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Application of Mutated Acetolactate Synthase Genes to Herbicide Resistance and Plant Improvement

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1. Introduction

Herbicides have been used to enhance the productivity of plants including crops by killing the weeds which compete with the growth of cultivated plants. They have also been utilized as a tool to improve plants by means of genetic engineering, whereby transformed plants containing genes for enzymes which impart tolerance to herbicides are selected. These genes are designated as "selectable markers" and are utilized for the production of genetically-modified (GM) plants. Selectable markers used for the selection of plants in which genes of interest are successfully integrated into the genome of host plants include genes that impart tolerance to antibiotics or herbicides, the majority of which are derived from bacteria: genes for neomycin phosphotransferase II (*nptII*) from Tn5 in *Escherichia coli*, 5-enoylpyruvate shikimate-3-phosphate synthase (*epsps)* from *Agrobacterium* sp. CP4, phosphinothricine acetyltransferase (*pat, bar* for bialaphos resistance) from *Streptomyces viridochromogenes*, and aminoglycoside-3"-adenyltransferase (*aadA*) for spectinomycin resistance from *Shigella flexneri* (Hare and Chua, 2002). The safety of genes used for antibiotic resistance is questionable given the possibility that these genes might be transferred into pathogenic bacteria that may be converted to antibiotic-resistant strains. A search for appropriate selectable markers from plants is therefore desirable.

The impact on the environment is another important factor that must be considered, and efforts need to be made to minimize so-called "genetic pollution" or detrimental effects on the ecosystem. The transfer of foreign genes into other non-transgenic plants is most reliably performed via pollen. Apprehension associated with this process is dispelled when considering the transformation of plastids such as chloroplasts. Since genes in plastids of most plant species are inherited maternally, they represent genes that are not transferred

into other plants via pollen. Therefore, the development of methodologies based on the genetic manipulation of plastid genomes, in addition to that of nuclear genomes where the engineering has already been established, is necessary for ecological safety. Our efforts have focused on the use of acetolactate synthase (ALS, EC 2.2.1.6), also referred to as acetohydroxyacid synthase (AHAS), an enzyme involved in the biosynthesis of branched-chain amino acids in chloroplasts in higher plants. This enzyme is uniquely suited for use in biotechnology and basic research.

2. ALS and ALS-inhibiting herbicides

ALS is a common enzyme that catalyzes the first step of the biosynthetic pathway of the branched-chain amino acids valine, leucine and isoleucine. ALS is the primary target site for at least five structurally distinct classes of herbicides including sulfonylureas (SUs), imidazolinones (IMs), triazolopyrimidine sulfonamides (TPs), pyrimidinylsalicylates (pyrimidinylcarboxylates, PCs), and sulfonylaminocarbonyl-triazolinones (Figure 1, see the chapter written by Sato et al.) (Shimizu et al., 2002). ALS-inhibiting herbicides are widely used in agriculture given their high weed control efficacy, high crop-weed selectivity, low use rates and low levels of mammalian toxicity (Sharner & Singh 1997).

Plant ALS genes encoding the catalytic (large) subunits were first isolated from Arabidopsis and tobacco utilizing the yeast ALS gene as a heterologous hybridization probe (Mazur et al., 1987). Since then, some plant ALS genes encoding catalytic subunits have been cloned and characterized (Bernasconi et al., 1995; Fang et al., 1992; Grula et al., 1995; Rutledge et al., 1991). The plant ALS regulatory (small) subunit has been shown to enhance the catalytic activity of the large subunit and to confer sensitivity to feedback inhibition by branched-chain amino acids (Lee & Duggleby 2001). Plants and cultured plant cells resistant to SU- and IM-type ALS-inhibiting herbicides have been generated using conventional mutation breeding methods and *in vitro* cell selection (Hart et al., 1993; Newhouse et al., 1991; Rajasekaran et al., 1996). ALS genes encoding catalytic subunits have been cloned from some

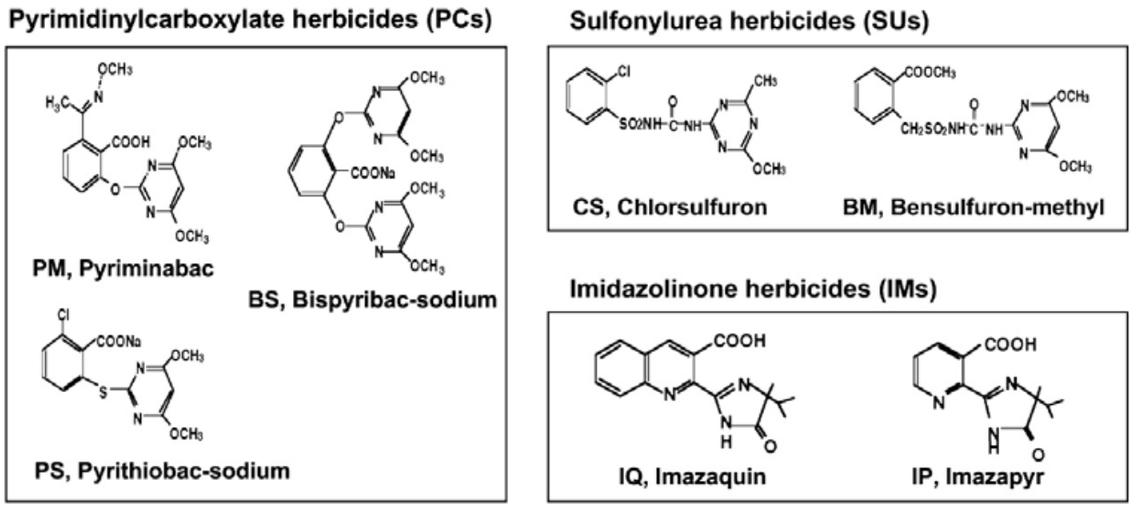

Fig. 1. Herbicides that inhibit ALS activity. These herbicides can be classified into the three classes PC, SU and IM. BS, PS and PM belong to the PC class of herbicides, CS and BM to the SU class of herbicides, and IQ and IP to the IM class of herbicides.

of these plants, and their sequences were found to possess single or double mutations. These mutated ALS (mALS) genes have been revealed to confer resistance to ALS-inhibiting herbicides.

