Generation and characterization of herbicide-resistant soybean plants expressing novel phosphinothricin N-acetyltransferase genes

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Herbicide resistance genes have been widely used in plant biotechnology both for commercial applications and as selectable markers. Phosphinothricin N-acetyltransferase (PAT) detoxifies the nonselective herbicide phosphinothricin (PPT, the ammonium salt of which is known as glufosinate), which is the active ingredient in bialaphos. Two novel PAT genes, hpat and mat, have been isolated from PPT-resistant soil bacteria, and we have now introduced these genes under the control of the 35S promoter of cauliflower mosaic virus into soybean [Glycine max (L.) Merrill] by particle bombardment and subsequent hygromycin selection. The expression of each enzyme was confirmed in leaves of the transgenic soybean plants by immunoblot analysis with specific antibodies and by measurement of PAT activity. The transgenic plants showed complete resistance to a commercial formulation of PPT at the recommended concentration. Our results suggest that these PAT genes may prove useful as markers for soybean transformation as well as in the production of herbicide-resistant transgenic plants.

Key Words: Basta, glufosinate, herbicide resistance, particle bombardment, phosphinothricin N-acetyltransferase, soybean, transformation.

Introduction

Soybean is one of the most important summer crops and is widely used as a staple for human food, animal feed, and industrial products worldwide. Control of weeds that compete with soybean plants for water, nutrients, and sunlight is important for maximization of the yield of high-quality soybeans, especially under minimal tillage or no-till farming conditions (Kapusta 1979). For effective weed control, herbicides are selected on the basis of several factors including weed spectrum, lack of crop injury, and cost. Few herbicides that are currently available satisfactorily meet all such requirements. This problem has been addressed by the generation of herbicide-resistant transgenic soybean lines, which have been adopted widely during the past dozen years. In 2007, herbicide-tolerant transgenic soybean lines accounted for 64% of the global crop acreage of soybean (James 2007). Multiple applications of glyphosate over the years, however, have been associated with the development of glyphosate resistance in weeds (Powles 2008).

Phosphinothricin [N,N-homoalanine-4-yl-(methyl)-phosphonic acid, or PPT], the ammonium salt of which is known as glufosinate, is the herbicidal component of bialaphos, a tripeptide antibiotic produced by the soil bacterium, Streptomyces hygroscopicus, and consisting of PPT and two L-alanine residues (Seto et al. 1982). PPT is an analog of L-glutamic acid and a potent inhibitor of glutamine synthetase, an enzyme that plays a central role in the assimilation of ammonia and in the regulation of nitrogen metabolism in plants (Miflin and Lea 1977, Skokut et al. 1978). Inhibition of glutamine synthetase results in the rapid accumulation of ammonia in plant cells and their consequent death (Tachibana et al. 1986). Intracellular peptidases of plants remove the alanine residues of bialaphos and release the active PPT. A resistance gene, bar (bialaphos resistance gene), has been isolated from a bialaphos biosynthetic gene cluster of S. hygroscopicus (Thompson et al. 1987), and a similar gene, designated pat (phosphinothricin acetyltransferase gene), has been isolated from Streptomyces viridochromogenes (Wohlleben et al. 1988). Glufosinate-resistant transgenic plants have been generated by the introduction of either bar or pat (Block et al. 1987, Thompson et al. 1987, Strauch et al. 1988). Both these genes have also been widely used in plant genetic engineering as selectable markers (Cao et al. 1987).
Cetyltmycin phosphotransferase (hpt) enzymes in the transgenic soybean plants was confirmed by no. DD355820, which were isolated from different bacterial strains resistant to bialaphos. Expression of active PAT enzymes in the transgenic soybean plants was confirmed by immunoblot analysis with specific antibodies and by spectrophotometric measurement of PAT activity. The transgenic plants were resistant to the herbicide Basta, a commercial formulation of PPT, at the recommended application concentration of the herbicide resistance trait was shown to be inherited together with the transgene by their progeny.

Materials and Methods

Plasmid construction and generation of transgenic plants

The bacterial N-acetyltransferase genes hpat and mat, which were isolated from the bialaphos-resistant soil bacteria Streptomyces sp. strain AB3534 and Nocardia sp. strain AB2253, respectively, were used to confer herbicide resistance in soybean. Each gene was inserted between the 35S promoter of cauliflower mosaic virus (CaMV) and the 3′ terminator sequence of the nopaline synthase gene (nos) in pBI221 (Clontech, Mountain View, CA) to generate an expression cassette. The expression cassette was digested with EcoRI and HindIII, and the released fragment was inserted into the same restriction sites of pUHG(SK) (Khalafalla et al. 2005) between two plant marker genes, those for hygromycin phosphotransferase (hpt) and for a modified green fluorescent protein [SGFP(S65T)]. The resulting plasmids, designated pUHG:hpat and pUHG:mat (Fig. 1), were amplified in Escherichia coli DH5α and subsequently harvested with the use of a Plasmid Midi Kit (Qiagen, Valencia, CA). Transformation of soybean with the purified plasmids by particle bombardment and subsequent plant regeneration were performed as described previously (Khalafalla et al. 2005).

