Molecular Characterization of True and Ectopic Gene Targeting Events at the Acetolactate Synthase Gene in *Arabidopsis*

Masaki Endo, Keishi Osakabe, Hiroaki Ichikawa and Seiichi Toki.

Precise modification of plant genomes via gene targeting (GT) is important for the study of gene function in vivo. A reliable GT system using the protoporphyrinogen oxidase (PPO) gene in *Arabidopsis* was reported 4 years ago; however, there are no subsequent successful reports of GT in *Arabidopsis*. A previous study showed ectopic gene targeting (EGT) of the endogenous gene in two-thirds of GT plants, which was an obstacle to efficient true gene targeting (TGT). The endogenous acetolactate synthase (ALS) gene is involved in the biosynthesis of branched chain amino acids in plants and is the site of action of several herbicides. To confirm the generality of the GT system in *Arabidopsis*, and to characterize the EGT event in plants in detail, we converted *ALS* from a herbicide (imazapyr)-susceptible to a -resistant form by GT. We obtained two imazapyr-resistant plants following GT. One of the targeting events was TGT while the other was EGT. After detailed Southern blotting, PCR and nucleotide sequence analysis of the EGT plant, we determined the genomic position and structure of the ectopically targeted site. Based on our findings, we discuss the possible mechanisms of EGT in plants.

**Keywords:** Acetolactate synthase — *Arabidopsis* — Ectopic gene targeting — Gene targeting — True gene targeting.

Introduction

Gene targeting (GT) is a general method applied in yeast (Hinnen et al. 1978) and mouse embryonic stem cell research (Capellesi 1989), and has recently been extended to research on *Physcomitrella* (Schafer and Zryd 1997), *Drosophila* (Rong and Golic 2000), sheep (McCreath et al. 2000) and human somatic cells (Hanson and Sedivy 1995, Sedivy et al. 1999). In the case of flowering plants, numerous trials of GT-mediated modification of endogenous genes (Lee et al. 1990, Lebel 1994, Miao and Lam 1995, Kempin et al. 1997) have been conducted. However, reproducibility was limited and putative targeting plants were not analyzed thoroughly at the nucleotide sequence level.

Recent studies by Hanin et al. (2001) and Terada et al. (2002) presented reliable and reproducible endogenous GT systems. Hanin et al. (2001) used *Arabidopsis* protoporphyrinogen oxidase (PPO) as a target gene and obtained products of true gene targeting (TGT), in which the wild-type gene was modified as expected, and those of ectopic gene targeting (EGT), in which the modified PPO sequence seemed to be integrated elsewhere in the genome. On the other hand, Terada et al. (2002) used two flanking diphtheria toxin genes lethal to plants for counter-selection against random, non-targeted integration events, and confirmed the expected recombinant events in rice. Hanin et al. (2001) reported possible replacement of specific amino acids by GT. Terada et al. (2002) showed that the targeting strategy is, in principle, applicable to any gene and may prove useful for generating knockouts and assigning gene function. These two reports indicate the potential use of GT technology for functional analysis of plant genes and molecular breeding. However, there are no subsequent reports of successful GT in flowering plants, despite great efforts (Jelesko et al. 2005).

To improve the GT system, more than one model GT system is preferable. Accordingly, we established a detection system of GT events at the endogenous acetolactate synthase (ALS) locus of *Arabidopsis* and analyzed the GT candidate plants in detail. ALS catalyzes the initial step common to the biosynthesis of branched chain amino acids leucine, isoleucine and valine. ALS is a target of sulfonlurea and imidazolinon herbicides (LaRossa and Schloss 1984), although several single base pair changes in the ALS coding sequence resulted in a herbicide-resistant form of the enzyme (Lee et al. 1988). ALS is a suitable target for GT because it exists as a single copy gene in the *Arabidopsis* genome and a single amino acid change results in a herbicide-resistant phenotype, so that targeted plants can be selected easily by the herbicide imazapyr (IM).