3. ALS mutations interfering with herbicide actions

Herbicide-resistant ALS genes are useful not only for the generation of transgenic plants that express resistance to the corresponding herbicide, but also for introducing foreign traits into plants as selectable markers. We have isolated a double-mutated ALS gene from rice cells (*OsALS-W548L/S627I*) (Figure 2), which confers a high level of resistance to the PC-type ALS-inhibiting herbicide bispyribac-sodium (BS) (Figure 1), and demonstrated that it could be used as a selectable marker for generating transgenic rice plants (Kawai et al., 2007a; Kawai et al., 2007b). We also found that the single amino acid substitution S627I in the ALS gene (*OsALS-S627I*) imparts high levels of resistance to the PCs pyrithiobac-sodium (PS) and pyriminobac (PM). It was postulated that these mALS genes coupled with the PC-type ALS-inhibiting herbicides might be promising selectable markers for various plant species. Indeed, it has been shown that *OsALS-W548L/S627I* works as an effective selectable marker gene for the transformation of wheat (Ogawa et al., 2008) and soybean (Tougou et al., 2009).

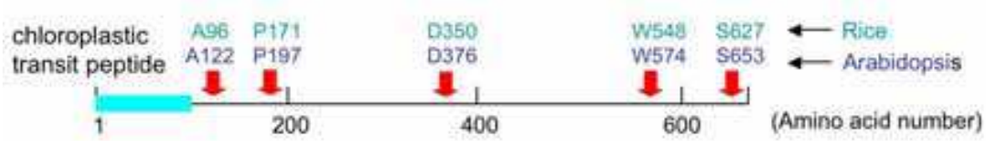

Fig. 2. Schematic representation of amino acid mutations conferring resistance to ALS-inhibiting herbicides. Amino acid residue numbers shown under the peptide are those of rice ALS.

Recombinant Arabidopsis mALSs, AtALS-W574L/ S653I and -S653I, were expressed in *Escherichia coli* cells. These recombinant mALSs exhibited resistance to PCs, and showed similar sensitivity against herbicides to rice recombinant ALSs with the corresponding mutations (Table 1) (Kawai et al., 2008). We have shown that these Arabidopsis ALS genes can also be utilized as selectable markers for the genetic transformation of Arabidopsis (Kawai et al., 2010). It has been revealed that selection by PCs can clearly distinguish resistant seedlings from non-resistant seedlings of Arabidopsis at very low concentrations of herbicide compared with kanamycin selection (Figure 3). The concentrations of PCs employed for the selection were about 1000-fold lower than that of kanamycin. We performed *in vivo* ALS assays in an effort to determine whether the Arabidopsis seedlings, selected by resistance to PCs, were indeed transformants. Although the *in vivo* ALS assay was originally developed for the analysis of ALS-resistant weeds (Gerwick et al., 1993), we reasoned that the procedure could be applied for the evaluation of transformants. PC-type ALS-inhibiting herbicide-resistant seedlings showing *in vivo* ALS activity were further analyzed to verify integration of the T-DNA region within the genome by PCR. Results suggested that the assay could reliably be used to evaluate transformation. The advantage of using mALS genes over other selectable markers is that the *in vivo* ALS assay confirms both integration of the mALS gene and expression of the corresponding protein in the selected plants. Furthermore, the *in vivo* ALS assay allows for a larger number of samples to be easily

tested at relatively lower costs compared to PCR-based screening methods. Differences in levels of acetoin accumulation were observed between the independent transgenic lines. This observation may reflect copy number differences or differential expression of ALS due to positional effects in the Arabidopsis genome. If so, transgenic plants expressing a high or desirable level of the gene of interest may be identified at an early stage of transformation.

Herbicide [b]	RS ratio [a]			
	AtALS-S653I	OsALS-S627I	AtALS-W574L/ S653I	OsALS-W548L/ S627I
CS	4.2	2.4	4,400	200
BM	43	73	>9,100	>14,000
IQ	21	6.8	>56	>45
IP	>14	>10	>14	>10
BS	83	41	>17,000	>16,000
PS	350	200	>2,900	>9,100
PM	3,300	2,500	>8,300	>13,000

[a] RS ratios for mutated ALSs were obtained by calculating the ratio of the I_{50} value for each mutated ALS to the I_{50} value for the wild-type.

[b] SUs: CS, chlorsulfuron; BM, bensulfuron-methyl; IMs: IQ, imazaquin; IP, imazapyr; PCs: PM, pyriminobac; PS, pyrithiobac-sodium; BS, bispyribac-sodium.

