Selectable Tolerance to Herbicides by Mutated Acetolactate Synthase Genes Integrated into the Chloroplast Genome of Tobacco[1][OA]

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Strategies employed for the production of genetically modified (GM) crops are premised on (1) the avoidance of gene transfer in the field; (2) the use of genes derived from edible organisms such as plants; (3) preventing the appearance of herbicide-resistant weeds; and (4) maintaining transgenes without obstructing plant cell propagation. To this end, we developed a novel vector system for chloroplast transformation with acetolactate synthase (ALS). ALS catalyzes the first step in the biosynthesis of the branched amino acids, and its enzymatic activity is inhibited by certain classes of herbicides. We generated a series of Arabidopsis (*Arabidopsis thaliana*) mutated ALS (*mALS*) genes and introduced constructs with *mALS* and the aminoglycoside 3'-adenyltransferase gene (*aadA*) into the tobacco (*Nicotiana tabacum*) chloroplast genome by particle bombardment. Transplastomic plants were selected using their resistance to spectinomycin. The effects of herbicides on transplastomic mALS activity were examined by a colorimetric assay using the leaves of transplastomic plants. We found that transplastomic *G121A*, *A122V*, and *P197S* plants were specifically tolerant to pyrimidinylcarboxylate, imidazolinon, and sulfonylurea/pyrimidinylcarboxylate herbicides, respectively. Transplastomic plants possessing mALSs were able to grow in the presence of various herbicides, thus affirming the relationship between mALSs and the associated resistance to herbicides. Our results show that *mALS* genes integrated into the chloroplast genome are useful sustainable markers that function to exclude plants other than those that are GM while maintaining transplastomic crops. This investigation suggests that the resistance management of weeds in the field amid growing GM crops is possible using (1) a series of *mALSs* that confer specific resistance to herbicides and (2) a strategy that employs herbicide rotation.

Plastid transformation was first reported in studies using the unicellular alga *Chlamydomonas reinhardtii* (Boynton et al., 1988) and was followed by investigations involving tobacco (*Nicotiana tabacum*; Svab et al., 1990). Genetic engineering approaches that utilize chloroplasts possess a number of benefits in comparison with nuclear transformation, including (1) a high level of transgene expression (DeCosa et al., 2001); (2) the delivery of multiple genes in a single transformation event (Daniell and Dhingra, 2002); (3) the absence of gene silencing (Lee et al., 2003); (4) the absence of position effects due to site-specific transgene integration (Daniell et al., 2004); and (5) the absence of pleiotropic effects since transgene products are localized within the chloroplast (Daniell et al., 2001). These benefits were exploited by chloroplast transformation trials utilizing a variety of plants, including Arabidopsis (*Arabidopsis thaliana*; Sikdar et al., 1998), potato (*Solanum tuberosum*; Sidorov et al., 1999), rice (*Oryza sativa*; Khan and Maliga, 1999), tomato (*Solanum lycopersicum*; Ruf et al., 2001), oilseed rape (*Brassica napus*; Hou et al., 2003), carrot (*Daucus carota*; Kumar et al., 2004a), cotton (*Gossypium hirsutum*; Kumar et al., 2004b), soybean (*Glycine max*; Dufourmantel et al., 2004), lettuce (*Lactuca sativa*; Lelivelt et al., 2005), and cabbage (*Brassica capitata*; Liu et al., 2007). However, transplastomic plants were not stable in Arabidopsis, oilseed rape, or rice. Recently, Lee et al. (2006) made

progress in rice. *Lesquerella fendleri* (oilseed Brassicacea) belongs to the mustard family (Brassicaceae), as does Arabidopsis and oilseed rape. Skarjinskaia et al. (2003) demonstrated that transplastomic *Lesquerella* plants were fertile and produced seed. Methods and selection conditions for the chloroplast transformation of these species were summarized by Verma and Daniell (2007). The basic plastid transformation vector consists of flanking sequences and chloroplast-specific expression cassettes. Flanking sequences are species specific and function as regions for homologous recombination. Chloroplast-specific expression cassettes have selectable markers and genes of interest for additional functions in plants (Verma and Daniell, 2007).

Transformation technology utilizing nuclear genomes was developed in an effort to eliminate antibiotic marker genes (Yoder and Goldsbrough, 1994) and is referred to as Nuclear genome-Clean Gene Transformation Technology (N-CGTT). This approach has recently been applied to genetic engineering studies utilizing chloroplasts and is referred to as Chloroplast genome-Clean Gene Transformation Technology (C-CGTT) in which antibiotic marker genes, such as the aminoglycoside 3'-adenyltransferase gene (*aadA*) introduced into the chloroplast genome, are eliminated (Iamtham and Day, 2000; Ye et al., 2003). Use of these methodologies prevents the transfer of antibiotic genes to surrounding weeds and microorganisms in the soil and to bacteria in the gut following oral intake. Integration of foreign genes into the plastid genome enhances gene containment because plastids are inherited maternally in many crop plants, thereby avoiding the pollen-mediated spread of transgenes (Maliga, 1993; Daniell et al., 1998; Scott and Wilkinson, 1999). Homologous recombination in plastids allows for accurate gene targeting into a well-characterized genome and the elimination of bacterial vector sequences (Svab et al., 1990). High levels of gene expression have been observed for a variety of foreign genes when located within plastids (McBride et al., 1995; Staub et al., 2000; Kanamoto et al., 2006).

