

## A novel mutant acetolactate synthase gene from rice cells, which confers resistance to ALS-inhibiting herbicides

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A two-point mutated gene of acetolactate synthase (ALS) was isolated from rice cells, which was cultured together with an ALS-inhibiting herbicide, bispyribac-sodium (BS). The mutations involved residues of tryptophan at position 548 to leucine (W548L) and serine at position 627 to isoleucine (S627I). The ALS expressed in *Escherichia coli* from this gene showed resistance to multiple herbicides including pyrimidinylcarboxylate (PC), sulfonyleurea and imidazolinone herbicides, and showed stronger resistance to PC herbicides than to other herbicides. BS, a PC herbicide, had almost no effect on the enzyme even at 100  $\mu$ M, which is an approximately 10,000-fold higher concentration than the concentration required for 50% inhibition of the wild-type. The resistance level of W548L/S627I mutating ALS to BS was stronger than the additive effect predicted from the degree of resistance of each single amino acid mutated ALS. Transformed rice cells carrying this gene and a regenerated rice plant expressed resistance to BS, suggesting that this gene is useful as a selectable marker for introducing foreign traits into rice when used with PC herbicides. © Pesticide Science Society of Japan

**Keywords:** acetolactate synthase, acetohydroxyacid synthase, ALS, AHAS, pyrimidinylcarboxylates, bispyribac-sodium.

### Introduction

Acetolactate synthase (ALS, EC2.2.1.6; also referred to as acetohydroxyacid synthase, AHAS) is the first common enzyme in the biosynthetic pathway to the branched-chain amino acids: valine, leucine and isoleucine. ALS is the primary target site of action for at least five structurally distinct classes of herbicides including pyrimidinylcarboxylates (PCs), sulfonyleureas (SUs), imidazolinones (IMs), triazolopyrimidine sulfonamides and sulfonylaminocarbonyltriazolinones.<sup>1)</sup>

Plant ALS genes coding for their catalytic subunits were first isolated from Arabidopsis and tobacco utilizing the yeast ALS gene as a heterologous hybridization probe.<sup>2)</sup> Since then, several plant ALS genes have been cloned and characterized. The deduced amino acid sequences are well conserved among plants. They are similar to the catalytic subunit of bacterial

and yeast ALSs, except for the *N*-terminal signal peptide sequences, which are required for translocation of the protein to the chloroplast.<sup>3)</sup> The gene coding for the regulatory subunit of ALS has also been cloned and characterized.<sup>4)</sup> The deduced amino acid of the regulatory subunit is over twice as large as that of bacteria and has two domains assumed to accept feedback inhibitors.

Plants and cultured plant cells resistant to SUs and IMs have been generated using both the conventional mutation breeding method and *in vitro* cell selection. ALSs from these herbicide-resistant plants have been shown to be insensitive to the herbicides. ALS genes encoding the catalytic subunit have been cloned from some of those plants, and their sequences are shown to differ from those of their wild-types. In most cases, the molecular basis for the herbicide resistance is due to a single or double amino acid change from the wild-type enzyme.<sup>1)</sup> A herbicide-resistant ALS gene has been shown to be useful not only for the generation of transgenic plants that express resistance to the corresponding herbicides but also for introducing foreign traits into plants as a selectable marker.<sup>5)</sup>

Recently, we have obtained a mutant ALS gene from PC-resistant rice cells, which confer resistance to one of the PC

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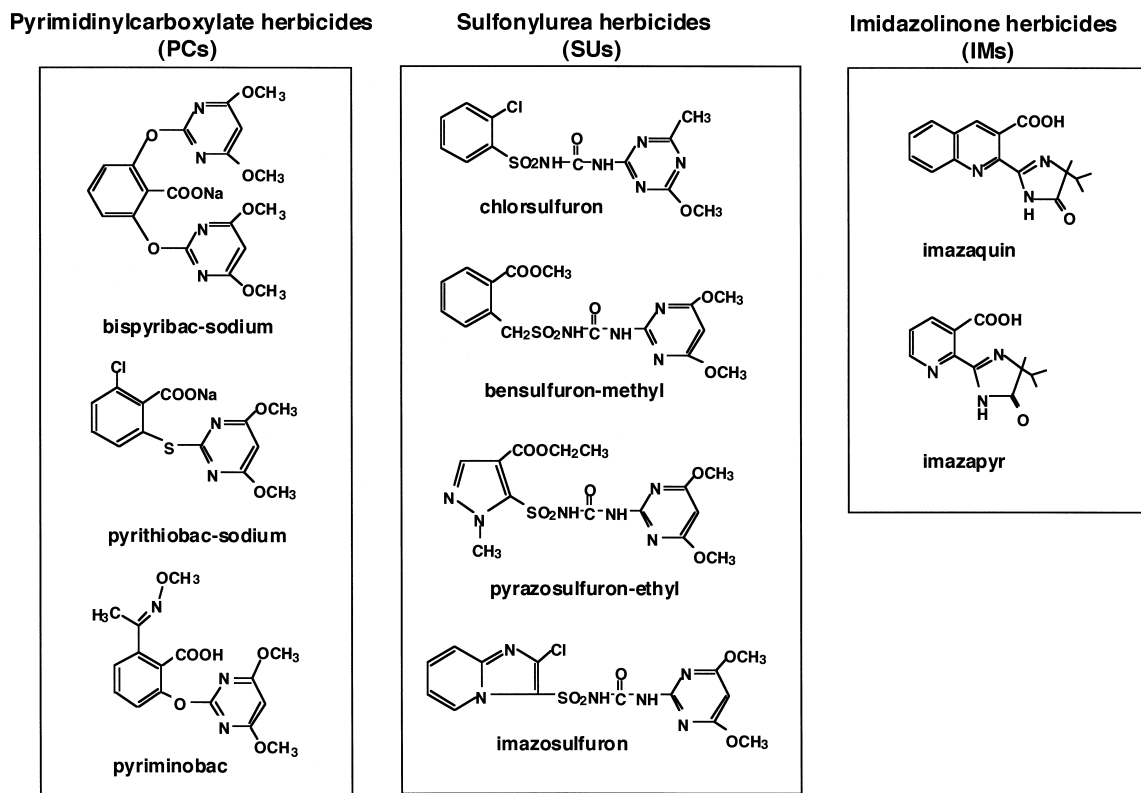


Fig. 1. ALS-inhibiting herbicides.

herbicides, bispyribac-sodium (BS). In this paper we describe the processes for isolation of the gene and show the properties of the gene and the protein expressed from the gene to use as a selectable marker for genetic transformation of plants.