Table 1. Degree of resistance of recombinant mALSs to ALS-inhibiting herbicides

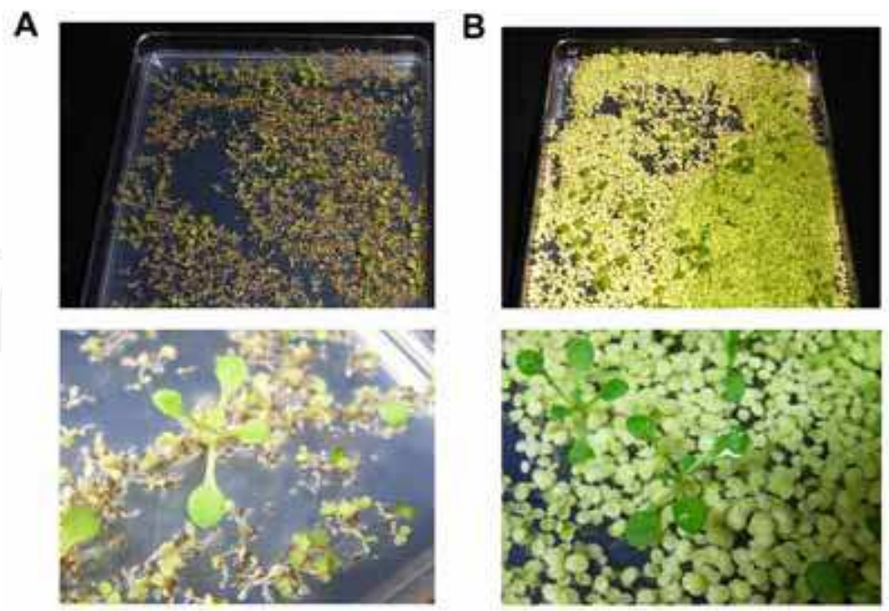

Fig. 3. Comparison of screening seedlings of Arabidopsis transgenic with *AtALS-W574L/S653I* in pMLH7133 binary vector (Kawai et al., 2010) encoding *nptII* by BS or kanamycin. A, 0.045 ppm BS; B, 50 ppm kanamycin.

4. Species-specific properties of ALS mutations

As mentioned above, the degrees of resistance of Arabidopsis and rice recombinant ALS proteins with identical mutations to PCs are very similar. However, the sensitivity of transgenic Arabidopsis to PCs indicated that the degree of resistance to PCs of transformants expressing Arabidopsis mALSs was greater than those of transformants expressing rice mALSs (Figure 4). It is known that plant ALSs have a signal peptide that is required for translocation of the protein into the chloroplast (Duggleby & Pang, 2000), although the exact size of the signal peptide remains to be determined experimentally. Several reports have indicated that the size of the signal peptide ranges between 70 and 85 amino acids (Chang & Duggleby, 1997; Rutledge et al., 1991; Wiersma et al., 1990). If the cleavage site of the signal peptide is assumed to be at position 85, then the sequence homology of rice and Arabidopsis ALS is only 23%. Therefore, signal peptide processing and transport of the protein into the chloroplast may be involved in limiting rice ALS enzyme activity in Arabidopsis. We also considered another potential reason for the observed difference in ALS activity. It has been shown that Arabidopsis ALS is composed of four catalytic subunits and four regulatory subunits (Lee & Duggleby, 2001; McCourt et al., 2006). Thus, ALS derived from transformants expressing rice ALS will presumably be chimeric, *i.e.*, composed of both rice and Arabidopsis catalytic subunits. As a result, the enzyme activity may be reduced compared with that of ALS composed of only Arabidopsis enzyme. The full-length amino acid sequences of ALSs derived from monocotyledonous and dicotyledonous plants were clearly divided into two distinct clusters in the phylogenetic

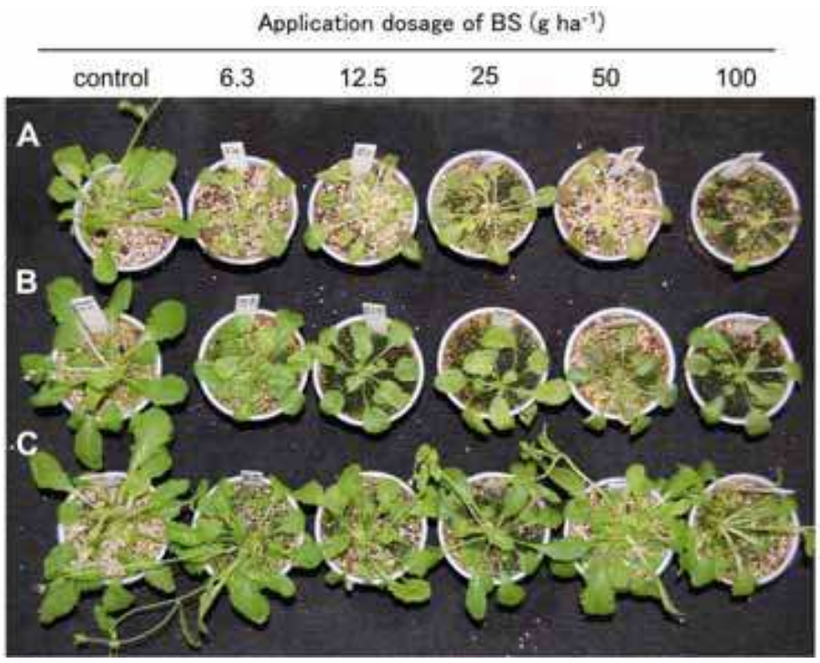

Fig. 4. Comparison of sensitivity to BS of Arabidopsis wild-type and T3 transformants. Plants, planted in pots (9-cm diameter), were sprayed with 6.3 to 100 μg mL^{-1} (approximately 14 to 220 μM) BS with an application dose of 6.3 g to 100 g ha^{-1}. The photograph was taken 2 weeks after spraying. A, wild-type; B, *OsALS-W548I/S627I* 30-8; C, *AtALS-W574L/S653I* 1-3.

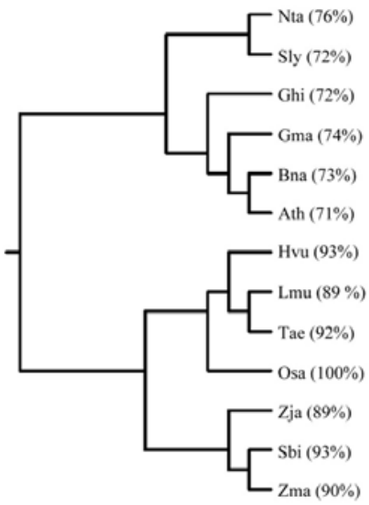

Fig. 5. Phylogenetic analysis with full-length amino acid sequences of ALSs. The NJ-tree was constructed using the ClustalW program (http:/ / align.genome.jp/). The percentage indicates the amino acid homology of each plant with rice (Osa). Amino acid sequences of corn (Zma), wheat (Tae) (lacking the N-terminal region), barley (Hvu) (lacking the N-terminal region), Italian ryegrass (Lmu), Arabidopsis (Ath), tobacco (Nta), cotton (Ghi) and rapeseed (Bna) were obtained from the GenBank database. Putative amino acid sequences of ALS from sorghum (Sbi) (lacking the N-terminal region) and soybean (Gma) were identified through a BLAST search of the JGI database (http:/ / genome.jgi-psf.org/) and that from tomato (Sly) was identified through a BLAST search of the Tomato SBM (http:/ / www.kazusa.or.jp/ tomato/). The nucleotide sequences of ALS of Japanese lawn grass (Zja) have been determined by genome walking using a DNA Walking SpeedUp Kit (Seegeen, Inc., Korea, unpublished data). Lack of the N-terminal region slightly increased the amino acid homology of sorghum, wheat and barley with rice compared to the complete ALS protein sequences since the homology in this region was very low.

