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ORIGINAL PAPER

Inheritance and molecular characterization of broad range tolerance to herbicides targeting acetohydroxyacid synthase in sunflower

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Abstract *Ahasl1* is a multilallelic locus where all the induced and natural mutations for herbicide tolerance were described thus far in sunflower (*Helianthus annuus* L.). The allele *Ahasl1-1* confers moderate tolerance to imidazolinone (IMI), *Ahasl1-2,* and *Ahasl1-3* provides high levels of tolerance solely to sulfonylurea (SU) and IMI, respectively. An Argentinean wild sunflower population showing plants with high level of tolerance to either an IMI and a SU herbicide was discovered and used to develop an inbred line designated RW-B. The objectives of this work were to determine the relative level and pattern of cross-tolerance to different AHAS-inhibiting herbicides, the mode of inheritance, and the molecular basis of herbicide tolerance in this line. Slight or no symptoms observed after application of different herbicides indicated that RW-B possesses a completely new pattern of tolerance to AHAS-inhibiting herbicides in sunflower. Biomass response to increasing doses of metsulfuron or imazapyr demonstrated a higher level of tolerance in RW-B with respect to *Ahasl1-1/Ahasl1-1* and *Ahasl1-2/Ahasl1-2* lines. On the basis of genetic analyses and cosegregation test, it was concluded that tolerance to imazapyr in the original population is inherited as a single, partially dominant nuclear gene and that this gene is controlling the tolerance to four different AHAS-inhibiting herbicides. Pseudo-allelism test permitted us to conclude that the tolerant allele present in RW-B is an allelic variant of *Ahasl1-1* and was designated as *Ahasl1-4*. Nucleotide and deduced amino acid sequence indicated that the *Ahasl1-4* allele sequence of RW-B has a leucine codon (TTG) at position 574 (relative to the *Arabidopsis thaliana* AHAS sequence), whereas the enzyme from susceptible lines has a tryptophan residue (TGG) at this position. The utilization of this new allele in the framework of weed control and crop rotation is discussed.

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Introduction

Acetohydroxyacid synthase (AHAS, EC 4.1.3.18, also known as acetolactate synthase, ALS) is the first enzyme in the pathway for the synthesis of the branched chain amino acids valine, leucine, and isoleucine (Singh 1999). This enzyme has been shown to be the site of action for different classes of herbicides collectively known as AHAS inhibitors: sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TZ), pyrimidinyloxybenzoates (POB), and sulfonylaminocarbonyl-triazolinones (SCT, Tranel and Wright 2002; Tan et al. 2005). Plants resistant to one or more of these herbicides have been successfully produced from seed, microspore, pollen, and callus mutagenesis, or somatic cell selection in *Arabidopsis thaliana* (L.). Heynh., canola (*Brassica napus* L.), cotton (*Gossypium hirsutum* L.), maize (*Zea mays* L.), soybean [*Glycine max* (L.) Merr.], sugar beet (*Beta vulgaris* L.), sunflower (*Helianthus annuus* var. *macrocarpus* Ckll.), tobacco (*Nicotiana tabacum* L.), and wheat (*Triticum aestivum* L.). Tolerance in most of these cases is due to a form of the AHAS large subunit enzyme (AHASL) that is less sensitive to herbicide inhibition and is conferred by a single, partially dominant nuclear gene (Tranel and Wright 2002; Tan et al. 2005). This reduction in herbicide binding is caused by mutations at key sites in the genes coding for the catalytic subunit of AHAS. Several authors have reviewed known mutations of

the AHAS genes that confer tolerance to AHAS-inhibiting herbicides in plants (Preston and Mallory-Smith 2001; Tranel and Wright 2002). Imidazolinone- and/or sulfonylurea-tolerant plants with altered AHAS genes and enzymes have been discovered in many species, including weeds and crops, which permitted the development and commercialization of several herbicide-tolerant crops (Tan et al. 2005).

Based on molecular studies, Kolkman et al. (2004) identified and characterized three genes coding for the AHAS catalytic subunits in sunflower (*Ahasl1*, *Ahasl2* and *Ahasl3*). *Ahasl1* is a multiallelic locus and the only member of this small family where all the induced and natural mutations for herbicide tolerance were described thus far in sunflower (Sala et al. 2008b). *Ahasl1-1* (also known as *Imr1* or Ar_{pur}, Bruniard and Miller 2001; Kolkman et al. 2004, respectively) harbors a C-to-T mutation in codon 205 (*A. thaliana* nomenclature) which confers a moderate tolerance to imidazolinones, *Ahasl1-2* (also known as Ar_{kan}) shows a C-to-T mutation in codon 197 conferring high levels of sulfonylurea tolerance (Kolkman et al. 2004), and *Ahasl1-3* presents a G-to-A mutation in codon 122 which confers high levels of imidazolinone tolerance (Sala et al. 2008b).

It is known that sunflower lines developed to tolerate some AHAS-inhibiting herbicides are susceptible to foliar applications and, in many cases, to soil residues of other AHAS-inhibiting herbicides (Howatt and Endress 2006). With the aim of developing a trait that confers high levels of cross-tolerance to different families of AHAS inhibitors, we developed from a wild sunflower population a sunflower inbred line—designated as RW-B—with high tolerance to imidazolinones and sulfonylureas. In this sense, the objectives of this work were to determine the pattern of cross-tolerance to different AHAS-inhibiting herbicides, the mode of inheritance, and the molecular basis of herbicide tolerance in this line.