## Materials and Methods

### 1. Compounds

Three chemical compounds, bispyribac-sodium (BS), pyrithiobac-sodium (PS), and pyriminobac (PM), were used as pyrimidinylcarboxylate herbicides. Four chemical compounds, chlorsulfuron (CS), bensulfuron-methyl (BM), pyrazosulfuron-ethyl (PE) and imazosulfuron (IM), were used as sulfonylurea herbicides. Two chemical compounds, imazaquin (IQ) and imazapyr (IP), were used as imidazolinone herbicides (Fig. 1). These chemical compounds were synthesized by KI Chemical Research Institute Co., Ltd. (Shizuoka, Japan).

### 2. Generation of rice cultured cells resistant to BS

Chaff-removed rice seeds (*Oryza sativa* var. Kinmaze) were immersed in 70% ethanol, and then immersed in about 5% sodium hypochlorite, followed by washing several times with sterile distilled water. The seeds were then cultured in Uchimiya & Murashige<sup>6)</sup> solid medium under sterile conditions. The obtained calli were sub-cultured successively in a liquid medium supplemented with BS.

### 3. Sensitivity of rice cells to herbicides

Approximately 1.5 g (wet weight) of rice cells were transplanted to a 200 ml of Erlenmeyer flask containing 50 ml of Uchimiya & Murashige liquid medium supplemented with a herbicide (BS or CS) at an appropriate concentration, followed by culturing for an appropriate period. The wet weight of the callus was measured periodically. The relative growth of calli was determined based on the wet weight of the transplanted cells.

### 4. Partial purification of ALS from rice cells

ALS protein was partially purified at below 4 °C as follows. Approximately 150 g of wild-type or BS-resistant cells, which were prepared by liquid culture without BS, were homogenized in three volumes of 100 mM potassium phosphate buffer (pH 7.5) containing 20% (v/v) glycerol, 0.5 mM thiamin pyrophosphate (TPP), 10 mM flavin adenine dinucleotide (FAD), 0.5 mM MgCl<sub>2</sub>, and 15 g of polyvinylpolypyrrolidone (buffer-1) using Hiscotron homogenizer (Nichion-Irikakikai-Seisakujo). The homogenate was filtered through nylon gauze, and then centrifuged at 15,000×g for 20 min. Ammonium sulfate was added to the supernatant to 50% saturation, and then allowed to stand on ice for approximately 1 hr. This homogenate was again centrifuged at 15,000×g for 20 min, and then the precipitate was dissolved in approximately 30 ml of 10 mM Tri-HCl buffer (pH 7.5) containing 20% (v/v) glycerol, 0.5 mM TPP and

0.5 mM MgCl<sub>2</sub> (buffer-2). The solution was again centrifuged at 15,000×g for 20 min, and then the supernatant was applied to a Sephadex G-25 (GE Healthcare Bio-Sciences KK) column. Approximately 40 ml of the pass-through fraction was collected as a crude enzyme solution. The protein concentration of this solution was measured by the Bradford method. This solution was then filtered through a Whatman 25 mm GD/X Sterile Syringe Filter, and applied to three vertically-connected HiTrap Q columns (GE Healthcare Bio-Sciences KK) using an FPLC (GE Healthcare Bio-Sciences KK). After proteins were adsorbed, the columns were washed with buffer-2. The adsorbed protein was then eluted using 150 ml of buffer-2 with a linear concentration gradient (0 to 0.4 M) of KCl. The eluate was fractionated into 5 ml each in test tubes including 0.5 ml of buffer-2 containing 20 mM sodium pyruvate to stabilize ALS.

#### 5. Assay of ALS and direct acetoin forming activity

ALS activity was measured as follows. A reaction mixture (1 ml) was prepared by mixing an enzyme solution with a solution comprising 20 mM sodium pyruvate, 0.5 mM TPP, 0.5 mM MgCl<sub>2</sub>, 10 mM FAD and 20 mM potassium phosphate buffer (pH 7.5). After the enzyme solution was added, the reaction was conducted at 30 °C for 40 to 60 min. The reaction was then stopped by the addition of 0.1 ml of 6 N sulfuric acid (for the ALS activity) or 0.25 N sodium hydroxide (for direct acetoin-forming activity). The mixture was incubated at 60 °C for 10 min, thereby converting acetolactate to acetoin. Then, 1 ml of 0.5% (w/v) creatine and 1 ml of 5% (w/v)  $\alpha$ -naphthol dissolved in 2.5 N sodium hydroxide were added to the mixture, followed by incubation at 60 °C for 10 min. Acetoin was then quantified by color comparison of the mixture at absorbance of 525 nm. Reaction-time zero was used as the control.

#### 6. Cloning of the wild-type and the mutant ALS gene

A rice partial cDNA clone (accession No. C72411), which was an ALS homolog-retaining plasmid, pBluescript II SK+ with an 1.6 kb insert DNA, was obtained from the MAFF DNA bank. The entire nucleotide sequence of the insert was determined with an ABI PRISM 310 genetic analyzer. Its homology to the nucleotide sequence of corn was 84.7%, revealing that the insert cDNA was a partial cDNA of the rice ALS gene. Thus, the insert DNA was digested with *Sal* I and *Not* I, and used as a probe for cloning ALS genes. The probe DNA was labeled by both the RI and non-RI method. RI labeling of the probe was performed using a BcaBEST DNA labeling kit (Takara Shuzo Co., Ltd.). Non-RI labeling of the probe was performed by DNA labeling reagent (peroxidase) and glutaraldehyde, which are provided in an ECL direct DNA/RNA labeling and detection system (GE Healthcare Bio-Sciences KK). Messenger RNAs were prepared by the standard method from approximately 200 g of wild-type and BS-resistant rice cells using an oligo dT cellulose (GE Healthcare Bio-Sci-

ences KK) column. Complementary DNAs were synthesized using 2 mg of mRNAs, so that cDNA  $\lambda$  phage libraries derived from wild-type and BS-resistant cells were constructed. ALS cDNAs were isolated by the standard method and cloned into pBluescript II SK+ that had been digested with *Eco* RI. *Escherichia coli* strain JM 109 was transformed with this plasmid. The obtained transformants were subjected to liquid culture, and then plasmids were prepared by the standard method. The nucleotide sequences of ALS cDNAs in the plasmids were determined with an ABI PRISM 310 genetic analyzer by primer walking.