PCR and Southern blot analyses of transgenic plants

Total DNA was extracted with an automatic DNA isolation system (PI-50x; Kurabo Industries, Osaka, Japan) and was subjected to polymerase chain reaction (PCR) analysis in order to examine the presence of transgenes in regenerated plants and segregating progeny. PCR was performed in a 50-μl reaction mixture containing 10 ng of total DNA, 0.2 μM of each deoxynucleoside triphosphate, 0.2 μM of each primer, and 2.5 U of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) in polymerase buffer. The incubation protocol comprised an initial denaturation step of 10 min at 95°C; 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C; and a final elongation step of 7 min at 72°C. The primers included hpat-F (5′-GGTGGAGCCGCGATCACATT-3′) and hpat-R (5′-GGGACCTCCTTCTCGTACC-3′) for amplification of a 431-bp fragment of hpat, as well as mat-F (5′-GAGATCCTGTAGTACCAAC-3′) and mat-R (5′-GAAGGTCCGTTCATGAGC-3′) for amplification of a 450-bp fragment of mat.

Southern blot analysis was performed to confirm the stable integration of hpat or mat. Total DNA (10 μg) isolated from young leaves of T1 plants by the CTAB method (Murray and Thompson 1980) was digested overnight with HindIII, and the resulting fragments were fractionated by electrophoresis through a 1% agarose gel and then transferred to a Hybond N+ membrane (GE Healthcare Bio-Science, Piscataway, NJ). Fragments of hpat or mat amplified from pUHG:hpat or pUHG:mat by PCR with the above primers served as hybridization probes. Each probe was labeled and detected with the use of an ECL kit (GE Healthcare Bio-Science). The blots were exposed to x-ray film for more than 1 h.

Detection of HPT or MAT protein by immunoblot analysis

To detect HPAT and MAT proteins, we prepared specific antibodies by injecting rabbits with corresponding synthetic peptides (see Fig. 3). A crude protein fraction was extracted from powdered fresh leaves cooled with liquid nitrogen by grinding in a solution containing 50 mM Tris-HCl (pH 8.0), 0.2% SDS, 5 M urea, 2% 2-mercaptoethanol, and 2% (v/v) plant protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) in the proportion of 1 g of leaves to 1 ml of solution. The homogenate was mixed well, boiled for 5 min, and centrifuged at 13,000 × g for 10 min at 4°C. The protein concentration of the supernatant was determined with the use of an RC/DC protein assay kit (Bio-Rad, Richmond, CA) and with bovine serum albumin as a standard. The extract (20 μg of protein) was subjected to SDS-polyacrylamide gel electrophoresis on a gel containing 12% acrylamide and 0.2% bisacrylamide. The separated proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (GE Healthcare Bio-Science), which was then exposed to rabbit polyclonal antibodies to HPAT or to MAT at a dilution of 1:6000. Immune complexes were detected with the use of an ECL Advance Western Blotting Detection Kit (GE Healthcare Bio-Science).

Spectrophotometric assay of N-acetyltransferase activity

PAT enzymatic activity was measured as described previously (Block et al. 1987) with slight modifications. Fresh leaves (1 g) were homogenized in 1 ml of ice-cold extraction buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, and 2% plant protease inhibitor cocktail. The homogenate was centrifuged at 13,000 × g for 10 min at 4°C, and the resulting supernatant was applied to a PD-10 column (GE Healthcare Bio-Science) that had been equilibrated with 25 ml of extraction buffer. A crude enzyme extract was eluted from the column with 3.5 ml of extraction buffer. The protein concentration of the extract was determined with an RC/DC protein assay kit and was adjusted to 1 mg/ml with extraction buffer. PAT activity was assayed at 30°C in the
well of a 96-well microtiter plate (light path of 1 cm). The 350-μl reaction mixture contained 50 μl of the crude enzyme preparation, 100 mM Tris-HCl (pH 8.0), 0.1 mM acetyl-CoA, and 5,5′-dithiobis-2-nitrobenzoic acid (0.4 mg/ml), and the reaction was initiated by the addition of PPT to a final concentration of 0.1 mM. The increase in absorbance at 412 nm per minute was divided by 13.6 to give activity values expressed in micromoles of PPT acetylated per min at 30°C, the activity values are also equivalent to the number of units of enzyme in the reaction mixture.

