Phosphinothricin (PPT) is the active ingredient in bialaphos, which specifically inhibits glutamine synthetase in land plants. We isolated a novel PPT-resistant gene from a soil bacterium, *Nocardia* sp., and characterized it. The encoded protein, consisting of 177 amino acids, showed significant similarity to bacterial N-acetyltransferases, and we originally designated the gene *MAT* (methionine sulfone N-acetyltransferase). The recombinant *MAT* protein exhibited functions as a methionine sulfone and PPT N-acetyltransferase in *vitro*. The PPT N-acetyltransferase activity reached the maximum at pH 8–8.5, indicating that the protein might optimally function in chloroplasts. We therefore constructed a *MAT* gene, encoding the enzyme with a chloroplast-localizing signal in its amino-terminus. Plant transformation with the construct resulted in the generation of PPT-resistant rice and *Arabidopsis*. Furthermore, the transformed *Arabidopsis* was selectable in a synthetic medium containing PPT. The *MAT* gene thus facilitated establishment of herbicide-resistant plants, and as a new selectable marker gene.

Key words: acetyltransferase; bialaphos; phosphinothricin; herbicide-resistant plant; selectable marker gene

Herbicide-tolerant crops modified by genetic engineering are widely cultivated in many countries. Most commercialized herbicides target plant enzymes. Among these, enzymes relating to amino acid biosynthesis are considered desirable targets because many of them do not exist in animal cells. One of the candidates is glutamine synthetase (GS), which catalyzes the conversion of L-glutamate to L-glutamine by providing the only efficient pathway for the conversion of inorganic nitrogen to the organic form. This reaction is required for the assimilation of ammonia generated by a variety of physiological processes in different subcellular organelles in plants. GS is ubiquitous in the plant kingdom, and it occurs in two major forms. One form plays a role in the cytosol (GS1) and the other in the chloroplast (GSII). GSII is considered to be the dominant form in land plants, and is also present in root plastids and other non-green plant tissues.

Bialaphos, L-2-amino-4-[(hydroxy)(methylphosphinoyl)] butyl-L-alanyl-L-alanine, is a tripeptide antibiotic produced by *Streptomyces hygroscopicus*. It consists of phosphinothricin, a glutamate analog, and two L-alanine residues. Phosphinothricin (PPT, also known as glufosinate) is a potent GS inhibitor. GS is the only enzyme in plants that is able to detoxify ammonia, and thus inhibition of GS by PPT causes rapid accumulation of ammonia and impairment of photosynthesis in plants.

PPT N-acetyltransferase enzymes derived from soil bacteria detoxify PPT by acetyllating the free amino group of PPT (Fig. 1), resulting in the tolerance of transgenic plants. Two PPT N-acetyltransferase proteins, which are encoded by the *bar* from *Streptomyces hygroscopicus* and *pat* from *Streptomyces viridochromogenes*, have been used to generate PPT-tolerant commercial crops such as corn, rice, and tobacco. The *bar* and *pat* genes have also been widely used as effective selectable markers in many types of plant transformation. Both genes encoded a polypeptide of 183 amino acids, and showed 85% homology at the amino acid sequence level and 87% homology at the nucleotide sequence level, although they belong to different species. However, no other PPT N-acetyltransferases have been applied to engineer herbicide resistance in plants by expressing an enzyme that detoxifies PPT.

In this study, we identified a novel bacterial PPT N-acetyltransferase, from which we engineered a chloroplast-localizing gene, *MAT* (methionine sulfone N-acetyltransferase). Transformation and expression of the *MAT* gene in plants allowed the plants to grow in the presence of PPT. Furthermore, *MAT* has been proven useful as a selectable marker in the transformation of plants. This novel gene should be added to the collection of genetic tools available for the genetic engineering of plants.

