

Molecular tools for the diagnosis of resistance to herbicides inhibiting acetyl-CoA carboxylase in three grass weeds

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Summary Herbicide resistance patterns associated with the presence of point mutations within chloroplastic acetyl-CoA carboxylase (ACCase) were determined using PCR-based assays in three grass weeds (*Setaria viridis*, *Alopecurus myosuroides* and *Lolium rigidum*). PCR associated with a fast and easy DNA extraction procedure enabled the detection of resistant ACCase alleles to be achieved within one day from any kind of plant material. PCR was successfully used to detect resistant ACCase genes in a field population of *A. myosuroides*. However, PCR do not enable prediction of the herbicides to which the weed populations remain sensitive. Thus, PCR is a highly effective tool for the specific detection of resistance genes, but should be coupled with bioassays to conduct optimal resistance management strategies.

Keywords Point mutation, PCR, black-grass, rye grass, green foxtail, field.

INTRODUCTION

Resistance is a factor that considerably reduces efficacy of herbicides. Thus, managing resistance in weed populations requires rapid and accurate methods to identify resistant biotypes. The most widespread method for detecting herbicide resistance is bioassay, which is also the only method when the resistance genes are unknown. Bioassays generally involve measuring the differences in the development of weed seedlings/young plants exposed to herbicides (e.g. Letouzé and Gasquez 1999). Consequently, bioassays most often require collecting viable weed seeds/plants, are time-consuming, and do not discriminate between different resistance mechanisms conferring similar resistance patterns. Furthermore, bioassays are generally not quick enough to permit an adaptation of the spraying program during the growing season when resistance is detected.

However, when resistance genes have been identified, development of fast and accurate DNA-based diagnosis tools is possible. In particular, where point mutations in genes encoding herbicide target enzymes have been identified, the polymerase chain reaction (PCR) technique can be exploited to detect resistant genotypes. In this work, we considered target

resistance of three major weeds, *Setaria viridis* L. Beauv. (green foxtail), *Alopecurus myosuroides* Huds. (black-grass) and *Lolium rigidum* Gaud. (annual ryegrass), to herbicides inhibiting chloroplastic acetyl-coenzyme A carboxylase (ACCase). ACCase, a vital enzyme in fatty acid biosynthesis, is inhibited by aryloxyphenoxypropionates (APPs) and cyclohexanediones (CHDs).

Although resistance to ACCase inhibitors has been reported since the end of the 1980s, it was only recently that an isoleucine-leucine substitution at codon 1781, within ACCase carboxyl-transferase (CT) domain was demonstrated to confer resistance to APP and CHD herbicides in *L. rigidum* (Zagnitko *et al.* 2001), *S. viridis* (Délye *et al.* 2002a) and *A. myosuroides* (Délye *et al.* 2002b). The resistant, ¹⁷⁸¹Leu ACCase allele is dominant (Délye *et al.* 2002a, b). Only two substitutions (A to T or A to C at the first nucleotide of an isoleucine codon) are possible to turn an isoleucine residue into a leucine residue. We developed easy and rapid PCR-based assays that detect ¹⁷⁸¹Leu alleles of ACCase in *S. viridis*, *A. myosuroides* and *L. rigidum*. Our objectives were i) to determine the cross-resistance pattern associated with the presence of ¹⁷⁸¹Leu ACCase allele(s) in a plant, and ii) to determine whether PCR can be used to detect resistance genes in the field.

MATERIALS AND METHODS

Plant material Seeds from 21 French field populations of *A. myosuroides* were collected in 2000. Seeds from five French field populations of *L. rigidum* were collected between 1995 and 1999. Seeds from *A. myosuroides* and *L. rigidum* were collected in fields that had predominantly been sprayed for at least four years with the APP herbicides fenoxaprop and diclofop-methyl, respectively. In all fields, satisfactory weed control was not achieved.

Resistance to ACCase inhibitors has not been reported in France in *S. viridis*. Three resistant or sensitive Canadian and two sensitive French populations of *S. viridis* were used in this study. Seeds from the resistant *S. viridis* line UM131 were provided by Dr. I.N. Morrison (University of Manitoba, Canada).