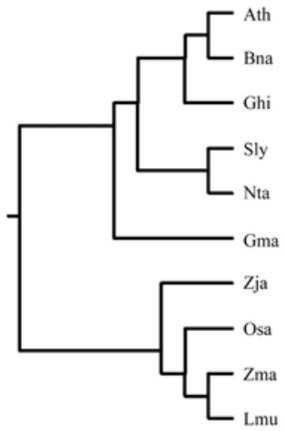

Fig. 6. Phylogenetic analysis with amino acid sequences of the putative signal peptide region of ALSs. Seventy amino acid residues from the first methionine of complete ALS protein sequences were used for the analysis.

tree, each cluster being highly conserved (Figure 5). The putative signal peptide amino acid sequences of ALSs were also divided into two clusters in the phylogenetic tree (Figure 6). These findings suggest it would be best to use rice and Arabidopsis mALS genes for generating monocotyledonous and dicotyledonous transgenic plants, respectively. Given differences in the sensitivity to PCs and in the expression level of induced mutant ALSs between plant species, the preparation of various combinations of mALS genes and PCswould be an effective strategy in applying this selection system to a broad range of plant species.

We artificially generated other types of mALS genes of Arabidopsis, which yielded recombinant ALS proteins with A122V, P197S, W574L, S653N and P197H/ R198S mutations. Recombinant ALS proteins from these genes were prepared as glutathione S-transferase-fused proteins, and the sensitivity of the proteins to ALS-inhibiting herbicides were examined. It was found that the level of resistance of these recombinant ALS proteins to ALS-inhibiting herbicides varied for the compounds tested (Table 2), while mALS-P197S, W574L and P197H/ R198S proteins showed similar sensitivity to herbicides to that of rice ALS proteins with the corresponding mutations (Kawai et al., 2008). These results indicated that some Arabidopsis-mALS genes are useful as selectable marker genes for the genetic transformation of plants when used together with ALS-inhibiting herbicides to which mALSs express high resistance.

Herbicide [b)]	RS ratio [a)]				
	A122V	P197S	W574L	S653N	P197H/ R198S
CS	10	300	>8,300	4.3	2,400
BM	190	9,100	>9,100	72	>9,100
IQ	>55	25	>56	>55	1.8
IP	>14	3.7	>14	>14	>14
BS	20	5.3	2,800	53	80
PS	1	56	>2,900	68	2
PM	140	13	6,500	700	13

[a)] RS ratios for mutated ALSs were obtained by calculating the ratio of the I_{50} value for each mutated ALS to the I_{50} value for the wild-type.

[b)] SUs: CS, chlorsulfuron; BM, bensulfuron-methyl; IMs: IQ, imazaquin; IP, imazapyr; PCs: PM, pyriminobac; PS, pyrithiobac-sodium; BS, bispyribac-sodium.

Table 2. Degree of resistance of recombinant Arabidopsis mALSs to ALS-inhibiting herbicides

5. Nuclear gene-targeting as an ultimate clean technology

More than two decades have passed since the basic technology of plant transformation was established (Herrera-Estrella et al, 1983). Although a variety of GM crops have been cultivated world-wide, they have not been fully accepted by consumers, especially in Japan and Europe, leading to the conclusion that the technology may not satisfy consumers' desires. Selectable-marker genes in addition to the genes of interest necessary to improve plant growth and resistance are always required for plant transformation. With respect to

selectable markers, the use of antibiotic-resistant genes may not obviate the possibility of generating antibiotic-resistant bacteria in the intestines of cattle. Most imported GM crops are generated with selectable-marker genes derived from microorganisms and promoters taken from plant pathogens such as cauliflower mosaic virus and *Agrobacterium* spp., and are therefore less accepted by consumers and the general public. In an effort to overcome these problems, strategies for excising selectable-marker genes from transgenic plants have been developed (Hare & Chua, 2002). Eventually, strategies employing a combination of selectable markers originating from plants and the use of herbicides harmless to the environment and humans are worth investigating to shorten the time it takes to create GM crops compared to technologies employing selectable-marker excision. Another approach to allay consumer anxiety is the employment of plant-derived DNA sequences including selectable markers without their excision. When DNA sequences are introduced into plant species from which they are derived, the transformed plants are designated as "intragenic" (Nielsen, 2003). Such an approach has been successfully performed with a mALS gene in Arabidopsis (Ahmad et al., 2009). Furthermore, if we can replace an internal wild-type gene with its point-mutated gene by gene-targeting, the resultant plants are completely equivalent to those generated by conventional breeding or mutagenesis. One gene for ALS has been reported to be present in the genome of Arabidopsis (Endo et al., 2006) or rice (Endo et al., 2007), and replacement of the internal wild-type gene with a mutated species which confers herbicide resistance on those plants has been successfully demonstrated.

