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Metabolic engineering of medicinal plants: Transgenic *Atropa belladonna* with an improved alkaloid composition

(scopolamine/hyoscyamine 6β-hydroxylase)

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ABSTRACT **The tropane alkaloid scopolamine is a medicinally important anticholinergic drug present in several solanaceous plants. Hyoscyamine 6β-hydroxylase (EC 1.14.11.11) catalyzes the oxidative reactions in the biosynthetic pathway leading from hyoscyamine to scopolamine. We introduced the hydroxylase gene from *Hyoscyamus niger* under the control of the cauliflower mosaic virus 35S promoter into hyoscyamine-rich *Atropa belladonna* by the use of an *Agrobacterium*-mediated transformation system. A transgenic plant that constitutively and strongly expressed the transgene was selected, first by screening for kanamycin resistance and then by immunoscreening leaf samples with an antibody specific for the hydroxylase. In the primary transformant and its selfed progeny that inherited the transgene, the alkaloid contents of the leaf and stem were almost exclusively scopolamine. Such metabolically engineered plants should prove useful as breeding materials for obtaining improved medicinal components.**

The use of recombinant DNA technology for the manipulation of metabolic processes in cells promises to provide important contributions to basic science, agriculture, and medicine (1). Secondary metabolism is a particularly attractive target for the improvement of yields of desirable products, without markedly affecting basic cellular functions. The production of the antibiotic cephalosporin C by a fungal production strain has been improved by giving increased gene dosages of a rate-limiting enzyme (2). Several novel antibiotics have been produced by transferring all or part of their biosynthetic pathways to heterologous host microorganisms (1), as well as by targeted disruption of a biosynthesis gene (3). In plants, flavonoid pigments in ornamental flowers appear to be the most suitable for genetic modification because the flavonoid biosynthetic pathways and the genes involved are relatively well understood and because any changes in color and pigmentation patterns have potential commercial value (4). A great variety of pharmaceutical and antimicrobial compounds derived from plants also stand to benefit from yield improvement produced by genetic engineering, but a lack of understanding of the regulation of biosynthetic pathways and the general unavailability of cloned biosynthesis genes severely limit this approach at present. None of the introduced genes that are expected to function in target biosynthetic pathways have produced a considerable increase in the desired phytochemicals in transgenic plants (5–7).

The tropane alkaloids hyoscyamine (its racemic form being atropine) and scopolamine are used medicinally as anticholinergic agents that act on the parasympathetic nerve system. Because they differ in their actions on the central nervous system, currently there is a 10-fold higher commercial demand for scopolamine, in the *N*-butylbromide form, than there is for hyoscyamine and atropine combined. Several solanaceous species have been used as the commercial sources of these alkaloids, but the scopolamine contents in these plants often are much lower than those of hyoscyamine (8). For this reason there has been long-standing interest in increasing the scopolamine contents of cultivated medicinal plants. Naturally occurring and artificial interspecific hybrids of *Duboisia* have high scopolamine contents and are cultivated as a commercial source of scopolamine in Australia and other countries (9, 10). Anther culture combined with conventional interspecific hybridization also has been used to breed high scopolamine-containing plants in the genera *Datura* (11) and *Hyoscyamus* (12), but without much success.

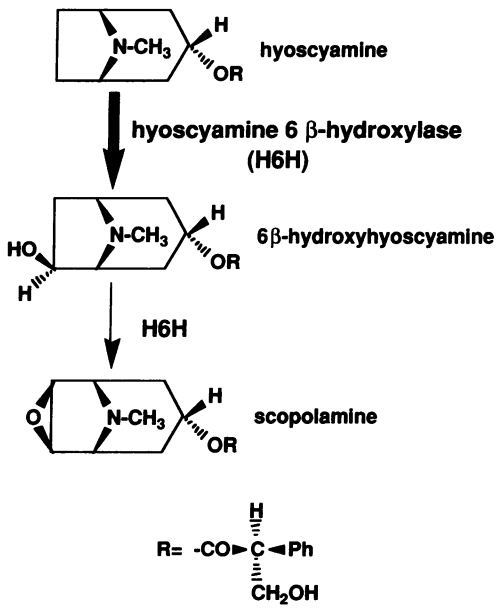

FIG. 1. Biosynthetic pathway from hyoscyamine to scopolamine. Scopolamine is formed from hyoscyamine via 6β-hydroxyhyoscyamine. H6H catalyzes the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine, as well as the epoxidation of 6β-hydroxyhyoscyamine to scopolamine.

