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Enhancement of metabolizing herbicides in young tubers of transgenic potato plants with the rat *CYP1A1* gene

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Abstract A rat P450 monooxygenase gene (*CYP1A1*) was introduced into potato plants to enhance the metabolism of the environmental contaminants in subterranean organs. The CYP1A1 gene was kept under the control of the potato patatin promoter to enhance tuber-specific expression. A total of 106 transgenic plants (PAT1A1 plants) were obtained following selection by a resistance test to kanamycin and PCR analysis. PAT1A1 plants treated with 10% exogenous sucrose showed a higher activity of monooxgenase in the leaves than the non-transgenic plants. This indicated that the activity enhanced by 10% sucrose was due to the patatin promoter containing the sucrose-inducted elements. One representative transgenic plant, Ag2197, was selected on the basis of monooxgenase activity in the leaves and Western blot analysis. Ag2197 was found to accumulate a large amount of CYP1A1 mRNA and protein in the developing tuber but not in the mature tuber. The residual herbicides, atrazine and chlortoluron, were analyzed in the micro-tubers of Ag2197 and non-transgenic plants. The amount of residual herbicides in Ag2197 was much lower than that in the non-transgenic plant, indicating that the transgenic plant metabolized the herbicides to a detoxified form. The transgenic plants produced in this study might be useful for the phytoremediation of chemical pollution in the soil.

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Introduction

Several cytochrome P450 monooxygenases in the microsomes of mammalian liver metabolize the xenobiotics to less toxic derivatives. These P450 species show a broad and overlapping substrate specificity and also metabolize phytotoxic chemicals such as herbicides in transgenic plants expressing the P450 genes. Transgenic plants harboring these P450 genes have been shown to enhance the ability of metabolizing the herbicides to detoxified compounds and have a high level of tolerance toward the herbicides (Shiota et al. 1994; Inui et al. 1998, 2000). The incorporation of the ability of mammalian P450-dependent detoxification into the plants would enable the production of herbicide-resistant crops, and the transgenic plants also might enhance the metabolism of various environmental contaminants (Ohkawa et al. 1998). Doty et al. (2000) demonstrated that transgenic tobacco harboring a P450 species *CYP2E1* gene derived from mammals showed a dramatic enhancement in the metabolism of halogenated hydrocarbons. In the transgenic plants, the integrated genes were usually kept under the control of strong and universal promoters, such as the cauliflower mosaic virus 35S (CaMV 35S) promoter. However, because most of the environmental contaminants are in the soil, it would be better that the introduced P450 genes be mainly expressed in the tissue under the ground. Therefore, it is necessary to develop a specific useful promoter that transcribes the transgenes in the roots or subterranean stem or tuber organs.

Patatin is a family of glycoproteins and major soluble proteins in potato tubers (Racusen and Foote 1980; Park et al. 1983) that is encoded by two classes of genes, class I and class II (Mignery et al. 1988). A promoter of class-I patatin is specifically activated in the tuberized stolon and the developing tuber (Pickard et al. 1987; Rocha-Sosa et al. 1989; Wenzler et al. 1989). Therefore,

the promoter of class-I patatin gene is expected to control the expression of transgenes specifically in the developing tuber (Park et al. 1988). The improvement of tuber contents and resistance against soft-rot have been reported to result from the expression of exogenous genes under control of the class-I patatin promoter (Oakes et al. 1991; Shewmaker et al. 1994; Wegener et al. 1996).

In the study reported here, we attempted to integrate a rat cytochrome P450 gene (*CYP1A1*) under the control of patatin promoter into potato in order to decrease the residual agrochemicals in the tuber and soil for phytoremediation. We also discuss the tuber-specific expression of transgenes in the transgenic plants and the ability of these plants to degrade residual agrochemicals in the tuber.

