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SNP markers for black-grass (*Alopecurus myosuroides* Huds.) genotypes resistant to acetyl CoA-carboxylase inhibiting herbicides

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Abstract Chloroplastic acetyl CoA-carboxylase (ACCase) is the target of widely used, specific graminicide herbicides: cyclohexanediones (CHDs) and aryloxyphenoxypropionates (APPs). Resistance to these compounds is a worldwide, increasing problem. Population genetic studies aimed at understanding the dynamics of this situation and the diffusion of resistance genes within and between weed populations are challenging because biological assays are not adequate for this purpose, and because different mechanisms of resistance confer a similar resistance phenotype. Molecular markers for specifically detecting resistance genes are therefore urgently needed to conduct such studies. For this purpose, we cloned and sequenced the whole gene encoding chloroplastic ACCase in *Alopecurus myosuroides* Huds. (Black-grass). We identified two point mutations at nucleotide 5,341 that both cause an isoleucine-leucine substitution at position 1,781. Three bi-directional allele-specific PCR assays were developed, each detecting two distinct ACCase alleles with a single PCR reaction. The sensitivity of 1,190 seedlings of *A. myosuroides* to one CHD and one APP was determined. Genotyping revealed that, although resistant plants were only selected by APPs, the ^{1,781}Leu ACCase allele is a widespread, dominant gene of resistance to both APPs and CHDs. No other ACCase allele associated with resistance could be identified in this work. Useful applications of allele-specific PCR markers are population genetic studies as well as routine molecular diagnosis of herbicide resistance.

Keywords Acetyl-Co A carboxylase · Herbicide · Resistance · Single nucleotide polymorphism

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Introduction

Acetyl-Co A carboxylase (ACCase, EC 6.4.1.2) is a biotin-dependent carboxylase catalysing the first step of fatty acid biosynthesis in eukaryotes and prokaryotes (Harwood 1988). Two forms of ACCase have been identified in plants and are located in the chloroplast, the primary site of plant fatty acid biosynthesis, and in the cytosol (Sasaki et al. 1995, Konishi et al. 1996), respectively. In most plants, chloroplastic ACCase is a multisubunit enzyme (Konishi et al. 1996). In the Gramineae (Konishi et al. 1996) and in the Geraniaceae (Christopher and Holtum 2000), chloroplastic ACCase is a multidomain enzyme of around 250 kDa. The cytosolic ACCase in all plants studied so far is of the multidomain type. In the Gramineae, two distinct nuclear genes encode cytosolic and chloroplastic ACCases (Gornicki et al. 1994, 1997; Podkowinski et al. 1996).

Chloroplastic ACCase is a key enzyme in fatty acid biosynthesis, and thus a vital site, of plant metabolism (Ohlrogge and Jaworski 1997). Two chemically dissimilar classes of herbicides, aryloxyphenoxypropionates (APPs) and cyclohexanediones (CHDs), block fatty acid biosynthesis by inhibition of multidomain-type chloroplastic ACCase, causing plant death (Burton et al. 1989). The chloroplastic, multisubunit-type ACCase and the cytosolic, multidomain-type ACCase, are insensitive and significantly less-sensitive, respectively, to CHDs and APPs than the chloroplastic, multidomain-type ACCase of the Gramineae (Egli et al. 1993; Alban et al. 1994). Thus, APPs and CHDs are effective selective graminicide herbicides.