In the present study, we obtained two IM-resistant plants after transformation of IM-sensitive wild-type plants with a T-DNA containing a fragment of the herbicide-resistant form of the ALS gene via in planta transformation. Results of initial molecular analyses of these two IM-resistant plants suggested...
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the occurrence of an EGT event in one of the two plants. Previously, an EGT model was proposed for the unexpected modification of targeting constructs. However, detailed analysis of EGT of endogenous genes has not been available to date. Then, in the next step, we analyzed the TGT and EGT events at the nucleotide sequence level. The result of our analysis was explained by a classical EGT model for the unexpected modification of a targeting construct and subsequent ectopic integration of the modified construct. In addition, another possibility of the random integration of the targeting construct and subsequent homologous recombination between targeting construct and the endogenous ALS locus has remained for the explanation of the EGT event. Detailed analysis of the EGT event enhanced our understanding of the molecular mechanism of GT events in flowering plants.

Results

Selection of IM-resistant plants after in planta transformation of an ALS vector series

The IM resistance mutation used in this experiment was a Ser (AGT)→Asn (AAC) change at amino acid 653 (S653N). Spontaneous IM-resistant mutants, which occur at a frequency of 10⁻⁶ (Chang and Duggleby 1998), can be distinguished from those resulting from homologous recombination (HR) by detection of two successive base pair changes to make the S653N mutation.

We constructed a series of binary vectors containing various lengths of Arabidopsis ALS genomic DNA sequences with or without S653N mutation. Wild-type IM-sensitive Arabidopsis plants were transformed using an in planta transformation method (Bechtold et al. 1993) with Agrobacterium containing the T-DNA constructs. The restriction sites used in the ALS vector construction are shown in Fig. 1A. IM-resistant T₁ plants were obtained by growth of seeds on aseptic IM-containing MS germination medium (Fig. 1B) or by spraying soil-grown seedlings with a commercial formulation of IM (Fig. 1C) (see materials and methods). The results of selections of IM-resistant plants are summarized in Table 1.

The negative control vector ALSWT comprises a 6.6 kb ClaI fragment containing 1 kb of promoter sequence, 0.3 kb of 5′-untranslated region (UTR) sequence, 2 kb of coding...

Fig. 1 Generation of IM-resistant plants by T-DNA-mediated GT of the ALS locus. (A) Experimental design of targeted modification of the Arabidopsis ALS locus. The ALS coding region is indicated by the blue box. The thick lines represent flanking plant genomic DNA. The mutation conferring IM resistance (S653N) is marked by red triangles at position +1,960 below the ALS coding region. The restriction sites used in the ALS vector construction are shown. RB, right border of T-DNA; LB, left border of T-DNA. (B) Selection of IM-resistant plants on MS germination medium supplemented with 5 µM IM for ALSWT, ALSΔN and ΔproALSΔN. (C) Selection of IM-resistant plants for Δ5′ALSΔN. Four-day-old soil-grown seedlings of Δ5′ALSΔN seedlings were sprayed with 6.25×10⁻⁴ % arsenal (BASF, Japan), a commercial formulation of IM (the final concentration of IM was 5 µM). The arrowhead indicates the IM-resistant plant.

