J. Amer. Soc. Hort. Sci. 148(6):304–314. 2023. https://doi.org/10.21273/JASHS05309-23

# Identification of Tropane Alkaloid Chemotypes and Genotypes in *Hyoscyamus niger* L.

**Lawrence Kramer and Sastry Jayanty**
*Department of Horticulture and Landscape Architecture, Colorado State University, 218 Nutrien Building, 301 University Avenue, Fort Collins, CO 80523, USA*

**David A. Reckhow**
*Department of Civil and Environmental Engineering, University of Massachusetts, 130 Natural Resources Road, Amherst, MA 01003, USA*

**Vidyasagar Sathuvalli**
*Hermiston Agricultural Research and Extension Center, Oregon State University, Hermiston, OR 97838, USA*

**Keywords.** hyoscyamine, hyoscyamine 6β-hydroxylase scopolamine, polymorphism, tropane alkaloids

**Abstract. Tropane alkaloids (TA) are compounds widespread in the Solanaceae family. The genera *Atropa*, *Brugmansia*, *Hyoscyamus*, and *Scopolia,* produce the pharmaceuticals hyoscyamine (Hy) and scopolamine (Sc), which are valued for their antimuscarinic and anticholinergic actions. The enzyme hyoscyamine 6β-hydroxylase (H6H) (EC 1.14.11.11) catalyzes both the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine and the epoxidation of the latter, leading to scopolamine (Hashimoto et al. 1993). During the examination of three genes in the TA biosynthetic pathway, the first committed step, the path branch point, and the final step in 13 accessions of *Hyoscyamus niger* from North America and Europe, genetic variations were found to be absent except in the *h6h* gene locus (GenBank: D26583.1). Quantification of TA showed average concentrations of 26 to 520 µg/g of dry leaf tissue among the accessions. From a monohybrid cross of the expected (Pennsylvania accession Ames 3103, *aa*) and novel (Netherlands accession PI 641691, *bb*) genotypes, the F2 population (n = 104) leaf and root tissues were extracted, analyzed for Hy and Sc contents, and compared with the *h6h* genotypes (*aa*, *ab*, *bb*). The polymorphism showed Mendelian inheritance. The presence of the polymorphic gene *bb* showed a marginally significantly greater concentration of hyoscyamine in the leaf tissue ($P = 0.0675$) and significantly greater concentration in root tissue ($P = 0.0436$), along with increased concentration of scopolamine in the root tissue ($P = 0.0494$) compared with the *aa* genotype. The increase in overall TA in the root tissue of the genotype *bb* was accompanied by a reduction in scopolamine in the foliar tissue. The 694-bp *b* amplicon has been sequenced for comparison with the expected 550-bp *a* amplicon and can be a useful enzymatic variant for TA metabolic engineering.**

Tropane alkaloids (TAs) are important natural compounds that are abundant in the Solanaceae, Erythroxylaceae, Convolvulaceae, Brassicaceae, and Euphorbiaceae families. These compounds are mostly toxins, and some have widespread pharmacological use (Kohnen-Johannsen and Kayser 2019). There are several tropane alkaloids. The major ones are L-hyoscyamine or the racemic mixture of L-hyoscyamine and D-hyoscyamine, atropine, which is used to dilate the pupil for retinal examinations and treat toxicity from organophosphate insecticide, nerve gas, and the toxic principles of the fungus *Amanita muscaria*. Another important TA, scopolamine, is used for motion sickness; additionally, it is used with morphine as a sedative during labor and as a remedy for stomach pain and cramping (Ullrich et al. 2017). Other important TAs include cocaine and calystegines, which are commonly used as a central nervous system stimulant and a β-glucosidase inhibitor, respectively (Dräger et al. 1994).

TAs have scattered distribution among angiosperm families. In species of the Solanaceae, which produce compounds such as atropine and scopolamine, the final steps in TA biosynthesis are catalyzed by a 2-oxogluterate-dependent enzyme (Hashimoto and Yamada 1986). Scopolamine is converted from hyoscyamine through a two-step process catalyzed by the enzyme hyoscyamine 6β-hydroxylase (H6H) (EC 1.14.11.11) (Kanegae et al. 1994). This enzyme catalyzes both the hydroxylation of hyoscyamine, transforming to 6β-hydroxyhyoscyamine, and the epoxidation of the latter, leading to scopolamine (Hashimoto et al. 1993).

