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The mutant form of acetolactate synthase genomic DNA from rice is an efficient selectable marker for genetic transformation

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Abstract

The proper use of a marker gene in a transformation process is critical for the production of transgenic plants. However, consumer concerns and regulatory requirements raise an objection to the presence of exogenous DNA in transgenic plants, especially antibiotic-resistant genes and promoters derived from viruses. One approach to overcome this problem is the elimination of marker genes from the plant genome by using several site-specific recombination systems. We propose an alternative method to solve this problem using a marker gene exclusively derived from the host plant DNA. We cloned a genomic DNA fragment containing regulatory and coding sequences of acetolactate synthase (ALS) gene from rice, and mutagenized the ALS gene into a herbicide-resistant form. After transfer of this construct to the rice genome, transgenic plants were efficiently selected with a herbicide, bispyribac-sodium salt, which inhibits the activity of wild type ALS. We also analyzed the regulatory feature of the rice ALS gene promoter with the *gusA* reporter gene and revealed that GUS expression was observed constitutively in aerial parts of rice seedlings and root tips. The marker system consisted exclusively of host plant DNA and enabled efficient selection in a monocot crop plant, rice. The selection system can potentially be applied to generate transgenic plants of other crop species and can be expected to be publicly acceptable.

Abbreviations: 4-MUG – 4-methylumbelliferyl β-D-glucuronide; CaMV35S – Cauliflower Mosaic Virus 35S; GUS – β-glucuronidase; X-gluc – 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

Results and discussion

The development of transgenic crops with desirable traits is an important strategy in modern plant breeding systems. However, consumers oppose the use of transgenic crops for a variety of reasons, such as the presence of plant virus derived promoter and antibiotic-resistant genes. In order to

minimize the public concerns, researchers have tried to eliminate selection marker genes from transgenic plants using site-specific recombinases (See review, Hare and Chua 2002). Although some success has been reported (Gleave et al. 1999; Sugita et al. 2000; Zuo et al. 2001), the establishment of the system in crop plants is relatively complex and several problems remain to be overcome for efficient excision. In this communication, we report an alternative approach to obtain public acceptance involving the development of a new marker gene cassette derived from the rice genomic DNA sequence of acetolactate synthase (ALS), and evaluated it for the *Agrobacterium*-mediated transformation of rice.

Acetolactate synthase (ALS; EC4.6.3.8, also referred to as acetohydroxy acid synthase; AHAS) is the first common enzyme in the biosynthetic pathway of branched-chain amino acids, isoleucine, leucine and valine (See review, Chipman et al. 1998). ALS is the target site of sulfonylurea and imidazolinone herbicides (Shimizu et al. 2002), and several mutations in ALS genes confer herbicide-resistance in several plant species (Tan et al. 2005). Thus, we postulated that the mutated ALS gene is a good candidate for a selectable marker cassette system coupled with appropriate herbicides.

The ALS cDNA from Japonica type rice, *OsALS* was cloned by Shimizu et al. (GenBank Accession Number AB049822). After surveying the Indica rice genome database (http://btn.genomics.org.cn/rice) by using the *OsALS* cDNA sequence as a query, we found a contig sequence containing 5′-upstream and N-terminal regions of the *OsALS* sequence. We designed PCR primers according to the contig sequence information of the Indica type rice ALS gene and amplified a 2.9-kb fragment of 5′-upstream sequence of *OsALS* with the genomic DNA isolated from Japonica type rice (Nipponbare) as a template. A 2.0-kb of 3′-downstream region of the *OsALS* sequence was cloned with the Universal Genome Walker Kit (Clontech Laboratories, CA). Finally, a 7.1-kb of the *OsALS* genomic DNA containing 2.9-kp 5′-upstream, 2.3-kb coding and 1.9-kb 3′-downstream regions, was amplified and cloned into pBluescript KS (−) vector. The nucleotide sequence of the cloned 7.1-kb fragment was sequenced and compared with the sequence determined by direct sequencing of the PCR product. Recently, a BAC clone (OSJNBa0052M16), which contained the *OsALS* gene, was determined by the Rice Genome Research Program (RGP) (GenBank Accession Number AP005841). The sequence of the ALS gene in the BAC DNA sequence completely matched our ALS genomic DNA sequence. *OsALS* lacked introns (Figure 1), similar to the ALS genomic DNA from tobacco (Lee et al. 1988) and *Arabidopsis* (Sathasivan et al. 1990).

