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PCR-based detection of resistance to acetyl-CoA carboxylase-inhibiting herbicides in black-grass (*Alopecurus myosuroides* Huds) and ryegrass (*Lolium rigidum* Gaud)

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Abstract: A simple method based upon allele-specific PCR was developed to detect an isoleucine-leucine substitution in the gene encoding chloroplastic acetyl-coenzyme A carboxylase (ACCase) in two gramineous weeds: *Lolium rigidum* Gaud and *Alopecurus myosuroides* Huds. Analysis of 1800 *A myosuroides* and 750 *L rigidum* seedlings showed that the presence of ACCase leucine allele(s) conferred cross-resistance to the cyclohexanedione herbicide cycloxydim and to the aryloxyphenoxy-propionate herbicides fenoxaprop-P-ethyl and diclofop-methyl. Seedlings containing ACCase leucine allele(s) could be either sensitive or resistant to the aryloxyphenoxypropionate herbicides haloxyfop-P-methyl and clodinafop-propargyl. Successful detection of resistant plants in a field population of *A myosuroides* was achieved using this PCR assay. Using it with basic molecular biology laboratory equipment, the presence of resistant leucine ACCase allele(s) can be detected within one working day.

Keywords: acetyl-CoA carboxylase; herbicide; resistance; mutation; *Alopecurus*; *Lolium*

1 INTRODUCTION

Resistance is a factor that considerably decreases the efficacy of herbicide treatments. Diagnosing resistance in weed populations requires rapid and accurate methods to identify resistant biotypes. The cornerstone for detecting herbicide resistance has long been—and still is—bioassay, which also remains the only method when nothing is known about resistance genes. Such assays are often simple and generally consist in measuring the differences in the development of weed seedlings exposed to herbicide solutions (see Letouzé and Gasquez[1] for an example). Therefore, bioassays most often require the collection of viable weed seeds, are time-consuming and labour-intensive, and do not discriminate between different resistance mechanisms conferring similar resistance patterns. The latter point is a major drawback in obtaining data for population genetics studies or predictive modelling. Furthermore, bioassays are generally not quick enough to permit an adaptation of the spraying programme during the growing season when resistance is detected. When resistance genes have been identified, development of fast and accurate DNA-based assays 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 two major weeds of cereal crops, *Alopecurus myosuroides* Huds (black-grass) and *Lolium rigidum* Gaud (annual ryegrass), to herbicides inhibiting chloroplastic acetyl-coenzyme A carboxylase (ACCase, EC 6.4.1.2). Chloroplastic ACCase is a key enzyme in fatty acid biosynthesis, and thus a vital point of plant metabolism.[2] Two chemically dissimilar classes of herbicides, aryloxyphenoxypropionates (APPs) and cyclohexanediones (CHDs), block fatty acid biosynthesis by inhibition of chloroplastic ACCase, causing plant death.[3] It has long been reported that both types of compound inhibit the transfer of carbon dioxide to acetyl-CoA that is catalysed by the carboxyl-transferase (CT) domain.[4] It was only recently that a single point mutation conferring resistance to APP and CHD herbicides was identified in *L rigidum*,[5] *A myosuroides*[6] and *Setaria viridis* L Beauv (green foxtail).[7] This mutation causes an isoleucine-leucine substitution within chloroplastic ACCase CT domain that is homologous in all three weeds. The resistant ACCase allele is dominant.[6,7] 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

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residue. A leucine ACCase allele due to an A-to-T substitution was identified in *L rigidum*[5] and *S viridis*,[7] while the two possible leucine ACCase alleles were found in *A myosuroides*.[6]

Here, we report the development of a simple and rapid PCR-based assay that detects the two leucine alleles of chloroplastic ACCase in *A myosuroides* and *L rigidum*. We have assessed cross-resistance to APP and CHD herbicides, and demonstrated the feasibility of using this method to detect resistant plants in the field.

2 EXPERIMENTAL

2.1 Plant material

Seeds from nine French populations of *A myosuroides* were collected in 2000 (Table 1). The herbicide-sensitive, reference population BGSA98, collected in 1998, was also used in experiments (Table 1). Seeds from five French populations of *L rigidum* were collected between 1995 and 1999 (Table 2). Seeds from both weeds were collected in wheat fields that had been sprayed with APP herbicides, and where control was not satisfactory. Seeds from *A myosuroides* were collected in fields that had predominantly been sprayed for at least 4 years with fenoxaprop-P-ethyl. Seeds from *L rigidum* were collected in fields that had predominantly been sprayed for at least 4 years with diclofop-methyl.

