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ORIGINAL ARTICLE/CONTROL

Characterization of the Acetolactate synthase (ALS) gene and Molecular Assay of Mutations Associated with Sulfonylurea Herbicide Resistance of *Monochoria vaginalis*

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Abstract

This research aims to contribute the characterization of acetolactate synthase (Ec 4.1.3.18; ALS) and the resistance mechanism by sequence analysis of ALS gene of the sulfonylurea-resisatnt and -susceptible *Monochoria vaginalis*. The ALS gene was obtained from susceptible (S) and resistant (R) *M. vaginalis* to sulfonylurea herbicides (SUs). The 815 bp the fragment and the genomic DNA sequence coding for acetolactate synthase (ALS) of S and R biotypes of *M. vaginalis* were cloned and sequenced. Nineteen clones were divided greatly into 4 groups as result of sequencing. The first group was not difference to S type, the second group was amino acid of P197S which found point mutations causing substitution of serine for proline at amino acid 197, the third group was observed greatly other part of 6 places than group 1, and the fourth group appeared the intergrade of group 1 and 3. Therefore, it could be assumed what ALS gene of various types can be one plant. The peptide of the 13 amino acid Domain A region for ALS genes from R biotype of *M. vaginalis* differed from that of the S biotype by one base substitution at proline codon of Domain A. It could also be confirmed that point mutation of serine for proline at amino acid 197.

Key words ALS, herbicide, resistance, *Monochoria vaginalis*, sulfonylurea

Introduction

Acetolactate synthase (ALS; EC 4.1.3.18), also named as acetohydroxyacid, catalyzes the first common step in the biosynthesis of the branched chain amino acids isoleucine, valine and leucine in plants (Umbarger, 1978). It is the ALS as the target site for five herbicide chemical families: SUs (Ray, 1984), imidazolinones (Shaner *et al.* 1984), triazolopyrimidines (Gerwick *et al*. 1990), pyrimidinythiobenzoates (Babczinski, 2002). It has been widely and rapidly adopted because it has the combined advantages of low use rates, low mammalian toxicity, effective, and prolonged control of broad-spectrum weeds. ALS-resistance has been described in more than 90 weed species worldwide, and this number is increasing at a linear rate (Heap, 2003).

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 *et al.* 1984). Initially, these point mutations were characterized using mutants generated in the laboratory e.g. (Haughn *et al*. 1988 and Lee *et al*. 1988) and later confirmed in field-resistant plants using biochemical methods e.g. (Bernasconi *et al*. 1995, Guttieri *et al*. 1992 and Gutieri *et al*.1996). Base changes in, at least, four protein domains have been associated with in vitro resistance in field plants (Wright *et*

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al. 1998). The most common biotypes selected by SUs is in highly conserved Domain A site that codes for 13 amino acids, where any alteration of the codon for Pro confers resistance; which is primarily due to the SUs and triazolopyrimidines (Guttieri *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 and Woodworth 1996). In Domain C, an Ala→Thr mutation appears to confer resistance only to imidazolinones (Bernasconi *et al*. 1995), while an Ala→Val substitution in Domain D is reported to confer broad cross-resistance (Woodworth, 1996), as in the case of the mutation in Domain B. Amino acid substitutions at 10 different sites within the yeast ALS protein are known to confer resistance to one or more class of ALS-inhibiting herbicide (Mazur *et al*. 1989). However, in higher plants, amino acid substitutions that confer resistance have been observed at five different sites in laboratory-generated and field mutants (Tranel *et al*. 2002). Each site is located within a highly conserved region of the ALS enzyme. Ala 122 occurs within a conserved region consisting of 18 amino acids and when substituted by Thr resistance to imidazolinone herbicides occurs (Bernasconi *et al*. 1995) Pro 197 and occurs within a conserved region of 13 amino acids (Wiersma *et a*l. 1989). Substitution of any one of a number of amino acids for Pro results in an ALS with resistance to Sus and triazolopyrimidine herbicides, and to a lesser extent, to imidazolinone herbicides (Tranel *et al*. 2002 and Devine *et al*. 2000). Ala 205 occurs within a conserved sequence of six amino acids and when substituted by Val resistance to Sus and imidazolinone herbicides occurs (Woodworth *et al* 1996). Trp 574 occurs within a conserved sequence of four amino acids. Substitution of Leu for Trp confers resistance to all three of the above chemical classes (Devine *et al* 1997). Lastly, Ser 653 occurs within a conserved region of six amino acids. Substitution of either Asp or Thr for Ser at this position confers resistance to imidazolinone herbicides (Sathasiven *et al*. 2003 and Diebold *et al* .2003). Certain mutations within ALS are more commonly reported in weed species selected in the field than are other possible mutations (Tranel *et al*. 2002). This remains to be solved but this could be associated with probably relates to differential fitness of the different mutations. For this to occur, mutations within ALS must have other effects on enzyme function. Understanding how naturally occurring modifications of ALS affect enzyme function may provide insights into the evolutionary processes that keep these mutations rare in unselected populations, but not so rare that they are rapidly selected by herbicide users (Christopher *et al.* 2005).