#### 7. Expression, preparation and purification of recombinant ALSs

The pBluescript II SK+ retaining ALS cDNA was digested with *Eco* RI. cDNAs of wild-type and mutated ALS genes were incorporated into *Eco* RI sites of pGEX 2T and pGEX 4T-3 (or pGEX 6P-1) vectors respectively. *E. coli* strain JM 105 was transformed with these vectors. The colonies obtained by transformation were subjected to liquid culture, then plasmids were extracted, and the orientation of the insert DNA was confirmed by the restriction enzyme cleavage pattern and sequencing. Clones, where the orientation of the insert DNA was as expected, were selected, and then cultured in an LB medium containing ampicillin. Using 1 ml of the pre-culture solution, culturing was performed in 50 ml or 250 ml of an LB medium containing ampicillin. After culturing overnight, 1 mM IPTG was added, and then GST-fused protein was induced for 3 to 4 hr. A pellet of the transformed *E. coli* was suspended in 2.5 ml of potassium phosphate buffer pH 7.5 containing 30% glycerol and 0.5 mM MgCl<sub>2</sub>. The suspension was subjected to ultrasonication (Heat Systems-Ultrasonics, Sonicator W-225R), and subjected to centrifugation at 15,000×g for 20 min, thereby obtaining the supernatant as a crude enzyme solution. GST-fused ALS protein was purified from the crude enzyme solution by the following method. The crude enzyme solution (12.5 ml) that had been prepared from the pellet (obtained from 250 ml of the culture solution) was applied to a glutathione sepharose 4B affinity column (1 ml of bed volume) (GE Healthcare Bio-Sciences KK) pre-equilibrated with 10 ml of phosphate-buffered saline (PBS). The column was washed with 10 ml of PBS, and then the adsorbed GST-ALS was eluted (4 times) with 2 ml of 10 mM glutathione solution. GST and ALS activities of each fraction were measured, and fractions with high activity were collected. GST activity was measured by rate assay using 1-chloro-2,4-dinitrobenzene as a substrate. The purified ALS free from GST was prepared by protease (thrombin or prescission protease) treatment.

#### 8. Preparation of protein expression vectors retaining single point mutated ALS cDNAs, and expression of recombinant ALSs

The mutant ALS gene having the W548L single mutation was

prepared as follows. First, PCR was performed using a sense primer named ALS-Rsp6 (5'-CATCACCAACCACCTCTT-3') and an anti-sense primer named ALS-RspF (5'-ACACG-GACTGCAGGAATA-3'), and using pBluescript II SK+ retaining the double-point mutant ALS gene as a template, thereby amplifying a DNA fragment having the W548L mutation but no S627I mutation. Meanwhile, a DNA fragment containing a region encoding serine at position 627 was amplified using a sense primer named ALS-RspE (5'-TTA-CAAGGCGAATAGGGC-3') and an M13R anti-sense primer (5'-GGAAACAGCTATGACCATG-3') and using pBluescript II SK+ retaining the wild-type ALS gene as a template. Next, a large DNA fragment was obtained from the two DNA fragments by linking them using the overlapping extension method. The obtained DNA fragment was digested with *Acc* I and *Eco* RI, thereby obtaining an *Acc* I-*Eco* RI fragment. After pGEX 2T plasmid containing the incorporated wild-type ALS gene (pGEX 2T-wALS) was digested at the *Acc* I site, partial digestion was performed with *Eco* RI at 37 °C for 1 min, thereby obtaining a partial fragment of pGEX 2T plasmid. Then, ligation of the *Acc* I-*Eco* RI fragment and the partial fragment of pGEX 2T plasmid resulted in a plasmid having the W548L mutant ALS gene. On the other hand, the mutant ALS gene having only S627I was obtained according to the method by which the W548L mutant ALS gene was prepared; that is, the S627I mutant ALS gene could be obtained in the same manner as employed for the above W548L mutant ALS gene except that ALS-RspE and M13R were used as primers in PCR performed using pBluescript II SK+ as a template, and ALS-Rsp6 and ALS-RspF were used as primers in PCR performed using pBluescript II SK+ retaining the wild-type ALS gene as a template. A plasmid having the W548L mutant ALS gene and a plasmid having the S627I mutant ALS gene were separately transferred into *E. coli* strain JM109. Full sequencing of the ALS gene was performed for the obtained *E. coli* colonies, so that the single-point mutation of W548L mutation and that of S627I mutation could be confirmed.

#### 9. Sensitivity of ALS to herbicides

The sensitivity of the partially purified ALSs from rice cells and the recombinant ALSs expressed in *E. coli* to ALS-inhibiting herbicides was examined as follows. Solutions containing the herbicides at certain concentrations (BS and PS were aqueous solutions and the others were acetone solutions) were added to the reaction mixture before addition of the ALS protein. The final concentration of acetone was 1%. Since a recombinant ALS expressed in *E. coli* is unsusceptible to valine,<sup>7)</sup> an assay using a crude preparation of the GST-ALS was performed under conditions in which the ALS activity derived from *E. coli* was inhibited with 10 mM valine.

#### 10. Transformation of rice with the double mutated ALS cDNA

The W548L/S627I mutant ALS cDNA was incorporated into a binary vector, pMLH7133,<sup>8)</sup> which was developed for transformation of a rice plant, thereby obtaining pMLH7133-ALS. The obtained pMLH7133-ALS was transferred into *E. coli* strain JM109. The resulting single colony was subjected to liquid culture, and then a plasmid was prepared by the alkali SDS method. This binary vector was introduced into cells of *Rhizobium radiobacter* (*Agrobacterium tumefaciens*) EHA 105 by electroporation.