The alkaloid synthesis in plants is species-specific (Chezem and Clay 2016). The structure of the alkaloids observed in various genera exhibits a degree of similarity depending on the closeness of their relationship within a family (Manske 1950). *Atropa belladonna*, *Datura stramonium*, *Duboisia myoporoides*, *Datura Leichhardt*, and *H. niger* are members of the family Solanaceae used by the pharmaceutical industry to produce TA for the manufacture of diagnostic and therapeutic drugs. These plants are harvested commercially for pharmaceutical and pesticide products (Bourgaud et al. 2001). Recently, researchers used ethyl methanesulfonate-based mutagenesis to increase the accumulation of scopolamine and hyoscyamine in *H. niger* L. (Chen et al. 2019).

*H. niger* is native to Eurasia and is now distributed globally. Historically, *H. niger* has been used as an anesthetic and because of its psychoactive properties (Kohnen-Johannsen and Kayser 2019). It continues to be used in folk medicine and pharmaceutical preparations of the active components hyoscyamine, scopolamine, and other tropane alkaloids (Roberts 2013). In this study,

Received for publication 13 Mar 2023. Accepted for publication 16 Oct 2023. Published online 15 Dec 2023.
We thank Dr. Lyle Craker, Department of Plant and Soil Science, University of Massachusetts, Amherst, MA, USA, for guidance and encouragement during the study.
S.J. is the corresponding author. E-mail: sastry.jayanty@colostate.edu.

*H. niger* was selected based on its plant size, growth habit, short life cycle, and availability of genetic material. *H. niger* is a monoecious plant that is easily emasculated before anthesis to prepare the selected female parents for crossing and amenable for genetic engineering.

All TA-producing plants have common biosynthetic pathways that begin with the synthesis of putrescine derived from the polyamine pool containing either ornithine or arginine (Hashimoto and Yamada 1994). The first committed step to alkaloid synthesis occurs with the formation of N-methylputrescine from putrescine by the enzymatic activity of putrescine N-methyltransferase (PMT) (EC 2.1.1.53) (Hashimoto and Yamada 1994). From this point, several diverging pathways are observed among various organisms, including those that lead to spermine, spermidine, and other alkaloids such as nicotine and cocaine (Kohnen-Johannsen and Kayser 2019). A key branch of alkaloids biosynthesis after the spontaneous formation of the N-methyl-Δ1-pyrrolinium cation is the conversion of tropinone to tropine by tropinone reductase I (TRI) (EC 1.1.1.206) (Dräger 2006). Tropine and phenyllactylglucose (derived from phenylalanine) react to produce littorine involving the enzyme littorine synthase (Qiu et al. 2020), leading to the production of hyoscyamine from reactions involving several recently identified enzymes (Huang et al. 2021; Li et al. 2006). The final significant transformation occurs with the intramolecular epoxide formation by H6H (EC 1.14.11.11) (Hashimoto and Yamada 1986). Figure 1 shows a summary diagram of TA biosynthesis.

During this study, accessions of *H. niger* from the United States, Eastern Europe, and Russia were assayed for polymorphic loci in the biosynthetic pathway. The novel allele was sequenced and annotated. The tropane alkaloid profile of the leaf and root tissues of the accessions and the $F_2$ population of a monohybrid cross was assayed. A chemotype × genotype analysis led to a discussion of improved TA production in *H. niger* and related species.

This study sought to determine tropane alkaloid concentrations and ratios of the available germplasm resources of *H. niger* from North America, Eastern Europe, and Russia to identify elite groups and individuals possessing higher TA concentrations, along with high scopolamine-to-hyoscyamine ratios. The plants were assessed to determine gene polymorphisms by focusing on three loci responsible for TA biosynthesis: the first committed step to alkaloid biosynthesis (*pmt*); the branch points to the tropane alkaloids hyoscyamine and scopolamine (*tr*I); and the final transformation from hyoscyamine to scopolamine (*h6h*) (Fig. 1). An assessment of the correlation between the presence of allelic forms with tropane alkaloid concentrations and ratios and the inheritance behavior in the segregating population was also performed.

