

DETECTION OF ACCASE TARGET-SITE RESISTANT *ALOPECURUS MYOSUROIDES* HUDS. (BLACK-GRASS) IN BELGIAN POPULATIONS

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SUMMARY

Black-grass is a common grass weed, widely spread in Northern Europe and also in Belgium. For ages, it has been an increasing problem in industrial crops, especially winter cereals. The first case of resistance in Belgium was reported in 1996 by Robert Bulcke (Eelen *et al.*, 1996). Yet the resistance mechanism was not specified. Since then, no more information was published about the evolution Belgium, while research continued in the United Kingdom and in France. Moreover, during the last decade, progress in molecular biology allowed to highlight the mechanism of target-site resistance. A simple PCR method allows to detect the mutation conferring resistance to herbicide.

After two years of resistance monitoring in Belgium, mostly in the Walloon part, some populations have been clearly identified as highly resistant to ACCase inhibitor. These populations have been tested by molecular biology so as to detect the single nucleotide polymorphism (SNP) involved in this case. The method employed was the Polymerase Chain Reaction Allele Specific Assays (PASA: Délye, 2002a) for the mutation Ile-1781-Leu that confers a target-site resistance to ACCase inhibitors. Those analyses were performed on plant material issued from bioassays, either in glasshouses or in Petri dishes. Leaves have been collected from plants which survived a fenoxaprop-P treatment applied in a glasshouse single dose assay. Seedlings from resistant populations grown in Petri dishes containing either fenoxaprop-P or cycloxydim provided the second type of sample. Ile1781 mutants were discovered within three populations. Each mutant plant was heterozygote. Five of those samples have been sequenced to confirm PASA results and everyone was matching. Moreover, they were all issued from Petri dishes containing cycloxydim, known to be unaffected by enhanced metabolism, confirming that these populations are indeed target-site resistant.

Key words: Black-grass, ACCase, Target-Site Mutation

INTRODUCTION

The repetitive use of herbicides induces a modification in the weed flora of the cultivated fields. Furthermore, it can lead to a selection of the most resistant plants within the original population. Year in, year out, the herbicide treatment turn out to be inefficient. The first cases of resistant weeds have been reported since 1972 (Heap I, 2009) for triazines and since 1982 for ACCase inhibitors. Today, 189 weed species are listed, among them 113 dicots and 79 monocots. The first case of resistant black-grass in Belgium was published in 1996 by R. Bulcke (Eelen *et al.*, 1996). Two main resistance mechanisms have been reported: enhanced metabolism and target-site resistance. Yet, it is important to distinguish cross-resistance, which is a resistance to two or more herbicides involving only one resistance mechanism, from multiple resistance, which results from several resistance mechanisms in the same plant. Contrary to enhanced metabolism resistance (EMR)

that can lead to progressive resistance levels, due to the ability of the plant to metabolise the herbicide, target-site resistance (TSR) induces directly an inefficacy of the herbicide treatment, since the active ingredient cannot fix to its target anymore. Therefore, increasing the herbicide dose is useless in case of TSR. Thus, it is particularly important to identify the resistance mechanism to implement the most appropriate management method to get rid of this problem as soon as possible (Moss *et al.*, 2007).

Rapid and simple DNA methods have already been developed and published by C. Délye *et al.* (2002a) and Kaundun *et al.* (2006) to detect ACCase inhibitors mutations. They worked respectively on French and English grass weeds populations (*Alopecurus myosuroides* HUDS., *Lolium spp.*, *Avena fatua* L., ...). The aim of this study was to implement one of these methods in our lab at the Pesticides Research Department of the Walloon Agricultural Research Centre (CRA-W) and try to detect one of the ACCase gene mutations in Belgian populations collected and tested within the framework of a large-scale monitoring in Belgium. In a way to confirm the results obtained with the rapid PCR method, sequencing was performed on some samples at the Biotechnology Department of the CRA-W.