We first analyzed the putative promoter region of the *OsALS* gene by using *gusA* as a reporter gene. A 2.2-kb *Kpn*I–*Nco*I fragment containing 5′-upsream sequence of *OsALS* was inserted into a *Kpn*I–*Nco*I site of the binary vector pSMAHd-N628omega-M2GUS (Ichikawa et al. unpublished

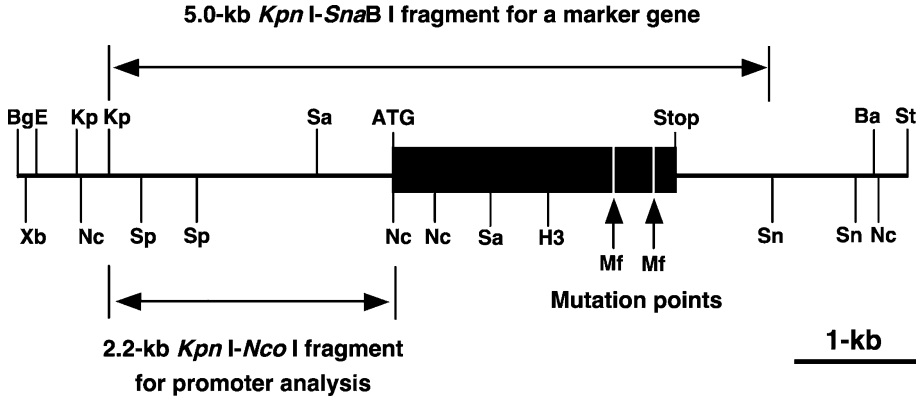


Figure 1. Schematic representation of the *OsALS* genomic DNA. The thick line represents 5′ and 3′ non-coding regions. The black box represents the coding region. Black arrows show two *Mfe*I sites introduced by site-directed mutagenesis. Ba; *Bam*HI, Bg; *Bgl*II, E; *Eco*RI, H3; *Hind*III, K; *Kpn*I, M; *Mfe*I, Nc; *Nco*I, Sa; *Sal*I, Sp; *Spe*I, Sn; *Sna*BI, St; *Stu*I, Xb; *Xba*I.

