**Original Paper**

# Transformation of *Arabidopsis* by mutated acetolactate synthase genes from rice and *Arabidopsis* that confer specific resistance to pyrimidinylcarboxylate-type ALS inhibitors

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**Abstract** Previously, we showed that four mutated acetolactate synthase (ALS) genes derived from rice and *Arabidopsis* (W548L/S627I*Os*ALS, S627I*Os*ALS, W574L/S653I*At*ALS and S653I*At*ALS) confer high levels of resistance to pyrimidinylcarboxylate type ALS inhibitors (Kawai et al. 2008). Mutated ALS genes of rice were obtained from rice cells cultured in the presence of an ALS-inhibitor. The mutated ALS genes of *Arabidopsis*, which have the same amino acid substitutions as those of rice, have been generated artificially. Here, we demonstrate that these mutated genes function as effective selectable markers for transformation of *Arabidopsis*. Specifically, we studied expression of the mutated ALSs in *Arabidopsis* and their effect on the sensitivity of transgenic *Arabidopsis* plants to the ALS inhibitors. Our results show that the degree of resistance to the ALS inhibitors of transformants expressing *Arabidopsis* mutated ALSs was greater than those of transformants expressing rice mutated ALSs. The amino acid sequences of ALSs derived from monocotyledonous plants and those derived from dicotyledonous plants were clearly divided into two clusters in a phylogenetic tree. Based on these results, it would be preferable to use rice and *Arabidopsis* mutated ALS genes for generating monocotyledonous and dicotyledonous transgenic plants, respectively. Moreover, our findings are particularly useful when generating transgenic plants with a known ALS nucleotide sequence. In such cases, their own ALS gene carrying these mutations could be used as a selectable marker because amino acid residues at the point of mutation are conserved among plant species.

**Key words:** Acetolactate synthase, pyrimidinylcarboxylate, selectable marker gene.

Acetolactate synthase (ALS; EC 2.2.1.6, also referred to as acetohydroxy acid synthase; AHAS) is a common enzyme that catalyzes the first step of the biosynthetic pathway of 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 sulfonylureas (SUs), imidazolinones (IMs), triazolopyrimidine sulfonamides, pyrimidinyl-salicylates (pyrimidinylcarboxylates, PCs), and sulfonyl-aminocarbonyltriazolinones (Shimizu et al. 2002). ALS-inhibiting herbicides (ALS inhibitors) are widely used in agriculture because of their high weed control efficacy, high crop-weed selectivity, low use rates and low levels of mammalian toxicity (Sharner and Singh 1997).

The plant ALS genes encoding for their catalytic (large) subunits were first isolated from *Arabidopsis* and tobacco utilizing the yeast ALS gene as a heterologous hybridization probe (Mazur et al. 1987). Since then, some plant ALS genes corresponding to the catalytic subunits have been cloned and characterized (Bernasconi et al. 1995; Fang et al. 1992; Grula et al. 1995; Rutledge et al. 1991). Interestingly, the plant ALS regulatory (small) subunit has been revealed to enhance catalytic activity of the large subunit and to confer sensitivity to feedback inhibition by the branched-chain amino acids (Lee and Duggleby 2001).

Plants and cultured plant cells resistant to SU- and IM-type ALS inhibitors have been generated using both a conventional mutation breeding method and *in vitro* cell selection (Hart et al. 1993; Newhouse et al. 1991; Rajasekaran et al. 1996). ALS genes encoding the catalytic subunits have been cloned from some of these plants, and their sequences were found to have mutations.

In our previous report, we isolated a novel mutated ALS gene from rice cells (W548L/S627I*Os*ALS), which confers a high level of resistance to a PC-type ALS

Abbreviations: ALS, acetolactate synthase; BS, bispyribac-sodium; IM, imidazolinone; PC, pyrimidinylsalicylate; PS, pyrithiobac-sodium; SU, sulfonylurea

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inhibitor, bispyribac-sodium (BS), and demonstrated that it could be used as a selectable marker for generating transgenic rice plants (Kawai et al. 2007a; Kawai et al. 2007b). We also found that a single amino acid substitution (S627I) in the ALS gene (S627I*Os*ALS) displays high levels of resistance to other PC-type ALS inhibitors; pyrithiobac-sodium (PS) and pyriminobac (PM). In addition, artificially generated W574L/S653I and S653I *Arabidopsis* ALS genes (W574L/S653I*At*ALS and S653I*At*ALS) corresponding to the rice mutated ALS genes showed similar sensitivity to PC-type ALS inhibitors (Kawai et al. 2008). We postulated that these mutated ALS genes coupled with the PC-type ALS inhibitors might be promising selectable markers for various plant species. Indeed, previous reports have shown that W548L/S627I*Os*ALS works as an effective selectable marker gene for transformation of wheat (Ogawa et al. 2008) and soybean (Tougou M et al. 2009).

In this paper, we generated transgenic *Arabidopsis* via agrobacterium-mediated transformation with rice mutated ALS genes (W548L/S627I*Os*ALS and S627I*Os*ALS) and *Arabidopsis* mutated ALS genes (W574L/S653I*At*ALS and S653*At*ALS). We then compared the levels of resistance of each transgenic *Arabidopsis* expressing the respective mutated ALS gene. Based on our results, we discuss the efficient application of these genes as selectable markers for transformation of a wide range of plant species.

## Materials and methods

### Plant material

*Arabidopsis thaliana* (Col-0) was used throughout this study. Plants were grown in soil at 22°C under 12 hr light/12 hr dark conditions.

### Chemical compound

Bispyribac-sodium (BS) and pyrithiobac-sodium (PS) were used as pyrimidinylcarboxylate (PC)-type ALS inhibitors. These chemical compounds were synthesized by KI Chemical Research Institute Co., Ltd (Iwata, Shizuoka, Japan).

