Conferred Resistance to an Acetolactate Synthase-inhibiting Herbicide in Transgenic Tall Fescue (*Festuca arundinacea* Schreb.)

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Abstract. Herbicide-resistant turfgrass can be an efficient tool that will allow easier turf maintenance. Acetolactate synthase (ALS) is the first common enzyme in the biosynthetic pathways leading to the branched-chain amino acids, and amino acid substitutions in ALS have been known to confer resistance to ALS-inhibiting herbicides. A two-point mutated rice *ALS* gene [*OsALS* (dm)] has been shown to confer strong resistance to bispyribac-sodium (BS), an ALS-inhibiting herbicide. In this study, we introduced into turf-type tall fescue (*Festuca arundinacea* Schreb.) the *OsALS* (dm) gene by using *Agrobacterium*-mediated transformation for conferring herbicide resistance. Stable integration of the transgene was confirmed by Southern blot analysis. Transgenic and wild-type plants were sprayed on the leaves with herbicide containing BS; approximately half of the transgenic plants were unaffected by the treatment and showed resistance to the herbicide, whereas the wild-type plants died. ALS activity in the leaf tissue of transgenic-resistant plants incubated with BS was almost equivalent to that in wild-type plants without BS and was higher than in wild-type plants incubated with BS. These indicate that the transgenic-resistant plants actively produced *OsALS* (dm) protein under herbicide treatment. This is the first report of herbicide-resistant transgenic tall fescue after introduction of a mutated *ALS* gene.

Tall fescue (*Festuca arundinacea* Schreb.) is a major cool-season perennial grass species. It is widely used not only as forage in pastures, but also as turf for lawns, golf courses, athletic fields, roadsides, and other places. Tall fescue is an outcrossing, open-pollinated, and highly self-incompatible grass species; therefore, generally genetic improvement is difficult and takes a long time. Genetic transformation can help to overcome these problems and facilitate grass improvement. In turfgrass, weed management is very important, and herbicide resistance can be used as an efficient tool to allow easier maintenance. Herbicide resistance in turfgrass facilitates control of weed species and contributes to reducing cost, labor, and the wastage of chemical spray.

Acetolactate synthase (ALS, electrical conductivity 2.2.1.6; also referred to as aceto-hydroxyacid synthase) is the first common

enzyme in the biosynthetic pathways leading to the branched-chain amino acids (isoleucine, leucine, and valine). It is the target of at least five structurally distinct classes of herbicides, including pyrimidinylcarboxylates, sulfonylureas, imidazolinones, triazolopyrimidine sulfonamides, and sulfonylaminocarbonyltriazolinones (Shimizu et al., 2002). ALS-inhibiting herbicides are used for controlling weed species at relatively low application rates and have both foliar and soil residual activity. Furthermore, ALS does not exist in mammals, and thus ALS-inhibiting herbicides are thought to be less toxic to mammals.

Resistance to ALS-inhibiting herbicides in plants has in most cases been conferred by either single or double amino acid substitutions at a particular position in ALS (Kawai et al., 2007b; Okuzaki et al., 2007). Mutated *ALS* genes can be used not only for the generation of herbicide-resistant plants, but also as a selectable marker. Because mutated *ALS* genes arising by spontaneous mutation are able to be isolated from plant DNA, the use of these genes instead of bacterial or viral genes would contribute to public acceptance of the transgenic plants. Different types of

mutation have been found to confer resistance to different classes of herbicide (Kawai et al., 2007b), and it is therefore possible to produce various kinds of herbicide-resistant plants. A cultivation system that depends on application of a single type of herbicide would increase the frequency of emergence of herbicide-resistant weed species. The use of a combination of several herbicides and plants resistant to those herbicides could inhibit the generation of herbicide-resistant weed species.

