3730 DNA Analyzer at the Nevada Genomics Center (University of Nevada, Reno). Alignments were generated using CodonCode software (Boston, Mass). Gene-specificity was confirmed by alignments with previously sequenced *Helianthus annuus* L *AHAS* genes (GenBank accession no. AY541451-AY541458) (Kolkman et al. 2004). Sequences were aligned for a total of 46 sunflower lines (Table 3) including the 27 lines sequenced in this study and an additional 19 lines previously sequenced by Kolkman et al. (2004). Haplotypes were identified within each set of alignments.

Table 3. Genotypes used to calculate sunflower nucleotide diversity in the AHAS gene family. Common names, plant introduction numbers, germplasm groups, origins, and herbicide resistance or susceptiblity for 46 sunflower (*H.annuus* L.) germplasm accessions.

Table 3. (Continued)

Common name	Number	Germplasm group	Origin	Herbicide resistance
PI-UT[a]	PI468619	Wild population	Utah	Unknown
PI-OK[a]	PI435619	Wild population	Oklahoma	Unknown
PI-NV[a]	PI468596	Wild population	Neveda	Unknown
PI-CA[a]	PI435593	Wild population	California	Unknown
PI-OR[a]	PI531015	Wild population	Oregon	Unknown
PI-AZ[a]	PI468575	Wild population	Arizona	Unknown
PI-MX[a]	PI413123	Wild population	Mexico	Unknown
PI-SD[a]	PI413039	Wild population	South Dakota	Unknown
PI-MT[a]	PI531022	Wild population	Montana	Unknown
ANN1811[a]	PI494567	Wild population	Texas	Unknown
ANN1238[a]	--	Wild population	Nebraska	Unknown
Abendsonne Red[a]	PI490316	Wild population	Germany	Unknown
Arikara[a]	PI369357	Domesticated	North Dakota	Unknown
Hopi[a]	PI369359	Domesticated	Arizona	Unknown
Havasupai[a]	PI369358	Domesticated	Arizona	Unknown
Seneca[a]	PI369360	Domesticated	New York	Unknown
HA89[a]	PI599773	Domesticated	USDA-ARS	S
HA383[a]	PI578872	Domesticated	USDA-ARS	S
HA372[a]	PI534658	Domesticated	USDA-ARS	S
HA369[a]	PI534655	Domesticated	USDA-ARS	S
HA370[a]	PI534656	Domesticated	USDA-ARS	S
HA407[a]	PI597371	Domesticated	USDA-ARS	S
RHA274[a]	PI599759	Domesticated	USDA-ARS	S
RHA801[a]	PI599768	Domesticated	USDA-ARS	S
RHA409[a]	PI603990	Domesticated	USDA-ARS	S
RHA417[a]	PI600000	Domesticated	USDA-ARS	S
RHA373[a]	PI560141[e]	Domesticated	USDA-ARS	S
RHA280[b]	PI552943	Domesticated	USDA-ARS	S
RHA266[b]	PI599755	Domesticated	USDA-ARS	S
HA425[b]	PI617098	Domesticated	USDA-ARS	R[d]
IMISUN-1[b]	PI607927	Domesticated	USDA-ARS	R[d]
IMISUN-2[b]	PI607928	Domesticated	USDA-ARS	R[d]
SURES-1[b]	PI633749	Domesticated	USDA-ARS	R[c]
SURES-2[b]	PI633750	Domesticated	USDA-ARS	R[c]
NMS373[b]	PI560141	Domesticated	USDA-ARS	S
ZENR1[b]	--	Domesticated	ADVANTA	S
ZENR7[b]	--	Domesticated	ADVANTA	S
ZENB9[b]	--	Domesticated	ADVANTA	S
ZENB13[b]	--	Domesticated	ADVANTA	S
ZENR13[b]	--	Domesticated	ADVANTA	S
ZENR16[b]	--	Domesticated	ADVANTA	S
ZENR17[b]	--	Domesticated	ADVANTA	S
CAS3[b]	--	Domesticated	ADVANTA	S
24311[b]	--	Domesticated	ADVANTA	S
29023[b]	--	Domesticated	ADVANTA	R[d]
32450[b]	--	Domesticated	ADVANTA	S

[a]Genotypes sequenced in current study using primers identified in Table 4
[b]Previously described genotypes in Kolkman et al. 2004
[c]Resistance to sulfonylurea herbicides
[d]Resistance to imidazolinone herbicides
[e]PI number of the restorer line RH373

Table 4. DNA sequences of the primers used for PCR screening and sequencing for gene diversity. Listed is the paralog- specific primer set, the primer sequence, the reference allele used to design the primer and location of the primer in *Helianthus annuus*.

Primer Name[a]	Paralog Targeted	Primer Sequence (5'-3')	Location (nt)
AHAS1-F3	*AHAS1*	CTCCTTCAAACCACCGTCAC	27-47[b]
AHAS1-R3	*AHAS1*	GAAAAATCAAGATTAGTCACCGAATTC	1242-1269[b]
AHAS2-F1	*AHAS2*	TCCTCCCCACCCTTCCATCA	15-35[c]
AHAS2-R3	*AHAS2*	CCTTCCTCCAATTTGAAAAATCAAGAGAATTT	1224-1256[c]
AHAS3-F1	*AHAS3*	ATGGCAGTCCCCCTCACTTT	1-20[d]
AHAS3-R3	*AHAS3*	GTGAGAAATCGAAATCAAGATCTTCA	1221-1247[d]

[a] Primers used for amplification and for final sequencing.

[b] Relative to nucleotide positions of the *AHAS1* haplotype1 allele in *Helianthus annuus*. (GenBank accession no. AY541451)

[c] Relative to nucleotide positions of the *AHAS2* haplotype1 allele in *Helianthus annuus*. (GenBank accession no. AY541456)

[d] Relative to nucleotide positions of the *AHAS3* haplotype1 allele in *Helinathus annuus*. (GenBank accession no. AY541458)

Statistics

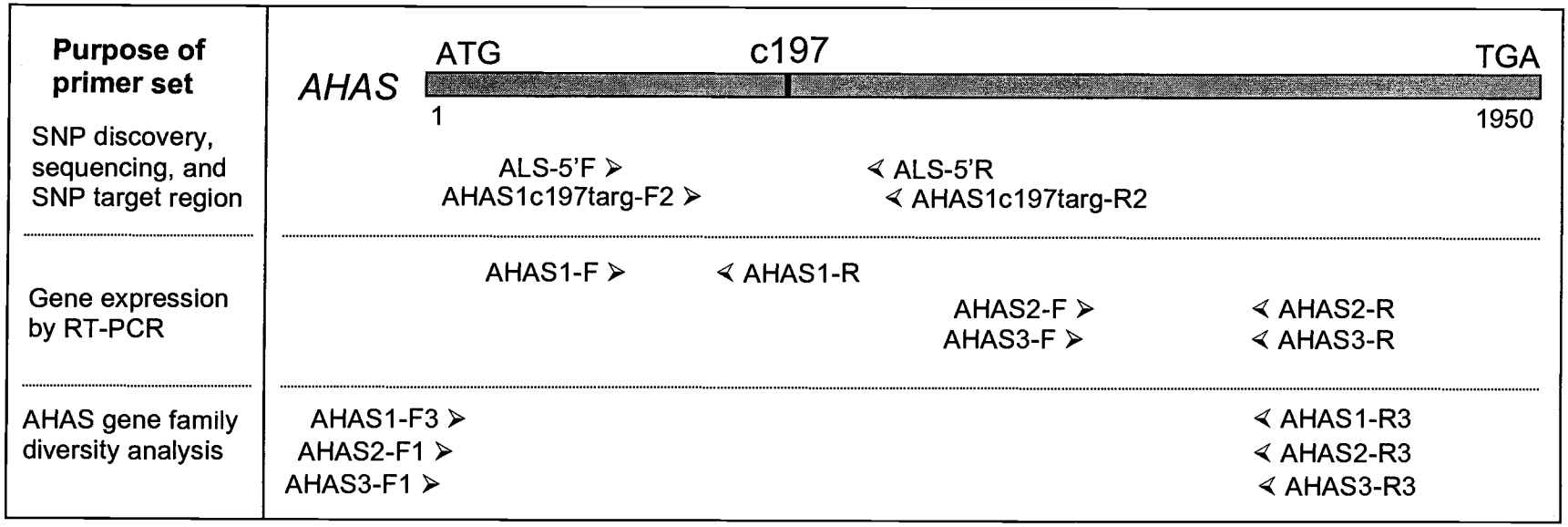
Haplotype (gene) diversity was estimated using the following equation: $H = 1 - \sum_{i=1}^{1} p_i^2$ where the number of codominant alleles at the locus was equal to 1 and p is the frequency of the i-th allele (Nei 1987).