### Construction of binary vector pMLH7133 harboring mutated ALS genes

Construction of a pMLH7133 vector plasmid harboring the W548L/S627I mutated rice ALS gene, pMLH7133-W548L/S627I*Os*ALS, was described in our preceding paper (Kawai et al. 2007). A pMLH7133 harboring S627I mutated rice ALS gene, pLML7133-S627I*Os*ALS was prepared as follows. A pGEX-2T vector plasmid harboring S627I mutated rice ALS gene was digested with BamHI. The liberated ALS gene was subcloned into the pMLH7133-W548L/S627I*Os*ALS at the BamHI site, resulting in substitution of the ALS fragment. Two pMLH7133 plasmids harboring mutated forms of the *Arabidopsis* ALS gene (W574L/S653I or S653I) were prepared as follows. A pBlueScript vector plasmid containing W574L/S653I or S653I mutated *Arabidopsis* ALS gene were digested with XbaI and blunted, followed by digestion with SacI. The mutated ALS genes were then subcloned into the pMLH7133-W548L/S627I *Os*ALS at the BamHI (blunted)/SacI site, resulting in substitution of the ALS fragment. The newly generated binary vectors harboring the W574L/S653I or S653I mutations were named pMLH7133-W574L/S653I*At*ALS and pMLH7133-S653I*At*ALS, respectively. The T-DNA region of the constructed binary vectors is shown in Figure 1.

### Transformation of Arabidopsis

*Arabidopsis thaliana* was transformed by the floral-dip method (Clough and Bent 1998) with *Agrobacterium tumefaciens* strain GV3101 harboring pMLH7133-W548L/S627I *Os*ALS, S627I*Os*ALS, W574L/S653I*At*ALS and S653I*At*ALS, respectively. After infection, the $T_0$ plants were grown to maturity and $T_1$ seeds were harvested. About 3000 seeds per plate were sown on a selection medium containing MS salts with Gamborg's B5 vitamins, 1% sucrose, 0.1% Hyponex (N : P : K = 6 : 10 : 5, Hyponex Japan, Osaka, Japan) supplemented with 0.1 μM BS or PS. Selection medium supplemented with 50 mg $l^{-1}$ (95 μM) hygromycin was also used for comparison. Experiments were repeated three times and transformation frequency was calculated as the total number of resistant seedlings divided by total number of seeds sown on selection medium.

### Evaluation of ALS resistance to ALS inhibitors in transgenic Arabidopsis by in vivo ALS assay

Acetolactate accumulation *in vivo* was determined by the procedure of Uchino *et al*. (Uchino et al. 1999) with some minor modification. The basis for this assay is the comparison of acetoin accumulation depending on ALS enzyme activity in plant tissues treated with a ketol-acid reductoisomerase (KARI) inhibitor alone or a KARI inhibitor plus an ALS inhibitor. Acetolactate synthesized by ALS is subsequently converted to dihydroxyisovalerate by KARI in the branched-chain amino acid biosynthetic pathway. In the presence of 1,1-cyclopropanedicarboxylic acid (CPCA) as a KARI inhibitor, conversion of acetolactate to dihydroxyisovalerate is inhibited. As a result, the acetolactate accumulates in both transgenic plants showing the ALS-inhibitor resistant trait and the non-transformants. However, in the presence of both CPCA and ALS inhibitor, accumulation of acetolactate only occurs in the transgenic plants where acetolactate synthesis is not affected by ALS inhibitor. The accumulated acetolactate can be converted

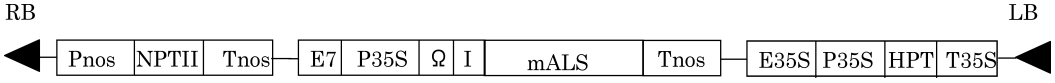


Figure 1. T-DNA region of a binary vector pMLH7133-mALS. RB, right border; LB, left border; Pnos, nopaline synthase promoter; NPTII, neomycin phosphotransferase gene; Tnos, nopaline synthase terminator; E7, seven replicated enhancers of CaMV 35S promoter; P35S, CaMV 35S promoter; Ω, tobacco mosaic virus omega sequence; I, first intron of phaseolin gene; mALS, mutated ALS genes of rice or *Arabidopsis*; E35S, enhanced CaMV 35S promoter; HPT, hygromycin B phosphotransferase gene; T35S, CaMV 35S terminator.

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to acetoin. The distinct color generated by a colorimetric reaction reveals whether acetoin does (red for transformants) or does not (yellow for non-transformants) accumulate in the resultant reaction mixture.

*In vivo* ALS assay was performed using 6-cm petri dishes. Rosette leaves of *Arabidopsis* plants (0.1 g) were cut into small pieces and incubated in 4 ml of 25% Murashige and Skoog salt medium containing 500 μM CPCA and various concentrations of ALS inhibitors. Petri dishes, containing leaf samples, were placed in a cultivation chamber at 22°C under fluorescent light for 24 hr. After incubation, leaves were transferred into a 1.5 ml tube. To determine ALS activity, 300 μl of deionized water was added to the samples and they were heated to 60°C for 5 min. The samples were then sonicated (40 kHz, 375 W) for 20 min. Aliquots of 200 μl were taken and mixed with 20 μl of 5% (w/v) $H_2SO_4$. The acidified solutions were incubated at 60°C for 30 min to facilitate the decarboxylation of acetolactate to acetoin. Then, 100 μl of 0.05% (w/v) creatin and 100 μl of 0.5% (w/v) 1-naphthol dissolved in 2.5 N NaOH were added to the samples. To allow color development, the samples were incubated at 37°C for 30 min, and absorbance measured at 530 nm. A reference absorbance was measured for a sample in which 2.5 N NaOH was added in place of the 5% (w/v) $H_2SO_4$. Experiments were repeated three times. The inhibitory activity of ALS inhibitors was represented as an inhibitor concentration ($I_{50}$) required for 50% inhibition. RS ratios for mutated ALSs were obtained from calculation of the ratio of the $I_{50}$ value for each mutated ALS to the $I_{50}$ value for the wild-type.

