Evolution and diversity of the mechanisms endowing resistance to herbicides inhibiting acetolactate-synthase (ALS) in corn poppy (Papaver rhoeas L.)

Christophe Délyea, Fanny Pernina, Laura Scarabel

INRA, UMR1210 Biologie et Gestion des Adventices, 17 rue Sully, F-21000 Dijon, France

Istituto di Biologia Agroambientale e Forestale - CNR, Agripolis, viale dell'Università 16, I-35020 Legnaro (PD), Italy

A R T I C L E   I N F O
Article history:
Received 30 July 2010
Received in revised form 5 October 2010
Accepted 7 October 2010
Available online 16 October 2010

Keywords:
ALS (acetolactate synthase)
dCAPS
 evolution
herbicide resistance
nucleotide variation
Papaver rhoeas

A B S T R A C T
We investigated the diversity of mechanisms conferring resistance to herbicides inhibiting acetolactate synthase (ALS) in corn poppy (Papaver rhoeas L.) and the processes underlying the selection for resistance. Six mutant ALS alleles, Arg197, His197, Leu197, Ser197, Thr197 and Leu574 were identified in five Italian populations. Different alleles were found in a same population or a same plant. Comparison of individual plant phenotype (herbicide sensitivity) and genotype (amino-acid substitution(s) at codon 197) showed that all mutant ALS alleles conferred dominant resistance to the field rate of the sulfonylurea tribenuron and moderate or no resistance to the field rate of the triazolopyrimidine florasulam. Depending on the allele, dominant or partially dominant resistance to the field rate of the imidazolinone imazamox was observed. Putative non-target-site resistance mechanisms were also likely present in the populations investigated. The derived Cleaved Amplified Polymorphic Sequence assays targeting ALS codons crucial for herbicide sensitivity developed in this work will facilitate the detection of resistance due to mutant ALS alleles. Nucleotide variation around codon 197 indicated that mutant ALS alleles evolved by multiple, independent appearances. Resistance to ALS inhibitors in P. rhoeas clearly evolved by redundant evolution of a set of mutant ALS alleles and likely of non-target-site mechanisms.

© 2010 Elsevier Ireland Ltd. All rights reserved.

1. Introduction
Weeds are plants adapted to agricultural ecosystems [1]. By competing with crop plants for nutrients, water and light, weeds have always been, and still are, the major cause for crop losses [2]. Thus, to secure crop yield, weed demography needs to be restrained in arable fields. Herbicides that can kill up to 98% weed plant in a field (e.g. ref. [3]) are the most effective way of controlling weeds in arable fields. Herbicides and other selective pressures in arable fields are understood, so that the processes driving weed adaptation to herbicides and other selective pressures in arable fields are understood and controlled [9].

While many studies have focused on resistance diagnosis, few have considered the diversity of resistance mechanisms occurring within weed populations, and only a handful have considered the evolutionary dynamics of resistance selection [9]. This has in particular resulted into the emergence of the concept of ‘biotype’ in weed science. A biotype is considered to be a group of individuals in a weed species sharing a resistant phenotype and/or genotype. An assumption found in numerous studies investigating resistance to herbicides is that a single ‘resistant biotype’ is present in a given field where resistance has arisen. This assumption has been challenged by recent studies conducted on several weed species that identified a diversity of resistant phenotypes or genotypes among individual plants in a same field (e.g. ref. [10–13]).

Papaver rhoeas L. (corn poppy) is a widespread, annual, diploid, dicotyledonous species that is insect-pollinated and self-incompatible [14]. P. rhoeas is a major weed of cereal crops in Southern Europe. Its competitive nature makes it especially nox-

Abbreviations: ALS, acetolactate-synthase; dCAPS, derived cleaved amplified polymorphic sequences.

* Corresponding author. Tel.: +33 380 693 185, fax: +33 380 693 262.
E-mail address: delye@dijon.inra.fr (C. Délye).

0168-9452/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2010.10.007
Table 1

<table>
<thead>
<tr>
<th>Code</th>
<th>Location of origin</th>
<th>Year collected</th>
<th>SELECTING HERBICIDE(S)</th>
<th>% PLANTS RESISTANT TO A</th>
<th>% MUTANT PLANTSb</th>
<th>MUTANT ALS ALLELES</th>
</tr>
</thead>
<tbody>
<tr>
<td>02-24</td>
<td>Lucera</td>
<td>2002</td>
<td>Tribenuron (SU)</td>
<td>50.0 (25/50)</td>
<td>44.0 (22/50)</td>
<td>0.0 (0/48)</td>
</tr>
<tr>
<td>02-26</td>
<td>Capalbio</td>
<td>2002</td>
<td>Tribenuron (SU), chlorsulfuron (SU)</td>
<td>86.0 (43/50)</td>
<td>83.0 (39/47)</td>
<td>62.0 (31/50)</td>
</tr>
<tr>
<td>04-38</td>
<td>Venosa</td>
<td>2004</td>
<td>Tribenuron (SU), other</td>
<td>100.0 (49/49)</td>
<td>94.0 (47/50)</td>
<td>22.0 (11/50)</td>
</tr>
<tr>
<td>04-41</td>
<td>Troia</td>
<td>2004</td>
<td>Tribenuron (SU), other</td>
<td>100.0 (43/43)</td>
<td>97.8 (45/46)</td>
<td>42.0 (21/50)</td>
</tr>
<tr>
<td>04-44</td>
<td>Milena</td>
<td>2004</td>
<td>Triasulfuron (SU)</td>
<td>82.0 (41/50)</td>
<td>83.4 (40/48)</td>
<td>16.7 (8/48)</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td></td>
<td></td>
<td>83.4 (201/242)</td>
<td>80.1 (193/241)</td>
<td>28.9 (71/246)</td>
</tr>
</tbody>
</table>