## Materials and Methods

**General experimental procedures.** The TA and DNA from leaf and root tissues were extracted from 14 accessions of *H. niger*. The dry mass content of hyoscyamine and scopolamine were analyzed using high-performance liquid chromatography-ultraviolet or ultrahigh-performance liquid chromatography-tandem mass spectrometry in the selected $F_2$ population. Primers were designed and used to identify polymorphisms at genes involved in TA biosynthesis, particularly the *h6h* gene locus. A statistical analysis of the gene polymorphism effect on the hyoscyamine and scopolamine concentrations in leaf and root tissue was performed.

**Plant material.** Germplasm was acquired from United States Department of Agriculture, Northcentral Regional Plant Introduction Station, Ames, IA, USA (USDA 2018). Fourteen accessions originating from the United States (n = 7) and Eastern Europe (n = 7) (Table 1) were cultivated for the study.

Seeds of each accession were sown in Promix BX soilless sterile medium in 0801 cell packs under 15% shadecloth from germination to emergence (7–13 d). After an additional 2 weeks of growth, the plantlets were transplanted to 4.5-inch square or round pots in the same medium. The plant were grown in a random plot design in the greenhouse with 21 °C day and 18 °C night temperatures and supplemented with 400 W high-pressure sodium light with a minimum of 350 photosynthetic active radiation (PAR) during 16-h days and 8-h nights for 4 weeks. Fertilizer was applied with watering three times per week using Jack's Professional General Purpose 20–20–20 water-soluble fertilizer adjusted to 150 parts per million of nitrogen (J.R. Peters Inc., Allentown PA, USA). On alternate days and as needed, the plants were watered with tap water containing no fertilizer.

An array of crosses were made between individuals among the accessions (data not shown). The anthers of female parents were excised before flower anthesis; anthers of male parents were removed upon full anthesis. The pollen was applied to the stigma of the freshly emasculated female parent flowers. One-third of the leaf tissue was collected 28 d after the anthesis of each plant, preserving the plant for seed development, and analyzed to determine hyoscyamine and scopolamine. After the analysis of the alkaloid proportions in the leaf tissue, four lines of seed developing from the crosses were propagated for the $F_1$ generation. After the genotype analysis, one line was rejected as having the same allele form *bb* in both parents. Line 03-4 × 91-1 was selected for a segregation analysis of the $F_2$ population based on the genotype and chemotype of the parents and their accession means (Table 1). The $F_2$ population was generated from artificially self-pollinated $F_1$ individuals 03-4-5 × 91-1-2. Leaf and root tissues were collected from the $F_2$ population 28 d after anthesis (n = 104) and freeze-dried; then, the tropane alkaloids and DNA were extracted.

### Chemotyping

**Extraction of alkaloids.** Modifications of four methods were used for the extraction and quantification of TA (Fliniaux et al. 1993, Häkkinen et al. 2005, Kamada et al. 1986, Mroczek et al. 2006) to accommodate the available instrumentation and experimental design.

Accessions were extracted as described by Kamada et al. (1986) with modifications: ground plant material was added to chloroform:methanol:25% ammonium hydroxide (15:5:1; 10 mL per 100 mg of powdered sample) and placed for 1 h on an orbital shaker at 40 °C; then, the extract was held for 1 h in an ultrasonic bath. After holding at room temperature for 1 h, the extract was filtered and washed with 1 to 2 mL chloroform and evaporated to dryness at 40 °C under a stream of nitrogen gas. The residue was resolved in 5 mL chloroform and 2 mL of 0.5 M sulfuric acid with thorough mixing. After removing the chloroform, the alkaloid fraction was maintained on ice and adjusted to pH 10 to 11 with 28% ammonium hydroxide. Then, alkaloids were isolated once with 2 mL of chloroform and twice with 1 mL of chloroform each. After adding anhydrous sodium sulfate, filtering, and washing the residue with 1 to 2 mL of chloroform, the extracted