### *Identification of mutated ALS genes in Arabidopsis*

DNAs were isolated from leaves using DNeasy Plant Mini Kit (QIAGEN, Inc., Valencia, CA) and subjected to PCR analysis. Forward (3-1-4: 5′-AGGTGTCACAGTTGTTG-3′) and reverse (4-83-3, 5′-GCTTTGCCAACATACAG-3′) primers were used for amplifying mutated rice ALS DNA fragments. Forward (ARALP-8, 5′-TGTGCAAGAGCTAGCCACTATTCG-3′) and reverse (pMLH-1, 5′-GACCGGCAACAGGATTCAATCT-3′) primers were used for amplifying a DNA fragment containing the junction region between the mutated *Arabidopsis* ALS and the Nos terminator. PCR amplification was performed as follows: initial denaturation for 2 min at 95°C, followed by 35 cycles of denaturation for 30 sec at 95°C, annealing for 1 min at 54°C, and extension for 1 min at 72°C. The final extension step was for 10 min at 72°C.

### *Evaluation of whole plant resistance of Arabidopsis to ALS inhibitors*

BS at the rate of 6.3 to 100 g a.i. $ha^{-1}$ or PS at the rate of 12.5 to 100 g a.i. $ha^{-1}$ were sprayed over the leaves of transgenic *Arabidopsis* plants, which were grown in pots (9-cm diameter) for 30 days. Two weeks after spraying, the growth of herbicide-treated plants was assessed.

### *Comparison of amino acid sequence of ALSs among plant species*

Amino acid sequences of corn (*Zea mays*), wheat (*Triticum aestivum*) (lacking the N terminal region), barley (Hordeum vulgare) (lacking the N terminal region), tobacco (Nicotiana tabacum), cotton (Gossypium hirsutum), Italian ryegrass and rapeseed were obtained from the GenBank database. Putative amino acid sequences of ALS from sorghum (lacking the N terminal region) and soybean were identified through a BLAST search of the JGI database (http://genome.jgi-psf.org/) and that from tomato was identified through a BLAST search of the Tomato SBM (http://www.kazusa.or.jp/tomato/). The nucleotide sequences of ALS of Japanese lawn grass have been determined by genome walking using DNA Walking SpeedUp Kit (Seegeen, Inc., Korea, unpublished data). Phylogenic analysis was conducted using the resultant sequences with ClustalW.

## Results

### *Selection of Arabidopsis harboring mutated rice ALS genes*

BS and PS at a concentration of 0.1 μM completely inhibited the growth of wild-type *Arabidopsis* seedlings in the selection medium, while BS and PS lower than 0.1 μM gave partial inhibition (Figure 2). Therefore, BS and PS at a concentration of 0.1 μM was employed for selection of transformants throughout this study.

In the case of transformation of *Arabidopsis* by pMLH7133-W548L/S627I *Os*ALS, a clear distinction between resistant and non-resistant seedlings was observed by BS selection, while the growth of non-resistant seedlings was not completely inhibited by hygromycin (Figure 3A, 3B). However, the growth of resistant seedlings was suppressed on the medium containing BS. In particular, extension of roots was significantly suppressed (Figure 3D). The transformation frequency obtained by BS selection or that obtained by hygromycin selection were 0.8% and 1.2%, respectively (Table 1). Selection of *Arabidopsis* transformed with pMLH7133-S627I*Os*ALS was performed in a similar way except that PS was used as a selection agent. The transformation frequency was almost the same as that of BS in combination with W548L/S627I*Os*ALS (Table 1).

### *Selection of Arabidopsis harboring mutated Arabidopsis ALS genes*

Selection of *Arabidopsis* harboring W574L/S653I*At*ALS was performed in a similar way as described above. As a result, it was revealed that BS selection in combination with W574L/S653I*At*ALS was functional as is the case with W548L/S627I*Os*ALS. Furthermore, unlike the W548L/S627I*Os*ALS, resistant seedlings grew normally on the selection medium (Figure 3C, D) and the transformation frequency was nearly equal to that of the hygromycin selection (Table 1). Tendency of selection by PS in combination with S653I*At*ALS was almost the same as that of selection by BS in combination with W574L/S653I*At*ALS.

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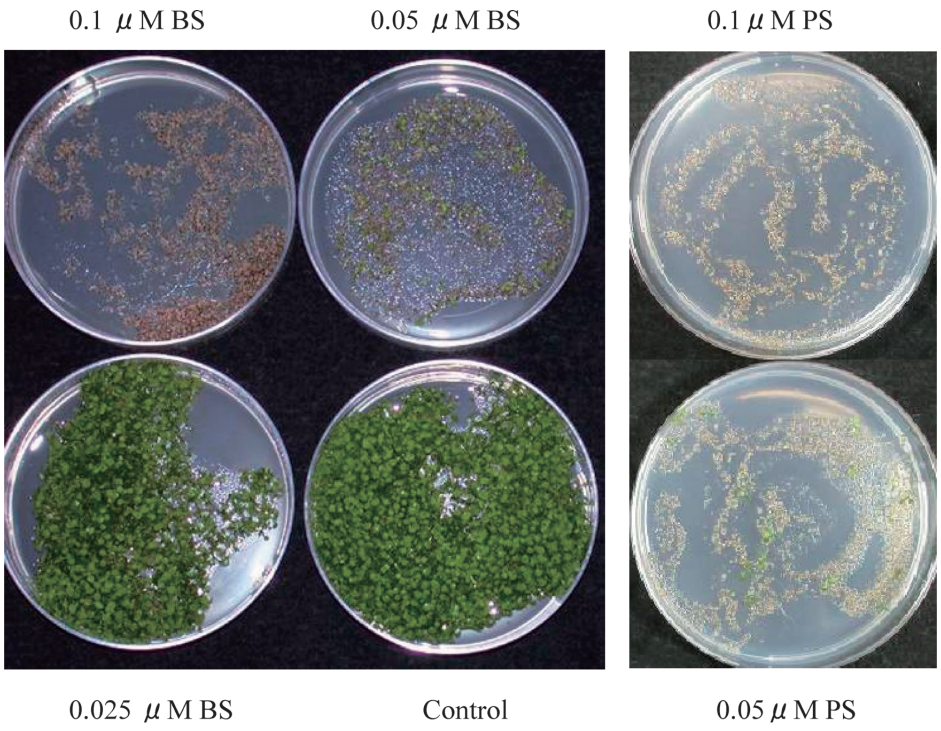