* Resistant includes moderately resistant plants. The number of resistant plants and the total number of plants tested with each herbicide are given between brackets (e.g., (25/50)).

b Plants containing at least one mutant ALS allele. All plants tested with an herbicide were genotyped. The total number of mutant plants and of plants genotyped are given between brackets.

c SU, sulfonylurea; IMI, imidazolinone; TP, triazolopyrimidine.

### 2. Materials and methods

#### 2.1. P. rhoeas populations

Five populations of *P. rhoeas* collected in 2002 or in 2004 in Italian fields where resistance occurred were used in this study (Table 1). They were selected because previous works revealed a high frequency of plants resistant to ALS inhibitors in each population [26, Scarabel, unpublished data].

*P. rhoeas* populations were collected in durum wheat fields by harvesting mature capsules from about 100 plants per field. The seed samples were cleaned from capsule debris, and stored at room temperature.
Table 2
dCAPS primers and restriction enzymes.

<table>
<thead>
<tr>
<th>Primersa</th>
<th>Sequence (5′–3′)b</th>
<th>Target codonb, c</th>
<th>Tm used in PCR, primer concentration</th>
<th>Restriction enzyme, recognition site</th>
<th>dCAPS patterns (fragment sizes in bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR122F</td>
<td>CAAGCTTATAAACCCTGAAAACCC</td>
<td>Ala122, GC</td>
<td>60 °C, 0.4 μM</td>
<td>BsmL, GAATGC</td>
<td>43, 169, 212</td>
</tr>
<tr>
<td>PR122R</td>
<td>TACTCTGAGCTCAGTAACCAATGCTG</td>
<td>GCa</td>
<td>65 °C, 0.2 μM</td>
<td>KpnI, GGTACCC</td>
<td>41, 186, 227</td>
</tr>
<tr>
<td>PR197F</td>
<td>GCTGAGGATTTATGTTCCATGTTG</td>
<td>Pro197, CTC</td>
<td>60 °C, 0.1 μM</td>
<td>BsmL, GAATGC</td>
<td>43, 166, 209</td>
</tr>
<tr>
<td>PR197R</td>
<td>GACATCTTTATGACTGCAAAATGTC</td>
<td>GC</td>
<td>60 °C, 0.2 μM</td>
<td>BstXI, CCAAGTG</td>
<td>40, 204, 244</td>
</tr>
<tr>
<td>PR205F</td>
<td>TTCTCTGAGGATTTATGTTCCATGTTG</td>
<td>Ala122, GC</td>
<td>60 °C, 0.2 μM</td>
<td>MwoI, GCN</td>
<td>GC</td>
</tr>
<tr>
<td>PR205R</td>
<td>TTAAGATCAGTCTGCTG</td>
<td>Ala653, G</td>
<td>60 °C, 0.2 μM</td>
<td>MwoI, GCN</td>
<td>GC</td>
</tr>
</tbody>
</table>

a Primer name ending with F, forward primer; primer name ending with R, reverse primer.
b Nucleotide(s) in bold in the primer sequence are modified from the P. rhoeas ALS sequence in order to create a recognition site for the restriction enzyme used. Nucleotides belonging to the enzyme restriction site are underlined in the primer sequence and in the target codon sequence.
c Nucleotide in the sequence of the target codon which substitution does not cause any amino-acid replacement is indicated in lowercase.
d Wild-type.
e dCAPS primer.
f A C-to-T substitution was introduced in the sequence of this primer to remove a MwoI restriction site naturally occurring in P. rhoeas ALS sequence (boxed).

2.2. Whole-plant herbicide sensitivity assessment

Seed germination was as described [26]. Seedlings were first grown in a growth chamber (12 °C/23 °C night/day with a 12 h photoperiod). After 10 days, seedlings were transplanted into plastic boxes (24 cm × 30 cm × 9 cm) filled with a substrate containing 60% silt-loam soil, 15% sand, 15% perlite and 10% peat by volume and placed in a greenhouse at Legnaro, north-eastern Italy (45°21′N, 11°58′E). The experiment was performed in March and April 2009, when natural temperature and light conditions were optimal for the growth of P. rhoeas.

Ten days after transplanting, seedlings were thinned down to 25 plants per box and two boxes were sprayed per herbicide for each population. At the 3-to-5-leaf stage, a leaf fragment (1 cm²) from each plant was collected for subsequent molecular analyses. Two days later, plants were sprayed with an ALS inhibitor at the recommended field rate. The sulfonylurea tribenuron-methyl (Granstar, DuPont, 75% by weight, soluble powder) was applied at 7.5 g ha⁻¹, at a prespray concentration of 1000 L ha⁻¹. dCAPS patterns were visualised by electrophoresis on 2% (wt/vol) agarose gels run in TBE buffer.

