Characterisation of the acetolactate synthase (ALS) gene of *Raphanus raphanistrum* L. and implications for weed management

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Summary The ALS gene of susceptible and resistant wild radish (*Raphanus raphanistrum* L.) plants was sequenced and found to consist of 1758 base pairs, with 81 base polymorphisms. Two adjacent nucleotide polymorphisms accounted for three possible amino acids, indicating there are at least three alleles of the ALS gene in *R. raphanistrum*. Only seven of the 81 mutations resulted in amino acid changes, five of which occurred randomly amongst the ALS resistant and susceptible populations. The other two mutations were observed only in the ALS resistant populations. Three of the four resistant populations examined had a mutation which disrupted the Pro codon in Domain A, a conserved region widely reported as conferring resistance to sulfonylurea herbicides in weeds. The fourth resistant population had a mutation that caused a Trp → Leu substitution in Domain B that is apparently associated with broad resistance to all four classes of ALS inhibiting herbicides. Some plants were heterozygotes for the resistant ALS gene allele characterised. Implications of the findings for management of *R. raphanistrum* are considered.

Keywords Herbicide resistance, point mutation, target site, ALS inhibitor, PCR, cross-resistance.

INTRODUCTION

Several related classes of herbicides are known to inhibit ALS, such as the sulfonylureas, imidazolinones, triazolopyrimidine sulfonanilides and pyrimidinyl oxybenzoates. Biotypes in at least 20 monocots and 44 dicot species, including *R. raphanistrum*, have naturally evolved resistance to several of the ALS inhibiting herbicides, prior to any selection by these herbicides.

Resistance in many cases has been attributed to single point mutations, which can occur at multiple sites in the ALS gene, resulting in a variable pattern of cross-resistance between the classes of ALS inhibitors (Shaner 1999). Base changes in at least four protein domains have been associated with *in vivo* resistance in field plants (Wright *et al.* 1998). The most common mutation in biotypes selected by sulfonylureas is in the highly conserved Domain A site that codes for 13 amino acids, where any alteration of the codon for Pro confers resistance, primarily to the sulfonylureas and triazolopyrimidines (Guttierii *et al.* 1992). A Trp → Leu mutation in Domain B has been associated with broad cross-resistance to representatives of all four families of ALS inhibiting chemicals (Bernasconi *et al.* 1995, Woodworth *et al.* 1996a). In Domain C an Ala → Thr mutation appears to confer resistance only to imidazolinones (Bernasconi *et al.* 1995), whilst an Ala → Val substitution in Domain D also appears to confer broad cross-resistance (Woodworth *et al.* 1996b).

The objective of the reported work was to characterise the ALS gene of some field populations of *R. raphanistrum* and search for known sequence mutations (Domains A to D) associated with ALS type resistance.

MATERIALS AND METHODS

Seed samples from fields located throughout southern Australia, consisted of four field-selected accessions of *R. raphanistrum* and two canola (*Brassica napus* L.) cultivars, known to be resistant to sulfonylurea herbicides (Table 1). Ten plants were germinated for each of the four resistant *R. raphanistrum* populations and five plants for 28 non-resistant populations.

Alignment was undertaken of the DNA and amino acid information of ALS gene sequences for wall cress (*Arabidopsis thaliana* (L.) Heynh.), *B. napus*, tobacco (*Nicotiana tabacum* L.), kochia (*Bassia scoparia* (L.) Schrad.), and red algae (*Porphyra purpurea* (Roth) C.Agardh). Conserved homologous regions in the gene of about 1800 bp were targeted for the design of three sets of primers for optimal PCR (Table 2).

Duplicate leaf sections were excised and ground in liquid nitrogen in Eppendorf tubes, suspended in extraction buffer, incubated, then extracted twice with an equal volume of chloroform:isoamyl alcohol. Total nucleic acids were precipitated and the pellet resuspended in TE buffer, treated with RNase A, twice extracted with an equal volumes of chloroform:isoamyl alcohol and resuspended in TE buffer.

Each PCR reaction consisted of deoxynucleotides (dATP, dTTP, dGTP, dCTP), primer pairs, genomic DNA and Taq DNA Polymerase in a buffer. Amplified DNA fragments were visualised by electrophoresis. The PCR products were purified for sequencing by precipitation using an equal volume of PEG

precipitation mix and resuspended in TE buffer. Full methods are given in Tan and Medd (2002).

RESULTS AND DISCUSSION

The coding sequence of the ALS gene for 12 plants of *R. raphanistrum* was found to be 1758 bp with 81 base polymorphisms, 74 of which resulted in neutral changes (Tan and Medd 2002). These diverse polymorphisms are probably the result of *R. raphanistrum* being an outcrossing species. In contrast, no neutral polymorphisms were observed in 530 bases of the ALS gene among four biotypes of Indian hedge mustard (*Sisymbrium orientale* L.), (Boutsalis *et al.* 1999), possibly because it is self-fertilising.