Because of their frequent use, spontaneous resistance has appeared in 25 gramineous weed species (Heap 2001). Although resistance may occur through de-toxification (e.g. Cocker et al. 1999), alteration of the chloroplastic multidomain-type ACCase is a frequent cause of resistance. This has been extensively documented based on biochemical evidence (Devine and Shukla 2000; see also Heap 2001 for a compilation of references). The molecular mechanism of ACCase inhibition by APPs

and CHDs is as yet unknown. It has been reported that both types of compounds inhibit the transfer of CO_2 to acetyl-CoA which is catalysed by the carboxyl-transferase (CT) domain (reviewed in Gronwald 1991). It was also found that a 400 amino-acid region, encompassing most of the CT domain of wheat chloroplastic ACCase, is involved in insensitivity to both CHD and APP herbicides (Nikolskaya et al. 1999). More recently, we identified a single nucleotide polymorphism (SNP) within the CT domain of the autogamous gramineous weed *Setaria viridis* (L. Beauv.) chloroplastic ACCase that confers a high level of resistance to a CHD herbicide and a lower level of resistance to APPs (Délye et al. 2002).

Alopecurus myosuroides Huds. (Black-grass) is a major, allogamous, annual winter weed in northern Europe, where farming conditions have led to the selection of numerous resistant populations (Heap 2001). Although resistance to herbicides is an increasing problem, only a few population genetic studies have been conducted to find out how resistance appears and spreads within and between populations. This is in part due to: (1) classical herbicide sensitivity bioassays, being time-consuming and destructive, and (2) different resistance mechanisms conferring similar resistance phenotypes. In this work, we searched for point mutations conferring resistance to CHDs and/or APPs within the CT domain of *A. myosuroides* chloroplastic ACCase, with the aim of developing SNP-based molecular markers suitable for population genetics study.

Materials and methods

Plant material

Seeds from 20 French populations of *A. myosuroides* were collected in 2000 from wheat fields that were sprayed with APP herbicides, and where control of *A. myosuroides* was not satisfactory (Tables 1 and 2). The herbicide-sensitive reference population SA98, collected in 1998, was also used in experiments (Table 1).

Herbicide sensitivity bioassay

Sensitivity of *A. myosuroides* seedlings to two ACCase-inhibiting herbicides was assessed as described by Letouzé and Gasquez (1999). For each *A. myosuroides* population (Tables 1 and 2), seedlings were assayed with one APP herbicide, fenoxaprop-P ethyl (Puma LS, 69 gl^{-1} active ingredient, AgrEvo France, Gif-sur-Yvette, France), and one CHD herbicide; cycloxydim (Stratos ultra, 100 g l^{-1} active ingredient, BASF France, Levallois-Perret, France). The concentrations discriminating resistant from sensitive seedlings were 30 μM for fenoxaprop-P ethyl and 6.5 μM for cycloxydim (Letouzé 1999). To obtain plant material for ACCase cloning and sequencing experiments, seedlings were collected from bioassays, rinsed with de-ionised water, transplanted into plastic trays filled with two-thirds loamy soil from the experimental station at Dijon and one-third sand, and cultivated in the greenhouse until they had 3–4 fully expanded leaves.

Cloning the gene encoding *A. myosuroides* chloroplastic ACCase

Total RNA was extracted from one single plant of *A. myosuroides* population SA98, and single-stranded cDNA was prepared as de-

Table 2 Cycloxydim sensitivity of and nucleotide present at position 5,341 on each copy of the gene encoding chloroplastic ACCase in *A. myosuroides* seedlings from 13 French populations. Thirty seedlings were assayed per population

Code	Location of origin	Cycloxydim sensitivity	
		S[a]	R[a]
V11	Rilly sur Vienne	2 A/A	26 A/T, 2 T/T
V15	Bleigny le Carreau	2 A/A	7 A/T, 21 T/T
V23	Nogent le Bernard	30 A/A	–[b]
V24	Aussonce	27 A/A	1 A/C, 2 A/T
V26	Le Bouchon	2 A/A	12 A/T, 16 T/T
V30	Neauphlette	27 A/A	3 A/T
V59	Hénaménil	11 A/A	19 A/T
V61	Voves	1 A/A	8 A/T, 21 T/T
V64	Montlevon	29 A/A	1 A/T
V65	Bruyère et Montberault	16 A/A	11 A/T, 3 T/T
V68	Closfontaine	9 A/A	17 A/T, 4 T/T
V92	Lucq	12 A/A	15 A/T, 3 T/T
V99	Oinville sous Auneau	26 A/A	4 A/T