Scopolamine is formed from hyoscyamine via 6β-hydroxyhyoscyamine (Fig. 1). Hyoscyamine 6β-hydroxylase (H6H; EC 1.14.11.11) catalyzes the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine, as well as the epoxidation of 6β-hydroxyhyoscyamine to scopolamine (13–15). Although the epoxidation activity of H6H is much lower than its hydroxylation activity, indirect evidence suggests that the epoxidation reaction may not be a limiting step *in planta*. 6β-Hydroxyhyoscyamine usually does not accumulate in scopolamine-producing plants (16). Moreover, a rough cor-

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Abbreviations: H6H, hyoscyamine 6β-hydroxylase; CaMV, cauliflower mosaic virus.

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relation has been found between H6H activity and the ratio of scopolamine to hyoscyamine in scopolamine-producing cultured roots (16). H6H therefore is a promising target enzyme which, if expressed strongly in hyoscyamine-accumulating plants, would result in increased scopolamine contents in the transformants.

Recently, an H6H cDNA clone was obtained from *Hyoscyamus niger* (17). We have introduced the H6H gene into, and expressed it in, *Atropa belladonna*, a typical hyoscyamine-rich plant. In the transformed plants conversion of hyoscyamine to scopolamine was highly efficient, evidence that the metabolic engineering of medicinal plants for the production of better pharmaceutical constituents is feasible.

MATERIALS AND METHODS

H6H Expression Vector. The *Xho* I fragment of the H6H cDNA insert was isolated from pBHH1 (17), filled-in with Klenow DNA polymerase, and ligated to a *Sal* I linker. The resulting DNA fragment was subcloned in pCaMVCN (Pharmacia) between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase terminator. The chimeric 35S–H6H gene then was excised as an *Xba* I fragment and subcloned in plant expression vector pGA482 (Pharmacia); the resulting binary vector is referred to as pHY8.

Plant Transformation. Seeds of *A. belladonna* were obtained from the Tsukuba Medicinal Plant Research Station (Ibaraki, Japan). The binary vectors (pHY8 and pGA482) were transferred to *Agrobacterium tumefaciens* LBA4404 by the direct transfer method (18). Exconjugants were used to transform belladonna leaf explants, basically as described for tobacco leaf disk transformation (18). All the leaf disks used were prepared from young leaves of a single seedling grown *in vitro*. After calli had formed on B5 agar medium (19) containing 10 μM 1-naphthaleneacetic acid and 1 μM 6-benzyladenine, shoots were induced on medium with 0.1 μM 1-naphthaleneacetic acid and 10 μM 6-benzyladenine and then transferred to hormone-free medium for root formation. All the culture media contained kanamycin at 500 μg/ml, carbenicillin at 500 μg/ml, and cefotaxime at 250 μg/ml. A total of 27 putative transformants that showed kanamycin resistance were screened for the expression of the H6H polypeptide by immunoblot analysis of the leaf samples. One plantlet that expressed the H6H polypeptide strongly in its leaves was selected and propagated from cuttings. Three plants propagated from the single primary transformant were planted in soil, then placed in a growth chamber, and grown at 20°C with a 14 hr/10 hr day/night regime and 80% humidity. Kanamycin-resistant plants regenerated from leaf disks treated with the LBA4404 strain harboring the empty vector pGA482 were the controls. Wild-type plants were grown in the same growth chamber. The T_0 primary transformant was self-pollinated, after which its seeds were collected. T_1 progeny were grown under the same conditions.

DNA Blot Analysis. DNA was isolated from belladonna leaves by the cetyltrimethylammonium bromide procedure (20). Southern hybridization was done as described (21). Genomic DNA was digested with *Bam*HI, then electrophoresed in a 0.7% (wt/vol) agarose gel, and blotted onto GeneScreen*Plus* (DuPont). DNA gel blots were probed with a 1.4-kilobase-pair (kbp) *Xho* I fragment from pBHH1 that contained the H6H gene (17) or with a 2-kbp *Bam*HI–*Hin*dIII fragment from pGA482 that contained the neomycin phosphotransferase II gene. The probes were labeled by random priming to a specific activity of $\approx 3 \times 10^7$ Bq/μg of DNA. The filters were hybridized with probe in 1.08 M sodium chloride/6 mM sodium phosphate, pH 6.8/6 mM EDTA/0.5% SDS/50% formamide containing salmon sperm DNA (100 μg/ml) at 42°C for 24 hr. The blots were washed in 0.3 M sodium chloride/0.03 M sodium citrate, pH 7.0/1% SDS at 65°C.