In this study, we used *Agrobacterium*-mediated transformation to introduce into turf-type tall fescue a two-point mutated rice *ALS* gene, *OsALS* (dm). The *OsALS* (dm) gene has been reported to confer strong resistance to bispyribac-sodium (BS), one of the pyrimidinylcarboxylates (Kawai et al., 2007b; Osakabe et al., 2005). The aim of this study is to describe the effectiveness of a mutated *ALS* gene in conferring herbicide resistance in tall fescue.

Materials and Methods

Agrobacterium strain and binary vector. *Agrobacterium tumefaciens* strain EHA105 carrying the binary vector pMLH7133-*OsALS* (dm) (Kawai et al., 2007a) was used for the transformation experiment. The pMLH7133-*OsALS* (dm) consisted of the *OsALS* (dm) gene and the hygromycin phosphotransferase gene (*hpt*) under the control of the enhanced cauliflower mosaic virus (CaMV) 35S promoter. The *OsALS* (dm) gene was created when two-point mutations in the *OsALS* gene were introduced by site-directed mutagenesis and two new *MfeI* sites were produced at the mutation sites (Osakabe et al., 2005). The mutations involve the residues of tryptophan at position 548 being substituted with leucine and the serine at position 627 being substituted with isoleucine.

Genetic transformation. Genetic transformation procedure by *Agrobacterium* and all media used in this study were as described previously (Sato and Takamizo, 2006). Embryogenic calli were induced from shoot tips of the turf-type tall fescue cultivar Tomahawk germinated in vitro. Infected calli were selected by incubation with 100 mg·L⁻¹ hygromycin in the dark at 25 °C for 8 to 10 weeks. Hygromycin-resistant calli were then placed on a regeneration medium containing 100 mg·L⁻¹ hygromycin under short-day conditions (8-h light/16-h dark) at 25 °C. Regenerated plants were transferred to soil and grown in a greenhouse at 20 °C. They were vernalized in a cold room at 4 °C for 8 weeks and then transferred to the greenhouse. T₁ progenies were obtained by crossing two transgenic plants.

Polymerase chain reaction analysis. Genomic DNA was extracted from leaf tissues using the cetyltrimethylammonium bromide method (Murray and Thompson, 1980) with some minor modifications. Polymerase chain reaction (PCR) was performed using the following primers: 5'-ATCCAGCAGAGATTGGAAAG-3' (forward) and 5'-AACAAGT

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ATGGCCCTGGAGT-3' (reverse) for *OsALS* (dm), and 5'-CGAAGAATCTCGTGCTTT CA-3' (forward) and 5'-TCCATCACAGTT TGCCAGTG-3' (reverse) for *hpt*. The *OsALS* (dm) primers were designed to cover one *MfeI* site, and the PCR products were subsequently digested with *MfeI*. PCR amplification was carried out in a 10 µL reaction mixture containing 100 ng of genomic DNA, 1 µL of 10× Ex Taq buffer, 1 µL of each 20 µM primer, 0.8 µL of dNTP mixture (with each dNTP at 2.5 mM), and 0.25 U TaKaRa Ex Taq (TaKaRa, Shiga, Japan). PCR conditions were: initial denaturation at 94 °C for 5 min; 30 cycles of 30 s at 94 °C (denaturation), 30 s at 60 °C (annealing), and 45 s at 72 °C (extension); and a final extension at 72 °C for 7 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel.

Southern blot analysis. Genomic DNA (15 µg) was digested with *XbaI*, fractionated on a 0.8% agarose gel at 30 V for 12 h, and blotted onto a positively charged nylon membrane (Roche, Mannheim, Germany). The probe for *hpt* was amplified from pMLH7133-*OsALS* (dm) by using the PCR DIG probe synthesis kit (Roche) and the same primers as those used in the PCR analysis. Southern blot analysis was carried out as described in the DIG manual.