Notwithstanding the advantages associated with C-CGTT, use of this technology remains problematic in terms of potential effects in the field. Genetically modified (GM) and non-GM plants must be distinguished correctly and easily. Although use of the PCR method is the most convenient for the determination of contamination in bulk samples, it is unsuitable for examining single seeds or plants in terms of efficiency and resources. Although the use of herbicides was proposed as a suitable method to resolve this problem, the generation of herbicide-resistant plants must be considered. Studies indicate that herbicides inhibiting ALS (acetohydroxyacid synthase [AHAS]; EC 2.2.1.6) have accelerated the generation rate of tolerant weeds and crops (Preston and Powles, 2002; Shimizu et al., 2002; Tranel and Wright, 2002; Tranel et al., 2007). Rotating the supply of some herbicides was proposed as a countermeasure against the occurrence of herbicide-resistant weeds (Gressel, 1984).
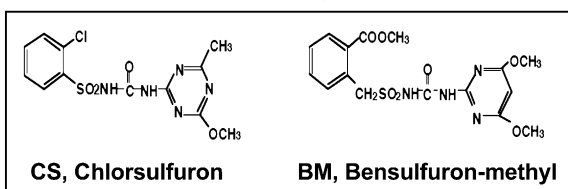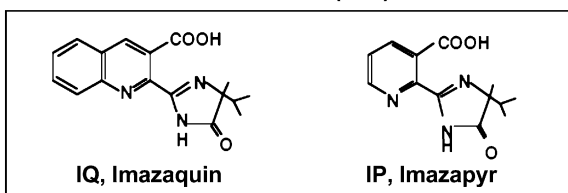
The solution to this problem was investigated in this investigation through the use of an herbicide-tolerant gene of plant origin. ALS catalyzes the first step in the biosynthesis of the branched amino acids Val, Leu, and Ile, and is composed of catalytic and regulatory subunits. Plant ALSs possess catalytic subunits similar to the equivalent bacterial and yeast (*Saccharomyces cerevisiae*) enzymes, except for the N-terminal signal peptide sequences required for translocation of the protein to the chloroplast (Ott et al., 1996). Genes that express the plant regulatory subunit have been cloned and characterized (Hershey et al., 1999). The deduced amino acid sequences are as much as twice the length of bacterial sequences and comprise two domains thought to be involved in feedback inhibitor mechanisms. It is well known that mutations at several amino acid residues in ALS are responsible for tolerance to herbicides shown in Figure 1. Herbicide-tolerant plants have been reported for rice, tobacco, and Arabidopsis (Chang and Duggleby, 1998; Shimizu et al., 2002; Tan et al., 2005; Kawai et al., 2007; Okuzaki et al., 2007). Several mutated species of Arabidopsis ALS have been expressed in *Escherichia coli* and sensitivity to inhibitors has been examined (Kawai et al., 2008). These results showed that, in the ALS polypeptide, P197S and S653I changes can produce sulfonylurea (SU) resistance, whereas W574L and P197H/R198S (double mutations of P197H and R198S) changes can produce imidazolinon (IM) resistance, and W574L/S653I (double mutations of W574L and S653I) changes can produce resistance to SU, IM, and pyrimidinylcarboxylate (PC). However, little is known about plant growth tolerance to herbicides in specimens with mALSs. Nuclear genetic engineering studies have shown that mutation of W548L/S627I and G95A in rice ALS confers tolerance to the aforementioned herbicides, including PC herbicides (Kawai et al., 2007; Okuzaki et al., 2007). The introduction of mALSs into the chloroplast genome had not been attempted because ALS was thought to be unsuitable as a selectable marker as in the case of 5-enol-pyruvyl shikimate-3-P synthase (EPSPS; Ye et al., 2003).

We report here the introduction of *mALS* genes into the chloroplast genome and investigate the sensitivity of transformants with respect to ALS-inhibiting herbicides. The *mALS* genes proved useful as sustainable markers that functioned to exclude nontransformed plants while maintaining the transplastomic plants. These markers showed selectable tolerance to various herbicides. Our findings suggest that rotation of the herbicide supply to transgenic plants harboring different sustainable markers can be an effective strategy in countering the occurrence of herbicide-resistant weeds.