Figure 2. Sensitivity of wild-type *Arabidopsis* to bispyribac-sodium (BS) and pyrithiobac-sodium (PS). Wild-type *Arabidopsis* seeds were sown on a medium with various concentrations of BS or PS. The photograph was taken 10 days after sowing.

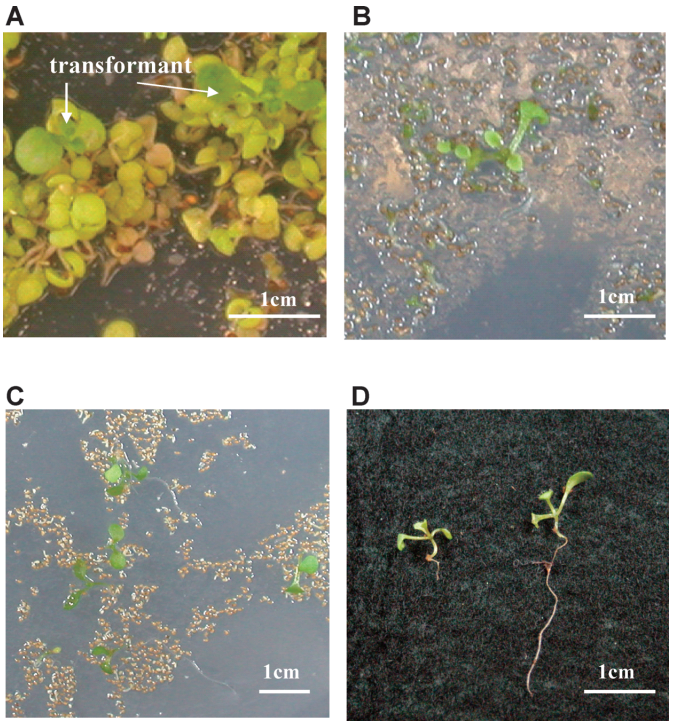


Figure 3. Screening of resistant seedlings by BS or hygromycin. (A) Selection of $T_1$ *Arabidopsis* seedlings harboring W548L/S627I*Os*ALS with 50 ppm (95 μM) hygromycin. (B) Selection of $T_1$ *Arabidopsis* seedlings harboring W548L/S627I*Os*ALS with 0.1 μM BS. (C) Selection of $T_1$ *Arabidopsis* seedlings harboring W574L/S653I*At*ALS with 0.1 μM BS. (D) Transgenic *Arabidopsis* seedling harboring W548L/S627I*Os*ALS (left) and W574L/S653I*At*ALS (right) grown on medium containing 0.1 μM BS for 10 days.

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### *Evaluation of the expression level of mutated ALS by in vivo ALS assay and detection of the mutated ALS genes in transformed Arabidopsis*

To confirm the expression level of mutated ALS protein in *Arabidopsis* selected by BS or PS, we performed an *in vivo* ALS assay. Figure 4 shows the result of the *in vivo* ALS assay in each ten independent $T_1$ transgenic lines harboring S627I*Os*ALS and S653I *At*ALS. A difference in the level of acetoin accumulation was observed among the independent transgenic lines. S653I*At*ALS lines showed a relatively high level of acetoin accumulation compared with the S627I*Os*ALS lines. Comparison between the W548L/S627I*Os*ALS lines and W574L/S653I*At*ALS lines revealed the latter accumulated a relatively high level of acetoin compared with the former (data not shown). These results showed that mutated ALS genes of *Arabidopsis* were more functional than those of rice plant in *Arabidopsis*.

All $T_1$ lines of *Arabidopsis* that were judged to have S627I*Os*ALS, S653I*At*ALS, W548L/S627I*Os*ALS and W574L/S653I*At*ALS, respectively by *in vivo* ALS assay, were confirmed to be positive for integration of each mutated ALS by PCR with a primer set described in *Materials and Methods* (data not shown).

### *Evaluation of ALS resistance to ALS inhibitors in transformed Arabidopsis by in vivo ALS assay*

Lines with single locus insertions, estimated by segregation for herbicide resistance, were selected in $T_2$ generations (Table 2). These lines were self-pollinated and $T_3$ homozygous plants were then selected and tested for resistance to BS or PS (Table 3). The degree of

Table 1. Transformation frequency of *Arabidopsis*.

| Binary vector | Selection agnet | Transformation frequency (%) |
|---|---|---|
| pMLH7133-W548L/S627I Os ALS | BS | 0.8 |
| | hygromycin | 1.2 |
| pMLH7133-W574L/S653I At ALS | BS | 1.2 |
| | hygromycin | 1.3 |
| pMLH7133-S627I Os ALS | PS | 0.7 |
| | hygromycin | 1.3 |
| pMLH7133-S653I At ALS | PS | 1.1 |
| | hygromycin | 1.3 |

In each experiment, about 3000 seeds were sown on the selection medium.

