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ORIGINAL PAPER

Molecular characterization of *Als1*, an acetohydroxyacid synthase mutation conferring resistance to sulfonylurea herbicides in soybean

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Abstract
Key message **The AHAS gene family in soybean was characterized. The locus *Als1* for sulfonylurea resistance was mapped and the resistant allele was characterized at the molecular level.**
Abstract Sulfonylurea (SU) resistance in soybean is controlled by *Als1*, a semi-dominant allele obtained by EMS mutagenesis over the cultivar Williams 82. The overall objective of this research was to map *Als1* in the soybean genome and to determine the nucleotidic changes conferring resistance to SU. Four nucleotide sequences (*GmAhas1–4*) showing high homology with the *Arabidopsis thaliana* acetohydroxyacid synthase (AHAS, EC 4.1.3.18) gene sequence were identified by in silico analysis, PCR-amplified from the SU-resistant line BTK323STS and sequenced. Expression analysis showed that *GmAhas1*, located on chromosome 4 by in silico analysis, is the most expressed sequence in true leaves. $F_{2:3}$ families derived from the cross between susceptible and resistant lines were evaluated for SU resistance. Mapping results indicate that the locus *als1* is located on chromosome 4. Sequence comparison of *GmAhas1* between BTK323STS and Williams 82 showed a single nucleotide change from cytosine to thymine at position 532. This transversion generates an amino acid change from proline to serine at position 197 (*A. thaliana* nomenclature) of the AHAS catalytic subunit. An allele-specific marker developed for the *GmAhas1* mutant sequence cosegregated with SU resistance in the F_2 population. Taking together, the mapping, expression and sequencing results indicate that the *GmAhas1* sequence corresponds to the *Als1* gene sequence controlling SU resistance in soybean. The molecular breeding tools described herein create the basis to speed up the identification of new mutations in soybean AHAS leading to enhanced levels of resistance to SU or to other families of AHAS inhibitor herbicides.

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Introduction

Soybean (*Glycine max* (L.) Merr.) is one of the world's most important crops, offering a stable source of materials for food, feed for livestock and aquaculture, industrial, and pharmaceutical applications, assuming also an important part of traditional foods in many Asian countries (Masuda and Goldsmith 2009). Soybean seeds contain a high content of nutritious protein, oil, and they are also abundant in physiologically active metabolites. Soybean also contributes to soil fertility through nitrogen fixation via symbiotic root bacteroids (Ishimoto and Harada 2012).

The advent of glyphosate-resistant (GR) soybeans revolutionized farming practices in the most important producer countries. The widespread adoption of GR crops was a consequence of the highly effective, environmentally sound, profitable, and excellent weed control method that provides this herbicide. This, in turn, determined an unprecedented, often exclusive, use of glyphosate over very large areas. Moreover, the adoption of GR crops and intensive glyphosate usage often results in the cessation of use of alternative herbicides (Shaner 2000) and/or tillage, and, therefore, there is a lack of diversity in weed control practices, resulting in almost complete reliance on glyphosate. The

persistent use of glyphosate over genetically diverse weed populations resulted in an intense selection pressure and, consequently, in the rapid rise in the populations of several troublesome weeds that are tolerant or resistant to this herbicide (Vila-Aiub et al. 2008; Powles 2008). This may signify that the useful lifetime of this economically important weed management trait will be cut short (Powles and Yu 2010). This prompted the commercial development of soybean cultivars which combines GR with the resistance to other types of herbicides with different modes of action (Green et al. 2008).

Sulfonylureas (SUs) are a family of compounds with high herbicidal activity and low mammalian toxicity (Beyer et al. 1988). SUs are effective in killing susceptible plants by inhibiting acetohydroxyacid synthase (AHAS; EC 4.1.3.18), also known as acetolactate synthase (ALS) (Duggleby and Pang 2000), which is a key enzyme in the biosynthesis of the branched chain amino acids such as valine, leucine and isoleucine in eukaryotes and prokaryotes (Umbarger 1978; Singh 1999; McCourt et al. 2006). Inhibition of AHAS leads to plant death primarily because of amino acid starvation, even though other secondary effects of AHAS inhibition have also been implicated in the mechanism of plant death (Shaner 1991). Although commercial SU herbicides offer a wide variety of weed control spectra, very few of them can be used on soybean due to crop sensitivity. Seed mutagenesis of the soybean cultivar 'Williams 82' followed by selection for resistance with the SU herbicide chlorsulfuron permitted to obtain 'W20', a soybean line which showed a high degree of tolerance to both postemergence and preemergence applications of a variety of SU herbicides (Sebastian et al. 1989). Biochemical tests indicated that the mechanism of tolerance is reduced sensitivity of AHAS to SU inhibition (Sebastian et al. 1989; Simpson and Stoller 1996). Inheritance studies of SU tolerance in W20 indicated that this trait is determined by a semi-dominant allele which was designated *Als1* (Sebastian et al. 1989).

As typical for AHAS-inhibiting herbicides, SU herbicides exert strong selection pressure over weed populations because of their high activity on sensitive biotypes at the rates used, their soil residual activity, and their widespread usage (Tranel and Wright 2002). For these reasons, weed populations resistant to this family of herbicides are frequent (Tranel and Wright 2002; Heap 2013), which makes unsuitable a weed management system based solely on sulfonylurea-tolerant (ST) soybeans. Nevertheless, in recent years and stacked with the GR trait, the *Als1* mutation greatly enhanced the weed control options available to soybean farmers and extends the effective lifetime of biotechnology-based weed management strategies (Green et al. 2008). In most weed populations, individuals naturally resistant to more than one herbicide mode of action (MOA) will be rare. On the other hand, assuming that no single allele confers cross resistance to multiple herbicides, and different resistance alleles are at unlinked loci, the probability of an individual plant being resistant to multiple MOAs is the product of the frequencies of resistance to each single MOA (Gressel and Siegel 1990; Wrubel and Gressel 1994). For these reasons GR/ST stacked soybeans allow farmers to combine recommended rates of different herbicide MOAs, simultaneously, sequentially, or annually, greatly reducing the likelihoods of survival and reproduction of the resistant individuals (Norsworthy et al. 2012). In recent years, these considerations prompted the continuous registration of GR/ST soybean cultivars, as well as several herbicides to be specifically used on them (for example, Synchrony®, Ligate® and Finesse®). Moreover, ST soybeans are more tolerant to carryover of residual SU applied to the preceding crop (Anderson and Simmons 2004) and this characteristic, together with the possibility of controlling weeds during the fallow period, was the main drivers in the initial commercialization of the stacked GR/ST soybeans (Rossi 2012). Finally, in addition to its role in weed management, it was demonstrated that ST soybeans yield almost 5 % more than their conventional isolines because of a greater seed set in the ST soybean (Santone et al. 2013).

In spite of its relevance, however, the number of *AHAS* gene copies in the soybean genome, their position, pattern of expression, and the mutation conferring SU resistance in *Als1* remain unknown. For this reason, the goals of the present study were to (1) characterize the *AHAS* gene family in soybean, (2) determine the pattern of expression of its members in true leaves (3) map the locus *Als1*, and (4) identify the *AHAS* mutation that confers resistance to SU.

Materials and methods

Plant materials and DNA isolation

Fully expanded leaves were harvested for DNA isolation from SU-resistant line BTK323STS, the susceptible cultivar NA4613RG, and 120 F_2 plants derived from crossing both lines. The herbicide resistance gene donor for BTK323STS was the mutant line W20, obtained by Sebastian et al. (1989) using EMS treatment. Williams 82, the cultivar employed to do the mutagenic treatment, was also used. 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 Dellaporta et al. (1983).