RESULTS

Development and Validation of a SNP Genotyping Assay Diagnostic for an *AHAS1* Mutation (Pro_{197}) Conferring Resistance to Sulfonylurea Herbicides

We sequenced partial *AHAS1* alleles from proprietary SU resistant (PH-SUR) and susceptible (PH-SUS) inbred lines by amplifying DNA fragments (nt 255 to 685) spanning three codons (c122, c197, and c205) that when mutated are known to confer resistance to SU and IMI herbicides in plants (Tranel and Wright, 2002; White et al., 2003; Kolkman et al., 2004). Alleles were amplified using the ALS-5'F/ ALS-5'R primer pair (Figure 1; Table 1). PH-SUR was developed by EMS mutagenesis of HA89 (Gabard and Huby, 2001), a susceptible inbred line, and we anticipated a G/C-to-A/T transition from the EMS induced mutation. Greene et al. (2003) analyzed 1,900 EMS-induced mutations in 192 *Arabidopsis* genes and found that >99% were G/C-to-A/T transitions. The herbicide resistance phenotype exhibited by PH-SUR suggested that PH-SUR might carry an induced proline (CCC) to leucine (CTC) mutation in codon 197 (Pro_{197} mutation) identical to the spontaneous Pro_{197} mutation discovered in *AHAS1* in the wild sunflower population ANN-KAN (Kolkman et al., 2004). The ANN-KAN Pro_{197} mutation was introgressed into elite oilseed inbred lines (SURES-1 and SURES-2) and conferred resistance to the sulfonylurea herbicide tribenuron [ethyl 2-[[[[(4-chloro-6-methoxypryimidin-2-yl)amino]carbonyl]amino]sulfonyl]benzoate] (Fabie and Miller, 2002; Miller and Al-Khatib, 2004). However, in a comparative study, Fabie and Miller (2002) found that an SU resistant hybrid supplied by Pioneer Hi-Bred and an SU resistant USDA line

Figure 1. Segments of *AHAS* genes or cDNAs amplified by indicated primers.



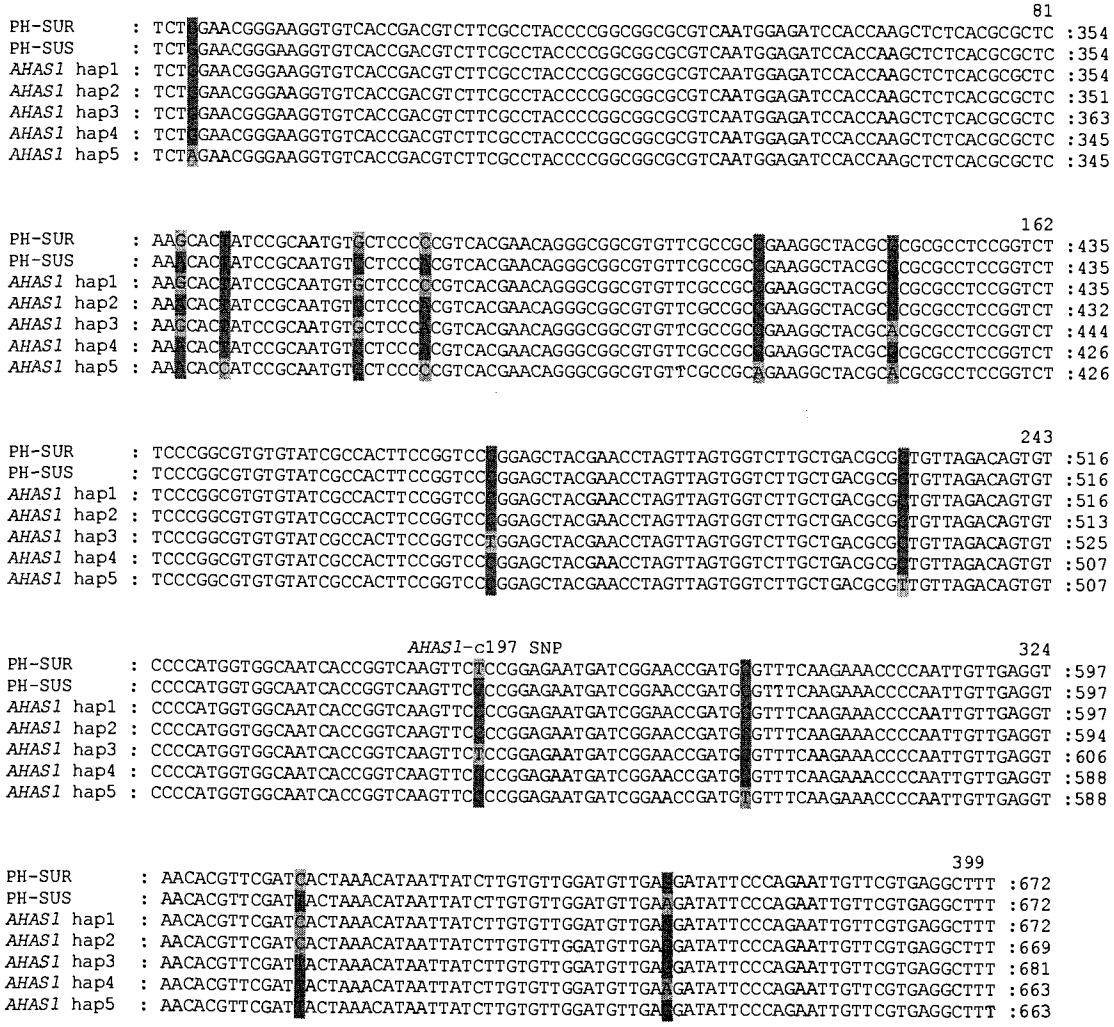
➤ represents forward primers; ➤ represents reverse primers.

derived from the wild population ANN-KAN differed in their response to the SU herbicides applied in that the Pioneer hybrid was some what less resistant to the SU herbicides.

PH-SUR and PH-SUS *AHAS1* partial allele sequences were aligned with SURES-1 (*AHAS1* haplotype 3), SURES-2 (*AHAS1* haplotype 3), HA89 (*AHAS1* haplotype 1), and additional sunflower *AHAS1* allele sequences. The PH-SUR sequence was identical to *AHAS1* haplotype 1 of its progenitor, HA89 (Kolkman et. al., 2004), except for a single nucleotide difference in the Pro_{197} codon (Figure 2). PH-SUS carried haplotype 4 as identified by Kolkman et al. (2004). We identified five additional SNPs between the PH-SUR and PH-SUS alleles. Four of these differences were not predicted to change amino acids and the fifth corresponded to the *AHAS1* haplotype 1 of PH-SUR's progenitor, HA89. The PH-SUR haplotype differed from ANN-KAN (haplotype 3) identified by Kolkman et al. (2004) because the latter originated in a wild population and carries additional SNPs.

We developed a single-base extension SNP marker assay diagnostic for the Pro_{197} mutation in *AHAS1* found in SURES-1, SURES-2, and PH-SUR (Figure 3). The forward and reverse target primers (AHAS1c197targ-F2 and AHAS1c197targ-R1, respectively; Figure 1; Table 1) were selected to amplify a 318 bp DNA fragment (nt 427 to 745) flanking the SNP from *AHAS1*, while the SNP detection primer (AHAS1c197snp-D) was positioned immediately downstream of the SNP. Since the AHAS1c197snp-D primer was positioned downstream, approaching from the 3' end, of the C/T SNP the acyclo-dNTPs incorporated were G or A. Target primer sites were chosen by aligning *AHAS1*, *2*, and *3* DNA sequences (GenBank accession no.

Figure 2. Nucleotide alignment of a 5' portion of the *AHAS1* gene. Partial sequences from PH-SUR, PH-SUS, HA89 (represented by *AHAS1* hap1), SURES-1 and SURES-2 (represented by *AHAS1* hap3) sunflower lines were aligned. Single-nucleotide polymorphisms (SNPs) are highlighted and the mutation conferring herbicide resistance (codon 197) is underlined and labeled.