2.2 Herbicide sensitivity bioassay

Sensitivity of *A myosuroides* and *L rigidum* seedlings was assessed to four and three ACCase-inhibiting herbicides, respectively. Herbicide sensitivity of 50 seedlings per population and per herbicide was determined using the seed bioassay described elsewhere.[1] For each *A myosuroides* population (Table 1), seedlings were assayed with three APP herbicides: fenoxaprop-P-ethyl (Puma LS, 69 g AI litre^{-1}, Aventis France, Gif-sur-Yvette, France), clodinafop-propargyl (Célio, 100 g AI litre^{-1}, Evolya, Rueil-Malmaison, France) and haloxyfop-P-methyl (Éloge, 104 g AI litre^{-1}, Bayer SA, Puteaux, France), and with one CHD herbicide: cycloxydim (Stratos ultra, 100 g AI litre^{-1}, BASF France, Levallois-Perret, France). The concentrations discriminating resistant from sensitive seedlings were 30 μM for fenoxaprop-P-ethyl, 15 μM for clodinafop-propargyl, 7 μM for haloxyfop-P-methyl and 6.5 μM for cycloxydim.[1,8] For each *L rigidum* population (Table 2), seedlings were assayed with two APP herbicides: diclofop-methyl (Illoxan CE, 360 g AI litre^{-1}, AgrEvo France) and clodinafop-propargyl, and with one CHD herbicide: cycloxydim. The concentrations discriminating resistant from sensitive seedlings were 115 μM for diclofop-methyl, 60 μM for clodinafop-propargyl and 6.5 μM for cycloxydim.[1,8]

2.3 Rapid DNA extraction procedure

Herbicide-resistant and sensitive seedlings were identified as described in Section 2.2. 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 (Tris-HCl, 100 mM pH 9.5, KCl 1 M, EDTA 10 mM).[9] Because sensitive seedlings do not develop a first leaf in the bioassay used, the whole seed was used for DNA extraction. The leaf sections or the seeds were roughly crushed using disposable micropipette tips. Tubes were closed and placed in a water bath at 95 °C for 6 min, transferred into ice for 5 min and vortexed for 15 s. DNA extracts were kept at −20 °C prior to PCR analysis.

2.4 Allele-specific PCR

The full DNA sequence encoding *A myosuroides* chloroplastic ACCase (EMBL accession number AJ310 767)[6] was aligned with four partial ACCase sequences from *L rigidum* (GenBank accession num-

Table 1. Herbicide sensitivity of, and ACCase allele(s) present in, *Alopecurus myosuroides* seedlings from nine French populations. Fifty seedlings were assayed per population and per herbicide

		CHD herbicide Cycloxydim		APP herbicides Fenoxaprop-P-ethyl		Clodinafop-propargyl		Haloxyfop-P-methyl	
Code	*Origin*[a]	*S*[b]	*R*[b]	*S*[b]	*R*[b]	*S*[b]	*R*[b]	*S*[b]	*R*[b]
BGSA98	21	50 Ile[c]	—[d]	50 Ile	—	50 Ile	—	50 Ile	—
BG003	08	—	50 T-Leu	2 Ile	48 T-Leu	2 Ile, 21 T-Leu	27 T-Leu	2 Ile, 39 T-Leu	9 T-Leu
BG010	86	50 Ile	—	8 Ile	42 Ile	48 Ile	2 Ile	46 Ile	4 Ile
BG017	57	50 Ile	—	50 Ile	—	50 Ile	—	50 Ile	—
BG018	54	50 Ile	—	8 Ile	42 Ile	50 Ile	—	50 Ile	—
BG023	72	41 Ile	9 Ile	4 Ile	46 Ile	2 Ile	48 Ile	23 Ile	27 Ile
BG029	51	50 Ile	—	50 Ile	—	50 Ile	—	50 Ile	—
BG032	80	18 Ile	32 T-Leu	6 Ile	6 Ile, 38 T-Leu	12 Ile, 3 T-Leu	3 Ile, 32 T-Leu	16 Ile, 20 T-Leu	14 T-Leu
BG049	08	—	8 Ile, 41 C-Leu, 1 C-Leu T-Leu	5 Ile	43 C-Leu, 1 T-Leu, 1 C-Leu T-Leu	2 Ile, 6 C-Leu	42 C-Leu	7 Ile, 2 T-Leu, 22 C-Leu	3 Ile, 15 C-Leu, 1 T-Leu
Total		309 Ile	82 T-Leu, 41 C-Leu, 1 C-Leu T-Leu, 17 Ile	183 Ile	87 T-Leu, 43 C-Leu, 1 C-Leu T-Leu, 136 Ile	24 T-Leu, 6 C-Leu, 266 Ile	59 T-Leu, 42 C-Leu, 53 Ile	61 T-Leu, 22 C-Leu, 294 Ile	24 T-Leu, 15 C-Leu, 34 Ile