6. Plastid transformation

Plastid transformation was first achieved in 1988 for the unicellular alga *Chlamydomonas reinhardtii* by Boynton *et al.* (Boynton et al., 1988), and was followed in 1990 by the transformation of tobacco by Maliga *et al* (Svab et al., 1990). Genetic engineering approaches utilizing chloroplasts possess a number of attractive advantages compared with nuclear transformation, and include: (i) a high level of transgene expression (Daniell et al., 2002), (ii) delivery of multiple genes in a single transformation event (Daniell & Dhingra, 2002), (iii) the absence of gene silencing (DeCosa et al., 2001), (iv) the absence of position effects due to site-specific transgene integration (Daniell et al., 2004), and (v) the absence of pleiotropic effects given localization of the transgene products inside the chloroplast (Daniell et al., 2001). These advantages have lead to trials of chloroplast transformation in many plants such as Arabidopsis (Sikdar et al., 1998), potato (Sidorov et al., 1999), rice (Khan & Maliga, 1999), tomato (Ruf et al., 2001), *Lesquerella fendleri* (Skarjinskaia et al., 2003), oilseed rape (Hou et al., 2003), carrot (Kumar S et al., 2004a), cotton (Kumar S et al., 2004b), soybean (Dufourmantel et al., 2004), lettuce (Lelivelt et al., 2005), and cabbage (Liu et al., 2007). The successful recovery of genetically-stable transplastomic plants is dependent on the ability to selectively amplify the plastid genomes, which are quite low in copy number, following delivery of the genes by particle bombardment. The key factor affecting transformation efficiency is the choice of selectable marker. There are two types of plastid selectable marker genes: 'primary selectable markers' to be used for direct selection (*aadA*, *nptII* and *aphA-6* for aminoglycoside phosphotransferase), and 'secondary selectable markers' (*bar* and *epsps*) that are not suitable for direct selection when only a few copies of plastid DNA (ptDNA) have settled down, but will allow selection when many copies of ptDNA are integrated (Maliga, 2004; Lutz et al., 2007). The 'primary selective markers' are of bacterial origin and confer resistance to an antibiotic: *aadA* to spectinomycin and streptomycin (Svab and Maliga, 1993;

Zoubenko et al., 1994) and *neo* (Carrer et al., 1993) and *aphA-6* (Huang et al., 2002) to kanamycin. The 'secondary selective markers', *bar* and *epsps*, are also derived from bacteria and confer resistance to herbicides such as phosphinothricin (Lutz et al., 2001) and glyphosate (Ye et al., 2003), respectively. To date, introduction of mALSs to the chloroplast genome has not been attempted perhaps because ALS was thought to be unsuitable as a selectable marker, as in the case of *bar* or *epspe* (Cao et al., 1992, Ye et al., 2003). We therefore attempted to utilize mALSs in chloroplast engineering strategies.

7. Clean gene transformation technology for chloroplast engineering

Transformation technologies of nuclear genomes have been developed to eliminate antibiotic marker genes, an approach referred to as nuclear genome-clean gene transformation technology (CGTT) (Yoder et al., 1994). Over the past several years, consumer and environmental organizations have expressed ethical and biosafety concerns about the use of antibiotic- and herbicide-resistance genes derived from microorganisms (Miki & McHugh, 2004). This concept has also been applied to chloroplast genetic engineering, in which antibiotic-resistant genes such as *aadA* were eliminated from the chloroplast genome, resulting in marker-free transplastomic plants or replacement with plant-derived marker genes. To date, four strategies have been developed for the generation of marker-free transplastomic plants: (i) homology-based excision via direct repeated regions (Iamtham & Day, 2000); (ii) cotransformation–segregation (Carrer & Maliga, 1995); (iii) transient co-integration of marker genes (Klaus et al., 2004), and (iv) excision by phage site-specific recombinases (Corneille et al., 2001). On the other hand, two marker genes applied to tobacco have been reported to be derived from plants: genes for betaine aldehyde dehydrogenase (BADH) from spinach (*Spinacia oleracea*) (Daniell et al., 2001) and feedback-insensitive anthranilate synthase α-subunit (ASA2) from tobacco (Barone et al., 2009). However, use of the *BADH* gene has not been consistently reproduced (Maliga, 2004; Whitney & Sharwood, 2008).

Use of such technologies prevents the transfer of antibiotic-resistant genes to surrounding weeds and microorganisms in soil, and to bacteria in animal guts after oral intake. Integration of foreign genes into the plastid genome strengthens gene containment since plastids are inherited maternally in many crop plants, avoiding the pollen-mediated spread of transgenes (Maliga, 1993; Daniell et al., 1998; Scott & Wilkinson, 1999). Homologous recombination in plastids allows for accurate gene targeting into a well-characterized genome and elimination of bacterial vector sequences (Svab et al., 1990). High levels of gene expression have resulted from an increasing number of foreign genes being located in plastids (McBride et al, 1995; Staub et al., 2000; Kanamoto et al., 2006). Notwithstanding all of the advantages associated with marker-free technology in chloroplast transformation, there remains one outstanding problem that should be resolved in the field. GM and non-GM plants must be clearly and easily distinguished from one another. Although PCR-based methods are the most convenient for ascertaining contamination in bulk samples, they are unsuitable for checking a single seed or plant in terms of efficiency and use of resources. The use of herbicides was proposed as an appropriate method to solve this problem, although the generation of herbicide-resistant plants must be considered. Some reports have indicated that herbicides which inhibit ALS or acetyl-CoA carboxylase, such as glyphosate and others, accelerated the generation rate of weeds and crops tolerant to the herbicide (Preston & Powles, 2002; Shimizu et al., 2002; Tranel & Wright, 2002; Tranel et al., 2007,

Heap, 2010). Employing a rotation supply of some herbicides was proposed as a countermeasure against the occurrence of herbicide-resistant weeds (Gressel, 1984).