Herbicide application. Transgenic plants and T₁ progenies were sprayed on the leaves twice with 3% Nominee commercial herbicide containing 2% BS (Kumiai Chemical Industry Co., Ltd. Tokyo, Japan) in the greenhouse. Their response to the herbicide was observed 45 d after herbicide treatment.

Measurement of acetolactate synthase activity. ALS activity was analyzed by the colorimetric enzymatic assay (Osakabe et al., 2005) with some minor modifications. Leaves (50 mg) were cut into small pieces and incubated in 4 mL of pretreatment solution [25% MS basal medium (Murashige and Skoog, 1962), 500 µM 1,1-cyclopropanedicarboxylic acid, and 10 mM pyruvic acid sodium salt] with or without 0.1 µM BS under fluorescent light at 30 °C for 24 h. Only the leaf tissues were transferred to a new tube and frozen for 1 h. Subsequently, 220 µL of 0.025% Triton X-100 solution was added, and the tube was heated at 60 °C for 10 min. After incubation, 200 µL of the supernatant was mixed with 20 µL of 5% H₂SO₄ and incubated at 60 °C for 30 min. Then, 100 µL of 5% 1-naphthol dissolved in 2.5 N NaOH and 100 µL of 0.5% creatine were added to the mixture, and the mixture was incubated at 37 °C for 30 min. The color of the reaction mixture was observed, and the absorbance at 530 nm was measured by a spectrophotometer.

Results

Production of transgenic tall fescue. A total of 17 regenerated plants were obtained from five hygromycin-resistant calli through *Agrobacterium*-mediated transformation. Introduction of *OsALS* (dm) and *hpt* genes was confirmed by PCR analysis. The PCR products

amplified by the *OsALS* (dm) primers from both regenerated and wild-type plants were equivalent in size to a 721-bp fragment amplified from the binary vector pMLH7133-*OsALS* (dm). Because the *ALS* genes are well conserved in plants, the PCR product from the wild-type plant would be amplified from the endogenous tall fescue *ALS* gene (*FaALS*). In the *OsALS* (dm) gene, two new *MfeI* sites are produced at the mutation sites, and thus the primers were designed to cover one *MfeI* site to distinguish the *OsALS* (dm) gene from the *FaALS* gene (Fig. 1A). After the PCR products were digested with *MfeI*, the regenerated plants and pMLH7133-*OsALS* (dm) yielded two fragments (528 bp and 193 bp), whereas wild-type plant yielded the 721-bp single fragment (Fig. 1B). With *hpt* primers, a 408-bp fragment was amplified from both regenerated plants and pMLH7133-*OsALS* (dm), but not from the wild-type plant (Fig. 1C). The copy number of integrated genes was estimated by Southern blot analysis and ranged from one to five (Fig. 2). Some of the transgenic plants originated from the same callus showed the similar hybridization patterns, suggesting that they originated from the same transgenic cell.

Herbicide resistance of transgenic plants.

Transgenic plants were sprayed on the leaves with an ALS-inhibiting herbicide containing BS. Wild-type plants were confirmed to die completely after herbicide treatment (Fig. 3A). Nine transgenic plants (1a, 1d, 2a, 2b, 2c, 4a, 4b, 4c, 4d) were unaffected by the treatment and showed resistance to the herbicide (Fig. 3B). The other eight plants (1b, 1c, 3a, 3b, 3c, 3d, 3e, 5) died after herbicide treatment (Fig. 3C). In contrast to resistant plants, multiple integrated genes were observed in susceptible plants (Fig. 2).