MATERIAL AND METHODS

PCR Allele-Specific Assay

The PCR Allele-Specific Assay has been developed to detect the presence of one or two mutant alleles at the 1781¹ position of the ACCase gene, in one individual (Délye *et al.*, 2002a). Reaction conditions were transposed from this paper. The specificity of PASA conducted on blackgrass plantlets was verified by sequencing DNA samples, which had been found to be "A/A", "A/T", "T/T", "C/C" and "A/C" (No "T/C" was found). Sequencing was performed on five plantlets to confirm the obtained results.

DNA extraction was performed with a fast extraction kit from the brand OmegaBio-tek² (*E.Z.N.A. Plant DNA Extraction Mini Kit*). The foliage material was either fresh or frozen. Some leaves had been conserved after a glasshouse bioassay and frozen for further analyses. These leaves came from plantlets which had resisted a fenoxaprop-P spraying.

To perform PCR, we used the primers described by C. Délye in his paper published in 2002a. We ordered them from IDTDNA (Leuven, Belgium). They are presented at Table 1, with Délye's name and the whole sequence. The DNA polymerase was the *GoTaq[®] Hot Start Polymerase*, from PROMEGA (Leiden, Nederland), with a colored 5x concentrated reaction buffer (*5x Green GoTaq[®] Reaction Buffer*), containing 7,5mM MgCl₂, which gives a final concentration of 1,5 mM. A mix of deoxynucleotides (dNTP), with total concentration of 10 mM, was provided by VWR (Leuven, Belgium).

The PCR cycle consisted in a first denaturing step of 3 minutes at 95°C, followed by 37 cycles of denaturation (15s, 94°C), primers hybridation (30s, 63°C), elongation (60s, 72°C) and a final step of elongation during 10 minutes at 72°C. The brand of the thermocycler we used is VWR (UnoCycleur, 96 puits).

The migration of the PCR product containing amplified fragments was performed on agarose 1,5%, in TAE 1x buffer, in horizontal mini-cuves, from VWR, with a 80V voltage for small gels (10x8 cm, 12 lanes) and 110 V for bigger gels (16x15 cm, 32 lanes). The molecular marker was a 100pb DNA ladder, from APPLICHEM (Darmstadt, Germany). The gel was stained after the electrophoresis (*post-staining*) in a

GelRed 3x solution⁴ (BIOTIUM, Hayward CA, USA). Then, the visualization was performed with the gel documentation system GenoSmart from the brand VWR, in combination with a UV transilluminator at a wavelength of 312 nm.

Sequencing

DNA samples previously tested by PCR and which seemed to present a mutation were sequenced so as to verify the accuracy of the method and to confirm the obtained results. We performed these analyses at the Biotechnological Department of the Gembloux Agricultural Research Centre, equipped with a LI-COR sequencer. DNA was specifically amplified with the previously used primers, ACIL 1-3R for A alleles, ACIL 2C/T-4R for alleles C or T. PCR amplified fragments were separated with an agarose gel electrophoresis (TBE 1x; 1.5% agarose). Five DNA samples were tested and 8 fragments were obtained, because there were 3 heterozygote (A/T and A/C). Agarose bands containing the DNA fragments were cut out of the gel and then purified with an *Agarose Gel Extraction DNA Kit* (ROCHE, Vilvoorde, Belgium). The DNA presence was tested with a short gel migration. Then, the fragments of 330 or 508 bp were ligated to a plasmid vector: *pJET 1.2/Blunt*, thanks to a *CloneJET PCR Cloning Kit* (FERMENTAS, St. Leon-Rot, Germany). The ligation mixture containing the plasmid insert was transferred in a competent bacterial culture, *E. coli* DH5a, by "Heat-shock". These bacteria were grown on a "LB" medium at 37°C overnight (14h), in Petri dishes.