8. Mutated ALS genes as plastid sustainable markers

The employment of some plant-origin genes for herbicide tolerance has solved the problem and allayed the public's anxiety. We have focused on the use of mALS genes as sustainable markers. It is well known that ALS imparts herbicide tolerance by mutation at several amino acid residues (Figure 2). Herbicide-tolerant plants have been reported for rice, tobacco and Arabidopsis (Chang et al., 1998; Tan et al., 2005; Shimizu et al., 2002; Kawai et al., 2007b; Okuzaki et al., 2007). Several mutated species of Arabidopsis ALS have been expressed in *Escherichia coli*, and their sensitivity to inhibitors was examined (Tables 1 and 2) (Kawai et al., 2008). These results showed that P197S, W574L, S653I, P197H/ R198S and W574L/ S653I were resistant to SUs, IMs, SUs, IMs, and all three types of herbicides, respectively. However, little is known about the effect of herbicides on the growth of plants with mALSs. It has recently been reported that mutation of W548L/ S627I and G95A in rice ALS imparts tolerance to all three types of herbicides and pyrimidinylcarboxylate herbicides, respectively (Kawai et al., 2007b; Okuzaki et al., 2007). We examined whether introduction of mALSs into the chloroplast genome can be applied to a strategy involving the rotation supply of different herbicides by characterizing the transplastomic lines with respect to: (i) the influence of hyper-expression of mALSs on plant growth, (ii) feedback regulation by the regulatory subunit *in vivo*, (iii) the dependency of herbicide resistance on each mutation similarly observed *in vitro* (Tables 1 and 2) (Kawai et al., 2008; Okuzaki et al., 2007), and (iv) the availability of multiple combinations of different mutations and herbicides. We have reported on the introduction of some mALS genes into the chloroplast genome and examined the sensitivity of transformants to ALS-inhibiting herbicides. The results indicated that mALS genes are useful as sustainable markers, which function to exclude non-transformed crops while maintaining transformed plants. These markers have shown selectable tolerance to different types of herbicide**.** We have proposed that the rotation supply of different herbicides can be effective when used with transgenic plants harboring mALS genes (Shimizu et al., 2008).

The chloroplast transformation vectors pLD201- mALS (Figure 7A), possessing the *aadA* and *mALS* (transit peptide truncated) genes inserted between tobacco sequences *rbcL* for the large subunit of ribulose-1,5-bisphosphate carboxylase/ oxygensae and *accD* for homologous recombination, were introduced by particle bombardment (Figure 7A). The integration of *mALS* into the chloroplast genome in regenerated tobacco plants was confirmed by PCR using the 5 primer sets shown in Figure 7A. Tobacco chloroplast transformation was performed using pLD-201-mALS harboring *G121A, A122V, P197S, P197S/S653I* or *W574L/S653I*. G121A in Arabidopsis ALS corresponds to G95A in rice (Okuzaki et al., 2007). The resultant transplastomic plants were maintained on hormone-free Murashige and Skoog (MS) medium (Figure 7B). It is concluded that the chloroplast genome in these transgenic plants were almost transplastomic (Figure 7C).

We investigated the involvement of the regulatory subunit in ALS activity. The regulatory subunit plays a role in feedback regulation by Val, Ile and Leu and in general enzyme activity (Lee & Duggleby, 2001). The determination of ALS activity in leaves, where regulatory subunit molecules are present, has been performed in the presence of 1,1-cyclopropanedicarboxylic acid, which blocks acetolactate metabolism, resulting in no

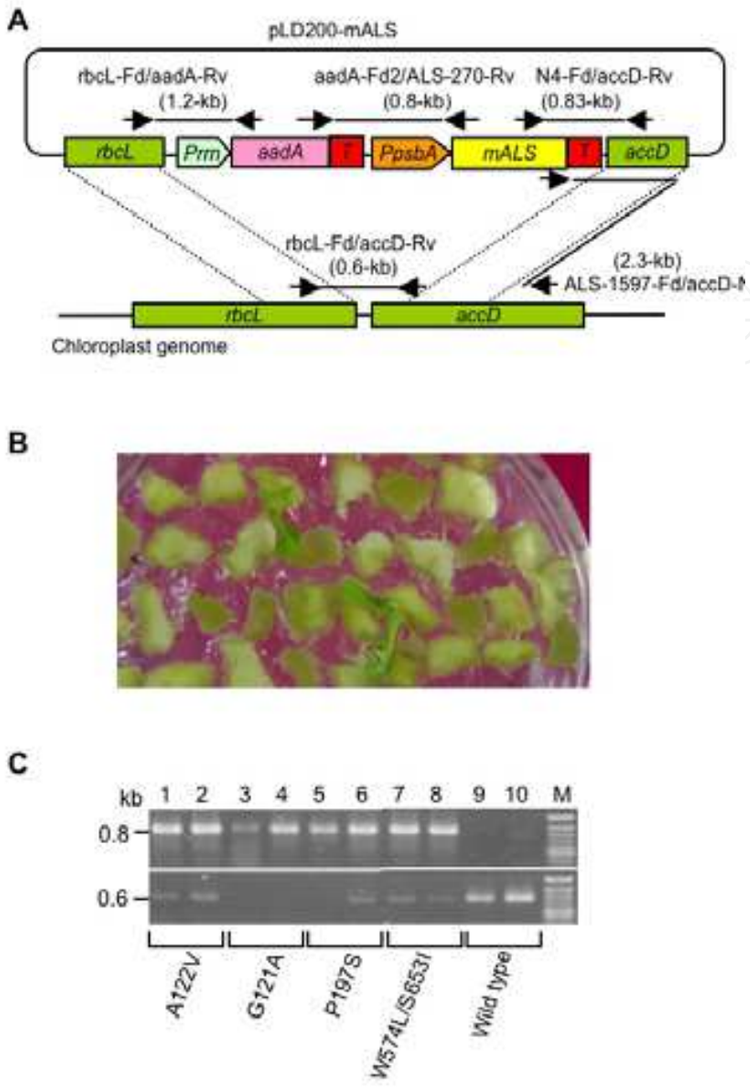

Fig. 7. Transformation of tobacco chloroplast with *aadA* and mALS. A, Structure of the chloroplast transformation vector. *Prrn-aadA* and *PpsbA-mALS* show the transgenes introduced into the chloroplast genome. The region located between *rbcL* and *accD* may be integrated into the chloroplast genome by homologous recombination. PCR was performed to confirm transplastomic integration using the primer sets shown, and the expected sizes of the PCR products are shown in parentheses. B. Following bombardment, leaf slices were grown on RMOP (Shimizu et al., 2008) containing 0.5 mg L^{-1} spectinomycin. A spectinomycin plate after 6 weeks is shown. Regenerated plants represent candidate transformants. C. Population of the transformed chloroplast genome. PCR analysis of different lines of each chloroplast transformant harboring A122V (lanes 1 and 2), G121A (lanes 3 and 4), P197S (lanes 5 and 6), P197S/ S653I (lanes 7 and 8), W574L/ S653I (lanes 9 and 10), and wild-type (lanes 11 and 12). PCR reactions were performed using 25 cycles. The 0.8-kb product represents part of the transgene introduced into the chloroplast genome, and the 0.6-kb product is derived from endogenous chloroplast genome without a transgene insert.