RESULTS

Generation and Analysis of Transplastomic Plants

Chloroplast transformation vectors, pLD200-mALS possessing the *aadA* and *mALS* (transit peptide truncated)

Pyrimidinylcarboxylate herbicides (PCs)

PM, Pyriminabac

BS, Bispyribac-sodium

PS, Pyrithiobac-sodium
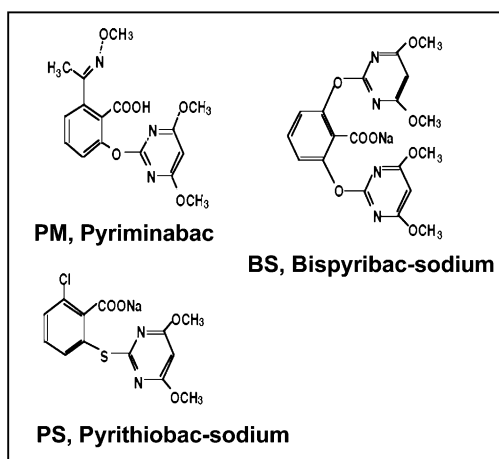
Sulfonylurea herbicides (SUs)

CS, Chlorsulfuron   **BM, Bensulfuron-methyl**

Imidazolinone herbicides (IMs)

IQ, Imazaquin   **IP, Imazapyr**

Figure 1. Herbicides inhibiting ALS activity. These herbicides can be classified into the three classes PC, SU, and IM. BS, PS, and PM belong to the PC class of herbicides, CS and BM to the SU class of herbicides, and IQ and IP to the IM class of herbicides.

genes inserted between tobacco *rbcL* (gene for the large subunit of Rubisco) and *accD* (gene for acetyl-CoA carboxylase) sequences for homologous recombination, were introduced by particle bombardment. The integration of *mALS* into the chloroplast genome of regenerated tobacco plants was confirmed by PCR using five primer sets as shown in Figure 2A. Tobacco chloroplast transformation was performed with 10 shots of bombardment on 10 different plates per vector pLD200-mALS harboring *mALS* comprising *G121A*, *A122V*, *P197S*, or *W574L/S653I* (double mutant). The resultant transplastomic candidates (10 per vector) were maintained on hormone-free Murashige and Skoog medium (Fig. 2B). PCR was performed with primer sets comprising rbcL-Fd (annealing to the homologous region of *rbcL*) and ALS-433-Rv to generate 0.83-kb products in lanes 1 to 4, 6, and 10 (Fig. 2C, bottom; results of *P197S*), and ALS-1597-Fd and accD-Rv (annealing to the homologous region of *accD*) to generate 1.2-kb products in lanes 1 to 4, 6 to 8, and 10 (Fig. 2C, middle; results of *P197S*), confirming the presence of transgenes in candidate lines. To discriminate between nuclear and chloroplast transgenic lines, an accD-N-Rv primer was designed that would anneal to the endogenous chloroplast genome and enable a 2.3-kb PCR product to be generated when paired with an internal ALS primer, ALS-1597-Fd (Fig. 2A). Transplastomic lines were identified by PCR with the primer set accD-N-Rv and ALS-1597-Fd, with 2.3-kb products visible in lanes 1 to 4, 6, and 10 (Fig. 2C, top; results of *P197S*), confirming site-specific integration of the transgenes into the tobacco chloroplast genome. The size of the PCR product amplified in lanes 7 and 8 was 1.2 kb, not 2.3 kb and 0.8 kb, indicating that the mALS region was not integrated into the chloroplast genome. The overall results indicate that plant samples shown in lanes 1 to 4, 6, and 10 were derived from genuine transplastomic lines containing full-length

transgenes. The same PCR approach was employed for all candidates transformed with the other vectors and confirmed the production of several transplastomic lines.

To examine the amount of transplastomic chloroplast genome in each line, the population representing the native chloroplast genome was determined by PCR with rbcL-Fd and accD-Rv. Two different lines transformed with the same construct were subjected to PCR analysis. The wild-type lines markedly amplified the 0.6-kb product (Fig. 2D, top; lanes 9 and 10), whereas the 0.6-kb products were barely amplified in lanes 1 to 8 (Fig. 2D, top), indicating that the native regions amplified by rbcL-Fd and accD-Rv were split by the transgenes. The internal insert sequence was detected to a greater extent using primers aadA-Fd2 and ALS-270-Rv in all transgenic plants (Fig. 2D, bottom). However, the 0.8-kb products were not detected in wild-type plants. Therefore, it is concluded that the majority of the chloroplast genome in these transgenic plants was transplastomic. Subsequent studies used the transplastomic plants of lane 2 for A122V, lane 4 for G121A, lane 6 for P197S, and lane 8 for *W574L/S653I*.