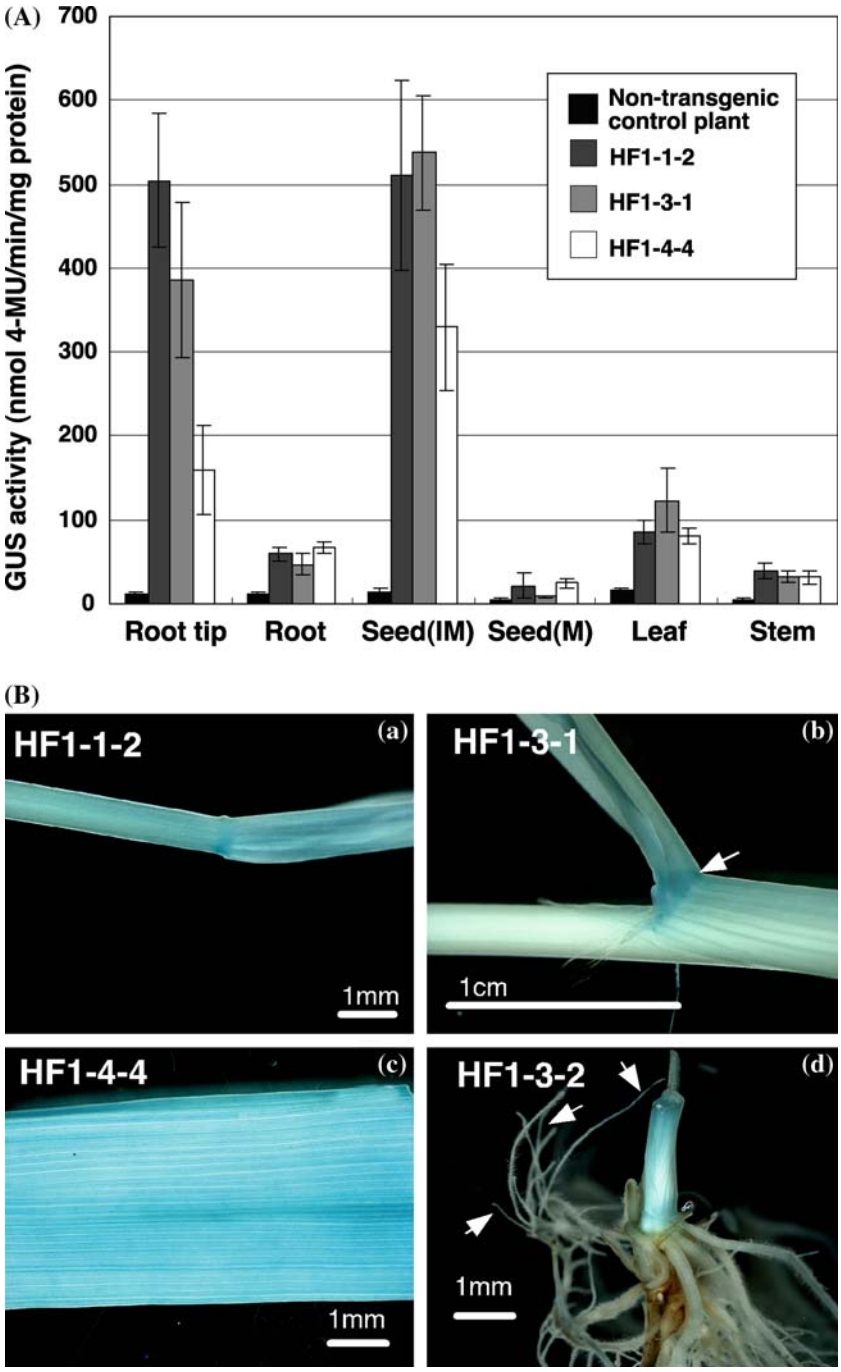
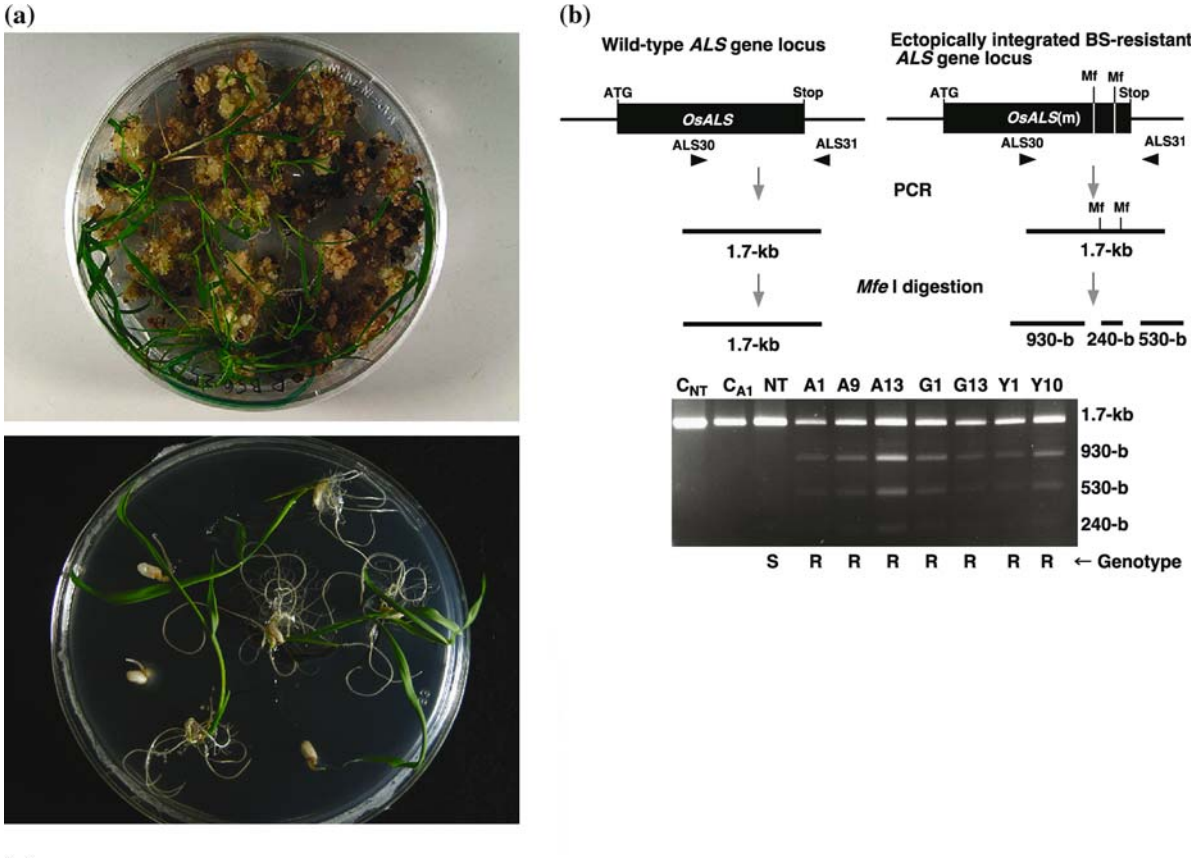