Mutations found in susceptible and resistant biotypes There were seven loci where base changes resulted in amino acid changes in the *R. raphanistrum* ALS gene (Table 2). Five of these amino acid polymorphisms at nt 823, 1015, 1034, 1079 and 1080 appeared to be uncorrelated with the herbicide resistance (Table 3).

Alignment of the *R. raphanistrum* ALS sequences with those of *B. napus*, *N. tabacum*, *A. thaliana*, *B. scoparia* and *P. purpurea* revealed that the mutation at nt 823 of *R. raphanistrum* resided in a highly conserved region of 15 amino acids, QMLGMHGTVYANYAV. The base, Ala, was found in all of the documented ALS genes, as well as in the ALS gene in the chloroplast genome of *P. purpurea.* Base polymorphism at nt 823 in *R. raphanistrum* ALS sequences was T → G, which caused the substitution of Ser_{275} by Ala (Table 2). Ser was present in a much higher ratio (3/4) than Ala, and thus appeared to be the native amino acid at this locus for the species. The population 161653 had both polymorphisms at this locus.

Polymorphisms at nt 1015 involved $A_{1015} \rightarrow G$ causing the substitution of Lys by Glu**.** As for changes at nt 823, both base polymorphisms occurred in population 161653 and as well in 161789, 161654 and 161825.

Another polymorphism, $C_{1034} \rightarrow G$ involved the substitution of Ala by Gly (Table 3), where the Ala residue was observed in all but two plants from the same population 161829. The amino acid residue at this site was found to be variable among the documented ALS genes (Ala for *B. scoparia*, Ala and Arg for *B. napus*, Glu for *N. tabacum* and Phe for *P. purpurea*).

Base polymorphisms were also observed at adjacent positions at nt 1079 and nt 1080. A polymorphism involving one base change, $G_{1079} \rightarrow A$ and another involving adjacent base changes, $G_{1079}C_{1080} \rightarrow AA$ were observed (Table 3). These base polymorphisms resulted in three possible amino acids, Ser, Asn and Lys in this codon. Alignment of the amino acid sequences of the ALS genes found that this codon site was very variable, with Asn for *B. scoparia*, Thr for *N. tabacum*, and Ser and Glu for *B. napus*. The three possible amino acids found at this position in *R. raphanistrum* may thus be attributed to three alleles of the ALS gene, and could possibly be members of a multi-gene family, as is the case in *B. napus* (Rutledge *et al.* 1991).

Table 1. Plant population collection details.

Population number	Resistance status[1]	Locality
R. raphanistrum		
161631	S	Toolleen, Vic.
161632	S	Charlton, Vic.
161639	S	Sea Lake, Vic.
161647	S	Newstead, Vic.
161650	S	Mullewa, WA
161651	S	Walkaway, WA
161653	S	Merredin, WA
161654	R	Coorow, WA
161657	S	Dimboola, Vic.
161659	S	Bundoora, Vic.
161661	S	Nuriootpa, SA
161662	S	Balhannah, SA
161789	S	Tomingley, NSW
161790	S	Forest Reefs, NSW
161793	S	Cowra, NSW
161824	S	Raglan, NSW
161825	R	Mukinbudin, WA
161826	S	Horsham, Vic.
161829	R	Yuna, WA
161831	S	Wagga Wagga, NSW
161833	S	Avondale, WA
161836	S	Wongan Hills, WA
161837	S	Mullewa, WA
161839	S	Giles Corner, SA
161840	S	Giles Corner, SA
161841	S	Springton, SA
161842	S	Springton, SA
161843	S	Piccadilly Valley, SA
161855	R	Mullewa, WA
161857	S	Burracoppin, WA
161941	S	Bakers Swamp, NSW
161944	S	Noonbinna, NSW
B. napus		
162159	R	cv. 44C71, ITCANQQ1
162160	R	cv. 44C72, ITCANQQ2

[1] S indicates ALS-susceptible field populations and R indicates populations that contain ALS resistant individuals, based on field observations.

Table 2. Primers for ALS gene of *R. raphanistrum*.

Primers	Sequence 5' → 3'
ALS1F	TTCRTCTCCCGMTACGCTCCC
ALS1B	CAARCTGYTGCTGAATATC
ALS2F	GATGTTCCTAAGGATATTC
ALS2B	CTGATGYTGYCCAACACC
ALS3F	GGRGAAGCCATTCCTCC
ALS3B	TCARTACTWAGTGCKACCATC

Table 3. List of nucleotide polymorphisms (bold font) observed in the ALS gene sequences of *R. raphanistrum* plants that resulted in amino acid substitutions.