Experiments were repeated three times. Transformation frequency was calculated as follows:

(number of resistant plants/number of sown seeds on selection medium) × 100.

Table 2. Segregation of the herbicide resistant trait in $T_2$ transgenic *Arabidopsis*.

| Transformant | Selection agent | Number of $T_2$ plants | | $\chi^2$ (3 : 1) |
|---|---|---|---|---|
| | | Resistant | Susceptible | |
| W548L/S627I *Os* ALS 30 | BS | 255 | 79 | 0.32 |
| W574L/S653I *At* ALS 1 | BS | 345 | 127 | 0.91 |
| S627I *Os* ALS 12 | PS | 118 | 32 | 1.07 |
| S653I *At* ALS 14 | PS | 107 | 35 | 0.009 |

Sterile seeds were sown on the selection medium with 0.1 $\mu$M BS or 0.1 $\mu$M PS. Ten days after sowing, transgenic plants were scored for their resistance or susceptibility to each chemical. $\chi^2$ values are based on an expected 3 : 1 ratio of resistant and susceptible seedlings. 3 : 1 ratio gave the smallest $\chi^2$ value for each experiment.

Table 3. Sensitivities and RS ratios of mutated ALSs expressing in transgenic *Arabidopsis* to ALS inhibitors.

| Plant | ALS inhibitor | $I_{50}$ ($\mu$M) | RS Ratio |
|---|---|---|---|
| Wild-type | BS | 0.003 | — |
| W548L/S627I OsALS 30-8 | BS | 2.6 | 880 |
| W574L/S653I At ALS 1-3 | BS | 170 | 58,000 |
| Wild-type | PS | 0.03 | — |
| S627IOs ALS 12-2 | PS | 0.12 | 4 |
| S653IAtALS 14-5 | PS | 0.56 | 19 |

$I_{50}$ value and RS ratio were determined with a dose-response curve obtained using the *in vivo* ALS assay.

$I_{50}$, concentration of ALS inhibitor that was required for 50% inhibition of the ALS activities;

RS Ratio, resistance to sensitivity ratio ($I_{50}$ mutated ALS/$I_{50}$ wild-type ALS).

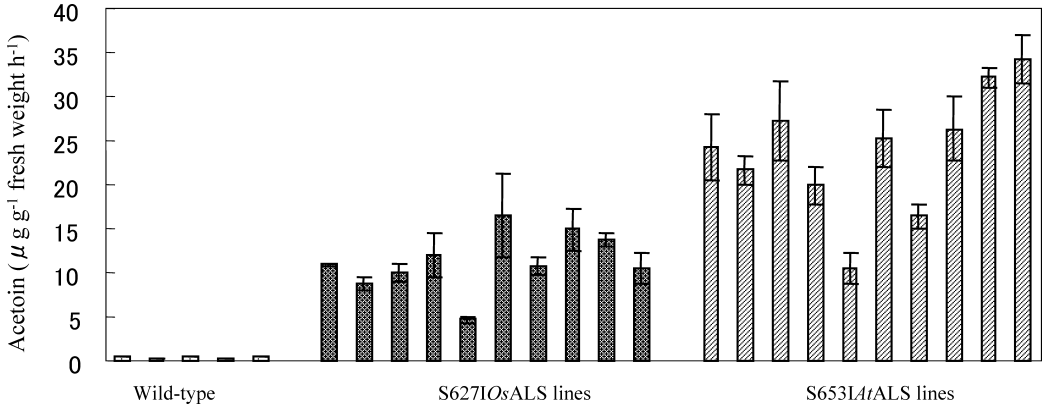


Figure 4. *In vivo* ALS assay of $T_1$ transgenic *Arabidopsis*. Individual leaves of wild-type and transgenic plants (S627I*Os*ALS and S653I*At*ALS lines) were treated with CPCA plus 0.1 μM PS, and then the amount of accumulated acetoin was measured. Results represent data obtained from three independent experiments, and error bars indicate standard error.

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resistance of these $T_3$ *Arabidopsis* to BS or PS was evaluated by comparing RS ratio obtained from the *in vivo* ALS assay. All of these $T_3$ homozygous *Arabidopsis* harboring each mutated ALS gene accumulated acetoin in the presence of BS or PS, suggesting the ALS inhibitor-resistant trait of transgenic plants was inherited by the progeny in a Mendelian manner. The maximum increase in BS resistance was observed in W574L/S653I*At*ALS 1-3 line (RS ratio of 58000), while the lowest increase in herbicide resistance was observed in S627I*Os*ALS 12 line (RS ratio of 4). The resistance levels of these transformants expressing mutated *Arabidopsis* ALSs to BS or PS were higher than those of corresponding transformants expressing mutated rice ALSs.

### *Sensitivity of transgenic Arabidopsis to ALS inhibitors*

BS or PS was sprayed over the leaves of $T_3$ homozygous plants expressing mutated ALSs. Wilting symptom was observed in wild-type at the lowest concentration tested (6.3 g a.i. $ha^{-1}$), whereas the growth of W574L/S653I *At*ALS 1-3 line was not inhibited by BS even at the highest concentration tested (100 g a.i. $ha^{-1}$). Although the W548L/S627I*Os*ALS 30-8 line showed resistance to BS compared with wild-type, growth was suppressed by BS even at the lowest concentration tested. Furthermore, wilting symptom was observed by BS at a rate of 50 g $ha^{-1}$ or more (Figure 5). Similarly, the transformants expressing single mutated ALSs, S627*Os*ALS 12-2 line and S653I*At*ALS 14-5 line, showed resistance to PS compared with wild-type, and S653I*At*ALS 14-5 line showed greater resistance to PS than S627I*Os*ALS 12-2 line (Figure 6). These results were entirely consistent with those obtained from the *in vivo* ALS assay. Thus, the strength of resistance to ALS inhibitors in transgenic *Arabidopsis* correlated with the mutated ALS enzyme activity.