Figure 2. Promoter analysis of the *OsALS* gene. Transgenic rice plants were produced with the *OsALS* promoter + GUS construct. Six independent transformed plants (HF1-1-2, HF1-2-1, HF1-3-1, HF1-3-2, HF1-4-4, HF1-4-5) were used for analyses of GUS activities. (A) Fluorometric assay of the GUS activity in transgenic rice plants with 4-MUG as a substrate. Data represent mean ± SD of three independent experiments. Root tip: root tips from 2-month-old plants, root: roots eliminated root tip from 2-month-old plants, seed(IM): immature seeds, seed(M): mature seeds, leaf: the 10th to 12th leaves from 2-month-pld plants, stem: stems appeared the 10th to 12th leaves. (B) Histochemical analysis of GUS activity of transgenic plants with X-gluc as substrate. Panel a: the 1st leaf and internode of HF1-1-2; panel b: the 2nd leaf and internode of HF1-3-1. White arrow indicates the collar; panel c: the 3rd leaf of HF1-4-4; panel d: Roots and the mesocotyle of HF1-3-2. White arrows indicate root tips.



data) to yield p2.2ALS-GUS. p2.2ALS-GUS was transformed into rice according to the method reported previously by Toki (1997). To determine tissue-specific expression of *OsALS*, the expression of GUS activity was analyzed by fluorometric and histochemical assays using 4-MUG and X-gluc as substrates, respectively (Jefferson et al. 1987). Figure 2a shows the variation in expression levels of the *OsALS* promoter in different tissues. The highest expression level was observed in root tips and immature seeds. GUS activity was also detected throughout the aerial part of the seedlings

