JOURNAL OF PLANT PROTECTION RESEARCH Vol. 51, No. 2 (2011)

DOI: 10.2478/v10045-011-0022-z

MOLECULAR BASIS OF BLACKGRASS (*ALOPECURUS MYOSUROIDES* HUDS.) RESISTANCE TO SULFONYLUREA HERBICIDES

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Received: August 24, 2010
Accepted: February 4, 2011

Abstract: Blackgrass biotype resistant to the mesosulfuron + iodosulfuron mixture has been found in Poland and was investigated in this study. Seedlings that survived double-dosed herbicide treatment were submitted to molecular analysis in order to explain the mechanism of resistance. Domains A and B of the acetolactate synthase gene were amplified by PCR and then sequenced. Biotypes which were both resistant and susceptible to mesosulfuron+iodosulfuron were analyzed. The comparison of the obtained sequences was made on a nucleotide and aminoacid level. The comparison revealed a substitution of proline codon to histidine codon in position 197 in each resistant plant. Mutation Pro197His is the basis of the target site resistance of *A. myosuroides* to sulfonylureas. There were no other mutations in *als* gene of the biotype that might modify the level of resistance to these herbicides.

Key words: Acetolactate synthase (ALS) inhibitors, iodosulfuron, mesosulfuron, mutations, Pro197, resistance, *Alopecurus myosuroides*

INTRODUCTION

Chemical weed management is efficient and economically reasonable. There are negative sides though, one of which is the selection of herbicide resistant weeds. The first case of resistance was found in USA in 1968: *Senecio vulgaris* L. had become tolerant to triazines (Ryan 1970). Since then, 193 weed species have developed biotypes resistant to those herbicides having diverse modes of action (International Survey of Herbicide Resistant Weeds 2010a). The occurrence of a resistant plant is a result of two major phenomena: 1) DNA mutations which underpin the natural variability of organisms, and 2) Selection by constant use of the same mode-of-action herbicides.

There are two major types of herbicide resistance. Target site resistance is usually based on a single gene mutation, which encodes the enzyme that is the herbicide's target. Substitutions in the aminoacid sequence alter the protein structure in a way which prevents the inhibitor molecule to bind with the enzyme. This kind of resistance was already observed in blackgrass (Délye and Boucansaud 2008), as well as in other monocot and dicot weeds (Tranel *et al*. 2010). Biotype can gain the metabolic type of resistance, which is a potential for rapid xenobiotic detoxication, *e.g*. by enhanced activity of cytochrome P450 (Siminszky 2006). The two mechanisms are separate and independent, but may be found in one biotype simultaneously (Burnet *et al.* 1994).

Acetolactate synthase (ALS) inhibitors are one of the world's most important herbicides used for protection of cereals. They are mainly represented by sulfonylureas in Poland. Selectivity, low toxicity, and high biological efficacy with use of very small doses underlies the market success of herbicides from group B (singled out by Herbicide Resistance Action Committee – HRAC). However, ALS inhibitors are also the most resistance-prone herbicide group known. Up till now, 107 weed species worldwide are known to have developed biotypes resistant to ALS inhibitors (International Survey of Herbicide Resistant Weeds 2010a). Four species with resistance to HRAC B herbicides were found in Poland: *Conyza canadensis* L. (International Survey of Herbicide Resistant Weeds 2010b), *Centaurea cyanus* (Rola and Marczewska 2002), *Apera spica-venti* L. (Rola and Marczewska 2002; Krysiak *et al.* 2007) and *Alopecurus myosuroides* Huds.

Blackgrass is a troublesome weed in cereal crops. Its intensive infestation may cause a decrease in yield, reaching 45% (Moss 1987). *A. myosuroides* resistance to sulfonylureas is a dangerous phenomenon, because this group of herbicides is highly effective in controlling blackgrass (Domaradzki and Rola 2006).

The target site resistance may be detected by molecular analyses that focus on DNA mutations in the gene encoding the target protein. The simplest way is the partial amplification and sequencing of the gene. If the mutations conferring resistance are determined, molecular marker-based approaches may be useful for high throughput tests. In Cleaved Amplified Polymorphic Sequences (CAPS) and dCAPS (derived CAPS) marker

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techniques, restriction endonucleases cleave the amplicon depending on the occurrence of the mutation that confers resistance (Kaundun and Windass 2006, Yu *et al.* 2008). Mutations in resistant plants can also be detected by allele-specific polymerase chain reaction, where amplicon is produced only for resistant or wild-type sequence (Delyé *et al.* 2002). For ALS inhibitor resistance, it is crucial to analyze the sequences of two regions in *als* gene: domain A, covering codons from 122 to 205, and domain B with codons 574–653 (numbering of codons and aminoacids is standardized to the precursor of ALS from *Arabidopsis thaliana*). Mutations that confer resistance to ALS inhibitors are most likely to be localized in these two domains (Gressel 2002; Tranel and Wright 2002).