Quality and quantity of the extracted DNA were assessed by electrophoresis in 1.5 % agarose gels. DNA was quantified by fluorometry using Qbit® dsDNA BR kit

(Cat# Q23850, Qiagen). The DNA was diluted to a final concentration of 60 ng/μl.

In the cross NA4613RG × BTK323STS, the susceptible line was used as female parent to allow the detection of the hybrid condition of F_1 seeds. To do this, putative F_1 plants were sprayed with the SU herbicide chlorsulfuron [1-(2-chlorophenylsulfonyl)-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea] at a rate of 10 g a.i. ha^{-1}. Surviving plants were transplanted into potting media consisting of equal parts of vermiculite, soil and sand in 20-by-20-by-30 cm pots and cultivated in a greenhouse under natural light conditions supplemented with 400 W halide lamps to provide a 16-h daylength. One hundred and sixty seeds from a single F_1 plant were harvested and sown in pots as described above.

Ahas gene discovery

Identification of *Ahas* gene sequences in soybean was performed by retrieving from NCBI GenBank database the nucleotide sequence of the catalytic subunit of *Arabidopsis thaliana* AHAS (Accession NM_114714.2) and blasting it to the *Glycine max* genome in the database Phytozome v8.0 (Goodstein et al. 2012). Four sequences were identified (Accession No. Glyma04g37270, Glyma06g17790, Glyma13g31470 and Glyma15g07860) and the Primer3 program (Rozen and Skaletsky 2000) was used for designing specific primers to amplify each of them (Table 1). PCRs were carried out in 15 μl final volume, using 1.5 μl of Buffer 10× High Fidelity PCR Buffer, 0.1 mM of dNTPs, 4.2 mM $MgCl_2$, 1 U proof reading Platinum *Taq* polimerase High Fidelity (Invitrogen, Life Technologies), 0.13 μM of each oligonucleotide primer and 2 μl of genomic DNA input at 60 ng/μl. PCRs were conducted on PTC200 thermocycler as follows: heat denaturation at 95 °C for 2 min followed by 40 cycles of 95 °C 1 min, 55 or 60 °C 20 s (according to the combination of primers used, Table 1), 72 °C 2 min and a final extension step at 72 °C for 5 min. The PCR products were separated by electrophoresis in agarose gels [1.5 % (w/v) in 1× TBE pH 8.3 (Tris-acid boric-EDTA: 0.089 M Tris base, 0.089 mM boric acid, 0.002 M EDTA)] for 45 min to 60–80 V. Agarose gels were stained with Sybr® SAFE (Cat# S33100, Invitrogen, Life Technologies). A 1 kb molecular weight marker (Cat# G571A, Promega) was used to estimate the size of the amplified fragments. The PCR products were visualized in Safe Imager™ 2.0 Blue Light Transilluminator (Invitrogen, Life Technologies) and photographed. DNA fragments were submitted for direct sequencing (Macrogen, USA). Internal coding sequences were completed by internal primers (Table 1) to obtain overlapped fragments.

Sequence trace files were imported into ContigExpress (Invitrogen, Carlsbad, CA, USA), trimmed, aligned, and evaluated for sequence specificity by comparing with the corresponding control accessions from Phytozome. Nucleotide and amino acid multiple sequences 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 *A. thaliana* (GenBank accession no. X51514, Sathasivan et al. 1991). Gene sequences reported herein have been

Table 1 Primers for soybean AHAS

Primer ID	Purpose	Sequence	Tm	Amplicon size (pb)
GmAhas1-F	Gene discovery	**ATG**GCGGCCACCGCTTC	59	2,041
GmAhas1-R		ACAGGCCAAATCCTGCAACTAGGAC	60.5	
GmAhas2- F	Gene discovery	**ATG**GCGGCCACAGCTTCCAG	61.8	1,936
GmAhas2-R		TCAGTACCTCGTTCTACCGTCTCCCTCC	60.2	
GmAhas3-F	Gene discovery	TTTAGATTATTGTGGTATTGGAAG**ATG**	52.4	2,105
GmAhas3-R		GAATATTTAGTACTAAAAGAAACCAACATC	51.3	
GmAhas4-F	Gene discovery	ACCTTTTGGTGCTATTTGAAA**ATG**	53.6	2,122
GmAhas4-R		ACATATAATTAACAAAAATAACCAACATTG	52.6	
int-GmAhas1-GmAhas2-R	Sequencing	TGGAAACTTGTGTTTCTGCACATTAATCTC	60.7	
int-GmAhas1-GmAhas2-F	Sequencing and RT-PCR	GCAGTTTGAATTCCAGTGCTGAATTG	59.9	
int-GmAhas3-GmAhas4-F	Sequencing and RT-PCR	GGTTGGTTATGGAATCTAAGAAACC	50.2	
int-GmAhas3-GmAhas4-R	Sequencing	CAAACGCAAGCAAAAGATCAG	52.9	
GmAhas1-532C	TaqMan assay	VIC-5′-CCAGGTCCCCCGCCGG-3′-BHQ	60	
GmAhas1-532T		FAM-5′-CCAGGTCTCCCGCCGG-3′-BHQ	60	
TQF		AGGAAGCGCCGACCAC	60	378
TQR		GCGTCGGTGCCGATCAT	60	

submitted to GenBank, with accession numbers KC254821 to KC254825 for *GmAhas1*, *GmAhas2*, *GmAhas3* y *GmAhas4* from BTK323STS and *GmAhas1* from Williams 82, respectively.

A dendrogram showing the relationships among the soybean *Ahas* paralogs and the *Ahas* gene from *A. thaliana* was constructed by the maximum likelihood method based on the JTT matrix-based model using MEGA5 (Tamura et al. 2011).

Genetic mapping of SU resistance

The AHAS-inhibiting herbicide resistance locus *Als1* was scored in the described segregating population by inferring *Als1* genotypes of F_2 plants from herbicide resistance phenotypes of $F_{2:3}$ families. One hundred and twenty $F_{2:3}$ families from the cross NA4613RG × BTK323STS were phenotyped for resistance to SU. To do this, 20 seeds of each family were sown in 20-by-20-by-30 cm pots and when plants reached the V3–V4 stage of development (Fehr et al. 1971), they were sprayed with chlorsulfuron at a dose of 10 g a.i. ha^{-1}. Ten days after treatment, plants were rated visually on a scale of 1–4 for herbicide injury. Plants in which the apex had died or with a severe necrosis 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. Families with an average score <1.5 were taken as susceptible (genotype *als1/als1*), those with and average score between 2 and 3 were considered as segregating and those with an average score >3.5 were taken as resistant (*Als1/Als1*). The fit of observed to expected segregation ratios for the *Als1* locus was tested using χ^2-statistics.

Bulked segregant analysis (BSA, Michelmore et al. 1991) was performed to identify molecular markers putatively linked to *Als1*. DNA from 10 resistant (R) and 10 susceptible (S) F_2 plants were mixed in equal amounts to form resistant and susceptible bulks. DNA samples of the two parents and bulks were screened for polymorphism by SSR markers which were chosen based on their proximity to the *Ahas* gene sequences identified by in silico analysis.

The SSR amplification reaction was conducted with 1 U of *Taq* Platinum DNA polymerase, 40 ng DNA input, 1 µg/ml of bovine serum albumin, 0.1 mM of each dNTP, 0.16 µM of each primer, 90 mM Tris–HCl pH 8, 20 mM $(NH_4)_2\ SO_4$ and 2.5 mM $MgCl_2$, in 15 µl final volume. The PCR program began with a denaturation step at 95 °C for 2 min, followed by 33 cycles of: 94 °C 25 s, 47 °C 25 s, and 68 °C 25 s. Finally, an extension step of 5 min at 68 °C.