Materials and Methods

Plant materials and growth conditions. Rice (*Oryza sativa* cv. *Nipponbare*) plants and calli were grown as described previously. Pea (*Pisum sativum*, cv. Toyonari; Sakata’s Seeds) plants were grown at 25 °C under a 16 h-light/8 h-dark cycle using artificial light (150–170μmol m⁻² s⁻¹). *Arabidopsis thaliana* ecotype Columbia was used.
for transformation. *Arabidopsis* seeds were sown on soil and stratified at 4°C for 3 d before transfer to a growth chamber under long-day (16 h light, 8 h dark) conditions. Temperature and humidity were maintained at 23°C and 50%.

**Bacterial strains and PPT-resistant gene cloning.** The soil bacteria strains used for screening in this study were collected from various places in Japan. Bialaphos-resistant bacteria screening was conducted using a medium containing 1% glucose, 0.2% t-asparagine, 0.03% NaCl, 0.05% MgSO₄, 0.05% KH₂PO₄, 0.5% (v/v) trace element solution (ZnCl₂ 40 mg/l, FeCl₃ 2H₂O 200 mg/l, CaCl₂ 2H₂O 20 mg/l, MnCl₂ 4H₂O 20 mg/l, NH₄Cl 20 mg/l, MoO₃ 4H₂O 20 mg/l, CoCl₂ 6H₂O 20 mg/l, CaCl₂ 2H₂O 100 mg/l), 1.8% agar, and 50 mg/l bialaphos for 1 week at 27°C. Genomic DNA from selected soil bacteria was extracted and digested with *Bgl*II, and the obtained fragments were ligated into the plasmid vector pIJ70.13 The ligated plasmids were transformed into *S. lividans*, as described previously,14 and transformants that showed bialaphos resistance were selected. Based on restriction enzyme analyses, subcloning was performed using pUC18 and *E. coli* JM109 to estimate the location of the PPT-resistant gene.

**Enzyme assay.** Assays for N-acetyltransferase activity were performed by spectrophotometry at 412 nm, followed by the formation of 2-thio-2-nitrobenzoic acid, a yellow-colored product, produced by the reaction of 2-nitrobenzoic acid (0.2 mM) in a volume of 0.25 ml. Absorbance changes were measured using a BioSpec-1600 spectrophotometer (Shimadzu, Kyoto, Japan).

**Construction of a gene encoding plastid- and cytosol-targeting MAT protein.** The translation initiation codon of the MAT gene in *Nocardioides* sp. strain AB2253 is GTG. To guarantee appropriate translation in a plant, an ATG initiation codon is required. Therefore, the translation initiation codon of the MAT protein.

**Plant transformation.** The constructs pYT105-MAT and pYT105-cTP-MAT were introduced into *A. tumefaciens* strain C58C1 by the freeze-thaw method,23 and transformation of *A. thaliana* (Columbia) by the resulting bacteria was performed by *in planta* infiltration.24 Transformed seedlings (*T₂*) were selected on solid MS medium (Murashige and Skoog plant salt mixture, Gamborg's B5 vitamin salt mixture, 2,4-dichlorophenoxyacetic acid (2 mg/l), casamino acid (1 g/l), and 0.2% (w/v) Gelrite (Wako, Osaka, Japan). For elicitor treatment, calli were subcultured in an R2 liquid medium26 as described previously.27 Constructs pUB-Hm-MAT and pUB-Hm-cTP-MAT were introduced into A. tumefaciens strain EHA101 by the freeze-thaw method,23 and transformation of seed calli of rice by the resulting bacteria was performed as described previously.27 Selection and plant regeneration were performed as described previously.23