The research described in this paper focused on identifying the molecular basis for resistance of blackgrass biotype to sulfonylurea herbicides.

MATERIALS AND METHODS

Plant material

Two biotypes of blackgrass were investigated. Biotype "R" was found in 2007 (Bieganów, Lubuskie voivodship) in a field where winter wheat was cultivated in monoculture. For weed management, urea and sulfonylurea herbicides were applied regularly. In the Spring of 2007, mesosulfuron + iodosulfuron (Atlantis 04 WG) and iodosulfuron + amidosufluron (Sekator 6.25) WG were applied timely and properly in recommended doses. *A. myosuroides* survived the treatment, while *Apera spica-venti* and broadleaf weeds did not. Up to 2007, a growing lack of control of *A. myosuroides* by sulfonylureas had been observed. The resistance of biotype "R" to mesosulfuron + iodosulfuron was confirmed in greenhouse experiments, where 33.8 g/ha of mesosulfuron + iodosulfuron was required to destroy 50% of plants (data not shown). As the control, susceptible biotype "S" was used, in which 50% of plants were destroyed by mesosulfuron + iodosulfuron at the rate of 6.84 g/ha (data not shown).

Selection of resistant plants

Seeds of "R" and "S" biotypes were sown in separate pots, in turf-based soil substrate, supplemented with micro- and macroelements. After germination, 15 plants per pot were left, and in BBCH 12-14 stage they were submitted to herbicide spraying with mesosulfuron+iodosulfuron mixture (Atlantis 04 WG). The applied dose was 28.8 g active substance (a.s.)/ha (triple registered dose for blackgrass control) with the addition of adjuvant: Olbras 88 EC in a rate of 1.0 l/ha. Plants of "R" and "S" biotype, not treated with herbicide, were used as the control. After 3 weeks, treated plants from "R" biotype, with no phytotoxicity symptoms (*e.g.* inhibited growth, chlorotic or necrotic spots) were harvested separately and freeze-dried for the following molecular analysis. As the control for molecular analysis, untreated plants of biotype "S" were also sampled.

DNA isolation

The total plant DNA was extracted with the use of CTAB according to the Doyle and Doyle and Doyle (1987) method with minor modifications: CTAB concentration 3%, 20 min. of incubation in 65°C of plant material in isolation buffer.

Primers design and amplification of domains A and B

Domains A and B were amplified by PCR. Primers used for the reaction (Table 1) had been previously designed for *als* gene in *A. spica-venti* (Krysiak *et al.* 2009). For domain A PCR reagents concentrations were as follows: 0.8 µM each primer, 25 mM MgCl2 and 0.2 mM dNTP. In each 30 µl reaction mixture, 200 ng of template DNA and 1 U of High Fidelity PCR Enzyme Mix (Fermentas) were used. In order to provide high quality and yield of amplicon, PCR enhancer CES5x was used in a concentration of 20% in the reaction mix (Ralser *et al.* 2006). PCR program consisted of 5 min of initial denaturation in 95°C, followed by 40 cycles of: 1 min in 94°C (denaturation), 30 s in 63°C (annealing) and 30 s in 72°C (DNA elongation). Final elongation lasted 10 minutes in 72°C.

Table 1. Sequences of primers used for PCR-based amplification of *als* gene analyzed fragments

Primers for domain A amplification	
A3-F	AAG GGC GCC GAC ATC CTC
A3-R	CGA GGT AGT TGT GCT TGG TGA
Primers for domain B amplification	
B4-F	CAG GTG TCA CGG TTG TTG AC
B4-R	GCA AAA CAC ATG CTT TAT TAG TTG A

Domain B was amplified in PCR with reagent concentrations: 1 µM of each primer, 25 mM MgCl2, 0.2 mM dNTP. Each 30 µl reaction mixture included 200 ng of template DNA, 20% CES5x and 1 U of High Fidelity PCR Enzyme Mix (Fermentas). PCR started with 5 min in 95°C (initial denaturation), and each of the following 35 cycles consisted of: 1 min in 94°C (denaturation), 30 s in 63°C (annealing) and 40 s in 72°C (elongation). The reaction was finalized by 10 min incubation in 72°C.

The effects of amplification were checked by electrophoresis of 5 µl of PCR product in 1.5% agarose gel stained with ethidium bromide. The rest of the product was purified and sequenced by Genomed Sp. z o.o. in Warsaw, with the use of A3-F and B4-F primers. Nucleotide sequences were translated to aminoacid sequences in the Transeq program and compared in the ClustalW program.