Immunoblot Analysis. Leaves, stems, main roots, and branch roots were collected, frozen with liquid nitrogen, and homogenized. The homogenate was suspended in 100 mM phosphate buffer, pH 7.5/3 mM dithiothreitol and centrifuged at 13,000 × *g* for 20 min. The supernatant was precipitated with ammonium sulfate of 80% saturation. The precipitate obtained after centrifugation was dissolved in 50 mM Tris·HCl, pH 7.8/1 mM dithiothreitol and then desalted on a PD-10 column (Pharmacia).

The crude cell extracts (30 μg of protein per lane) were subjected to SDS/PAGE in a 12.5% separating gel (22), and the separated proteins were transferred electrophorectically to Immobilon (Millipore). Immunoblotting was done as described (23) with the anti-H6H monoclonal antibody mAb5.

Enzyme Assay. Gas–liquid chromatography was used to assay the crude cell extracts for H6H enzyme activity by measuring the formation of 6β-hydroxyhyoscyamine from hyoscyamine (16).

Alkaloid Analysis. Tropane alkaloids were extracted from plant tissues and then purified and quantified by gas–liquid chromatography, as described (24). Alkaloids were identified by comparing their mass spectra with those of authentic samples (25).

RESULTS

Transfer and Expression of the *H. niger* H6H Gene in *A. belladonna*. Tropane alkaloids, including hyoscyamine, are synthesized mainly in the root, after which they are translocated to the leaf (26). Ectopic expression of the H6H gene introduced into tissues other than at the site of biosynthesis may give the H6H enzyme access to its alkaloid substrates, hyoscyamine and 6β-hydroxyhyoscyamine, during the translocation and storage of these alkaloids in the aerial plant parts. We therefore used the CaMV 35S promoter to drive the expression of the H6H gene from *H. niger* in a wide variety of cell types. The chimeric 35S–H6H gene was introduced by a leaf-disk transformation system into *A. belladonna*, which accumulates hyoscyamine as its main alkaloid. A total of 27 kanamycin-resistant primary transformants were screened by immunoblot analysis for the expression of the H6H polypeptide in the leaf.

One transformant that expressed H6H particularly strongly was chosen, and after it had grown to maturity it was self-pollinated to give T_1 progeny. The primary T_0 transformant and its 10 randomly selected T_1 progeny, as well as the wild-type belladonna, were analyzed for the presence of the H6H gene by Southern hybridization of *Bam*HI-digested genomic DNA. An endogenous belladonna H6H gene was detected as an ≈10-kbp *Bam*HI fragment in all the samples (Fig. 2). The T_0 plant contained three copies of the introduced 35S–H6H chimeric gene as shown by three hybridizing bands at approximately 18, 20, and 23 kbp. These hybridizing bands segregated, apparently at random, in the T_1 progeny. Similar results were obtained when a neomycin phosphotransferase II gene was the probe (data not shown).

Next, four plant parts (leaf, stem, main root, and branch root) were analyzed by immunoblotting and by enzyme assays (Fig. 3). In the T_0 (data not shown) and T_1 plants, there was strong expression of the H6H polypeptide and H6H enzyme activity in all the parts. In wild-type belladonna, however, weak expressions of the polypeptide and enzyme activity were found only in the branch root. Transgenic belladonna plants that constitutively expressed *H. niger* H6H showed no obvious differences in growth and development when compared with the control and wild-type plants.