2.3. DNA extraction

Each leaf fragment collected from the seedlings was placed in a 0.2 mL microcentrifuge tube. DNA was extracted using a rapid procedure [27]. DNA extracts were kept at −20 °C prior to genotyping or sequencing.

2.4. Detection of mutant ALS alleles by genotyping

The P. rhoeas ALS sequence deposited in Genbank/EMBL (accession AJ577316) was used to design derived cleaved polymorphic amplified sequence (dCAPS) [28] assays to detect nucleotide substitutions causing amino-acid replacements at codons critical for sensitivity to ALS inhibitors. It must be noted that, in wild-type sensitive ALS in P. rhoeas, codon 653 encodes an alanine residue and not a serine residue as in most other species [26].

The dCAPS technique uses PCR to create a restriction enzyme recognition site in a sequence where none exists, detecting nucleotide polymorphisms on the basis of the gain (amplicon digested) or loss (amplicon undigested) of this site. The dCAPS primer is located close to the variable nucleotide position(s) and contains mismatched nucleotide(s) so as to create a restriction enzyme recognition site encompassing the variable nucleotide(s) [28]. Primers targeting codons Ala122, Pro197, Ala653, Trp574 and Ala653 were generated using the software dCAPS Finder 2.0 [29] so that only amplicons containing wild-type codons were cut. The second primer in each assay was selected so that the amplicons generated were 200–250 nucleotide long. Using dCAPS primers about 40 nucleotide long was expected to enable easy discrimination of undigested and digested (about 40 bp removed) amplicons by standard agarose gel electrophoresis. To avoid false detection of mutant, resistant ALS alleles, we ensured that, in each dCAPS assay, all nucleotides in the restriction enzyme recognition site were exclusively located in the dCAPS primer sequence and in the part of the targeted codon where any variation would cause amino-acid replacement. When several restriction enzymes could be used, we selected the cheapest one among those with no other recognition site in the amino-acid sequence.

PCR mixes were as described [30]. Primers concentration in PCR mixes are indicated in Table 2. Cycling programs consisted of 95 °C 5 min followed by 35 cycles of 95 °C 5 s, annealing temperature (Tm, Table 2) 10 s and 72 °C 30 s. Digestions were performed 3 h at 37 °C, except for BstXI (55 °C). The 10−μL digestion mixes contained 4 μL of the PCR mixes, 5 U enzyme (Fermentas, Vilnius, Lithuania), 0.5 μL 10× provided enzyme buffer and 4 μL water. dCAPS patterns were visualised by electrophoresis on 2% (wt/vol) agarose gels run in 0.5× TBE buffer.
All sprayed plants were genotyped using the five dCAPS assays. When no ALS allele mutant at codon 122, 197, 205, 574 or 653 was detected in a plant classified as resistant or moderately resistant to one ALS inhibitor, the full coding sequence of its ALS was amplified using primers ALP1 (5'-CTCTCAACTTCTCTGGAGATC-3') and PR197R with a proofreading polymerase as described [31], and directly sequenced on both strands.

Sequence analysis was conducted with the software BioEdit [32]. Some plants contained two different ALS haplotypes (heterozygous plants), as deduced from the occurrence of double-peaks on the corresponding sequence chromatograms. The sequences of individual haplotypes were statistically inferred with the program PHASE, which implements a Bayesian method for estimating haplotypes from population sequence data [33,34]. Haplotype inferring was performed on the basis of the sequences obtained from the plants we sequenced that were homozygous. Nucleotide diversity analyses and differentiation among populations calculated as FST after [35] were computed using DNAsp 4.2 [36].

3. Results

3.1. Herbicide resistance

A total of 729 individual P. rhoeas plants were screened for resistance to one of three herbicides (Table 1). The majority of the plants assayed with tribenuron or imazamox were classified as resistant, while the majority of plants assayed with florasulam were classified as sensitive. Of the 201 plants classified as tribenuron-resistant, 18 plants (9.0%) were moderately resistant (dead leaves but re-growth from the centre of the rosette). They originated from populations 02-24 (eight plants), 02-26 (two plants) and 04-44 (eight plants). Of the 193 plants classified as imazamox-resistant, 15 plants (7.8%) were moderately resistant (dead leaves but re-growth from the centre of the rosette). They originated from populations 02-24 (eight plants), 02-26 (two plants) and 04-44 (one plant). In contrast, of the 71 plants classified as florasulam-resistant, 45 plants (63.4%) were moderately resistant. They originated from populations 02-26 (13 plants), 04-38 (nine plants), 04-41 (15 plants) and 04-44 (eight plants). No plant in population 02-24 was resistant to florasulam (Table 1).

3.2. dCAPS genotyping

Primers for dCAPS assays were designed for codons Ala122, Pro197, Ala205, Trp574 and Ala653. No dCAPS assays were designed for codons Asp376 or Gly654 because (1) involvement of mutations at these codons in resistance was first reported after we designed the dCAPS assays and (2) our results showed that mutations at these codons were very unlikely to play a substantial role in resistance to ALS inhibitors in P. rhoeas.