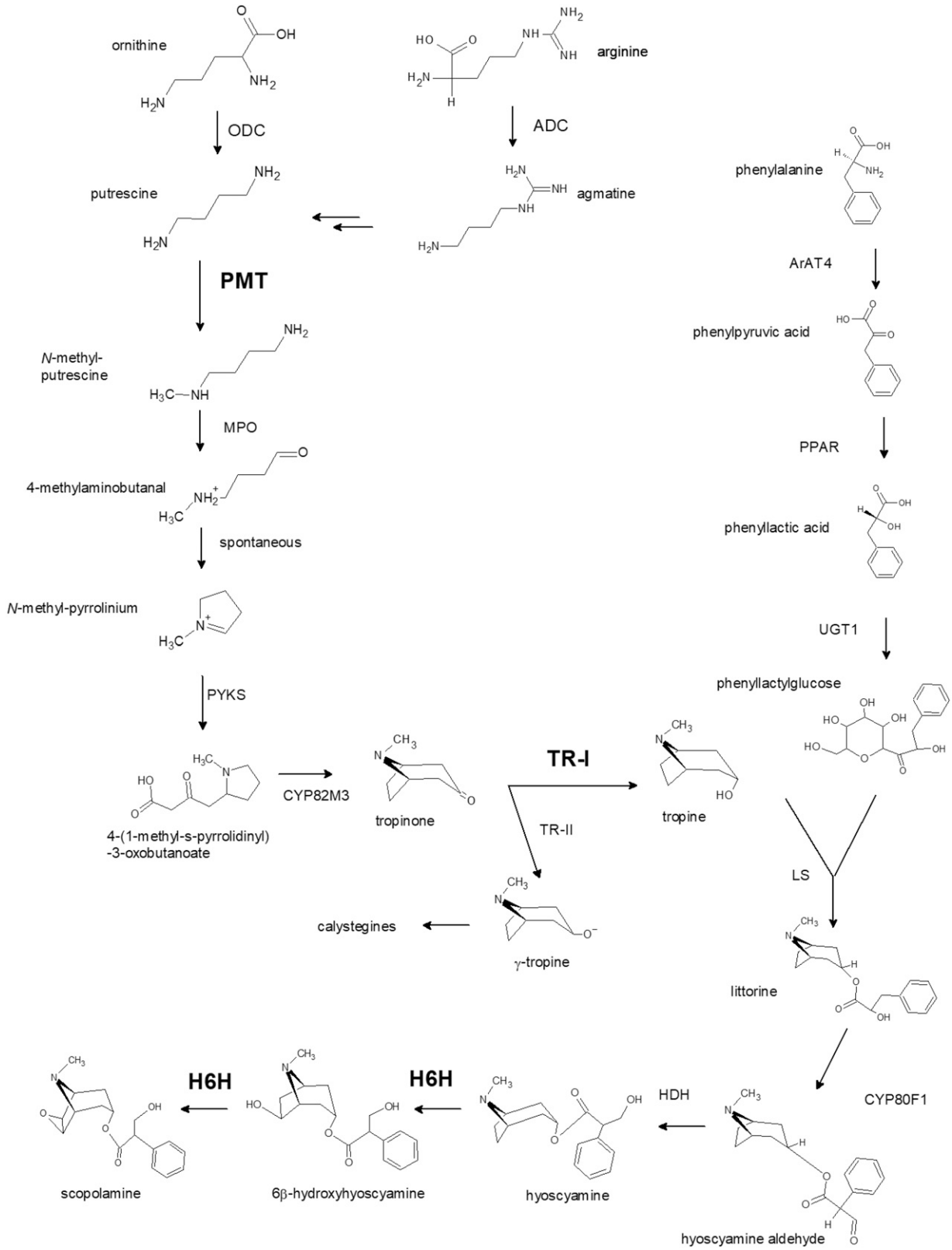


Fig. 1. Tropane alkaloid biosynthesis in *Hyoscyamus niger*. ADC = arginine decarboxylase; ArAT4 = aromatic amino acid aminotransferase; CYP80F1 = littorine mutase; CYP82M3 = tropinone synthase; H6H = hyoscyamine 6β-hydroxylase; HDH = hyoscyamine dehydrogenase; LS = littorine synthase; MPO = *N*-oxidase; ODC = ornithine decarboxylase; PMT = putrescine *N*-methyltransferase; PPAR = phenylpyruvic acid reductase; PYKS = pyrrolidine ketide synthase; TRI = tropinine reductase I; TRII = tropinone reductase II; UGT1 = phenyllactate UDP-glycosyltransferase. The gene sequences for the enzymes in bold were addressed in this study.

