Pattern and molecular basis of resistance to acetolactate synthase (ALS) inhibiting herbicides in two *Amaranthus* species

M. Sibony and B. Rubin
Department of Field Crops, Vegetable and Genetics, Institute of Plant Sciences and Genetics, Faculty of Agriculture, Food and Environmental Science, The Hebrew University of Jerusalem, Rehovot 76100, Israel

Summary The pattern of resistance to various ALS inhibiting herbicides was investigated in biotypes of *Amaranthus retroflexus* L. (SuR) collected from Ganot and *A. blitoides* S.Watson (SuR/TR) from Ganot and Sorek. The triazine resistant *A. blitoides* biotype (SuS/TR) was collected from a corn field at Yavne. The whole plant response to the herbicide was determined following a post-emergence treatment as compared to wild type plants (SuS and SuS/TS) from Kfar Shmuel. Based on the dose response curves and ED_{50} values we calculated the resistance ratio (R/S). The resistant biotype of both weed species exhibited high R/S ratios to all ALS inhibitors tested. Studies with isolated ALS enzyme have shown that all SuR biotypes were resistant due to less sensitive target site. Genomic DNA isolated from SuR *A. retroflexus* biotype have shown that *als* gene confers a point mutation in domain A, leading to an exchange of pro188 in the wild type to leu. This mutation conferred resistance to all four herbicide sub-groups tested. In *A. blitoides*, however, two point mutation were discovered in the SuR/TR biotypes, one in domain A that led to an exchange of pro 188 to ser, and a second mutation in domain B that led to an exchange of try 569 to leu. The pro to ser substitution in SuR/TR resulted in resistance to sulfonylureas, triazolopyrimidines, and pyrithiobac but not to the imidazolinones. On the other hand, the mutation in domain B conferred resistance to all four groups. In spite of the similar selection pressure imposed on the weeds from Ganot and Sorek, the *A. blitoides* population from Ganot was a mixture of two different mutants, SuRA/TR and SuRB/TR whereas the population from Sorek was SuRA/TR only. All *A. blitoides* plants from both sites confer also resistance to triazines (TR) as evident from the mutation detected in the chloroplastic *psb*A gene leading to ser 264 for gly exchange in D1 protein. The fact that individual plant within a population may confer multiple resistance and different pattern of cross-resistance due to different point mutation, emphasises the importance of molecular analyses in herbicide-resistance weed management.

Keywords *Amaranthus retroflexus*, *Amaranthus blitoides*, ALS, sulfonylurea-resistance, triazine-resistance.

INTRODUCTION

Acetolactate synthase (ALS, EC 4.1.3.18), which catalyses the first common step in the biosynthesis of the branched-chain amino acids in plants, is a target of several herbicide groups, thereby blocking the biosynthesis of Val, Leu and Ile. The popularity of these herbicides can be attributed to their efficacy at low use rate against a broad spectrum of weeds, multi-crop selectivity, lack of mammalian toxicity and favourable environmental profile (Saari *et al.* 1994). Worldwide, ALS herbicide resistance has been observed in 72 weed species (Heap 2002). The evolution of resistant weed populations was generally attributed to continuous application of sulfonylurea or imidazolinone herbicides for more than three years (Rubin, 1996).

Sulfometuron-resistant *A. retroflexus* and *A. blitoides* were first discovered in 1991 in Ganot (Sibony and Rubin 1996), following repeated application for three years of sulfometuron (75 g ha^{-1}) and simazine (2.5 kg ha^{-1}). ALS resistance has been reported also for *A. rudis* J.Sauer (common waterhemp) (Hinz and Owen 1997, Foes *et al*. 1998), *A. hybridus* (smooth pigweed) (Manley *et al.* 1999), *A. palmeri* (Palmer amaranth) (Sprague *et al*. 1997, Wetzel *et al*. 1999). Resistance to ALS inhibiting herbicides is mostly due to an altered target site, namely, point mutations that occur within discrete conserved domains of the *als* gene (Saari *et al*. 1994, Devine and Eberlein 1997).

