Metabolic engineering of plant alkaloid biosynthesis

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Plant alkaloids, one of the largest groups of natural products, provide many pharmaceutically active compounds. Several genes in the biosynthetic pathways for scopolamine, nicotine, and berberine have been cloned, making the metabolic engineering of these alkaloids possible. Expression of two branching-point enzymes was engineered: putrescine N-methyltransferase (PMT) in transgenic plants of Atropa belladonna and Nicotiana sylvestris and (S)-scoulerine 9-O-methyltransferase (SMT) in cultured cells of Coptis japonica and Escscholzia californica. Overexpression of PMT increased the nicotine content in N. sylvestris, whereas suppression of endogenous PMT activity severely decreased the nicotine content and induced abnormally formed morphologies. Ectopic expression of SMT caused the accumulation of benzylisoquinoline alkaloids in E. californica. The prospects and limitations of engineering plant alkaloid metabolism are discussed.

berberine | nicotine | polyamine | sanguinarine | scopolamine

Higher plants constitute one of our most important natural resources. They provide not only foodstuffs, fibers, and woods, but many chemicals, such as oils, flavorings, dyes, and pharmaceuticals. Although plants are renewable resources, some species are becoming more difficult to obtain in sufficient amounts to meet increasing demands. Destruction of natural habitats and technical difficulties in cultivation also are driving the drastic reductions in plant availability. For example, it is claimed that the demand for paclitaxel, a potent anticancer compound, could endanger forests of Taxus brevifolia (Pacific yew) because of the low paclitaxel content (40–100 mg/kg of bark) in and slow growth of the trees (1).

For many natural chemicals it is possible to synthesize alternatives from petroleum, coal, or both. The economic limitations of chemical synthesis and the pollution that accompanies this type of chemical synthesis, however, have led to the development of cell culture and molecular engineering of plants for the production of important and commodity chemicals. Plant cell and organ culture offer promising alternatives for the production of chemicals because totipotency enables plant cells and organs to produce useful secondary metabolites in vitro (2). Cell culture is also advantageous in that useful metabolites are obtained under a controlled environment, independent of climatic changes and soil conditions. In addition, the products are free of microbe and insect contamination. Fermentation technology also can be used to produce desired metabolites and can be optimized to maintain high and stable yields of known quality by cellular and molecular breeding techniques to further improve productivity and quality. After extensive empirical trials, some metabolites are now being produced by large-scale cell culture (e.g., shikokin and berberine; ref. 2), but the numbers of compounds that are producible commercially by cell culture technology are still very few. The main limitations are low productivity and the necessity of the downstream processing of the desired compounds. Molecular engineering of secondary metabolites has the potential to increase productivity and improve product composition.

The Solanaceae produce a range of biologically active alkaloids that include nicotine and the tropane alkaloids (3). Tropane alkaloids, such as hyoscymamine (atropine) and scopolamine (hyoscymine), which are found mainly in Hyoscyamus, Daboisit, Atropa, and Scopolia species, together with their semisynthetic derivatives, are used as parasympatholytics that competitively antagonize acetylcholine. Both the tropane ring moiety of the tropane alkaloids and the pyrrolidine ring of nicotine are derived from putrescine by way of N-methylputrescine (MP) (Fig. 1). Because putrescine is metabolized to polyamines such as spermidine and spermine, the N-methylation of putrescine catalyzed by putrescine N-methyltransferase (PMT) is the first committed step in the biosynthesis of these alkaloids.