Materials and methods

Plant materials

The oilseed maintainer inbred lines RW-B, HA425, SuBL, HA89 and B770, and the oilseed restorer inbred lines RHA426 and SuRL were used. HA425 and RHA426 are inbred lines developed and released by the USDA and express the imidazolinone-tolerant trait Imisun ([A205V], Miller and Al-Khatib 2002). SuBL and SuRL were obtained by inbreeding and selection from the genetic stocks SURES-1 and SURES-2, respectively. These sulfonylurea-tolerant genetic stocks [P197L] were developed and released by the USDA (Miller and Al-Khatib 2004). HA89 is an herbicide-susceptible line released by the USDA and Texas Agricultural Experiment Station in 1971, and B770 is a susceptible proprietary line from Nidera S.A. RW-B is a maintainer line developed at the Biotechnology Department of Nidera S.A. as described below.

Breeding procedure to develop RW-B

During the Summer of 2004 and 2005 we collected seed samples of wild sunflower populations growing along the roadsides in the provinces of Córdoba, La Pampa and Buenos Aires, Argentina. Two groups of 50 plants each of the 46 collected populations were screened under greenhouse conditions with a SU and an IMI herbicide. One group was treated with metsulfuron at 5 g a.i ha^{-1}, and the other with imazapyr at 80 g a.i. ha^{-1} together with susceptible checks. Only one of these populations, collected 22 km from Jovita (Province of Córdoba, Argentina), showed plants with no injury in both groups. Three-hundred seeds of this population were sown under greenhouse conditions together with the control lines HA89, B770 (susceptible, "S") and RHA426 (tolerant, "T"). At the V6–V8 stage (Schneiter and Miller 1981) plants were sprayed with imazapyr at a rate of 80 g a.i.ha^{-1}. Two weeks after treatment, all the plants were evaluated for herbicide injury by visual inspection. Lines HA89 and B770 were killed by the treatment whereas the tolerant line RHA426 showed signs of chlorosis. In total, 159 plants of the wild population resisted the herbicide treatment and 129 were killed. Further, 18 out of the 159 tolerant plants showed no symptoms of herbicide injury. One of these plants, coded as RW-73, was used for the genetic analyses and for introgressing the tolerance trait into the susceptible line B770 by a marker-assisted backcrossing selection procedure after detecting tolerant plants in each generation by imazapyr treatments. The converted line, coded as RW-B, was used for conducting the tolerance level assays. RW-B was fixed for the tolerance trait since its selfed progeny did not segregate for susceptibility after imazapyr application.

Inheritance of imazapyr tolerance in RW-73

RW-73 was crossed as pollen donor with the susceptible inbred lines HA89 and B770. F_1 hybrid plants derived from these crosses were self-pollinated and backcrossed to the susceptible parents to obtain the F_2 and BC_1F_1 generations, respectively. Seeds from the susceptible parents and the F_1, F_2, and BC_1F_1 generations were sown under greenhouse conditions and were challenged with 80 g a.i. ha^{-1} of imazapyr at the V2–V4 stage. Phenotypes were scored 14 days after treatment (DAT) as either T, S, or intermediate (I). Plants were scored as T if they showed no herbicide damage, S if they died, or I if they displayed height reduction or chlorosis.

Twenty F_2 plants derived from the cross HA89/RW-B were randomly selected, left untreated, and selfed to produce $F_{2:3}$ families for conducting a test to evaluate the co-segregation of tolerance to IMI, SU, TZ, and POB. To do this, 20 seeds of each $F_{2:3}$ family were sown in pots and sprayed at V2–V4 stage of development with imazapyr (80 g a.i. ha^{-1}), chlorsulfuron (25 g a.i. ha^{-1}), cloransulam-methyl (67 g a.i. ha^{-1}), or bispiribac-Na (80 g a.i. ha^{-1}). Fourteen DAT each plant was scored either as tolerant (T) if it did not show any symptoms or it showed slight injury, and susceptible (S), if it died.

Pseudo-allelism test

Since sunflower has three *Ahasl* genes which map to different linkage groups (Kolkman et al. 2004), we carried out a pseudo-allelism test in order to determine if the factor that governs herbicide tolerance in RW-B is allelic to *Ahasl1-1* and *Ahasl1-2*, the mutations already described in imidazolinone and sulfonylurea-tolerant inbred lines, respectively. To do this, RW-B plants were crossed with RHA426 and SuRL. F_1 hybrid plants from the cross RW-B/RHA426 were self-pollinated and backcrossed to RHA426 to obtain the F_2 and BC_1F_1 generations, respectively. Seeds of the parents and F_1, F_2 and BC_1F_1 were challenged with 80 and 320 g a.i. ha^{-1} of imazapyr at the V2–V4 stage and scored as described above. F_2 plants from the cross RW-B × SuRL, their parental lines, and F_1 hybrid plants were sprayed with 25 g a.i. ha^{-1} of chlorsulfuron and phenotypically scored for herbicide injury 14 DAT.