ALS Activity in Leaves of Transplastomic Plants

The activity of native ALS from wild-type tobacco in the absence of ALS-inhibiting herbicides was determined by a colorimetric assay and appeared as a red color in samples (Fig. 3A). The red color changed to a transparent or pale yellow color following the addition of SU herbicide (0.1 μM bensulfuron-methyl [BM]), PC herbicide (0.1 μM pyrithiobac-sodium [PS]), and IM herbicide (5 μM imazapyr [IP]; Fig. 3A), indicating that these herbicides inhibited ALS activity. This assay was employed for the evaluation of mALS activity in transplastomic plants (*G121A*, *A122V*, *P197S*, and
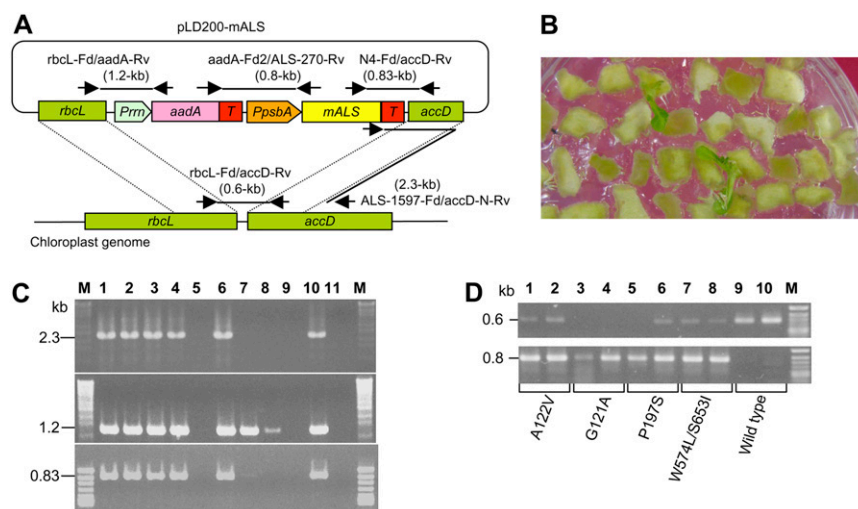
Figure 2. Chloroplast transformation with *aadA* and mutated ALS (*mALS*). A, Structure of the chloroplast transformation vector. *Prrn-aadA* and *PpsbA-mALS* represent transgenes introduced into the chloroplast genome. The region located between *rbcL* and *accD* may be integrated in the chloroplast genome by homologous recombination. The primer set rbcL-Fd and aadA-Rv was used to amplify *rbcL-aadA* in the transformation vector, generating a 1.2-kb product. The primer set N4-Fd and accD-Rv was used for the *mALS-accD* region, yielding a 0.83-kb product. The primer set ALS-1597-Fd (forward primer of mALS) and accD-N-Rv (reverse primer for endogenous *accD*) generated a 2.3-kb product. The primer set aadA-Fd2 and ALS-270-Rv was used for the *aadA-mALS* region, generating a 0.8-kb product. The primer set rbcL-Fd and accD-Rv was used to amplify the endogenous region between *rbcL* and *accD* of the chloroplast genome, generating a 0.6-kb fragment. B, Following bombardment, leaf slices were grown on RMOP containing 0.5 g L^{-1} SP. A SP plate following a 6-week growing period is shown. The regenerated plants represent potential transformants. C, Confirmation of transgenes integrated into the chloroplast genome by PCR. PCR analysis was performed using three primer sets. Lanes 1 to 10, DNA from regenerated plants; lane 11, nontransformed tobacco; M, size markers. The indicated sizes of 2.3 kb, 1.2 kb, and 0.83 kb are products derived from the use of primer sets ALS-1597-Fd and accD-N-Rv, rbcL-Fd and aadA-Rv, and N4-Fd and accD-Rv, respectively. D, The population representing the transformed chloroplast genome. PCR analysis of two different lines of each chloroplast transformant harboring *mALS*: *A122V* (lanes 1 and 2), *G121A* (lanes 3 and 4), *P197S* (lanes 5 and 6), *W574L/S653I* (lanes 7 and 8), and the wild type (lanes 9 and 10). PCR was performed in 25 cycles. The 0.6-kb product represents the endogenous chloroplast genome without a transgene insert, whereas the 0.8-kb product represents part of the transgenes introduced into the chloroplast genome.

W574L/S653I), as determined by the PCR methods described above. The ALS activity of *G121A* plants was strongly resistant to PS, weakly resistant to BM and sensitive to IP (Fig. 3B), whereas *A122V* plants were specifically resistant to IP (Fig. 3B), and *P197S* plants were strongly resistant to BM, middle resistant to PS, and sensitive to IP (Fig. 3B). The ALS activity of *W574L/S653I* plants was strongly resistant to PS, BM, and IP (Fig. 3B). These results show a mALS-mediated herbicide-specific resistance resulting from a transgene introduced by chloroplast transformation.