Since the first discovery of triazine resistance, 63 weed species has been reported worldwide (Heap 2002), mostly due to altered binding site of the herbicides in the D1 protein (Hirschberg and McIntosh 1983). A ser 264 to gly mutation in the D1 protein renders the plant to be resistant to triazines and several other PSII inhibitors (Yaacoby *et al*. 1986, Gronwald 1994, Lior *et al.* 2000). Multiple resistance to triazines and to ALS inhibitors were previously reported also in other *Amaranthus* spp. (Heap 2002).

The aims of this study were to characterise the resistance, and to determine the extent of cross- and multiple-resistance and their molecular basis to atrazine and different sub-groups of ALS-inhibitors in the *A. blitoides* and *A. retroflexus* biotypes.

MATERIALS AND METHODS

Whole plant studies Sulfonylurea resistant *A. retroflexus* (SuR) and multiple resistant *A. blitoides* to both sulfonylurea and triazines (SuR/TR) seeds were collected during the summer of 1995 from Ganot and during 1999 from Sorek, Israel. The seeds were pooled from many plants that survived an annual treatment with a mixture of sulfometuron at 75 g ha^{-1} and simazine at 2.5 kg ha^{-1} that was applied in the fall for three successive years. Seeds of the wild type populations – sulfonylurea and triazine susceptible (SuS and SuS/TS) were collected from a nearby location (Kfar Shmuel) that was never treated with herbicides. A sulfonylurea-sensitive and triazine resistant *A. blitoides* (SuS/TR) population was collected in a corn field (Yavne).

Experiments were conducted in a net house under local summer conditions. Whole plant and enzyme assays were conducted as described in Sibony *et al*. (2001). Herbicides (Table 1) at various rates were applied post-emergence to seedlings at the three to five leaf stage. ALS was extracted and assayed according to Ray (1984) with modifications. The resistance ratios (R/S) were calculated from the ED_{50} values.

Molecular studies Total plant genomic DNA was extracted from leaves as described by Bernatzky and Tanksley (1986). Two regions of the *als* gene were amplified using specific primers based on the published sequence (Woodworth *et al*. 1996). Region 1 with a 308-bp with domain A, and a 362-bp fragment of region 2 with domain B. The PCR purified products from the R and S biotypes were compared for the identification of mutations.

PCR-amplification and restriction enzyme digestion of a 985-bp fragment of the *psb*A gene was carried out as described by Stankiewicz *et al.* (2001). The PCR-products were exposed to the restriction enzyme *Bst*XI that cuts *psb*A gene and forms two fragments (783 bp and 202 bp) from the triazine resistant (TR) biotype as compared to one fragment (985 bp) in the triazine susceptible (TS) biotype.

Statistical analysis All the experiments were arranged in a complete randomised block design with 5 replicates and repeated at least twice. Dose-response experiments were analysed by a non-linear regression using the model of Streibig (1988). Dose response curves were calculated using the NLIN-procedure of SAS (SAS Institute Inc., Cary, NC, USA). An F-test (P = 0.01) was used to test significant differences of the regression parameter. Resistance ratios were calculated from the ED_{50} values ($ED_{50}R/ED_{50}S$).

RESULTS

Large differences were revealed in the response of *A. blitoides* and *A. retroflexus* populations to the tested ALS inhibiting herbicides (Table 1). The resistant biotypes survived high rates of the tested herbicides with R/S ratios ranging from 4 to 790 depending on the herbicide.

The SuR/TR and the SuS/TR exhibited high level of resistance to atrazine, whereas SuS/TS plants were controlled at a rate of 40 g ha^{-1}. Fluorescence measurements and PSII electron transport in isolated chloroplasts studies further confirmed the triazine resistance and its nature in the resistant biotypes (Data not shown).