Relative tolerance level of RW-B to AHAS-inhibiting herbicides

Seeds of the utilized lines were sown in pots under greenhouse conditions. Plants were grown under natural light conditions supplemented with 400 W halide lamps to provide a 14 h day length. Day/night temperatures were 25 and 20°C, respectively. At V2–V4 stage of development at least 20 plants of each line were sprayed with different doses of three SU herbicides (25 g a.i. ha^{-1} chlorsulfuron, 100 g a.i. ha^{-1} nicosulfuron, and 24 g a.i. ha^{-1} foramsulfuron), three IMI (100 g a.i. ha^{-1} imazamox, 100 g a.i. ha^{-1} imazapic, and two rates of imazapyr: 160 and 320 g a.i. ha^{-1}), a POB (80 g a.i. ha^{-1} bispyribac-Na), and a TZ (67 g a.i. ha^{-1} cloransulam-methyl). Doses of imazamox and imazapyr are twice those recommended for weed control in Imisun sunflowers. In the other cases, utilized doses correspond to labeled doses for broadleaf weed control in different crops. Twenty plants of each line were left as untreated controls. Fourteen DAT plants were scored phenotypically using a Phytotoxicity Index (PI). PI is a phenotypic scale from 0 to 9 that was assessed for each pot by visual inspection. Plants without any symptoms were recorded as "0," increasing levels of stunting and yellowing with respect to the untreated control plants were recorded as "1" to "4," increasing levels of leaf abnormalities and leaf necrosis were recorded from "5" to "8," and dead plants with total necrosis of the apex were recorded as "9." Lines showing an average PI < 0.5 with respect to a given herbicide were taken as tolerant, a PI from 1 to 4 was considered as moderately tolerant, a PI from 5 to 8 as moderately susceptible, and a PI > 8.5 was taken as susceptible.

Dose–response experiments

Dose–response assays were conducted to evaluate the tolerance of RW-B to imazapyr and metsulfuron on a whole-plant level in comparison with the lines HA425, SuBL, and B770. Lines were sown and grown under greenhouse conditions as described above. Experiment 1 consisted of six doses of imazapyr (0, 40, 80, 160, 320, and 480 g a.i. ha^{-1}) and Experiment 2 consisted of six doses of metsulfuron (0, 2.5, 5, 10, 15, and 20 g a.i. ha^{-1}). Both experiments were arranged as a randomized block design with a full factorial (sunflower line × treatment) arrangement of treatments. Each plot consisted in ten plants randomly assigned to each treatment and both experiments were repeated twice.

On the day of herbicide application ten plants of each line were cut at the cotyledonal node and dried at 60°C for 48 h for the time-zero dried weight determination. The remaining plants were maintained for 14 DAT at which time above ground dry biomass was recorded. The data from each line were converted to biomass accumulation following application by subtracting the appropriate average time-zero biomass from each sample. Dry biomass data were converted to percentages of the untreated control plants within each line to allow direct comparisons among lines and subjected to an ANOVA procedure. Means were separated using Fisher's protected least significant difference at the 0.01 probability level. Statistical analysis of dose–response curves followed the procedure outlined by Seefeldt et al. (1995). Data were fit to a log-logistic model given by

$$Y = 100/\left[1 + (X/\mathrm{GR}_{50})^{b}\right]$$

where y = shoot biomass (expressed as the percent of the untreated control), x = herbicide dose (g a.i. ha^{-1}), b is a rate parameter (slope) related to the response to increasing herbicide dose, and GR_{50} is the herbicide dose that caused a 50% of reduction in shoot biomass accumulation. Regressions were performed on all data using nonlinear least square regression procedure (PROC NLIN, SAS 2004). Adequacy of model fit was determined by

significance of the model approximate F-statistic and the coefficients of determination.

PCR amplification and sequencing of *HaAHASL1* gene sequence from RW-B

Genomic DNA was extracted from sunflower leaf tissue using Qiagen's DNeasy 96 Plant kit (catalog no. 69181). *HaAHASL1* gene sequence was PCR-amplified in three fragments and direct-sequenced by Macrogen USA. PCR amplification was accomplished with Qiagen's Hotstart Taq DNA polymerase and associated reagents (catalog no. 203205). The PCR primers for the three fragments were as follows: AHAS 1F1 (forward primer at base pair 1–19 of the sunflower public sequence AY541451) 5′ATGGCG GCTCCTCCCAACC3′,

AHAS 1R1 (reverse primer at base pair 757–777 of the sunflower public sequence AY541451) 5′CGGTAACCTC ATCGGTTCATCS3′, AHAS 1F2 (forward primer at base pair 757–777 of the sunflower public sequence AY541451) 5′GATGAACCGATGAGGTTACCGS3′, AHAS 1R2 (reverse primer at base pair 1,794–1,814 of the sunflower public sequence AY541451)

5′TCCGCCTTTTGGGTCACTCGAS3′, AHAS1F3 (forward primer at base pair 1,248–1,269 of the sunflower public sequence AY541451) 5′GGTGACTAATCTTGATTTTT CG3′ and AHAS 1 R3 (reverse primer at base pair 1,949–1,968 of the sunflower public sequence AY541451) 5′TCA ATATTTCGTTCTGCCAT3′.

The three PCR fragments cover all the mutation sites known to confer resistance to the AHAS-inhibiting herbicides (Tranel and Wright 2002). Alignment of the obtained nucleotide sequences was performed and the resulting chromatographs were examined for polymorphisms between the wildtype lines HA89, HA372 and RHA280 (GenBank accessions AY541451, AY541452, and AY541454, respectively; Kolkman et al. 2004) and the herbicide-tolerant line RW-B. The AHAS gene sequence from *A. thaliana* (GenBank accession AY124092; Jander et al. 2003) was used as reference and for numbering of amino acids. 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). Gene sequence reported herein has been deposited in GenBank with accession number JF895115.