Isoquinoline alkaloids are some of the major metabolites successfully produced by plant cell culture. So far, about 60 have been isolated from plant cell cultures (ref. 4 and references therein). One example is berberine, a benzylisoquinoline alkaloid obtained from Coptis (Ranunculaceae) that is used as an antibacterial agent. Berberine biosynthesis in plant cells has been well investigated at the enzyme level (5–7). The biosynthetic pathway leading from L-tyrosine to berberine has 13 different enzymatic reactions that involve a norcoclaurine synthase, an N-methyltransferase, three O-methyltransferases (OMTs), a hydroxylase, a berberine bridge enzyme, a methylenedioxy ring-forming enzyme, and a tetrahydroprotoberberine oxidase (Fig. 2). cDNAs of several enzymes in this pathway have been isolated and characterized: norcoclaurine 6OMT (8), a hydroxylase (9), 3′-hydroxy-N-methyl-coalurine 4′OMT (8), a berberine bridge enzyme (10), and (3′)-scoulerine 9-O-methyltransferase (SMT) (11).

We previously reported that overexpression of hyoscymamine 6β-hydroxylase in Atropa belladonna efficiently converts this species’ main alkaloid, hyoscyamine, to scopolamine (12). This successful metabolic engineering of a medicinal plant has raised prospects for biotechnological applications of secondary metabolite production, but fundamental difficulties remain in transforming the host plants (e.g., Catharanthus; ref. 13). Our recent attempts to improve the production of putrescine-derived alkaloids and isoquinoline alkaloids by molecular engineering are reported.

Materials and Methods

Vector Construction. Tobacco PMT cDNA (14) with an introduced Ncol site at the first ATG, which had been cloned in pcDNAIIZ (Invitrogen), was excised as an Ncol–BamH I fragment and cloned into pRTL2 (15) under the CaMV35S promoter with the duplicated enhancer (16), then excised as an XbaI–BamH I site at the first ATG, which had been cloned in a binary vector pGA482 (Amerham Pharmacia). Coptis SMT cDNA in pETSMT (11) was excised as an XbaI–XhoI fragment and cloned into modified binary vector pBIKXII6 with the kanamycin-resistant gene after the CaMV35S promoter with the duplicated enhancer (16), giving pBIEX. cDNA in pETSMT also was cloned into modified pBIKXII6, yielding pBHES with a hygromycin-resistant

Abbreviations: PMT, putrescine N-methyltransferase; SMT, (S)-scoulerine 9-O-methyltransferase; MP, N-methylputrescine; OMT, O-methyltransferase.

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gene (K.T., K.-B.C., H.F., and F.S., unpublished observations). These binary vectors were introduced into Agrobacterium tumefaciens LBA4404 and A. rhizogenes 15834 by electroporation (17).

**Plant Transformation.** A. belladonna and Nicotiana sylvestris were transformed by a leaf disk method that used A. tumefaciens or A. rhizogenes, basically as described (17). Transgenic plants were regenerated from the leaf discs, grown to maturity in a greenhouse, and selfed. Hairy roots were cultured as described (18).

Cultured Coptis cells (7 days old) (19) were harvested and sonicated in Linsmaier–Skog medium (20) for 2 min and then cocultured in Linsmaier–Skog medium (pH 5.2) containing 10 μM 1-naphthylacetic acid, 1 μM benzyladenine, and 100 μM acetosyringone with A. tumefaciens harboring pBIES for 7–13 days. Thereafter, transformed cells were selected on Linsmaier–Skog medium containing 10 μM 1-naphthylacetic acid, 0.01 μM benzyladenine, 20 μg/ml geneticin, and 200 μg/ml cefotaxin. Aseptically grown seedling segments of Eschscholzia californica were transformed by the procedure used for the Coptis cells, except that coagulation with A. tumefaciens with pBHES was for 2 days, and selection was on Linsmaier–Skog medium containing 10 μM 1-naphthylacetic acid, 1 μM benzyladenine, 20 μg/ml hygromycin, and 200 μg/ml cefotaxin.