Fig. 2 Sequence analysis of the ALS locus in IM-resistant plants. PCR was performed using TF1 + TR4 (PCR fragment 1) and TF3 + TR6 (PCR fragment 2) as primers. Sequence analysis of purified PCR fragment 1 using primer TF3 detected the S653N mutation in Δ4-1 and Δ4-2 but did not detect any trace of left border sequence on Δ4-1 and Δ4-2 by using primer TR2. Sequence analysis of cloned PCR fragment 2 using primer TF3 detected the S653N mutation in Δ4-2 but not in Δ4-1. Sequence analysis using primer TF5 did not detect any trace of right border sequence on Δ4-1 and Δ4-2.
sequence and 3.2 kb of terminator sequence of the wild-type ALS gene. IM-resistant plants were not obtained from seeds that settled in ALSWT-transformed plants. The positive control vector ALSN is similar to ALSWT except for the presence of the S653N mutation. ALSN contains an IM-resistant form of the ALS coding sequence with its own 5′-, 3′-regulation sequence, and thus random integration of this construct should confer IM resistance. Accordingly, the emergence rate of IM-resistant plants represents the transformation efficiency. The transformation efficiency was estimated to be ~0.4% based on the result of ALSN transformation (Table 1). ΔproALSN is the promoter deletion construct of ALSN; a ClaI–PmII fragment containing 1 kb of promoter sequence and 144 bp of 5′-UTR sequence was deleted from ALSN. When gene trap or promoter trap occurred by this construct, transgenic plants showed IM resistance. The calculated promoter trap efficiency was ~16% (Table 1). Although we checked the junction sequences from 201 ΔproALSN transformants, no GT plants were identified (data not shown). The targeting vector Δ5′ALSNSN is the 5′-coding region deletion construct of ALSN; a ClaI–SacI fragment containing 1 kb of promoter sequence, 308 bp of 5′-UTR sequence and 156 bp of 5′-coding region was deleted from ALSN. Because Δ5′ALSNSN retains the S653N mutation but lacks the 5′-coding region corresponding to the first 52 amino acids including the chloroplast targeting signal of ALS, the ALS genes on these targeting vectors are truncated and non-functional. IM-resistant plants can result from HR between the introduced fragment of the ALS mutant gene and the chromosomal genomic ALS locus. Finally, two IM-resistant individual plants (Δ4-1, Δ4-2) were obtained from a total of 7.8×105 Δ5′ALSNSN-transformed seeds.

Confirmation of S653N mutation in GT candidate (Δ4-1, Δ4-2) using PCR and sequence analysis

DNAs extracted from the T1 seedlings of Δ4-1 and Δ4-2 were used as templates for PCR and sequence analysis. As shown in Fig. 2, amplification using primer TF1, which anneals to the promoter region of the ALS gene (absent in Δ5′ALSNSN), and primer TR4 yielded a PCR product of 2.8 kb (PCR fragment 1) from Δ4-1 and Δ4-2. These fragments were used as templates for direct sequencing. Sequencing of the PCR fragment 1 from Δ4-1 and Δ4-2 using primer TF3 detected the S653N mutation, namely AGT to AAC on the state of wild-type/mutational heterozygous, as expected. Neither insertion of the left border sequence nor deletion of the ALS gene was identified at the position of the left border by sequencing using primer TR2 in PCR fragment 1 of Δ4-1 and Δ4-2. These results suggest the occurrence of a GT event in Δ4-1 and Δ4-2.

Table 1 Selection of IM-resistant plants after in planta transformation

<table>
<thead>
<tr>
<th>Vector</th>
<th>No. of seeds applied for selection (a)</th>
<th>No. of IM-resistant plants (b)</th>
<th>b/a ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALSWT</td>
<td>3,500</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ALSN</td>
<td>5,000</td>
<td>20</td>
<td>4.00×10⁻³</td>
</tr>
<tr>
<td>ΔproALSNSN</td>
<td>320,000</td>
<td>201</td>
<td>6.28×10⁻⁴</td>
</tr>
<tr>
<td>Δ5′ALSNSN</td>
<td>780,000</td>
<td>2 (1')</td>
<td>1.28×10⁻⁶</td>
</tr>
</tbody>
</table>

Transformation efficiency.
Promoter trap efficiency.
True gene targeting efficiency.
Number of true gene targeting plants.