[a] S sensitive, R resistant
[b] No resistant seedling was found in this population

Table 1 Herbicide sensitivity of and nucleotide present at position 5,341 on each copy of the gene encoding chloroplastic ACCase in *A. myosuroides* seedlings from eight French populations. Fifty seedlings were assayed per population and per herbicide. The CT domain of chloroplastic ACCase was sequenced in two seedlings per population

Code	Location of origin	Herbicides			
		Fenoxaprop-P ethyl (APP)		Cycloxydim (CHD)	
		S[a]	R[a]	S[a]	R[a]
SA98	Saint Apollinaire	50 A/A	–[b]	50 A/A	–[b]
V03	Rémilly-Aillicourt	2 A/A	25 A/T, 23 T/T	–[b]	23 A/T, 27 T/T
V10	Basses	10 A/A	40 A/A	50 A/A	–[b]
V17	Silly en Saunois	50 A/A	–[b]	50 A/A	–[b]
V18	Hénaménil	8 A/A	42 A/A	50 A/A	–[b]
V29	Lavannes	50 A/A	–[b]	50 A/A	–[b]
V32	Moyencourt les Poix	6 A/A	5 A/A, 29 A/T, 10 T/T	8 A/A	30 A/T, 12 T/T
V49	Charbogne	10 A/A	4 A/T, 23 A/C, 12 C/C, 1 C/T	8 A/A	2 A/T, 20 A/C, 19 C/C, 1 C/T

[a] S sensitive, R resistant
[b] No sensitive/no resistant seedling was found in this population

Table 3 Primers

Primer	Sequence (5′ to 3′)	Location[a]	Experiment
NotIdT	AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTTTTTTTTT	Not applicable	Reverse-transcription
ACVII1R	GGCAGTGACTAAATCTCCCATGCTCTC	3,438 to 3,412	″
ACC2	GGTGTTTCTGCTGTTTGGCCTGGTTG	652 to 677	Degenerate PCR
ACVII2R	ATGCTTGTGCGGAGTTCGCTGAGCTTGG	3,329 to 3,302	″
ACVII4	CCTGCAAACTTCTTCGTTTCTTGATTGCC	2,345 to 2,373	Inverse PCR
ACVII7R	TCCTCAGGTATCGAGTCCAAACAAAGTTC	887 to 859	″
ACVII1	GAAGCTGCAGTGCAGAAGTCGTGGTCTAAC	4,777 to 4,806	Gene-specific PCR
ACVII8	AGGACACGCAGAGGAACCTCTTTCATTTAC	4,212 to 4,241	″
ACVII10R	GCAGCTGCCTCAGAAGCCAAGTACC	6,722 to 6,698	″
ACVIIAR	TCAATGCCACTGATGGCTGCACGAGG	5,774 to 5,749	″
VRDIC+	GGACTAGGTGTGGAGAACC	5,323 to 5,341	Allele-specific PCR
VRDITR	CAATAGCAGCACTTCCATGTAA	5,362 to 5,341	″
VRDIT	GACTAGGTGTGGAGAACT	5,323 to 5,341	″
VSDIR	CAATAGCAGCACTTCCATGTAT	5,362 to 5,341	″

[a] The first nucleotide in *A. myosuroides* ACCase coding sequence is number 1

scribed (Délye et al. 2002) using either the poly-dT primer NotIdT, or the gene specific primer ACVII1R (Table 3). PCR mixes were as described (Délye et al. 2002). Primers ACA1 and ACV1R (Délye et al. 2002) were used at a final concentration of 0.2 μM each, to generate a single amplicon of about 2,100 bp from the NotIdT reverse-transcription mix that was cloned into the vector pGEM-T (Promega). To exclude PCR errors, three different clones were sequenced on both strands using an extension of specific primers.