feedback regulation. The activity of native ALS from wild-type tobacco in the absence of ALS-inhibiting herbicides was determined using a colorimetric assay, and yielded a red color in the samples. The red color changed to a transparent or pale yellow color following the addition of SU herbicide (0.1 μM BM), PC herbicide (0.1 μM PS), and IM herbicide (5 μM IP), indicating that these herbicides inhibited ALS activity. This assay was employed for the evaluation of mALS activity in transplastomic plants (*G121A*, *A122V*, *P197S* and *W574L/S653I*). The ALS activity of *G121A* plants was strongly resistant to PS, weakly resistant to BM and sensitive to IP (Figure 8), whereas *A122V* plants were particularly resistant to IP (Figure 8), and *P197S* plants were strongly resistant to BM, showed medium resistance to PS, and were sensitive to IP (Figure 8). The ALS activity of *W574L/S653I* plants was strongly resistant to PS, BM and IP (Figure 8). The selectable tolerance of plants transplastomic with *G121A*, *A122V* and *W574L/S653I* (Figure 9) were similar to those obtained when using the same recombinant mALSs that only expressed the catalytic subunit in *E. coli*, to which endogenous *E. coli* regulatory subunits, it was concluded, were not associated (Tables 1 and 2) (Kawai et al., 2008; Okuzaki et al., 2007). Therefore, the regulatory subunits do not affect the sensitivity of these mALSs to herbicides in transplastomic plants. On the other hand, the behavior of mALS P197S differed from that of the aforementioned 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 (Figure 8), whereas mALS P197S expressed in *E. coli* was resistant to SU but not to PC herbicides (Table2) (Kawai et al., 2008). This result suggests that the regulatory subunit contributes towards imparting mALS P197S with resistance to PC herbicides.

Fig. 8. Inhibition of ALS activity with herbicides in plants transplastomic with *mALSs*. ALS activity in tobacco transplastomic with *mALSs* was colorimetrically examined. ALS activity of tobacco, wild-type and plants transformed with *G121A, A122V, P197S, P197S/S653I* or *W574L/S653I* was determined in the presence of 0.1 μM BM, 0.1 μM PS or 5 μM IP.

In an effort to investigate the influence of feedback regulation caused by hyper-expression of the ALS gene, transplastomic plants were grown on medium containing 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., 2007b). The tolerance of *P197S*-plants to PC and SU herbicides was also demonstrated during plant growth (Figure 9). Additionally, two other transplastomic plants (*G121A* and *A122V*) showed sensitivity to herbicides with respect to the activity in leaves (Figure 8) and during plant growth (Figure 9). Our results provide evidence to suggest 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 & Duggleby, 2001). Therefore, the ALS activity of transplastomic plants was almost equivalent to that of wild-type plants in the absence of herbicide.

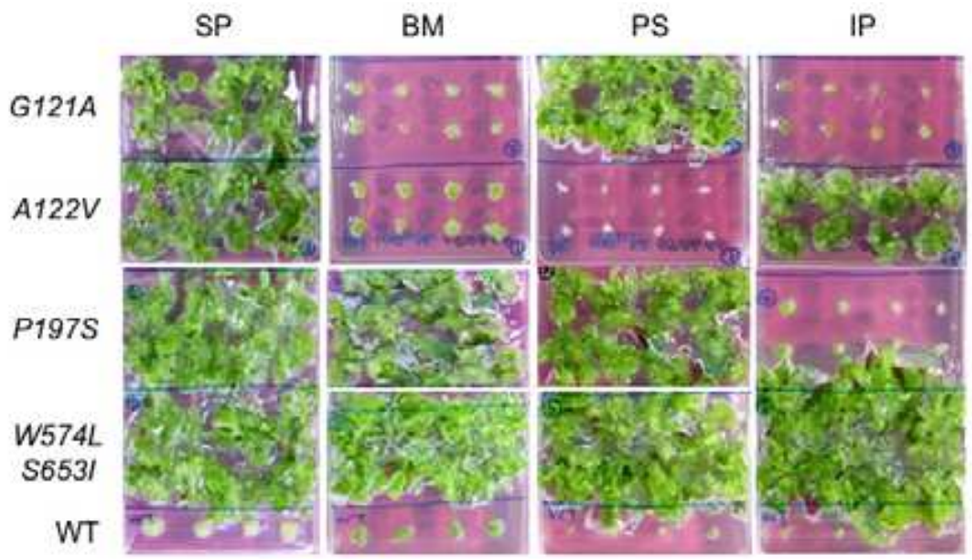

Fig. 9. Regeneration of transplastomic plants on medium containing ALS-inhibiting herbicides. Plants transgenic with A122V, G121V, P197S, P197S/ S653I or W574L/ S653I were regenerated on RMOP medium (Shimizu et al., 2008) containing 0.5 g L^{-1} spectinomycin (SP), 0.1 μM BM, 0.1 μM PS or 1 μM IP.