In paddy fields of Korea, the biotypes of SU-resisatnt *M. vaginalis* have been identified in southwestern area Korea (Kwon *et al*. 2000). These paddy fields have cultivated in monoculture rice production and have been routinely treated with a SU-based mixture for many consecutive years since 1990. Fifty percent of growth reduction dosage (GR_{50}) of imazosulfuron against the R biotype, showed a cross-resistant to the other SU herbicides, was 3,172 times higher than that of the susceptible (Kuk *et al.* 2003). The purpose of this study is to identify the characteristics of the ALS gene of *M. vaginalis* population to SUs, and the molecular assay of mutations associated with herbicide resistance.

Materials and Methods

Plant materials

Seeds of SU-resistant *M. vaginalis* were collected from experimental rice paddies of Chonnam Agricultural Research and Extension Service, Korea, in 2003. These fields had been treated with SUs-based mixtures such as pyrazosulfuron -ethyl +molinate for 11 consecutive years. And seeds of the susceptible biotype were collected from nearby areas which were never treated with herbicides. The seeds of the two biotypes were kept at 4 ºC to break dormancy for 2 month.

Genomic DNA extraction

Genomic DNA was extracted from individual plants collected from R (R1, R2, R3, R4, R5) and S biotypes of *M. vagnalis*. Duplicate sections of leaf, approximately 2 cm of each section, were excised from individual plants at the four to six leaf state of development and placed into Eppendorf tubes which were kept refrigerated prior to freezing and grinding in liquid nitrogen. The ground leaf powder was suspended in 400 μl of extraction buffer {Grind buffer; 4Homogenization buffer (Tris-Cl 0.03 M, pH 8.0, NaCl 0.7

M, Sucrose 0.2 M, EDTA 0.01 M) + 1 Phage lysis buffer (0.5 M Tris-HCl, pH 9.2, EDTA 0.25 M SDS 2.5% [w/c])} and incubate at 65 ºC for 30 min. The solution was then mixed with 13 μl K-acetate 3M (pH 4.7) on ice for 30min and centrifuged for 20 min 4ºC. The supernatant was mixed with an equal volume of isopropanol, incubated for 10 min and centrifuged for 5 min at room temperature. The precipitate was rinsed with 70% ethanol, dried briefly and dissolved 30 μl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

DNA amplification, cloning and sequence analysis

Fragments of ALS gene were amplified from the extracted genomic DNA of two biotypes, respectively, by *Taq* ploumerase (Pfu) with 34cycle of 30s at 94 ºC, 30s at 58ºC, and 60s at 72ºC. A 815 bp fragment of the *M. vaginalis* ALS gene was amplified by PCR described by Guang-Xi Wang and Ying Lin using their 2 primers, M13 F and M13R of TOPO-vector primer. PCR products were visualized in agarose gels and extracted using the QIAquick Gel Extraction Kit (Quagen, Cat No. 28704) based methods. Amplified fragments were sub-cloned to PCR-TOPO and transformed into TOPO E.*coli*. For sequencing, plasmids were isolated using the Promega plasmid isolation kit (Wizard® plus SV Minipreps, Cat. No. A1460) according the manufacturer's protocol. We picked up 24clones from each biotype for sequence analysis with ABI 1.0 genetic analyzer.