In the following, all primers are shown in Table 3. Primer ACC2 corresponds to a sequence of 26 nucleotides that was identical between the coding sequences of wheat, maize and foxtail millet eukaryotic-type chloroplastic ACCase (EMBL Database accession numbers AF029895, U19183 and AF294805, respectively), and was as close as possible to the 5′-end of these genes. This primer was used together with primer ACVII2R that was designed based on the 5′-end sequence of the fragment obtained from primers ACA1 and ACV1R, at a final concentration of 0.2 μM each. The cycling program consisted of 37 cycles with 30 s at 95 °C, 30 s at 60 °C and 2 min at 72 °C, followed by a final step of 10 min at 72 °C. A single amplicon of about 2,700 bp was obtained from a ACVII1R reverse-transcription mix. Cloning and sequencing were as before.

The 3′ end of the *A. myosuroides* chloroplastic ACCase gene was obtained using primer NotIdT and primer ACVII1, designed based on the 3′ end-sequence of the fragment using primers ACA1 and ACV1R, at a final concentration of 0.5 μM and 0.2 μM, respectively. The cycling program was as for primer pair ACC2/ACVII2R. A single amplicon of about 2,700 bp was obtained from a NotIdT reverse-transcription mix. Cloning and sequencing were as before. Inverse-PCR was used to clone the 5′-end of the *A. myosuroides* chloroplastic ACCase gene. A unique *Vsp*I restriction site was found close to the 5′-end of the fragment obtained using primers ACC2 and ACVII2R. Total genomic DNA was extracted as described by Doyle and Doyle (1987) from a leaf of the same sensitive plant. Five hundred micrograms of DNA were digested with *Vsp*I (MBI Fermentas, Vilnius, Lithuania). The restriction mix was heated at 75 °C for 10 min and deposited on a 0.025-μm dialysis membrane (Millipore SA, Molsheim, France) floating onto sterile distilled water. After 1-h dialysis, the restricted DNA was circularised using T4 DNA ligase (MBI Fermentas), and employed for PCR using primers ACVII4 and ACVII7R at a final concentration of 0.2 μM each. The cycling program consisted of 37 cycles with 10 s at 95 °C, 15 s at 70 °C and 2 min at 72 °C, followed by a final step of 10 min at 72 °C. A single amplicon of about 2,500 bp was obtained, which was cloned and sequenced as before.

Nucleotide sequence assembling and translation were performed using the BioEdit software (Hall 1999).

Sequence comparison of CT regions from *A. myosuroides* chloroplastic ACCase

Mutations possibly associated with herbicide resistance were searched for in the CT domain of ACCase. Primers ACVII8 and ACVII10R were used at a final concentration of 0.2 μM each to amplify a cDNA fragment of 2,511 bp encompassing the entire CT domain from resistant and sensitive seedlings in eight *A. myosuroides* populations (two seedlings per population). The 16 seedlings studied consisted of one cycloxydim-resistant seedling in populations V03, V32 and V49, one cycloxydim-sensitive seedling in the five remaining populations (Table 1), one fenoxaprop-P ethyl-resistant seedling in populations V03, V10, V18, V32 and V49, and one fenoxaprop-P ethyl-sensitive seedling in the three remaining populations (Table 1). The cycling program consisted of 37 cycles with 10 s at 95 °C, 15 s at 60 °C and 2 min at 72 °C, followed by a final step of 10 min at 72 °C. Cloning and sequencing were as before. Because we could not predict whether the seedlings used for the sequencing experiments would contain two identical or two different copies of the ACCase gene, at least five different cDNA inserts were sequenced on both strands for each seedling.

Bi-directional allele-specific PCR

Herbicide-resistant and -sensitive seedlings were identified as described in the “Herbicide sensitivity bioassay” section. A 2-cm section cut from the first leaf of each resistant seedling, or the whole seed and the first leaf from sensitive seedlings, were placed into a microcentrifuge tube. DNA extraction was as described (Délye et al. 2002), except that the DNA solutions obtained from seeds were diluted with 200-μl of sterile, distilled water. DNA extracts were kept at –20 °C prior to PCR analysis.