**DNA Gel Blot Analysis.** Genomic DNAs of the transformed Coptis cells were prepared by the cetyltrimethylammonium bromide method as described (21). After HindIII digestion, the digested DNAs were blotted on Hybond N membranes (Amersham Pharmacia) and hybridized with SMT cDNA fragments amplified by PCR. Hybridization was done at 42°C in buffer containing 1 M NaCl, 10% dextran sulfate, 20 mM Tris·HCl (pH 7.5), 1% SDS, 5× Denhardt’s solution, 50% formamide, and 100 μg/ml salmon sperm DNA. The membranes were washed in 0.1× SSC (0.015 M NaCl/1.5 mM sodium citrate, pH 7.0), 0.2% SDS at 65°C. Hybridization, washing, and signal detection were done as reported (21).

**RNA Gel Blot Analysis.** Total RNAs were extracted from plant tissues, electrophoresed on 1% formaldehyde agarose gels, and then blotted on Hybond N membranes (Amersham Pharmacia), as described (14). RNA probes were radiolabeled with a Riboprobe Combination System (Promega) or Random Prime kits (Roche Molecular Biochemicals). Hybridization, washing, and signal detection were as reported (14).

**Enzyme Assay and Protein Gel Blot Analysis.** The transfer of the 3H-labeled methyl group of S-adenosyl-L-[methyl-3H]methionine (NEN Life Science Products) to the product was measured to quantify the enzymatic activity of SMT. The reaction conditions and protein gel blot analysis have been described (11).

**Alkaloid and Polyamine Analyses.** Tropane alkaloids and nicotine were analyzed by gas-liquid chromatography as described (14, 22). Hygrine was identified by gas chromatography–mass spectrometry (JOEL JMS 700) after comparison of its mass spectrum with that of an authentic sample. Polyamines first were divided into their dansyl derivatives (23). Cellular polyamine contents were expressed as the sum of both forms.

Isoquinoline alkaloids extracted with HCl-acidified methanol were analyzed by HPLC with 50 mM tartaric acid and 10 mM SDS/acetonitrile/methanol (4:4:1) as described (19). Mass spectra were obtained with an API165 (Perkin–Elmer) after reverse-phase HPLC separation [mobile phase, linear gradient of 10–70% acetonitrile/H2O containing 1% acetic acid for 15 min; column, TSK-gel ODS-80 (Toso-Soda; 3.9 × 280 mm); flow rate, 1.0 ml/min], with enzymatically prepared columbamine.
(oxidized form of tetrahydrocolumbamine) and sanguinarine as the standards.

Results and Discussion

Overexpression of PMT in A. belladonna. Tobacco PMT cDNA was overexpressed under a dual CaMV35S promoter in belladonna. One transgenic line (35S::PMT) that had a high PMT transcript level in the leaf was selfed, and its five siblings with the transgene were analyzed. PMT transcript levels in the 35S::PMT roots were 1.3- to 3.3-fold those in the wild-type roots. Regardless of the variation in transgene expression level, all of the 35S::PMT transformants were phenotypically normal and had hyoscyamine as the principal leaf alkaloid at levels (4.6–8.3 nmol y mg dry weight) comparable to those for the wild-type and vector controls. The only differences were the accumulation of MP (0.9–2.6 nmol y mg dry weight) and the occasional presence of hygrine (up to 0.2 nmol y mg dry weight) in the 35S::PMT leaves, which was not found in the control leaves. These metabolites may be produced in situ in transgenic leaves by ectopic PMT activity. Transgenic hairy root clones, which had a 5-fold increase in the PMT transcript level as compared with the wild-type hairy roots, also were produced, but their alkaloid profiles were quantitatively and qualitatively similar to those of the wild type. The unchanged alkaloid profiles in the transgenic belladonna plants and hairy roots indicate that a severalfold increase in PMT expression in the root was not sufficient to boost tropane alkaloid synthesis late in the pathway.

