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Transformation a mutant *Monochoria vaginalis* acetolactate synthase (ALS) gene renders *Arabidopsis thaliana* resistant to ALS inhibitors

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ABSTRACT

Acetolactate synthase (ALS) genes from *Monochoria vaginalis* resistant (R) and susceptible (S) biotypes against ALS inhibitors found in Korea revealed a single amino acid substitution of Proline (CCT), at 169th position based on the *M. vaginalis* ALS sequence numbering, to serine (TCT) in conserved domain A of the gene (equal to the proline 197 in *Arabidopsis thaliana* ALS gene sequence). *A. thaliana* plants transformed with the single mutated (Pro_{169} to Ser) *M. vaginalis* ALS gene (including transit signal peptide) showed cross-resistance patterns to ALS-inhibiting herbicides, like as sulfonylurea-herbicide bensulfuron methyl (R/S factor of 9.5), imidazolinone-herbicide imazapyr (R/S factor of 5.1), and triazolopyrimidine-herbicide flumetsulam (R/S factor of 17.6) when measuring hypocotyls' length of *A. thaliana*. The ALS activity from the transgenic *A. thaliana* plants confirmed the cross-resistance pattern to these herbicides like as R/S factor of 8.3 to bensulfuron methyl, 2.3 to imazapyr, and 13.2 to flumetsulam.

1. Introduction

Acetolactate synthase (ALS, EC 2.2.1.6.) catalyzes the first step in the biosynthesis of branched-chain amino acids (valine, leucine, and isoleucine) in plants, fungi, and bacteria. The branched-chain amino acids are synthesized in both microorganisms and plants, but not in animals. This makes ALS as an attractive target site of five herbicide families known as ALS inhibitors: sulfonylureas (SU), imidazolinones (IM), triazolopyrimidines (TP), pyrimidinyl thiobenzoates (PT) and sulfonyl amino carbonyl triazolinones (SC). During the past 20 years, the worldwide use of these herbicides has been rapidly increased because of their advantages, e.g., efficacy at low application rates (grams per hectare) against various weed species with low mammalian toxicities. Unfortunately, use of ALS-inhibiting herbicide use has been plagued by the evolution of herbicide resistance. The first case of resistance was reported in *Lactuca serriola* [1,2] after only 5 years use of the chlorsulfuron commercialized in 1982. Since then, the number of species resistant to the herbicide has been steadily increased (to date 101 species) [3,4] mainly due to the repetitive use of the herbicides and monoculture cropping systems.

Sulfonylurea herbicide-based mixtures have been widely used to control weeds in rice paddies in Korea. As a result, the first recorded resistant weed species, *Monochoria korsakowii*, was identified in the Sousan reclaimed area of the western coast [5]. SU-resistant biotypes of *M. vaginalis* were identified from paddy fields in Chonnam province of southwestern Korea; these paddy fields were in monoculture rice production and have been routinely treated with a sulfonylurea herbicide-based mixture for 8 consecutive years since 1990 [6]. *M. vaginalis* is one of the most common annual weeds grown in rice paddy field in Korea and the resistant biotypes were nation-widely distributed at present time [3,4]. The molecular investigation of the SU-resistant biotypes of *M. vaginalis* was performed and found a mutation at the position of 197, proline (Pro_{197}), in the ALS gene to serine or leucine in the sulfonylurea resistant biotypes [7,8].

In plants, any alterations of six amino acids (Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Trp_{574}, and Ser_{653} numbered based on the ALS gene in *A. thaliana*) in the ALS gene was known to confer ALS inhibitor resistance [9,10]. Also, eight different amino acid substitutions for Pro_{197} and two for Ser_{653} have also been reported in ALS inhibitor-resistant populations [11]. Most of the ALS inhibitor-resistant populations have been discovered by whole plant responses and any substitutions in ALS gene was reported as results from the comparing gene sequences between resistant and susceptible biotype. However, the expression of ALS inhibitor-resistant gene in transgenic plants might be required for verifying the role of these substitutions in ALS gene.