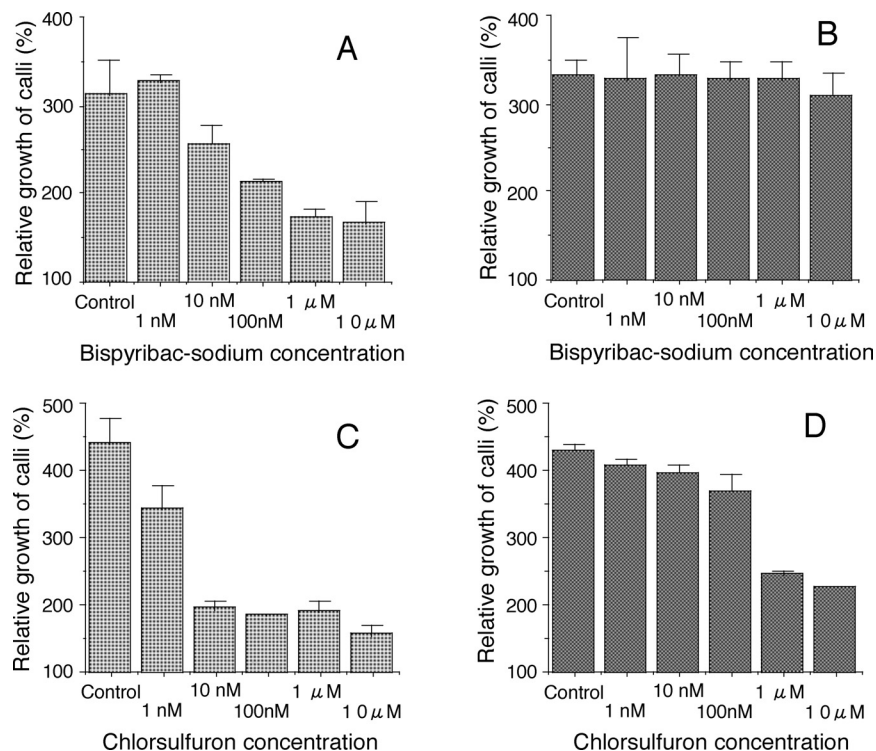
Calli, which had been induced from seeds (cv. Nipponbare) and pre-cultured in an N6D medium, were infected by *Agrobacterium* harboring pMLH 7133-ALS with the standard method. The calli were placed on an N6D solid medium containing 500 mg/l carbenicillin and 50 mg/l hygromycin. Hygromycin-selected calli were transplanted on re-differentiation media followed by transplantation onto hormone-free media. When regenerated plants spread over the Petri dish, the plants were potted into culture soil. Transformed rice plants were appropriately selected, and 1 kg a.i./ha BS supplemented with 0.2% surfactant K was sprayed over the leaves and stems. Growth was surveyed 40 days after spraying. Some of the calli before differentiation were sub-cultured on solid media, followed by liquid culture, and then BS sensitivity was examined. Nucleotide sequences of selected calli and re-generated plants were analyzed as follows. Genomic DNA was prepared from calli and plants using a DNeasy Plant Kit (QIAGEN Inc.). PCR was then performed with a sense primer (3-1-4: 5'-AGGTGTCACAGTTGTTG-3') and an anti-sense primer (4-83-3: 5'-GCTTTGCCAACATACAG-3') that flank the double-point mutated portion using genomic DNA as a template. The amplified DNA fragment was purified, and then the nucleotide sequence was analyzed with a sense primer, 3-1-4, and an anti-sense primer, ALS-Rsp2 (5'-AGTCCTGC-CATCACCATCCAG-3').

### Results

#### 1. Generation of rice (cv. Kinmaze) cultured cells resistant to BS

After approximately two months of cultivation with 1  $\mu$ M of BS, non-discolored cells were obtained from culture cells, most of which had died and become discolored to brown. These cells were isolated and sub-cultured, so that a cell line that could proliferate in the presence of 2  $\mu$ M of BS was obtained. This cell line was subsequently cultured while elevating the concentration of BS. Consequently, a cell line that grew in the presence of 100  $\mu$ M BS was obtained. The sensitivity of the cell line to ALS-inhibiting herbicides was then compared to the wild-type. The growth of wild-type cells was not inhibited by 1 nM BS, but was inhibited by 10 nM or more BS; however, that of the BS-resistant cell line was hardly affected even by 10  $\mu$ M BS. On the other hand, the growth of the wild-type was inhibited by an SU herbicide, CS to almost



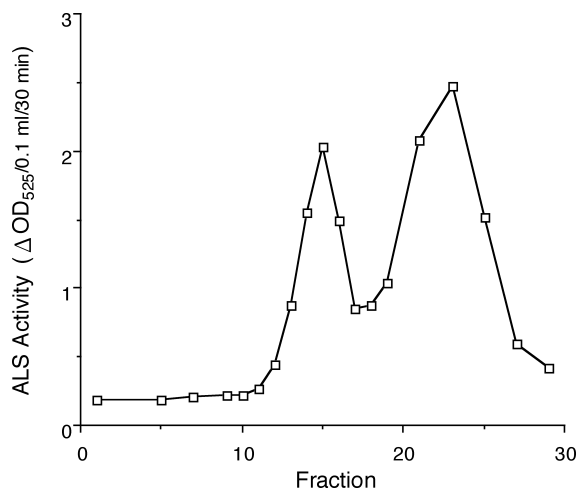


**Fig. 2.** Sensitivity of rice cells to bispyribac-sodium and chlorsulfuron. A and C: wild-type; B and D: mutant.

the same extent as BS, while that of the resistant cell line was strongly inhibited by 1 μM CS (Fig. 2). These results showed that the BS-resistant cell line possessed resistance specific to BS.