Herbicide sensitivity bioassay We investigated weed sensitivity using the seed bioassay described by Letouzé and Gasquez (1999). Four APP herbicides: fenoxaprop (Puma LS, 69 g a.i. L⁻¹, Aventis), clodinafop-propargyl (Célio, 100 g a.i. L⁻¹, Évolva), diclofop (Illoxan CE, 360 g a.i. L⁻¹, AgrEvo) and haloxyfop (Éloge, 104 g a.i. L⁻¹, Bayer), and three CHD herbicide: cycloxydim (Stratos ultra, 100 g a.i. L⁻¹, BASF), sethoxydim (Fervinal, 192 g a.i. L⁻¹, Schering-Plough) and clethodim (Centurion 240 EC, 240 g a.i. L⁻¹, Sipcarn-Phyteurop) were used for this study. The concentrations discriminating resistant from sensitive seedlings were 30 µM for fenoxaprop, 15 µM for clodinafop-propargyl, 115 µM for diclofop, 7 µM for haloxyfop, 6.5 µM for cycloxydim, 33 µM for sethoxydim and 4 µM for clethodim.

DNA methods A 1 cm section of the first leaf of each resistant seedling was cut and placed into a 0.5 mL microcentrifuge tube containing 150 µL of extraction buffer (100mM Tris-HCl, pH = 9.5, 1M KCl, 10mM EDTA). Because sensitive seedlings did not develop a first leaf in the bioassay used, the whole seed was used for DNA extraction. Plant material was roughly crushed using disposable micropipette tips. Tubes were placed at 95°C for six min, transferred into ice for five min and vortexed for 15 s. DNA extracts were kept at -20°C prior to PCR analysis. The set-up of bidirectional allele-specific PCR assays enabling to detect ¹⁷⁸¹Leu ACCase alleles is described elsewhere (Délye *et al.* 2002a,b). PCR primers are listed in Table 1. Because of highly similar nucleotide sequences, it was possible to set up one single allele-specific PCR assay to detect the two possible ¹⁷⁸¹Leu ACCase alleles in both *A. myosuroides* and *L. rigidum*. In the very limited number of *S. viridis* populations we studied, only one ¹⁷⁸⁰Leu ACCase allele, due to an A-to-C substitution, was recorded. *S. viridis* ACCase gene sequence was not enough conserved to use the primers designed for *A. myosuroides* and *L. rigidum*. Specific primers were

thus designed to genotype *S. viridis* plants. PCR reaction mixes were as described (Délye *et al.* 2002a). In each allele-specific PCR assay, all four primers were used at a final concentration of 0.2 µM each. Cycling program consisted of one step of 30 s at 95°C, followed by 37 cycles of 5 s at 95°C, 10 s at annealing temperature and 30 s at 72°C. Annealing temperature was 63°C for the assay targeting *A. myosuroides* and *L. rigidum*, and 59°C for that targeting *S. viridis*.

Analysing various plant tissues using allele-specific PCR

In order to validate the PCR assay in the field, a total of 360 distinct *A. myosuroides* adult plants were analysed from a field in Burgundy where resistance to cycloxydim was suspected, but not demonstrated. Plants were sampled at 5 m intervals along 18 transects separated by 7 m intervals. For each plant, two samplings, each consisting of one dead and one green leaf fragment (1 cm²), one stem section (1 cm long), two green and two mature seeds were collected. One sampling was subjected to DNA extraction and PCR analysis immediately, while the second one was dried during 10 days between newspaper sheets prior to PCR analysis. This was done to determine whether PCR patterns could be obtained from any kind of starting plant material collected in the field and mailed to the lab.

RESULTS

Herbicide bioassays and allele-specific PCR Sethoxydim bioassay revealed that 80 out of the 400 *S. viridis* seedlings tested were resistant. PCR analysis revealed that all 80 resistant seedlings, but none of the 320 sensitive seedling, contained ¹⁷⁸⁰Leu ACCase allele(s). The sensitivity of a total of 2250 seedlings from 21 *A. myosuroides* populations to five ACCase inhibitors and of a total of 750 seedlings from five *L. rigidum* populations to three ACCase inhibitors is shown in Table 2. All *A. myosuroides*, *L. rigidum* and *S. viridis* seedlings were genotyped using the primers listed in Table 1 (see Figure 1 for examples of amplicon patterns).

Table 1. Primers.