We previously reported the resistance to acetolactate synthase inhibitors in a biotype of *M. vaginalis* discovered in Korea. The resistant factors and patterns of *M. vaginalis* to ALS inhibitors were investigated in a greenhouse condition. Also, the pI_{50} value of ALS inhibitors to the wild/resistant type of *M. vaginalis* was investigated in vitro aceolactate synthase activity, and in vivo acetolactate accumulation in *M. vaginalis* treated with five different ALS-inhibiting herbicides, bensulfuron-methyl, pyrazosulfuron-ethyl, cyclosulfamuron, imazaquin, and flumetsulam [6].

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In this study, we investigated the genetic differences between susceptible and resistant biotypes of *M. vaginalis* against ALS-inhibiting herbicides in Korea. Also, ALS gene from resistant biotype of *M. vaginalis* was cloned and transformed to the Arabidopsis thaliana plants. The role of ALS-resistant gene was confirmed by transgenic *A. thaliana* plants for investigating resistance factor and pattern to the ALS inhibitors.

2. Materials and methods

2.1. Plant materials and genomic DNA preparation

Seeds of SU-resistant *M. vaginalis* were collected from in Chonnam Agricultural Research and Extension Service, Korea. The fields had been treated with sulfonylurea herbicide-based mixtures of bensulfuron-methyl/molinate and pyrazosulfuron-ethyl/molinate for 8 consecutive years, hereafter referred to as resistant biotype (R). Seeds of SU-susceptible seeds were harvested from the experimental field without any SU-herbicide treatments in Korea Research Institute of Chemical Technology, hereafter referred to as susceptible biotype (S). To break dormancy, these seeds were water-soaked and stored in a cold room at 4 °C, and water was changed twice monthly to remove any germination inhibitors. Seeds from the R and S of *M. vaginalis* were germinated on 0.5× Murashige-Skoog (MS) medium containing 0.8% (*w*/*v*) agar for genomic DNA preparation. Growth condition was 25 °C/16 h, light period. About 0.1 g of leaves collected from the R and S of *M. vaginalis* plant leaves were ground to fine powder under liquid nitrogen using mortar and pestle, respectively. Genomic DNA was isolated using a DNeasy Plant Mini Kit (Qiagen).

2.2. Isolation and sequencing of M. vaginalis ALS gene

To isolate the *M. vaginalis* ALS genes (*MvALS*) by PCR amplification, primer set was designed from Gen Bank Accession AB243613 of *MvALS* nucleotide sequence. Their sequences were 5′-ATGGCTGCTTCGAAGCCCTCTCCATT-3′ (forward) and 5′-ACTAGTGCACTGTGCTCCCATCTCCAT-3′ (reverse). PCR amplification was performed in a total volume of 20 μL containing 2.5 units (U) of TaKaRa EX *Taq* DNA polymerase (Takara Bio Inc., Japan), reaction buffer, 200 μM dNTPs, 20 pmol primers, and 25 ng of total DNA from each R or S biotypes. The PCR conditions were as follows: denaturing at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. Amplified fragments were cloned into pGEM-T Easy plasmid vector (Promega, WI, USA), transformed to *Escherichia coli* (strain JM109, Promega, WI, USA), and analyzed sequences. The plasmids were extracted using a Plasmid Mini kit (RBC Bioscience, Taipei, Taiwan). Sequencing service was provided by MACROGEN Company (Seoul, Korea). The sequences were aligned using Clustal W program which was integrated in BIOEDIT Software provided by North Carolina State University and BLAST program.