Figure 3. (a) Transgenic rice plants selected on selection medium containing BS. *Top*: regenerating rice shoot from callus (T_0 generation). *Bottom*: transgenic rice plants resistant to BS (T_1 generation). Both were selected on MS medium containing 0.5 μM of BS. (b) Schematic representation of PCR-*Mfe*I digestion genotyping assay. PCR was performed with 2.5 units of KOD Dash® (Toyobo, Osaka, Japan) as a thermostable DNA polymerase in 50 μl of reaction mixture containing 10 nmol of each deoxyribonucleotide triphosphate, 10 pmol of forward and reverse primers indicated by black arrows, and 30 ng of genomic DNA from non-transgenic or transgenic plants. The thermal cycle program was as follows: 95 °C for 1 min, 30 cycles of (98 °C for 10 s, 65 °C for 10 s, 72 °C for 1 min), and 72 °C for 7 min. PCR products (10 μl from 50 μl reaction mix) were digested with 10 units of *Mfe*I (New England Biolabs Inc., MA). PCR products were separated and analyzed on 1.5% agarose gel. The bottom panel indicates typical results of genotyping assay with non-transgenic (Lane NT) and seven transgenic plants (Lane A1, A9, A13, G1, G13, Y1 and Y10). Lane C_{NT} and C_{A1}: PCR products without *Mfe*I digestion from non-transgenic and transgenic (Line A1) plants, respectively. Genomic DNA from other transgenic lines also gave a single 1.7-kb band (data not shown). (c) PCR–*Mfe*I digestion genotyping assay of transgenic plants, Y1 and Y10. 40 seeds of both lines were tested by genotypic and phenotypic analyses. Top left panel (Y1) and bottom left panel (Y11) show results of the genotypic analysis (each panel shows results of 20 plants). Top and bottom of each lane indicate genotype (R = type R, S = type S) and phenotype (s = sensitive, r = resistant), respectively. Tables at right side of panels summarize genotypic and phenotypic analyses of Y1 and Y10.

(Figure 2a,b), although the activity in the stem and the older part of the root were relatively low. Thus, we decided to use this promoter region for the expression of the herbicide-resistant form of *OsALS*.

For the construction of the herbicide-resistant form of *OsALS* gene cassette, the 5.0-kb *Kpn*I–SnaBI fragment containing 2.2-kb 5′-upstream, 2.3-kb coding and 0.5-kb downstream regions, was subcloned into pBluescript KS(−). To produce a herbicide-resistant *OsALS* gene, we introduced two point mutations in *OsALS* by site directed mutagenesis with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, CA). The mutation points were selected according to the analysis of a herbicide-resistant rice callus produced by somatic mutation during tissue culture (Shimizu et al. unpublished results), and the mutations involve the residues of tryptophan 548 to leucine (W548L) and serine 627 to isoleucine (S627I). Introduction of these mutations produced two new *Mfe*I sites in the *OsALS* gene (Figure 1). The 5.0-kb fragment containing the herbicide-resistant type of ALS, *OsALS*(m), was further subcloned into *Kpn*I/blunt-ended-*Hind*III site of the binary-vector pPZP200 (Hajdukiewicz et al. 1994) to yield the pPZP OsALS(m). *Agrobacterium*-mediated transformation of rice (Oryza sativa L. cv. Nipponbare, cv. Kitaake) by using the pPZP OsALS(m) was performed as described by Toki (1997). Immediately after transformation, infected calli were transferred to the fresh callus-inducing medium containing 0.25 or 0.5 μM of bispyribac-sodium (BS, Kumiai Chemical Industry Co., Tokyo, Japan). Calli grown vigorously on the selection medium were transferred to regeneration medium containing the same concentration of BS (Figure 3a, the top panel). The regenerated plants were further grown on hormone-free medium to facilitate root growth and then planted in soil and grown to maturity. These transgenic plants (T_0 generation) were self-fertilized and T_1 seeds were used in a herbicide resistance test (seven independent lines). Seeds from T_0 generation of transgenic plants exhibited resistance to 0.5 μM of BS, and segregated at a ratio three resistant: one sensitive on the selection medium (Figure 3a, the bottom panel). These results of efficient selection during regeneration and germination from T_1 seeds indicated that the 2.2-kb promoter expressed sufficient amounts of herbicide-resistant *OsALS* proteins in rice cells for selection, although the promoter activity of *OsALS* was relatively low in leaves, stems and immature seeds (Figure 2a).