Primer	Sequence (5' to 3')	Target weed(s)	Target ACCase allele(s)
ACVRG1	AATGGGTCGTGGGGCACTCTATAATTCC		All
ACVRG1R	GCTGAGCCACCTCAATATATIAGAAACACC	<i>A. myosuroides</i> and <i>L. rigidum</i>	All
VRDIC+	GGACTAGGTGTGGAGAACC		¹⁷⁸¹ Leu (A-to-C change)
VRDITR	CAATAGCAGCACTTCCATGTAA		¹⁷⁸¹ Leu (A-to-T change)
ACSA	GGGCTCAATGACATTGGCATGGTAGC		All
ACSAR	ACCACACTTGTCAGCCGAGGAAC	<i>S. viridis</i>	All
SETS1	TGGGCTTGGTGTGAGAATA		¹⁷⁸⁰ Ile
SETR1R	AGCAGCACTTCCATGTAG		¹⁷⁸⁰ Leu (A-to-C change)

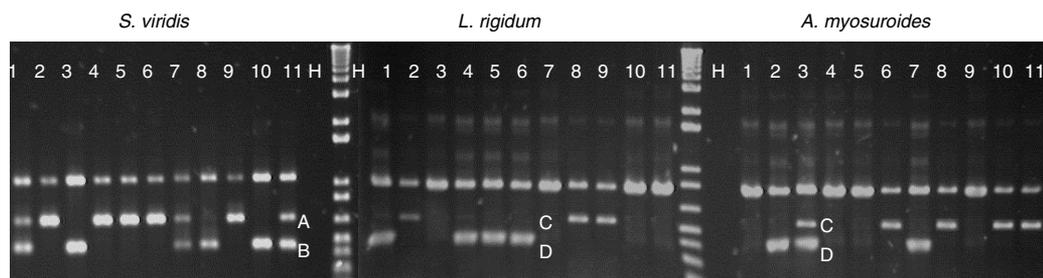


Figure 1. Allele-specific PCR patterns of 11 seedlings per weed. *S. viridis*: lanes 1, 3, 7, 8, 10, 11, seedlings resistant to sethoxydim; lanes 2, 4 to 6, 9, sensitive seedlings. Amplicons: A, ¹⁷⁸⁰Ile ACCase allele, B, ¹⁷⁸⁰Leu ACCase allele (A-to-C substitution). *L. rigidum*: lanes 1, 2, 4 to 6, 8, 9, seedlings resistant to cycloxydim; lanes 3, 7, 10, 11, sensitive seedlings. *A. myosuroides*: lanes 2, 3, 6 to 8, 10, 11, seedlings resistant to cycloxydim; lanes 1, 4, 5, 9, sensitive seedlings. Amplicons: C, ¹⁷⁸¹Leu ACCase allele (A-to-T substitution), B, ¹⁷⁸¹Leu ACCase allele (A-to-C substitution). Lanes H, H₂O control (no DNA).

Table 2. Herbicide sensitivity of and ACCase allele(s) present in seedlings from *A. myosuroides* and *L. rigidum*.

		<i>A. myosuroides</i>		<i>L. rigidum</i>	
		Leu	Ile	Leu	Ile
Cycloxydim	R	124	17	94	–
	S	–	309	–	156
Clethodim	R	21	4	–	–
	S	113	312	–	–
Fenoxaprop	R	131	136	–	–
	S	–	183	–	–
Diclofop	R	–	–	87	123
	S	–	–	–	40
Clodinafop	R	101	53	83	111
	S	30	266	7	49
Haloxypop	R	39	34	–	–
	S	83	294	–	–
Total		642	1608	271	479
Total		2250		750	

***Alopecurus myosuroides* field sample analysis** From the 360 plants sampled, a total of 360 fresh and 360 dried stem fragments, 360 fresh and 360 dried green leaf fragments, 360 fresh and 360 dried dead leaf fragments, 720 fresh and 720 dried green seeds, and 720 fresh and 720 dried mature seeds were analysed. All fresh and dried leaf and stem fragments, either dead or green, yielded clear allele-specific PCR patterns. This was also true for all fresh or dried green seeds. Amplicon patterns were much weaker or even absent when fresh or dried mature seeds were used as starting

material. Adding 200 µL sterile, distilled water to those samples greatly enhanced the quality of allele-specific PCR patterns, enabling to analyse 1397 of the 1440 mature seeds collected.

PCR analysis of leaf and stem fragments revealed that a total of 12 plants out of 360 sampled contained ¹⁷⁸¹Leu ACCase allele(s). All other plants contained ¹⁷⁸¹Ile ACCase alleles only. All seeds issued from the 12 ¹⁷⁸¹Leu plants and yielding clear amplicon patterns contained ¹⁷⁸¹Leu allele(s). A total of nine seeds containing ¹⁷⁸¹Leu ACCase allele(s) were found among the seeds issued from the 348 ¹⁷⁸¹Ile plants.