Allele-specific primers were designed by using the fact that a 3′ mismatch does not prime a PCR at a specific annealing temperature (Sommer et al. 1992). Primers VRDIT and VRDITR were designed to specifically prime *A. myosuroides* ACCase sequences containing T at nucleotide position 5,341. Primers VRDIC+ and VSDIR were designed to specifically prime *A. myosuroides* ACCase sequences containing C or A at nucleotide position 5,341, respectively. Primer pairs VRDIC+ and VRDITR, VRDIC+ and VSDIR, or VRDIT and VSDIR were used together with primers ACVII1 and ACVIIAR at a final concentration of 0.2 μM for each of the four primers. PCR amplifications were performed in 20 μl. 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 program consisted of one denaturation step of 30 s at 95 °C, followed by 37 cycles of 10 s at 95 °C, 15 s at 63 °C and 30 s at 72 °C.

Table 4 Allele-specific PCR patterns expected from *A. myosuroides* individuals with different ACCase alleles. The sizes of the expected amplicons are given in base pairs. Plants containing nucleotide X at position 5,341 on one copy of the gene encoding the chloroplastic ACCase and nucleotide Z at position 5,341 on the second copy are designated "X/Z"

Primers	Genotype					
	A/A	A/T	T/T	T/C	C/C	A/C
ACVII1/VRDIT/VSDIR/ACVIIAR	998, 586	998, 586, 452	998, 452	998, 452	998	998, 586
ACVII1/VRDIC+/VRDITR/ACVIIAR	998	998, 586	998, 586	998, 586, 452	998, 452	998, 452
ACVII1/VRDIC+/VSDIR/ACVIIAR	998, 586	998, 586	998	998, 452	998, 452	998, 586, 452

Primers were designed to generate up to three distinct sizes of amplicons depending on the ACCase alleles present within one plant (Table 4). Primers ACVII1 and ACVIIAR yielded a 992-bp fragment. Amplification with primer ACVII1 and either primer VSDIR or primer VRDITR yielded a 564-bp fragment. Amplification with primer ACVIIAR and either primer VRDIC+ or primer VRDIT yielded a 433-bp fragment.

Results

Structure of *A. myosuroides* chloroplastic ACCase

The fragment amplified from genomic DNA using inverse PCR was 2,512 bp long, of which 121 bp encompassed the region between the first nucleotide of the primer ACVII4 annealing position and the *Vsp*I restriction site. An open reading frame of 2,264 bp interrupted by four introns of 569, 640, 83 and 83 bp, respectively, was identified on the fragment. Intron sequences were removed, and a 7,589-bp nucleotide sequence was obtained after assembling the sequences of the fragments amplified from genomic DNA and cDNA. It was deposited in the EMBL Nucleotide Sequence Database (accession number AJ310767). A coding sequence starting at nucleotide 157 encoded a polypeptide of 2,320 amino acids, with a calculated molecular mass of 256 kDa. When compared with eukaryotic-type ACCases, the amino-acid sequence of the ACCase from *A. myosuroides* showed 90%, 85% and 84% identity with the chloroplastic eukaryotic-type ACCases from wheat, foxtail millet and maize, respectively. Those sequences are the three only other complete sequences of chloroplastic eukaryotic type ACCases from the Gramineae reported so far.

The general organisation of the *A. myosuroides* chloroplastic ACCase protein was the same as those of other known eukaryotic-type ACCases. Amino-acid positions 1 to 103 at the N-terminal end of the deduced protein contained a high number of hydroxylated and small hydrophobic amino acids, typical of chloroplast transit peptides (Schleiff and Soll 2000). The four most-conserved amino-acid regions among the eukaryotic-type ACCases, the biotin-carboxylase domain, the biotin-carboxyl carrier domain and the β- and α-domains of the CT (Gornicki et al. 1994), were located at amino-acid positions 139 to 636, 665 to 844, 1,659 to 1,708 and 1,937 to 1,970, respectively. Searches for homologies in databases located the CT domain of *A. myosuroides* chloroplastic ACCase at amino-acid positions 1,639 to 2,204.