Acetolactate synthase activity in transgenic plants. ALS activity in the transgenic plants was analyzed by colorimetric enzymatic assay. This assay is able to estimate ALS activity in plant tissues with or without herbicide treatment based on a comparison of acetoin accumulation (Gerwick et al., 1993). Red or pink coloration indicates a high accumulation of acetoin produced by the ALS activity, and yellow or brown indicates a low accumulation of acetoin. When the leaf tissues were incubated without BS, both wild-type and transgenic plants produced pink coloration (Fig. 4A). When incubated with BS, only transgenic-resistant plants produced pink coloration, and both the wild-type

Fig. 1. Polymerase chain reaction (PCR) analysis of transgenic plants. (A) Schematic representation of PCR analysis coupled with *MfeI* digestion of the *OsALS* (dm) gene. (B) Detection of the *OsALS* (dm) gene. (C) Detection of the *hpt* gene. P = pMLH7133-*OsALS* (dm); WT = wild-type plant. For the transgenic plants, the numbers indicate the hygromycin-resistant callus from which the plant was generated, and the letters indicate independent plants regenerated from that callus.

Fig. 2. Southern blot analysis of transgenic plants. WT = wild-type plant. Transgenic plants are labeled as described in Figure 1. Genomic DNA was digested with *XbaI* and hybridized with the *hpt* probe. The phenotype with respect to herbicide, either resistant (R) or susceptible (S), is indicated at the bottom of each line. Plant 5 had 5 copies of transgene.

Fig. 3. Herbicide application to wild-type and transgenic plants. (A) Wild-type plant. (B) Transgenic-resistant plant (1a). (C) Transgenic susceptible plant (3b). The pictures were taken 45 d after herbicide treatment.

Fig. 4. Colorimetric enzymatic assay in leaves of wild-type and transgenic plants. The leaf tissues were incubated with (+) or without (–) bispyribac-sodium (BS). All transgenic plants of each phenotype, resistant (R) or susceptible (S), were analyzed. (A) Comparison of acetoin accumulation. (B) Measurement of acetolactate synthase activity. Error bars represent the SE for wild-type plants (n = 3), transgenic plants (R) (n = 9), and transgenic plants (S) (n = 8).

and transgenic susceptible plants produced brown or light pink coloration (Fig. 4A).

When ALS activity with BS was measured, the ALS activity in resistant plants (0.84) was almost equivalent to that in wild-type plants without BS (0.98) and showed higher activity than in either wild-type plants (0.35) or susceptible plants (0.59) (Fig. 4B). In the assay without BS, the ALS activity tended to be higher in resistant plants (1.13) than in wild-type plants (0.98) and susceptible plants (0.87) (Fig. 4B), because OsALS (dm) protein would be produced in addition to the endogenous FaALS protein. The resistant plants showed lower ALS activity with BS than without BS, probably because the FaALS protein was inhibited by BS treatment. These results indicated that the transgenic-resistant plants actively produced OsALS (dm) protein under herbicide treatment.

Analyses of *T*₁ progenies. *T*₁ progenies were obtained by crossing two resistant plants carrying a single transgene (1a and 2b); *T*₁ seeds were harvested from plant 2b. With herbicide application, 11 of 13 *T*₁ plants showed resistance, whereas the other two

plants (T1, T8) died. PCR analysis coupled with *M*feI digestion showed the *OsALS* (dm) gene to be present in all resistant plants, but not in the two susceptible plants (Fig. 5). In resistant plants, Southern blot analysis detected a single hybridizing band identical in size to that of the two parent plants (Fig. 2); susceptible plants did not hybridize with the *hpt* probe (Fig. 6). *T*₁ progenies that did not inherit the transgene were generated because both parents were hemizygous for the transgene. We think that *T*₁ progenies with both transgenes derived from the parents were not generated because the number of *T*₁ progenies was very small. We need to confirm further the genetic segregation of the transgene in the next generation. The phenotypic and molecular analyses indicated stable expression and inheritance of the transgene in *T*₁ progenies.