Effects of Herbicides on Plant Growth

Herbicide resistance was examined using a combination of three different herbicides and a variety of mALSs. In an effort to examine the in vivo effect of each herbicide on transplastomic plants, the leaves of plants were transferred to regeneration medium containing 0.5 g L^{-1} spectinomycin (SP), 0.1 μM BM, 0.1 μM PS, or 1 μM IP, and cultured for 3 weeks. Wild-type tobacco was unable to grow on medium containing any of the herbicides. On the other hand, *G121A* plants regenerated on 0.1 μM PS medium (Fig. 4), and *A122V* plants regenerated on 1 μM IP medium (Fig. 4).

*P197S* plants were tolerant to 0.1 μM BM and 0.1 μM PS (Fig. 4). *W574L/S653I* plants regenerated on medium containing 0.1 μM BM, 0.1 μM PS, or 1 μM IP (Fig. 4).

T1 seeds, the self-pollinated progeny of *A122V* plants, and wild-type seeds were planted on medium supplemented with 1 μM IP or 0.5 g L^{-1} SP. Both seed types were able to grow on Murashige and Skoog (Fig. 5A). Although wild-type plants were sensitive to IP and SP, all *A122V* seeds were uniformly resistant to SP and IP (Fig. 5, B and C). Green tissues of *A122V* plants on 1 μM IP medium grew in a manner similar to that of wild-type tissue grown on herbicide-free medium (Fig. 5C), although the root length of the former was shorter. The accumulation of mALS protein did not appear to be sufficient to impart resistance to the herbicide in root plastids because mALS expression was driven by the *psbA* promoter, which is known to be a strong promoter in green tissues.

### DISCUSSION

ALS Activity in Transplastomic Plants

Herbicide-specific resistance conferred by mALSs and directed by transplastomic genes in the chloro-
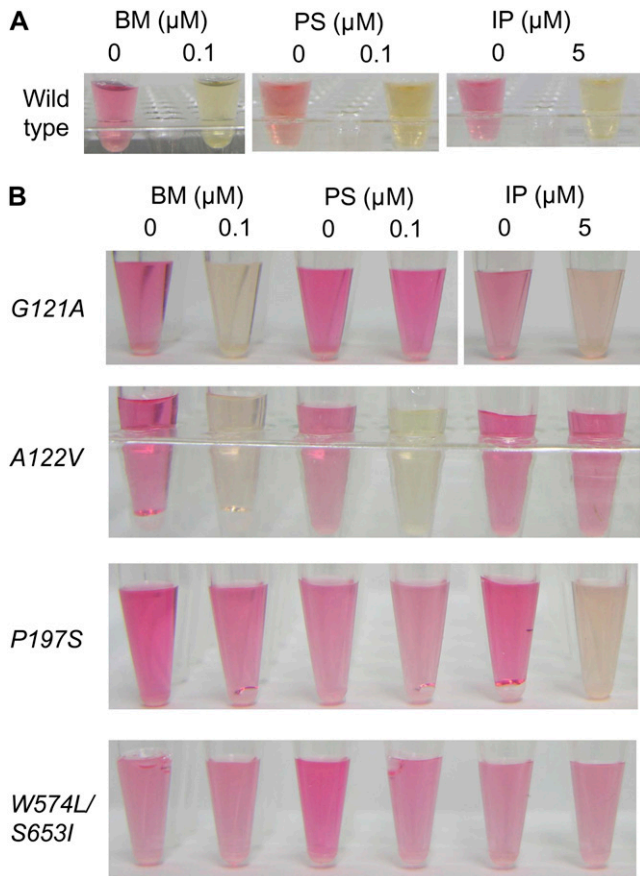
Figure 3. Inhibition of ALS activity with herbicides in transplastomic plants with *mALSs*. ALS activity in transplastomic tobacco plants with *mALSs* was determined using a colorimetric assay. ALS activity of tobacco, wild-type (A), and *mALS*-transformed plants *G121A*, *A122V*, *P197S*, and *W574L/S653I* (B) was determined in the presence of 0.1 μM BM, 0.1 μM PS, or 5 μM IP. Experimental details are described in "Materials and Methods."

plast genome has been demonstrated by this investigation, even though *mALS* genes had previously only been delivered into the nuclear genomes of certain species (Kawai et al., 2007; Okuzaki et al., 2007). ALS activity is thought to be controlled by the regulatory subunit, which also plays a role in feedback regulation by Val, Ile, and Leu (Lee and Duggleby, 2001). Although the tobacco mosaic virus 35S promoter has frequently been used in nuclear transformation studies, our study used the *psbA* promoter because it is the strongest known promoter in chloroplasts (Hayashi et al., 2003). Moreover, given that 100 to 1,000 copies of the chloroplast genome exist in a cell, it would be interesting to determine whether highly expressed mALSs in chloroplasts can influence plant growth. This study has revealed that transplastomic plants with *mALSs* grow normally on Murashige and Skoog medium without significant differences when compared to wild-type plants, showing that hyperexpression of mALSs does not influence plant growth.