Results and Discussions

PCR amplification and identification of ALS gene of *M. vaginalis*

To identify the presence of ALS gene in WT of *M.vaginalis* about 815 bp, the central region of ALS gene, PCR was amplified using primer (Fig. 1, Table 1). The 4 clones from 1 species of S biotype and 15 clones from 5 species of R biotype were obtained. The amino acid sequence of ALS derived from the DNA codes was organized as FASTA format, and the file was inputted into BioEdit to carry out the alignment manually (Wang *et al.* 2004). The sequencing alignment of *M. vaginalis* ALS fragment with the other ALS of the same location fragment sequences reported in GenBank confirmed the presence of two conserved domains from A and D, in which the mutation(s) conferring resistance to ALS-inhibitors is usually located (Devine *et al*. 1997). Fig. 2 shows the alignment of sequence for 4 cloned ALS genes from S biotype of *M. vaginalis*. It reveals high homology (S1-1; 81%, 80% and 97%, S2-1; 81%, 80% and 97%, S2-2; 81%, 80% and 97%, S2-4; 80%, 79% and 95%) with of *A. thaliana* (GenBank No.NM 114714.2), *R. raphanistrum* (GenBank No. AJ344986) and *M. vaginlis* of Japan (Wang *et al.* 2004-8) between 138 and 408 amino acid residues, which cover conserved Domains A and D (Table 2.).

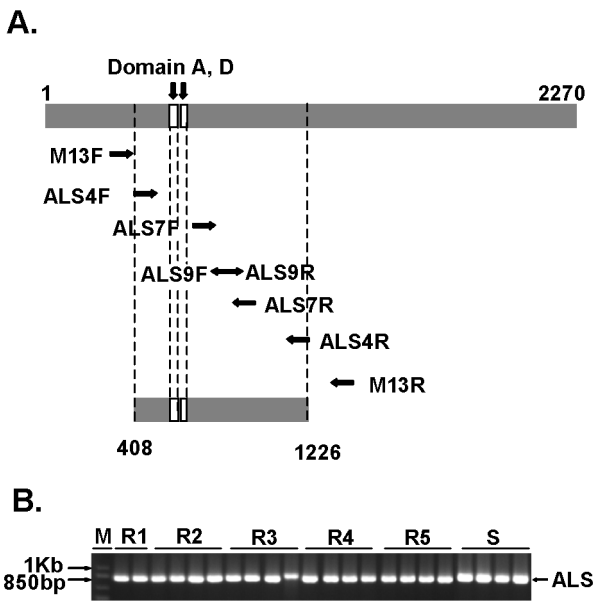

Fig. 1. Scheme of the ALS gene of *A. thaliana*. 815 bp were sequenced by genomic DNA walking of *M. vaginalis* (GenBank No. NP 190425 of *A. thaliana*). A; Location or primers ALS4F/R, ALS7F/R, ALS9F/R and M13F/R used for polymerase chain reaction analysis and sequencing is indicated by arrowheads. The white boxes, A (nt 570-609) and D (nt 613-630) represent two highly conserved domains (Boutsalis *et al*. 1999) of the ALS gene. B; B showed colony PCR of ALS gene from WT (S) and R type of *M. vaginalis*.

Table 1. List primers designed for the amplification of the ALS gene of *M. vaginalis*

Primers	Sequence
4F	5' GCAACGTGCTGCCACGTCACGAGCAGGG 3'
4R	5' ACATGAGGCTGCTTGTTCTTTCCAATCTCAGC 3'
7F	5' ATGATC GGCACTGACGCC 3'
7R	5' GAATGTCATCGACGTCGAG 3'
9F	5' GAATGTCATCGACGTCGAG 3'
M13 F	5' GTAAAACGACGGCCAG 3'
M13 R	5' CAGGAAACAGCTATGAC 3'

Fig. 2. Deduced amino acid sequences of ALS proteins from WT *M. vaginalis* (S1-1Kor, S2-1Kor, S2-2 Kor and S2-4 Kor; *M. vaginalis* of Korea) aligned with the amino acid sequences of *A. thaliana*, *Raphanus raphanistrum (R. raphanistrum)* and *M. vaginalis of* Japan. Amino acids that differ from those of *A. thaliana* ALS protein are shown. Asterisks indicate amino acids that are the same as *A. thaliana*. Two points and one point indicated insertions introduced to maximize sequence homology. Boldfaces indicate the differences between the biotypes. The proline site of Domain A (underlined) is indicated in 197.