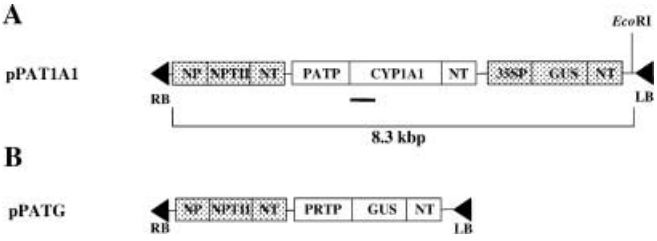


Fig. 1A, B Structure of the expression plasmids used for the transformations. **A** pPAT1A1, **B** pPATG. *RB* Right border, *LB* Left border, *NP* Nos promoter, *NPT II* Neomycin phosphotransferase II, *NT* Nos terminator, *PATP* patatin promoter, *CYP1A1* rat CYP1A1 cDNA, *35SP* CaMV 35S promoter *GUS* ß-glucronidase. *Eco*RI indicates the restriction site of the *Eco*RI enzyme in the expression plasmids. The DNA fragment used for probes in Southern blot analysis is shown beneath the expression plasmids as a *bold line*

Materials and methods

Expression plasmid construction

A rat CYP1A1 cDNA was cloned from rat liver tissues (Yabusaki et al. 1984). Patatin class-I promoter was cloned by the polymerase chain reaction (PCR) using patatin-promoter-specific primers, 5′-TTGAGTCTAGAAATCATAATGTT-3′ and 5′-TTGTTTTATTTGTCATGTTAGTCC-3′, as described by Rocha-Sosa et al. (1989). The rat CYP1A1 cDNA was joined under the potato patatin promoter. This chimeric gene was inserted into the *Cla*I site with Nos terminator between neomycin phosphatransferase II (NPTII) and β-glucuronidase (GUS) units in plasmid pBI121 (pPAT1A1, Fig. 1A). The plasmid vector, pPATG, was prepared by replacing the CaMV 35S promoter with the patatin class-I promoter in pBI121 (Fig. 1B).

Plant materials and transformation

Solanum tuberosum cv. May Queen was used for the transformation. Each pPAT1A1 and pPATG was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. The formation of micro-tubers and the transformation of tuber disks by the *Agrobacterium* method were carried out by the methods reported by Ishige et al. (1991). In kanamycin-resistant plants, integration of the CYP1A1 gene was confirmed by PCR analysis.

GUS assay for the GUS gene under the control of the patatin promoter

After inoculation with *A. tumefaciens* LBA4404 harboring pPATG, one representative transgenic plant Ag2620 was selected. This plant was used as an indicator of the gene expression induced by a high concentration of sucrose and in tuber-specific expression. The in vitro shoots grown in liquid MS medium (Murashige and Skoog 1962) supplemented with 10% sucrose and in vivo shoots in the growth chamber were used for the GUS assay (fluorescence method, Jefferson 1987).

Assay for 7-ethoxycoumarin *O*-deethylase (ECOD) activity

After the PCR-positive plants transformed with pPAT1A1 were cultured in liquid MS medium supplemented with 10% sucrose for 1 month, the fresh leaves were collected. The leaf tissues were incubated in the liquid MS medium containing 400 μ*M* of 7-ethoxycoumarin for 3 days in vitro at 20°/15 °C (day/night) under a 14/10-h (day/night) photoperiod. ECOD activity was measured by the method described by Inui et al. (2000).

Southern blot analysis

Total DNA was isolated from fresh leaves (2–3 g) by the method of Drapper et al. (1988). Polysaccharides were removed by ethanol precipitation (Michaels et al. 1994) after RNaseA treatment. The fragment of rat CYP1A1 synthesized by PCR (Fig. 1A) was used as the probe. Probe labeling, hybridization and detection were carried out using the ECL direct nucleic acid labeling and detection system (Amersham, UK).

Northern blot analysis

Total RNA was isolated from fresh leaves and young and mature tubers by the method described by Lagrimini et al. (1987). Riboprobe-labeling with digoxigenin-dUTP, hybridization and immunological detection were carried out according to the supplier's instruction (Boehringer Mannheim, Germany).

Western blot analysis

The microsomal fractions were prepared from fresh leaves and young and mature tubers by the methods of Shiota et al. (1994). Blotted proteins were immunologically detected using a polyclonal antibody against rat CYP1A1 (Daiichi Pure Chemicals, Japan) and a donkey anti-goat IgG conjugated with horseradish peroxidase as a secondary antibody. Detection was carried out using ECL Western blotting detection system (Amersham).