### *Comparison of amino acid sequences of ALSs*

The amino acid residues corresponding to tryptophan at position 548 and serine at position 627 of rice are identical among the plants listed in Figure 7B. Phylogenetic analysis showed that the full-length amino acid sequences of ALSs derived from monocotyledonous plants and those derived from dicotyledonous plants formed two separate highly conserved clusters (Figure 8). Lack of the N terminal region slightly increased the amino acid homology of *S. bicolor, T. aestivum*, *H. vulgare* (incomplete ALS protein sequences) with *O. sativa* by comparison with that of the complete sequences (Figure 8) because the homology of this region (putative signal peptide region) was very low (Figure 7A). Despite the low level of homology at the N terminal region, monocotyledonous and dicotyledonous plants were divided into two clusters in the phylogenetic tree even when analyzing the amino acid sequences of the putative signal peptide region (Figure 9).

## Discussion

We demonstrated here that four mutated ALS genes derived from rice and *Arabidopsis* could be used as selectable markers in combination with PC-type ALS inhibitors. In addition, we revealed that selection by PC-type ALS inhibitors can clearly distinguish the resistant seedlings from non-resistant seedlings at a very low concentration of herbicide compared with hygromycin selection. Indeed, the concentrations of BS and PS for selection were about 1000-fold lower than that of hygromycin.

We performed *in vivo* ALS assay to identify whether the *Arabidopsis* seedlings, selected by resistance to BS or PS, were transformants. Although the *in vivo* ALS assay was originally developed for the analysis of ALS resistant weeds (Gerwick et al. 1993), we reasoned that the procedure could be applied to evaluate the transformants. Indeed, BS or PS resistant seedlings showing *in vivo* ALS activity were further analyzed to verify integration of the T-DNA region in their genome by PCR. The results from this analysis suggest that the assay could be reliably used to evaluate transformation. This is an advantage of using mutated ALS genes over other selectable markers because the *in vivo* ALS assay confirms both integration of the mutated ALS gene and expression of the corresponding protein in the selected plants. Furthermore, the *in vivo* ALS assay allows a large number of samples to be easily tested at relatively low cost by comparison with PCR-based screening methods.

Differences in the levels of acetoin accumulation were observed among the independent transgenic lines. This observation may reflect copy number differences or differential expression of the ALS due to positional effects in the *Arabidopsis* genome. If so, transgenic plants expressing a high level, or a desired level, of the gene of interest may be identified at an early stage of transformation. However, further analysis is needed to verify this hypothesis.

The results of *in vitro* ALS assay in our previous paper have indicated that the degrees of resistance to PC-type ALS inhibitors (RS ratio) of rice and *Arabidopsis* recombinant ALS proteins with identical mutations are very similar (Kawai et al. 2008). However, the results obtained in this report concerning the *in vivo* ALS assay and sensitivities of transgenic *Arabidopsis* to PC-type ALS inhibitors show that the degree of the resistance to PC-type ALS inhibitors of transformants expressing *Arabidopsis* mutated ALSs were greater than those of transformants expressing rice mutated ALSs. It is known that plant ALSs have a signal peptide that is required for

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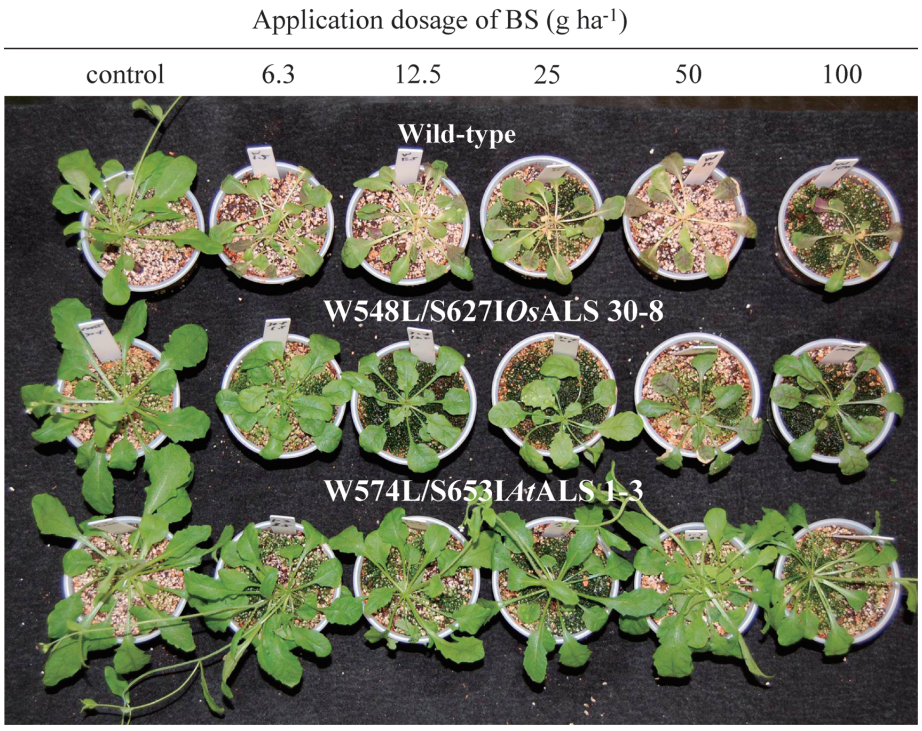


Figure 5. Comparison of sensitivities to BS of *Aranbidopsis* among wild-type and $T_3$ transformants. Plants, planted in pots (9 cm diameter), were sprayed with 6.3 to 100 μg $ml^{-1}$ (approximately 14 to 220 μM) BS at a application dose of 6.3 g to 100 g a.i. $ha^{-1}$. The photograph was taken 2 weeks after spraying.