**Northern and Southern blot analyses.** For RNA analysis, total RNA was extracted from homozygous *Arabidopsis* transformant seedlings (T₃) using an RNEasy Plant Mini Kit (Qiagen, Hilden, Germany), and 5 μg of the RNA was subjected to RNA gel-blot hybridization. A digoxigenin-labeled antisense RNA probe for the detection of *MAT* transcripts was prepared using a DIG RNA labeling kit (Roche, Mannheim, Germany). A 300-bp fragment of the *MAT* ORF (nucleotides 10 to 309) was amplified by PCR with primers MAT3 (5'-CATCGGCGACCTCTATTG-3') and MAT4 (5'-TAAATCACTCTCTATAGCGGACACCGCCGGCATGAAAT-3'), T7 promoter sequence underlined). The antisense RNA probe was synthesized from the fragment amplified as above by *in vitro* run-off transcription with T7 RNA polymerase in the presence of digoxigenin-labeled UTP. RNA gel-blot hybridization was performed as described previously.28
For Southern hybridization, genomic DNA was extracted from rice plants by the cetritrimethylammonium bromide method. Then 2.5 μg of genomic DNA was digested with SacI, fractionated by electrophoresis through TAE agarose gel, and transferred to a Hybond-N+ membrane, which was then subjected to hybridization. A DNA probe was synthesized by labeling with [α-32P]dCTP by the random primer method (Takara, Tokyo) with primers MAT5 (5’-CCCGATACGCTCCGGCGCCGCCGATCCG-3’) and pUB-Hm-RB (5’-ACGGATGGGAACCTGAGGACGC-3’).

Herbicide resistance test. Wild-type and transgenic Arabidopsis lines were grown for 3 weeks on an MS medium agar plate supplemented with a range of concentrations of PPT (0, 50, 100, 150, and 200 mg/l) to confirm their PPT resistance. Pieces of leaves (1 cm × 1 cm) of wild-type and transgenic rice plants were cultured for 1 week on MS medium at one-third strength containing 3% sucrose and 0.3% Gelrite supplemented with a range of concentrations of PPT (0.0, 0.5, 0.75, and 1.0 g/l) at 26 °C under a 16-h light/8-h dark cycle using artificial light (50–60 μmol m−2 s−1) was used to determine their PPT resistance. The herbicide resistance of the transgenic rice plants was also evaluated under glasshouse conditions, and was compared with the wild-type rice plant. Two-month-old transgenic and wild-type rice plants were sprayed with a 3-fold recommended field dosage of the commercial herbicide Basta, equivalent to 2.8 mg/l PPT, and the phenotype was scored 2 weeks later.

Results

Cloning of a novel phosphinothricin N-acetyltransferase gene

Five hundred different soil bacteria strains, collected from various places in Japan, were screened for bialaphos resistance. One bialaphos-resistant strain was isolated, and was identified as the Nocardioid sp. strain AB2253 after precise taxonomical analysis. A novel gene, which encodes a PPT-acetyltransferase, was isolated from Nocardioid sp. strain AB2253 by expression screening of a plasmid library constructed in Streptomyces lividans. The nucleotide sequence of the gene revealed an open reading frame (ORF) of 534 nucleotides starting with an initiation codon GTG and ending with a termination codon TGA, which encoded a protein of 177 amino acids with a calculated molecular mass of 19.6 kDa (GenBank accession no. E02494).

The gene was subcloned into pUC18 plasmid, and the plasmid was introduced in E. coli strain JM109. The expressed protein was found to be able to utilize not only PPT but also methionine sulfone, another inhibitor of glutamine synthetase. The Km values of MAT were 14.31 and 2.2 mM for methionine sulfone and PPT, respectively. We therefore designated the gene MAT (methionine sulfone N-acetyltransferase). MAT catalytic activity reached a maximum at pH 8 to 8.5 and decreased to 15% of the maximum value at pH 6.5 (Fig. 2), indicating that MAT activity exhibited a pronounced pH-dependence with a pH optimum of approximately 8.5.

The MAT protein was found to have 32.1%, 33.3%, and 29% homology at the amino acid sequence level with known PPT-N-acetyltransferases, BAR from S. hygroscopicus, PAT from S. viridochromogenes, and ScPAT from Streptomyces coelicolor A3(31) respectively. Although the homology among these gene products was low, 10 putative acetyl-CoA binding domain residues were conserved in all four proteins (Fig. 3).