Analysis of progeny of putative GT plants by Southern blot

We next investigated the correlation between IM resistance and ALS gene structure. Using a portion of leaves from randomly selected T2 plants of Δ4-1 and Δ4-2, we assessed the IM-resistant trait by the ability of leaves to form a callus after their incubation for 3 weeks on callus induction medium containing 0.5 µM IM. Simultaneously, we subjected DNA prepared from leaves of the same T2 plants to Southern blot analysis to characterize their ALS gene structure. In Fig. 3, IM-sensitive plants are denoted by the red numbers while IM-resistant plants are represented by the blue numbers. For Δ4-1, plants 1, 3, 7 and 10 were IM sensitive and plants 2, 4, 5, 6, 8 and 9 were IM resistant (Fig. 3A). The genomic DNAs from these T2 plants were digested with EcoRI and hybridized with PCR-labeled digoxigenin (DIG) probe A. Using this probe, a 5 kb band was the signature of the wild-type ALS gene (Fig. 3E). As shown in Fig. 3B, an ectopic transgenic insertion was detected as an additional 12 kb band in all IM-resistant plants. All Δ4-1 progeny were IM resistant (Fig. 3C). All Δ4-2 progeny contained the 5 kb band, indicating a wild-type copy of the ALS gene, meaning that the endogenous ALS locus was not modified. This result suggests the occurrence of EGT in Δ4-1 as discussed later. For Δ4-2, plants 1, 9 and 10 were IM sensitive and plants 2–8 were IM resistant (Fig. 3C). All Δ4-2 progeny contained either a single band of 5 kb (plant 5) or a 5 kb band plus multiple other bands (plants 1–4 and 6–10) (Fig. 3D). Furthermore, Δ4-2 plant 5 was IM resistant. This result indicates that TGT occurs at the ALS locus and that several T-
DNA fragments are simultaneously integrated at another single locus, as a result of non-homologous end joining (NHEJ).

Confimation of EGT and TGT events in Δ4-1 and Δ4-2 by PCR and DNA sequencing

Sequence analysis using the T1 generation of putative GT plants was performed to confirm that TGT occurred on Δ4-2 and EGT occurred on Δ4-1. As shown in Fig. 2, amplification using primers TF3 and TR6, which anneal 3.5 kb downstream of the S653N position that is absent in the targeting vector, yielded a PCR product of 3.7 kb (PCR fragment 2) in Δ4-1 and Δ4-2. These fragments were cloned and used as templates for sequencing. Sequence analysis of PCR fragment 2 using primer TF3 detected the S653N mutation in Δ4-2. Neither insertion of the right border sequence nor deletion of the Arabidopsis genome was found at the position of the right border in Δ4-2 based on the sequence analysis using primer TF5. These results confirm that TGT occurred on Δ4-2. On the other hand, in PCR fragment 2 of Δ4-1, only the wild-type sequence was detected at the site of S653N, although several clones were sequenced. This suggests that the mutational type ALS sequence is not located on the original ALS locus in Δ4-1. After further PCR and nucleotide sequence analysis, we found a functional ALS gene that contained the S653N mutation at the locus 700 kb distant from the original ALS locus. In this locus, 5 kb upstream of the ALS gene missing on Δ4′ALS and the whole binary vector sequence (6.8 kb) following the right border sequence were detected (Fig. 4 lower panel). The 12 kb band shown in Fig. 3B can be explained by this genome structure. These results suggest the occurrence of an EGT event in Δ4-1.

Fig. 3 Correlation between IM resistance and ALS structure. Leaf cuttings of randomly selected Δ4-1 (A) and Δ4-2 (C) T2 plants were grown on callus induction medium containing 0.5 µM IM for 3 weeks. For Southern blot analysis of Δ4-1 (B) and Δ4-2 (D) T2 plants, genomic DNA was cleaved with EcoRI, separated by electrophoresis on a 0.8% agarose gel, and blotted onto a nylon membrane. The membrane was then hybridized with a PCR-labeled DIG probe A. The DNA sample in each lane corresponds to the plants in (A) and (C). Red-colored numbers, IM-sensitive plants; blue-colored numbers, IM-resistant plants. The sizes of fragments produced after EcoRI digestion of the endogenous ALS gene and the position of the probe are shown in (E). WT, Columbia wild type.
Reconfirmation of EGT on Δ4-1 by Southern blot analysis

To confirm the modified structure of the ALS genome suggested by the PCR and subsequent DNA sequence analysis of Δ4-1, Southern blot analysis was performed with probe B (Fig. 4). The DNA samples correspond to the plants shown in Fig. 3A and B. Thus, IM-sensitive plants are those denoted by the red numbers and IM-resistant plants are those denoted by blue numbers. All Δ4-1 progeny showed a band of 3.6 kb, indicating the presence of the wild-type ALS gene at the genomic ALS locus. Furthermore, IM-resistant Δ4-1 progeny showed an additional band of 9.1 kb, which was predicted from the junction sequence analysis of the ectopically inserted ALS construct and subsequent database analysis.