Table 2. List of homology observed the fragment ALS genes of WT *M. vaginalis* of Korea aligned with *A. thaliana, R. raphanistrum* and *M.vaginalis* of Japan

Accession No. / clone No	*A. thaliana* (%) (# NM-190425)	*R. raphanistrum* (%) (# AJ344986)	*M. vaginalis* (%) (Jap [a])	*M. vaginalis* of Korea			
				S1-1 (%)	S2-1 (%)	S2-2 (%)	S2-4 (%)
A. thaliana	100	-	-	-	-	-	-
R.raphani strum	92	100	-	-	-	-	-
M.vaginalis-Jap [a]	81	81	100	-	-	-	-
S1-1 Kor	81	80	97	100	-	-	-
S2-1 Kor	81	80	97	99	100	-	-
S2-2 Kor	81	80	97	100	99	100	-
S2-4 Kor	80	79	95	98	98	98	100

a Refer to Wang G.X. et al. (2004).

The gene families encoding ALS genes have been characterized in *A. thaliana*, *N. tabacum, B. napus* and *Gossypium hirsutum*. *A. thaliana* contains a single ALS gene (Mazur *et al.,* 1987). *Nicotiana tabacum* contains two ALS genes that are expressed in consecutive manner (Keeler *et al*., 1993). *Brassica napus* and *G. hisutum* contain five and six ALS genes, respectively (Rutledge *et al*. 1991and Grula *et al.* 1995). The ALS genes of *B. napus* and *G. hirsutum* were demonstrated to have divergent expression patterns and have different functions (Ouellet *et al*. 1992; Grula *et al.* 1995).

Characterization of ALS gene from SU-resistant biotypes

The majority of examples of resistance to ALS-inhibiting herbicides are the result of amino acid changes within the target enzyme ALS. Mutation within ALS resulting in resistance to herbicides is known at five different sites in plants (Devine et al. 1997). Many of these carry an amino acid modification at Pro 197 within a highly conserved region of the ALS (Saarum et al.1994). It appears that any amino acid substitution at this Pro will result in an active enzyme that is resistant to herbicides (Guttieri et al.1995). NCBI research came up with fifteen clones sequenced of R type, which conformed with that sequenced SU-R biotype, in Japan. NCBI has already registered ALS genes [GenBanK No.; AB243611 (I), AB243612 (II), AB243619 (III), AB243631 (IV)]. In all plants of sulfonylurea-resistant *M. vaginalis*, sequence analysis demonstrated the alteration of a single

nucleotide substitution of ALS genes. The 19 clones obtained from among 4 clones from 1 specie of S biotype and 15 clones from 5 species R biotype. The cloned genes were classified into 4 groups for R biotypes, namely: ALS1, ALS2 and ALS3 as shown in Fig. 3. The first group nearly resembled sequence of R types that belong R3-7, R4-3 and R5-3-1. The second group is found in Fig. 3 and Table 3 showing part of P197S and belong to R1-8, R2-2, R2-5, R2-8, R4-6 and R5-1, and differed from the first group. We presumed that ALS gene to *M. vaginalis* of the Korea also has several genes that divide into 4 groups. Therefore, we can conclude that 4 plants from among the 5 plants of R biotype cause a single nucleotide substitution from P197S (CCT→TCT). In the case of the third group, it was observed that eight amino acid formed part of nucleotide substitution, Asn268Asp (AAT→GAT), Glu290Glu (CAA→GAA), Glu291Arg (CAG→CGG), Val293Ile (GTC→ATC), Ile295Leu (ATA→CTC), Gly342Ala (GGT→GCT), Pro343His (CCT→CAT) and Ala360Ser (GCT→TCC) were changed two clone ALS sequences, and belong to R2-7 and R5-2. The differences