The next day, a bacterial colony has been inoculated in LB medium, also containing Ampicilline (100mg) in each tube. Six colony of each bacterial mix were transferred in those tubes. Then these 48 tubes were put in growing up at 24°C during 24h with shaking. This step allows selecting only the recombinant clones, since only the bacteria carrying the insert may grow in the medium added with antibiotic. A PCR was performed on this mix with *pJET* plasmid specific primer in order to check the presence of the plasmid and so of the DNA fragments.

According to the PCR results, two colonies per fragment were chosen and the plasmid was extracted from the bacterial colony using an extraction kit (*GeneJET Plasmid Miniprep Kit* FERMENTAS). A PCR was performed with the cloned insert, a forward primer tagged with an IRD 800 Fluorochrom, TaqPolymerase, dNTP and ddNTP (FERMENTAS). Then, after a short step of denaturation, the sequencing was achieved with the LiCor sequencer device (LICOR Biosciences, Bad Homburg, Germany) and the *Thermo Sequenase DYEnamic Direct Cycle Sequencing Kit* (AMERSHAM Biosciences, Otelfingen, Switzerland). Those sequencing reactions were separated with a PolyAcrylamid Gel Electrophoresis (PAGE). The obtained sequences were analysed with the software eSeq and then compared between each of them with the software DNASIS.

Table 1. List of primers used for PASA assays

Name	Délye's Name	Sequence 5'-3'	Position 1 st base	Length in bases	Annealing Temp. T _m
ACIL 1	ACVRG1	aat ggg tcg tgg ggc act cct ata att cc	5023	29	88
ACIL 2T	VRDIT	tgg act agg tgt gga gaa ct	5478	20	60
ACIL 2C	VRDIC+	tgg act agg tgt gga gaa cc	5478	20	62
ACIL 3AR	VSDIR	caa tag cag cac ttc cat gta t	5531	22	62
ACIL 3TR	VRDITR	caa tag cag cac ttc cat gta a	5531	22	62
ACIL 4R	ACVRG1R	gct gag cca cct caa tat att aga aac acc	5808	30	86
Distance between primers					
1 – 4R : 785 pb		1 – 3R : 508 pb		2 – 4R : 330 pb	

DISCUSSION

Primers are presented at Figure 1 in a linear way, with the different combinations used for the PASA, giving different sizes of PCR fragments. The forward primer 1 and reverse 4 fix at a fairly long distance from the mutation site, without specificity towards the allele, and they give a 765 base pair fragment. The reverse primers 3AR and 3TR fix downstream from the mutation, the last 5' base of the primer being respectively a T or an A, to be able to hybridize on an A individual (susceptible) or a T individual (resistant). Together with the primer 1 they give a 508 bp fragment. Then, the forward primers 2C and 2T, fix just upstream from the mutation, the last 3' base of the primer being voluntarily modified in order to fix respectively to a C or a T. These two primers only fix on a mutant individual and thus amplify a 330 bp fragment, together with the reverse primer 4.

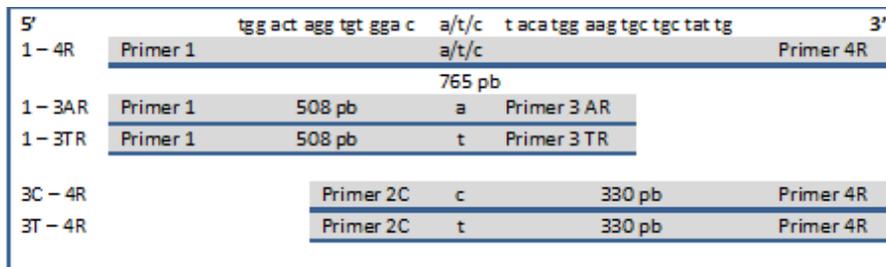


Figure 1. Linear representation of Primers around the mutation point

According to the genotype and the two pairs of primers used for the PCR amplification, 3 type of bands can be revealed. The correspondence between the electrophoresis results and the genotype is presented at Table 2. When observing the results of two reactions, it is possible to determine precisely the genotype.