[a] French département number.

[b] S: sensitive, R: resistant.

[c] T-Leu and C-Leu: detection of at least one leucine ACCase allele caused by a A-to-T or a A-to-C substitution at nucleotide position 5341, respectively. Ile: No detection of leucine ACCase allele(s).

[d] No sensitive/no resistant seedling was found in this population.

Table 2. Herbicide sensitivity of, and ACCase allele(s) present in, *Lolium rigidum* seedlings from five French populations. Fifty seedlings were assayed per population and per herbicide

		CHD herbicide Cycloxydim		APP herbicides Diclofop-methyl		APP herbicides Clodinafop-propargyl	
Code	Origin[a]	S[b]	R[b]	S[b]	R[b]	S[b]	R[b]
RG95002	11	22 Ile[c]	28 T-Leu	20 Ile	5 Ile, 25 T-Leu	26 Ile, 3 T-Leu	2 Ile, 19 T-Leu
RG97002	21	50 Ile	—[d]	7 Ile	43 Ile	6 Ile	44 Ile
RG98003	21	50 Ile	—	5 Ile	45 Ile	9 Ile	41 Ile
RG99008	30	—	50 C-Leu	—	50 C-Leu	2 C-Leu	48 C-Leu
RG99007	31	34 Ile	16 T-Leu	8 Ile	30 Ile, 1 C-Leu, 11 T-Leu	8 Ile, 2 T-Leu	24 Ile, 16 T-Leu
Total		156 Ile	44 T-Leu, 50 C-Leu	40 Ile	36 T-Leu, 51 C-Leu, 123 Ile	5 T-Leu, 2 C-Leu, 49 Ile	35 T-Leu, 48 C-Leu, 111 Ile

[a] French département number.
[b] S: sensitive, R: resistant.
[c] T-Leu and C-Leu: detection of at least one leucine ACCase allele caused by a A-to-T or a A-to-C substitution at nucleotide position 5341, respectively. Ile: No detection of leucine ACCase allele(s).
[d] No sensitive/no resistant seedling was found in this population.

bers AF359 513 to AF359 516).[5] The 24 base pairs located upstream and the 28 base pairs located downstream of nucleotide 5341 in *A myosuroides* sequence were identical in all five sequences. Allele-specific PCR primers VRDIC+, 5′ GGA CTA GGT GTG GAG AAC C 3′, and VRDITR, 5′ CAA TAG CAG CAC TTC CAT GTA A 3′, were designed to specifically prime *A myosuroides* and *L rigidum* ACCase sequences respectively containing C or T instead of a A at nucleotide position 5341. Both changes cause an isoleucine-leucine substitution in ACCase coding sequence that confers resistance to ACCase inhibitors.[5–7] Allele-specific PCR primers were designed by using the fact that a 3′ mismatch does not prime in a PCR at a specific annealing temperature.[10]

Primers VRDIC+ and VRDITR were used together with primers ACVRG1, 5′ AAT GGG TCG TGG GGC ACT CCT ATA ATT CC 3′ and ACVRG1R, 5′ GCT GAG CCA CCT CAA TAT ATI AGA AAC ACC 3′ at a final concentration of 0.2 μM for each of the four primers. Primers ACVRG1 and ACVRG1R targeted nearly identical nucleotide sequences in *A myosuroides* and *L rigidum* ACCase genes. Primers were designed to generate up to three distinct sizes of amplicons depending on the ACCase allele(s) present within one plant (Fig 1). Primers ACVRG1 and ACVRG1R yielded a 785-bp fragment that would be amplified from all ACCase alleles (internal positive control). Amplification with primer ACVRG1 and primer VRDITR, indicating the presence of an ACCase allele with a T at nucleotide position 5341, yielded a 495-bp fragment. Amplification with primer ACVRG1R and primer VRDIC+, indicating the presence of an ACCase allele with a C at nucleotide position 5341, yielded a 329-bp fragment.