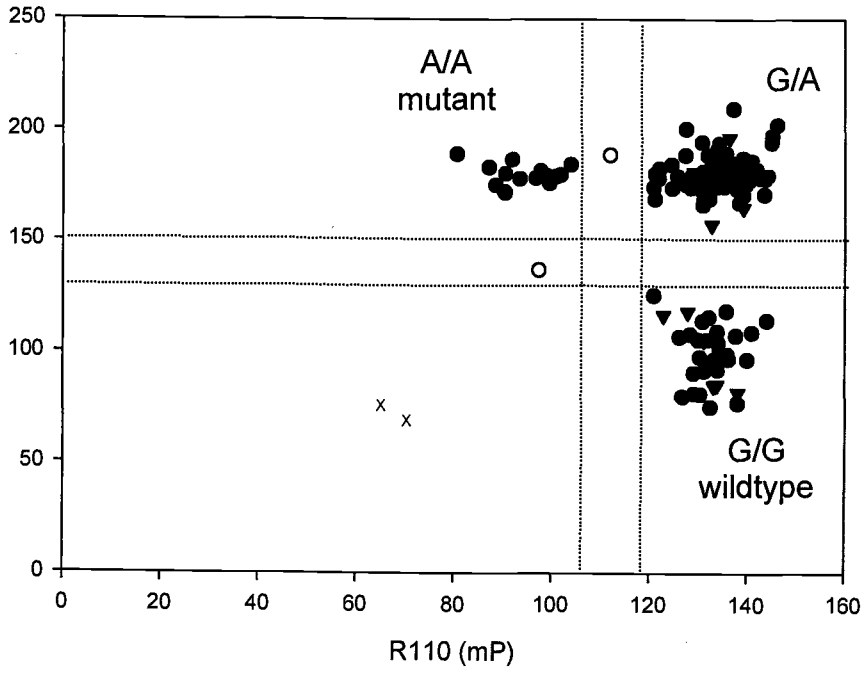


AY541451- AY541458) and identifying paralog- specific DNA sequences. The number of target primer sites unique to each member of the gene family was limited because the three paralogs are highly conserved, particularly in the segment targeted for SNP marker development (Kolkman et al., 2004). Gene specificity of the AHAS1c197targ-F2 and AHAS1c197targ-R1 primer pair was assured by designing primers which have a nucleotide specific only to *AHAS1* at the 3' end of each primer, in addition each primer had another nucleotide mismatch with *AHAS2* and four mismatches with *AHAS3*.

The SNP marker assay was initially tested on SURES-1, HA441 (a susceptible line), and a small panel of progeny from a cross of R X S inbred lines to assess the magnitude of separation among A/A (resistant), G/A (heterozygous), and G/G (susceptible) genotypes. Genotype clusters were separated by 25 mP on the TAMRA and 15 mP on the R110 axis and the SNP assay produced the expected genotypes in all cases.

To further investigate the utility of the c197 SNP assay, we performed a blind test using DNA samples from 25 herbicide resistant inbred and hybrid lines, 43 susceptible inbred and hybrid lines, and 116 single-cross hybrids between resistant and susceptible lines. Other than PH-SUR and PH-SUS, the *AHAS1* alleles carried by the inbred lines and hybrids were not known *a priori*. The inbred lines and hybrids had been assigned to homozygous resistant or susceptible and heterozygous classes by Pioneer based on screening the parental lines for resistance to chlorsulfuron in the field. The AHAS1c197 SNP marker identified 48 G/G homozygotes, 25 A/A homozygotes, and 111 heterozygotes among the 184 unknowns (Figure 3). We

Figure 3. SNP genotyping assay for the C/T (G/A) SNP in codon 197 of *AHAS1*. The *AHAS1*-c197 SNP marker was genotyped on 184 hybrid and inbred lines. Data points marked o were not assigned a genotype. Data points marked ▼ were the discrepancies between the SNP assay and Pioneer's predicted calls. Data points marked X are blanks.



observed 20 discrepancies between Pioneer's calls based on parental phenotyping and the genotypic calls of the SNP assay, an error rate of 10% (Table 5). Pioneer had identified 12 of these as heterzygotes of which seven were classified as G/G and 5 as A/A according to the SNP assay. Additionally, 6 discrepancies were called homozygous resistant by Pioneer and heterozygous by the SNP assay. The pedigrees of the discrepancies (Table 5) show four different parental lines (B-1, B-2, B-3, and PH-SUR) in multiple crosses.

The reproducibility and reliability of the SNP genotyping assay for the *AHAS1* Pro_{197} mutation was further investigated by performing independent analyses, in triplicate, on three lines (HA89, SURES-1 and HA434) with known genotypes identified by allele sequencing and six non-discrepant and 11 discrepant samples from the blind test. Genotyping calls in each replicate in the second test were identical to the first. The concordance between genotypes identified by the SNP assay and allele sequencing was 100%. We concluded that the 20 discrepancies observed in the blind test were apparently caused by misclassifying inbreds or hybrids after the parental phenotypic test, errors made in crossing, or mistakes in tissue collection.

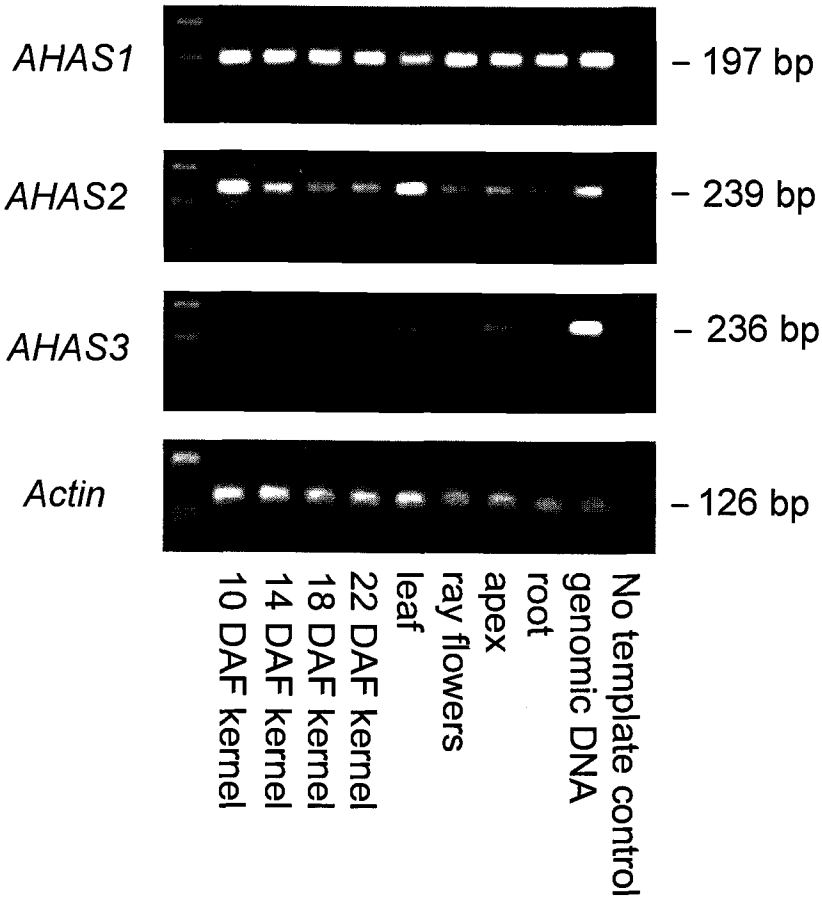
Expression of Three AHAS Genes in Selected Tissues of Sunflower

The patterns of expression of the *AHAS* gene family were surveyed using non-quantitative RT-PCR (Figure 4). Paralog specific primers (Table 2) were designed to amplify a region in the mature protein flanking 197 to 239 bp in the reference allele sequence (Figure 1). The control primer set, actin, was designed to amplify 126 bps of

Table 5. Discrepancies between the SNP assay and Pioneer phenotypic calls. In a blind assay 20 discrepancies were observed among the 184 sunflower lines screened. The parent lines used in the cross are listed as the pedigree.

SNP assay genotypic call	Pioneer phenotypic call	Pedigree
gg	ga	A/**B-1**
gg	ga	C/**B-1**
gg	ga	D/**B-1**
gg	ga	E/**B-1**
gg	ga	**B-2**/F
gg	ga	**B-3**/G
gg	ga	**B-3**/H
aa	ga	**PHB-R**/I
aa	ga	**PHB-R/PHB-S**
aa	ga	J/K
aa	ga	L/M
aa	ga	N/O
ga	aa	P/**B-1**
ga	aa	**B-2/B-1**
ga	aa	**B-2**/q
ga	aa	inbred (**B-2**)
ga	aa	inbred (X)
ga	aa	inbred (Y)
aa	gg	T/U
ga	gg	V/W

Figure 4. The *AHAS* gene family is expressed in eight tissues at different levels. Expression of *AHAS1*, *2*, and *3* genes in developing kernels and somatic tissues of sunflower assayed by non quantitative RT-PCR (PCR 35 cycles). DAF, days after flowering.



the sunflower *actin* gene. The specificities of the primers were confirmed by sequencing genomic DNAs amplified from RHA373, an inbred line with known allele sequences. *AHAS1* and *AHAS2* were expressed in each of the tissues sampled, whereas *AHAS3* was only expressed in leaves and apices and was more weakly expressed than the other two genes in those tissues (Figure 4). *AHAS1* seemed to be more strongly expressed than *AHAS2* in the tissues sampled other than leaves and developing seeds 10 DAF.