The specific activity of the crude enzyme extracted from R and S plants was similar. The resistance level of ALS isolated from Ganot population was consistent with the results of the whole plants studies and supports our hypothesis that the resistance mechanism in the two *Amaranthus* spp. is based on an altered target site (Table 2).

Two forms of plant response were discovered in *A. blitoides* from Ganot, one form (SuRA/TR), *ca.* 50% of the plants, was sensitive to imidazolinones and resistant to the other three herbicide sub-groups tested,

Table 1. Resistance ratio (R/S) of *A. retroflexus* and *A. blitoides* biotypes based on the ED_{50} values.

	R/S ratio	
Herbicide	*A. retroflexus*	*A. blitoides*
Amidosulfuron	11	16
Chlorsulfuron	127	135
Sulfometuron	17	790
Imazamethabenz	12	25
Imazapyr	4	36
Imazethapyr	19	10
Flumetsulam	35	8
Pyrithiobac	11	18

Table 2. Resistance ratio (R/S) of the ALS crude enzyme extracted of *A. retroflexus* and *A. blitoides* biotypes.

	R/S ratio	
Herbicide	*A. retroflexus*	*A. blitoides*
Chlorsulfuron	114	164
Sulfometuron	147	155
Imazapyr	25	82
Flumetsulam	46	53
Pyrithiobac	39	45

whereas the other form (SuRB/TR) was resistant to all ALS inhibitors tested. The SuR/TR plants from Sorek population were defined as SuRA/TR. The SuS/TR population from Yavne was resistant to atrazine only.

Molecular studies have shown that nucleotide alignments of region 1 of the *als* gene were highly identical across the studied populations. The SuS/TS, SuS/TR, SuRB/TR differed from SuRA/TR plants only in one nucleotide of domain A (Figure 1). A substitution of cytosine by thymine was observed at position 337 leading to the exchange of pro 188 by ser in the SuRA/TR *A. blitoides*, resistant to three sub-groups of ALS inhibitors but sensitive to imidazolinones.

SuRB/TR plants were different from SuS/TS, SuS/TR and SuRA/TR in the nucleotide sequences in region 2 only. In SuRB/TR, a substitution of guanine by thymine (susceptible: TGG; resistant: TTG) at position 1721 occurred in domain B (Figure 1). This led to an exchange of a trp 569 by leu in the resistant plants. The SuRB/TR was resistant to all four herbicide sub-groups tested.

In *A. retroflexus*, the SuS from Kfar Shmuel and SuR from Ganot differed in the second nucleotide of the codon (susceptible: CCT; resistant: CTC) leading to the substitution of pro 188 to leu, conferring cross-resistance to all four-herbicide sub-groups.

PCR amplification of the *psb*A gene with the specific primers produced a single band of expected length of 985 bp. Digestion of the PCR product with *Bst*XI recognised the mutant allele (GGT), producing two fragments, which could only occur if the specific

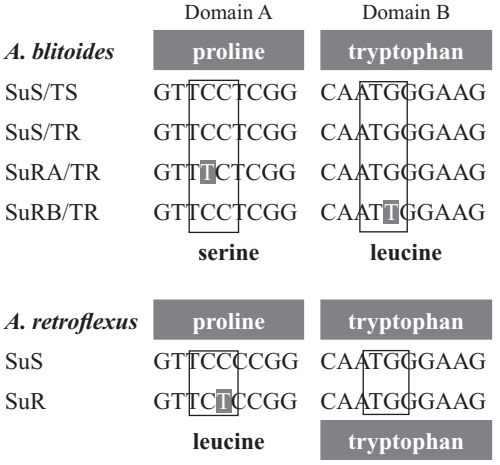

Figure 1. Alignment of the nucleotide sequences of ALS-specific PCR products region 1 domain A and region 2 domain B. The box indicates the nucleotide encoding for pro 188 and try 569.

codon mutation in the R biotypes genomic DNA was present. The *A. blitoides* resistant biotypes from Ganot, Sorek and Yavne formed two different fragments of 783 bp and 202 bp, whereas the susceptible biotypes and the resistant *A. retroflexus* from Ganot did not possess the *Bst*XI recognition site and gave a single band of 985 bp (Data not shown). These results confirmed the molecular basis for the observed resistance of these biotypes to atrazine.