Table 1. Chemotype grouping of *Hyoscaymus niger* accessions. Parental individuals were selected from the highlighted accessions based on the Duncan grouping of scopolamine (Sc)-to-hyoscyamine (Hy) ratio and total tropane alkaloids (Sc+Hy) in leaf tissue among henbane (*Hyoscyamus niger* L.) accessions. The accessions used in the experiment are highlighted.

| Accession | Origin | N | Mean Sc:Hy | SD | Duncan grouping | Mean Sc+Hy | SD | Duncan grouping |
|---|---|---|---|---|---|---|---|---|
| Ames 15563 | Netherlands | 15 | 1.6 | 2.286 | F | 5.6 | 4.1 | DE |
| Ames 15565 | Germany | 18 | 1.3 | 1.68 | F | 7.6 | 5.0 | D |
| Ames 3098 | Maryland, USA | 27 | 7.0 | 9.153 | C | 19.3 | 12.4 | B |
| Ames 3102 | Maryland, USA | 42 | 5.7 | 5.277 | CD | 14.2 | 18.6 | C |
| Ames 3103 | Pennsylvania, USA | 34 | 20.1 | 45.108 | A | 15.9 | 22.5 | BC |
| Ames 3104 | Maryland, USA | 24 | 4.5 | 5.916 | DE | 5.8 | 2.7 | DE |
| Ames 3105 | Maryland, USA | 24 | 2.0 | 1.518 | F | 6.5 | 1.8 | DE |
| Ames 3155 | Massachusetts, USA | 21 | 2.9 | 1.53 | EF | 3.6 | 2.8 | E |
| PI 596383 | Russian Federation | 8 | 2.4 | 1.622 | F | 3.5 | 1.9 | E |
| PI 596388 | Russian Federation | 22 | 5.1 | 5.352 | D | 12.5 | 11.9 | C |
| PI 641691 | Netherlands | 22 | 17.0 | 24.913 | B | 52.0 | 66.4 | A |
| PI 649671 | Germany | 20 | 2.2 | 1.464 | F | 2.6 | 1.2 | E |
| PI 649672 | Finland, Turku ja Pori | 3 | 1.9 | 1.18 | F | 13.7 | 21.1 | C |

N = number of extractions. Sc:Hy = [Sc]/[Hy]. Sc+Hy = [Sc]+[Hy] in $\mu g \cdot gdm^{-1}$.

TAs were evaporated to dryness and resolved as a 2-mL mobile phase for the high-performance liquid chromatography-ultraviolet analysis.

After the isolation of alkaloids using a solid phase extraction method (Mroczek et al. 2006), the $F_2$ population was analyzed with ultra-performance liquid chromatography tandem mass spectrometry. Lyophilize and powdered plant material were sonicated for 15 m in 10 mL of 1% tartaric acid in methanol per 100 mg of tissue. After filtering through Whatman no. 5 filter paper and washing the filtride with 2 mL of 1% tartaric acid in methanol, the extract was evaporated to dryness under reduced pressure at 40 °C. The residue was resolved in 5 mL 0.05 M HCl and sonicated briefly until dissolved. The solid phase was extracted with a Waters Oasis MCX 6-mL 30-μm cartridge. Conditioning with 5 mL of 0.5 M HCl on a vacuum manifold adjusted to 0.01 MPa (approximately one drop every 3 s) resulted in a 2-mm layer of 0.05 M HCl over the sorbent. The extract was applied to the cartridge on a vacuum manifold adjusted to 0.01 MPa (approximately one drop every 3 s) and the eluate was discarded. The sorbent was washed with 4 mL of methanol and dried at full vacuum for 1 min. The TA was eluted with 10 mL methanol–10% ammonium hydroxide 3:1 (volume/volume). The eluant was evaporated to dryness under a gentle stream of nitrogen gas and resolved as a liquid chromatography mobile phase for analysis.

**Detection and quantification of alkaloids.** Alkaloid extracts from accessions and their $F_1$ progeny were resolved in 5 mL of a mobile phase consisting of acetonitrile–0.3% phosphoric acid adjusted to pH 2.2 with triethylamine (1:7). Analytic samples were filtered through a Whatman 13-mm × 2-μm nylon syringe filter into 2-mL screw cap autosampler vials. The chromatographic system consists of a Waters Alliance 2690/5 separation module, a Waters 996 photodiode array ultraviolet/Vis detector, and Waters Empower 2 Pro software. The separation column was a Supelco Adventis 15-cm × 4.3-mm C18 reverse-phase column with 2.6-μm packing. Two mobile phase formulations were used. The first mobile phase carrier solvent (Fliniaux et al. 1993) was 12.5% aqueous acetonitrile with 0.3% phosphoric acid adjusted to pH 2.20 with triethylamine. This mobile phase was set at a flow rate of 1 mL/min. The second mobile phase (Zárate et al. 2006) was 78% 50 mM potassium phosphate monobasic ($KH_2PO_4$) adjusted to pH 3.0 with phosphoric acid and 22% acetonitrile. The flow rate for this mobile phase was 0.8 mL/min.