Table 2. PCR with the 3 primers combination and the corresponding genotypes and phenotypes

PCR Result	Fragments size (bp)	Genotype	Phenotype
PCR A : ACIL 1 – ACIL 2C – ACIL 3TR – ACIL 4R			
1 Band	765	A/A	Susceptible A
2 Bands	765 + 508	A/T + T/T	Resistant Mutant T
2 Bands	765 + 330	A/C + C/C	Resistant Mutant C
3 Bands	765 + 508 + 330	T/C	Resistant Heterozygote T/C
PCR B : ACIL 1 – ACIL 2T – ACIL 3AR – ACIL 4R			
1 Band	765	C/C	Resistant Mutant C
2 Bands	765 + 508	A/A + A/C	
2 Bands	765 + 330	T/T + T/C	Resistant Mutant T/C
3 Bands	765 + 508 + 330	A/T	Resistant Heterozygote A/T
PCR C : ACIL 1 – ACIL 2C – ACIL 3AR – ACIL 4R			
1 Band	765	T/T	Resistant Mutant T
2 Bands	765 + 508	A/A + A/T	
2 Bands	765 + 330	C/C + T/C	Resistant Mutant T/C
3 Bands	765 + 508 + 330	A/C	Resistant Heterozygote A/C

RESULTS

PCR Allele Specific Assay

DNA was extracted from 51 plantlets. '1781' mutation was discovered in 3 populations coming from different regions. Population 14, coming from Yves-Gomezée, in the south of Namur province, presents at least one copy of the mutant allele, changed from Adenine to Thymine at the 5341 position in the nucleotide sequence. Population 69, harvested in weeding trials in Slijpe, in the Polders region, contains some individuals carrying the mutation of Adenine to Cytosine. Population 71, coming from weeding trials in Pecq, nearby Tournai region, also contains some mutant individuals from Adenine to Thymine. Those results were confirmed by sequencing. It's important to note that every mutant resisted the cycloxydim treatment in Petri Dishes. This confirms the hypothesis that the resistance is present historically in a weak proportion of the population and this is the herbicide use that reveals it. For each of these 3 populations, plantlets issued from the untreated Petri dishes, those containing fenoxaprop-P, were tested but none of them was carrying the mutation. Moreover, since most of the mutants are heterozygote, we can suppose that the mutation is not yet fixed in the population.

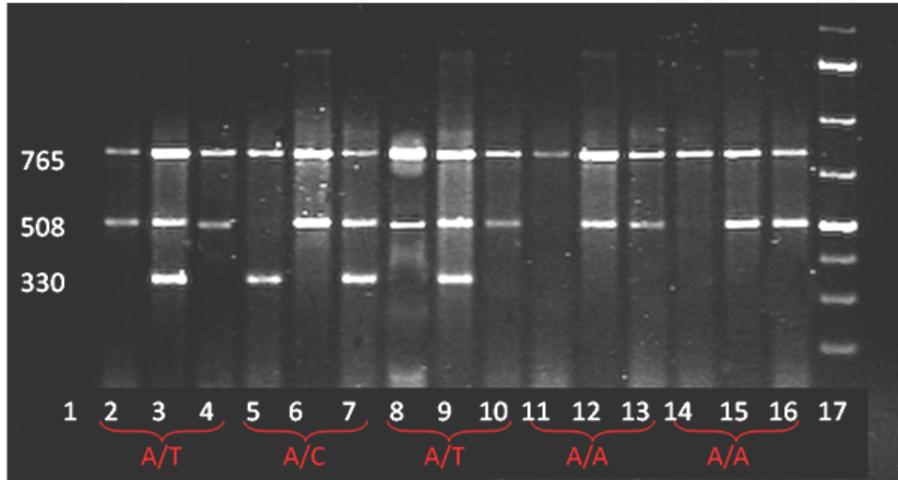


Figure 2. Photography of the agarose gel. The 5 DNA samples sequenced are here presented