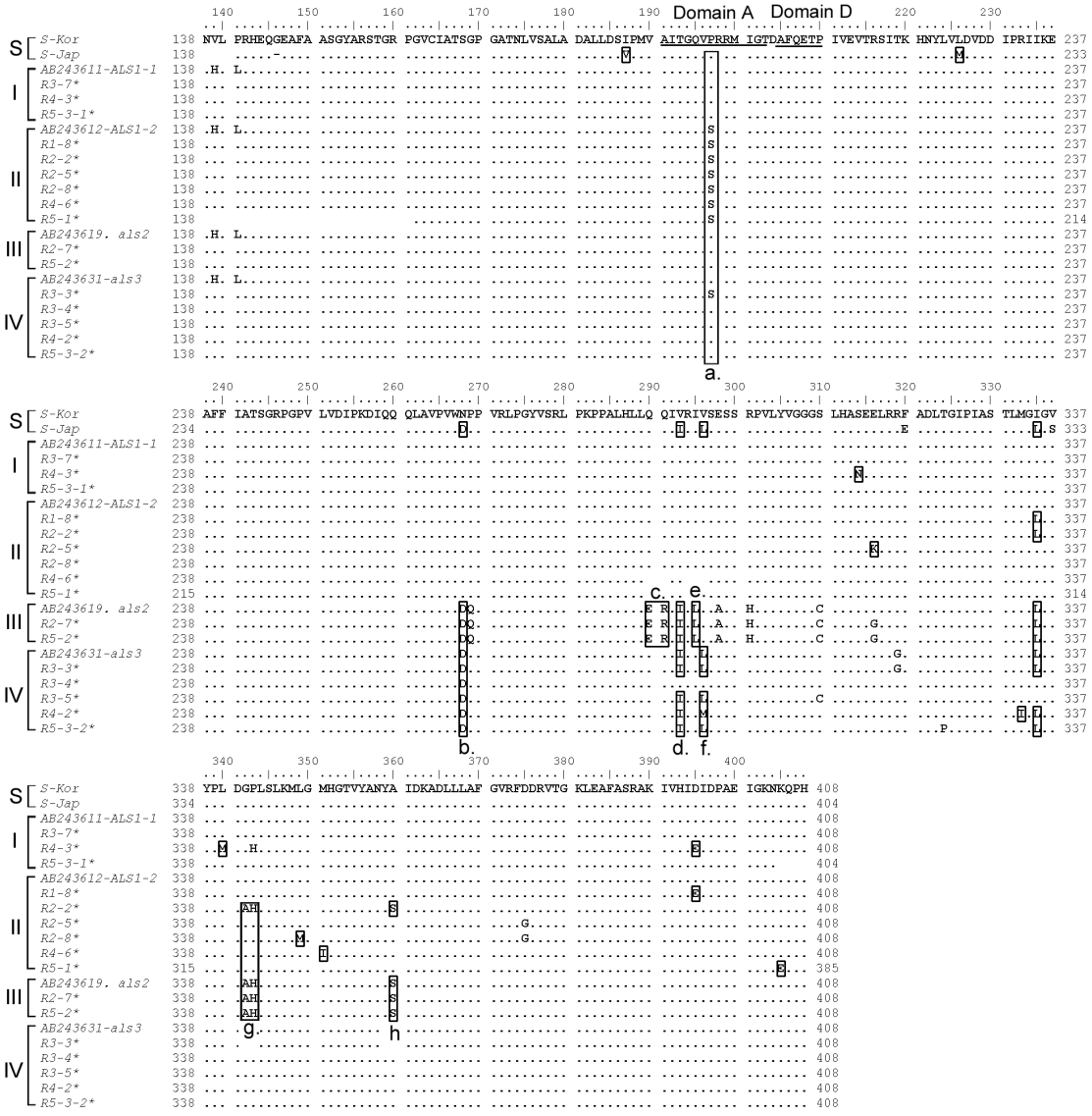

Fig. 3. Deduced amino acid sequences of ALS proteins from WT of M. vaginalis (S-Korea, S-Japan) aligned with the amino acid sequences of R type (R1-8, R2-1, R2-2, R2-4, R2-5, R2-8, R3-3, R3-4, R3-5, R3-7, R4-1, R4-2, R4-3, R4-6, R5-3-1, R5-2, R5-3-2) and results of Blast search R type of AB243611, AB243612, AB243619, and AB243631. R types divided into four groups; I; R3-7, R4-3, R5-3-1, II; R1-8, R2-2, R2-5, R4-6, R5-1, III; R2-7, R5-2, IV; R3-3, R3-4, R3-5, R4-2, R5-3-2. Boxes (a, b, c, d, e, f, g, h) indicate the differences between WT and four groups of R types.