DISCUSSION

Detection of ¹⁷⁸¹Leu ACCase allele(s) and herbicide resistance We developed a quick PCR-based method to detect ¹⁷⁸¹Leu ACCase alleles in *A. myosuroides*, *L. rigidum* and *S. viridis*. This allele was present in 642 *A. myosuroides* seedlings out of 2250 analysed, in 271 *L. rigidum* seedlings out of 750 analysed, and in 80 *S. viridis* seedlings out of 400 analysed. Putting together the results obtained from *A. myosuroides*, *L. rigidum* and *S. viridis*, we found that plants containing ¹⁷⁸¹Leu ACCase allele(s) were cross-resistant to the CHD herbicides cycloxydim and sethoxydim, and to the APP herbicides fenoxaprop and diclofop. According to the bioassay used, the presence of ¹⁷⁸¹Leu ACCase allele(s) did not confer cross-resistance to the APP herbicides clodinafop-propargyl and haloxypop, nor to the CHD herbicide clethodim.

PCR-based test versus bioassay for the diagnosis of herbicide resistance When compared to bioassays, PCR-based diagnosis has the advantages of being:

- i) Fast: the whole procedure, from DNA extraction to allele-specific PCR pattern observation, can

be performed within the same day. Twelve plants containing ^{1781}Leu ACCase allele(s) were detected in a field without the need to wait for viable seeds to be produced, as would have been the case for herbicide bioassay. Immediate adaptation of the spraying program was thus possible to avoid further selection of resistant plants.

- ii) Not demanding, with respect to the plant material used: detection of ^{1781}Leu ACCase allele(s) was successfully achieved from living or dead, fresh or dried plant material.
- iii) Accurate: one resistance gene, that confers a given cross-resistance pattern, is detected.
- iv) Non-destructive: the same DNA sample can be re-used for other PCR-based analysis. Furthermore, the rapid DNA extraction procedure described here and allele-specific PCR assays can be performed with minimal laboratory equipment.

However, specific detection of a given resistance gene, the greatest advantage of a PCR-based assay, is also its major drawback. In this work, we found that ^{1781}Ile *A. myosuroides* and/or *L. rigidum* seedlings could be resistant to at least one of the herbicides used in this study (Table 2). This is in agreement with previous works demonstrating that, in those weeds, resistance to ACCase-inhibiting herbicides may be due to an altered target enzyme and/or to enhanced herbicide metabolism. This illustrates the limits of DNA-based assays targeting a given resistance gene: detecting ^{1781}Leu ACCase allele(s) in a plant means that this plant is resistant to cycloxydim, sethoxydim, fenoxaprop and diclofop. It also means that no conclusion can be drawn regarding the sensitivity of this plant to clodinafop, haloxyfop and clethodim. Furthermore, no conclusion can be drawn from the detection of the ^{1781}Ile allele only. Thus, developing a fully PCR-based herbicide resistant survey would imply to set up one PCR assay per existing resistance gene, which is obviously not possible. A more realistic possibility would be to develop DNA-based assays detecting the most widespread resistance genes for immediate adaptation of spraying programs, and to use bioassays to search for 'rare', or new, resistant biotypes.

Where to, now? Only one mutation conferring resistance has been found to date within the gene encoding chloroplastic ACCase in gramineous weeds. Although the ^{1781}Leu ACCase allele seems to be present in rather high frequency in European *A. myosuroides* populations (Délye *et al.* 2002b), studies conducted upon gramineous weed species have shown that other alleles of chloroplastic ACCase exist (e. g. Tardif *et al.* 1996). We are currently examining candidate ACCase alleles in *A. myosuroides* and in *L. rigidum* that are likely

to confer cross-resistance to APPs but not to CHDs. Those data will enable to develop more PCR-based assays similar to the one described here. Apart from early resistance diagnosis, molecular markers for herbicide resistance will be of immense value for population genetics studies aiming at understanding how resistant genes appear, maintain and spread between and within weed populations. Such studies, associated with the assessment of cropping systems controlling or reducing weed demographic increase (Chauvel *et al.* 2001), will be used to develop and improve integrated weed management strategies.

ACKNOWLEDGMENTS

This work was supported by a grant from the Conseil Régional de Bourgogne (Grant number HCP 01/5112/12).

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