We investigated the involvement of the regulatory subunit in ALS activity. Regulatory subunits play roles in feedback regulation and full enzyme activity (Lee and Duggleby, 2001). The determination of ALS activity in leaves where regulatory subunits exist was done in the presence of 1,1-cyclopropanedicarboxylic acid, which blocked the acetolactate metabolism, resulting in no feedback regulation. The selectable tolerance of plants transplastomic with *G121A*, *A122V*, and *W574L/S653I* (Fig. 3) were similar to those obtained when using the same recombinant mALSs that only expressed the catalytic subunit in *E. coli*, with which endogenous *E. coli* regulatory subunits was not concluded to be associated (Kawai et al., 2008). Therefore, the regulatory subunits do not affect the sensitivity of these mALSs to herbicides in transplastomic plants.
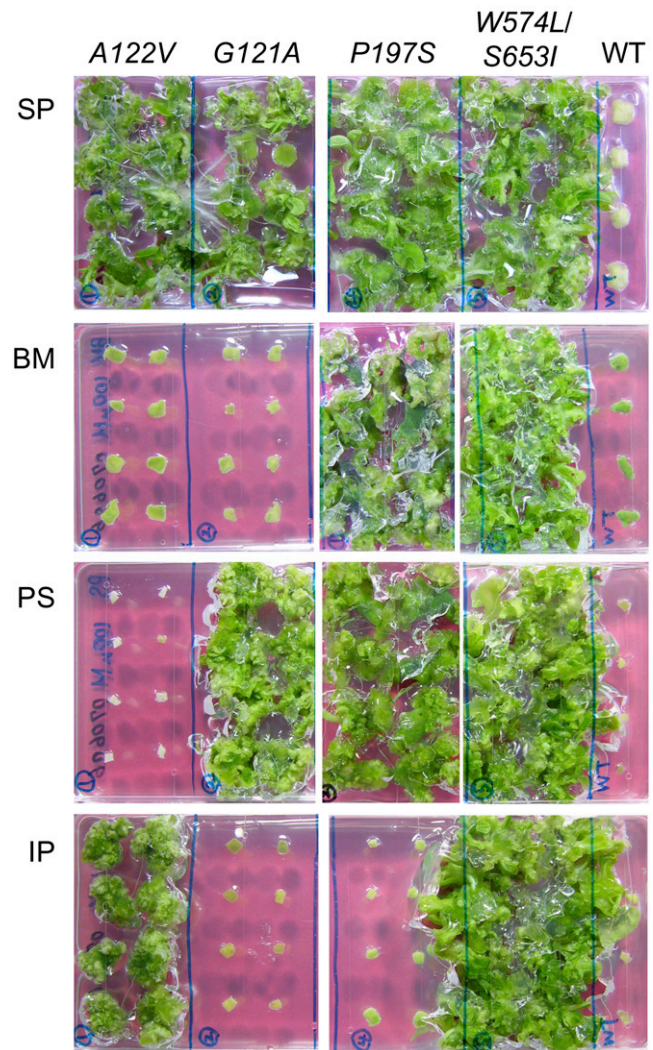
Figure 4. Regeneration of transplastomic plants on medium containing ALS-inhibiting herbicides. Transgenic plants comprising A122V, G121V, P197S, or W574L/S653I mALSs were regenerated on RMOP medium supplemented with 0.5 g L^{-1} SP, 0.1 μM BM, 0.1 μM PS, or 1 μM IP.
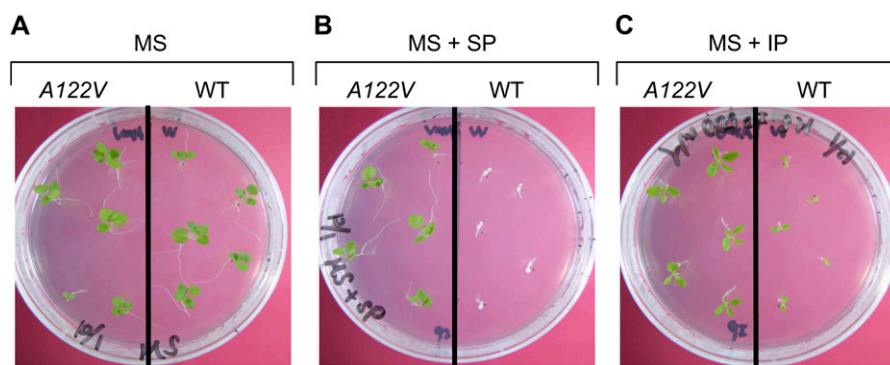
Figure 5. Influence of *psbA* promoter on the growth of transplastomic plants. T1 seeds transplastomic with *PpsbA-A122V* (on the left in all images, *A122V*) and wild-type (on the right in all images, WT) seeds were germinated on Murashige and Skoog medium alone (A, MS), or on medium containing 0.5 g L^{-1} SP (B, MS + SP) or 1 μM IP (C, MS + IP).