To confirm the presence of the *OsALS*(m) gene at the molecular level, we performed PCR analysis coupled with *Mfe*I digestion (PCR-*Mfe*I digestion genotyping assay). We amplified a 1.7-kb PCR fragment corresponding to the C-terminal region of *OsALS* with primers ALS30 (5′- GCCGGTCTGGGACACCTCGATGAATCTA-3′) and ALS31 (5′- GATCGACCGAAGAGAGGGAAAACAGTAGATG3′) (Figure 3b). PCR products from either non-transgenic plants or transgenic plants with the *OsALS*(m) construct were digested with *Mfe*I. No *Mfe*I sites exist in the wild-type *ALS* locus of the rice genome. Thus, *Mfe*I digestion yielded a single 1.7-kb band from the PCR products amplified from genomic DNA from non-transgenic plants (Figure 3b, type S). On the other hand, *Mfe*I digestion yielded four bands (1.7-, 0.93-, 0.53- and 0.24-kb bands) from the PCR products amplified from genomic DNA from transgenic plants (Figure 3c, type R). The

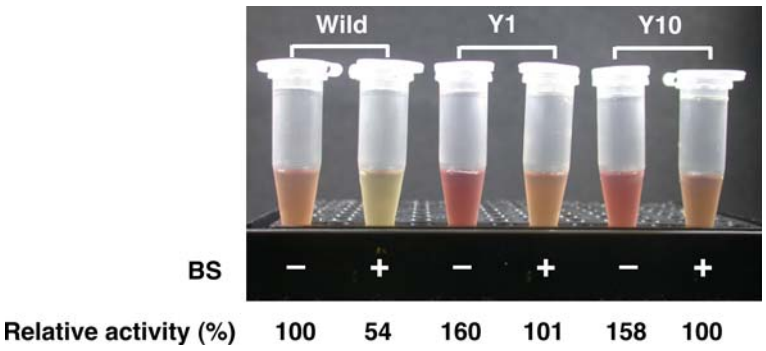


Figure 4. Colorimetric enzyme assay of ALS from transgenic and non-transgenic plants. Leaves (150 mg) cut into small pieces were incubated with or without BS (0.15 μM) in 4 ml of pre-treatment solution (1/4 concentration of MS salts liquid medium, 500 μM 1,1-cyclopropanedicarboxilic acid (CPCA) as a inhibitor of ketol-acid reductoisomerase (KARI) (Gerwick et al. 1993), 0.125% Triton X-100). After incubation at 30 °C under fluorescent light (160 μmol/m^2/s) for 24 h, leaf samples were transferred into 300 μl extraction buffer (1/4 concentration of MS salts liquid medium, 0.025% Triton X-100, 3.3% (w/v) polyvinylpolypyrrolidone), heated at 60 °C for 5 min, and sonicated. After centrifugation, 200 μl of supernatant was mixed with 20 μl of 5% H_2SO_4. After incubation the acidified mixture at 60 °C for 30 min to facilitate the decarboxylation of acetolactate to acetoin, 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 mixture. To allow color development, the mixture was incubated at 37 °C for 30 min. Absorbance was measured by spectrophotometer at 525 nm. + or − downside of each reaction tube indicates the pretreatment with 0.165 μM BS or without BS, respectively. Relative activities are expressed as percentages relative to the specific activity of the non-transgenic plant pretreated without BS.