PCR amplifications were performed in 20-μl reaction mixes, as described elsewhere.[11] To add DNA to PCR mixes, a sterile, disposable pipette tip was dipped first into a DNA solution, then into the corresponding PCR mix. The cycling programme consisted of one denaturation step of 30 s at 95 °C, followed by 37 cycles of 5 s at 95 °C, 10 s at 63 °C and 30 s at 72 °C. Fifteen microlitres of each reaction mix were loaded onto 1.3 mg litre^{-1}, 48-well, 10 × 11-cm agarose gels run for 30 min in Tris-borate EDTA buffer.

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 lanes separated by 7-m intervals. A leaf fragment (1 cm^2) was collected on each plant, placed in a microcentrifuge tube and analysed using PCR as described.

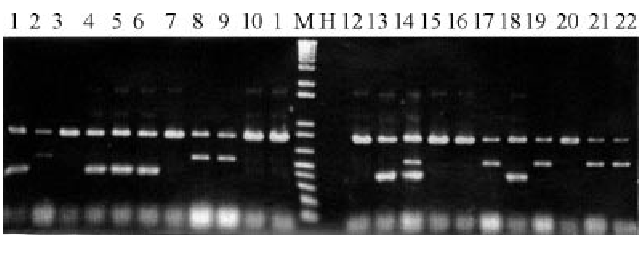

Figure 1. Allele-specific PCR analysis of 11 *Lolium rigidum* seedlings (lanes 1 to 11) and 11 *Alopecurus myosuroides* seedlings (lanes 12 to 22) using the four primers ACVRG1/VRDIC+/VRDITR/ACVRG1R. Lanes 1, 2, 4 to 6, 8, 9, 13, 14, 17 to 19, 21 and 22, seedlings resistant to cycloxydim; lanes 3, 7, 10, 11, 12, 15, 16 and 20, seedlings sensitive to cycloxydim. Lane H, water control (no DNA); lane M, molecular mass marker (1 kb plus DNA ladder, Gibco BRL). The sizes of the amplified fragments are from top to bottom: 785, 495 and 329 bp.

3 RESULTS

3.1 Allele-specific PCR

In the following, plants containing at least one leucine ACCase allele resulting from a A-to-X substitution will be designated 'X-Leu' plants. Other plants will be designated 'Ile' plants. Allele-specific PCR assays were developed to detect the presence of leucine ACCase allele(s) in A *myosuroides* and *L rigidum*. Reaction conditions were set up using plasmids with a partial, 2511-bp cDNA insert from *A myosuroides* chloroplastic ACCase coding sequence containing C, T or A at nucleotide position 5341. Specificity of allele-specific PCR amplifications obtained from DNA samples were verified by sequencing from seedlings that were genotyped as 'Ile', 'T-Leu' or 'C-Leu' using allele-specific PCR. Sequencing was performed for two seedlings per genotype. All electrophoregrams were checked to detect heterozygote plants. All sequences obtained were in agreement with allele-specific PCR results.

3.2 Herbicide bioassay and allele-specific PCR

The sensitivity of a total of 1800 seedlings from nine *A myosuroides* populations to four ACCase inhibitors is shown in Table 1. The sensitivity of a total of 750 seedlings from five *L rigidum* populations to three ACCase inhibitors is shown in Table 2. All seedlings were genotyped using primers ACVRG1/VRDIC+/VRDITR/ACVRG1R (see Fig 1 for an example of amplicon patterns).

In *A myosuroides* (Table 1), all 309 cycloxydim-sensitive and all 183 fenoxaprop-P-ethyl-sensitive seedlings were Ile. The 141 cycloxydim-resistant seedlings consisted of 124 Leu and 17 Ile seedlings. The 267 fenoxaprop-P-ethyl-resistant seedlings consisted of 131 Leu and 136 Ile seedlings. Seedlings sensitive to clodinafop-propargyl consisted of 30 Leu and 266 Ile seedlings. Seedlings resistant to this molecule consisted of 101 Leu and 53 Ile seedlings. Seedlings sensitive to haloxyfop-P-methyl consisted of 83 Leu and 294 Ile seedlings. Seedlings resistant to this molecule consisted of 39 Leu and 34 Ile seedlings.