The PCR products were separated by electrophoresis in 4 % Metaphor (Cat# 54184, Lonza) agarose gels, in 1× TBE buffer to 100 V for 3 h, visualized and photographed as described before. When the polymorphism was barely detected using the described system, amplification products were resolved on denaturing polyacrylamide gels and visualized by silver staining (Sambrook et al. 1989).

SSR markers putatively linked to *Als1* detected by the BSA were screened on each of the 120 individuals of the mapping population. Phenotypic and molecular data were analyzed using the program MAPMAKER/EXP 3.0 (Lander et al. 1987) to construct a linkage map. For this analysis, the value of minimal LOD score of 3.0 and a maximum distance of 30 cM was used to identify linkage between markers. Map in centimorgans distances was calculated using the Kosambi function. The image of the map was done by MapChart (Voorrips 2002).

Ahas gene expression analysis

The presence of *AHAS* transcripts for the sequences identified in silico was determined in leaf tissue retrieved from soybean plants with two true leaves by RT-PCR. Harvested tissue was placed in liquid nitrogen and total RNA was extracted using RNeasy plant kit (Cat# 74904, Qiagen) according to the manufacturer's instructions. The quality and integrity of the RNA were assessed on 1.5 % agarose gels. RT-PCR was performed by a two-step protocol using Illustra Ready-To-Go RT-PCR Beads kit (Cat# 27-9259-01, GE Healthcare Life Science). For the synthesis of the first cDNA, the oligo $(dT)_{12–18}$ was reconstituted to a final concentration of 0.5 µg/µl. The same amount of RNA input was used in each tube of a RT-PCR bead reaction and incubated during 30 min at 42 °C. For the second step, the primer combination for specific amplification of each transcript was added to each tube. Hence, for *GmAhas1* cDNA, the primers int-GmAhas1-GmAhas2-F and GmAhas1-R were used; for *GmAhas2* cDNA, the primers int-GmAhas1-GmAhas2-F and *GmAhas2*-R; for *GmAhas3* cDNA, the primers int-GmAhas3-GmAhas4-F and GmAhas3-R; and for *GmAhas4* the primers int-GmAhas3-GmAhas4-F and GmAhas4-R (Table 1). The primer combinations amplified a PCR fragment of approximately 1 kb targeting the half 3′ end of each *GmAHAS* transcript. Since *GmAHAS* sequences are intronless, this fragment is enough to determine if the transcript is being expressed or not. Control of cDNA contamination was performed using RNA in the reaction without the use of primer $(dT)_{12–18}$. Genomic DNA input was used as positive control for each primer combination. Also the positive control of the reagents was performed with the positive control provided by the kit following manufacturer's instructions. The second step of the RT-PCR and the PCR (genomic DNA) was performed under the following conditions: 5 min at 94 °C, 40 cycles of: 94 °C 1 min, 60 or 50 °C 30 s (depending on the primers combination, see Table 1) and 72 °C 1 min and a final extension step of 72 °C

during 5 min. RT-PCR products were electrophoresed in 1.5 % agarose gels and stained with Sybr® SAFE (Cat# S33100, Invitrogen, Life Technologies). A 1 kb molecular weight marker (Cat# G571A, Promega) was used to estimate the size of the amplified fragments. PCR products were visualized in Safe Imager™ 2.0 Blue Light Transilluminator (Invitrogen, Life Technologies), photographed and the image inverted for better resolution. PCR products were sequenced to confirm gene-specific amplification.

SNP marker development for the *Als1* resistant allele

An allele-specific assay designed for detection of C/T polymorphism present in codon 197 of the *GmAhas1* was performed using a TaqMan assay (Applied Biosystems, Foster City, CA, USA). TaqMan allele-specific oligonucleotide and primers were developed by Applied Biosystems by design (AbD) (Table 1). Because *GmAhas1* and *GmAhas2* sequences are 93 % identical, a pre-amplification PCR for *GmAhas1* was performed with primer combination Gm1-F/Gm1-R following the PCR conditions specified above. After the pre-amplification step, two dilutions of the *GmAhas1* PCR product were tested, 1:10 and 1:50. All experiments were conducted using the appropriate negative and no template controls.

Real-time PCRs were performed with the iQCycler thermocycler iQ4 (Bio-Rad). The PCR was carried out in 25 µl final volume using 2× TaqMan SNP Genotyping (PE Applied Biosystems), 20× SNP genotyping assay, 5 µl of 1:10 or 1:50 of the *GmAhas1* PCR product dilution from BTK323STS, NA4613RG and their F_1, using the 2 step PCR program at 95 °C 15 s followed by 45 cycles at 60 °C 30 s. All PCRs were performed in triplicate for each sample. For each assay, Ct-values were averaged and the standard deviation was calculated to enable the scoring of *GmAhas1* haplotypes. Analysis of cosegregation of SU resistance and the haplotype-specific markers developed above was carried out on the 120 plants of the F_2 population already described.

Results

The *Ahas* gene family in soybean

Blast analysis on the database Phytozome v 8.0 identified four sequences as highly homologous to the catalytic subunit of the *Ahas* gene cloned from *A. thaliana*. These sequences are located on soybean chromosomes 4, 6, 13 and 15, and were designated as *GmAhas1*, *GmAhas2*, *GmAhas3* and *GmAhas4*, respectively. Two more sequences were identified, but because of the low score and the increased *E* value displayed they were discarded.

Sequences of soybean *Ahas* genes were isolated by designing specific primers complementary to nucleotide sequences of each *GmAhas*. The primer design was performed to span the complete coding sequence of each gene sequence. Each amplicon showed the expected size as described in Table 1. No introns were found in any of the soybean *Ahas* genes, as is the case of other plant species (Tranel and Wright 2002; Kolkman et al. 2004).

The nucleotide sequences of the soybean *Ahas* genes were aligned with the catalytic subunit of *A. Ahas* gene (Fig. 1). Nucleotide sequences alignment of *GmAhas* sequences showed from 78 to 94 % similarity among them. The nucleotide sequences of *GmAhas1* and *GmAhas2* shared 93 % of sequence homology, whereas the nucleotide sequences of *GmAhas3* and *GmAhas4* were 94 % identical. *GmAhas1* and *GmAhas2* showed the lesser similarity with respect to *GmAhas3* and *GmAhas4* (79 and 78 %, respectively). When these sequences were compared to the *Ahas* sequence of *A. thaliana* (*CSR1*), the similarity dropped to 70–72 %.

An alignment of deduced amino acid sequences among GmAHAS showed from 81 to 94 % sequence homology. GmAHAS1 and GmAHAS2 were 94 % identical, and GmAHAS3 and GmAHAS4 shared a 93 % identity. GmAHAS1 was 81 % identical when compared to GmAHAS3 and 82 % identical to GmAHAS4. GmAHAS2 was 82 % identical to GmAHAS3 and GmAHAS4. When GmAHAS sequences were compared to the CSR1 from *A. thaliana*, the identity ranged between 74 and 76 %.

The relationship among the four paralogous *Ahas* sequences identified in soybean and *CSR1* from *A. thaliana* used as outlier is shown in Fig. 2. Clearly, the soybean *Ahas* sequences are divided into two groups: one consisted of *GmAhas1* and *GmAhas2*, and the other included *GmAhas3* together with *GmAhas4*, and this is the closest group to *CSR1* from *A. thaliana*.