On the other hand, P197S mALS exhibited different behavior from the above mutations. The novel tolerance of *P197S* plants to PC and SU herbicides was demonstrated with regard to mALS activity in response to herbicides in leaves (Fig. 3), whereas P197S mALS expressed in *E. coli* was resistant to SU herbicides, but not to PC herbicides (Kawai et al., 2008). This result suggested that the regulatory subunit contributed to the acquisition of resistance of P197S activity to the PC herbicides.

In an effort to investigate the influence of feedback regulation, transplastomic plants were grown on medium containing an herbicide. We analyzed herbicide resistance in transplastomic plants harboring four different mALSs. *W574L/S653I* plants showed synergistic tolerance, similar to that observed when the corresponding *mALS* gene was introduced into the nuclear genome of rice (Kawai et al., 2007). The tolerance of *P197S* plants to PC and SU herbicides was demonstrated also in plant growth (Fig. 4). In addition, two other transplastomic plants (*G121A* and *A122V*) showed sensitivities to herbicides both in the activity in leaves (Fig. 3) and in plant growth (Fig. 4). Our results provide evidence suggesting that the sensitivity of mALSs to herbicides in plants is not affected by feedback regulation. The highly expressed mALS molecules may not be fully active due to the resultant stoichiometrically insufficient number of regulatory subunits (Lee and Duggleby, 2001). Therefore, the ALS activity of transplastomic plants was almost equivalent to that of wild-type plants in the absence of herbicides. It is concluded that transplastomic mALSs are useful as sustainable markers that can be employed to distinguish GM and non-GM plants when using appropriate herbicides in the field.

Benefit of mALSs as Sustainable Markers

Herbicide-resistant weeds have been reported in many countries (Tranel and Wright, 2002; Tranel et al., 2007) and include weeds resistant to ALS-inhibiting herbicides. New technology is required to assist in the management of weeds resistant to these herbicides. We propose a strategy involving herbicide rotation to overcome the aforementioned problem. To this end, we have developed transplastomic plants that possess tolerance to PC, IM, and SU/PC. Although it is known that some ALS mutations are associated with plant resistance to a single herbicide, there have been no reports detailing the relationship between a single ALS mutation and resistance to the three classes of herbicide PC, SU, and IM. In this article, transplastomic plants were generated and their resistance to herbicides was characterized. We determined three kinds of ALS mutations that conferred specific resistance to the three classes of herbicides used with the transplastomic plants and showed that *G121A*, *A122V*, and *P197S* plants were resistant to PC, IM, and SU/PC

Table I. *List of primer sequences*

Name	Nucleotide Sequence (5′ to 3′)
G121A Fd	GGCGCTGATATCCTGGTCGAAGCATTAGAACGTCAAGGCGTAG-AAACCGTATTCGCTTACCCTGGAGCTGCATCAATGG
G121A Rv	CTCAATAATCCTAGGAATATCTTCAACATCCATCACAAG
ALS Fd	TCATGACATTTATCTCTCCCGATTCGCTCCAGATCAACC
ALS Rv	CCGGTTTGAGCTCTTAGTATTTAATCCGGCCATCTCC
rbcL Fd	AGCAGTGGACGTTTTGGATAA
aadA Rv	ATCTCGCCTTTCACGTAGTGG
N4-Fd	GAGAATCTTCCAGTGAAGG
pLD200-Rv	TATGTCGTGTCAAATTCGCATCG
ALS-1597-Fd	GTGGATATTGACGGAGATGGAAGC
accD-N-Rv	CAACATACCAAAACTAGCTGTCACTCC
aadA-Fd2	GGACAAGAAGAAGATCGCTTGG
ALS-270-Rv	TCCCGATTCGCTCCAGAT

herbicides, respectively (Fig. 4). Use of these transplastomic markers in crop plants would enable a new strategy based on the rotation of three or more combinations of herbicides. The advanced technology described in this article allows for the efficient and strict management of weeds resistant to ALS-inhibiting herbicides.

Investigations concerning herbicide resistance have been made using chloroplast transformation. For example, the petunia *EPSPS* gene was introduced into the tobacco chloroplast genome and resulted in transplastomic plants resistant to glyphosate (Daniell et al., 1998). Similarly, the *bar* gene for phosphinothricin resistance was used to investigate the resulting plant phenotype (Lutz et al., 2001). Because this gene is derived from microorganisms and not plants, it is less suitable for use in C-CGTT-based approaches. However, EPSPS is worthy of consideration in strategies involving herbicide rotation schemes as described above because EPSPS is present in higher plants. The glyphosate and ALS-inhibiting herbicides are thought to be nontoxic to living organisms, except plants and microorganisms (Peterson and Shama, 2005). Plant-derived EPSPS might be useful as an additional tool for use in an herbicide rotation system for the management of herbicide-resistant weeds. The technology developed in this study may be employed in C-CGTT-based methodologies in association with *aadA* elimination following transformation.