Manipulation of PMT levels in N. sylvestris. Tobacco PMT cDNA was expressed under the same CaMV35S promoter in N. sylvestris plants to assess the impact of altered PMT expression in another, shorter, alkaloid pathway. Three transgenic lines that expressed 4- to 8-fold higher PMT transcript levels in their roots were selected (Fig. 3A), as was a cosuppression line whose PMT expression was approximately 16% that of the wild type (Fig. 3B). These overexpression lines had an approximately 40% increase in leaf nicotine contents as compared with the wild type and accumulated MP in the leaf, whereas the spermidine and spermine contents were somewhat decreased (Fig. 3C). In contrast, the cosuppression line accumulated only a very small amount of nicotine (about 2% that of the wild type). Instead it had increased amounts of putrescine and spermidine (Fig. 3C), indicating that the efficient inhibition of PMT activity shifted the nitrogen flow from nicotine to polyamine synthesis. Interestingly, the reduction of PMT activity in the root caused the accumulation of polyamines in the leaf. One explanation for this correlation is that polyamines, like nicotine, may be transported from the root to the aerial parts of the plant.

The overexpression lines were phenotypically normal, but the cosuppression line showed several distinct phenotypes in the T0 and T1 generations: neighboring leaves were fused in the basal region, forming a continuous spiral sheet along the stem (Fig. 3D); inflorescent stems often were branched, and self-pollinated flowers produced only a small seed set (less than...
10% that of the wild type). Reciprocal cross-pollination between the cosuppressed and wild-type flowers indicated that reduced fertility was due to a pollen defect in the transgenic plants. These phenotypic abnormalities may be caused by the increased accumulation of putrescine and spermidine, which may have hormonal functions during plant development (24). Alternatively, a reduction in the MP supply might cause the accumulation of precursors for the nicotinic acid moiety of nicotine, and, at high levels, these precursors might be responsible for the abnormalities.

These morphological changes caused by reduced PMT expression in tobacco were not previously reported in tobacco nic1nic2 mutants, even though PMT expression was down-regulated to 7% that of the wild-type value in mutant roots (13). In the low-nicotine mutant, genes for ornithine decarboxylase and quinolinic acid phosphoribosyltransferase, the two primary metabolism enzymes in nicotine biosynthesis (Fig. 1), also were down-regulated (T. Shoji, and T.H., unpublished results). Accumulation of pathway intermediates therefore may be less pronounced in the nic mutant than in PMT-suppressed plants.

Overexpression of SMT in Coptis Cells. The above results with engineered PMT expression suggest that modification of enzyme activity at a branch point with short subsequent steps to the target products may be used for efficient metabolic engineering. Previous findings suggest that SMT activity in Coptis cells was not correlated with the berberine/coptisine ratio, suggesting that SMT at the branch point (Fig. 2) cannot effectively compete for a rate-limiting intermediate in the biosynthesis of berberine and coptisine (25). To directly evaluate the contribution of SMT at this branch point for the regulation of metabolic flow in berberine and coptisine biosynthesis, we overexpressed Coptis SMT in cultured Coptis 156-1 cells that had been selected as high berberine producers (19). After several empirical trials, transformed Coptis cells with additional Coptis SMT genes were obtained. Digestion of genomic DNAs with HindIII, which has no restriction site in the probe, and DNA gel blot analysis showed that this transformant has three additional copies of the SMT gene (Fig. 4A). Transformation efficiency was very low, one per ca. 160 million cells (16 g fresh weight cells). Because the characteristics of the cell line used were fairly stable (19), the transformant could be used to evaluate the effect of the ectopic expression of Coptis SMT. RNA gel blot analysis indicated that the SMT transformant had a steady increase in SMT transcript (30–50% more than the wild type) in PMT-suppressed plants.

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HPLC analysis of the alkaloids in wild-type and transgenic Coptis cells indicated that the relative amounts of berberine and columbamine were 91% in the transgenic cells as compared with...
Coptis cells, whereas the wild-type E. californica was less than that in cultured Coptis cells. The increase in berberine and columbamine activity at the branch point affects the metabolite composition. The enzyme activity at the branch point is important for metabolite composition. The increase in berberine and columbamine (ca. 20%) and SMT activity (ca. 79%) in the wild-type ones (Fig. 4C). This difference indicates that the ectopic expression of SMT in the transgenic Coptis cells increased the metabolic flow to berberine and columbamine.