Analysis of residual herbicides in micro-tuber

Shoots of both May Queen and transgenic plants were cultured in liquid MS medium containing 2% sucrose. After 3 weeks, these shoots were exposed to either 40 μ*M* atrazine or 200 μ*M* chlortoluron in liquid MS medium supplemented with 10% sucrose. Following culture for 1 month under dark conditions, micro-tubers grown in vitro were harvested and analyzed for residual herbicides. The residuals of atrazine and chlortoluron in micro-tubers were measured by gas chromatography–mass spectrometry (GC–MS) using the methods of Iijima et al. (1997).

Results

Transformation, regeneration and selection of transgenic plants

One hundred and eighty regenerants were obtained from tuber disks inoculated with *A. tumefaciens* LBA4404

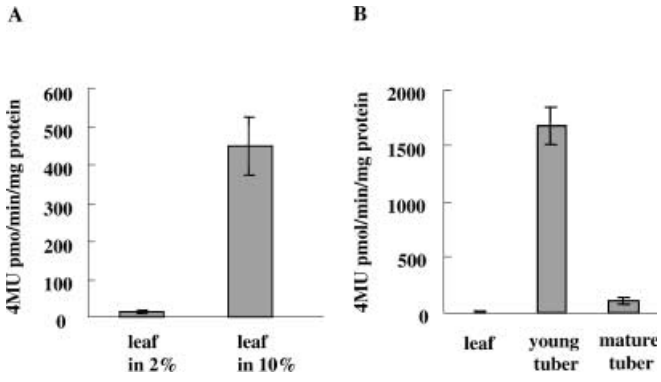


Fig. 2A, B GUS activity in the transgenic plant Ag2620 harboring patatin-GUS gene. **A** The GUS activity of leaves in Ag2620 grown in MS medium containing 2% or 10% sucrose. **B** The GUS activity of leaves, young (developing) tubers and mature tubers in Ag2620 grown in a growth chamber. The data represent the results of three independent experiments with standard errors

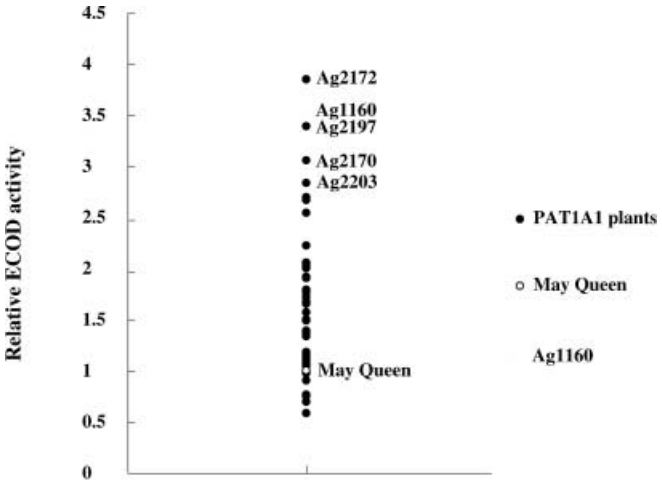


Fig. 3 Relative activity of 7-ethoxycumarin *O*-deethylation (*ECOD*) in transgenic plants against the activity of May Queen. Plants with high ECOD activity are shown by numbered plant (Ag2170, Ag2172, Ag2197 and Ag2203). Ag1160 is a transgenic plant harboring the rat CYP1A1 gene under the control of CaMV 35S promoter

harboring the expression plasmid pPAT1A1. After confirming the integration of the rat CYP1A1 gene by PCR in kanamycin-resistant plants, we selected 106 transformants (PAT1A1 plants).

GUS activity in transgenic plant Ag2620

GUS activity in Ag2620 was measured in leaves from plants grown for 40 days in liquid MS medium containing either 2% sucrose or 10% sucrose. The plants grown in MS medium containing 10% sucrose showed a much higher GUS activity in their leaves than those grown in 2% sucrose (Fig. 2A). These facts indicate that ideal plants that had a strong activity of rat CYP1A1 enzyme could be selected on the basis of ECOD activity in leaves treated with 10% sucrose. The developing tuber-specific expression of GUS gene was confirmed in Ag2620 plants grown in a growth chamber. Although GUS activity in the leaf and mature tuber was low, higher GUS activity was observed in the developing tuber (Fig. 2B).