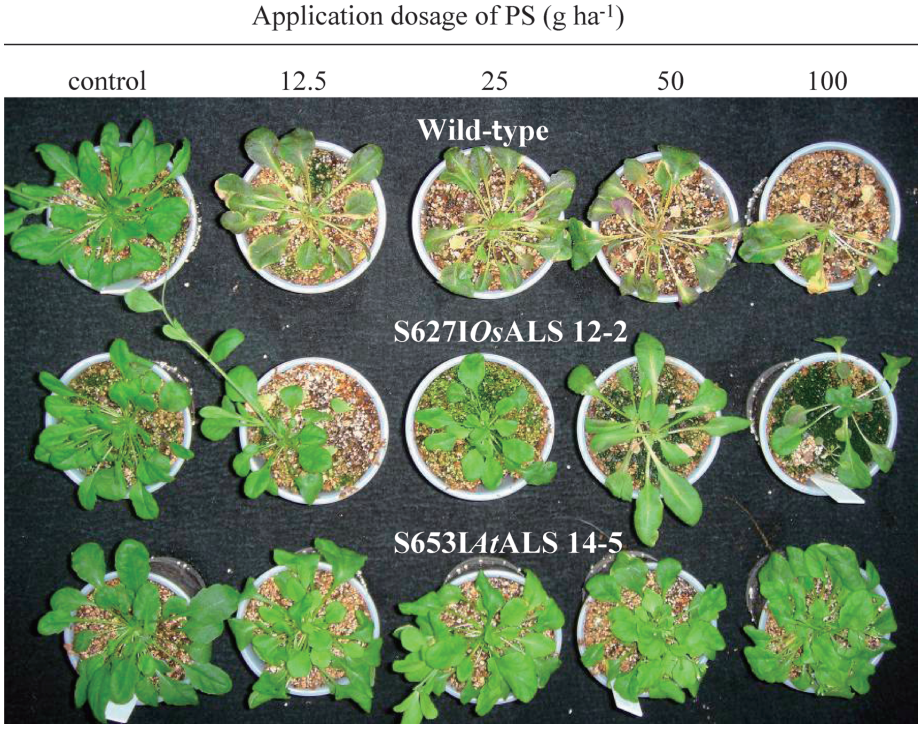


Figure 6. Comparison of sensitivities to PS of *Aranbidopsis* among wild-type and $T_3$ transformants. Plants, planted in pots (9 cm diameter), were sprayed with 12.5 to 100 μg $ml^{-1}$ (approximately 36 to 290 μM) PS at a application dose of 12.5 to 100 g a.i. $ha^{-1}$. The photograph was taken 2 weeks after spraying.

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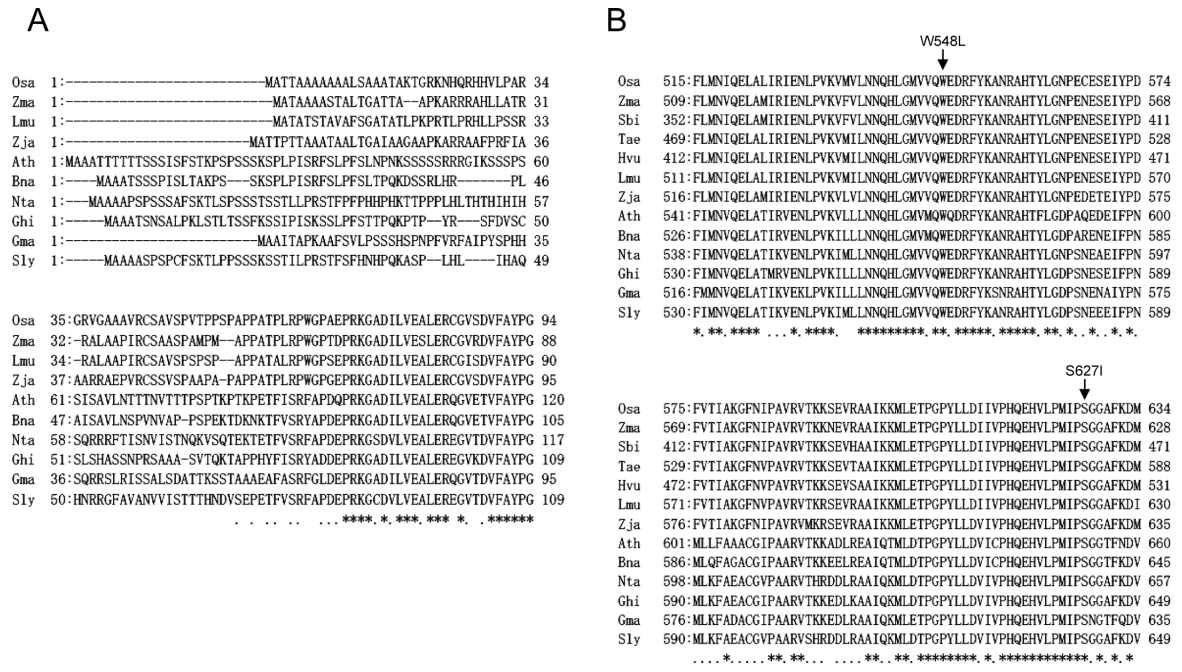


Figure 7. Amino acid sequence alignments of plant ALSs. A, Amino acid sequence alignments of putative signal peptide region. Multiple alignments were constructed with only complete ALSs. B, Amino acid sequence alignments of mutation region. Identical or similar amino acid residues are indicated by asterisks or dots, respectively. The residues of tryptophan at position 548, serine at position 627 of rice are conserved among plants listed here. Predicted ALSs with GenBank accession number or name in individual database are as follows: Osa, *Oryza sativa* (BAB20812); Zma, *Zea mays* (Q41768); Sbi, *Sorghum bicolor* (Sb04g020680 from JGI (http://genome.jgi-psf.org/)); Tae, *Triticum aestivum* (AAO53548); Hvu, *Hordeum vulgare* (AAC14572); Zja, *Zoysia japonica* (AB513331); Ath, *Arabidopsis thaliana* (CAB62345); Nta, *Nicotiana tabacum* (P09342); Gma, *Glycine max* (Glyma13g31470.1 from JGI); Ghi, *Gossypium hirsutum* (CAA87083); Sly, *Solanum lycopersicum* (SlSBM_S00638_04.30 from Tomato SBM (http://www.kazusa.or.jp/tomato/)); Bna, *Brassica napus* (P27818).