Genetic mapping of SU resistance in soybean

The resistance or susceptibility of plants to SU was determined by observation of symptoms and comparison with the parental lines used as controls. Resistant plants showed a uniform green color, without chlorosis or necrosis in the apex or in the leaves as it was observed for the resistant parental line BTK323STS. Instead, susceptible plants showed severe phytotoxicity symptoms (leaf abnormalities, yellowing, and leaf necrosis) or were dead with total necrosis of the apex. Each plant was scored visually and an average rating was obtained for each $F_{2:3}$ family, so they were categorized as resistant (R), segregant (Seg) or susceptible (S) according to herbicide injury. Observed segregation ratio among $F_{2:3}$ families was 32 R: 61 Seg: 27 S, which fitted the expected 1:2:1 ratio ($p = 0.84$), confirming that SU

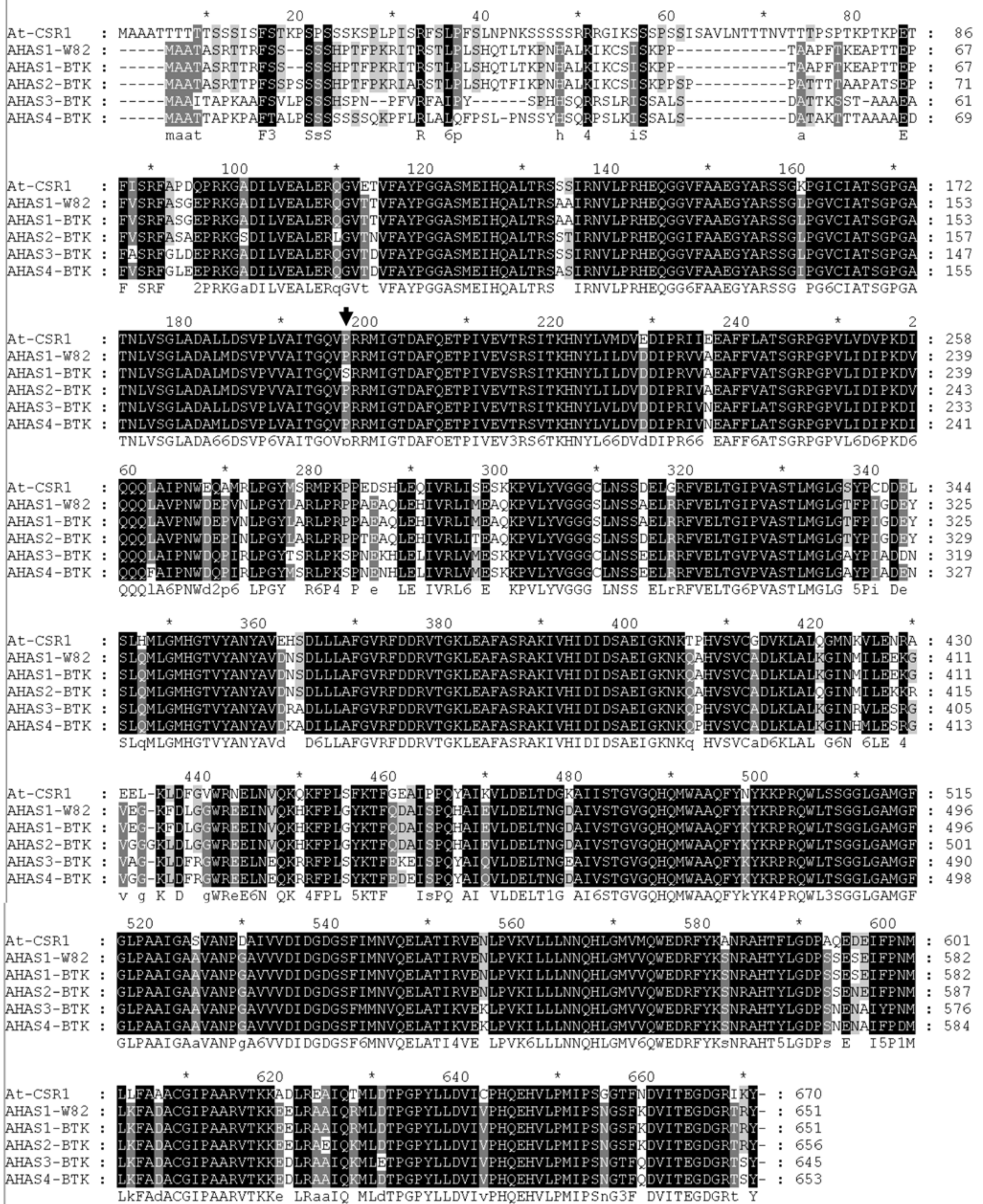

Fig. 1 Deduced amino acid sequence of AHAS proteins from BTK323STS (AHAS1, AHAS2, AHAS3 and AHAS4) aligned with the AHAS amino acid sequence of *A. thaliana* (CSR1) and the AHAS1 from Williams 82. The *arrowhead* indicates the amino acid substitution conferring resistance

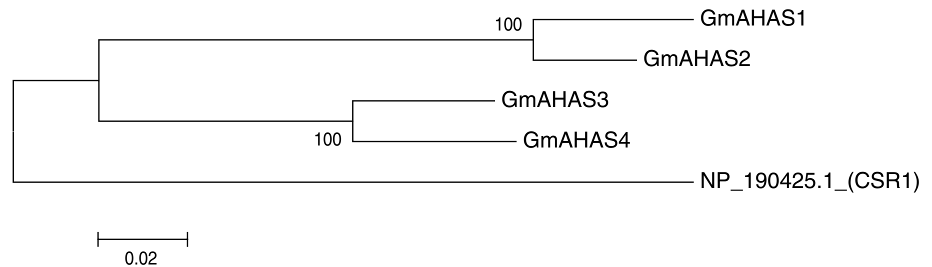

Fig. 2 Dendogram showing the similarity relationships among paralogous *Ahas* sequences of soybean and the *Ahas* gene *CSR1* from *Arabidopsis thaliana* (Acc. No. NP_190425.1)

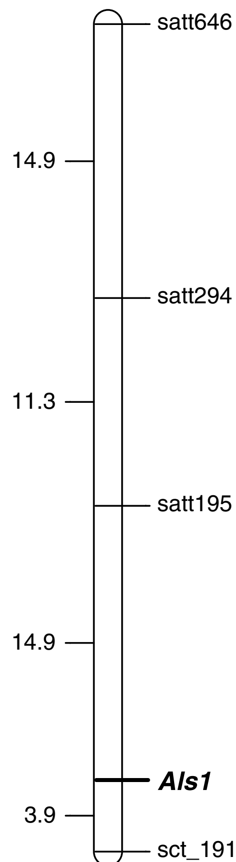

Fig. 3 Linkage map for the *locus Als1* on soybean chromosome 4

resistance derived from W20 is controlled by one dominant allele.

Parental lines were polymorphic for 19 SSRs located on the four candidates regions detected by in silico analysis. However, only two of them were identified as associated with the SU-resistant phenotype by bulk segregant analysis: sct_191 and satt294. Both markers map to the chromosome 4 of the soybean integrated linkage map (Hyten et al. 2010) and for this reason polymorphism for additional SSR markers on this chromosome was screened. By this way, another two markers were found to be associated with the SU-resistant phenotype. All the individuals of the F_2 population were genotyped with these four markers and the information was used to construct a map of the genomic region around *Als1* covering 45 cM. The SSR marker sct_191 is the closest marker distal to this locus with a genetic distance of 3.9 cM (Fig. 3).