Results

Inheritance of imidazolinone tolerance in RW-73

At an imazapyr application rate of 80 g a.i. ha^{-1}, plants from B770 and HA89, their F_1 hybrids with RW-73, F_2 and BC_1F_1 populations could easily be scored into one of three discrete phenotypic classes (T, I, or S) 14 DAT. The imidazolinone-tolerant line HA425 was used as a control in all experiments and consistently produced a tolerant phenotype when sprayed with 80 g a.i. ha^{-1} of imazapyr, whereas HA89 and B770 were killed.

F_1 plants from the cross HA89/RW-73 and B770/RW-73 were phenotypically intermediate between their parents since they showed a reduction in height when compared with untreated F_1 control plants (Table 2). Observed segregation in the F_2 populations resulting from susceptible × tolerant crosses fitted a 1:2:1 T:I:S ratio ($P = 0.17$ and 0.68), indicating monogenic inheritance for resistance to imazapyr. To confirm these results from the F_2 populations, F_1 plants were test-crossed to the susceptible parents and the resulting progeny were evaluated for reaction to imazapyr. Observed segregation ratios in BC_1F_1 populations fitted an expected 1:1 I:S ratio ($P = 0.82$ and 0.42), confirming the single-locus hypothesis (Table 2).

Test for co-segregation of tolerance to four different AHAS inhibiting herbicides was conducted over 20 $F_{2:3}$ families derived from the cross HA89/RW-B. Five of the evaluated families were tolerant to the four analyzed herbicides, four families were completely susceptible, and eleven of them showed segregation for tolerance to each one of the analyzed herbicides (Table 3). This indicates that the same genetic factor in RW-73 is controlling the tolerance to all the AHAS inhibiting herbicides used.

Pseudo-allelism test

To determine the relationship between the tolerance gene present in RW-B and *Ahasl1-1*, F_1, F_2, and BC_1F_1 populations from the cross RW-B/RHA426 were evaluated at two herbicide applications rates (80 and 320 g a.i. ha^{-1}) of imazapyr. No susceptible plants were observed in the F_2 and BC_1F_1 populations resulting from this cross when progeny were evaluated at the lower herbicide rate (Table 4). When F_2 and BC_1F_1 populations were scored at the higher herbicide rate (320 g a.i. ha^{-1}) which discriminates both parents (Table 1), segregation for susceptibility was observed. Only two phenotypic classes could be detected, a tolerant class composed of plants without any injury or slight symptoms and a susceptible phenotype that was killed like the control line RHA426. Observed segregation ratios over 508 F_2 plants screened were not significantly different from a 3:1 segregation ratio ($P = 0.84$, Table 4). To confirm these results, F_1 plants were backcrossed to RHA426 and the resulting BC_1F_1 plants were screened at 320 g a.i. ha^{-1} of imazapyr. Observed segregation ratios fitted a 1:1 T:S ratio ($P = 0.77$), confirming that the tolerant gene in RW-B is different from the tolerant gene in RHA426 and that both of them are allelic variants

Table 1 Average phytotoxicity index of seven sunflower lines after spraying with eight AHAS-inhibiting herbicides belonging to four different families

Genotype	Mutation	Sulfonylureas			Imidazolinones			Pyrimidinyl-thiobenzoates	Triazolpyrimidines
		Foramsulfuron (24 g a.i. ha^{-1})	Chlorsulfuron (25 g a.i. ha^{-1})	Nicosulfuron (100 g a.i. ha^{-1})	Imazamox (100 g a.i. ha^{-1})	Imazapyr (160 g a.i. ha^{-1})	Imazapyr (320 g a.i. ha^{-1})	Imazapic (100 g a.i. ha^{-1})	Bispyribac-Na (80 g a.i. ha^{-1})	Cloransulam-methyl (67 g a.i. ha^{-1})
B770	WT	9.0 ± 0.0	8.8 ± 0.2	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0
HA89	WT	8.7 ± 0.2	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	8.5 ± 0.3	9.0 ± 0.0
SuBL	P197L	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	8.0 ± 0.2	9.0 ± 0.0
SuRL	P197L	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	8.5 ± 0.3	9.0 ± 0.0
Rha426	A205 V	6.5 ± 0.1	6.0 ± 0.2	8.6 ± 0.2	5.4 ± 0.3	2.0 ± 0.1	9.0 ± 0.0	6.5 ± 0.1	7.0 ± 0.1	9.0 ± 0.0
HA425	A205 V	7.2 ± 0.3	5.7 ± 0.3	9.0 ± 0.0	5.2 ± 0.1	2.5 ± 0.1	9.0 ± 0.0	5.6 ± 0.2	7.7 ± 0.2	9.0 ± 0.0
RW-B	W574L	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.1	0.0 ± 0.0	2.0 ± 0.2

Plants without any symptoms were recorded as "0"; increasing levels of stunting and yellowing with respect to the untreated control plants were recorded as "1" to "4"; increasing levels of leaf abnormalities and leaf necrosis were recorded from "5" to "8"; dead plants with total necrosis of the apex were recorded as "9"

of the locus *Ahasl1*. A close linkage between both genes cannot be ruled out as an explanation of these results, but it is unlikely considering that the three *Ahasl* genes in sunflower belong to different linkage groups. This new allele was named *Ahasl1-4*.

To further confirm that *Ahasl1-4* also controls sulfonylurea tolerance, F_2 plants from the cross RW-B × SuRL were challenged with chlorsulfuron and phenotypically scored 14 DAT. No susceptible plants were observed among 316 analyzed F_2 individuals, confirming that *Ahasl1-4* governs the broad range tolerance to AHAS-inhibiting herbicides observed in RW-B.