In an effort to confirm that the herbicide-related traits of the transplastomic plants were inherited by the next generation, T1 seeds, the self-pollinated progeny of the transplastomic lines, were planted on medium containing the corresponding herbicide or spectinomycin. Both seed types were able to grow on MS medium (Figure 10). Although wild-type plants were sensitive to IP and SP, all *A122V* seeds were uniformly resistant to SP and IP (Figure 10). This study revealed that transplastomic plants with *mALS*s grow normally on MS medium without significant differences compared to wild-type plants, indicating that hyper-expression of mALSs does not influence plant growth. These transplastomic plants containing mALS were able to grow in the presence of the corresponding herbicide, indicating that mALSs are useful as sustainable markers in the field, and lending support to proposals that involve the rotation of three or more combinations of herbicide and

transplastomic plants. The advanced technology described here would allow for the efficient and controlled management of weeds resistant to ALS-inhibiting herbicides.

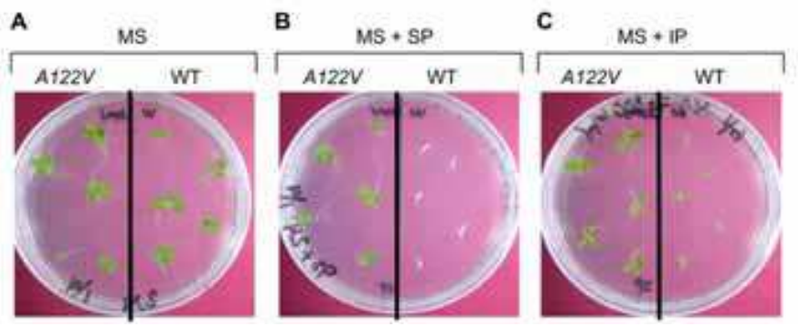

Fig. 10. Inheritance of herbicide tolerance in the seed progeny of chloroplast transgenic plant. The T_1 seeds transplastomic with *PpsbA-A122V* (the left in all panels, A122V) and wild-type (the right in all panels, WT) were germinated on MS medium alone (panel A, MS), or medium containing 0.5 mg L^{-1} spectinomycin (panel B, MS+SP) or 1 μM IP (panel C, MS+IP).

9. Application of mALSs integrated into plastid genomes

Herbicide-resistant weeds have been reported in many countries (Tranel & Wright, 2002; Tranel et al., 2007, Heap, 2010) including 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. We identified three types 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 herbicides, respectively (Figure 9). Use of these transplastomic markers in crop plants could allow for the implementation of a new strategy based on the rotation of three or more combinations of herbicides. The advanced technology described in this review provides the basis for the efficient and strict management of weeds resistant to ALS-inhibiting herbicides. Investigations concerning herbicide resistance have been performed 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). Since this gene is derived from microorganisms and not plants, it is less suitable for use in CGTT-based approaches. However, epsps is worthy of consideration in strategies involving herbicide rotation schemes as described above since 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 & Shama, 2005). Plant-derived epsps might be useful as an additional tool for use in a herbicide rotation system for the management of herbicide-resistant weeds. We have tried to adapt *mALSs* for use as selectable markers in chloroplast transformation but have not succeeded to date. As with *epsps* and *bar* (Cao et al., 1992, Ye et al., 2003), mALSs might be unsuitable for use as selectable markers. The technology

described here may be employed in CGTT-based applications in association with *aadA* elimination following transformation.

10. Conclusion

A number of genes have been employed for the generation of genetically-modified crops possessing tolerance to herbicides in an effort promote crop growth and discourage the growth of competing plants such as weeds. Herbicide-resistant genes are also invaluable for use as selectable markers in the genetic transformation of plants. The majority of herbicide-resistant genes are derived from soil bacteria such as *Agrobacterium* and *Streptomyces*, organisms which have never been utilized as ingredients in products for human consumption. With respect to the use of plant-derived genes for herbicide tolerance, attention may be paid in order to facilitate public awareness and acceptance of the technologies involved. These genes are also useful in strategies involving intragenic transformation through homologous recombination to generate plants free from any exogenous DNA fragments. Our research efforts have focused on ALS. Use of this gene has several advantages including: (i) a single locus is present in Arabidopsis and rice, thus allowing for the straightforward implementation of gene targeting strategies, (ii) multiple classes of herbicides which interfere with different domains of ALS molecules are available, thereby providing the opportunity to generate plants with selected tolerance so as to reduce the occurrence of herbicide-resistant weeds in programs employing the rotation supply of different herbicides, and (iii) availability as a sustainable marker in chloroplast transformation in addition to a selectable marker for nuclear transformation. We have introduced the mutations G121A, A122V, P197S, P197H, R198S, W574L, S653I and others into Arabidopsis ALS and delivered these genes into nuclear and chloroplast genomes of plants. Use of these nuclear and transplastomic markers in crop plants would facilitate the implementation of a new strategy based on the rotation of multiple combinations of herbicides and mALSs to prevent the generation of herbicide-resistant weeds. Furthermore, the use of mALSs in gene-targeting for nuclear transformation and homologous recombination in plastid engineering would bring us closer to our goal of an ultimate clean technology, and allow for the production of GM plants in which only the ALS gene is mutated without integration of any other external DNA sequences.

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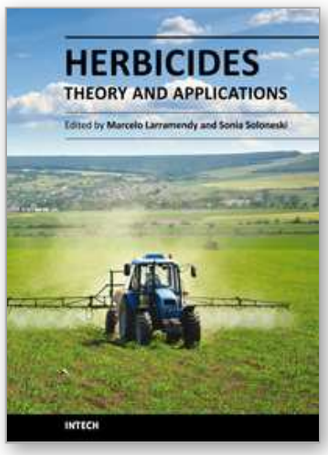

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The content selected in Herbicides, Theory and Applications is intended to provide researchers, producers and consumers of herbicides an overview of the latest scientific achievements. Although we are dealing with many diverse and different topics, we have tried to compile this "raw material" into three major sections in search of clarity and order - Weed Control and Crop Management, Analytical Techniques of Herbicide Detection and Herbicide Toxicity and Further Applications. The editors hope that this book will continue to meet the expectations and needs of all interested in the methodology of use of herbicides, weed control as well as problems related to its use, abuse and misuse.

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