Expression analysis of *Ahas* genes

Seventeen ESTs fragments showing high homology to the described *Ahas* sequences were identified in the soybean EST database: 6 of them for *GmAhas1* (AW830274.1, CX706242.1, CX703162.1, DB958432.1, CX707353.1, BI786456.1), 2 for *GmAhas2* (EV271682.1, CD418463.1), 4 for *GmAhas3* (CA938887.1, BM187985.1, BW652157.1, BW669216.1), and 5 for *GmAhas4* (FG997997.1, BW673087.1, FG997998.1, BG047162.1, FG998285.1). This information indicates that these four sequences are expressed in certain tissues or developmental stages of soybeans.

AHAS inhibitor herbicides are usually utilized in postemergence. Under this condition, leaves are the target organs for these herbicides. For this reason, the expression of *GmAhas* sequences was analyzed in true leaves of soybean plantlets. Using a two-step RT-PCR analysis with gene-specific primers, only transcripts putatively from *GmAhas1* were detected in true leaves at the V2 developmental stage (Fehr et al. 1971; Fig. 4). Sequencing results of this fragment indicated that it has the expected size of 1.12 kb and 100 % sequence homology with *GmAhas1* confirming its identity. No expression for *GmAhas2*, *GmAhas3* and *GmAhas4* was detected by this analysis. This indicates that even though four *Ahas* genes seem to be expressed in soybean, *GmAhas1* is the only paralog highly expressed in true leaves at V2 stage of development.

SU-resistant soybeans carry a mutation in *Ahas1* which cosegregate with resistance

Resistance to SU herbicides was mapped on soybean chromosome 4, where also the *GmAhas1* gene sequence, which is highly expressed in true leaves of soybean plantlets, is located. As a consequence, *GmAhas1* nucleotide sequences from BTK323STS and that from Williams 82 were compared. The alignment of both sequences revealed

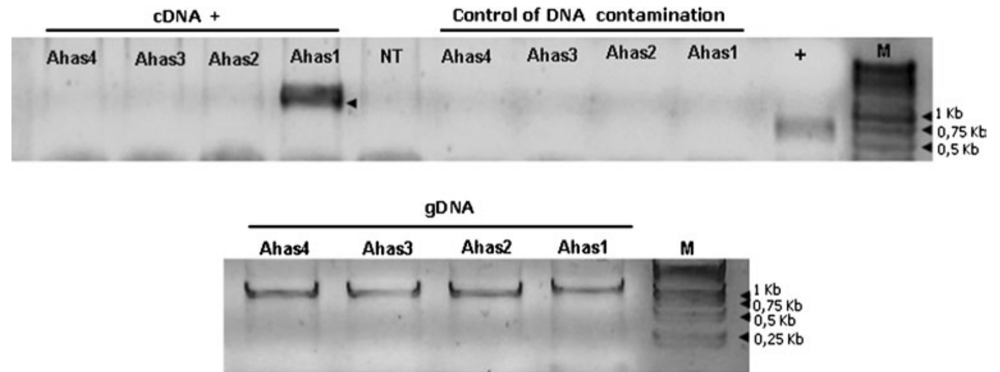

Fig. 4 Analysis of *GmAhas* expression in true soybean leaves at V2 developmental stage by RT-PCR. NT: negative control (water); *plus symbol* positive control provided in the RT-PCR kit; gDNA: genomic DNA; *M* 1 Kb DNA ladder

Table 2 Cycle threshold (Ct) mean values for two Taqman assays to detect a SNP in *GmAhas1* and corresponding genotypes for the locus *als1*

Pre-amp dilution	GmAhas1-532T allele		GmAhas1-532 callele		Genotype
	Ct-value	Mean ± SD	Ct-value	Mean ± SD	
BTK323STS 1:10	17	17.0 ± 0.10	ND	ND	*Als1/Als1*
	17.1		ND		
	16.9		ND		
BTK323STS 1:50	27.7	28.3 ± 0.51	ND	ND	*Als1/Als1*
	28.7		ND		
	28.4		ND		
NA 4613RG 1:10	ND	ND	21.9	21.8 ± 0.31	*als1/als1*
	ND		22.1		
	ND		21.5		
NA 4613RG 1:50	ND	ND	26.7	26.3 ± 0.32	*als1/als1*
	ND		26.1		
	ND		26.2		
F_1 1:10	25.7	25.67 ± 0.06	22.2	21.8 ± 0.51	*Als1/als1*
	25.7		21.2		
	25.6		21.9		

ND not detectable

the existence of a single DNA polymorphism: a change of cytosine to thymine (C/T) at nucleotide position 532.

An alignment of the predicted amino acid sequences of the *GmAhas1* nucleotide sequences of BTK323STS and Williams 82 is provided in Fig. 1. Relative to the AHAS1 amino acid sequence of Williams82, the AHAS1 amino acid sequence of BTK323STS has a proline (**C**CC) to serine (**T**CC) substitution at amino acid position 197 of the *A. thaliana* sequence.

The SNP (C/T) present at the position 532 of *GmAhas1* was targeted to develop a TaqMan assay with a pre-amplification step procedure using DNA from BTK323STS (*Als1/Als1*), NA4613RG (*als1/als1*) and their F_1 hybrid (*Als1/als1*). The assay for the *GmAhas*-532T allele gave amplification signals for the line BTK323STS and the F_1 hybrid, whereas no signal was detected for the cultivar NA4613RG. On the other hand, the assay for the *GmAhas*-532C allele only showed amplification signals for the susceptible cultivar and the F_1 hybrid. Both pre-amplification dilutions gave essentially the same results (Table 2).

The cosegregation of resistance to SU and *GmAhas1* haplotypes was assessed on the 120 F_2 plants derived from the cross NA4613RG × BTK323STS. Ninety-three plants showed amplification signal with the *GmAhas*-532T assay, whereas 88 plants gave signal with the *GmAhas*-532C assay. Both haplotypes completely cosegregated with phenotypes for resistance/susceptibility to SU (Table 3). Apart from allowing genotyping of homozygous and heterozygous individuals for *Als1*, these results confirm that the sequence of *GmAhas1* corresponds to the gene sequence of *Als1*.

Discussion

The soybean genome sequence was assembled and made available in late 2008 (Schmutz et al. 2010) which enabled the development of several genome browsers (SoyBase,

Table 3 Reaction of soybean plants to the herbicide chlorsulfuron [resistant (R), segregant (Seg) and susceptible (S)] and *GmAhas1* haplotype segregation (*A* = *GmAhas*532-T, *B* = *GmAhas*532-C) in a F_2 population derived from the cross NA4613RG × BTK323STS

Reaction to chlorsulfuron	Genotype	No. of plants	Haplotype segregation	
			A	*B*
S	*als1/als1*	27	0	27
Seg	*Als1/als1*	61	61	61
R	*Als1/Als1*	32	32	0
Total		120	93	88

Legumebase, Soybean knowledge base, Soybean Functional Genomics Database, Phytozome, Legume Integrative Platform, among others) each with different specializations and capabilities. In this work, phytozome was used to identify soybean genome sequences highly similar to the *A. thaliana* AHAS gene sequence. By this way, four sequences were identified, each one of them belonging to a different soybean chromosome. This number is not surprising since it is well known that the number of genes controlling AHAS activity varies greatly among plant species. The diploid species *A. thaliana,* sugar beet (*Beta vulgaris*), chicory (*Cichorium intybus*), rice (*Oryza sativa*), and poppy (*Papaver rhoeas*) present only one *Ahas* copy in their genomes constitutively expressed (Mazur et al. 1987; Wright and Penner 1998; Dewaele et al. 1997; Scarabel et al. 2004; Sikdar and Kim 2010). In the allotetraploids tobacco (*Nicotiana tabacum*) and maize (*Zea mays*), two *Ahas* genes have been identified and characterized (Mazur et al. 1987; Fang et al. 1992). Also, the allohexaploid wheat (*Triticum aestivum*) possesses three homeologous *Ahas* catalytic subunit genes (Ascenzi et al. 2003). The number of *Ahas* genes does not reflect exactly the ploidy level of a given species, as three highly homologous *Ahas* genes are present in the paleotetraploid sunflower (*Helianthus annuus*, Kolkman et al. 2004), five in the allotetraploid canola (*Brassica napus*, Wiersma et al. 1989; Ouellet et al. 1992), and six in the allotetraploid cotton (*Gossypium hirsutum*, Grula et al. 1995).