Import of the chimeric MAT protein into chloroplasts

To target the MAT protein to chloroplasts in plants, we generated a chimeric MAT protein with an N-terminal chloroplast transit peptide (cTP, 58 amino acid residues) derived from the tobacco ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit (RbcS). To determine whether the fused cTP gene was functional, we performed an in vitro protein import assay with freshly prepared pea chloroplasts using chimeric cTP:MAT and MAT proteins labeled with [35S]methionine and [35S]cysteine. The labeled proteins were incubated with intact chloroplasts, after which the reaction mixture was treated with the protease thermolysin to digest unimported protein. Analysis by SDS–PAGE and autoradiography of lysed chloroplasts revealed that the cTP:MAT (25.3 kDa) chimeric protein had been proteolytically processed to a product of 19.6 kDa that was identical to the MAT protein (Fig. 4). Subsequent treatment with thermolysin resulted in digestion of the fused protein but not of the processed product, indicating that the latter had been translocated into the chloroplasts. In contrast, a control MAT protein was digested entirely by thermolysin. These results support the notion that the chimeric cTP:MAT protein is targeted to chloroplasts and is subsequently processed to MAT protein on translocation into these organelles.

Introduction of the chimeric MAT gene into plants

To express the MAT and cTP:MAT proteins in Arabidopsis thaliana, MAT and cTP were cloned into binary T-vector pYT105 (pYT105-MAT and pYT105-cTP:MAT) (Fig. 5A), and the constructs were introduced separately into Agrobacterium tumefaciens strain C58C1. Transformation of A. thaliana (Columbia) by the resulting bacteria was performed by in planta infiltration. Kanamycin-resistant transformed plants (T1) (five each) were selected as the putative transgenic plants. Finally, we obtained two independent homozygous T3 plants.
MAT and cTP:MAT were also introduced individually into the T-vector (pUB-Hm-MAT and pUB-Hm-cTP:MAT) under the control of the maize Ubi1 promoter for high-level expression in rice (Oryza sativa cv. Nipponbare) (Fig. 5C). Transformed calli and regenerated plants were established by infection with Agrobacterium tumefaciens strain EHA101 harboring each construct. From the hygromycin-resistance selection, many independent transformants and transgenic plants were generated.

**Analysis of transgenic plants**

We obtained two independent homozygous Arabidopsis lines from the MAT and cTP:MAT transgenic plants (T₃). The accumulation of MAT and cTP:MAT transcripts in the seedlings of these lines was demonstrated by RNA gel-blot hybridization, their abundance being almost equal in all lines (Fig. 5B). cTP:MAT transcripts (1.1 kb) were longer than those of MAT (0.9 kb). This indicates that the fused gene, cTP:MAT, was successfully transcribed in plants. We detected no marked differences in morphology or growth rate among these four homozygous lines and wild-type plants (data not shown).

We also generated many independent transformants and transgenic rice plants by selection with regard to hygromycin resistance. We then assayed for import into plastids by incubation with intact pea chloroplasts in vitro. The reaction mixtures were subsequently treated (or not) with thermolysin before analysis by SDS–PAGE and autoradiography. The input of MAT and cTP:MAT protein into the assay is shown in the first and fourth lanes. The positions and sizes of the cTP:MAT and MAT proteins are indicated on the right.

**Herbicide resistance of transgenic plants**

The herbicide (PPT) tolerance of transgenic Arabidopsis lines was evaluated. We used seeds of T₃ homozygous transformant lines from MAT-32 and cTP:MAT-74. Wild-type and transgenic seeds (12 each) were germinated and grown on an MS medium agar plate containing various concentrations of PPT (0, 50, 100, 150, and 200 mg/l) for 3 weeks. Germination of wild-type seeds was not observed at any concentration of PPT. On the other hand, both MAT and cTP:MAT transgenic seeds germinated under all the tested conditions. However, after germination, the MAT transgenic seedlings showed growth retardation compared with the cTP:MAT transgenic seedlings in a medium containing 200 mg/l PPT (Fig. 6A), and the MAT and cTP:MAT transgenic seeds did not germinate in a medium containing PPT, at more than 500 mg/l (data not shown).
Fig. 5. Plant Expression Vector Construction and Expressed Gene Analysis of Transformed Plants.