In *L rigidum* (Table 2), all 156 cycloxydim-sensitive and all 40 diclofop-methyl-sensitive seedlings were Ile. All 94 cycloxydim-resistant seedlings were Leu. The 267 diclofop-methyl-resistant seedlings consisted of 87 Leu and 123 Ile seedlings. Seedlings sensitive to clodinafop-propargyl consisted of seven Leu and 49 Ile seedlings. Seedlings resistant to this molecule consisted of 83 Leu and 111 Ile seedlings.

3.3 Field sample analysis

All 360 samples collected from *A myosuroides* plants in the field yielded clear allele-specific PCR patterns. A total of 12 C-Leu plants were identified. All other plants were Ile. Analysing seeds from C-Leu plants using herbicide bioassay thereafter confirmed resistance to cycloxydim.

4 DISCUSSION

We have developed a quick PCR-based method to detect leucine alleles of the chloroplastic ACCase gene in *A myosuroides* and *L rigidum*. This allele was previously shown to confer resistance to ACCase-inhibiting herbicides.[5–7] It was present in 508 *A myosuroides* seedlings out of 1800 analysed, and in 271 *L rigidum* seedlings out of 750 analysed. Assessment of the sensitivity of 1800 *A myosuroides* and 750 *L rigidum* seedlings to CHD and APP herbicides followed by PCR analysis enabled to determine cross-resistance patterns conferred by chloroplastic ACCase leucine allele (Tables 1 and 2). The results obtained both on *A myosuroides* and *L rigidum* showed that plants containing ACCase leucine allele(s) were cross-resistant to the CHD herbicide cycloxydim and to the APP herbicides fenoxaprop-P-ethyl and diclofop-methyl. According to the bioassay used, the presence of the isoleucine-leucine substitution did not seem to confer cross-resistance to the APP herbicides clodinafop-propargyl and haloxyfop-P-methyl. Ile seedlings were found among seedlings resistant to each herbicide used in this study. This is in agreement with previous work demonstrating that, in *A myosuroides* and in *L rigidum*, resistance to ACCase-inhibiting herbicides may be due to an altered target and/or to enhanced metabolisation of the herbicides.[12–14]

This illustrates both the advantages and the limits of DNA-based assays targeting a given resistance gene: detection of ACCase leucine allele(s) in *A myosuroides* or *L rigidum* implies resistance to cycloxydim, fenoxaprop-P-ethyl and diclofop-methyl, but no conclusion can be drawn from detection of the isoleucine allele only.

The only ACCase-inhibiting herbicides sprayed in the fields where all populations of *A myosuroides* and *L rigidum* were collected were APPs (mostly fenoxaprop-P-ethyl and diclofop-methyl). Plants cross-resistant to the CHD cycloxydim because of the presence of ACCase leucine allele(s) were therefore selected by APPs only, as was previously reported in *L rigidum* populations.[15] Thus, cross-resistance patterns may not be easily predicted from the selection pressure applied.

A total of 360 *A myosuroides* plants were analysed in three days using allele-specific PCR. Twelve resistant plants were detected, 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 programme was thus possible to avoid selection of resistant plants. The PCR assay described in this study has the advantages of being non-destructive (the same DNA sample can be re-used for other PCR-based analysis), accurate (identification of the ACCase allele(s) present in a single plant is achieved) and fast (the whole procedure, from DNA extraction to allele-specific PCR pattern observation, can be performed within the same day). The inexpensive and rapid extraction procedure and the allele-specific PCR assay described here can be used in any molecular biology laboratory, with minimal equipment. Adapting these methods to take advantage of automated micro-titre plate technologies may greatly improve the number of samples processed.

Only one mutation conferring herbicide resistance has been found to date within the gene encoding chloroplastic ACCase in gramineous weeds.[5–7] Studies conducted on gramineous weed species such as ryegrasses (*Lolium* spp) have shown that other alleles of chloroplastic ACCase may exist that would confer cross-resistance to APPs but not to CHDs.[4,12,13,15]

Determination of the mutation(s) involved would enable to develop more PCR-based detection assays similar to the one described here. The use of PCR diagnostic for early detection of herbicide resistance genes, coupled with population genetics studies and risk evaluation, will be of immense value for further resistance risk assessment and validation of anti-resistance strategies.

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