One of the most striking features of the soybean genome is the presence of a whole-genome duplication (WGD). The WGD has been dated to between ~5 and ~13 Mya (Doyle and Egan 2010; Schmutz et al. 2010) and this resulted in a high similarity between coding sequences in paralogous genes. Besides the WGD, the soybean genome has also been strongly shaped by at least two previous rounds of genome duplications: one at around 58 million years ago, near the origin of the papilionoid legume subfamily; and a genomic triplication that occurred before the radiation of the Rosid or Fabid clade, more than 130 million years ago (Schlueter et al. 2007). All together, these polyploidy events resulted in up to 12 homeologous genomic copies of any given genomic region. Typically, a genomic region will be closely related to one other region (via the more recent WGD duplication); more distantly related to two other regions (via the early legume duplication plus the WGD); and showing faint similarity to up to eight other regions (via the pre-Fabid triploidy, the legume duplication and the *Glycine* WGD). This means that, assuming no gene losses or additional duplications, a gene whose function has been identified in *A.* may have four equidistant paralogs in soybean and eight somewhat more distant paralogs via the Fabid triplication (Severin et al. 2011; Cannon and Shoemaker 2012). This is exactly the case for the soybean *Ahas* paralogs described in this work: four copies were identified and they show a similarity with *A. Ahas* gene sequence ranging from 69 to 76 %.

Another consequence of the soybean genomic duplication history is related to identity among paralogous genes. It has been shown that paralogous genes from the *Glycine* WGD typically have ~93–94 % identity; meanwhile paralogs from the early legume WGD typically have ~75–79 % identity (Severin et al. 2011; Cannon and Shoemaker 2012). This is also the case for the soybean *Ahas* paralogs identified here. *GmAhas3* and *GmAhas4* have 93 % identity at the nucleotide level, and both of them show only 76 to 81 % identity with respect to *GmAhas1* and *GmAhas2*, respectively. In other words, each of the members of the two pairs of paralogs *GmAhas3–GmAhas4* and *GmAhas1–GmAhas2* may have arisen during the *Glycine* WGD, and the initial sequences originating each pair of paralogs may have arisen during the early legume genome duplication.

After genome duplication once fixed within species, alternative fates for duplicate genes are pseudogenization, neofunctionalization and subfunctionalization (Hurles 2004; Cusack and Wolfe 2007; Blanc and Wolfe 2004; Moore and Purugganan 2005). Despite carrying five *Ahas* copies, only three of them were shown to be expressed in *B. napus* (Rutledge et al. 1991; Ouellet et al. 1992). Four out of the six *Ahas* copies of *G. hirsutum* are organized as tandem pairs. The two genes that are not part of the tandem repeats encode the main housekeeping forms of *Ahas* in this crop. Among the four *Ahas* genes comprising the tandem pairs, at least two are functional and they exhibit a highly specific expression in reproductive tissue. Thus, they are considered tissue-specific paralogs (Grula et al. 1995). Soybean does not seem to be the exception to this pattern. In the soybean genome, about 75 % of the genes are present in multiple copies, and approximately 50 % of paralogs are differentially expressed and have undergone expression subfunctionalization, and only a small proportion of the duplicated genes have been neofunctionalized or non-functionalized (Roulin et al. 2012), suggesting that the main fate of duplicated genes was subfunctionalization.

In line with these observations, the results obtained in this work indicate that only one out of the four identified paralogs in the soybean genome is highly expressed in the leaves. This paralog, *GmAhas1*, was further investigated in SU-resistant and susceptible soybeans.

Sequencing results indicate that the sulfonylurea-resistant AHAS from BTK323STS has a serine residue (TCC) at position 197 (referring to nomenclature of *A. thaliana*), whereas the herbicide-susceptible enzyme from Williams 82 has a proline residue (CCC) at this position. This mutation (P197S) has been identified as the basis for AHAS-inhibition resistance in several species of different botanical families: Amaranthaceae (*Kochia scoparia*, Guttieri et al. 1995; *Amaranthus retroflexus*, Sibony et al. 2001; *Beta vulgaris*, Wright et al. 1998); Asteraceae (*Anthemis cotula*, Intanon et al. 2011; *Chrysanthemum coronarium*, Tal and Rubin 2004; *Conyza canadensis*, Zheng et al. 2011), Brassicaceae (*Capsella bursa-pastoris*, Cui et al. 2012; Jin et al. 2011, *Descurainia sophia*, Cui et al. 2011; *Raphanus raphanistrum*, Yu et al. 2003; *Sinapis arvensis*, Warwick et al. 2005), Cyperaceae (*Scirpus juncoides* var *ohwianus*, Uchino et al. 2007), Papaveraceae (*Papaver rhoeas*, Scarabel et al. 2004), Poaceae (*Apera spica-venti*, Krysiak et al. 2011; *Bromus tectorum*, Park and Mallory Smith 2004; *Hordeum leporinum*, Yu et al. 2007; *Lolium rigidum*, Yu et al. 2008) Ponteridaceae (*Monocoria vaginalis*, Wang et al. 2004) and Scrophulariaceae (*Lindernia dubia* var *major*, Uchino and Watanabe 2002). To the best of our knowledge, this is the first report of the P197S substitution in an *Ahas* gene sequence for a member of Fabaceae and also for a crop species.

The *Als1 locus* was located onto chromosome 4 of the consensus genetic map of soybean by mapping SU resistance in an experimental population derived from the cross NA4613RG × BTK323STS. *Als1* is flanked by markers sct_191 and satt 195 toward the distal and proximal ends, respectively. The mapping strategy used in this work started from in silico analysis to identify four candidate sequences selected by their degree of similarity to the AHAS sequence of the model species *A. thaliana*. The bulk segregant analysis was then focused on only the four genomic regions containing the candidate sequences. By this way, the mapping process became more efficient, which is particularly useful in a crop species such as soybean which has a low to intermediate level of polymorphism for SSRs markers. Unlike classical mapping procedures, the utilized strategy results not only in the identification of the genomic location of the *locus* of interest and a pair of markers flanking it, but also in the association of a gene sequence to the locus. Validation that this sequence corresponds to the *Als1 locus* was conducted by comparing the DNA and protein sequences of two lines differing for SU resistance. Observed nucleotidic change leading to a non-synonymous substitution at the amino acidic level in the resistant line BTK323STS with respect to the original line Williams 82 indicates that the obtained sequence corresponds to the *Als1 locus*. The fact that the observed substitution (P197S) was informed as the basis of SU resistance in several other species confirms this hypothesis.

The molecular breeding tools described herein create the basis for rapidly identifying new mutations in soybean *Ahas* leading to enhanced levels of resistance to SU or to other families of AHAS inhibitor herbicides. The autogamous nature of this crop and the fact that *GmAhas1* is the only member of the *Ahas* gene family that is expressed in true leaves preclude the utilization of different *Ahas* mutations in the same cultivar, as is the case in allogamous crops, like sunflower (Sala et al. 2012). Although the variability among soybean genotypes for the pattern of expression of different Ahas paralogs remains to be investigated, it appears that the only non-GMO strategy to exploit positive interactions among different *Ahas* mutations in soybean would be to obtain double or multiple mutations in the same haplotype, as was described for other crops (Hattori et al. 1992).