We subsequently quantified the expression of *AHAS1* and *AHAS2* using quantitative RT-PCR (Table 6). In preliminary real-time RT-PCR experiments we were unable to amplify *AHAS3*. *AHAS1* and *AHAS2* were more strongly expressed in leaves than other tissues. The expression of *AHAS1* was 6.1 to 61.6-fold greater in leaves than developing seeds, ray flowers, apices, and roots, whereas the expression of *AHAS2* was 3.8 to 11-fold greater in leaves than the other seven tissues (Figure 5; Table 6).

The absolute value of the slope of the standard curve, log input amount vs. ΔCt (AHAS1-AHAS2), was equal to 0.6 indicating a difference in primer efficiency and according to the ABI user bulletin #2 (2001) the absolute value should be <0.1 for equal efficiency. We, therefore, could not accurately quantify differences in expression among paralogs because of amplification efficiency differences of the primers. Due to the high conservation of this gene family it was challenging to design paralog-specific primers and so were unable to design primer sets with equal amplification efficiencies and paralog specificity.

Table 6. Expression levels and relative expression of AHAS1 (A) and AHAS2 (B) in eight select tissues. Relative expression values are based on data normalized to actin and reported in fold differences between leaf and the select tissue, with leaf having the highest level of expression in both genes.

A

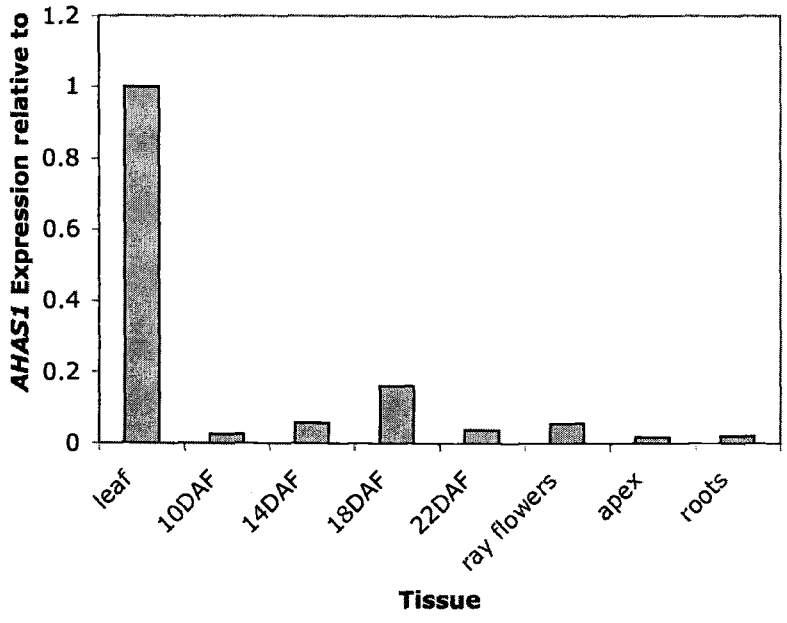
Tissue	AHAS1 Normalized to actin	Normalized AHAS1 relative to leaf
10DAF-HA341	0.48±0.12	40.08±9.67
14DAF-RHA345	1.11±0.10	17.52±1.65
18DAF-HA341	3.16±0.30	6.14±0.58
22DAF-HA341	0.73±0.13	26.53±4.57
leaves-HA341	19.37±1.32	1.00±0.07
ray flowers-HA89	1.07±0.18	18.06±3.01
Apex-HA441	0.31±0.08	61.62±15.83
roots-HA89	0.38±0.06	51.53±8.91

B

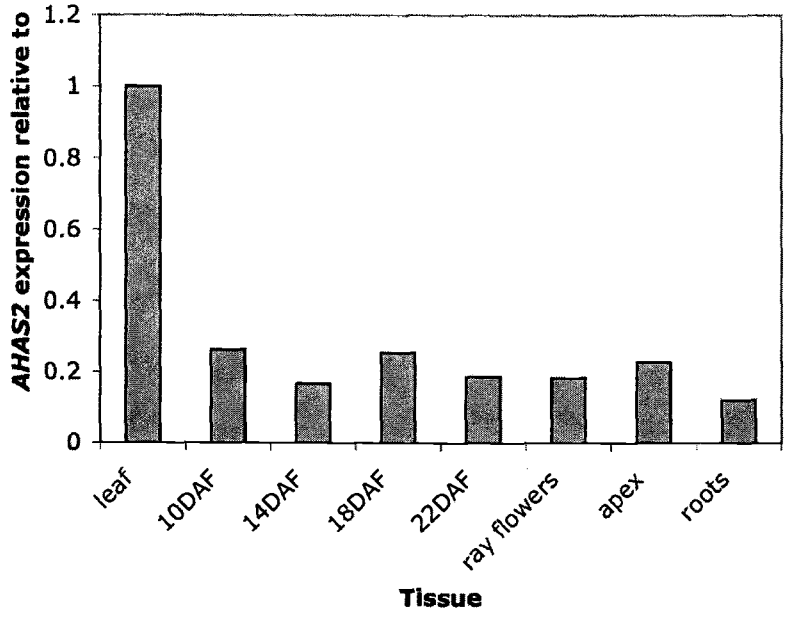
Tissue	AHAS2 Normalized to actin	Normalized AHAS2 relative to leaf
10DAF-HA341	1.54±0.38	3.81±0.94
14DAF-RHA345	0.97±0.04	6.01±0.26
18DAF-HA341	1.49±0.05	3.92±0.12
22DAF-HA341	1.11±0.06	5.29±0.27
leaves-HA341	5.85±0.59	1.00±0.10
ray flowers-HA89	1.07±0.16	5.45±0.80
Apex-HA441	1.33±0.22	4.39±0.72
roots-HA89	0.70±0.12	8.36±1.47

Figure 5. Expression levels of *AHAS1* and *AHAS2* quantified using real-time RT-PCR. The expression levels of *AHAS1* (A) and *AHAS2* (B) genes in the tissues of developing embryos at 10 DAF, 14 DAF, 18 DAF, and 22 DAF, ray flowers, apex and roots relative to leaf expression, if leaf expression equals 1.

A



B



Gene Diversity for the *AHAS* Gene Family in Domesticated and Wild Sunflower

To assess patterns of allelic diversity in domesticated and wild sunflower germplasm we amplified and sequenced partial *AHAS1*, *2*, and *3* alleles from 15 domesticated lines (including Native American land races and elite breeding lines) and 12 wild populations using paralog-specific primers flanking 926 to 1,120 bp in the reference allele sequences (Figure 1; Table 4; Table 3[a]). The DNA sequences targeted within each paralog flanked three of five codons (Ala_{122}, Pro_{197} and Ala_{205}) where *AHAS*-inhibiting mutations have been identified in plants (Tranel and Wright 2002; White 2003). Amplified segments of the three genes were selected to overlap as much as possible, but were not identical because primers had to be strategically placed in paralog-specific sequences. The allele sequences spanned nt 153-1165 in *AHAS1*, nt 149-502 and nt 682-1147 in *AHAS2*, and nt 67-1186 in *AHAS3*, as identified and numbered in *H. annuus AHAS* (Kolkman et al., 2004; GenBank accession no. AY541451, AY541456, AY541458). The locus specificities of the primers were confirmed by sequencing genomic DNAs amplified from lines with known allele sequences (HA89 and RHA801). Six sets of DNA sequences (two/locus) were produced from each DNA sample by single-pass sequencing *AHAS1*, *2*, and *3* amplicons in both directions. While some of the forward and reverse DNA sequences overlapped, we could not assemble contigs for every genotype; therefore, we separately aligned the DNA sequences produced in each direction, and performed statistical analyses on six different DNA sequence alignments (Table 7). The new allele sequences were aligned with previously described allele sequences (Table 3[b];

Table 7. Gene diversity in *AHAS1*, *AHAS2* and *AHAS3* of wild and domesticated sunflowers.