A. Structure of the T-region of the constructs pYT105-MAT and pYT105-cTP:MAT. RB and LB, right and left borders, respectively; P35S, 35S promoter of cauliflower mosaic virus; Prom and Ter, promoter and terminator of the nopaline synthase gene, respectively; NPTII, neomycin and kanamycin resistance gene. B, RNA gel-blot hybridization analysis of MAT and cTP:MAT mRNA in transgenic Arabidopsis lines. Total RNA (5 μg) from wild-type and transgenic (T1) seedlings was subjected to electrophoresis on a 1% agarose gel under denaturing conditions, and the separated molecules were transferred to a nylon membrane and subjected to hybridization with a digoxigenin-labeled probe for MAT mRNA. The 0.9-kb MAT and 1.1-kb cTP:MAT transcripts are indicated by arrowheads. The membrane was also stained with methylene blue for visualization of 25S and 17S RNA (arrowheads). C, Structure of the T-regions of the binary vectors pUB-Hm-MAT and pUB-Hm-cTP:MAT. Pubi, promoter of maize ubiquitin 1 gene; HTP, hygromycin phosphotransferase gene. D, Determination of copy numbers of MAT and cTP:MAT in transgenic rice by Southern hybridization. Genomic DNA (2 μg) was prepared from wild-type or transgenic rice leaves, and was analyzed by Southern blot hybridization after digestion with Sacl using a 32P-labeled MAT probe.

Two lines of transgenic rice, MAT-4 and cTP:MAT-105, were also evaluated for herbicide tolerance. Leaves (1 cm × 1 cm pieces) from wild-type and transgenic rice plants were cultured on MS medium at one-third strength, containing various concentrations of PPT (0, 0.5, 0.75, and 1.0 g/l), for 1 week. Among the leaves from the cTP:MAT transgenic rice, there was no significant difference in the tested concentrations of PPT. However, leaves from the wild-type and the MAT transgenic rice turned pale green at all tested concentrations of PPT (Fig. 6B). These results indicate that the chloroplast-localizing form of MAT protein detoxified PPT more efficiently than the cytosol-localizing form in the transgenic plants.

Transgenic rice plants regenerated from calli were transferred to soil in the greenhouse. Herbicide resistance was tested by spraying the transgenic and wild-type plants with Basta. The wild-type rice plants turned brown and died within 2 weeks of herbicide treatment. In contrast, the MAT and cTP:MAT transgenic plants exhibited tolerance to Basta, with normal subsequent growth and development (Fig. 6C). These results indicate that MAT is useful for the creation of herbicide-tolerant rice plants.

The MAT gene functions as selectable marker in Arabidopsis

To investigate the usefulness of the MAT gene as a selectable marker, constructs pYT105-MAT and pYT105-cTP:MAT (Fig. 5A) were used for Arabidopsis transformation, and their T1 seeds were primarily selected by PPT instead of kanamycin. Wild-type and T1 seeds were germinated in medium containing 50 mg/l PPT for 1 week, and the surviving plants were selected as putative transformants (Fig. 7). In the selected transformants, the introduction of the MAT or cTP:MAT genes was confirmed by PCR amplification using their genomic DNA (data not shown). The efficiency of the selection of transformants by a PPT-containing medium was similar to that by a kanamycin-containing medium (data not shown). These results indicate that the MAT gene is useful as a selectable marker in A. thaliana.

Discussion

In the present study, we identified a novel bacterial N-acetyltransferase (MAT) from a Nocardia sp. strain. The MAT sequence showed a low homology with the major PPT N-acetyltransferase BAR and PAT. There is another PPT N-acetyltransferase (ScPAT) from S. coelicolor A3(31) that showed low homology with BAR and PAT (about 30%); however, MAT also showed low homology (29%) with ScPAT (Fig. 3). This suggests that MAT may be categorized as a different type of PPT acetyltransferase to those from Streptomyces sp.