The increase in berberine and columbamine (ca. 15%) was very similar to the increase in SMT activity (ca. 20%), but the change was small. This finding suggests that the ratio of the enzyme activities at the branch point is important for metabolite composition late in the pathway and that even a small change in the activity at the branch point affects the metabolite composition.

Overexpression of SMT in California Poppy. Coptis SMT cDNA was introduced into California poppy (E. californica) cells that produce the benzophenanthridine alkaloid sanguinarine rather than berberine-type alkaloids (see Fig. 2) to modify the metabolite profile. Transformation of E. californica was confirmed by RNA and protein blot analyses of Coptis SMT. All of the transformants selected on hygromycin-containing media had an accumulation of the SMT transcript as high as that of the wild-type cells. The intracellular localization of target products is also an important target for metabolic engineering. The high toxicity of berberine in nonproducing plant species as compared with the low toxicity in high berberine-producing Coptis cells (26, 27) suggests that metabolic accumulation is crucial in the creation of a novel metabolite producer(s). Our current knowledge of metabolic accumulation is very limited; much research is needed. What must be carefully evaluated is the undesirable accumulations of pathway precursors and their metabolic effects on related pathways, such as we have found in PMT-suppressed N. sylvestris plants. Primary metabolism enzymes that supply amino acids or other precursors to particular secondary path-

Fig. 5. RNA and protein blot analyses and SMT activity in transformed E. californica cells. (A) RNA blot. (B) Protein blot. (C) Relative enzyme activity. CJWT, cultured Coptis cells; ECWT, nontransformed E. californica cells; ECSMT, transformed E. californica cells with Coptis SMT; E. coli SMT, recombinant SMT produced in Escherichia coli. 79% in the wild-type ones (Fig. 4C). This difference indicates that the ectopic expression of SMT in the transgenic Coptis cells increased the metabolic flow to berberine and columbamine. The increase in berberine and columbamine (ca. 15%) was very similar to the increase in SMT activity (ca. 20%), but the change was small. This finding suggests that the ratio of the enzyme activities at the branch point is important for metabolite composition late in the pathway and that even a small change in the activity at the branch point affects the metabolite composition.

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Prospects. Increases in the numbers of cloned biosynthetic genes and refinements in plant transformation techniques should enable researchers to improve the production of useful plant secondary metabolites by genetic engineering. Our findings point to the usefulness of single gene transfer for modifying metabolic flow. Although a substantial increase in productivity is feasible when a bona fide, rate-limiting enzyme is targeted (13), single rate-limiting steps may not exist in most biosynthetic pathways for secondary metabolites. In most pathways, overexpression of one enzyme renders subsequent reactions more limiting. After several intervening limiting steps, the effect of overexpression may be quickly dampened. Strategies should include fortification of the multiple steps by overexpression of multiple biosynthetic genes, manipulation of regulatory genes that control the expression of multiple pathway enzyme genes, or both.

Genes of unique enzymes isolated from diverse organisms are another promising genetic resource for the modification of metabolic pathways. The enormous number of reactions catalyzed by enzymes active in secondary metabolism provides unprecedented opportunities for the selection of suitable enzymes for use in metabolic engineering. Metabolic pathway inhibition may prove to be much more straightforward. Several efficient techniques are available for the suppression of endogenous gene expression: cosuppression, antisense suppression, RNA interference, and gene disruption by reverse genetics. These techniques should open up the field for the metabolic engineering of medicinal plants.

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Sato et al.

PNAS | January 2, 2001 | vol. 98 | no. 1 | 371

plant biology
ways often are regulated in concert with secondary metabolism enzymes and therefore may be genetically engineered for the optimal manipulation of secondary metabolism.

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