Table 7. (Continued)

Paralog	Group	Sequence Location (nt)	Allele Length (bp)[d]	n[e]	Number of SNPs	Number of INDELs	bp/SNP	S[f] (%)	NS[g] (%)	S/NS Ratio	Number of Haplotypes	Haplotype Diversity[h]
AHAS1[a]	Domesticated-1	153-567	414	24	8	1	52	50	50	1:1	3	0.6424
	Domesticated-2	564-1165	592	27	10	0	59	100	0	0	6	0.7133
	Wild-1	153-567	414	15	21	1	20	71.4	28.6	2.5:1	11	0.8711
	Wild-2	564-1165	592	18	39	0	15	92.3	7.7	12:1	14	0.9012
	Total-1	153-567	414	39	21	1	20	71	29	2.5:1	14	0.8455
	Total-2	564-1165	592	45	39	0	15	92	8	12:1	19	0.8810
AHAS2[b]	Domesticated-1	149-502	360	32	0	0	0	0	0	0	1	0.0000
	Domesticated-2	682-1147	566	33	1	0	566	100	0	0	3	0.1690
	Wild-1	149-502	360	11	3	1	120	33.3	66.7	1:2	5	0.5620
	Wild-2	682-1147	566	12	3	0	189	66.7	33.3	2:1	4	0.5139
	Total-1	149-502	360	43	3	1	120	33	67	1:2	6	0.4175
	Total-2	682-1147	566	45	3	0	189	67	33	2:1	6	0.5185
AHAS3[c]	Domesticated-1	67-652	586	16	5	0	117	100	0	0	2	0.1172
	Domesticated-2	653-1186	534	18	7	0	76	85.7	14.3	6:1	4	0.2963
	Wild-1	67-652	586	4	12	0	49	100	0	0	4	0.7500
	Wild-2	653-1186	534	12	25	0	21	84	16	5.25:1	12	0.9167
	Total-1	67-652	586	20	14	0	42	100	0	0	6	0.4250
	Total-2	653-1186	534	30	26	0	21	84.6	15.4	5.5:1	16	0.7333

[a] Sequence location relative to the *AHAS1* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541451; Kolkman et al., 2004)

[b] Sequence location relative to the *AHAS2* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541456; Kolkman et al., 2004)

[c] Sequence location relative to the *AHAS3* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541458; Kolkman et al., 2004)

[d] Number of sites in aligned sequences, excluding gaps

[e] Number of sequences sampled

[f] Synonymous nucleotide change

[g] Non-synonymous nucleotide change

[h] Haplotype (gene) diversity measured as $H = 1 - \sum_{i=1}^{1} P_i^2$ Nei 1987).

Kolkman et al., 2004). The number of allele sequences used in the alignments ranged from 4 to 39. Heterozygosity was observed in alleles from wild populations and land races within the domesticated lines but not in the inbreds within the domesticated lines.

We identified 21 SNPs and an ACC repeat polymorphism in *AHAS1* alignment 1 (Table 8, forward sequencing), and 39 SNPs in *AHAS1* alignment 2 (Table 9, reverse sequencing), comprising 14 and 19 haplotypes, respectively. In *AHAS2* alignment 1, we identified 3 SNPs and one 6 bp in-frame INDEL (Table 10, forward sequencing), and 3 SNPs in *AHAS2* alignment 2 (Table 11, reverse sequencing), comprising 6 haplotypes in each alignment. In *AHAS3* alignment 1, we identified 14 SNPs (Table 12, forward sequencing), and 26 SNPs in *AHAS3* alignment 2 (Table 13, reverse sequencing), comprising 6 and 16 haplotypes, respectively. *AHAS1* was significantly more polymorphic than *AHAS2* and 3. SNP frequencies were 3.3-fold greater in *AHAS1*, 6-fold greater in *AHAS2*, and 3.1-fold greater in *AHAS3* among wild than among domesticated germplasm accessions. Similarly, haplotype diversities were 1.3-fold greater in *AHAS1*, 3.0-fold greater in *AHAS2*, and 6.4-fold higher in *AHAS3* alignment 1 and 3-fold higher in *AHAS 3* alignment 2 among wild than domesticated germplasm accessions. Haplotype diversity was greater for *AHAS1* (0.8455 alignment 1 and 0.8810 alignment 2) than for *AHAS2* (0.4175 alignment 1 and 0.5185 alignment 2) and *AHAS3* (0.4250 alignment 1 and 0.7333 alignment 2). We observed 5 alleles among wild and 3 alleles among domesticated germplasm accessions for the $[ACC]_n$ repeat found in *AHAS1* (Kolkman et al., 2004). Heterozygosities for the SSR were 0.755 among wild and 0.642 among domesticated genotypes.

We identified 51 synonymous and 9 non-synonymous variants in *AHAS1*. Among the non-synonymous mutations were the functionally important Pro_{197} and Ala_{205} mutations found in *AHAS1* alleles in SURES-1 and 2, PH-SUR, and IMISUN-1 and 2 (Kolkman et al., 2004; Tranel and Wright, 2002; Table 7 and Table 8-9). The other seven non-synonymous SNP differences affected codons different from the five codons known to render plants resistant to AHAS-inhibiting herbicides (Tranel and Wright, 2002; Jander et al., 2003; White et al., 2003).

Table 8. Haplotypes of *AHAS1* in wild and domesticated sunflowers (alignment -1). Genotypes were grouped into the subsequent haplotype and the polymorphisms corresponding to the haplotype were identified.

Codon position*			79	82	83	89	92	106	112	114	133	134	38	140	150	154	155	161	170	175	183	183	197
Nucleotide position**			203	212	215	234	243	285	302	309	365	369	381	387	417	429	432	447	474	489	511	513	554
Amino acid change			P-R	P-Q	A-V				T-N		N-S												P-L
Haplotype	**Genotype**	**ACC repeat**																					
1	HA89, ZENB9, ZENR1, HA370, RHA274, HA369, RHA801, RHA409, HA407, RHA417	7	G	C	T	T	A	G	C	C	G	T	G	C	C	G	C	C	C	A	C	G	C
2	ZENB13, ZENR13, ZENR16, ZENR17, 32450, 24311,HA383, HA372, Arikara	6	C	A	C	G	T	G	C	C	A	T	C	A	C	G	C	C	C	A	C	G	C
3	SURES-1, SURES-2	10	C	A	C	T	T	G	C	C	G	T	G	A	C	A	C	C	T	A	C	G	T
4	RHA280, RHA266, CAS3, ZENR7, RHA373	4	C	A	C	T	A	G	C	C	A	T	C	A	C	G	C	C	C	A	C	G	C
5	IMISUN-1, IMISUN-2, HA425, 29023	4	C	A	C	K	A	A	C	C	A	C	C	C	A	A	C	C	C	A	T	G	C
6	PI-OK	7	S	C	Y	K	A	A	C	C	A	T	C	C	C	A	C	C	T	A	C	G	C
7	ANN1811	7	C	A	C	Y	A	R	C	C	A	T	C	A	C	A	C	Y	C	A	C	G	C
8	PI-MX	5	C	A	C	K	W	R	C	C	A	T	S	C	C	A	C	C	C	A	C	R	C
9	PI-UT	7	C	C	Y	T	A	A	C	M	A	T	C	C	C	A	C	Y	C	A	C	R	C
10	PI-AZ	6	C	C	T	T	A	A	C	C	G	K	G	A	C	A	C	C	C	A	C	G	C
11	PI-NV	6	C	A	C	T	W	G	C	C	G	T	G	C	C	A	G	C	T	T	C	A	C
12	PI-OR	6	C	A	C	G	T	G	C	C	A	T	S	C	C	R	C	C	T	A	C	R	C
13	PI-CA	6	C	A	C	T	A	G	M	C	A	T	S	C	C	A	Y	C	C	A	C	G	C
14	Abendsonne Red	6	C	A	C	G	T	G	C	C	A	T	C	A	C	G	C	C	C	A	C	G	C

* The codon position is relative to the *AHAS1* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541451)

**The nucleotide position is relative to the *AHAS1* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541451; Kolkman et al., 2004)

Table 9. Haplotypes of *AHAS1* in wild and domesticated sunflowers (alignment -2). Genotypes were grouped into the subsequent haplotype and the polymorphisms corresponding to the haplotype were identified.