Discussion

In this study, we developed a GT system for the Arabidopsis ALS gene and thoroughly characterized plants resulting from both TGT and EGT. GT of the ALS gene in tobacco with a similar system was reported previously (Lee et al. 1990). However, the system was more complex due to ploidy and the gene-targeted plants were not analyzed at a nucleotide sequence level. Furthermore, recent available data indicate that Lee et al. probably could not obtain TGT plants (see the review by Iida and Terada 2005). While the herbicide-resistant form of the Arabidopsis ALS gene is well characterized and frequently used as a selection marker gene for the production of transgenic plants (Sathasivan et al. 1990), successful GT of this gene has not yet been reported in Arabidopsis. In the report of Hanin et al. (2001), they argued that several single base pair changes (spontaneous mutations) in the ALS coding sequence, which provide herbicide resistance, result in a relatively high background level of herbicide resistance under stringent herbicide selection. However, in our system, GT events and spontaneous mutations could be distinguished by the detection of two successive base pair changes.

The basal transformation frequency in this study was estimated to be ~0.4% (Table 1) from the transformation experiment of ALS<sub>SN</sub> consisting of the IM-resistant form of the ALS coding sequence with its own 5′-, 3′-regulatory sequence. In this study, we obtained one TGT plant from a total of 7.8×10<sup>5</sup> Δ5′ALS<sub>SN</sub>-transformed seeds. Considering the transformation frequency of ~0.4%, one TGT event for every 3,120 non-homologous DNA insertions occurred. It is impossible to cal-
cule a meaningful GT frequency from the present work. For the estimation of statistically significant GT frequency, additional GT events should be identified and analyzed. In various GT experiments using different plants and transformation systems, a wide range of GT frequency has been recorded, ranging from $10^{-3}$ to $10^{-5}$ (for a review see Hohn and Puchta 2003).

Following molecular characterization of two GT plants (A4-1 and A4-2), a TGT event was confirmed in one of the targeting events (A4-2). The S653N mutation was introduced in the chromosomal ALS locus without further modifications. The modifications of the ALS locus and IM resistance were stably transmitted to the progeny, and plants homozygous for the modified ALS locus were phenotypically indistinguishable from the wild-type plants (data not shown). These results suggest that there is no significant alteration in the activity of the gene and/or enzymatic properties of its protein product.

Fig. 5 shows three types of anticipated transformants obtained from T-DNA-mediated GT experiments in plants. The genome structure of the ALS gene in A4-2 is explainable by the classical double-crossover model via HR (Fig. 5A). HR at the target locus leads to the desired gene replacement. Resolution of Holliday junctions leads to conversion and crossover events. The border sequences that have no homology to genomic DNA might be eliminated in this process.

Southern blot analysis and evaluation of IM resistance of A4-1 progeny showed that IM resistance was dependent on the existence of an ectopic ALS fragment. Such results may appear in the case of TGT via a one-sided invasion model (Fig. 5B) or EGT (Fig. 5C). In the case of a one-sided invasion model TGT (Fig. 5B), modifications occurred at the wild-type ALS locus, so that the band corresponding to the wild-type ALS copy should disappear in one of four T$_2$ plants on Southern blot analysis. Our results showed that all A4-1 progeny plants retained the 5 kb band corresponding to the wild-type copy of the ALS gene using probe A (Fig. 3B). This result suggests that A4-1 could not be explained by a one-sided invasion model TGT. In the case of the classical EGT model (Fig. 5C), sequence homology can be copied from the target locus to the vector, and the modified vector may then integrate elsewhere in the genome so that the original target locus is not modified.

Careful sequence analysis of A4-1 revealed the existence of a functional ALS gene that contained the S653N mutation at the locus 700 kb distant from the original ALS locus. In this locus, 5 kb upstream of the ALS gene missing on Δ5’ALS$^{SN}$ and the whole binary vector sequence (6.8 kb) following the right border were detected (Fig. 4, lower panel). Considered together, we concluded that the IM resistance of A4-1 was caused by an EGT event. It is not clear why the EGT event observed in this study used a T-DNA that had been irregularly processed from a binary vector. Concerning this type of processing, van der Graaff et al. (1996) reported that the transferred DNA consisted of complete binary vector sequences in addition to the T-region. They also showed that left borders acted as the start position for T-strand transfer (assuming here the role normally played by right borders).