Selection of transgenic plants by ECOD activity

ECOD activity was measured in the leaves of PAT1A1 plants grown in the medium containing 10% sucrose. 7-Ethoxycoumarin was generally used as substrate to measure the activity of P450 enzyme. ECOD activity has been shown to have a good correlation with the activity of the CYP1A1 enzyme (Inui et al. 2000). Figure 3 shows relative ECOD activity in the transgenic plants against the activity of May Queen. The relative ECOD activity varied from 0.7 to 3.8. On the basis of their high relative ECOD activity, four transgenic plants (Ag2170, Ag2172, Ag2197 and Ag2203) were selected.

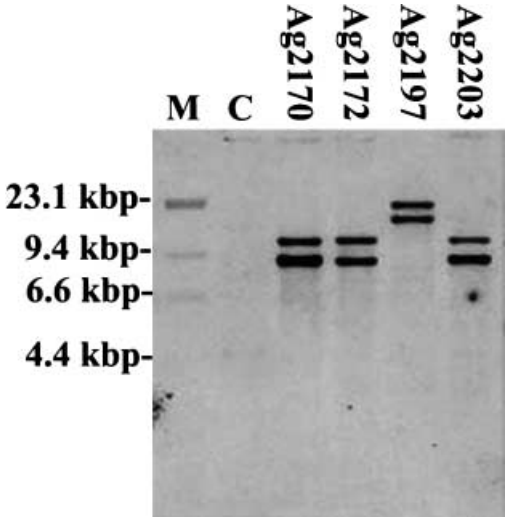


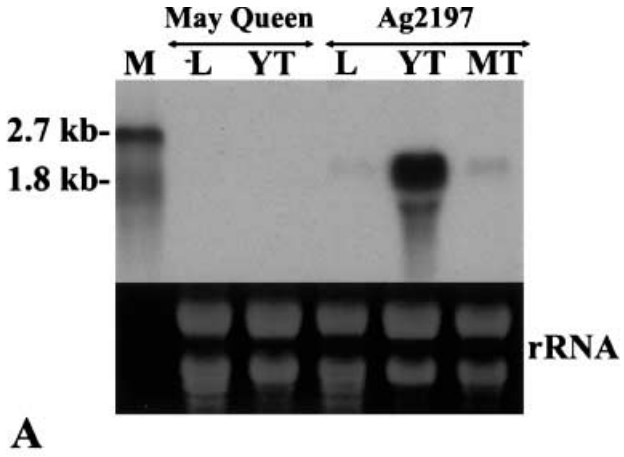
Fig. 4 Southern blot analysis in four transgenic plants. Genomic DNA was digested with *Eco*RI and probed with a labeled DNA fragment of the *CYP1A1* gene (see Fig. 1). *M* Molecular-weight marker (lambda DNA digested with *Hin*dIII), *C* control (May Queen)

Southern blot analysis in transgenic plants

Although the signal that hybridized with the CYP1A1-specific probe was not detected in May Queen, four plants (Ag2170, Ag2172, Ag2197 and Ag2203) showed a two-band pattern corresponding to the CYP1A1-specific probe (Fig. 4).

Western blot analysis of leaves of transgenic plants grown in the medium containing either 2% or 10% sucrose

Four transgenic plants and four May Queen plants were cultured in MS medium containing either 2% or 10% su-



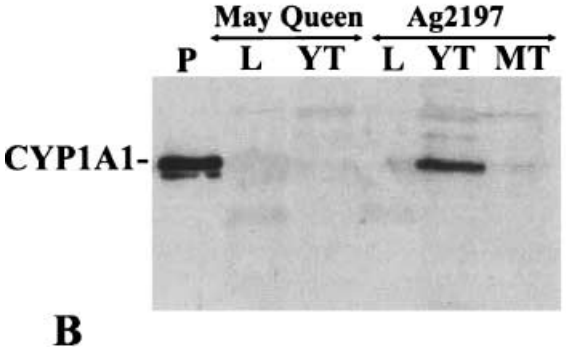


Fig. 5A, B Northern and Western blot analyses in May Queen and Ag2197. **A** Total RNA was extracted from the leaves (*L*), young tubers (*YT*) and mature tubers (*MT*) in both May Queen and transgenic plants. *M* RNA molecular-weight marker. Ten micrograms of total RNA was hybridized with rat CYP1A1 riboprobes. **B** Microsomal fraction was extracted from the leaves (*L*), young tubers (*YT*) and mature tubers (*MT*) in both May Queen and transgenic plants. Ten micrograms of protein was immunologically detected by rat CYP1A1 antibody. *M* rat CYP1A1 (CYP1A1, 59 kDa) protein as positive control

crose. The accumulation of a protein corresponding to CYP1A1 was confirmed only in the four transgenic plants grown in the medium containing 10% sucrose. Ag2197 was used for further experiments because the plant was found to accumulate the largest amount of CYP1A1 protein among the four transgenic plants and exhibited normal morphological characters.