Evaluation of herbicide resistance

The effects of Basta (Bayer CropScience, Monheim, Germany) were assessed by application of a 0.5% Basta solution [equivalent to a glufosinate (PPT, ammonium salt) concentration of 0.9 mg/ml] to the entire plant body or to the upper surface of young leaves. SPAD values of transgenic plants and nontransgenic plants were measured with a SPAD-502 chlorophyll meter (Minolta Camera, Osaka, Japan), which determines the relative content of chlorophyll (Markwell et al. 1995).

Statistical analysis

Data are presented as means±SE, as indicated, and were subjected to analysis of variance followed by the Tukey-Kramer honestly significant difference (HSD) test for pairwise comparisons with the use of JMP 7 software (SAS Institute, Cary, NC). The significance of differences between the observed and best-fitted segregation ratios was evaluated by the chi-square test. A P value of <0.05 was considered statistically significant.

Results

Generation of transgenic soybean lines harboring hpat or mat

We introduced the plasmid vectors pUHG:hpat or pUHG:mat (Fig. 1) into somatic embryos of soybean cultivar Jack by particle bombardment. From six culture dishes treated with pUHG:hpat, we obtained 57 green clumps of somatic embryos that exhibited green fluorescence (excitation at 455 to 490 nm, emission at >515 nm) of the sGFP(S65T) marker protein after selection for 5 weeks with hygromycin. Two independent transgenic lines, h-7 and h-9, were obtained through regeneration from the transgenic somatic embryos. Only one transgenic line (m-12) was recovered from six culture dishes treated with pUHG:mat, even though 95 green clumps of somatic embryos that exhibited green fluorescence were selected in the presence of hygromycin. All three R₀ plants appeared to grow normally and produced viable seeds in a greenhouse. Segregation of the transgene (hpat or mat) in the R₁ generation was confirmed by PCR analysis. The segregation ratios for R₁ seeds of h-9 [(19(+)) : 5(−), χ² = 0.22, P = 0.64] and m-12 [23(+) : 11(−), χ² = 0.98, P = 0.32] fitted well to 3(+) : 1(−), where + and − indicate the presence and absence of the transgene, respectively. In contrast, the R₀ plant of h-7 produced 49 seeds, the segregation ratio for which, 47(+):2(−), fitted well to 15(+):1(−) (χ² = 0.39, P = 0.53) for transgene insertion at two different loci.

Southern blot analysis was performed to assess the stable integration of the hpat or mat genes in the putative T₁ plants that gave a positive transgene signal by PCR analysis. Total DNA was digested with HindIII, which cuts each plasmid vector at only one position (Fig. 1). The resulting DNA fragments were subjected to hybridization with probes specific for hpat (h-7, h-9) or for mat (m-12). Each transgenic plant yielded a different banding pattern with more than two bands corresponding to each PAT gene (Fig. 2).

Detection of HPAT or MAT protein and enzymatic activity in transgenic plants

To assess the stable expression of the transgenes at the protein level, we first examined the abundance of HPAT or MAT protein in leaves of the transgenic plants by immunoblot analysis. Although HPAT and MAT belong to the same family of phosphinothricin N-acetyltransferases as do the bar and pat products, their amino acid sequences differ.

Fig. 1. Schematic representations of the plasmid vectors, pUHG:hpat and pUHG:mat, used for soybean transformation. The expression cassette of each PAT gene (hpat or mat) was immobilized in pUHG(SK). P35S, 35S promoter of CaMV; hpt, hygromycin phosphotransferase gene; Tnos, terminator of the nopaline synthase gene; sGFP, modified green fluorescent protein gene. Each plasmid is digested by HindIII at only one position.
leafflet of nontransgenic or T1 (h-7, h-9, and m-12) plants was painted with 0.5% Basta solution, a concentration recommended for foliar application in the field (glufosinate concentration of 0.9 mg/ml). The control plants showed an ~40% decrease in SPAD value after 4 days (Table 1), and the treated leaflets were totally blighted within 7 days of Basta application (Fig. 5A). All the transgenic plants were fully resistant to the Basta application: The painted leaflets did not show a significant change in SPAD value (Table 1) and they remained as green as those painted with water (Fig. 5A).

We confirmed the stability of herbicide resistance in the T2 generation. The growth of T2 plants harboring hpat or mat in the greenhouse appeared indistinguishable from that of nontransgenic control plants. Application of 0.5% Basta to the plant body killed control plants within 2 weeks but had no apparent effects on the T2 plants (Fig. 5B). Indeed, the transgenic plants that had been sprayed went on to flower normally and to set seeds.