MATERIALS AND METHODS

Construction of Plastid Transformation Vectors

Genes for the transit peptide-truncated ALS mutants A122V, P197S, and W574L/S653 placed in pBluescript (pBS; Stratagene; Kawai et al., 2008) were used as templates for PCR. ALS(G121A) was generated by replacement of the *Eco*RV-*Avr*II fragment of pBS-ALS(A122V) with a G121A-containing product amplified using G121A Fd and G121A Rv, followed by digestion with *Eco*RV and *Avr*II. pBS-ALS(G121A) was also used as a template for PCR. Fragments of ALS mutants were amplified using KOD-plus DNA polymerase for higher fidelity (Toyobo). Primers corresponding to the ALS coding region were ALS Fd and ALS Rv. The PCR reaction was performed by employing 30 cycles of denaturation for 30 s at 94°C, annealing for 20 s at 55°C, and extension for 2 min at 68°C. PCR-generated fragments were ligated to the *Sph*I site of pLD6 (GenBank accession no. CS165374; Adachi et al., 2007) harboring the 16S rRNA gene promoter-*aadA-psbA* gene terminator, *psbA* gene promoter-*Sph*I-TpsbA, and *Prrn-aadA*-TpsbA-PpsbA-*Sph*I-TpsbA to drive *mALS* gene expression using the *psbA* promoter. Sequencing analysis was performed using a BigDye terminator cycle sequence kit (Applied Biosystems). Vectors generated were named pLD6-mALS, pLD6-A122V, and so on. The *Sal*I-*Not*I region of pLD6-mALS was introduced into the *Sal*I and *Not*I sites of pLD200 (GenBank accession no. BD174938; Adachi et al., 2007), which possesses a sequence between *rbcL* and *accD* derived from tobacco for homologous recombination. The absence of unexpected mutations was confirmed by sequence analysis. Finally, each chloroplast transformation vector comprised two transgene constructs, *Prrn-aadA*-TpsbA and PpsbA-*mALS*-TpsbA, referred to as pLD200-mALS. The nucleotide sequences of primers used in this study are listed in Table I.

Transformation and Transgene Confirmation

Chloroplast transformation and the preparation of genomic DNA to confirm the presence of transgenes were performed using the tobacco (*Nicotiana tabacum*) cultivar Xanthi according to previously described methods (Adachi et al., 2007). SP-resistant shoots were selected on RMOP medium containing 0.5 g L^{-1} SP rooted on Murashige and Skoog agar and self-pollinated to obtain transplastomic seeds. PCR analysis was performed using the following five primer sets: the *rbcL-aadA* region of pLD200-mALSs with rbcL-Fd and aadA-Rv, the ALS-accD region with N4-Fd and accD-Rv, the insert integrated into the chloroplast genome with ALS-1597-Fd (annealed with *ALS* in the vector) and accD-N-Rv (for *accD* in the endogenous chloroplast genome, but not included in the vector), the *aadA-ALS* region with aadA-Fd2 and ALS-270-Rv, and the *rbcL* and *accD* regions of the endogenous chloroplast genome with rbcL-Fd and accD-Rv. The nucleotide sequences of primers used in this study are listed in Table I.

Herbicides Inhibiting ALS Activity

BS, PS, and PM were used as representative PC herbicides, chlorsulfuron (CS) and BM were used as representative SU herbicides, and imazaquin (IQ) and IP were used as representative IM herbicides (Fig. 1). These chemical compounds were provided by KI Chemical Research Institute Co., Ltd.

ALS Activity Assay in Leaves

ALS activity was determined as follows. A leaf section (50 mg) was floated and incubated on 25% Murashige and Skoog medium containing 0.5 mM 1,1-cyclopropanedicarboxylic acid, an inhibitor of acetolactate metabolism, with or without ALS-inhibiting herbicides for 42 h under the same conditions used for plant growth (Adachi et al., 2007). The sample was then placed at −80°C for 1 h, transferred into 200 μL of 0.025% Triton X-100, and incubated at 60°C for 5 min followed by incubation at room temperature for 60 min to effect extraction of acetolactate synthesized by ALS. The extract (100 μL) was transferred to a 1.5-mL tube, to which was added 10 μL of 1 N H$_2$SO$_4$. The sample was then incubated at 60°C for 30 min to convert acetolactate to acetoin. An aliquot (50 μL) of 0.5% (w/v) creatine and 50 μL of 5% (w/v) 1-naphthol dissolved in 2.5 N NaOH were added and the resultant mixture was subsequently incubated at 37°C for 30 min. The amount of acetoin formed was determined by a colorimetric assay.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers CS165374 (pLD6), BD174938 (pLD200), and BT020540 (ALS).

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