Glutamine synthetase (GS), the target of the herbicide bialaphos, localizes in the chloroplast and cytosol. The chloroplast stroma changes pH depending on light conditions. In illuminated conditions, the pH of the stroma increases up to 8 because of proton flux from the inside of the thylacoid membrane, which is due to electron transfer from water molecules in the oxygen-evolving complex, and is used for driving ATP-synthase. In fact, RbcS enzymes from tobacco have a narrow optimum pH range of 8.4 to 8.6 regulating carbon fixation in harmony with prevailing light conditions. As shown in Fig. 2, the optimum pH of the MAT was around 8.0 to 8.5, suggesting that the MAT is able to function fully in the stroma under light illumination. We therefore attempted to express MAT in...
the chloroplasts to increase herbicide tolerance in transgenic plants. The generation of herbicide-resistant plants by direct transformation to plastomes has been shown to be feasible, but the application of transformation methodology for engineering the plastid genome is still limited to model dicotyledonous plants. This technology is so far hard to apply to crop plants because many of them are regenerated from non-green embryonic cells rather than leaf cells.

In contrast, the targeting ability of the chloroplast transit peptide of tobacco RbcS-fused foreign protein to the chloroplast has been shown in vivo, and this method is applicable to all plant species that have an established transformation method. Our in vitro chloroplast import assay using cTP:MAT chimeric protein also clearly demonstrated that cTP:MAT targets the chloroplasts (Fig. 4).

To confirm and compare the herbicide resistance of transgenic plants that expressed MAT in the cytosol and chloroplasts, we introduced expression vectors pYT105-MAT and pYT105-cTP:MAT into Arabidopsis, and pUB-Hm-MAT and pUB-Hm-cTP:MAT into rice by an Agrobacterium-mediated method. The ability of herbicide resistance of the transgenic Arabidopsis and rice was evaluated using various concentrations of PPT. Supporting our hypothesis, the transgenic plants harboring the chloroplast-localizing form of MAT grew at higher concentrations of PPT than that harboring the MAT in the cytosol.
cytosol-localizing form of MAT (Fig. 6). Especially, the difference was more significant when pieces of leaves from transgenic rice plants were used in the evaluation of herbicide-resistance in the plants. This evaluation was carried out by monitoring senescence due to the loss of photosynthetic activity in the chloroplasts. Therefore, this result strongly suggests that PPT inhibits the activity of GS2, and that the chloroplast-localizing form of MAT is more effective for detoxifying PPT in the chloroplasts. We confirmed that the pH range of BAR is approximately 6.5 (data not shown), and it has been demonstrated that BAR works well in the cytosol.5.7 Moreover, the bar gene has been utilized in plastid gene transformation,35–37 indicating that BAR functions as PPT N-acetyl transferase in plastids as well. It is probable that BAR mainly functions in the dark cycle in contrast to MAT, which is activated in the light cycle. To clarify the limiting factor of MAT function in the cytosol, we may also need to consider the protein stability of MAT. There is no information regarding the turnover of the MAT or the BAR protein in cytosol and plastids.

The PPT N-acetyltransferase proteins BAR and PAT are widely used as selectable markers in plant transformation.6.11 Combined with the results obtained from the analysis of Arabidopsis transformants that expressed the MAT gene and acquired PPT resistance, we also confirmed the utility of the MAT gene as a selectable marker in Arabidopsis transformation (Fig. 7). Although we did not test the primary selection of MAT-transformed rice calli by PPT in this study, transformed calli, which were primarily selected by hygromycin resistance test, and wild-type calli were compared in the 10 mg/l PPT-containing medium. The wild-type calli did not grow in the PPT-containing medium, but transformants grew (data not shown). This indicates that the MAT gene might be useful for screening transformed rice calli and as a selectable marker. Further investigations are in progress to confirm the utility of the MAT gene as a selectable marker in the rice transformation system.

In conclusion, we identified a novel bacterial N-acetyltransferase, MAT, which can detoxify the herbicide PPT. MAT-expressing transgenic plants acquired herbicide resistance. Chloroplast expression of MAT resulted in higher herbicide resistance than expression in the cytosol. The MAT gene also showed potential for use as a selectable marker in the transformation of plants.

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References