Northern and Western blot analyses in Ag2197

The tissue-specific expression of the CYP1A1 gene was examined in plants grown in the growth chamber by both Northern and Western blot analyses. In the May Queen plants no bands hybridized with the CYP1A1 riboprobes in either fresh leaves or young tubers (Fig. 5A). On the other hand, Ag2197 had a strong signal corresponding to CYP1A1 in young (developing) tubers but not in leaves or mature tubers (Fig. 5A). No CYP1A1 protein was detected in May Queen plants, but Ag2197 was found to accumulate a large amount of CYP1A1 protein in the young tuber but not in the leaves or the mature tubers (Fig. 5B).

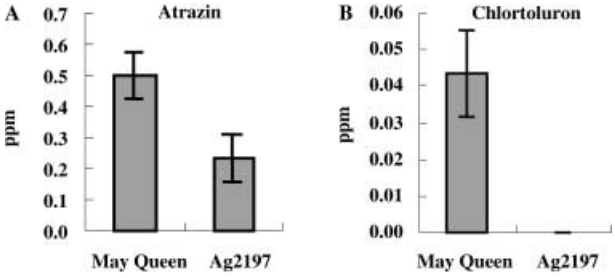


Fig. 6A, B The analysis of residual herbicides in the micro-tubers of both May Queen and Ag2197. **A** Atrazine, **B** chlortoluron. A concentration (ppm) indicates residual herbicide (milligram) per micro-tuber fresh weight (kilogram). The data are expressed as the results of three independent experiments with standard errors

Analysis of herbicides residues in the micro-tubers

Shoots of May Queen and Ag2197 plants were cultured in liquid medium containing atrazine or chlortoluron, and the harvested micro-tubers were used for the analysis of residual herbicides. The amount of atrazine detected in Ag2197 was approximately half of that found in May Queen after a 1-month culture (Fig. 6A). Although the residual chlortoluron was not detected in Ag2197, a small amount of chlortoluron still remained in May Queen (Fig. 6B).

Discussion

We tested for ECOD activity in leaves in order to select for transgenic plants expressing CYP1A1 in the tubers. Inui et al. (2000) reported a simple procedure of measuring ECOD activity in the leaves. However, the patatin promoter was mainly active in the developing tuber (Pickard et al. 1987; Rocha-Sosa et al. 1989). It was complicated to select for plants showing a high tuber-specific activity of CYP1A1 enzyme. The patatin promoter has also been reported to be active in leaves treated with a high concentration of exogenous sucrose (Wenzler et al. 1989). Grierson et al. (1994) showed that the sequences TTTCAAGTCTCATCACA and AATACTAAT in the patatin promoter are elements of a tuber-specific enhancer and AATAGAAAA, a sucrose-inducted element. The patatin promoter used in this study also included two copies of TTTCAAGTCTCATCACA and three copies of AATACTAAT and AATAGAAAA *cis* sequences (unpublished data). Consequently, the activity of ECOD in leaves induced by treatment with 10% sucrose was supposed to reflect the activity of CYP1A1 in the developing tuber. Compared with the activity of ECOD in May Queen plants, the relative ECOD activity of leaves varied in the range of 0.7

to 3.8 among the PAT1A1 plants cultured on the medium containing 10% sucrose (Fig. 3). The relative ECOD activity of four PAT1A1 lines (Ag2170, Ag2172, Ag2197 and Ag2203) was similar to that (the value 3.6) of the transgenic plant (Ag1160) harboring the CYP1A1 gene under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter (Fig. 3). One, Ag2197, produced a large amount of mRNA and protein in young tubers (Fig. 5A and B). That is, we could select for the transgenic plant showing a high activity of CYP1A1 enzyme in the tubers by measuring the ECOD activity in leaves of plants treated with 10% sucrose.