Lane 1	Nil (No DNA);
Lane 2-4	Sample N° 14 F (A/T), Yves-Gomezée, PCR A,B,C;
Lane 5-7	Sample N° 69F (A/C), Slijpe, PCR A,B,C;
Lane 8-10	Sample N° 71F (A/T), Pecq, PCR A,B,C;
Lane 11-13	Sample N° 69T (A/A), Slijpe, PCR A,B,C;
Lane 14-16	Sample N° 70P (A/A), Pecq, PCR A,B,C;
Lane 17	DNA Ladder 100pb

Sequencing

The first PCR was performed to amplify the fragments flanking the mutation, with the same primers as for the PASA, but one pair of primers at a time. We only needed to amplify one type of fragment, carrying the mutation. Each DNA sample was put in reaction with the 1-3R primers (508 pb) to amplify the wild-type A allele fragment and in another PCR tube, with the 2T- or 2C-4R primers (330 pb), which can fix only to a T or C mutant allele, respectively. An agarose gel electrophoresis was carried on to separate the amplicons. For both the A/A samples, this reaction did not give any strip, which confirms that they are susceptible. From the five DNA samples, 8 amplicons were obtained, extracted from the gel and renamed:

1 = 14F A; 2 = 14F T; 3 = 69F A; 4 = 69F C; 5 = 71F A; 6 = 71F T; 7 = 69T2 A; 8 = 70 P A.

Every further handling is detailed in the previous section. The results obtained for the sequencing with two replicates per fragment are presented at Figure 3 for wild-type fragments and at Figure 4 for mutant T or C alleles. PCR allele-specific amplification gave the same results, shown on the gel presented at Figure 2.

71. SEQ	401	GCAGCTAGAT	AGTGGCGAGA	ACAGGTGGGT	TATCGATTCT	ATTGTGGG	450
72. SEQ	401	GCAGCTAGAT	AGTGGCGAGA	ACAGGTGGGT	TATCGATTCT	ATTGTGGG	450
81. SEQ	401	GCAGCTAGAT	AGTGGCGAGA	ACAGGTGGGT	TATCGATTCT	ATTGTGGG	450
83. SEQ	401	GCAGCTAGAT	AGTGGCGAGA	ACAGGTGGGT	TATCGATTCT	ATTGTGGG	450
		460	470	480	490	500	
11. SEQ	451	AAAGGATGG	ACTAGGTGTG	GAGAACTAG	ATGGAACTGG	TGCTATTG	500
12. SEQ	451	AAAGGATGG	ACTAGGTGTG	GAGAACTAG	ATGGAACTGG	TGCTATTG	500
31. SEQ	451	AAAGGATGG	ACTAGGTGTG	GAGAACTAG	ATGGAACTGG	TGCTATTG	500
32. SEQ	451	AAAGGATGG	ACTAGGTGTG	GAGAACTAG	ATGGAACTGG	TGCTATTG	500
51. SEQ	451	AAAGGATGG	ACTAGGTGTG	GAGAACTAG	ATGGAACTGG	TGCTATTG	500
52. SEQ	451	AAAGGATGG	ACTAGGTGTG	GAGAACTAG	ATGGAACTGG	TGCTATTG	500
71. SEQ	451	AAAGGATGG	ACTAGGTGTG	GAGAACTAG	ATGGAACTGG	TGCTATTG	500
72. SEQ	451	-----	-----	-----	-----	-----	500
81. SEQ	451	AAAGGATGG	ACTAGGTGTG	GAGAACTAG	ATGGAACTGG	TGCTATTG	500
83. SEQ	451	AAAGGATGG	ACTAGGTGTG	GAGAACTAG	ATGGAACTGG	TGCTATTG	500
		510	520	530	540	550	

Figure 3. Extract of the sequence of wild-type fragments: 11-12, 2 replicates of Sample 14 F; 31-32, 2 replicates of sample 69 F; 51-52, 2 replicates of Sample 71 F; 71-72, 2 replicates of Sample 69 T; 81-83, 2 replicates of Sample 70 P. 5341 nucleotide wild-type A is squared in red. Comparison made with DNASIS software.