RESULTS

Selection of resistant plants.

Plants of biotype S and "R" differed in their response to the herbicide treatment. All the "S" plants were severely damaged, while plants of biotype "R" showed diverse responses to herbicide treatment: either no injuries were observed, or multiple chlorotic, necrotic spots or/and inhibited growth occurred.

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                                                    His197
R1   CTCCATCCCGATGGTTGCTATCACGGGACAGGTTCACCGCCGCATGATAGGCACGGACGC
R2   CTCCATCCCGATGGTTGCTATCACGGGACAGGTTCACCGCCGCATGATAGGCACGGACGC
R3   CTCCATCCCGATGGTTGCTATCACGGGACAGGTTCACCGCCGCATGATAGGCACGGACGC
R4   CTCCATCCCGATGGTTGCTATCACGGGACAGGTTCACCGCCGCATGATAGGCACGGACGC
R5   CTCCATCCCGATGGTTGCTATCACGGGACAGGTTCACCGCCGCATGATAGGCACGGACGC
S1   CTCCATCCCGATGGTCGCCATCACGGGGCAGGTTCCCCGCCGCATGATAGGCACGGACGC
                                                    Pro197
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Fig. 1. Fragment of comparison of the domain A sequences in *als* gene. R1, R2, R3, R4 and R5: plants from resistant biotype of blackgrass. S1: plant from susceptible biotype. Mutating codons are marked with bold type and grey background. The number above the mutation is the changed aminoacid's position in protein. Below, the aminoacid of susceptible biotype is marked (the numbers are standardized to the numeration of aminoacids of *A. thaliana*'s ALS precursor protein)

Molecular analysis

Primers that had been designed to amplify domains A and B in *A. spica-venti*'s *als* gene, provided successful amplification of the same domains in blackgrass, due to high similarity of the two genes. For biotype "R", 5 amplicons from 5 plants were sequenced; for biotype "S" one plant was analyzed. Alignment of sequences revealed that in domain A, each plant from biotype "R" showed Pro197→His mutation: codon CCC changed into CAC (Fig. 1). Concerning this mutation, plants were homozygous. Comparison of nucleotide and aminoacid sequences of domain B did not reveal any mutations that might have caused blackgrass resistance to sulfonylurea herbicides.

DISCUSSION

Mutation of proline in position 197 in *als* gene was found in each plant from biotype R, that survived herbicide treatment in increased dose. Substitutions in position 197 were previously described for *A. myosuroides* biotypes originated from France (Délye and Boucansaud 2008), as well as in biotypes of other species: *Bromus tectorum* L. (Park and Mallory-Smith 2004), *Hordeum leporinum* (Yu *et al.* 2007), *Schoenoplectus juncoides* var. *Ohwianus* (Uchino *et al.* 2007) and *Lolium rigidum* Gaudin (Yu *et al.* 2008). In each mentioned case, mutation of Pro197 conferred a high level of resistance to sulfonylureas – which corresponds perfectly with the results of the experiment described in this paper.

The results of the research confirm that blackgrass has developed target site resistance to sulfonylureas. *A. myosuroides* tolerance may become a major problem. This troublesome weed causes serious decrease in cereal yield in the moderate climate zone, and at one time it had been possible to control *A. myosuroides* by sulfonylureas spraying.

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POLISH SUMMARY

MOLEKULARNE PODSTAWY ODPORNOŚCI WYCZYŃCA POLNEGO (*ALOPECURUS MYOSUROIDES* HUDS.) NA HERBICYDY SULFONYLOMOCZNIKOWE

Odnaleziono w Polsce biotyp wyczyńca polnego odporny na mieszaninę mezosulfuronu i jodosulfuronu. Siewki traktowano herbicydem w trzykrotnej zalecanej dawce polowej, a rośliny, które wykazały odporność na preparat poddano analizom molekularnym w celu wyjaśnienia mechanizmu odporności. Wykorzystując technikę PCR, namnożono domenę A i B genu syntazy acetylomleczanowej osobników odpornych i wrażliwych na mieszaninę mezosufluronu i jodosulfuronu. Uzyskane produkty PCR następnie zsekwencjonowano. Porównanie sekwencji na poziomie nukleotydowym i aminokwasowym ujawniło zamianę kodonu proliny w pozycji 197 na kodon histydyny w każdym osobniku odpornym. Mutacja Pro197His powoduje odporność typu „w miejscu działania" na pochodne sulfonylomocznikowe. W badanym biotypie nie wykryto innych mutacji genu *als* mogących modyfikować poziom odporności na te preparaty.