**2. Herbicide sensitivity of ALS partially purified from BS-resistant cells**

Two ALS activity peaks were detected by negative ion exchange FPLC of the crude enzyme preparation from BS-resistant cells (Fig. 3). It was found that a fraction eluted earlier, which expressed no direct acetoin-forming activity, corre-



**Fig. 3.** Chromatogram of negative ion exchange chromatography of the crude enzyme preparation from BS-resistant rice cells.

sponded to the mutant ALS protein. The wild-type ALS protein was also partially purified by the same method. Using these mutant and wild-type ALS proteins, sensitivities to BS, CS and IQ were examined. The mutant ALS showed a relatively high ALS activity even in the presence of herbicides, when compared to the wild-type ALS. In particular, the most significant difference between the mutant and the wild-type ALS was the sensitivity to BS, indicating that the mutant ALS possessed especially high resistance to BS (Table 1).

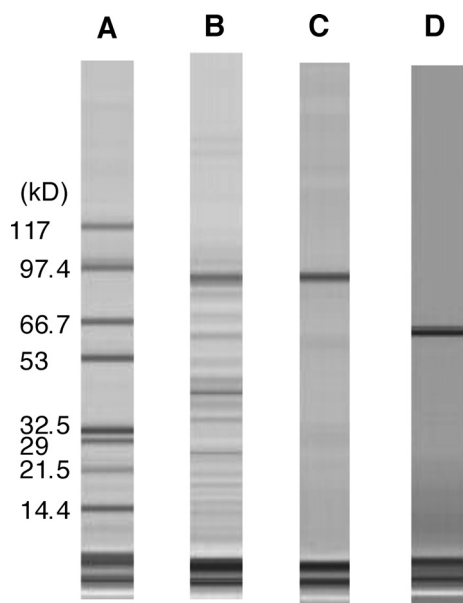
**3. Cloning of wild-type and mutant ALS cDNAs**

One full-length ALS cDNA, of which the translational region was 3 nucleotides longer than the initiation codon, was isolated from a cDNA library of wild-type rice cells. Two kinds of full-length ALS cDNA, of which the non-translation region was 47 nucleotides and 31 nucleotides longer than the initia-

**Table 1.** Sensitivity of ALSs from rice calli to ALS-inhibiting herbicides

Herbicide	I <sub>50</sub> (nM) <sup>a)</sup>		RS Ratio <sup>b)</sup>
	Wild	Mutant	Mutant/Wild
Bispyribac-sodium (BS)	5.63	421	74.8
Chlorsulfuron (CS)	17.3	92.8	5.36
Imazaquin (IQ)	1480	16700	11.3

<sup>a)</sup> Concentration required for 50% inhibition. <sup>b)</sup> RS ratio = I<sub>50</sub>(mutant)/I<sub>50</sub>(wild type).



**Fig. 4.** Gel images of electrophoresis with the Agilent Bio-analyzer. A, molecular markers; B, crude enzyme preparation; C, purified GST-ALS; D, purified GST free ALS.

tion codon, were isolated from a cDNA library of BS-resistant cells (accession numbers: AB049822 and AB049823). One of these ALS cDNAs had an identical sequence to that derived from wild-type cells; thus this was considered to encode the wild-type ALS protein. The deduced amino acid sequence of this cDNA is highly conserved compared with corn (accession number: X63553 and X63554)<sup>9)</sup> and barley (accession number, AF059600/partial cDNA). The other ALS cDNA derived from BS-resistant cells was assumed to encode a mutant ALS. The mutations of this cDNA involved residues of tryptophan at position 548 to leucine (W548L) and serine at position 627 to isoleucine (S627I).

#### 4. Expression of ALS proteins and their sensitivity to herbicides

The crude enzyme preparation from *E. coli* expressing the GST-fused wild-type ALS or the double-mutated ALS showed specific activity approximately 3- to 4-fold higher than that from the control containing no plasmid. The GST-fused ALS proteins expressed in *E. coli* were purified by GST affinity column chromatography. ALSs were prepared by protease treatment and the molecular weight of the ALS free from GST was evaluated using an Agilent bioanalyzer (Fig. 4). The GST-fused ALS from the double-mutated ALS cDNA expressed a similar specific activity to that of the wild-type (data not shown). The GST-fused ALS from the wild-type ALS cDNA showed similar sensitivity to ALS-inhibiting herbicides compared with that prepared from a natural source.<sup>10)</sup> On the other hand, the GST-fused ALS from the mutant ALS cDNA expressed resistance to different classes of ALS-inhibiting herbicides including PCs: BS, PS, PM, SUs: CS, BM, PE, IS,

**Table 2.** Sensitivity of recombinant ALSs to ALS-inhibiting herbicides

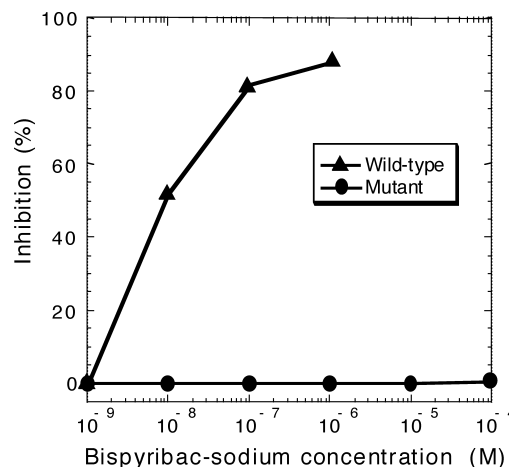
Class	Herbicide	I <sub>50</sub> (nM) <sup>a)</sup>		RS Ratio <sup>b)</sup>
		Wild	Mutant	Mutant/Wild
PC	Bispyribac-sodium	6.3	0.3% <sup>c)</sup>	>16000
	Pyriothiobac-sodium	11	32% <sup>c)</sup>	>9100
	Pyriminobac	8.0	13% <sup>c)</sup>	>13000
SU	Chlorsulfuron	13	2600	200
	Bensulfuron-methyl	7.0	22% <sup>c)</sup>	>14000
	Pyrazosulfuron-ethyl	20	50000	2500
	Imazosulfuron	25	65000	2600
IM	Imazaquin	2200	12% <sup>c)</sup>	>45
	Imazapyr	9600	27% <sup>c)</sup>	>10

<sup>a)</sup> Concentration required for 50% inhibition. <sup>b)</sup> RS ratio = I<sub>50</sub> (mutant)/I<sub>50</sub> (wild-type). <sup>c)</sup> Inhibition percentage by 0.1 mM of compounds.

and IMs: IQ, IP (Table 2), but it showed stronger resistance to BS than to the others. BS had no effect on the enzyme even at 100 μM, which is an approximately 10,000-fold higher concentration than that required for 50% inhibition of the wild-type enzyme (Fig. 5). Purified GST-fused ALSs and purified ALSs showed herbicide sensitivities similar to those of crude enzymes in the presence of 10 mM valine (data not shown).