Size	333 bp	Select	1					
			10	20	30	40	50	
21. SEQ	1	TGGACTAGGT	GTGGAGAACT	TACATGGAAG	TGCTGCTATT	GCCAGTGCCT	50	
22. SEQ	1	TGGACTAGGT	GTGGAGAACT	TACATGGAAG	TGCTGCTATT	GCCAGTGCCT	50	
41. SEQ	1	TGGACTAGGT	GTGGAGAACT	TACATGGAAG	TGCTGCTATT	GCCAGTGCCT	50	
42. SEQ	1	TGGACTAGGT	GTGGAGAACT	TACATGGAAG	TGCTGCTATT	GCCAGTGCCT	50	
61. SEQ	1	TGGACTAGGT	GTGGAGAACT	TACATGGAAG	TGCTGCTATT	GCCAGTGCCT	50	
62. SEQ	1	TGGACTAGGT	GTGGAGAACT	TACATGGAAG	TGCTGCTATT	GCCAGTGCCT	50	
			60	70	80	90	100	
21. SEQ	51	ATTCTAGGGC	GTACGAGGAG	ACATTTACAC	TTACATTTCGT	TACTGGACGA	100	
22. SEQ	51	ATTCTAGGGC	GTACGAGGAG	ACATTTACAC	TTACATTTCGT	TACTGGACGA	100	
41. SEQ	51	ATTCTAGGGC	GTACGAGGAG	ACATTTACAC	TTACATTTCGT	TACTGGACGA	100	
42. SEQ	51	ATTCTAGGGC	GTACGAGGAG	ACATTTACAC	TTACATTTCGT	TACTGGACGA	100	
61. SEQ	51	ATTCTAGGGC	GTACGAGGAG	ACATTTACAC	TTACATTTCGT	TACTGGACGA	100	
62. SEQ	51	ATTCTAGGGC	GTACGAGGAG	ACATTTACAC	TTACATTTCGT	TACTGGACGA	100	
			110	120	130	140	150	

Figure 4. Extract of the sequence of mutant fragments: 21-22, 2 replicates of Sample 14 F; 41-42, 2 replicates of Sample 69 F; 61-62, 2 replicates of Sample 71 F. Mismatching nucleotide is squared in red. Comparison made with DNASIS software.

CONCLUSION

Délye's method, described in his paper (2002 a), has been successfully transposed to our lab in Gembloux. The 1781 mutation of the ACCase gene has been discovered in three Belgian Blackgrass populations. Those populations are located in three different locations: the Polders, and in the South Western and the South Centre part of the Walloon Region. Both substitutions of the 5341 allele have been detected. Only heterozygote mutants have been discovered in those samples.

ACKNOWLEDGMENTS

This work was part of my Master Thesis, co-realised with the Gembloux Agricultural University (FUSAGx) and the Walloon Agricultural Research Centre (CRA-W). Many thanks to Dr. M. De Proft, director of the Pesticides Department of the CRA-W and Ir. G. Jacquemin, scientists from the CRA-W, for providing with some data and materials, and for their help to implement the analyses.

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FOOTNOTES

- ¹ Numbering based on the *Alopecurus myosuroides* chloroplastic ACCase gene sequence published at NCBI GenBank, accession number: AJ 310767
<http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=20975573>
- ² <http://www.omegabiotek.com/genomicplantmini.asp>
- ³ http://www.applichem.com/fileadmin/produktinfo/a3470_de.pdf
- ⁴ http://www.biotium.com/product/product_info/Protocol/PI-41003.pdf