All dCAPS assays were set up using six plants from a reference French P. rhoeas population that had never been sprayed with ALS inhibitors. Digested and undigested amplicons were loaded on adjacent lanes prior electrophoresis on 2% (wt/vol) agarose gels. After enzyme digestion, all plants yielded the expected wild-type patterns (fully digested amplicons) that were readily discriminated from undigested amplicons on agarose gels (not shown).

All 729 P. rhoeas plants which sensitivity to one ALS inhibitor had been determined (see previous section) were analysed using all five dCAPS assays. All plants genotyped yielded clear dCAPS patterns for all assays (not shown). No plant contained ALS allele(s) bearing a mutation at codons Ala122, Ala205 or Ala653. A total of 628 plants (86.1% of the plants genotyped) contained at least one ALS allele bearing a mutation at codon 197, of which 337 were homozygous for the mutant allele (examples of dCAPS patterns are illustrated Fig. 1). The frequency of mutant plants varied from 58.1% to 99.3% among the five populations investigated (Table 1). One single plant from population 04-41 contained one ALS allele bearing a mutation at codon 574 (0.14% of the plants genotyped). dCAPS result was checked by sequencing, which confirmed the occurrence of one G-to-T substitution at the second nucleotide in codon 574 in one of the two ALS alleles in this plant, causing a Trp-to-Leu replacement.

3.3. Cross-resistance associated to mutant ALS alleles

The only plant carrying one mutant Leu574 ALS allele was resistant to the sulfonylurea tribenuron, which is consistent with the literature [8,20].

Eight amino-acid replacements endowing resistance to ALS inhibitors are known at codon Pro197 [20]. A 362-bp amplicon encompassing codon Pro197 was sequenced in all 628 plants where mutant ALS allele(s) were detected using dCAPS to identify the amino-acid replacement(s) present in each plant. Five types of mutant ALS alleles (Arg197, His197, Leu197, Ser197, Thr197) were identified. One to four types of mutant ALS alleles were detected in...
a single population (Table 1). In populations where several types of mutant alleles were present, a total of 101 plants classified as homozygous mutant were actually trans-heterozygotes (i.e., they contained two different types of mutant ALS alleles). Five types of trans-heterozygotes were identified (Table 3).

To establish the cross-resistance pattern associated to each mutant ALS allele, we associated phenotype (i.e., resistant, moderately resistant or sensitive plants) with genotype (i.e., having one or two mutant ALS allele(s)) for each individual plant except trans-heterozygotes. Results varied with the herbicide and the mutant ALS allele (Table 3).

All plants carrying at least one mutant ALS allele survived tribenuron application. All homozygous mutant plants were classified as resistant to this herbicide. Heterozygous mutant plants carrying a single Thr197 allele were all classified as resistant to tribenuron. Heterozygous mutant plants carrying Ser197 allele(s) were classified as resistant to this herbicide. All homozygous mutant plants were classified as resistant, moderately resistant or sensitive to an herbicide (Table 3).

Table 3 Herbicide sensitivity to three ALS inhibitors applied at the field rate and ALS alleles identified in P. rhoeas plants.

<table>
<thead>
<tr>
<th>Genotype at codon 197</th>
<th>Phenotype a</th>
<th>Tribenuron (SLP)</th>
<th>Imazamox (IMP)</th>
<th>Florasulam (TPb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>r</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Arg/Pro</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>P(Arg/Arg)</td>
<td>&lt;10−3</td>
<td>&lt;10−3</td>
<td>&lt;0.01</td>
<td>1.00</td>
</tr>
<tr>
<td>P(Arg/Pro)</td>
<td>&lt;10−3</td>
<td>&lt;10−3</td>
<td>&lt;0.01</td>
<td>1.00</td>
</tr>
<tr>
<td>His/His</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>His/Pro</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P(His/His)</td>
<td>&lt;10−3</td>
<td>&lt;10−3</td>
<td>&lt;0.01</td>
<td>1.00</td>
</tr>
<tr>
<td>P(His/Pro)</td>
<td>&lt;10−3</td>
<td>&lt;10−3</td>
<td>&lt;0.01</td>
<td>1.00</td>
</tr>
<tr>
<td>Leu/Leu</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leu/Pro</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P(Leu/Leu)</td>
<td>&lt;10−3</td>
<td>&lt;10−3</td>
<td>&lt;0.01</td>
<td>0.24</td>
</tr>
<tr>
<td>P(Leu/Pro)</td>
<td>&lt;10−3</td>
<td>&lt;10−3</td>
<td>&lt;0.01</td>
<td>0.24</td>
</tr>
<tr>
<td>Ser/Ser</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ser/Pro</td>
<td>32</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>P(Ser/Ser)</td>
<td>&lt;10−3</td>
<td>&lt;10−3</td>
<td>&lt;0.01</td>
<td>0.24</td>
</tr>
<tr>
<td>P(Ser/Pro)</td>
<td>&lt;10−3</td>
<td>&lt;10−3</td>
<td>&lt;0.01</td>
<td>0.24</td>
</tr>
<tr>
<td>Thr/Thr</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thr/Pro</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P(Thr/Thr)</td>
<td>&lt;10−3</td>
<td>&lt;10−3</td>
<td>&lt;0.01</td>
<td>0.24</td>
</tr>
<tr>
<td>P(Thr/Pro)</td>
<td>&lt;10−3</td>
<td>&lt;10−3</td>
<td>&lt;0.01</td>
<td>0.24</td>
</tr>
<tr>
<td>Arg/Leu</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Arg/Thr</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Arg/Ser</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Ser/Leu</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ser/Thr</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pro/Pro</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>41</td>
</tr>
</tbody>
</table>

