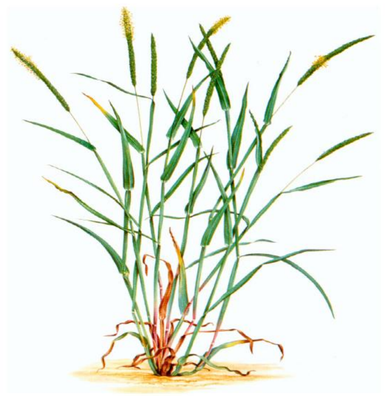
Detection of ACCase Target-Site Resistant *Alopecurus myosuroides* HUDS. (Black-Grass) in Belgian populations



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Abstract

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's Team (Eelen *et al.,* 1996). Yet the resistance mechanism was not specified. Since then, no more information was published about 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 theses populations are indeed target-site resistant.

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 1781[1] 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 OmegaBiotek[2] (*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 the IDT DNA[3] society. 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[4], with a colored 5x concentrated reaction buffer (*5x Green GoTaq® Reaction Buffer*), containing 7,5mM $MgCl_2$, which gives a final concentration of 1,5 mM. A mix of deoxynucleotides (dNTP), with total concentration of 10 mM, was provided by VWR[5].

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 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[6].
The gel was stained after the electrophoresis (*post-staining*) in a GelRed 3x solution[7]. 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.

Table 1: List of primers used for PASA assays.

Name	Délye's Name	Sequence 5'-3'	Position 1st 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 voluntary 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.
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.

5'		tgg act agg tgt gga c	a/t/c	t aca tgg aag tgc tgc tat tg	3'
1 – 4R	Primer 1		a/t/c		Primer 4R
			765 bp		
1 – 3AR	Primer 1	508 bp	a	Amorce 3R	
1 – 3TR	Primer 1	508 bp	t	Amorce 3R	
2C – 4		Primer 2	c	330 bp	Primer 4R
2T – 4		Primer 2	t	330 bp	Primer 4R

Figure 1: Linear reprensentation of Primer around the mutation point.

Table1: PCR with the 3 primers combination and the corresponding genotypes and phenotypes

PCR Results	Fragment Size	Genotype	Phenotype
PCR Reaction 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 mutated T
2 Bands	765 + 330	A/C + C/C	Resistant mutated C
3 Bands	765 + 508 + 330	T/C	Resistant heterozygote T/C
PCR Reaction B : ACIL 1 – ACIL 2T – ACIL 3AR – ACIL 4R			
1 Band	765	C/C	Resistant mutated C
2 Bands	765 + 508	A/A + A/C	
2 Bands	765 + 330	T/T + T/C	Resistant
3 Bands	765 + 508 + 330	A/T	Resistant heterozygote A/T
PCR Reaction C : ACIL 1 – ACIL 2C – ACIL 3AR – ACIL 4R			
1 Band	765	T/T	Resistant mutated T
2 Bands	765 + 508	A/A + A/T	
2 Bands	765 + 330	C/C + T/C	Resistant
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 regions, contains some individuals carrying the mutation of Adenine to Cytosine. Population 71, coming from weeding trials in Pecq, in the 'Tournaisis' 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, I also tested plantlets issued from the untreated Petri dishes, those containing fenoxaprop-P , 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.

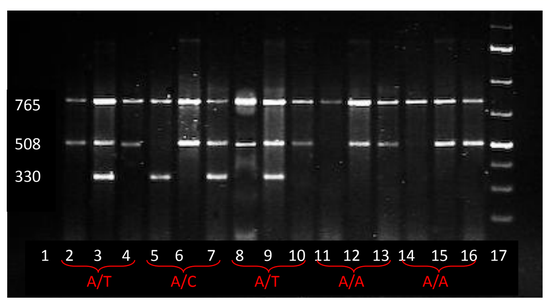

Figure 2: Photography of the agarose gel. The 5 DNA samples sequenced are here presented.
Lane 1 : Nil (No DNA) ;
Lane 2-4, Sample N°14 F (A/T), Yves-Gomezée, PCR A, B, C ;
Lane 5-7, Sample N° 69F (A/C), Slijpe, PCR A, B, C ;
Lane 8-10, Sample N° 71F (A/T), Pecq,¨ PCR A, B, C ;
Lane 11-13, Sample N° 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

MATERIAL AND METHODS

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 Center, 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[8]). The DNA presence was tested with a short gel migration. Then, the fragments of 330 or 508 bp was ligated to a plasmid vector : *pJET 1.2/Blunt*, thanks to a *CloneJET PCR Cloning Kit* (FERMENTAS[9]). 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 [10]) and the *Thermo Sequenase DYEnamic Direct Cycle Sequencing Kit* (AMERSHAM Biosciences [11]). 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.

RESULTS

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 (508pb) to amplify the wild-type A allele fragment and in another PCR tube, with the 2T- or 2C-4R primers (330pb), 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.

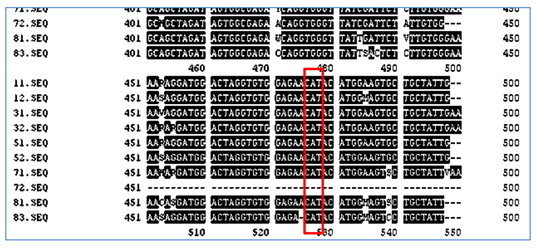

Figure 3 : Extract of the sequence of wild-type fragments :
11-12, 2 replicates of Sample 14 F ; 31-32, 2 replicates of Sample 69 F; 51-52, 2 replicates of Sample 71 F ; 71-72, 2 replicates of Sample 69 T ; 81-83, 2 replicates of Sample 70 P.
5341 nucleotide wild-type A is squared in red. Comparison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

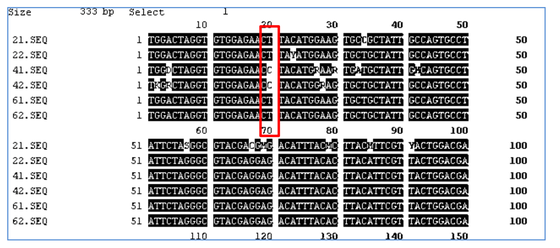

Figure 4 : Extract of the sequence of mutant fragments :
21-22, 2 replicates of Sample 14 F ; 41-42, 2 replicates of Sample 69 F;
61-62, 2 replicates of Sample 71 F
Mismatching nucleotide is squared in red. Comparison made with DNASIS software.

CONCLUSION

We can say that 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, "Tournaisis" (South Western) and South Center part of the Walloon Region. Both substitutions of the 5341 allele have been detected. Only heterozygote mutants have been discovered in those samples.

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Délye C, Wang T, Darmency H (2002b) An isoleucine-leucine substitution in chloroplastic acetyl-Co A carboxylase from green foxtail (*Setaria viridis* L. Beauv.) is responsible for resistance to the cyclohexanedione herbicide sethoxydim. *Planta* **214**: 421–427
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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=nuccore&id=20975573
[2]http://www.omegabiotek.com/genomicplantmini.asp
[3]Integrated DNA Technologies, Interleuvenlaan 12A B-3001 Leuven. http://www.idtdna.com
[4]Promega Benelux Schipholweg 1 PO Box 391 2300 AJ Leiden http://www.promega.com/nl
[5]VWR Belgium Geldenaaksebaan 464 3001 Leuven http://be.vwr.com
[6]AppliChem GmbH Ottoweg 4 D64291 Darmstadt Allemagne http://www.applichem.com/fileadmin/produktinfo/a3470_de.pdf
[7]http://www.biotium.com/product/product_info/Protocol/PI-41003.pdf
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