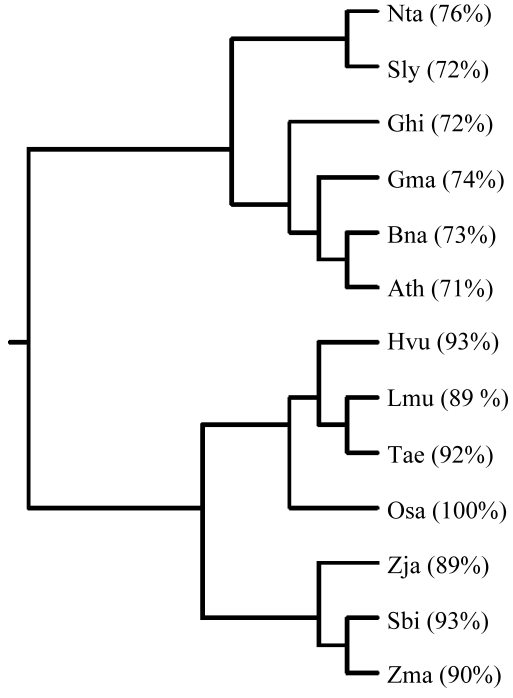


Figure 8. Phylogenetic analysis with full-length amino acid sequence of ALSs. The NJ-tree was constructed with ClustalW program at http://align.genome.jp/. The percentage indicates the amino acid homology of each plant with rice (Osa). Lack of the N terminal region slightly increased the amino acid homology of *S. bicolor, T. aestivum*, *H. vulgare* with *O. sativa* when compared to complete ALS protein sequences because the homology of this region was very low.

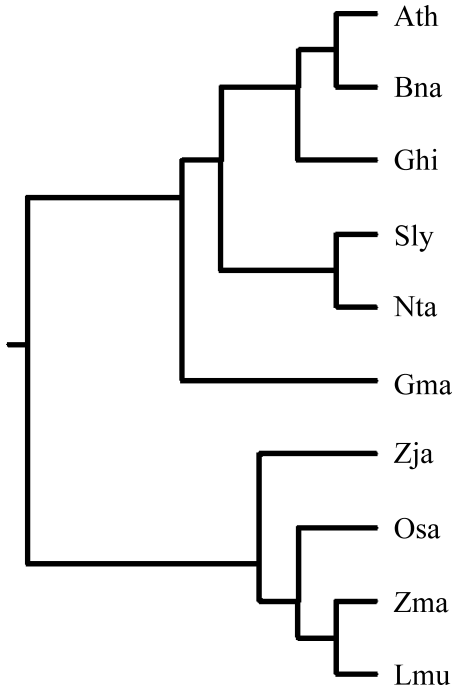


Figure 9. Phylogenetic analysis with amino acid sequence of putative signal peptide region of ALSs. Seventy amino acids from the first methionine of complete ALS protein sequences were used for the analysis.

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translocation of the protein into the chloroplast (Duggleby and Pang 2000). The exact size of signal peptide has not been identified experimentally. However several reports have indicated that the size of the signal peptide ranges between 70 and 85 amino acids (Chang and Duggleby 1997; Rutledge et al. 1991; Wiersma et al. 1990). If the cleavage site of the signal peptide is assumed to be at position 85, then the sequence homology of that of rice and *Arabidopsis* ALS is only 23%. Therefore, signal peptide processing and transport of the protein into the chloroplast may be involved in limiting rice ALS enzyme activity in *Arabidopsis*.

We also considered another potential reason for the observed difference in ALS activity. It has been shown that *Arabidopsis* ALS is composed of four catalytic subunits and four regulatory subunits (Lee and Duggleby 2001; McCourt et al. 2006). Thus, ALS derived from transformants expressing rice ALS will presumably be chimeric i.e., composed of both rice and *Arabidopsis* catalytic subunits. As a result, enzyme activity may be reduced compared with that of an ALS composed of only the *Arabidopsis* enzyme.

As shown in Figure 8, the full-length amino acid sequences of ALSs derived from monocotyledonous and dicotyledonous plants were clearly divided into two distinct clusters in the phylogenetic tree, each cluster being highly conserved. The putative signal peptide amino acid sequences of ALSs were also divided into two clusters in the phylogenetic tree (Figure 9). Therefore, our findings suggest it would be best to use rice and *Arabidopsis* mutated ALS genes for generating monocotyledonous and dicotyledonous transgenic plants, respectively. Given the differences in the sensitivity to PC-type ALS inhibitors and in the expression level of induced mutant ALSs among plant species, preparing various combinations of mutated ALS genes and PC-type ALS inhibitors is an effective strategy for applying this selection system to a broad range of plant species.

Transgenic *Arabidopsis* plants could be generated by using the host derived ALS gene in which the following amino acid substitutions had been engineered: W548L and/or S627I. In addition, these amino acid residues are conserved among various plants. Therefore, when generating transgenic plants whose nucleotide sequences of ALS have been determined, their own ALS genes carrying these mutations can be used as selectable markers.

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