Nucleotide sequence comparisons between tolerant and susceptible lines

Results of the pseudo-allelism test permitted to focus the sequence analysis on the *Ahasl1* gene. PCR products were sequenced to produce the *Ahasl1* nucleotide sequences for RW-B which was deposited in GenBank as accession number JF895115. The alignment of this sequence and the nucleotide sequences of three herbicide-susceptible sunflower lines and *A. thaliana Ahas* genes revealed several SNPs. The alignment of the corresponding deduced amino acid sequences of AHAS catalytic subunit genes showed that only one SNP leads to an aminoacidic change which differentiates RW-B from all the other sequences. In fact, RW-B has a leucine codon (TTG) at position 574 (relative to the *A. thaliana* AHAS sequence), whereas the enzyme from susceptible lines has a tryptophan residue (TGG) at this position.

Relative tolerance level of RW-B to AHAS inhibitor herbicides

Plants of the susceptible inbred lines HA89 and B770 died 6 DAT at any application rate of each one of the eight herbicides tested (Table 1). Lines carrying the *Ahasl1-1* allele (RHA426 and HA425) showed different levels of yellowing, stunting, leaf abnormalities, and necrosis according to the applied herbicide and dose. Both lines were scored as moderately susceptible to imazamox (PI = 5.4 and 5.2, for RHA426 and HA425, respectively), imazapyc (PI = 6.5 and 5.6), foramsulfuron (PI = 6.5 and 7.2), chlorsulfuron (PI = 6 and 5.7) and bispyribac-Na (PI = 7.0 and 7.7), and completely susceptible to nicosulfuron and cloransulam-methyl (PI > 8.5). In addition, they showed from moderate tolerance (PI = 2.0) to susceptibility (PI = 9.0) to imazapyr according to the applied dose. Lines SuBL and SuRL showed tolerance to the three SU (PI = 0) and susceptibility to the each of the other herbicides tested (PI > 8.5). In contrast with the patterns showed by Imisun and Sures lines, plants of RW-B were

tolerant and showed no symptoms of chlorosis or leaf deformations (PI = 0) to the tested SU, imazamox, bispyribac-Na, and the two doses of imazapyr. This line only showed a slight yellowing of the apex when challenged with imazapic or cloramsulam-methyl and, for this reason, it was scored as moderately tolerant (PI = 2) to both herbicides (Table 1).

Dose–response experiments

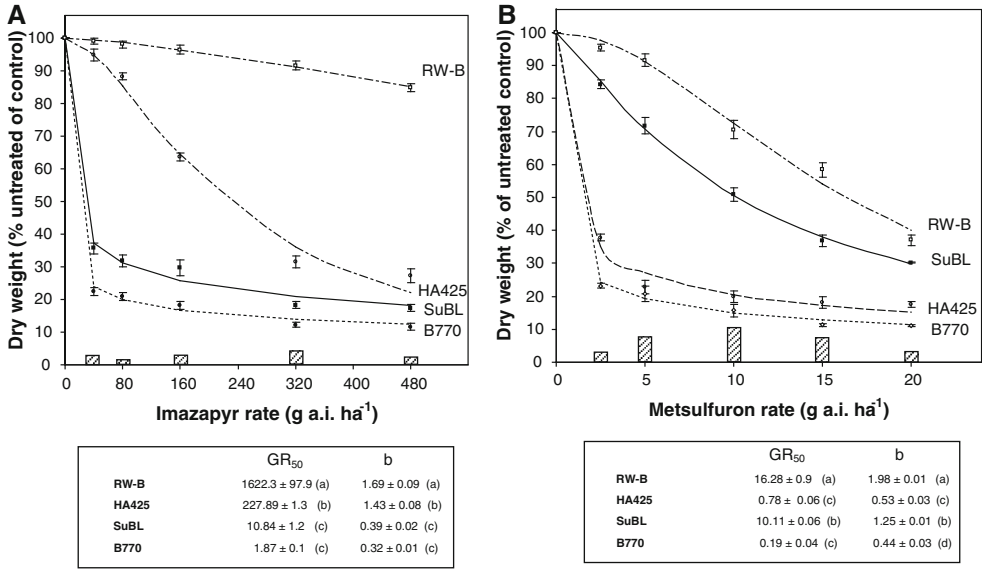
Lines, herbicide doses and their interaction significantly contributed ($P < 0.001$) to the variation in biomass accumulation 14 DAT with metsulfuron or imazapyr. This significant interaction suggested that there exist differences among genotypes for their response to increased doses of both herbicides. The log-logistic model accurately described biomass accumulation for susceptible and tolerant sunflower materials after imazapyr or metsulfuron applications (Fig. 1).

Estimates of the doses of imazapyr needed to reduce the biomass accumulation by the half (GR50) varied from 1.87 ± 0.1 to 1,622.3 ± 97.9 g a.i. ha^{-1} depending on the genetic material ($P < 0.001$). Biomass accumulation of the susceptible line was reduced to 50% with a dose of 1.87 ± 0.1 g a.i. ha^{-1} of imazapyr, which represents only 2.3% of the recommended rate for Imisun sunflower under field conditions (1× rate). On the other hand, GR50 estimate for the Imisun material was 227.9 ± 1.3 g a.i. ha^{-1}, a dose which almost corresponds to a 3× application rate under field conditions. In contrast, the line RW-B showed the higher GR50 value, more than 860 times greater than the susceptible line and 7 times greater than the Imisun material.