Based on the unique *Eco*RI restriction site in the inserted region (see Fig. 1A), the banding pattern in the Southern blot revealed that more than two copies of patatin-CYP1A1 gene were integrated into the genomic DNA in four transgenic plants. This indicated that the integration of several copy numbers of patatin-CYP1A1 gene might be associated with high ECOD activity.

Ag2197 treated with 10% sucrose showed the same level of ECOD activity in its leaves as did Ag1160 harboring the CaMV 35S-CYP1A1 gene (Fig. 3). Ag2620 harboring the patatin-GUS gene showed a much higher GUS activity in the young tubers than in the leaves of the plants treated with 10% sucrose (Fig. 2A). Jefferson et al. (1990) demonstrated that the activity of the GUS gene under the control of 35S promoter was lower in the tubers than in the other tissues such as leaves, roots and stems. These facts indicate that Ag2197 showed a much higher activity of the CYP1A1 enzyme in the young tuber than the plant harboring the CYP1A1 gene under the control of the 35S promoter.

The production of micro-tubers in medium containing a herbicide is good system for analyzing residual photosynthesis inhibitor herbicides such as atrazine and chlortoluron. The culture system consists of a high concentration of sucrose as plant nutrients and darkness so that the photosynthetic products are not used. Therefore, the residual herbicide can be analyzed in the plant body without damage by the herbicide. Hanioka et al. (1999) demonstrated that triazine herbicides – simazine, atrazine and propazine – can be metabolized to non-toxic compounds through *N*-dealkylation and deisopropylation by P450 species in the microsomes of a rat liver. They also reported that the *N*-dealkylation was mainly induced by rat P450 species CYP1A1, CYP1A2, CYP2B1 and CYP2B2, and deisopropylation by CYP2B1 and CYP2B2 (Hanioka et al. 1999). Inui et al. (1999) reported that transgenic potato plants harboring the human *CYP1A1* gene metabolized atrazine to detoxified compounds through deisopropylation and then *N*-dealkylation. The amount of residual atrazine in the micro-tubers of Ag2197 plants harboring the patatin-CYP1A1 gene decreased until it was about one-half of that found in the micro-tubers of May Queen plants (Fig. 6A), indicating that the CYP1A1 enzyme metabolizes atrazine to less toxic chemicals through *N*-dealkylation and/or deisopropylation in the micro-tubers.

The phenylurea herbicide chlortoluron is extremely stable to heat, acids and alkalis and is smoothly absorbed into the plants through the roots and the foliage (Tomlin et al. 1998). Therefore, absorbed chlortoluron is very stable in the plant body. There was no detectable residue of chlortoluron in the micro-tubers of Ag2197, indicating that Ag2197 metabolized chlortoluron to other chemicals in the developing micro-tubers as well as the metabolization of atrazine. The transgenic plants harboring the rat CYP1A1 gene metabolized chlortoluron through *N*-demethylation and ring-methyl hydroxylation to a detoxified form (Shiota et al. 1994; Inui et al. 1998). Ag2197 was also considered to convert chlortoluron to detoxified compounds through *N*-demethylation and ring-methyl hydroxylation. On the other hand, a small amount of chlortoluron (0.02 ppm) was detected in May Queen plants. Inui et al. (1998) demonstrated that while non-transgenic potato was also able to metabolize chlortoluron to *N*-demethylated metabolite this chemical still retained its phytotoxic characteristic to wither leaves. These indicated that both Ag2197 and May Queen converted chlortoluron to other compounds during the development of the micro-tuber under dark conditions but that Ag2197 could metabolize the chlortoluron to a detoxified form more rapidly than the non-transgenic plant.

We have demonstrated here that P450-dependent detoxification of herbicides with different chemical structures was incorporated into potato plants by the integration of the rat *CYP1A1* gene and that the function of detoxification was enhanced in the young tuber using the transgene under the control of the patatin promoter. The constitutive expression of the transgene is generally unacceptable and unnecessary in the field. Yamada et al. (2001) demonstrated the chemical inducible expression of the CYP1A1 gene under the control of the tobacco PR1a promoter. Considering the public acceptance of transgenic plants, young tuber-specific expression of transgene using patatin promoter is also acceptable and necessary. Since the CYP1A1 enzyme metabolizes other chemicals, including environmental pollutants (Inui et al. 2001), the transgenic plants produced in this study might be useful for the phytoremediation of chemical pollution in the soil.

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