a R, resistant; r, moderately resistant (dead leaves but re-growth from the centre of the rosette); S, sensitive (dead).
b SL, sulfonylurea; IMP, imidazolinone; TP, triazolopyrimidine.
c Number of plants in a given category of genotype and herbicide sensitivity.
d P-value of Fisher's exact test for the association of the genotype between brackets with survival to an herbicide.
e Trans-heterozygous mutant plants.
f Plant carrying one mutant Leu197 allele.

A 362-bp fragment containing codon Pro197 was sequenced from all 628 plants carrying ALS allele(s) bearing a mutation at codon 197. Primer sequences and flanking low-quality nucleotides were discarded, resulting in a 269-bp-long sequence encompassing nucleotides 422 to 690 that correspond to codons Phe141 to Ala230 in the coding sequence of P. rhoeas ALS (EMBL/Genbank accession AJ377316). A total of 21 polymorphisms were identified that discriminated 18 haplotypes. Polymorphisms consisted
of nine nucleotide substitutions, one of which had three alleles and one had four alleles (Table 4). Four substitutions were non-synonymous. Two of them occurred at codon Pro197, one of which had three alleles and one had four alleles.

Eight haplotypes encoded a Pro197 codon. Haplotype Pro-1 represented the majority of the Pro197 ALS sequences (presence in all five populations, 77.0% of Pro197 sequences, Table 4). Three Pro197 haplotypes were private (i.e., detected in a single population), of which one was a singleton haplotype (i.e., only detected once). The ten remaining haplotypes all bore a nucleotide substitution encoding an amino-acid replacement at codon Pro197. Pairwise FST values ranged from 0.066 to 0.5703 with an overall average value of 0.361. There was no significant correlation among pairwise FST values and geographical distances among populations (Mantel’s test, r = 0.278, P = 0.136).

4. Discussion

4.1. Diversity of the mechanisms endowing resistance to ALS inhibitors in P. rhoeas

4.1.1. Occurrence of mutations essentially at ALS codon 197

All mutant ALS alleles identified in this work after analysing a total of 729 P. rhoeas plants encoded an amino-acid replacement at codon 197, except one that encoded an amino-acid replacement at codon 574. This is the first report of a Leu574 allele in P. rhoeas. No mutation was detected at any other ALS codon known to be involved in resistance to ALS inhibitors (Tables 1 and 3). Our results are consistent with previous studies considering P. rhoeas, where mutation ALS alleles encoding amino-acid replacements were exclusively reported at codon 197 [16,25,26], even if these studies analysed a much more restricted number of plants. The resistance situation in P. rhoeas contrasts with that in other weed species where mutations at different codons in the ALS gene could be identified in the same population, or even in the same plant (e.g. ref. [11,37-39]). The P. rhoeas populations we investigated were essentially selected for by sulfonylurea herbicides (Table 1). Besides substitutions at codon 197, substitutions at codons 376 and 574 are known to confer a high level of resistance to ALS inhibitors (Tables 1 and 3).

Overall differentiation among populations computed as FST was high and highly significant (0.373; P < 10^-3), mostly because of differences in the respective frequencies of haplotypes encoding an amino-acid replacement at codon 197. Pairwise FST values ranged from 0.066 to 0.5703 with an overall average value of 0.361. There was no significant correlation among pairwise FST values and geographical distances among populations (Mantel’s test, r = 0.278, P = 0.136).
of the plant together with a substantial reduction in seed production has been associated with the presence of mutant Leu674 ALS in *Amaranthus powellii* [40]. Interestingly, in this species, codon 653 encodes an alanine residue, as is the case in *P. rhoes* [26]. It could be that an amino-acid replacement at codon 574, and possibly at codon 376, is not compatible with the occurrence of an alanine residue at amino-acid position 653. As we did not keep the only plant carrying Leu574 ALS for observation, this remains to be investigated in *P. rhoes*. It is however noteworthy that, in other weed species with an ALS gene encoding a serine residue at codon 653, plants carrying mutant Leu574 allele(s) do not differ in morphology from plants carrying only wild-type ALS alleles (Délye, Scarabel, unpublished observations on the monocotyledonous species *Alopecurus myosuroides* Huds. and *Schoenoplectus mucronatus* L.).