Conflict of interest The experiments described in this paper comply with the current laws of Argentina. The authors declare that they have no conflict of interest.

References

Anderson AH, Simmons FW (2004) Use of the sulfonylurea-tolerant soybean trait to reduce soybean response to prosulfuron soil residues. Weed Technol 18(3):521–526

Ascenzi R, Birk I, Ortlip C, Howie W, Pozniak C, Menard C, O'Donoughue L, Parker G, Hucl P, Singh BK (2003) Characterization and mapping of the acetohydroxyacid synthase genes of *Triticum aestivum* L. and the development of wheat varieties resistant to imidazolinone herbicides. In: Proceedings of the 7th International Congress of Plant Molecular Biology, Barcelona, Spain, 23–28 June

Beyer JREM, Duffy MJ, Hay JV, Schlueter DD (1988) Sulfonylureas. In: Kearney PC, Kaufmann DD (eds) Herbicides: chemistry, degradation and mode of action. Dekker, New York, pp 117–189

Blanc G, Wolfe KH (2004) Functional divergence of duplicated genes formed by polyploidy during *Arabidopsis* evolution. Plant Cell 16:1679–1691

Santone WD, Cairo, C, Sala CA, Rossi R (2013) Pleiotropic effects of the allele for sulfonylurea tolerance, *Als1*, on soybean (*Glycine max* (L) Merr.). In: Proc. IX World Soybean Research Conference, Durban, South Africa. http://www.proteinresearch.net/html_images/wsrc2013/18-february-session-3/359_santone-wd.pdf

Cannon SB, Shoemaker RC (2012) Evolutionary and comparative analyses of the soybean genome. Breed Sci 61:437–444

Cui HL, Zhang CX, Wei SH, Zhang HJ, Li XJ, Zhang YQ, Wang GQ (2011) Acetolactate synthase gene proline (197) mutations confer tribenuron-methyl resistance in flixweed (*Descurainia sophia*) populations from China. Weed Sci 59:376–379

Cui HI, Li X, Wang G, Wang J, Wei S, Cao H (2012) Acetolactate synthase proline (197) mutations confer tribenuron-methyl

resistance in *Capsella bursa-pastoris* populations from China. Pestic Biochem Phys 102:229–232

Cusack BP, Wolfe KH (2007) When gene marriages don't work out: divorce by subfunctionalization. Trends Genet 23:270–272

Dellaporta S, Wood L, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19–21

Dewaele E, Forlani G, Degrande D, Nielsen E, Rambour S (1997) Biochemical characterization of chlorsulfuron resistance in *Cichorium intybus* L. var. Witloof J Plant Physiol 151:109–114

Doyle JJ, Egan AN (2010) Dating the origins of polyploidy events. New Phytol 186:73–85

Duggleby RG, Pang SS (2000) Acetohydroxyacid synthase. J Biochem Mol Biol 33:1–36

Fang LY, Gross PR, Chen PH, Lillis M (1992) Sequence of two acetohydroxyacid synthases genes from *Zea mays*. Plant Mol Biol 18:1185–1187

Fehr WR, Caviness CE, Burmood DT, Pennington JS (1971) Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. Crop Sci 11:929–931

Goodstein DM, Shenggiang S, Russell H, Neupane R, Hays RD, Fazo J, Mitros T, Dirks W, Hellsten U, Putnam N, Rokhsor D (2012) Phytozome: a comparative platform for green plant genomics. Nucleic Acids Res 40:1178–1186

Green JM, Hazel CB, Forney R, Pugh LM (2008) New multiple-herbicide crop resistance and formulation technology to augment the utility of glyphosate. Pest Manag Sci 64:332–339

Gressel J, Siegel A (1990) Modeling the effectiveness of herbicide rotations and mixtures as strategies to delay or preclude resistance. Weed Tech 4:186–198

Grula JW, Hudspeth RL, Hobbs SL, Anderson DM (1995) Organization, inheritance and expression of acetohydroxyacid synthase genes in the cotton allotetraploid *Gossypium hirsutum*. Plant Mol Biol 28:837–846

Guttieri MJ, Eberlein CV, Thill DC (1995) Diverse mutations in the acetolactate synthase gene confer clorsulfuron resistance in kochia (*Kochia scoparia)* biotypes. Weed Sci 43:175–178

Hattori J, Rutledge R, Labbé H, Brown D, Sunohara G, Miki B (1992) Multiple resistance to sulfonylureas and imidazolinones conferred by an acetohydroxyacid synthase gene with separate mutations for selective resistance. Mol Gen Genet 232:167–173

Heap I (2013) The international survey of herbicide resistant weeds. Online publication available at http://www.weedscience.org

Hurles M (2004) Gene duplication: the genomic trade in spare parts. PLoS Biol 2:e206

Hyten DL, Choi I, Song Q, Specht JE, Carter TE, Shoemaker RC, Hwang EY, Matukumalli LK, Cregan PB (2010) A high density integrated genetic linkage map of soybean and the development of a 1536 universal soy linkage panel for quantitative trait locus mapping. Crop Sci 50:960–968

Intanon S, Perez-Jones A, Hulting AG, Mallory-Smith CA (2011) Multiple Pro197 ALS substitutions endow resistance to ALS inhibitors within and among mayweed chamomile populations. Weed Sci 59:431–437

Ishimoto M, Harada K (2012) Breeding Science Issue: soybean breeding genomic era. Breed Sci 61:435

Jin T, Liu J, Huan Z, Wu C, Bi Y, Wang J (2011) Molecular basis for resistance to tribenuron in shepherd's purse (*Capsella bursa-pastoris* (L.) Medik.). Pestic Biochem Phys 100:160–164

Kolkman JM, Slabaugh MB, Bruniard JM, Berry S, Bushman BS, Olungu C, Maes N, Abratti G, Zambelli A, Miller JF, Leon A, Knapp SJ (2004) Acetohydroxyacid synthase mutations conferring resistance to imidazolinone or sulfonylurea herbicides in sunflower. Theor Appl Genet 109:1147–1159

Krysiak M, Gawronski SW, Adamczewski K, Kierzek R (2011) ALS gene mutations in *Apera spica-venti* confer broad-range resistance to herbicides. J Plant Prot Res 51:261–267

Lander ES, Green P, Abrahamson J (1987) MAPMAKER: an interactive computer package for constructing primary genetic maps of experimental and natural population. Genomics 1:174–181

Masuda T, Goldsmith PD (2009) World soybean production: area harvested, yield, and long-term projections. Int Food Agribus Manag Rev 12:153

Mazur BJ, Chui CF, Smith JK (1987) Isolation and characterization of plant genes coding for acetolactate synthase the target enzyme for two classes of herbicide. Plant Physiol 85:1110–1117

McCourt JA, Pang SS, King-Scott J, Guddat LW, Duggleby RG (2006) Herbicide-binding sites revealed in the structure of plant acetohydroxyacid synthase. Proc Natl Acad Sci 103: 569–573

Michelmore R, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. PNAS 88:9828–9832

Moore RC, Purugganan MD (2005) The evolutionary dynamics of plant duplicate genes. Curr Opin Plant Biol 8:122–128