Alkaloid Analysis. The content and composition of tropane alkaloids are known to vary considerably during plant de-

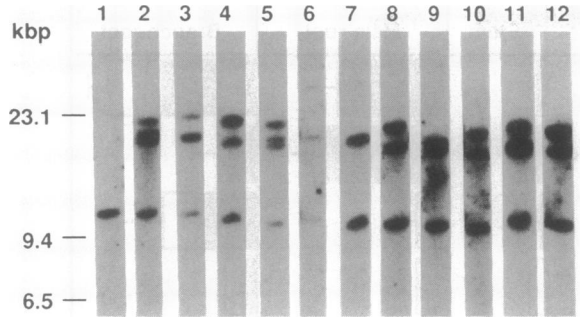

FIG. 2. Southern blot analysis of *A. belladonna* DNA digested with *Bam*HI. Lanes: 1, wild-type belladonna; 2, primary transformant T_0; 3, plant T_1-5; 4, plant T_1-11; 5, plant T_1-12; 6, plant T_1-13; 7, plant T_1-20; 8, plant T_1-24; 9, plant T_1-25; 10, plant T_1-26; 11, plant T_1-27; 12, plant T_1-28. T_1 plants are progeny of self-fertilized T_0. The blot was probed with the random-primed cDNA insert of pBHH1. Positions of size markers (*Hin*dIII-digested λ phage DNA) are shown at left.

velopment (27, 28). We found that the scopolamine content of *A. belladonna* was relatively high in the seedling and vegetative stages but progressively decreased toward the flowering stage, at which time total alkaloid content had considerably increased and hyoscyamine was the predominant alkaloid. We first analyzed the tropane alkaloids in the leaves of 3-month-old transformants during the preflowering stage (Table 1). The T_0 plant and 73 of the 74 T_1 plants showed a high-scopolamine phenotype, in which scopolamine made up >70% of the total alkaloid content. One T_1 plant (T_1-57) had a low percentage (35%) of scopolamine in its leaf alkaloids, which was comparable to the scopolamine percentages found in the wild-type and control plants during the corresponding developmental stages. This segregation ratio for the high-scopolamine phenotype in the T_1 progeny is expected from the estimation that three copies of the transgene are integrated in the T_0 transformant.

We next analyzed in detail the alkaloids present in mature plants after seeds had formed (Fig. 4). During this stage, >92% (average, 97%) of the total alkaloid contents of the leaf, stem, and main root of the wild-type and control plants was hyoscyamine. The branch roots of these plants contained relatively high amounts of scopolamine, which reflects the

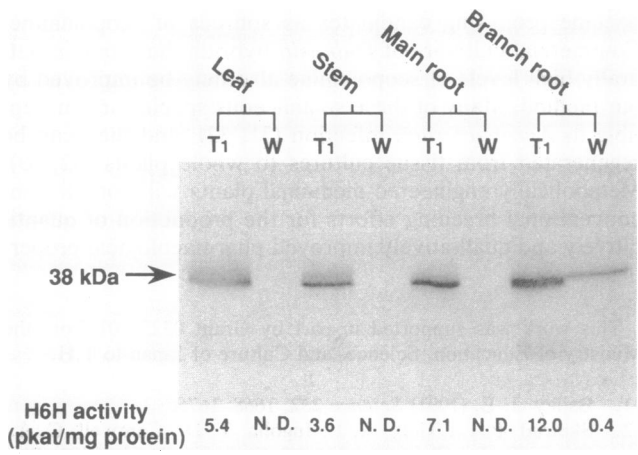

FIG. 3. Western blot analysis and H6H enzyme activity of *A. belladonna*. The immunoblot was probed with an H6H-specific antibody, mAb5. The position of the H6H polypeptide (38 kDa) is indicated. W, wild-type plant. T_1, pooled sample from transgenic plants T_1-14, -24, and -25. When 3-month-old plants were analyzed, >80% of total alkaloids in the leaves of these T_1 plants was scopolamine, as shown in Table 1. N.D., not detected.

Table 1. Distribution of scopolamine phenotypes among different 3-month-old belladonna plants

	Scopolamine percentage in total leaf alkaloids*					
	0–20	20–40	40–60	60–80	80–100	Total†
Wild-type and control plants	7					7
Primary transformants					3	3
T_1 progeny		1		1	72	74

*Scopolamine, hyoscyamine, and 6β-hydroxyhyoscyamine were identified in the leaf. In the plants with low scopolamine contents, hyoscyamine was the main alkaloid.
†Total number of plants analyzed.