Comparison of the sequences encoding the ACCase CT domain in *A. myosuroides* seedlings sensitive or resistant to fenoxaprop-P ethyl or cycloxydim

The complete cDNA sequence encoding the CT domain of *A. myosuroides* chloroplastic ACCase was determined from each of the three cycloxydim-resistant, five cycloxydim-sensitive, five fenoxaprop-P ethyl-resistant and three fenoxaprop-P ethyl-sensitive seedlings investigated. A total of 11 SNPs were recorded, nine of which were synonymous mutations. The two non-synonymous mutations both occurred at nucleotide position 5,341, within codon 1,781, causing an ATA codon (isoleucine) to be changed into either a TTA or a CTA codon. Both changes caused a substitution from isoleucine to leucine at amino-acid position 1,781. In the following, plants containing nucleotide X at position 5,341 on one copy of the gene encoding chloroplastic ACCase and nucleotide Z at position 5,341 on the second copy will be designated "X/Z" plants. The eight sensitive seedlings were A/A. The three cycloxydim-resistant seedlings from populations V03, V32 and V49 were T/T, A/T and A/C, respectively. The five fenoxaprop-P ethyl-resistant seedlings from populations V03, V10, V18, V32 and V49 were A/T, A/A, A/A, A/T and C/C, respectively. The synonymous SNPs observed enabled us to distinguish one ^{5,341}C, two ^{5,341}T and six ^{5,341}A different ACCase alleles.

Herbicide bioassay and allele-specific PCR

Allele-specific PCR assays were developed to detect the presence of one or two ^{1,781}Leu ACCase allele(s) in the same seedling. Reaction conditions were set up using plasmids with a cDNA insert containing C, T or A at nucleotide position 5,341. The specificity of allele-specific PCR amplifications obtained from DNA samples were verified by sequencing from seedlings that were genotyped as "A/A", "A/T", "T/T", "C/T", "C/C" and "A/C". Sequencing was performed for one seedling per genotype. All sequences obtained were in agreement with the allele-specific PCR results.

The sensitivity of a total of 800 seedlings from eight *A. myosuroides* populations to cycloxydim or fenoxaprop-P ethyl is shown in Table 1. All seedlings were first analysed using primers ACVII1/VRDIC+/VRDITR/ACVIIAR. No further analysis was necessary to deter-

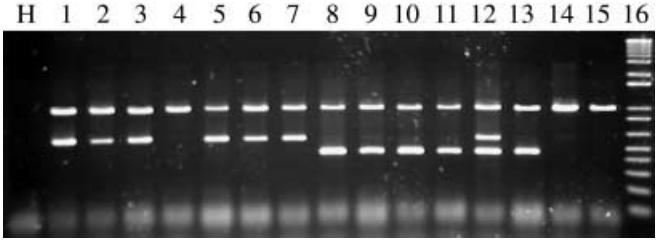

Fig. 1 Allele-specific PCR analysis of 15 *A. myosuroides* seedlings using the four primers ACVIIA/VRDIC+/VRDITR/ACVIIAR. *Lanes 4, 14 and 15* seedlings sensitive to cycloxydim; *lanes 1 to 3 and 5 to 13* seedlings resistant to cycloxydim. *Lane H* H_2O control (no DNA); *lane M* molecular-weight marker (1 kb plus DNA ladder, Gibco BRL). The sizes of the amplified fragments are, from top to bottom, 998, 586 and 452 bp