Norsworthy JK, Ward SM, Shaw DR, Llewellyn RS, Nichols RL, Webster TM, Bradley KW, Frisvold G, Powles SB, Burgos NR, Witt WW, Barrett M (2012) Reducing the risks of herbicide resistance: best management practices and recommendations. Weed Sci 31–62 (2012 Special Issue). doi:10.1614/WS-D-11-00155.1

Ouellet T, Rutledge RG, Miki BL (1992) Members of the acetohydroxydacid synthase multigene family of *Brassica napus* have divergent patterns of expression. Plant J 2:321–330

Park KW, Mallory Smith CA (2004) Physiological and molecular basis for ALS inhibitor resistance in *Bromus tectorum* biotypes. Weed Res 44:71–77

Powles S (2008) Evolved glyphosate-resistant weeds around the world: lessons to be learnt. Pest Manag Sci 64:360–365

Powles SB, Yu Q (2010) Evolution in action: plants resistant to herbicides. Annu Rev Plant Biol 61:317–347

Rossi R (2012) Historia del mejoramiento genético de la soja en la Argentina. In: Baigorri HEJ, Salado Navarro LR (eds) El cultivo de Soja en Argentina, 1st edn. Agroeditorial, Vicente López, pp 33–50

Roulin A, Auer PL, Libault M, Schlueter J, Farmer A, May G, Stacey G, Doerge RW, Jackson SA (2012) The fate of duplicated genes in a polyploid plant genome. Plant J 73:143–153

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132:365–386

Rutledge RG, Quellet T, Hattori J, Miki BL (1991) Molecular characterization and genetic origin of the *Brassica napus* acetohydroxyacid synthase multigene family. Mol Gen Genet 229:31–40

Sala CA, Bulos M, Altieri E, Weston B (2012) Response to imazapyr and dominance relationships of two imidazolinone-resistant alleles at the *Ahasl1* locus of sunflower. Theor Appl Genet 124:385–396

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, New York. ISBN 0-87969-309-6

Sathasivan K, Haughn GW, Murai N (1991) Molecular basis of imidazolinone herbicide resistance in *Arabidopsis thaliana* var Columbia. Plant Physiol 97:1044–1050

Scarabel L, Carraro N, Sattin M, Varotto S (2004) Molecular basis and genetic characterization of evolved resistance to ALS-inhibitors in *Papaver rhoeas*. Plant Sci 166:703–709

Schlueter JA, Lin JY, Schlueter SD, Vasylenko-Sanders IF, Deshpande S, Yi J, O'Bleness M, Roe BA, Nelson RT, Scheffler BE (2007) Gene duplication and paleopolyploidy in soybean and the implications for whole genome sequencing. BMC Genomics 8:330

Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J (2010) Genome sequence of paleopolyploid soybean. Nature 463:178–183

Sebastian SA, Fader GM, Ulrich JF, Forney DR, Chaleff RS (1989) Semidominant soybean mutation for resistance to sulfonylurea herbicide. Crop Sci 29:1403–1408

Severin AJ, Cannon SB, Graham MM, Grant D, Shoemaker RC (2011) Changes in twelve homoeologous genomic regions in soybean following three rounds of polyploidy. Plant Cell 23:3129–3136

Shaner DL (1991) Mechanisms of resistance to acetolactate synthase/acetohydroxyacid synthase inhibitors. In: Proceedings of the Western Society of Weed Science, pp 122–125

Shaner DL (2000) The impact of glyphosate-tolerant crops on the use of other herbicides and on resistance management. Pest Manag Sci 56:320–326

Sibony M, Michel A, Haas HU, Rubin B, Hurle K (2001) Sulfometuron resistant *Amaranthus retroflexus*: cross resistance and molecular basis for resistance to acetolactate synthase inhibiting herbicides. Weed Res 41:509–522

Sikdar MSI, Kim JS (2010) Functional analysis of a gene encoding threonine synthase from rice. Afr J Biotechnol 9:1122–1129

Simpson DM, Stoller EW (1996) Thifensulfuron and imazethapyr interaction at the ALS enzyme in sulfonylurea-tolerant soybean (*Glycine max*). Weed Sci 44:763–768

Singh BK (1999) Biosynthesis of valine, leucine and isoleucine. In: Singh BK (ed) Plant amino acids. Marcel Dekker, New York, pp 227–247

Tal A, Rubin B (2004) Occurrence of resistant *Chrysanthemum coronarium* to ALS inhibiting herbicides in Israel. Resist Pest Manag Newslett 13:31–33

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Tranel PJ, Wright TR (2002) Resistance of weeds to ALS-inhibiting herbicides: what have we learned? Weed Sci 50:700–712

Uchino A, Watanabe H (2002) Mutations in the acetolactate synthase genes of sulfonylurea-resistant biotypes of *Lindernia* spp. Weed Biol Manag 2:104–109

Uchino A, Ogata S, Kohara H, Yoshida S, Yoshioka T, Watanabe H (2007) Molecular basis of diverse responses to acetolactate synthase-inhibiting herbicides in sulfonylurea-resistant biotypes of Schoenoplectus juncoides. Weed Biol Manag 7:89–96

Umbarger HE (1978) Amino acid biosynthesis and its regulation. Annu Rev Biochem 47:533–606

Vila-Aiub MM, Vidal RA, Balbi MC, Gundel PE, Trucco F, Ghersa CM (2008) Glyphosate-resistant weeds of South American cropping systems: an overview. Pest Manag Sci 64:366–371

Voorrips RE (2002) MapChart: software for the graphical presentation of linkage maps and QTLs. J Hered 93:77–78

Wang G, Lin Y, Li W, Ito M, Itoh K (2004) A mutation confers *Monochoria vaginalis* resistance to sulfonylureas that target acetolactate synthase. Pestic Biochem Physiol 80:43–46

Warwick SI, Sauder C, Beckie HJ (2005) Resistance in Canadian biotypes of wild mustard (*Sinapis arvensis*) to acetolactate synthase inhibiting herbicides. Weed Sci 53:631–639

Wiersma PA, Schmiemann MG, Condie JA, Crosby WL, Moloney MM (1989) Isolation, expression and phylogenetic inheritance of an acetolactate synthase gene from *Brassica napus*. Mol Gen Genet 219:413–420

Wright T, Penner D (1998) Cell selection and inheritance of imidazolinone resistance in sugar beet (*Beta vulgaris*). Theor Appl Genet 96:612–620

Wright TR, Bascomb NF, Sturner SF, Penner D (1998) Biochemical mechanism and molecular basis for ALS-inhibiting herbicide resistance in sugarbeet (*Beta vulgaris*) somatic cell selections. Weed Sci 46:13–23

Wrubel RP, Gressel J (1994) Are herbicide mixtures useful for delaying evolution of resistance? A case study. Weed Tech 8:635–648

Yu Q, Zhang XQ, Hashem A, Walsh MJ, Powles SB (2003) ALS gene proline (197) mutations confer ALS herbicide resistance in eight separated wild radish (*Raphanus raphanistrum*) populations. Weed Sci 51:831–838

Yu Q, Nelson JK, Zheng MQ, Jackson M, Powles SB (2007) Molecular characterization of resistance to ALS-inhibiting herbicides in *Hordeum leporinum* biotypes. Pest Manag Sci 63:918–927

Yu Q, Han H, Powles SB (2008) Mutations of the ALS gene endowing resistance to ALS-inhibiting herbicides in *Lolium rigidum* populations. Pest Manag Sci 64:1229–1236

Zheng D, Kruger GR, Singh S, Davis VM, Tranel PJ, Weller SC, Johnson WG (2011) Cross-resistance of horseweed (*Conyza canadensis*) populations with three different ALS mutations. Pest Manag Sci 67:1486–1492