Responses to increasing doses of metsulfuron also varied widely among the four lines tested as revealed by the significant differences found among them for the parameters GR_{50} and b ($P < 0.001$, Fig. 1b). In this case, GR_{50} estimates varied from a minimum of 0.19 ± 0.04 g a.i. ha^{-1} for the susceptible material to a maximum value of 16.28 ± 0.9 g a.i ha^{-1} for the line RW-B. Between these extremes, GR_{50} estimate for the sulfonylurea tolerant line SuBL was 10.1 ± 0.06 g a.i. ha^{-1} and for the line HA425 was 0.78 ± 0.06 g a.i. ha^{-1}.

Discussion

Weeds compete with sunflower for moisture, nutrients, and depending on species for light and space. Weed competition causes substantial yield losses in sunflower, with reports ranging from 20 to 70%. The amount of yield



	GR_{50}	b
RW-B	1622.3 ± 97.9 (a)	1.69 ± 0.09 (a)
HA425	227.89 ± 1.3 (b)	1.43 ± 0.08 (b)
SuBL	10.84 ± 1.2 (c)	0.39 ± 0.02 (c)
B770	1.87 ± 0.1 (c)	0.32 ± 0.01 (c)

	GR_{50}	b
RW-B	16.28 ± 0.9 (a)	1.98 ± 0.01 (a)
HA425	0.78 ± 0.06 (c)	0.53 ± 0.03 (c)
SuBL	10.11 ± 0.06 (b)	1.25 ± 0.01 (b)
B770	0.19 ± 0.04 (c)	0.44 ± 0.03 (d)

Fig. 1 Dry weight of shoot biomass, as percentage of untreated control plants, of sunflower plants 14 days after application of different doses of imazapyr (**a**) and metsulfuron (**b**) in four lines carrying different alleles at the *Ahasl1* locus: B770 (susceptible, *ahasl1/ahasl1*), HA425 (*Ahasl1-1/Ahasl1-1*), SuBL (*Ahasl1-2/Ahasl1-2*), and RWB (*Ahasl1-4/Ahasl1-4*). Data points and *vertical bars* around each point represent the mean at each herbicide dose and its standard deviation, respectively. *Vertical gray shaded bars* in the lower part of each graphic represent the LSD value at the 0.01 probability level of the difference among lines for dry weight at each herbicide dose. *Lines* represent the fitted nonlinear regression. The regression equation is of the following form: shoot biomass (expressed as the percent of the untreated control) = $100/[1 + (x/GR_{50})^b]$, where x is the herbicide dose (g a.i. ha^{-1}), b is a rate parameter (slope) related to the response to increasing herbicide dose, and GR_{50} is the herbicide dose that caused a 50% reduction in shoot biomass accumulation. Average values and their standard deviations of each of both parameters of the equation are provided for each line. *Different letters* after each value indicate differences at a 0.01 probability level

Table 2 Inheritance of imidazolinone tolerance in RW-73

Cross	F_1			F_2					BC_1F_1				
	Number of plants			Number of plants			Ratio tested	χ^2 *P* value	Number of plants			Ratio tested	χ^2 *P* value
	R	I	S	R	I	S			R	I	S		
HA89/RW-73	0	23	0	129	247	102	1:2:1	0.17	0	157	161	1:1	0.82
B770/RW-73	0	31	0	152	305	165	1:2:1	0.68	0	317	297	1:1	0.42

Response of sunflower plants [Tolerant (R), Intermediate (I), Susceptible (S)] to imazapyr applied at a rate of 80 g a.i. ha^{-1} in F_1, F_2 and BC_1F_1 populations resulting from crosses between resistant line RW-73 and one of either of two susceptible lines, HA89 or B770, and Chi-square tests of single-locus model for control of resistance

Table 3 Cosegregation analysis of tolerance

$F_{2:3}$ family code	Chlorsulfuron (25 g a.i. ha^{-1})		Imazapyr (80 g a.i. ha^{-1})		Bispiribac-Na (80 g a.i. ha^{-1})		Cloransulam-methyl (67 g a.i. ha^{-1})		Pattern of segregation
	T	S	T	S	T	S	T	S	
3	15	4	15	4	13	5	14	4	Segregant
21	14	3	16	4	15	5	13	4	Segregant
50	17	0	18	0	20	0	20	0	Tolerant
54	0	17	0	18	0	18	0	18	Susceptible
71	0	14	0	17	0	16	0	19	Susceptible
124	12	4	13	3	12	4	15	3	Segregant
135	16	4	14	5	14	5	13	4	Segregant
164	20	0	19	0	19	0	18	0	Tolerant
198	14	6	15	5	13	5	15	4	Segregant
204	17	0	16	0	16	0	15	0	Tolerant
238	0	16	0	19	0	20	0	20	Susceptible
260	15	4	13	5	14	6	14	6	Segregant
317	15	5	16	4	15	4	16	4	Segregant
366	13	5	15	5	12	6	15	4	Segregant
373	20	0	19	0	19	0	20	0	Tolerant
389	16	4	15	5	14	4	14	4	Segregant
412	0	19	0	18	0	18	0	17	Susceptible
421	15	5	14	6	11	5	16	4	Segregant
436	16	0	18	0	19	0	20	0	Tolerant
452	15	5	13	5	15	3	13	6	Segregant

Twenty $F_{2:3}$ families from the cross RW-73/HA89 were sprayed either with four different AHAS-inhibiting herbicides and the progenies scored as tolerant (T) or susceptible (S) in order to determine the pattern of segregation of each family