mine the genotype of A/A and C/T seedlings (Table 1, see Fig. 1 for an example of amplicon patterns). In all other cases, and depending on the presence of a C or a T at nucleotide position 5,341, seedlings were analysed using primers ACVII1/VRDIC+/VSDIR/ACVIIAR or primers ACVII1/VRDIT/VSDIR/ACVIIAR, respectively, to discriminate homozygotes and heterozygotes (Table 1). All 266 cycloxydim-sensitive and all 186 fenoxaprop-P ethyl-sensitive seedlings were A/A. The 134 cycloxydim-resistant seedlings consisted of 55 A/T, 39 T/T, 1 T/C, 20 A/C and 19 C/C genotypes. The 214 fenoxaprop-P ethyl-resistant seedlings consisted of 87 A/A, 58 A/T, 33 T/T, 1 T/C, 23 A/C and 12 C/C genotypes. Preliminary experiments showed that only the fenoxaprop-P ethyl-resistant phenotype of A/A seedlings reverted to a sensitive phenotype when using inhibitors of the herbicide-detoxifying enzymes described by Letouzé (1999). The resistant phenotype of all seedlings containing at least one copy of the ^{1,781}Leu allele of the chloroplastic ACCase was not modified in the same experiment.

The sensitivity of 390 seedlings from 13 more *A. myosuroides* populations to cycloxydim was also determined in an effort to find cycloxydim-resistant genotypes that did not contain a ^{5,341}C nor a ^{5,341}T ACCase allele (Table 2). All 194 cycloxydim-sensitive seedlings were A/A. The 196 cycloxydim-resistant seedlings consisted of 125 A/T, 70 T/T and 1 A/C genotypes. No cycloxydim-resistant seedling was A/A.

Discussion

We sequenced a cDNA fragment of 2,511 bp encompassing the entire CT domain of a *A. myosuroides* chloroplastic ACCase from a total of 16 seedlings resistant or sensitive to the APP herbicide fenoxaprop-P ethyl or to the CHD herbicide cycloxydim. Two SNPs, representing the only two possible changes that may convert the isoleucine codon at position 1,780 into a leucine codon, discriminated all seedlings resistant to cycloxydim from sensitive seedlings. Within the 21 populations studied, all 261 seedlings genotyped using an allele-specific PCR that contained at least one ^{1,781}Leu ACCase allele were resistant to cycloxydim or to fenoxaprop-P ethyl. The ^{1,781}Leu ACCase allele is thus a dominant gene for resistance to both a CHD and an APP herbicide in *A. myosuroides*, as previously demonstrated in *Setaria viridis* (Délye et al. 2002).

The synonymous SNPs found within the sequence encoding the chloroplastic ACCase CT domain enabled us to distinguish three distinct ^{1,781}Leu ACCase alleles, suggesting that the ^{1,781}Leu ACCase alleles appeared independently in geographically distant populations. The only ACCase-inhibiting herbicides sprayed in the fields where all 21 populations of *A. myosuroides* were collected were APPs (mostly fenoxaprop-P ethyl). Plants cross-resistant to CHD cycloxydim and to APP fenoxaprop-P ethyl because of the presence of ^{1,781}Leu allele(s) were therefore selected by APPs only, as was also reported in annual ryegrass (*Lolium rigidum* Gaud.) (Heap and Knight 1990). Thus, cross-resistance patterns may not be easily predicted from the selection pressure applied.

Only one mutant ACCase allele conferring resistance to herbicides was recorded in this work. This allele, which was present in all populations where cross-resistance to cycloxydim and fenoxaprop-P ethyl was due to target mutation, is thus probably easily selected in the field. ACCase being a vital site of plant metabolism (Ohlrogge and Jaworski 1997), the number of possible amino-acid substitutions within the ACCase CT domain that may confer resistance to ACCase inhibitors without altering enzymatic activity is very likely limited. Biochemical data obtained from *S. viridis* revealed no significant differences between the catalytic properties and enzymatic activity of the Ile and Leu chloroplastic ACCase alleles (Shukla et al. 1997; Délye et al. 2002), probably because leucine and isoleucine have similar chemical structures.