Codon position*		203	205	218	229	234	242	243	255	255	256	257	265	268	270	279	279	281	287	299	300
Nucleotide position**		573	578	618	651	666	690	693	727	729	732	735	759	768	774	799	801	807	825	861	864
Amino acid change			A-V						P-A												
Haplotype	**Genotype**																				
1	HA89, ZENB9, ZENR1, HA370, RHA274, HA369, RHA801, RHA409, HA407, RHA417	C	C	C	G	T	G	T	C	G	A	T	G	T	G	A	A	G	G	G	G
2	ZENB13, ZENR13, ZENR16, ZENR17, 32450, 24311, HA383, HA372, Arikara, Abendsonne Red	C	C	C	G	T	G	T	C	G	A	T	G	T	G	A	A	G	G	G	G
3	SURES-1, SURES-2	C	C	T	G	T	G	T	C	G	A	T	G	T	G	A	A	G	G	G	G
4	RHA280, RHA266, CAS3, ZENR7, RHA373	C	C	T	A	T	G	T	C	G	A	T	G	T	G	A	A	A	G	G	G
5	IMISUN-1, IMISUN-2, HA425, 29023	C	T	T	G	T	G	T	C	G	A	T	A	T	G	A	A	A	C	G	A
6	Seneca	C	C	Y	G	T	G	T	C	G	A	T	G	T	G	A	A	G	G	G	G
7	Hopi	C	C	C	G	T	G	T	C	G	A	T	G	T	G	A	A	G	G	G	G
8	Havasupai	C	C	C	G	T	G	T	C	G	A	T	G	T	G	A	A	G	G	G	G
9	PI-SD	C	C	T	G	T	G	Y	C	K	R	T	G	T	G	A	A	G	G	R	G
10	ANN1238	C	C	T	G	T	G	T	C	G	A	T	G	T	G	A	A	R	G	G	G
11	PI-OK	C	C	T	G	A	G	T	C	G	A	T	G	T	G	A	A	A	G	G	G
12	ANN1811	C	C	T	G	T	K	C	C	G	A	T	G	T	G	A	A	G	G	R	G
13	PI-MX	C	C	T	G	T	G	T	C	G	A	T	G	Y	G	A	A	G	G	G	G
14	PI-MT	C	C	T	R	T	G	Y	C	K	R	T	G	T	G	A	A	R	G	R	G
15	PI-UT	C	C	T	G	T	K	T	C	G	A	C	G	T	G	A	A	R	G	R	G
16	PI-AZ	C	C	T	G	T	G	T	C	G	A	T	G	C	G	A	A	A	G	G	G
17	PI-NV	M	C	T	G	T	G	C	C	T	G	T	G	T	R	M	R	R	G	R	G
18	PI-OR	M	C	Y	G	T	G	Y	S	K	R	T	G	T	R	M	R	G	G	G	G
19	PI-CA	C	C	T	G	T	G	T	C	G	A	T	G	C	G	A	A	A	G	G	G

* The codon position is relative to the *AHAS1* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541451)

**The nucleotide position is relative to the *AHAS1* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541451; Kolkman et al., 2004)

Table 9 (Continued)

Codon position*		306	322	324	327	329	334	335	337	339	340	341	353	354	355	357	369	376	386	398
Nucleotide position**		882	930	936	945	951	966	969	973	981	984	987	1023	1026	1029	1035	1071	1092	1122	1158
Amino acid change									A-T											
Haplotype	**Genotype**																			
1	HA89, ZENB9, ZENR1, HA370, RHA274, HA369, RHA801, RHA409, HA407, RHA417	G	G	G	G	G	G	C	G			G		G		G	G			
2	ZENB13, ZENR13, ZENR16, ZENR17, 32450, 24311, HA383, HA372, Arikara, Abendsonne Red	G	G	G	G	G	G	C	G	G		G		G		G	G	C		G
3	SURES-1, SURES-2	G	G	G	G	G	G		G	G		G		G		G	G	C		
4	RHA280, RHA266, CAS3, ZENR7, RHA373	G	G	G	G	G	G	C	G	G		G	G		C	G	G	C		G
5	IMISUN-1, IMISUN-2, HA425, 29023	G	G	G	G	A	G	C	G			G	G		C	G	G	C		G
6	Seneca	G	G	G	G	G	G	C	G	K	Y	G		G		G	G	Y		K
7	Hopi	G	G	G	G	G	G	C	G			G		G		G	G			
8	Havasupai	G	G	G	G	G	G	C	G	K	Y	G		G		G	G	Y		K
9	PI-SD	G	G	R	R	G	G	C	G	G	Y	R	K	G		G	G	Y		G
10	ANN1238	G	G	G	G	G	G	C	G	G	C	G		G		G	G	C		G
11	PI-OK	G	G	G	G	G	G	C	G	G		G		G		G	G	C		G
12	ANN1811	S	G	R	R	G	G	C	R			G		G		G	G	Y	K	G
13	PI-MX	G	G	G	G	G	K	C	G	G	C	G	K	S		G	G	C		G
14	PI-MT	G	G	R	R	G	G	C	G	G	Y	G	K	K	Y	G	G	C		G
15	PI-UT	S	G	G	G	G	G	C	G	G		G		G		G	G	C		G
16	PI-AZ	G	G	G	G	G		C	G	G	C	G	G	C		G	G	C		G
17	PI-NV	G	G	G	G	R	G	C	G	G	Y	G		G	K	R	A	C		G
18	PI-OR	G	G	G	G	R	G	C	G	K		G		G	K	G	R	C		G
19	PI-CA	G	A	G	G	G		C	G	G	C	G		G		G	G			G

* The codon position is relative to the *AHAS1* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541451)

Table 10. Haplotypes of *AHAS2* in wild and domesticated sunflowers (alignment -1). Genotypes were grouped into the subsequent haplotype and the polymorphisms corresponding to the haplotype were identified.

Codon position*			76	112	163
Nucleotide position**			226	335	489
Amino acid change			P-term	T-M	
Haplotype	**Genotype**	**INDEL**			
1	RHA280, HA89, HA370, HA372, RHA373, HA383, RHA274, RHA417, HA407, HA369, RHA409, RHA801, RHA266, NMS373, SURES-1, SURES-2, IMISUN-1, IMISUN-2, ZENB9, ZENR1, ZENB13, ZENR13, ZENR16, ZENR17, ZENR7, CAS3, 32450, 24311, 29023, Seneca, Hopi, Havasupai	6bp DEL	C	C	G
2	ANN1238, PI-OK, ANN1811, PI-MX, PI-MT, PI-AZ, PI-CA	GCCACC	C	C	G
3	PI-SD	GCCACC	C	T	G
4	PI-UT	GCCACC	C	C	R
5	PI-OR	GCCACC	Y	C	G
6	Abendsonne Red	GCCACC	C	Y	G

* The codon position is relative to the *AHAS2* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541456)

**The nucleotide position is relative to the *AHAS2* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541456; Kolkman et al., 2004)

Table 11. Haplotypes of *AHAS2* in wild and domesticated sunflowers (alignment -2). Genotypes were grouped into the subsequent haplotype and the polymorphisms corresponding to the haplotype were identified.

Codon position*		228	268	347
Nucleotide position**		684	803	1041
Amino acid change			G-L	
Haplotype	**Genotype**			
1	RHA280, HA89, HA370, HA372, RHA373, HA383, RHA274, RHA417, HA407, HA369, RHA409, RHA801, RHA266, NMS373, SURES-1, SURES-2, IMISUN-1, IMISUN-2, ZENB9, ZENR1, ZENB13, ZENR13, ZENR16, ZENR17, ZENR7, CAS3, 32450, 24311, 29023, Arikara	C	G	G
2	PI-SD, ANN1238, PI-OK, ANN1811, PI-MX, PI-MT, PI-AZ, PI-NV	C	G	G
3	Havasupai	C	G	A
4	Seneca, Hopi, Abendsonne Red	C	G	R
5	PI-UT	C	K	G
6	PI-OR, PI-CA	S	G	G

* The codon position is relative to the *AHAS2* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541456)

**The nucleotide position is relative to the *AHAS2* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541456; Kolkman et al., 2004)

Table 12. Haplotypes of *AHAS3* in wild and domesticated sunflowers (alignment -1). Genotypes were grouped into the subsequent haplotype and the polymorphisms corresponding to the haplotype were identified.

Codon position*		64	82	85	88	98	115	118	141	146	155	176	178	200	217
Nucleotide position**		192	246	253	264	294	345	354	423	438	465	528	534	600	651
Amino acid change															
Haplotype	**Genotype**														
1	Seneca, SURES-2, IMISUN-1, IMISUN-2, HA383, HA369, HA370, HA372, HA407, HA89, RHA274, RHA373, RHA409, RHA801, RHA417	G	A	G	G	C	C	T	T	C	C	T	T	T	C
2	Arikara	G	A	G	R	C	C	[illegible]	Y	M	Y	Y	[illegible]	[illegible]	C
3	PI-MX	C	A	G	G	C	A	[illegible]	T	C	C	T	C	A	[illegible]
4	PI-MT	G	A	G	G	C	A	[illegible]	C	A	[illegible]	C	[illegible]	[illegible]	C
5	PI-NV	C	A	S	G	Y	A	Y	T	C	Y	T	T	A	G
6	PI-CA	C	C	G	G	C	A	[illegible]	T	C	[illegible]	T	T	A	G

* The codon position is relative to the *AHAS3* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541458)

**The nucleotide position is relative to the *AHAS3* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541458; Kolkman et al., 2004)

Table 13. Haplotypes of *AHAS3* in wild and domesticated sunflowers (alignment -2). Genotypes were grouped into the subsequent haplotype and the polymorphisms corresponding to the haplotype were identified.