#### 5. Expression of single-point mutated ALSs and their sensitivity to herbicides

ALS cDNAs possessing each single mutation were generated by PCR and the overlapping extension PCR using the wild-type and the double-mutated ALS cDNAs as templates. Each GST-fused ALS protein was expressed in *E. coli*, and its sensitivity to the herbicides was examined. Each single mutation of W548L and S627I in the ALS conferred resistance to BS,



**Fig. 5.** Sensitivity of recombinant ALSs to bispyribac-sodium.

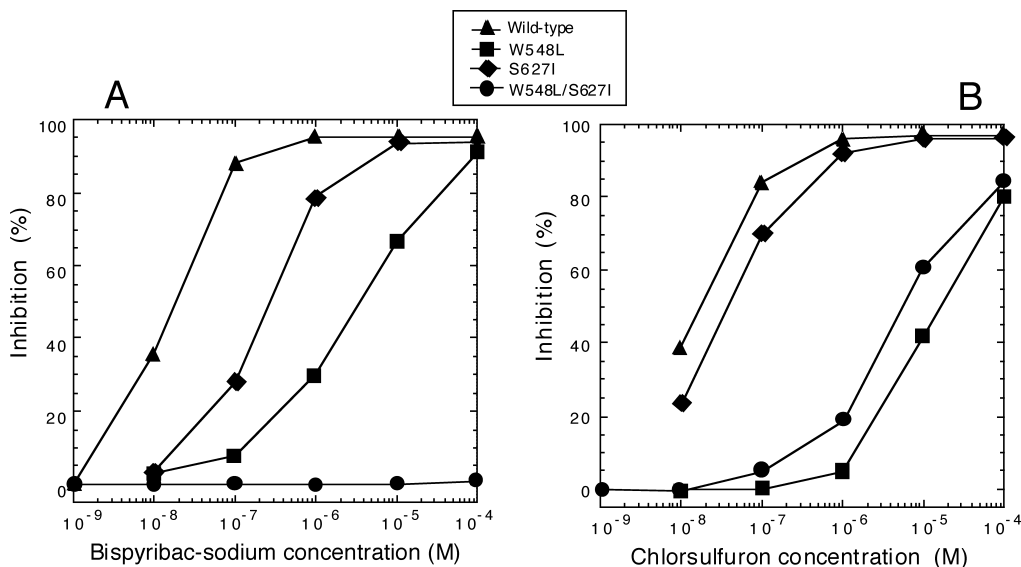


Fig. 6. Comparison of sensitivity of recombinant ALSs to bispyribac-sodium and chlorsulfuron.

and the degree of resistance was higher in W548L than in S627I. The level of resistance to herbicides among these single-mutated ALSs was all shown to be lower than that of the double-mutated ALS (Fig. 6). The W548L mutation alone conferred resistance to CS, while the S627I mutation alone conferred no obvious resistance to this herbicide. A comparison of the degree of resistance to CS between the W548L single mutant and the W548L/S627I double mutant revealed that they shared the same degree of resistance (Fig. 6). Therefore, the S627I mutation, which was first found in this study, was shown to drastically enhance especially BS resistance when it was introduced to an ALS gene carrying the W548L mutation.

#### 6. Transformation of rice with the double mutated ALS

Rice cells (cv. Nippon-bare) were transformed with the double-mutated ALS cDNA in the binary vector, pMLH 7133, and then the introduced cDNA of transformed cells, which were selected by hygromycin pressure, was analyzed. Heterologous sequences of the wild-type and the mutant were found in the nucleotide sequence of the ALS in the genomic DNA from the cells, indicating that the double-mutated ALS cDNA was incorporated into the genome. These cells grew well in the presence of 10  $\mu$ M BS as well as cells from which the double-mutated ALS cDNA was isolated (data not shown). Of 80 transformed rice plants regenerated from cells, 27 plants grew normally. These plants were shown to possess the mutant gene as indicated in the transformed cells. At the 5th leaf stage, four of these 27 plants containing the mutant ALS gene were appropriately selected, and 1 kg a.i./ha BS supplemented with 0.2% surfactant was sprayed over the leaves and stems. One of these plants grew normally, suggesting that the plant was highly resistant to BS (Fig. 7).

## Discussion

Mutations in the ALS of crops and Arabidopsis that have been reported to confer resistance to ALS-inhibiting herbicides are described in Table 3 using the rice ALS number shown in this paper. The most commonly encountered muta-

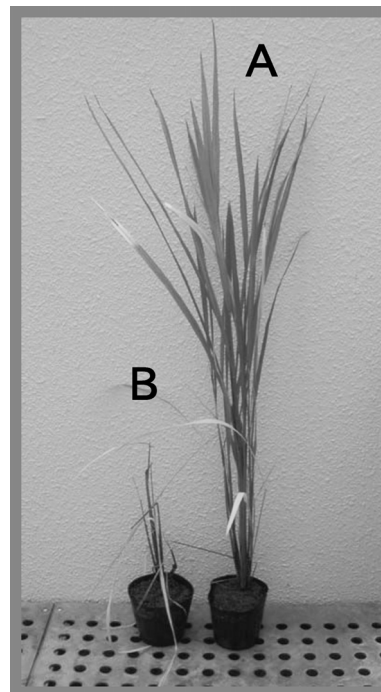


Fig. 7. Photograph showing bispyribac-sodium sensitivity of rice plants transformed with ALS genes (40 days after treatment of bispyribac-sodium at 1 kg a.i./ha). A, Rice plant transformed with the mutant ALS gene. B, Rice plant transformed with the wild-type ALS gene.