Table 4 Pseudo-allelism test

Herbicide rate	F_1		F_2		Ratio tested	χ^2 *P* value	BC_1F_1		Ratio tested	χ^2 *P* value
	R	S	R	S			R	S		
80 g a.i. ha^{-1}	22	0	520	0	–	–	280	0	–	–
320 g a.i. ha^{-1}	18	0	381	127	3:1	0.84	151	146	1:1	0.77

Response of sunflower plants [tolerant (R) and susceptible (S)] to imazapyr applied at rates of 80 and 320 g a.i.ha^{-1} in F_1, F_2 and BC_1F_1 generations resulting from crosses between RW-B and the inbred line carrying the imidazolinone tolerant gene *Ahasl1-1*, HA425

reduction varies depending on the weed species, weed density, time of weed and crop emergence, climatic conditions, and type of soil. Herbicides are the most desirable method for weed control; however, the availability of selective herbicides for the sunflower crop is quite limited and, due to the high cost of herbicide registration, new molecules of herbicides are unlikely to be specifically developed for weed control in this crop. For this reason, gene discovery and trait development for herbicide resistance in sunflower is one of the most important issues in raising the productivity and the competitive ability of this crop in the near future (Sala et al. 2011).

In this context, during the past decade three technologies of weed control that make use of the available mutations at the *Ahasl1* locus were developed for sunflower. The Clearfield® system is based on two genes (*Ahasl1-1* and an enhancer, Tan et al. 2005): ExpresSun®, based on a mutant allele similar to *Ahasl1-2* (Canadian Food Inspection Agency 2008) and the ClearfieldPlus® system, based on the allele *Ahasl1-3* (Sala et al. 2008c).These herbicide-tolerant alleles of the *Ahasl1* locus have been discovered either by prospecting wild sunflower populations (Al-Khatib et al. 1998; White et al. 2002) or as the result of mutagenesis breeding (Gabard 2004; Sala et al. 2008a). *Ahasl1-1* confers tolerance to imazethapyr, imazamox, slight tolerance to thifensulfuron and chlorimuron, but not tolerance to cloransulam-methyl, pyrithiobac or high doses of imidazolinones (Al-Khatib et al. 1998; Bruniard and Miller 2001; Baumgartner et al. 1999a; White et al. 2002; Sala et al. 2008b). Plants carrying the *Ahasl1-2* allele in homozygous condition show tolerance to tribenuron (Miller and Al-Khatib 2004), metsulfuron, and chlorsulfuron, but complete susceptibility to imazapyr, imazapic, and imazamox (Sala et al. 2008b). On the contrary, the allele *Ahasl1-3* confers high levels of tolerance to imidazolinones but complete susceptibility to sulfonylureas (Sala et al. 2008b). The results obtained in this work are consistent to the already known patterns of cross-tolerance of the first two alleles and indicate that the line RW-B presents a completely new pattern of cross-tolerance for sunflower, since it shows a broad range level of tolerance to different AHAS -inhibiting herbicides (IMI, SU, TZ and POB, Table 1). Furthermore, RW-B also presents a higher level of tolerance to IMI and SU than lines carrying the *Ahasl1-1* and *Ahasl1-2* alleles (Table 1, Fig. 1). Imisun lines (RHA 426 and HA425) showed tolerance to imazapyr at 160 g a.i. ha^{-1} which is consistent with that observed by Bruniard and Miller (2001) for other Imisun lines and with the current practice of weed control for Clearfield® Sunflowers, which are homozygous for the *Ahasl1-1* allele. However, at the higher rate of imazapyr (320 g a.i. ha^{-1}) application, plants of HA425 and RHA426 developed chlorosis, stunting and finally died with complete burning of the apex. In contrast, plants of RW-B were tolerant and showed no symptoms of chlorosis or necrosis 14 DAT even at the higher rates of application of this herbicide (Table 1). In fact, the GR_{50} estimated value after imazapyr application was significantly higher in RW-B than in the Imisun line HA425 (Fig. 1a). With respect to the response to sulfonylureas, lines SuBL, SuRL, and RW-B showed no phytotoxicity symptoms when challenged with three different SU herbicides (Table 1). However, when SuBL and RW-B were challenged with increasing doses of metsulfuron, the latter line presented a significantly higher GR_{50} value (Fig 1b).

The combined results of the genetic studies indicate that broad range AHAS-inhibiting herbicide tolerance in RW-73 or RW-B is inherited as a partially dominant trait conferred by a single-nuclear gene (Tables 2, 3). This pattern of inheritance is consistent with other findings that have reported the genetic control of resistance to AHAS-inhibiting herbicides. In maize, soybean, *A. thaliana*, wheat, tobacco, and CLPlus sunflower resistance to AHAS inhibitors is partially dominant and inherited as a single-nuclear gene (Chaleff and Ray 1984; Newhouse et al. 1991; Sathasivan et al. 1991; Sala et al. 2008a). In contrast with this result, the imazamox tolerance found in Imisun sunflower is not conferred by a single gene (Miller and Al-Khatib 2002). The *Ahasl1-1* (or *Imr1*) allele in sunflower does not confer complete tolerance to IMI herbicides (Bruniard and Miller 2001; Kolkman et al. 2004). In fact, a second gene (*Imr2*) with a modifier effect when the major gene *Ahasl1-1* is present is necessary to achieve complete tolerance (Bruniard and Miller 2001). Pseudo-allelism tests (Table 4) and sequencing results confirmed that the tolerant gene in RW-B is a new member of the multilallelic locus *Ahasl1* of sunflower and was named *Ahasl1-4*.