4.1.2. Cross-resistance associated to mutant ALS alleles

We characterised the cross-resistance to three families of ALS inhibitors associated to five different ALS alleles bearing a mutation at codon 197 in *P. rhoes* at the whole-plant level. For the first time, we also considered the dominance of the resistance associated with these alleles by discriminating homozygous mutant plants from heterozygous plants using a dCAPS assay. Ser197 ALS is the only mutant ALS isoform of *P. rhoes* for which detailed enzyme sensitivity data has been published so far. These data show a high reduction in sensitivity to tribenuron (36-fold lower than wild-type ALS) and a moderate reduction in sensitivity to imazamox (2.1-fold lower than wild-type ALS) [16]. Here, all plants carrying Ser197 ALS allele(s) were resistant to tribenuron, except one heterozygous plant out of the 32 assayed that was moderately resistant to this herbicide. Thus, resistance to tribenuron endowed by Ser197 ALS could be defined as dominant. All homozygous plants carrying Ser197 ALS alleles survived imazamox application, while 25% of the heterozygous plants were killed. On this basis and after enzyme data, we propose that Ser197 ALS conferred a moderate, partially dominant resistance to imazamox. Ser197 ALS was the only mutant ALS allele for which we observed plants resistant to florasulam. Yet, plants containing Ser197 ALS allele(s) could also be moderately resistant or sensitive to florasulam. Thus, Ser197 ALS alone did not confer resistance to this herbicide, although it significantly reduced the sensitivity of the plants carrying it.

All plants carrying Thr197 ALS allele(s) were resistant to tribenuron or to imazamox. Thus, Thr197 ALS conferred dominant resistance to both tribenuron and imazamox. Most plants carrying Thr197 ALS allele(s) were sensitive to florasulam. Only four homozygous plants carrying Thr197 alleles were moderately resistant to this herbicide. Thus, Thr197 ALS alone did not confer resistance to florasulam.

All plants carrying Leu197 ALS allele(s) survived tribenuron or imazamox application. Because some heterozygous plants were moderately resistant to tribenuron or imazamox, we proposed that resistance to tribenuron and imazamox endowed by Leu197 ALS was partially dominant. Most plants carrying Leu197 allele(s) were sensitive to florasulam. The plants carrying Leu197 allele(s) that survived this herbicide were all moderately resistant. Thus, Leu197 ALS alone likely reduced the sensitivity of plants to florasulam, but did not confer resistance to this herbicide.

All plants carrying His197 ALS allele(s) survived tribenuron application. Because some heterozygous plants were moderately resistant to this herbicide, we proposed His197 ALS conferred partially dominant resistance to tribenuron. All homozygous plants carrying His197 ALS alleles survived imazamox application, while some of the heterozygous plants were killed. We thus propose that His197 ALS conferred a moderate, partially dominant resistance to imazamox. All plants carrying His197 ALS allele(s) were sensitive to florasulam. Thus, His197 ALS did not confer resistance to this herbicide.

All plants carrying Arg197 ALS allele(s) survived tribenuron application. Because one heterozygous plant out of the six assayed was moderately resistant to this herbicide, we proposed Arg197 ALS conferred partially dominant resistance to tribenuron. All plants carrying Arg197 ALS allele(s) survived imazamox application except one homozygous plant. Few plants carrying Arg197 ALS allele(s) were identified in our study. Because the nine remaining homozygous plants and the two heterozygous plants assayed were all resistant to imazamox, we propose the homozygous plant that did not survive imazamox application may have died for other reasons than the treatment (i.e., root rot due to the presence of a clay lump in the growing substrate). In this hypothesis, it is likely that Arg197 ALS conferred a dominant or partially dominant resistance to imazamox. Because most of the plants carrying Arg197 ALS were sensitive to florasulam, this allele did not confer resistance to florasulam.

In conclusion, all five mutant alleles studied conferred dominant (Thr197 ALS) or partially dominant (Arg197, His197, Leu197 and Ser197 ALS) resistance to tribenuron. Only Ser197 ALS seemed to confer a significant decrease in sensitivity to florasulam, while the remaining four mutant alleles did not confer resistance to this herbicide. Thr197 ALS conferred dominant resistance to imazamox. His197, Leu197 and Ser197 ALS conferred partially dominant resistance to imazamox, with a more moderate level of resistance associated to His197 and Ser197 ALS than to Leu197 ALS. Arg197 ALS most probably conferred dominant or partially dominant resistance to imazamox. Our results are consistent with a previous study that proposed the levels of resistance endowed by His197, Ser197 or Thr197 alleles at the whole plant level would be relatively high for sulfonylureas and moderate for imidazolinones, while no resistance was conferred to triazolopyrimidines [26].

While the exact binding mode of triazolopyrimidines remains unknown, it is known that residue Pro197 is part of the binding site for sulfonylureas and has no direct interactions with imidazolinones [18]. Amino-acid substitutions at codon 197 are thus expected to destabilize the binding of sulfonylureas [18]. This is in agreement with our observing dominant resistance to tribenuron associated to all ALS alleles bearing a mutation at codon 197 we studied. Amino-acid substitutions at codon 197 have been proposed to obstruct the entry of imidazolinones in the tunnel where the herbicide binding site lies, with the efficacy of the obstruction depending on the substituted amino-acid side-chain [18]. This is a likely explanation to the differences in sensitivity to imazamox observed among the ALS alleles bearing a mutation at codon 197 studied here. Our observing no substantial resistance to florasulam endowed by these alleles suggests that Pro197 may not be part of the binding site for triazolopyrimidines. Herbicide efficacy in inhibiting ALS activity depends on the access and fitting of the herbicide molecule in its binding site into the substrate access tunnel [18]. From our results, it seems likely that the amino-acids substituted at codon 197 in the ALS alleles we studied have no or little effect in obstructing florasulam access to its binding site.