three, 0.93-, 0.53- and 0.24-kb bands were derived from the integrated *OsALS*(m) gene locus, and the 1.7-kb band was derived from the wild-type *OsALS* locus. Using this system, we analyzed genotypes of T_1 plants germinated on MS medium without herbicides. After sampling for genomic DNA isolation, seedlings were transferred onto MS medium containing 0.5 μM BS to check the phenotype of each plant. For this assay, we used two transgenic lines, Y1 and Y10. The results are summarized in Figure 3c. Both lines showed type R:type S = 3:1 segregation expected for a single Mendelian locus. Importantly, all plants defined as type R by the genotyping assay showed the BS-resistant phenotype, and plants defined as type S showed the BS-sensitive phenotype. These results indicated that the BS-resistant phenotype was caused by integration of the *OsALS*(m) gene, and both phenotype and genotype were inherited in a Mendelian manner.

We also analyzed the herbicide-resistant form of *OsALS* protein in transgenic plants by using a colorimetric assay based on the comparison of acetoin accumulation depending on ALS enzyme activity in plant tissues treated with or without a herbicide (Gerwik et al. 1993). The distinct color reveals whether acetoin does (red or pink:wild-type without BS or resistant plants with BS) or does not (yellow or brown:sensitive plants with BS) accumulate in the resultant reaction mixture. As shown in Figure 4, when samples were not pre-incubated with BS, crude extracts from transgenic plants yielded a deeper red colored reaction mixture than that from non-transgenic plants (Figure 4, (−) tube of wild-type vs. (−) tubes of transgenic plants). Moreover, crude extracts from transgenic plants incubated with BS resulted in red colored reaction mixtures (Figure 4, (+) tubes of transgenic plants), while the crude extract from the non-transgenic plant incubated with BS resulted in a yellow color mixture (Figure 4, (+) tube of wild-type). These results indicated that transgenic plants expressed the active and herbicide-resistant OsALS protein.

According to phenotypic, genotypic and enzymatic analyses as shown above, we concluded that the mutated *OsALS* gene cassette consisted only of the rice genome worked efficiently in the original host plant as a selection marker cassette. Although an extra copy of *OsALS* exists in transgenic plants, BS-resistant and sensitive plants emerged in T_1 generation of transgenic plants in a Mendelian manner. Therefore, gene silencing due to transformation or overexpression of the *OsALS* genomic DNA sequence in the rice genome, did not occur. We also introduced the herbicide-resistant form of OsALS driven by the CaMV35S promoter in rice plants, and confirmed that the higher

expression level of the herbicide-resistant form of OsALS by this promoter did not affect the growth and seed numbers (Shimizu et al. unpublished data). Furthermore, the expression level of the *gusA* gene driven by the ALS gene promoter was much lower than that driven by the CaMV35s promoter (data not shown). Thus, we concluded that overexpressed OsALS driven by the ALS promoter has less or no effect on the growth and amino acid metabolism in the transgenic rice plant.

Herbicide-resistant forms of ALS genes have been isolated and characterized in several plant species, and further used for the production of transgenic herbicide-resistant plants. However, resistant forms of ALS genes (cDNA) are usually used under the control of promoter sequences derived from virus or bacteria (Honma et al. 1993; Ray et al. 2004). Here, we provided evidences that the mutated ALS gene cassette consisting only of host plant genomic DNA could be used as an efficient selectable marker cassette in an important monocot crop plant, rice. Recent progress in genomic research has advanced functional and structural analyses of genes in economically important crop plants. Several mutations resulting in amino-acid substitutions of ALS proteins have been reported in several plant species, and some of these conferring-herbicide-resistance, are well conserved (Tan et al. 2005). In addition, Pang et al. analyzed the crystal structures of herbicide-resistant ALS proteins from yeast (2003) and *Arabidopsis* (2004). Once the ALS gene is identified from a target crop plant, these sequences and structural information will help to construct herbicide-resistant ALS gene, even if mutations that confer herbicide-resistance are not characterized in that plant. This marker system with the ALS gene tested in our study could be applied to such crop plants for the production of publically acceptable transgenic plants.

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