DISCUSSION

Our experiments have shown that *A. retroflexus* from Ganot and *A. blitoides* from Ganot and Sorek are cross-resistant to most ALS inhibitors, and that *A. blitoides* from Ganot and Sorek are also multiple-resistant to atrazine.

The resistance is based on an altered target site as exhibited by the response of SuR/TR populations to all ALS inhibitors and atrazine tested. In spite of the fact that the plants were exposed to similar intensity of selection pressure, the pattern of cross-resistance varied within and between the *Amaranthus* species. The *als* gene mutation, a substitution of pro 188 of domain A by leu in *A. retroflexus* resulted in resistance to all four herbicide groups tested. However, a mutation at the same location in *A. blitoides* resulted in exchange of pro188 to ser conferred resistance to three herbicide groups only but not to imidazolinones. Furthermore, two forms of resistant plants were identified within the Ganot population of *A. blitoides.* Approximately 50% of the plants (SuRA/TR) carried mutation in domain A (pro188 to ser) and the other 50%, (SuRB/TR) were mutated in domain B (trp 569 to leu). These differences result in a different pattern of cross resistance.

It should be noted that in spite of the similar selection pressure imposed on *A. blitoides* in the two sites (Ganot and Sorek), no resistance to imidazolinones (SuRB/TR) was found in Sorek.

The fact that the resistance evolved shortly after the introduction of sulfometuron into the market suggests that the initial frequency of both resistant mutants is relatively high. The process was facilitated by the lack of herbicide rotation, herbicide residual effect and a single mode of action.

We assume that multiple resistance has evolved gradually, first the *A. blitoides* acquired resistance to triazines (Benyamini *et al*. 1991), before sulfometuron was registered in Israel, followed by the evolution of the resistance to ALS inhibitors. The multiple resistance as well as the different pattern in cross resistance may complicate the planning of a rational resistance management, which is further complicated by the reduced ecological fitness associated with the triazine-resistance (Sibony and Rubin 2002).

ACKNOWLEDGMENTS

This study was supported by the Deutsche Forschungsgemeinschaft, as a part of the Trilateral German-Israeli-Palestinian Research Program: Alternative Practices in Near East Agriculture for Environmental Conservation and by the Chief Scientist Fund of the Ministry of Agriculture, Israel.

REFERENCES

Benyamini, Y., Schonfeld, M. and Rubin, B. (1991). Kinetics of chlorophyll fluorescence decay in triazine-resistant and -susceptible weeds. Brighton Crop Protection – Weeds 3, 1103-1110.

Bernatzky, R. and Tanksley, D. (1986). Methods for detection of single or low copy sequences in tomato on Southern blots. *Plant Molecular Biology and Reproduction* 4, 37-41.

Devine, M.D. and Eberlein, C.V. (1997). Physiological, biochemical and molecular aspects of herbicide resistance based on altered target sites. *In* 'Herbicide activity: toxicology, biochemistry and molecular biology,' eds R.M. Roe, J.D. Burton and R.J. Kuhr, pp. 159-186. (IOS Press, Amsterdam, The Netherlands).

Foes, M.J., Liu, L.X., Tranel, P.J., Wax, L.M. and Stoller, E.W. (1998). A biotype of common waterhemp (*Amaranthus rudis*) resistant to triazine and ALS herbicides. *Weed Science* 46, 514-520.

Gronwald, J.W. (1994). Resistance to photosystem II inhibiting herbicides. *In*. 'Herbicide resistance in plants: biology and biochemistry,' eds S.B. Powles and J.A.M. Holtum, pp. 27-60. (Lewis Publishers, Boca Raton, Florida, USA).