4.1.3. Other resistance mechanisms?

Previous studies considering target-site resistance to ALS inhibitors concluded that resistance varies according to the herbicide, the mutant ALS allele and the species [8,20]. Our study revealed resistance also varies with the individual plant. Part of this variation can be explained by the homozygous mutant or heterozygous mutant status of the plants. When mutant ALS alleles are partially dominant, homozygous mutant plants are expected to be more resistant than heterozygous plants. This is consistent with our observing lower proportions of resistant plants and higher proportions of moderately resistant or sensi-
tive plants among heterozygous plants than among homozygous mutant plants (Table 3). Yet, plants with the same genotype at ALS did not always display the same phenotype. For instance, plants containing Ser\textsubscript{197} ALS allele(s) could be resistant, moderately resistant or sensitive to florasulam, or plants containing one His\textsubscript{197} ALS allele could be resistant, moderately resistant or sensitive to imazamox (Table 3). This clearly suggests that mutant ALS alleles alone do not explain all the variation in sensitivity we observed. The most likely hypothesis explaining the variation in sensitivity not due to mutant ALS alleles is the occurrence of other mechanisms not related to ALS (non-target-site mechanisms) that would also reduce the sensitivity of \textit{P. rhoeas} plants to ALS inhibitors. The existence of non-target-site-based resistance mechanisms and of the associated variation in sensitivity in \textit{P. rhoeas} is further supported by our identifying three resistant or moderately resistant plants not carrying mutant ALS allele(s) (Table 3). Considering all plants analysed, variation in sensitivity among plants with identical genotypes at ALS was observed for 420 plants out of 729 (Table 3). This suggests non-target-site mechanisms could be widespread in \textit{P. rhoeas}. Occurrence of non-target-site mechanisms endowing resistance to ALS inhibitors has rarely been reported, and mostly in grass weeds [13,21–23]. To our knowledge, it had been reported to date in a single other dicotyledonous species, \textit{Sinapis arvensis} L. [24]. Whether the rarity of the reported non-target-site-based resistance cases in dicotyledonous weeds accurately reflects the situation in the field or is merely due to target-site-based resistance being both widespread and far easier to study [19] remains to be investigated. Anyway, it is quite likely that a diversity of combinations of mutant ALS allele(s) and of non-ALS-based resistance mechanisms in individual plants from the self-incompatible \textit{P. rhoeas} species is responsible for the variation in sensitivity we observed among plants.

4.2. Detection of target-site resistance to ALS inhibitors in \textit{P. rhoeas}

Given the few existing alternatives to ALS inhibitors for effective control of \textit{P. rhoeas} in cereals, rapid, reliable and proactive methods are needed to detect resistance to ALS inhibitors as early as possible, so as to prevent its spread. Bioassays using whole plants [41] or germinated seeds [42] have been developed to detect resistance to ALS inhibitors in \textit{P. rhoeas}. Yet, they are time- and space-consuming, and require viable, non-dormant seeds. Thus, and because ALS-based resistance is considered the major mechanism of resistance to ALS inhibitors, the techniques most broadly used for resistance diagnosis are molecular assays targeting resistant ALS alleles [19,27,37].

A PCR-RFLP assay targeting substitutions at codon Pro\textsubscript{197} had previously been developed in \textit{P. rhoeas}. It uses a restriction enzyme which recognition site encompasses not only the two first nucleotides in codon Pro\textsubscript{197}, where nucleotide substitutions do cause amino-acid replacements (Table 2), but also the following four nucleotides, where nucleotide substitutions leave codon Pro\textsubscript{197} unchanged [25]. There is therefore a significant risk for false detection of resistant alleles using this assay. dCAPS assays such as those we developed herein are therefore the only molecular method available so far that can be used with basic molecular biology equipment to detect mutations at given codons in \textit{P. rhoeas} ALS without preliminary sequence data and without the risk of false detection of mutant ALS alleles.