endogenous H6H activity expressed in this organ. The alkaloid contents in the aerial parts of the T_0 transformant and five randomly selected T_1 progeny [all confirmed by Southern hybridization to contain at least one copy of the 35S–H6H transgene (Fig. 2)] were composed almost exclusively of scopolamine. In the main roots of some T_1 progeny, and particularly in the branch roots of the T_0 and T_1 transformants, hyoscyamine conversion was not as efficient as in the aerial parts. Possibly because of unintended subtle differences in the growth conditions under which the groups of plants were grown (sometimes successively in one growth chamber), the total alkaloid contents may have varied markedly among the genotypes, with total leaf alkaloids tending to be higher in the T_1 progeny than in the other plants. Further tests in the greenhouse and in the field are necessary to assess the long-term performance of these transgenic scopolamine-type belladonnas, especially with regard to the total yields of alkaloids.

DISCUSSION

Transfer of the H6H gene into, and its constitutive expression in, hyoscyamine-rich belladonna converted the herbaceous perennial to a chemotype pharmacologically more valuable. The change in the alkaloid composition in transgenic belladonna was remarkably efficient: scopolamine was almost the only alkaloid present in the aerial parts. This almost exclusive presence of scopolamine in medicinal plants cannot be achieved by conventional breeding methods. From such plants, pure scopolamine can be isolated by recrystallization of the total alkaloid fraction from leaf samples, instead of by the conventional differential extraction and chromatography of each component alkaloid. Although the primary transformant and the majority of its T_1 progeny received more than one copy of the chimeric 35S–H6H gene, alkaloid analysis of the T_1-13 and T_1-20 progeny plants showed that a single copy of the transgene was sufficient to create an all-scopolamine chemotype in the leaf.

The H6H purified from the cultured roots of *H. niger* (15, 16) catalyzed two consecutive reactions (6β-hydroxylation and 6,7-epoxidation) in the conversion of hyoscyamine to scopolamine, but epoxidase activity inherently was very weak (about 2–5% of the hydroxylase activity). Nevertheless, when 0.2 mM hyoscyamine and 6β-hydroxyhyoscyamine were fed to wild-type tobacco plants that lacked the H6H gene and to transgenic tobacco plants that constitutively expressed the 35S–H6H transgene, both alkaloids were efficiently converted to scopolamine in the leaves of the transgenic tobacco, but not in those of the wild-type tobacco (unpublished work). H6H therefore must be responsible for the high-scopolamine chemotype in transgenic belladonna plants and, probably, in naturally scopolamine-rich species such as *H. niger* and *Duboisia myoporoides* as well.