**Table 3.** Mutations in ALS conferring resistance to ALS-inhibiting herbicides

Plant species	Mutation <sup>a)</sup>	Method <sup>b)</sup>	Selection
<i>Zea mays</i>	A96T	CMB	Imidazolinone
<i>Beta vulgaris</i>	A96T	SCM	Imidazolinone
<i>Arabidopsis thaliana</i>	A96V	SDM	
<i>Arabidopsis thaliana</i>	M98E	SDM	
<i>Arabidopsis thaliana</i>	M98I	SDM	
<i>Arabidopsis thaliana</i>	M98H	SDM	
<i>Arabidopsis thaliana</i>	P171S	CMB	Sulfonylurea
<i>Nicotiana tabacum</i>	P171Q	SCM	Sulfonylurea
<i>Nicotiana tabacum</i>	P171A	SCM	Sulfonylurea
<i>Nicotiana tabacum</i>	P171S	SCM	Sulfonylurea
<i>Beta vulgaris</i>	P171S	SCM	Sulfonylurea
<i>Brassica napus</i>	P171S	SDM	
<i>Arabidopsis thaliana</i>	P171deletion	SDM	
<i>Arabidopsis thaliana</i>	R173A	SDM	
<i>Arabidopsis thaliana</i>	R173E	SDM	
<i>Arabidopsis thaliana</i>	F180R	SDM	
<i>Zea mays</i>	W548L	CMB	Imidazolinone
<i>Nicotiana tabacum</i>	W548L	SCM	Sulfonylurea
<i>Brassica napus</i>	W548L	SCM	Sulfonylurea
<i>Oryza sativa</i>	W548L	SCM	Pyrimidinylcarboxylate
<i>Gossypium hirsutum</i>	W548S	SCM	Sulfonylurea
<i>Gossypium hirsutum</i>	W548C	SCM	Sulfonylurea
<i>Arabidopsis thaliana</i>	W548L	SDM	
<i>Nicotiana tabacum</i>	W548F	SDM	
<i>Arabidopsis thaliana</i>	W548S	SDM	
<i>Arabidopsis thaliana</i>	W548deletion	SDM	
<i>Zea mays</i>	S627D	CMB	Imidazolinone
<i>Arabidopsis thaliana</i>	S627N	SCM/SDM	Imidazolinone
<i>Zea mays</i>	S627N	SCM	Imidazolinone
<i>Oryza sativa</i>	S627I	SCM	Pyrimidinylcarboxylate
<i>Arabidopsis thaliana</i>	S627T	SDM	
<i>Arabidopsis thaliana</i>	S627F	SDM	
<i>Arabidopsis thaliana</i>	S627deletion	SDM	

<sup>a)</sup> Amino acids are described by one letters. <sup>b)</sup> Mutated ALS were obtained through conventional mutation breeding (CMB), somatic cell mutation (SCM) or site-directed mutagenesis (SDM).

tions involve the residues of alanine at position 96 (A96), proline at position 171 (P171), tryptophan at position 548 (W548) and serine at position 627 (S627). The mutation of W548L was first isolated together with the mutation of P171A in tobacco<sup>2)</sup> through selection by the SU. Since then, this mutation has been found in corn<sup>11)</sup> and canola.<sup>12)</sup> It was first shown that this mutation confers resistance to both SUs and IMs using the mutated gene generated by site-directed mutagenesis.<sup>13)</sup> This mutation confers resistance to different

classes of ALS-inhibiting herbicides. At this position, other amino acid substitutions, W548C and W548S, have been found in cotton.<sup>14)</sup>

On the other hand, a mutation of S627 was first found in IM-resistant *Arabidopsis*.<sup>15)</sup> In contrast to W548L, the mutation of S627N confers resistance to the IM, but not to the SU.<sup>16)</sup> This mutation as well as a different amino acid change, S627D, has been reported in IM-resistant corn.<sup>17,18)</sup> The mutations at this position leading to S627A, S627N, S627T and



S627F have been studied in *Arabidopsis* by site-directed mutagenesis.<sup>19)</sup> Based on the sensitivity of mutated enzymes expressed in *E. coli*, it has been suggested that the size of the amino acid chain at this position determines resistance.

Regarding double mutations, three combinations have been reported in addition to the double mutation of the P171A/W548L pair found in tobacco as described above; P171S/S627N in *Arabidopsis*,<sup>20,21)</sup> A96T/P171S in sugar beets<sup>22)</sup> and A96T/S627T in tobacco.<sup>23)</sup>

SU-resistant weeds were first found in kochia and prickly lettuce in the field with the repeated use of CS. Since then, many weed species have developed resistance to SUs and IMs.<sup>24)</sup> Weeds possessing the mutated ALS at the P171 position were found in fields with the repeated use of the SU, whereas that of S627 was found with the use of the IM. In addition, a mutation at the W548 position was found in weeds through selection by both IMs and SUs. These mutation patterns raised by herbicide applications are very similar to those of herbicide-resistant crops and *Arabidopsis* described above.

In this paper we found double mutations in the ALS gene of BS-resistant rice cells, which resulted in W548L/S627I mutations in its amino acid sequence. These mutations are a new combination of spontaneous mutations with a novel substitution at the S627 position. This ALS showed resistance to different classes of ALS-inhibiting herbicides, but showed the highest resistance to BS. The resistant level of the ALS to BS was extremely high as compared with those to SUs and IMs. Co-existence of W548L and S627I mutations resulted in synergistic resistance to BS, which could not be predicted from either W548L or S627I mutation alone. Such an effect was not, however, observed in the inhibition by CS. Transformed rice cells with the W548L/S627I double-mutated ALS gene grew normally in the presence of BS and the regenerated plant expressed resistance to BS, indicating that the mutated ALS gene works functionally in the plant when introduced by genetic engineering. This result showed the mutated ALS gene obtained here could be used as a selectable marker gene along with BS for genetic transformation of plants.