Nucleotide position	Codon - amino acid
Mutations in both susceptible and resistant biotypes	
823	**T**CT-Ser
	GCT-Ala
1015	**A**AG-Lys
	GAG-Glu
1034	G**C**A-Ala
	G**G**A-Gly
1079	A**G**C-Ser
	A**A**C-Asn
1079, 1080	A**AA**-Lys
Mutations occurring only in resistant biotypes	
334	**C**CT-Pro
	SCT-Pro and Ala
	RCT-Ala and Thr
	MCT-Pro and Thr
1466	T**G**G-Trp
	T**T**G-Leu
	T**K**G-Trp and Leu

Mutations found only in resistant biotypes In *R. raphanistrum*, Domain A was located at nt 316 to 354, Domain B at nt 1465 to 1476, Domain C at nt 88 to 144 and Domain D at nt 358 to 375. Mutations in Domains A or B were revealed in the four ALS resistant *R. raphanistrum* populations tested, whereas no mutations were found in Domains C or D (Table 3).

Sequencing analyses of *R. raphanistrum* ALS gene revealed base changes at nt 334 in Domain A of two plants from one population 161654 and one from 161829. These involved the modification of the first nucleotide of the Pro codon from C to G or A, resulting in an amino acid substitution of Pro by Ala or Thr respectively (Table 3). One plant each from two of the resistant populations (161654 and 161829) had both the mutated and the native base at this locus, suggesting the plants were heterozygotes. Previous studies have indicated the same Pro codon is substituted in some resistant biotypes. Mutations in the 1st or 2nd base of the Pro codon to any of the six possible combinations has been shown in prickly lettuce (*Lactuca serriola* L.), (Guttieri *et al.* 1992), *B. scoparia* (Guttieri *et al.* 1995) and *S. orientale* and Mediterranean turnip (*Brassica tournefortii* Gouan) (Boutsalis *et al.* 1999) to confer resistance to sulfonylureas. It also appears that the pattern of cross-resistance to other ALS inhibitor herbicides is determined by the specific amino acid substitution. Point mutations in the 1st or 2nd base of the Pro codon in Domain A may be the molecular basis for putative herbicide resistance in several of the *R. raphanistrum* plants used in this study.

A different mutation at $G_{1466} \rightarrow T$ in Domain B was found in two plants from the same population, 161825. This mutation caused the substitution of Trp by Leu. The same mutation has been observed in resistant biotypes of cockleburr (*Xanthium strumarium* L.), (Bernasconi *et al.* 1995), *Amaranthus* sp. (Woodworth *et al.* 1996), *B. scoparia* (Foes *et al.* 1999), and *S. orientale* (Boutsalis *et al.* 1999) as well as in three laboratory-generated mutants in *B. napus*, *N. tabacum* and maize (*Zea mays* L.), (Hattori *et al.* 1995, Lee *et al.* 1988, Boutsalis *et al.* 1999). Directed mutation at this site (Bernasconi *et al.* 1995) showed that this specific mutation yielded an active protein with broad herbicide tolerance. Further studies have shown that this single point mutation was sufficient to endow a broad resistance to all four classes of ALS inhibiting herbicides (Bernasconi *et al.* 1995, Foes *et al.* 1999). It is thus predicted that the Domain B mutation in population 161825 would confer the molecular basis for broad based cross-resistance patterns to the four classes of ALS inhibiting herbicides.

Implications for management of *R. raphanistrum* The identification of a mutation in Domain B in one *R. raphanistrum* population (161825) emphasises the need for discerning management of this weed, especially in designing herbicide and crop rotation strategies. A transgenic *B. napus* line (Hattori *et al.* 1995) and two imidazolinone tolerant *B. napus* cultivars (ITCANQQ1 and ITCANQQ2) (Table 1) had the same mutation as found in 161825. When grown together these related crop and weed species on occasions produce fertile hybrids through outcrossing, albeit at low frequencies (Lefol *et al.* 1997, Chevre *et al.* 2000), though hybrids are not always apparent (Rieger *et al.* 2000). Hence there is a remote possibility of resistance being transferred across species (Wetzel *et al.* 1999), but it is more probable that selection for this mutant type in *R. raphanistrum* will arise from the heavy dependence on, and poor rotation of, herbicides.

The two altered domains found in resistant biotypes of *R. raphanistrum* exist independently. Laboratory generated strains of *N. tabacum* and sugarbeet (*Beta vulgaris* L.) have been reported to have substitutions at two conserved domains on a single allele, where each mutation independently endows resistance to give a higher overall resistance (Hattori *et al.* 1995, Lee *et al.* 1988, Wright *et al.* 1998). Pollen flow between herbicide resistant biotypes of *R. raphanistrum* would present many opportunities for the natural

selection of resistant biotypes with two or more altered ALS domains on a single allele, potentially giving higher and broader resistance characteristics. Pollen transfer between *B. napus* biotypes resistant to different classes of herbicides has given rise to multiple-resistant *B. napus* hybrids (Hall *et al.* 2000).

Based on the point mutation findings outlined, there is potential to exploit molecular assays for rapid ALS resistance screening as a very important tool in the management of *R. raphanistrum* (Tan and Medd 2002).

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