Furthermore, the isoleucine residue at position 1,781 in the *A. myosuroides* chloroplastic ACCase is conserved in all known eukaryotic-type chloroplastic ACCases, whereas a leucine residue is found at this position in all other known eukaryotic-type ACCases (plant cytosolic ACCases, fungal and animal ACCases), which are much less sensitive to both APP and CHD herbicides. There are therefore converging data to propose that, among a few possible alleles of chloroplastic ACCase, the leucine allele may be one of the easiest to select, or even the easiest to select, that may confer resistance to ACCase-inhibiting herbicides without altering the fitness of resistant plants. Studies conducted upon closely related gramineous weed species such as ryegrasses (*Lolium* spp.) have shown that other alleles of chloroplastic ACCase exist that may for instance confer resistance to APPs but not to CHDs (Heap and Knight 1990; Gronwald 1991). Such alleles may be less-frequently encountered in *A. myosuroides* populations, perhaps because they also confer a reduced fitness, and the mutation(s) involved still remains to be elucidated.

Eighty seven seedlings from populations V10, V18 and V32 (Table 1) contained a ^{1,781}Ile allele and were re-

sistant to fenoxaprop-P ethyl. Deduced amino-acid sequences from the CT domain of ACCases from four fenoxaprop-P ethyl-resistant seedlings of those populations were identical to those of sensitive seedlings. Resistance to fenoxaprop-P ethyl in these seedlings was due to metabolisation of the herbicide. This is in agreement with previous findings demonstrating that, depending on the *A. myosuroides* population studied, resistance to fenoxaprop-P ethyl may be due to an altered target site and/or to enhanced metabolism of the herbicide (Cocker et al. 1999). The fenoxaprop-P ethyl-resistant A/A seedlings are thus a good example to illustrate the use of molecular tools to discriminate between distinct molecular mechanisms of resistance conferring similar resistance phenotypes.

Genotyping 1,190 seedlings collected in 21 different fields sprayed with APPs revealed contrasted allelic compositions between populations (Tables 1 and 2). The ^{5,341}C ACCase allele appeared to be far less frequently encountered than the ^{5,341}T allele. Although both alleles caused the same amino-acid substitution, and thus conferred the same resistant phenotype, the ^{5,341}C allele was detected in two populations only (populations V24 and V49, Tables 1 and 2), in a mixture with the ^{5,341}T allele. APP herbicides were applied in all the fields investigated. Between-population variations of the frequency of resistant ACCase alleles, as well as the proportion of homozygous resistant seedlings, is therefore the consequence of two factors. The first is the intensity of the selection pressure created by the cropping system, which may vary greatly from one field to another. The second is the number of generations elapsed since a resistant allele(s) has (have) appeared or has (have) been introgressed into the *A. myosuroides* population present in the field.

The molecular markers developed in this work will be useful to obtain data concerning parameters of importance for herbicide resistance management, such as the frequency of appearance of resistance genes in sensitive populations, the neighbourhood size within populations, the major factors controlling gene flow within and between populations, and the evolution of the frequency of resistance genes in populations undergoing different selection pressures. The ultimate aim of such studies will be the development of cultural practices aimed at reducing the frequency of resistant genes in weed populations, and/or to limit their dissemination. Such studies would also enable extrapolation from natural genes of resistance to herbicides into transgenes.

Primers ACVII1/VRDIC+/VSDIR/ACVIIAR and ACVII1/VRDIT/VSDIR/ACVIIAR will be useful for population genetics studies, where the genotype of each individual needs to be determined. Primers ACVII1/VRDIC+/VRDITR/ACVIIAR can be used as a molecular assay for the diagnosis of resistance to ACCase inhibitors. The molecular markers developed in this study have the advantages of being non-destructive (genotyping can be performed from a fragment of leaf or stem, or from a seed), accurate [identification of the ACCase allele(s) present in a 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 described elsewhere (Délye et al. 2002) and the allele-specific PCR assay described here can be used in any molecular biology laboratory, with minimal equipment.

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