Codon position*		222	233	236	237	247	260	265	268	271	272	276	283	293	296	299	302	306	318	319	321	327	332	335	345	349	353	357
Nucleotide position**		666	699	708	709	741	780	794	803	813	816	828	849	879	887	897	906	918	954	957	962	981	996	1005	1033	1047	1059	1071
Amino acid change								T-R	R-Q						G-A						D-G							
Haplotype	**Genotype**																											
1	Seneca, SURES-2, IMISUN-1, IMISUN-2, HA369, HA370, HA372, HA383, RHA417, RHA801, HA407, RHA274, RHA373, HA409, HA89,	C	A	G		A	G	C	G	A	C	A	T	T	G	A	C	G	G	G	A	A	G	G	T	G	T	G
2	Havasupai	C	A	A		A	G	C	G	R	C	A	T	T	G	A	C	G	G	G	A	A	G	G	T	G	T	G
3	Arikara	C	A	G		A	G	C	R	R	C	A	Y	T	G	A	C	G	G	G	A	W	G	K	T	C	T	G
4	Hopi	C	A	R		A	R	C	G	R	C	A	T	T	G	A	C	G	G	G	A	A	G	G	T	C	T	G
5	PI-SD	C	A	A		A	A	C	G	A	C	A	T	T	G	A	C	S	G	G	A	W	G	K	T	C	T	G
6	ANN1238		A	A		R	G	C	G	A	C	A	T	T	G	A	S	G	G	G	R	W	G	K	T	C	T	G
7	PI-OK		A	A		A	R	S	G	A	C	A	T	T	G	A	C	S	G	G	A	W	G	K	T	C	T	G
8	ANN1811	C	A	A		A	R	C	G	A	C	A	Y	T	G	A	S	G	G	G	A	W	G	K	T	C	T	S
9	PI-MX		A	A		A	G	C	G	A	C	A	T	T	G	A	C	G	G		A	T	G	T	T	C	T	G
10	PI-MT	C	A	A		A	G	C	G	A	C	A	C	T	G	A	C	G		G	A	T	G	T	C	C	T	G
11	PI-NV	C	A	A	Y	A	G	C	G	A	C	A	Y	C	S	A	C	G	G	G	A	W	K	T	T	Y	T	G
12	PI-UT	Y	A	A		A	R	C	G	A	C	W	Y	T	G	R	C	G	G	G	A	T	G	K	T	C	Y	G
13	PI-AZ		A	A		A	R	C	R	A	Y	A	T	T	G	A	C	G	G	G	A	T	G	T	T	C	T	G
14	PI-OR	Y	A	A		A	R	C	G	A	C	W	Y	Y	S	R	C	G	G	G	A	T	K	T	T	Y	T	G
15	PI-CA	C	A	A		A	G	C	G	A	C	A	T	C	C	A	C	S	G	K	A	T	K	T	T	Y	T	G
16	Abendsonne Red	Y	A	A		A	R	C	G	A	C	W	Y	T	G	R	C	G	G	G	A	W	G	T	T	Y	T	G

* The codon position is relative to the *AHAS3* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541458)

**The nucleotide position is relative to the *AHAS3* haplotype 1 allele in *Helianthus annuus* (GenBank accession no. AY541458; Kolkman et al., 2004)

DISCUSSION

SNP Genotyping Assay for *AHAS1*

This study identified a mutation in the sunflower *AHAS1* gene that confers resistant to the AHAS-inhibiting SU herbicides. We discovered a Pro197Leu mutation in a sunflower line (PH-SUR) developed by Pioneer Hi-Bred International by EMS mutagenesis of an elite inbred line, HA89. Prior research has identified a mutation of Pro197 in AHAS-inhibitor resistant sunflower and *Arabidopsis* lines (Jander et al., 2003; Kolkman et al., 2004). In sunflower, SURES-1 and SURES-2 confer resistance to SU herbicides and were developed by introgression of the mutation from the resistant wild sunflower ANN-KAN (Al-Khatib et al., 1999; Miller and Al-Khatib, 2000). Similarly, the *Arabidopsis* isolate studied by Jander et al. (2003) conferred resistance to SU herbicides; however, like PH-SUR, it was developed by an EMS induced mutation. The Pro197 mutation is one of the most common mutations found in SU resistant plants. Other plant species gaining herbicide resistance at Pro197 with an amino acid substitution include *Lactuca serriola* (Guttieri et al., 1992), *Kochia scoparia* (Guttieri et al., 1995), *Brassica tournefortii* (Boutsalis et al., 1999), *Sisymbrium orientale* (Boutsalis et al., 1999), and *Amaranthus retroflexus* (Sibony et al., 2001). The previous identification in sunflower and *Arabidopsis* in addition to many other plant species suggests that the SNP at codon 197 in the Pioneer SU resistant line, PH-SUR, is the mutation conferring resistance.

The SNP site at codon 197 was exploited to develop a robust DNA marker for the *AHAS1* genotype in the SU herbicide resistant Pioneer lines. The SNP assay was developed with clear segregation of homozygous resistant (A/A), homozygous susceptible (G/G), and heterozygotes (G/A). This was demonstrated in a blind assay using 184 hybrids and inbreds provided by Pioneer.

The blind assay identified some discrepancies between the SNP assay's genotypic calls and Pioneer's phenotypic calls. The reproducibility and reliability of the SNP assay were tested and the reproducibility was clearly demonstrated. The SNP assay is robust, suitable for large-scale genetic studies, simple to set up following the determination of the proper assay conditions, and convenient because the results are obtained in an electronic form minutes after the allele-discriminating reaction is performed (Chen et al., 1999). However, this can be a costly assay to establish due to the cost of the equipment in addition to the cost of the reagents required to perform the assay. Also, with a highly conserved multi-gene family, like that of the sunflower *AHAS* gene family, the initial set up of the SNP assay can be a challenge, as it is absolutely essential to establish gene-specific target primers within the conserved family.

The SNP genotyping assay has many advantages over a phenotypic assay because it: 1) does not kill the plants that are sampled, 2) can eliminate mistakes made during crossing earlier, and 3) identifies resistant plants that may be of interest earlier. The dominance of the Pro197Leu mutation is unknown; therefore, a phenotyping assay such as a herbicide spray test may not clearly differentiate between a homozygous resistant and a heterozygous plant. Previously performed research on an

SU resistant sunflower line showed in spray tests that the resistant lines are not completely resistant at the 1X field rate (Fabie and Miller, 2002). Partial dominance has been seen in the genes with a mutation conferring resistance to AHAS-inhibiting herbicides in several plant species (Sebastian et al., 1989; Newhouse et al., 1991; Hart et al., 1993; Wright and Penner, 1998; Foes et al., 1999; Kolkman et al., 2004). The calls by Pioneer were based on phenotypic data of the parental lines used to make the inbreds and hybrids of the blind assay. Evidence of additional genetic factors that influence herbicide resistance based on the *AHAS* gene has been presented (Fabie and Miller and Miller, 2002; Jander et al., 2003; Kolkman et al., 2004). Additionally, the degree of dominance of the c197 mutation has not been rigorously studied and may have contributed to misclassification of the parental lines based on phenotyping and therefore errant calls. The genotyping SNP assay used in addition to a phenotyping assay would yield the most reliable results.

Expression of Three AHAS Genes

This study showed that all three genes in the AHAS gene family are expressed in at least one of the tissues collected, 10 DAF, 14 DAF, 18 DAF, and 22 DAF kernels, leaf, ray flower, apex or root. Additionally it provided evidence for a divergent pattern of expression within the AHAS genes of sunflower. The pattern observed in the RT-PCR experiment was similar to the pattern of ESTs identified in the sunflower EST database for the three genes. Ten ESTs encoding *AHAS1* (QHA8G11, QHB10A23, QHB23I24, QHI1F03, QHE14F03, QHI10G24, QHE20B02, QHI4B16, QHE20P09, and QHI15 N13), one EST encoding *AHAS2*

(QHF14PO1) and no ESTs encoding *AHAS3* have been identified in the sunflower database that consists of 44,061 ESTs isolated from diverse tissues and development stages (Kozik et al., 2002; Kolkman et al., 2004). This suggests that *AHAS1* is more strongly expressed then *AHAS2* or *AHAS3*.

The results of both the non-quantitative RT-PCR and real-time RT-PCR suggest that *AHAS1,* and possibly *AHAS2,* code for housekeeping forms of AHAS in sunflower. It has been shown in other plant species with multiple genes encoding AHAS, such as *Brassica napus* (Ouellet et al., 1992), cotton (Grula JW, 1995) and tobacco (Keeler et al., 1993), that some *AHAS* paralogs function as housekeeping genes. Furthermore, in both *B.napus* and cotton it has been shown that at least one member of the *AHAS* gene family is expressed in a tissue-specific manner (Ouellet et al.,1992; Grula JW, 1995). The extensive expression analysis by Ouellet et al. (1992) of *B.napus* provides clear evidence for the sub-specialization of *AHAS* paralogs. In the Ouellet (1992) study, one *B.napus AHAS* paralog is shown be highly expressed in a tissue-specific manner exclusively in reproductive tissues, while two other paralogs are constitutively expressed. Similarly, in the sunflower *AHAS* gene family, our study showed that *AHAS3* was only expressed in leaf and apex whereas *AHAS1* and *AHAS2* showed expression in all the tissues examined.