2.3. Arabidopsis transformation

The ALS genes isolated from ALS R (*MvALS-R*) and S (*MvALS-S*) were then modified introduce flanking restriction site for the enzymes *Bam*HI (forward underlined) and XbaI (reverse underlined). Their sequences were 5′-CGGGATCCCGATGGCTGCTT-3′ and 5′-GCTCTAGAGCACTAGTGCACTGTGCT-3′. Each PCR reaction mixture contained 1 ng of template DNA which was pGEM-T-R or -S plasmid clone, 2.5 units (U) of EX *Taq* DNA polymerase (TaKaRa, Japan), reaction buffer, 200 μM dNTPs, and 20 pmol primers. After an initial denaturation step at 94 °C for 5 min, the reaction mixtures were subjected to 30 amplification cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 2 min with subsequent incubations for 10 min at 72 °C. The PCR product were double digested with *Bam*HI and *Xba*I, and ligated by using RBC rapid ligation Kit to the site *Bam*HI-*Xba*I of the pCambia3300 plant transformation vector containing a basta resistance marker and CaMV 35S promoter. Products of ligation were introduced into JM109 cells and selected on LB media containing kanamycin (50 mg/ml). The conformed Plasmids pCambia3300-35S::*ALS-R* and -*S* were introduced into *Agrobacterium tumefaciens* strain GV3101 by the heat shock method and transformed *A. tumefaciens* (GV3101) were selected from the plate containing YEP medium supplemented with kanamycin (50 mg/ml). The transformed *A. tumefaciens* (GV3101) were introduced into *A. thaliana* (columbia-0) by the floral-dip method (Clough and Bent, 1998). Plants were cold-treated for 48 h and grown in a growth chamber (22 °C, 50% relative humidity, 200 μmol m^{-2} s^{-1} of light intensity, and 16 h of light condition). Transformed plants containing basta marker were selected with spray of basta solution of 1:400 (*v*/*v*) at the four-leaf stage. Leaf discs were collected from the survived plants after basta treatment for DNA extraction. Extracted DNA was used as template for PCR detection by using the *MvALS* primers listed above, and plants containing the *MvALS* was transplanted and maintained. The process was repeated to obtain non-segregating T_3 transgenic lines.

2.4. Hypocotyls length measurement of transgenic A. thaliana T_3 plants

Wild type Arabidopsis (columbia-0 ecotype) and T_3 seeds of 35S::*ALS-R* and -*S* Arabidopsis were surface-sterilized for 10 min in a solution of 5% sodium hypochlorite and washed with sterilized water. Surface-sterilized seeds (20 seeds per plate) were sown on 0.5 times MS medium containing 0.8% (*w*/*v*) agar with or without three ALS-inhibiting herbicides of 200–2000 nM. The plates were kept in the dark at 4 °C for 72 h, and then transferred to 20 °C, dark conditions. For hypocotyls length measurements, the image of one week-old plants was captured using digital camera. Hypocotyls length was measured using the NIH image software (Bethesda, ND, USA).

2.5. ALS activity assay

ALS activity was determined as follows. A leaf section (three discs of 6 mm diameter) was floated and incubated on 1× MS medium containing 0.5 mM 1,1-cyclopropane dicarboxylic acid (CPCA), an inhibitor of acetolactate metabolism, with 1, 10, and 100 nM or without ALS-inhibiting herbicides for 48 h under the same conditions used for plant growth [12]. The sample was then placed at −70 °C for 1 h, transferred into 200 μl of 0.025% Triton X-100, and incubated at 60 °C for 5 min followed by incubation at room temperature for 60 min to effect extraction of acetolactate synthesized by ALS. The extract of 100 μl was transferred to a 1.5-mL tube, to which was added 10 μl of 1 N H_2SO_4. The sample was then incubated at 60 °C for 30 min to convert acetolactate to acetoin. Fifty microlitre of 0.5% (*w*/*v*) creatine and 50 μl of 5% (*w*/*v*) 1-naphthol dissolved in 2.5 N NaOH were added and the resultant mixture was subsequently incubated at 37 °C for 30 min. The amount of acetoin formed was determined with absorbance at 530 nm by a colorimetric assay. Bensulfuron methyl (BS) was used as representative sulfonylureas (SU), imazapyr as representative imidazolinones (IM), flumetsulam as representative triazolopyrimidines (TP).

3. Results and discussions

3.1. Identification of ALS gene of Monochoria vaginalis

The ALS gene was sequenced to determine the molecular basis for resistance. The amino acid sequence of ALS gene derived from