The FAO/WHO expert panels encourage the use of recombinant DNA without antibiotic-resistant marker genes, particularly those that could interfere with human and animal therapies,<sup>25)</sup> and the scientific panels on genetically modified organisms of the European Food Safety Authority have categorized antibiotic-resistant marker genes into three groups,<sup>26)</sup> one of which includes the neomycine phosphotransferase gene (NPT II, a kanamycin resistant gene)<sup>27)</sup> and the hygromycine phosphotransferase gene (HPT, a hygromycin resistant gene),<sup>28)</sup> where the use of genes is not prohibited either for field experiment of transgenic plants or for the purpose of placing plants on the market, but in the other two groups including other antibiotic-resistant genes, it is prohibited to use the genes in transgenic plants for marketing. Furthermore, the Codex guideline recommends that alternative transformation technologies that do not result in antibiotic-resistant marker

genes in foods should be used in the future development of recombinant-DNA plants.<sup>29)</sup> Therefore, the double-mutated ALS gene from rice is not only helpful for introducing useful rice genes into rice by self-cloning as a host-derived selectable marker gene but also can extinguish the scientific concern for antibiotic-resistant genes, leading to minimize public concern for this issue in transgenic plants. It is expected that this gene will be used widely together with PC herbicides, especially BS, as a selectable marker for plant genetic transformation in general. This gene is assumed to be safe to use because it has been isolated from BS-resistant rice cells that were spontaneously mutated through co-cultivation with BS.

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#### References

- 1) T. Shimizu, I. Nakayama, K. Nagayama, T. Miyazawa and Y. Nezu: "Herbicide Classes in Development: Mode of Action—Targets—Genetic Engineering—Chemistry" ed. by P. Böger, K. Wakabayashi and K. Hirai, Springer-Verlag, Berlin Heidelberg, pp. 1–41, 2002.
- 2) K. Y. Lee, J. Tepperman, M. Black, C. F. Chui, B. Mazur, P. Dunsmuir and J. Bedbrook: *EMBO J.* **7**, 1241–1248 (1988).
- 3) R. G. Duggleby and S. S. Pang: *J. Biochem. Mol. Biol.* **33**, 1–36 (2000).
- 4) H. P. Hershey, L. J. Schwartz, J. P. Gale and L. M. Abell: *Plant Mol. Biol.* **40**, 795–806 (1999).
- 5) Z. Li, A. Hayashimoto and N. Murai: *Plant Physiol.* **100**, 662–668 (1992).
- 6) H. Uchiyama and T. Murashige: *Plant Physiol.* **54**, 936–944 (1974).
- 7) M.-W. Kil and S.-I. Chang: *J. Biochem. Mol. Biol.* **31**, 287–295 (1998).
- 8) I. Mitsuhashi, M. Ugaki, H. Hirochika, M. Ohshima, T. Murakami, Y. Gotoh, Y. Katayose, S. Nakamura, R. Honkura, S. Nishimiya, K. Ueno, A. Mochizuki, H. Tanimoto, H. Tsugawa, Y. Otsuki and Y. Ohashi: *Plant Cell Physiol.* **37**, 49–59 (1996).
- 9) L. Y. Fang, P. R. Gross, C. H. Chen and M. Lillis: *Plant Mol. Biol.* **18**, 1185–1187 (1992).
- 10) T. Shimizu: *J. Pestic. Sci.* **22**, 245–256 (1997).
- 11) P. Bernasconi, A. R. Woodworth, B. A. Rosen, M. V. Subramanian and D. L. Siehl: *J. Biol. Chem.* **270**, 17381–17385 (1995); Correction, *J. Biol. Chem.* **271**, 13925–13926 (1996).
- 12) J. Hattori, D. Brown, G. Mourad, H. Labbe, T. Ouellet, G. Sunohara, R. Rutledge, J. King and B. Miki: *Mol. Gen. Genet.* **246**, 419–425 (1995).
- 13) J. M. Hand, B. K. Singh and R. S. Chaleff (American Cyanamid Company): *Eur. Pat. Appl.* EP 492113 (1992).
- 14) K. Rajasekaran, J. W. Grula and D. M. Anderson: *Plant Sci.* **119**, 115–124 (1996).
- 15) G. W. Haughn and C. R. Somerville: *Plant Physiol.* **92**, 1081–1085 (1990).

- 16) G. Mourad and J. King: *Planta* **188**, 491–497 (1992).
- 17) S. Bright, J. William, M. T. Chang, I. J. Evans and M. J. Macdonald (Imperial Chemical Industries PLC): *PCT Int. Appl.* WO 9208794 (1992).
- 18) G. E. Dietrich (American Cyanamid Company): *US Pat. Appl.* US 5731180 (1998).
- 19) Y. T. Lee, L. A. Chang and R. G. Duggleby: *FEBS Lett.* **452**, 341–345 (1999).
- 20) J. Hattori, R. Rutledge, H. Labbe, D. Brown, G. Sunohara and B. Miki: *Mol. Gen. Genet.* **232**, 167–173 (1992).
- 21) G. Mourad, G. Haughn and J. King: *Mol. Gen. Genet.* **243**, 178–184 (1994).
- 22) T. R. Wright, N. F. Bascomb, D. Penner and S. F. Sturmer: *Weed Sci.* **46**, 13–23 (1998).
- 23) C. Chom-Kyu and C. Jung-Do: *Biochem. Biophys. Res. Commun.* **279**, 462–467 (2000).
- 24) L. L. Saari, J. C. Cotterman and D. C. Thill: “Herbicide Resistance in Plants Biology and Biochemistry,” ed. by S. B. Powles and J. A. M. Haltum, CRC Press Boca Raton, FL, pp. 83–139, 1994.
- 25) [http://www.who.int/foodsafety/biotech/who\\_study/en/index.html](http://www.who.int/foodsafety/biotech/who_study/en/index.html)
- 26) <http://www.efsa.eu.int>
- 27) J. Velten, L. Velten, R. Hain and J. Schell: *EMBO J.* **3**, 2723–2730 (1984).
- 28) L. Gritz and J. Davies: *Gene* (Amsterdam), **25** 179–188 (1983).
- 29) [http://www.codexalimentarius.net/web/standard\\_list.do?lang=en](http://www.codexalimentarius.net/web/standard_list.do?lang=en)