Discussion

We were able to generate transgenic tall fescue that showed resistance to BS. This is the first report on resistance to an ALS-

inhibiting herbicide conferred by using a mutated *ALS* gene in tall fescue. On the other hand, approximately half of the transgenic plants did not show resistance to herbicide (Fig. 3C) and ALS activity in the susceptible plants was insufficient to confer herbicide resistance (Fig. 4B). In resistant plants, low copy of transgene was integrated, whereas more than four integrated genes were observed in the susceptible plants (Fig. 2). All resistant plants with a single transgene in *T*₁ progenies did not exhibit apparent gene silencing (Fig. 6). Some studies suggest that homology-dependent gene silencing is associated with the presence of either multiple copies of homologous transgenes and promoters (Matzke and Matzke, 1995) or a transgene and a homologous endogenous gene (Meyer, 1995). The *OsALS* (dm) has been used as a selectable marker in wheat and rice (Ogawa et al., 2008; Osakabe et al., 2005). However, no relationship between herbicide resistance and copy number was apparent in wheat transformation using the rice *ALS* promoter (Ogawa et al., 2008). Although we did not use a mutated *ALS* gene as a selectable marker in this study, gene silencing by copy number was not observed in transgenic plants with multiple copies when a mutated *ALS* gene driven by the rice *ALS* promoter was used as a selectable marker in tall fescue transformation (in preparation). It was assumed that the rice *ALS* promoter is not a strong one and is expressed in a tissue-specific manner (Osakabe et al., 2005). Okuzaki et al. (2007) reported that some transgenic rice calli with multiple copies of a mutated *ALS* gene driven by the maize ubiquitin promoter, which is a strong constitutive promoter, did not regenerate, whereas transgenic calli with only one or two transgenes did. In this study, the CaMV 35S promoter, which drives a strong and constitutive expression, was used for two genes [*OsALS* (dm), *hpt*] in the same binary vector and therefore the chances of gene silencing may increase by overuse of the CaMV 35S promoter. Consequently, herbicide resistance might be affected by the silencing of the transgene, which was caused by multiple integration of the transgene driven by the CaMV 35S promoter. Strong expression in all tissues by constitutive promoters tends to cause deleterious effects, and the use of the endogenous *ALS* promoter would be preferable for more stable expression.

Although the transgenic plants were confirmed to show herbicide resistance in the greenhouse, they should be further examined to ensure that herbicide resistance is stable under field conditions. However, because tall fescue is an outcrossing and anemophilous grass, it is possible that transgenes could be dispersed into the environment through pollen. Lee (1996) discussed two environmental risks associated with transgenic turfgrass. The first risk is the possibility that transgenes will be spread by crossing transgenic plants with weed species. The second is the chance that transgenic plants will themselves become weeds. Tall fescue produces large

Fig. 5. Detection of the *OsALS* (dm) gene in T₁ progenies by polymerase chain reaction analysis coupled with *Mfe*I digestion. P = pMLH7133-*OsALS* (dm); WT = wild-type plant; T₁–T₁₃ = T₁ plants.

Fig. 6. Southern blot analysis of T₁ progenies. WT = wild-type plant; T₁–T₁₃ = T₁ plants. Genomic DNA was digested with *Xba*I and hybridized with the *hpt* probe. The phenotype with respect to herbicide, either resistant (R) or susceptible (S), is indicated at the bottom of each line.

amounts of pollen-containing allergenic proteins that cause hay fever in susceptible people. To limit grass pollen allergy, plants with cytoplasmic male sterility have been developed (Fujimori et al., 2004). To minimize the risk of dispersal of transgenic pollen in the field, we plan to cross and evaluate such cytoplasmic male-sterile plants with our transgenic plants.

When we generate transgenic plants, it is desirable to use transgenes derived from host plant DNA as much as possible. It will be applicable to the self-cloning method with public acceptance. Some amino acid substitutions to confer herbicide resistance are well conserved in plant ALS (Kawai et al., 2007b). We can develop herbicide-resistant *ALS* gene

using this information even if mutations that confer herbicide resistance have not been characterized in the target plant. In future works, we will isolate the *ALS* gene from tall fescue and construct a herbicide-resistant *ALS* gene for tall fescue transformation.

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