4.3. Redundancy in the evolution of resistance in \textit{P. rhoeas}

Our study characterised the diversity of the ALS haplotypes present in five populations of \textit{P. rhoeas} by sequencing a fragment of the ALS gene encompassing codon 197 in a total of 628 individual plants. Although the fragment sequenced was only 269-bp long, we identified up to three different haplotypes bearing a given mutation at codon 197 (e.g., Ser\textsubscript{197} or Thr\textsubscript{197} haplotypes, Table 4). The different mutant haplotypes bearing a given mutation at codon 197 clearly evolved from distinct wild-type Pro\textsubscript{197} haplotypes by independent mutation events. Although their frequencies varied substantially among populations, identical haplotypes encoding both wild-type and mutant ALS were observed in different populations up to 425 km apart. It may be that haplotypes we found identical actually differ by mutations located outside the a 269-bp fragment we sequenced. Else, this raises questions about the origin of these haplotypes, to wit, a single appearance followed by dispersal versus multiple, independent local appearances. In plants, genes are dispersed by seeds and pollen. Natural seed dispersal in \textit{P. rhoeas} has a peak at 0.5 m and a mean distance of 1.1 m [43]. Seeds are easily sorted from cereal seeds due to their very light weight, and are unlikely to be efficiently dispersed by human action. The pollen of \textit{P. rhoeas} is dispersed by insects, and particularly insects in the \textit{Apidae} family (bees). Studies investigating pollen foraging in \textit{Apidae} indicated that, although pollen can be foraged up to 15–20 km from the nest, the mean flight distance between two plants is of the order of a few metres [44–46]. This data suggest that the capacity for \textit{P. rhoeas} to disperse genes across long distances is restricted. Furthermore, the populations studied herein were collected only four or six years after the first case of resistance to ALS inhibitor was reported in Italy (in 1998 [44]), when resistance to ALS inhibitors in \textit{P. rhoeas} was not widespread. Even considering that plants issued from pollen or seed carrying mutant ALS alleles would have been strongly advantaged by ALS inhibitor treatments, it is therefore not conceivable that gene flow can explain the occurrence of identical mutant ALS haplotypes in populations separated by an average distance of 317 km, with a minimum distance between two populations of 20 km. At the time scale we considered, these populations can thus be considered genetically isolated, with each one evolving according to its own evolutionary trajectory. Identical wild-type haplotypes shared among distant populations thus reflect ancient gene flow. On the other hand, a single \textit{P. rhoeas} plants is able to produce thousands of seeds (up to 100,000 seeds per plant [47]). This makes the probability for the appearance of one or even several mutant, resistant ALS allele(s) in a single field, where hundreds or thousands of plants are often present, high. Thus, data on the biology of \textit{P. rhoeas} strongly support the identical mutant haplotypes observed in the different populations we studied having evolved by multiple, independent appearances from identical wild-type haplotypes originally present in the populations investigated.

Evolution of target-site-based resistance to ALS inhibitors in \textit{P. rhoeas} in all populations studied occurred by selection for the same amino-acid replacements in ALS several times (Table 4). Given that variation in sensitivity among plants with the same genotype at ALS was observed in all populations studied, it seems that non-ALS-based resistance mechanisms might have evolved in a similar fashion. Thus, herbicide resistance in \textit{P. rhoeas} likely evolved according to a replicated pattern, that is, via redundant evolution [48]. This could be expected in geographically isolated plant populations undergoing similar selective pressures, the pressure here being the application of ALS inhibitors (mostly sulfonylureas, Table 1).

As resistance to a given ALS inhibitor in \textit{P. rhoeas} is endowed by different ALS alleles and possibly also by different genes endowing non-ALS-based resistance (Table 3), similar resistant phenotypes can be produced by different genotypes. Resistance in \textit{P. rhoeas} is thus also characterised by a high genetic redundancy, which implies cryptic genetic variation sensu Phillips [49]. This is of applied significance for non-ALS-based resistance, which can confer resistance to herbicides with different modes of action [8]. If \textit{P. rhoeas} populations were to exchange migrants, then mixing of
the different local non-ALS-based resistance genes could generate new resistant genotypes, and likely novel patterns of resistance at the individual plant level. Studies conducted at a more local level (neighbouring or adjacent fields) and identification of the genes involved in non-ALS-based resistance would help clarifying this point and defining the spatial scale where resistance should be managed in P. rhoeas.

From a more fundamental point of view, our results underline the need to consider weed adaptation at two nested, interacting geographical scales: the individual field, where adaptation to agricultural practices occurs, and likely a broader geographical scale where adaptive genes would be exchanged among weed populations according to the biology of the weed species and the landscape structure.

5. Concluding remarks

This work clearly demonstrated that the genetic basis for resistance to ALS-inhibiting herbicides in P. rhoeas populations we studied was far from simple. This clearly refutes the widespread misconception that the selection of a single ‘resistant biotype’ is responsible for the resistance observed in a given field. From a practical point of view, it appears that ALS alleles bearing a mutation at codon Pro197 represent the vast majority of the resistant P. rhoeas alleles selected for by ALS inhibitors in P. rhoeas. This should facilitate high-throughput genetic analysis of single nucleotide polymorphisms: experimental applications, and which would increase the capacity for resistance detection.

Another significant result from this study is our observing that part of the resistance to ALS inhibitors observed in P. rhoeas is most likely due to non-ALS-based mechanisms. This clearly needs to be further investigated. Indeed, although the variation in sensitivity we observed among plants suggests a number of plants could contain non-ALS-based resistance mechanisms besides mutant ALS alleles, the importance of the role played by non-ALS-based mechanisms in resistance to ALS inhibitors in P. rhoeas remains unknown. We identified very few resistant plants not containing mutant ALS alleles. Yet, the prevalence of non-ALS-based resistance mechanisms in P. rhoeas could be underestimated because these mechanisms could be present in plants also containing mutant ALS alleles. The presence of mutant ALS alleles would mask that of non-ALS-based resistance mechanisms in resistant plants. Furthermore, while non-ALS-based resistance mechanisms can confer resistance to herbicides with targets other than the ALS [8], they cannot be diagnosed to date. Only by identifying genes involved in non-ALS-based resistance, characterising their associated cross-resistance patterns and assessing their prevalence will a full understanding of resistance to ALS inhibitors in P. rhoeas emerge.

Acknowledgment

This work was supported by DuPont Solutions (Puteaux, France).

References