Discussion

We have assessed the herbicide resistance of transgenic soybean plants expressing the novel PAT genes hpat or mat. The well-characterized PAT genes bar and pat encode proteins of 183 amino acids that share an overall sequence identity of 84.7% (Wohlleben et al. 1988). In contrast, the proteins encoded by the novel PAT genes hpat and mat contain 174 and 177 amino acids, respectively, and show only 31.6 and 31.1% sequence identity, respectively, with BAR (Fig. 3B). The primary structure of HPAT resembles that of ScPAT of Streptomyces coelicolor A3 more than that of BAR and PAT (Bedford et al. 1991), whereas MAT shares low homology with PPT N-acetyltransferase proteins previously reported. All five proteins, however, conserved ten putative domain residues for acetyl-CoA binding (Dyda et al. 2000) (Fig. 3A). In addition, the recombinant MAT protein expressed in E. coli strain JM109 exhibited acetylation activity toward PPT (Yun et al. 2009). HPAT and MAT have thus been designated members of the phosphinothricin N-acetyltransferase family.

We introduced the two PAT genes into soybean without any modification for targeting to the chloroplasts as well as for plant codon usage (Yun et al. 2009). Nevertheless, two independent transgenic lines for hpat (h-7 and h-9) and one for mat (m-12) expressed the active PAT proteins at a level sufficient to confer resistance to glufosinate (PPT, ammonium salt). The constitutive expression of the PAT genes driven by the 35S promoter of CaMV did not appear to have any adverse effects on plant growth, and the herbicide resistance trait was inherited by the next generation together with the transgene. PPT or its ammonium salt (glufosinate) are the active ingredients of several commercial preparations including Basta and Liberty, which are used widely as nonselective herbicides. Utilization of transgenic plants resistant to PPT is one of the reliable solutions to the problem of weed damage in soybean production. Safety assessments indicate that the
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PAT enzyme is highly substrate specific and does not possess characteristics associated with food toxins or allergens (Hérouet et al. 2005). Similar evaluation of the safety of plants expressing HPAT or MAT for food, feed and biodiversity will be required before their practical application.

The herbicide-resistant transgenic soybean lines of the present study were generated by particle bombardment-mediated transformation of embryogenic tissue and subsequent hygromycin selection. Although soybean transformants are routinely produced by this method, the competence of somatic embryogenesis is a critical factor and is dependent on soybean genotype (Kita et al. 2007). The application of this transformation method has thus been limited to a few soybean varieties. Furthermore, the procedure tends to result in unexpected transgene silencing due to the complexity of transgene integration into the plant genome (El-Shemy et al. 2004). Improvements in Agrobacterium-mediated transformation of cotyledonal node tissue have been pursued to increase the efficiency of this procedure since its first description by Hinchee et al. (1988). The addition of antioxidant reagents such as L-cysteine and dithiothreitol has thus been shown to increase substantially the efficiency of

Fig. 3. Comparison of the amino acid sequences of the phosphinothricin acetyltransferases HPAT (DD555820), MAT (AB378291), BAR (X05822), PAT (M22827) and ScPAT (NP_627417). (A) Alignment of the primary structures of the five PAT proteins. The underlined sequences correspond to the synthetic peptides injected into rabbits for the production of antibodies specific for HPAT or for MAT. Dashes (−) represent gaps introduced to optimize alignment. Asterisks (*) indicate amino acids conserved among the five enzymes. Outline characters of amino acid residues indicate putative acetyl-CoA binding domain. (B) Phylogenetic relations among the four PAT proteins. The cluster dendrogram was generated by the unweighted pair-group method with the arithmetic mean (UPGMA).

Fig. 4. Detection of HPAT and MAT proteins by immunoblot analysis of leaf extracts from T₁ plants. Crude leaf extracts (20 μg of protein) were subjected to immunoblot analysis with antibodies specific for HPAT or for MAT, as indicated. Lanes 1 and 2 in the left panel correspond to T₁ plants transformed with pUHG:hpat (h-7 and h-9, respectively). Lane 1 in the right panel corresponds to a T₁ plant transformed with pUHG:mat (m-12). Lanes C in both panels correspond to non-transgenic control plants. The positions of molecular size markers are indicated on the left.

PAT enzyme is highly substrate specific and does not possess characteristics associated with food toxins or allergens (Hérouet et al. 2005). Similar evaluation of the safety of
transformation by this approach (Olhoft and Somers 2001, Olhoft et al. 2003). The bar gene has been widely applied as a selective marker in combination with glufosinate for the transformation of soybean cotyledonary node tissue (Zhang et al. 1999, Paz et al. 2004, Sato et al. 2004, Zeng et al. 2004, Paz et al. 2006). Yun et al. (2009) demonstrated the utility of the mat gene as a selective marker in Arabidopsis transformation and the PPT resistance in transformed rice calli harboring mat. The two novel PPT N-acetyltransferase genes, hpat and mat, therefore may be selective markers in soybean transformation under the control of appropriate promoters. We are currently confirming the application of hpat and mat as selective markers for Agrobacterium-mediated transformation in soybean.

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**Literature Cited**

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