found in groups one and two were due to multiple nucleotide substitution, but the codon for 197Pro remained unchanged. The fourth group came up with three amino acid of single nucleotide substitution, Asn268Asp, Val293Ile, and Val296Leu or Met were changed five clone ALS sequences, and go with R3-3, R3-4, R3-5, R4-2 and R5-3-2 (Fig. 3, Table 3). It is interesting to note that only R3-3 among the 5 clones of group 4 altered Pro197Ser. Therefore, it is concluded that seven colonies (R1-8, R2-2, R2-5, R2-8, R3-3, R4-6, R5-1) from the nucleotide sequences of the 39 bp Domain A region for the resistant-*M. vaginalis* biotype differed from that of the S biotype by a single base substitution at variable Pro codon of Domain A (CCT to TCT) as shown Fig. 3. However, another clones did not change Pro to Ser. It is also confirmed that all resistant biotypes of *M. vaginalis*, contained ALS genes in genome. Therefore, mutation of ALS gene known well as cause of weed herbicide in *M. vaginalis* of the Korea in this experiment, mutation of ALS attains by several single point mutations. The utmost concern of the weed molecular biology is the speedy identification of single nucleotide changes responsible for resistance without the need to sequence specific gene fragments. In this study, it is recommended to

Table 3. List of nucleotide polymorphisms observed in the ALS gene sequences of *M. vaginalis* plants that resulted in amino acid substitutions in the gene. A single base mutation in various parts of an amino acid difference between ALS gene from the WT (S) and four groups of R type of *M. vaginalis*

Classification	Clone names	#Accession No.	Homology (%)	Location	Observed Changes	
					Amino acid	Nucleotide
I	R3-7	AB243611	97	a	-	-
	R4-3		96			
	R5-3-2		96			
II	R1-8	AB243612	98	**a**	P-197-S (Pro→Ser)	CCT→**T**CT
	R2-2		97			
	R2-5		99			
	R2-8		98			
	R4-6		98			
	R5-1		99			
III	R2-7	AB243619	99	**b**	N-268-D (Asn→Asp)	AAT→GAT/c
	R5-2		99	**c**	QQ-290/291-ER (Gln Gln→Glu Arg)	CAACAG→GAACGG
				d	V-293-I (Val-lle)	GTC→ATC
				e	I-295-L (Ile-Leu)	ATA-CTC
				g	GP-342/343-AH (Gly Pro→Ala His)	GGTCCT→GCTCAT
				h	A-360-S (Ala-Ser)	GCT-TCC
IV	R3-3	AB243631	98	**b**	N-268-D Asn→Asp	AAT→GAT/C
	R3-4		98			
	R3-5		96	**d**	V-268-I (Val-Ile)	GTC→ATC
	R4-2		98	**f**	V-296-L/M (Val-Leu/Met)	GTG→CTG/ATG
	R5-3-5		96			

use of genomic DNA to produce distinct, precise modifications within the ALS gene of this *M. vaginalis* to S biotype and the molecular assay of mutations associated with herbicide resistance. Such approaches research us to distinguish between two biotypes from sequencing.

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물달개비의 Acetolactate synthase (ALS) 유전자의 특성과 Sulfonylurea 제초제 저항성과 관련 돌연변의 분자생물학적 접근

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요 약 본 연구는 한국에서 발생하고 있는 설포닐우레아계 제초제 저항성 및 감수성 물달개비의 ALS 염기서열 분석에 의한 ALS 유전자의 특성과 제초제 저항성 메카니즘을 구명하기 위하여 실시하였다. 감수성 및 저항성 두 계통의 biotype에서 815개의 염기서열을 분석하였다. 분석된 염기서열에서 감수성 biotype으로부터 4개 clone, 저항성 계통으로부터 15개 clone, 총 19개의 clone들을 크게 4 group으로 분류할 수 있었다. 첫 번째 group은 저항성 및 감수성 biotype의 ALS 유전자 염기서열이 차이가 없었으며, 두 번째 그룹은 아미노산 197번째의 proline이 DNA의 점돌연변이(point mutation)에 의해 serine으로 변화된 부분이다. 세 번째 group은 6곳에서 점돌연변이에 의한 아미노산치환이 발생하였으며, 네 번째 group는 첫 번째 group과 세 번째 group 중간적 특성으로 3곳에서 염기서열 점돌연변이에 의해 아미노산 치환이 있었다. 따라서 하나의 물달개비 식물체에서 여러 가지 형태의 ALS 유전자가 존재할 수 있으며, 물달개비 저항성 biotype의 ALS 유전자는 Domain A 부분에 있는 아미노산 197번째 proline이 염기서열 변화에 의해 serine으로 돌연변이 되었다.

색인어 물달개비(*Monochoria vaginalis*), 제초제, 저항성, ALS, sulfonylurea