Heap, I. (2002) International survey of herbicide resistant weeds. *Annual Report Internet*. http: /www.weedscience.com/.

Hinz, J.R.R. and Owen, M.D.K. (1997). Acetolactate synthase resistance in a common waterhemp (*Amaranthus rudis*) population. *Weed Technology* 11, 13-18.

Hirschberg, J. and McIntosh, L. (1983). Molecular basis of herbicide resistance in *Amaranthus hybridus*. *Science* 222, 1346-1349.

Lior, E., Sibony, M., Kigel, J. and Rubin, B. (2000). Polymorphism in the resistance of *Plantago lagopus* to herbicides. *Weed Research* 40, 457-466.

Manley, B.S., Singh, B.K., Shaner, D.L. and Wilson, H.P. (1999). Imidazolinone resistance in smooth pigweed (*Amaranthus hybridus*) is due to an altered acetolactate synthase. *Weed Technology* 13, 697-705.

Ray, T.B. (1984). Site of action of chlorsulfuron inhibition of valine and isoleucine biosynthesis in plants. *Plant Physiology* 75, 827-831.

Rubin, B. (1996). Herbicide-resistant weeds – the inevitable phenomenon: mechanisms, distribution and significance. *Zeit Pflanzenkrank Pflantzenschutz* XV, 17-32.

Saari, L.L., Cotterman, J.C. and Thill, D.C. (1994). Resistance to acetolactate synthase-inhibitor herbicides. *In* 'Herbicide resistance in plants: biology and biochemistry,' eds S.B. Powles and J.A.M. Holtum, pp. 83-139. (Lewis Publishers, Boca Raton, Florida, USA).

Sibony, M. and Rubin, B. (1996). Multiple resistance to ALS inhibitors and triazine herbicides in *Amaranthus* species. Proceedings of the International Symposium on Weed and Crop Resistance to Herbicides, Cordoba, Spain, pp. 112-114.

Sibony, M. and Rubin, B. (2002). The ecological fitness of *Amaranthus retroflexus* and *A. blitoides* resistant to acetolactate synthase (ALS) inhibitors and atrazine. Proceedings of the 13th Australian Weeds Conference, Perth.

Sibony, M., Michel, A., Haas, H.U., Rubin, B. and Hurle, K. (2001). Sulfometuron-resistant *Amaranthus retroflexus*: Cross-resistance and molecular basis for resistance to acetolactate synthase (ALS) inhibiting herbicides. *Weed Research* 41, 509-522.

Sprague, C.L., Stoller, E.W., Wax, L.M. and Horak, M.J. (1997). Palmer amaranth (*Amaranthus palmeri*) and common waterhemp (*Amaranthus rudis*) resistance to selected ALS-inhibiting herbicides. *Weed Science* 45, 192-197.

Stankiewicz, M., Gadamski, G. and Gawronski, S.W. (2001). Genetic variation and phylogenetic relationships of triazine-resistant and triazine susceptible biotypes of *Solanum nigrum* – analysis using RAPD markers. *Weed Research* 41, 287-300.

Streibig, J.C. (1988). Herbicide bioassay. *Weed Research* 28, 479-484.

Wetzel, D.K., Horak, M.J., Skinner, D.Z. and Kulakow, P.A. (1999). Transferal of herbicide resistance traits from *Amaranthus palmeri* to *Amaranthus rudis*. *Weed Science* 47, 538-543.

Woodworth, A.R., Rosen, B.A. and Bernasconi, P. (1996). Broad range resistance to herbicides targeting acetolactate synthase (ALS) in a field isolate of *Amaranthus* sp. is conferred by a Trp to Leu mutation in ALS gene. *Plant Physiology* 111, 1353.

Yaacoby, T., Schonfeld, M. and Rubin, B. (1986). Characcteristics of atrazine – resistant biotypes of three grass weeds. *Weed Science* 34, 181-184.