The real-time RT-PCR experiment detected expression of both *AHAS1* and *AHAS2* in all tissues, reproductive and somatic, but at varying levels. In the real-time assay *AHAS3* was not detected suggesting that it is weakly expressed. In *AHAS1* there was a trend in the expression level within the developing embryos. The increasing expression level from 10 DAF to 18 DAF may be due to the increased biosynthesis of

proteins during kernel development (Duggleby and Pang, 2000). *AHAS1* and *2* were both highly expressed in leaf, yet, the leaf *AHAS1* expression level was much greater relative to the other tissues then it was in *AHAS2* (Figure 5). This suggests that steady-state levels of *AHAS1* mRNA are higher than *AHAS2* mRNA levels. However, accurate quantification of relative expression was not possible due to primer efficiency differences.

Currently, all mutations conferring herbicide resistance in sunflowers have been identified only in *AHAS1* (White et al., 2003; Kolkman et al., 2004). Previous research where plants were sprayed with herbicides at the six leaf stage, in addition to the expression analysis presented here, provides evidence that the enzyme targeted by the herbicide is in leaves and is likely *AHAS1* (Fabie and Miller, 2002; Kolkman et al., 2004). This suggests that only mutations in *AHAS1* would provide enough of the herbicide resistant enzyme to allow plants to survive herbicide treatments, therefore explaining the observation that all mutations to date have been found in *AHAS1*.

Gene Diversity for the *AHAS* Gene Family in Domesticated and Wild Sunflower

Alignments made in this research provide a clear indication that there is variability in the diversity between the gene family members. The patterns of genetic diversity within the AHAS gene family observed in this study corroborated previous studies of genetic diversity that scanned marker diversity across the sunflower genome (Tang and Knapp 2003; Kolkman et al., unpublished data). The patterns observed in this study were consistent with marker patterns on the genetic linkage map (Yu et al.,

2003). Kolkman et al. (2004) mapped *AHAS1* to a hyperpolymorphic region of LG 9, which may be related to why this was found to be the most diverse gene of the three. *AHAS2* was mapped to LG 6 (Kolkman et al., 2004) that has a dearth of markers (Yu et al., 2003) and may therefore be why *AHAS2* showed the least amount of haplotype (gene) diversity. The total haplotype diversity of *AHAS1* is greater then the diversity for *AHAS3* and considerably more then that of *AHAS2* (Table 7).

Based on a survey of individuals from wild accessions and domesticated lines all three AHAS genes exhibited greater sequence diversity in wild sunflowers than among domesticated lines. In AHAS1 the SNP frequency was 3X greater in wild accessions than domesticated lines, in AHAS2 it was 6X greater and in AHAS3 it was 3X greater. Additionally wild accessions have 3X more non-synonymous SNPs than the domesticated lines. Haplotype diversity in domesticated lines was highest in *AHAS1*, ranging from 0.64 and 0.71, which is higher then the haplotype diversity found in the genomes of domesticated soybeans (0.52, Zhu et al., 2003) or maize (0.56, Ching et al., 2002). Previous research in sunflower demonstrated a dramatic narrowing of allelic diversity from wild sunflowers to domesticated sunflowers (Arias and Rieseberg, 1995; Tang and Knapp, 2003). While the results of this study were not unexpected this does reflect domestication and breeding bottlenecks.

This study provides further evidence that wild populations hold a wealth of genetic diversity. As suggested by Tang et al. (2003), the genetic diversity in wild sunflowers could be utilized to saturate the linkage map further in a wild x inbred cross by identifying more polymorphisms (Kolkman et al., unpublished data). The domesticated lines SURES-1, SURES-2, IMISUN-1, IMISUN-2, HA425, and

Advanta line 29023 carry mutations conferring resistance that were introgressed from wild sunflowers (Kolkman et al., 2004). Theses SNPs, at codon 197 and codon 205 in *AHAS1*, were only observed in these lines, and since they were introgressed from a wild population they were included in the wild subgroup for the *AHAS1* analysis. The mutation observed in these lines provided a strong selective advantage in an environment where herbicide was repeatedly applied (Al-Khatib et al., 1999), c197 and c205 mutations are only observed in these lines and not in any other wilds or domesticated lines therefore suggesting that they are not necessary for the plants function and were in fact a product of their in environment. Other SNPs observed only in the wilds were perhaps a result of the environment they were growing in and not essential for the development of the plant in the domesticated lines.

AHAS2 lacked the gene diversity observed in *AHAS1,* among wild accessions and domesticated germplasm. We observed a single 6-bp in-frame insertion in the wild group that did not appear in any of the domesticated lines. Additionally, only six SNPs were observed between the two alignment groups among 23 samples. This sharply contrasts with the total number of SNPs observed in the two alignments of *AHAS1* (60 SNPs) and in *AHAS3* (40 SNPs). This agrees with prior evidence demonstrating the lack of polymorphism in linkage group 6 of sunflower (Yu et al., 2003; Burke et al., 2005; Kolkman et al., unpublished data). In multiple studies LG 6 has shown reduced polymorphism that may have been the result of a selective sweep during domestication (Burke et al., 2005). Kolkman et al. (2004) identified two haplotypes in *AHAS2*, with one of the haplotypes based on a wild isolate and the other haplotype based on the alignment of 23 sequences of domesticated lines. In this study,

the wild accessions show limited diversity within the *AHAS2* gene (Table 10 and 11; Table 7), which is contrary to the evidence reported by Burke et al. (2005) suggesting that genetic diversity remains high on linkage group 6 even in the wild accessions. These conflicting results suggest further characterization of *AHAS2* is necessary to determine its pattern of evolution.

The total gene diversity of wild accessions and domesticated lines observed in *AHAS3* was greater than that of *AHAS2* but less than that of *AHAS1*. The greatest difference in gene diversity was observed in the domesticated lines that ranged from 0.11 to 0.29 in *AHAS3* and from 0.64 and 0.71 in *AHAS1* (Table 7). In contrast the gene diversity among the wild accessions was similar between *AHAS3*, ranging from 0.75 to 0.92, and *AHAS1*, ranging from 0.87 to 0.90. The gene diversity observed in these two genes supports evidence of a breeding bottleneck.

Previous research by Kolkman et al. (2004) identified two haplotypes in *AHAS3* based on sequencing nine alleles in elite lines. A single SNP at codon 581, a synonymous SNP, differentiated the two haplotypes. This region of the gene was not incorporated into the current study, which could have had an impact on the number of haplotypes observed in this gene. Also, the small number of alleles sequenced in *AHAS3* by Kolkman et al. (2004) in comparison to the 23 *AHAS1* alleles and 24 *AHAS2* alleles may have had an impact on the number of haplotypes observed in the previous work.

CONCLUSION

The *AHAS1* paralog is the most highly expressed and highly diverse gene in the *AHAS* gene family of sunflower and *AHAS1* was the site of a Pro197Leu mutation likely to confer SU resistance to a newly characterized sunflower line. Thus far, in the plant species that have been characterized all mutations conferring resistance that were sequenced have been single nucleotide polymorphisms. The polymorphism we discovered was used to develop a marker that can be utilized in a reliable SNP assay. The *AHAS1* paralog in sunflower has been the only paralog that mutations conferring resistance have been identified in. The expression pattern in *AHAS2* suggests that this paralog may possibly play a role in herbicide resistance and should be further explored. Real-time RT-PCR was used to show that *AHAS1* is highly expressed in all plant tissues. The high expression level of *AHAS1* suggests that this is the reason the paralog has been the target of all mutations resulting in resistance to AHAS-inhibiting herbicides in sunflower to date. Of the mutations identified thus far in sunflower, none have been found in *AHAS2* or *3* (Fabie and Miller, 2002; White et al., 2003; Kolkman et al., 2004).

The diversity study provides evidence for a connection among the three genes of the AHAS gene family and where they map to on the linkage groups and therefore the degree of diversity observed in the genes. These results provide clear evidence of a breeding bottleneck, and consequently wild sunflowers are the greatest source of genetic diversity.

The SNP assay eases genotyping and can be used to perform MAS for herbicide resistant sunflowers with C/T transitions at codon 197 of *AHAS1* and could

be applied to other mutations conferring herbicide resistance. The gene diversity revealed here could be used to further understand the evolution of the sunflower genome and it shows the promise for increasing the density of the molecular linkage map of sunflower using wild X elite crosses.

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