Ahasl1 is the only member of the family of AHAS genes in sunflower where all the induced and natural mutations for herbicide resistance were described thus far and also is the most polymorphic (Kolkman et al. 2004; Hawley 2005) and the most preponderant in the sunflower EST database (Kozik et al. 2002). Expression analysis showed that the *Ahasl1* and *Ahasl2* are expressed in all tissues, though the leaf *Ahasl1* expression level was much greater relative to other tissues than it was in *Ahasl2* and that *Ahasl3* is only weakly expressed in leaves and the apex (Hawley 2005). This suggests that the *Ahasl1* gene is the gene family member that encodes the AHAS enzyme with essential housekeeping functions in sunflower and that it is predominantly expressed in tissues affected by herbicide treatment. Divergent patterns of expression of different members of the AHAS multigene family are not observed in neopolyploid species (v.g. wheat, Pozniak and Hucl 2004), but it appears to be the rule in paleopolyploid species (*Brassica napus*, Ouellet et al. 1992; *Gossypium hirsutum*, Grula 1995) like sunflower.

Molecular characterization of *Ahasl1-4* was conducted by PCR amplification and sequencing of the *HaAhasl1*

gene sequence. The obtained results indicate that the AHAS inhibitor-tolerant enzyme of RW-B has a leucine residue (TTG) at position 574, whereas the herbicide-susceptible enzyme from three susceptible lines has a tryptophan residue (TGG) at this position. This mutation (W574L) has been identified as the basis for AHAS-inhibition resistance in several species of plants. For example, the mutant XA17 (also known as IR) of corn, arising from cell culture, has a W574L substitution conferring cross-tolerance to IMI, SU, POB, and TZ (Bernasconi et al. 1995). The mutant PM2 of oilseed rape, obtained by microspore mutagenesis, harbors this same mutation and shows cross-tolerance to IMI and SU (Swanson et al. 1989). Moreover, 22 weed species which are tolerant to at least one family of AHAS inhibitors have been reported to harbor a W574L mutation in the AHAS gene sequence (Tranel and Wright 2002; Tranel et al. 2011). A broad spectrum tolerance (i.e., tolerance to IMI, SU, POB, and TZ or SCT) was confirmed in six out of these 22 species: *Xanthium strumarium* (Bernasconi et al. 1995), *Amaranthus blitoides* (Moshe and Baruch 2003), *Camelina microcarpa* (Hanson et al. 2004), *Sinapis arvensis* (Christoffers et al. 2006), *Bidens subalternans* (Lamego et al. 2009), and *Scirpus mucronatus* (Scarabel et al. 2010). In fact, the W574 residue is not only important for defining the shape of the active-site channel of the AHAS protein, but it also serves to anchor IMI and SU molecules to this enzyme. Consequently, the mutation of this residue to leucine changes the shape of the herbicide-binding site of the enzyme and results in the loss of several interactions with the herbicide molecules (McCourt et al. 2006).

As far as we know, this new herbicide-tolerant allele for sunflower is the first example of a W574L substitution in a crop developed by prospecting wild genetic resources. Tolerant plants to AHAS inhibitor herbicides from wild populations of *Helianthus annuus* were reported in several opportunities, either as a weed of crops where IMI or SU herbicides are usually applied (Al-Khatib et al. 1998, Baumgartner et al. 1999b, White et al. 2002, Miller and Al-Khatib 2004, Zelaya and Owen 2004) or as accessions collected in natural habitats in its center of diversity (USA and Canada, Miller and Seiler 2005; Olson et al. 2004). Three wild sunflower species were naturalized in Argentina (Sala et al. 1990) covering an area of about 5 million hectares in the central part of the country (Poverene et al. 2009). Natural populations of one of these species, *H. annuus* ssp. *annuus*, show a great morphological diversity in this country, probably arisen—at least in part—by introgressive hybridization with cultivated sunflower (Cantamutto et al. 2010) since the naturalized wild annual populations are sympatric with the crop over an extensive area in Argentina (Poverene et al. 2004; Ureta et al. 2008). Our results indicate that the wild Argentinean sunflower germplasm not only shows a great morphological diversity but also variability for other important traits, like herbicide tolerance. In the context of gene flow from cultivated to wild species, and as were pointed out by Miller and Seiler (2005) and Olson et al. (2004), it is important to state that the tolerance to AHAS inhibitor herbicides occurred in natural populations of wild sunflowers before the release of herbicide tolerance traits in commercial varieties.

Even though the obtained results regarding the broad spectrum and high-tolerance level of *Ahasl1-4* are still preliminary since they are based on observations under greenhouse conditions, it appears that the herbicides specifically designed to be applied over Clearfield®, ExpresSun® and Clearfield Plus® sunflowers can also be applied over sunflowers carrying the *Ahasl1-4* allele. Moreover, this allele also allows the possibility to develop new types of AHAS-inhibiting herbicides for weed control in the sunflower crop (i.e., POB and TZ), different formulations of the herbicides currently used or the registration for sunflower of well-known herbicides used in other crops. Finally, and independently of the type of AHAS-inhibitor herbicide developed to be applied on top of the crop, the cross-tolerance of *Ahasl1-4* could allow sunflower hybrids carrying this allele to cope with the soil residues of other types of AHAS-inhibiting herbicides from the fallow or the previous crop.

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