According to the classical EGT model depicted in Fig. 5C, an EGT event in A4-1 can be explained as follows. Incomplete recognition of the right border occurred in Agrobacterium and consequent single-stranded T-DNA contained the whole vector sequence. This single-stranded T-DNA was transferred to the nucleus of the Arabidopsis cell and modified by the chromosomal template. The completed ALS gene containing the S653N mutation was integrated into the Arabidopsis genome via NHEJ. On the other hand, another model for the construction of this IM-resistant ALS structure cannot be excluded based on the evidence that the ectopically integrated ALS gene was located relatively close to the endogenous ALS gene with reverse orientation. In such a model, the 5’-truncated ALS sequence on T-DNA is integrated into the Arabidopsis genome by NHEJ, and then intrachromosomal HR between the endogenous ALS gene and ectopically integrated T-DNA occurred, although an accidental double-strand break should be induced at the ectopically integrated gene before HR. In this context, Shalev and Levy (1997) reported the occurrence of recombination between two homologous ectopic (non-allelic) sequences following double-strand breaks in transgenic tobacco plants. Recently, a reliable gene targeting system in Drosophila was developed (Rong and Golic 2000). In this system, the transgene was first integrated into the Drosophila genome via NHEJ, and finally recombined with the targeted gene via intrachromosomal recombination. Our results suggest that the system developed for Drosophila GT might be applied to flowering plant GT.

A deletion construct of selectable transgenes could serve as a rapid and inexpensive tool for identifying GT events. Arabidopsis ALS protein consists of 670 amino acids. To date, several amino acid substitutions in ALS at amino acids 124, 197, 199 and 653 that confer herbicide resistance have been identified (Chang and Duggleby 1998). In addition, the W574S mutation in Arabidopsis also seems to confer herbicide resistance (Chang and Duggleby 1998). The IM resistance mutation used in this experiment is a Ser→Asn change at amino acid 653. In order to maintain sequence homology near the target site, we prefer a 5’- to 3’-coding region deletion construct.

Recent analyses of the sequence of insert junctions of T-DNA indicate that T-DNA is preferentially integrated into promoters and AT-rich sequences of transcribed chromatin domains by NHEJ (Koncz et al. 1989, Mayerhofer et al. 1991, Brunaud et al. 2002, Szabados et al. 2002). Furthermore, VirD2, which acts as a pilot protein for T-DNA transfer and integration, interacts with TATA box-binding protein (Bako et al. 2003). These factors seem to affect the relatively high efficiency of promoter or gene trap. Our results of the ΔproALS$^{SN}$ transformation experiment support the statement of relatively high promoter or gene trap efficiency (∼16%) (Table 1). Assuming that T-DNA integration and GT involve a common process, such as T-DNA access to the genome and homology
search, the 3′-coding region deletion construct appears to be better than the 5′-coding region deletion construct as a targeting vector.

The induction of a point mutation(s) that alters amino acid residue(s) in ALS involved in herbicide resistance by a combination of various deletions in the coding sequence may improve GT efficiency.

Recently, crystalization and preliminary X-ray diffraction analysis of the catalytic subunit of Arabidopsis thaliana ALS in complex with the sulfonylurea herbicide chlorimuron ethyl were reported (Pang et al. 2004). This is the first report of the structure of any plant protein in complex with a commercial herbicide. This finding allowed us to design novel ALS proteins resistant to ALS-inhibiting herbicides. The combination of the protein design study and the GT system of the ALS gene presented here should be useful for the development of new herbicide-resistant plants.

During the preparation of this manuscript, Shaked et al. (2005) reported a high-frequency GT in Arabidopsis plants expressing the yeast RAD54 gene. It will be interesting to test GT of the AtALS gene with this hyper-gene targeting Arabidopsis plants.