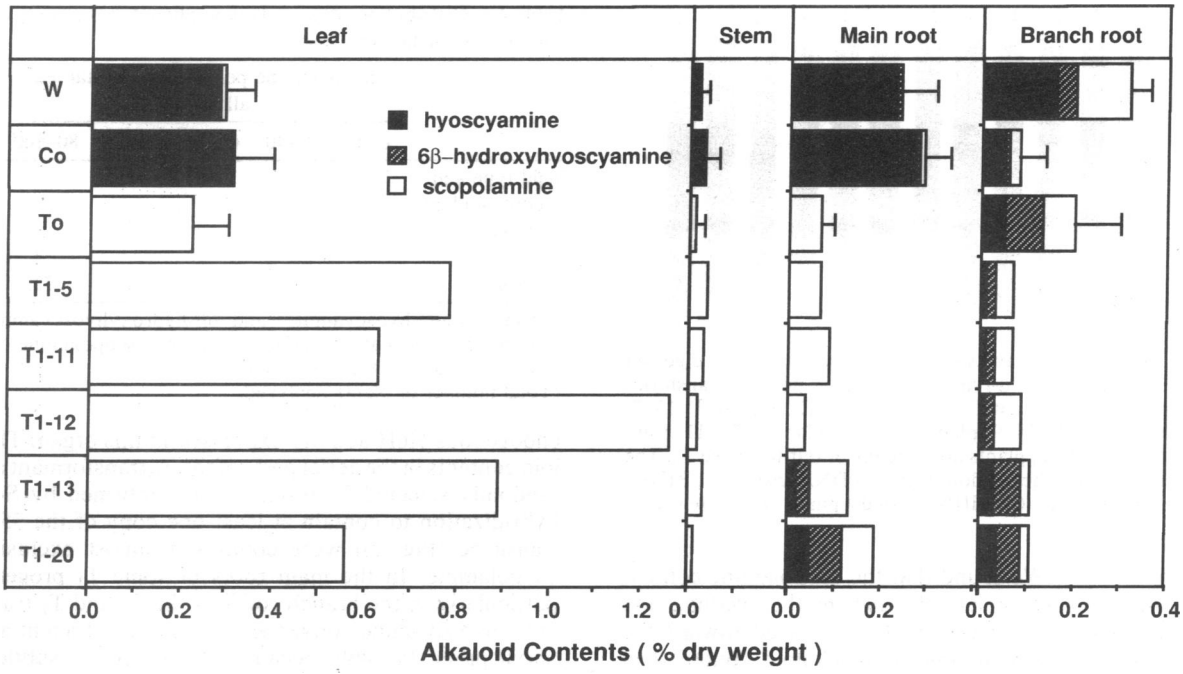

FIG. 4. Alkaloid contents in *A. belladonna*. W, wild-type plants; Co, control plants transformed with pGA482; T_0, transgenic plants transformed with pHY8; T_1, selfed progeny of T_0. After their seeds had been collected, the mature plants were harvested. All the T_0 and T_1 plants shown here had been confirmed to express the H6H polypeptide, at least in the leaves. Tropane alkaloid content was analyzed in the upper leaves, lower stem, main root, and branch root. Data for three independent plants each of W, Co, and T_0 were pooled and expressed as means ± SD.

In contrast to the highly efficient conversion of hyoscyamine to scopolamine in the aerial parts of the transgenic belladonna, the 6β-hydroxyhyoscyamine and scopolamine contents were enhanced only marginally in the branch roots. The H6H polypeptide and the enzyme activity were also expressed relatively strongly in the branch roots as well as in the aerial parts of the transgenic belladonna (Fig. 3). Although involvement of unidentified factors (e.g., novel enzymes) cannot be rigorously excluded, we suggest two alternative, nonexclusive possibilities for the difference in scopolamine content between aerial parts and roots. (*i*) Hyoscyamine may be synthesized in a restricted set of root cells in which the CaMV 35S promoter functions poorly. Specific localization of the endogenous H6H polypeptide in the pericycle cells of wild-type branch roots (23) indicates that its substrate hyoscyamine should be present, and may even be localized, in this cell type. The 35S promoter contains domain A (nucleotides −90 to +8), which drives the expression of the downstream gene in root tissue, including the pericycle (29). However, the strength of the entire 35S promoter in the pericycle cells, as compared with the other root cells, has not been reported. We analyzed the expression from the 35S promoter by using histochemical staining of the β-glucuronidase activity in another transgenic belladonna but found no evidence of poor expression of the reporter gene in the pericycle (data not shown).

(*ii*) Efficient conversion from hyoscyamine to scopolamine may occur during the translocation and storage of alkaloids. Classical reciprocal grafting experiments between alkaloid-producing and nonproducing solanaceous plants (26) showed that tropane alkaloids were synthesized mainly in the root and translocated to the aerial plant parts by a process also present in nonproducing plants. The feeding of labeled atropine (the racemic form of hyoscyamine) to steam-girdled belladonna plants indicated that tropane alkaloid translocation occurred through the xylem (T. Hartmann, personal communication). The alkaloids carried in the xylem sap ultimately are stored in various types of cells in the aerial parts, probably in the vacuole (30). The rate of alkaloid translocation in plants is not well understood but may be slow enough to allow the H6H expressed in the xylem or intervening tissues to act efficiently on alkaloids during translocation. The use of tissue-specific promoters to drive the expression of the H6H gene should provide more information about the tissues in which alkaloid conversion mainly takes place.

Although we used *A. belladonna* as the transgenic host, the metabolic engineering method reported here obviously is not limited to that species. Several medicinal plants that long have been known as rich sources of hyoscyamine (8) but that have been considered unattractive for commercial exploitation because of their low scopolamine contents may now become promising candidates as sources of scopolamine. Commercial cultivars of *Duboisia* hybrids that contain naturally high levels of scopolamine also may be improved by our method. Many of these solanaceous species are susceptible to *Agrobacterium* infection (31, 32), and they can be regenerated from tissue cultures to whole plants (32, 33). Metabolically engineered medicinal plants will complement conventional breeding efforts for the production of quantitatively and qualitatively improved pharmacological properties.

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