Materials and Methods

Plant growth conditions

Seeds of Arabidopsis (ecotype Columbia) were grown either in soil and maintained in a controlled growth chamber (approximately 22°C, 16 h light) or on MS germination medium (Murashige and Skoog 1962) supplemented with 0.3% gelrite and maintained in a controlled growth chamber (approximately 22°C, continuous light). For axenic growth, seeds were surface sterilized for 3 min with 10% (v/v) sodium hypochlorite containing a drop of 50% (v/v) Tween-20 per 5 ml, and then washed several times with sterile distilled water.

Construction of vectors

We isolated ALS genomic DNA of Arabidopsis (var. Col.) from a bacterial artificial chromosome (BAC) clone containing this gene. The herbicide-resistant form of this gene was genetically mapped on IGF (∆ALS). The 6.6 kb ∆ALS fragment containing the ALS genomic sequence was cloned into Bluescript KS vector and used as a target vector.

Two successive point mutations that converted Ser (AGT) to Asn (AAC) at codon 653 (S653N) were introduced into the ALS coding sequence of ALSWT using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) to generate ALSN. ALSN is similar to ALSWT except for the presence of the S653N mutation. ∆proALSN is the promoter deletion construct of ALSN; 1 kb of the promoter sequence and 144 bp of 5′-UTR sequence (between the Cda site and Pml site in Fig. 1A) are deleted in ∆proALSN from ALSN.

ΔSALS is a 5′-coding region deletion construct of ALSN; 1 kb of promoter sequence, 308 bp of 5′-UTR sequence and 156 bp of the 5′-coding region (between the Cda site and SacII site in Fig. 1A) are deleted in ΔSALS from ALSN.

Arabidopsis transformation

Agrobacterium tumefaciens strain EHA105 harboring binary vectors was used for transformation. Selection of transformed bacteria was carried out on plates containing 100 mg l⁻¹ spectinomycin. Agrobacterium used for in planta transformation of Arabidopsis were grown in Lucia-Bertani-rich medium (Lichtenstein and Draper 1986) at 28°C. Agrobacterium-mediated plant transformation of Arabidopsis (var. Col.) adult plants was performed by the inflorescence infiltration method (Bechtold et al. 1993).

Selection of imazapyr (IM)-resistant plants

The selection experiments were performed on aseptic MS germination medium (Murashige and Skoog 1962) supplemented with 0.3% gelrite and 5 µM IM (ALSWT, ALSN and ΔproALSN) or on soil-grown seedlings (ΔSALS). Four-day-old ΔSALS seedlings were sprayed with 6.25×10⁻⁶% arsenal (BASF, Japan), a commercial formulation of IM (the final concentration of IM was 5 µM). For T₃ segregation analysis, selection was carried out on aseptic MS germination medium supplemented with 0.3% gelrite and 0.5 µM IM. Preliminary experiments confirmed that 0.5 and 5 µM concentrations of IM produced comparable results (data not shown).

Southern blot analysis

DNAs were extracted from leaves of seedlings using the Nucleon Phytopure Extraction Kit (Amersham Pharmacia Biotech, UK) according to the instructions provided by the manufacturer. After endonuclease digestion and electrophoresis on 0.8% agarose gels, DNA fragments were transferred onto a positively charged nylon membrane (Roche, Basel, Switzerland). Probes were prepared by using PCR DIG Probe synthesis kit (Roche). Hybridization was performed according to the DIG Application Manual (Roche). Hybridization was performed at 42°C and washing was performed under high stringency conditions at 68°C.

Evaluation of IM resistance on leaves

Leaves from non-selective Δ4-1 and Δ4-2 T₃ plants were placed in callus induction medium (Murashige and Skoog salts supplemented with 20 g l⁻¹ glucose, 0.5 mg l⁻¹ 2,4-D, 5 mg l⁻¹ IAA, 0.3 mg l⁻¹ kinetin and 0.75% Bacto-agar; Difco, Detroit, MI, USA) containing 0.5 µM IM. Finally, the leaf cuttings were photographed after 3 weeks of incubation at 22°C under fluorescent light.

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