Standards purchased from Sigma Chemical Company (scopolamine hydrobromide; St. Louis, MO, USA) and MP Biomedicals Inc. (hyoscyamine hydrochloride; Solon, OH, USA) were used for calibration at 0, 10, 20, 40, 100, 200, and 400 ng per 20-μL injection. A combined stock solution for l-scopolamine HBr and l-hyoscyamine HCl standards was prepared and consisted of 1 μg/mL (1000 ppm) of each. From the stock, calibration standards were made by serial dilution.

Samples and calibration standards were maintained at 4 ± 2 °C in the sample chamber. Injections into the separation module had a volume of 20 μL and were performed by an autosampler with a run time of 45 min for the 0.3% phosphoric acid mobile phase and 30 min for the 50 mM potassium phosphate mobile phase. Photodiode array readings were configured to 200- to 400-nm spectral scans and detection of TAs at 204 nm absorbance. Absorbance values were recorded for 0 to 15 min for analysis after injection. Data collection and peak integration were performed using Empower 2 Pro Millenium 32 software (Waters Corporation, Milford, MA, USA) configured with peak purity matching and spectral library matching of the analytes and with automatic integration and quantification after generating a standard calibration curve. The calibration curve was a linear plot in ug/μg per gram dry mass (gdm) of standard injected vs. the area of absorbance units at 204 nm for the two peaks.

For the $F_2$ population, concentrations of scopolamine and hyoscyamine were determined by the liquid chromatography–mass spectrometry analysis. Chromatographic separation was performed using Waters Acquity ultra-performance liquid chromatography equipped with a BEH C18 column (100 mm × 1 mm, 1.7-μm particle size), 2996 photodiode array, and 2905 autosampler (Waters, Milford, MA, USA). Mobile phase eluent A was 0.5% volume/volume formic acid in methanol:water (5:95), and eluent B was 0.5% formic acid in methanol. The gradient was as follows: 3% B held for 0.48 min, 4.32 min at 95% B on a linear slope, 5.44 min at 95% B, 5.76 min 3% B on a linear slope, and, finally, 3% B at 8.00 min. The flow rate was 0.400 $mL \cdot min^{-1}$. The column was maintained at 40 °C.

The column outlet was connected to a MicroMass Quattro Premiere XE triple quadrupole mass spectrometer (Waters, Milford, MA, USA). The source electrospray inlet was set to 3.1 kV in the ESI+ mode. The source conditions were 550 $l \cdot h^{-1}$ desolvation and 50 $l \cdot h^{-1}$ cone nitrogen gas. Voltages were 25 V (cone), 3 V (extractor), and 0.4 V (radiofrequency). Temperatures were set to 340 °C for desolvation and 140 °C for the source. The compounds were identified in the multiple reaction monitoring mode with the argon collision gas set to 0.15 $mL \cdot min^{-1}$ ($3.8 \times 10^{-3}$ kPa) and a collision energy of 23 V. Under these conditions, the analytes were detected at 2.38 min (scopolamine) and 2.62 min (hyoscyamine).

A combined stock standard solution containing 1 $\mu g \cdot mL^{-1}$ each scopolamine HBr and hyoscyamine HCl was directly infused into the mass spectrometer to optimize the detection parameters. Multiple reactions monitoring tandem mass spectrometry acquisition was used to detect and confirm scopolamine at the transitions of 304.33 > 137.91 and 304.33 > 156.24 and hyoscyamine at the transitions of 290.12 > 124.33 and 290.12 > 92.75 (Fig. 2). Calibration standards were prepared by serial dilution to achieve concentrations of 50, 25, 12.5, 6.25, 3.125, and 1.5625 $ng \cdot mL^{-1}$. The injection volume of standards and samples into the separation module were 5 μL, with a run time of 8 min. TA concentrations were quantified using external standards to calculate the calibrations and concentrations.

**DNA EXTRACTION AND GENOTYPING.** DNA from the accessions and $F_2$ plants and the parents were extracted using a modified mini-prep CTAB method described by Bernatzky and Tanksley (1986). A 1.5-mL microcentrifuge tube half-filled with powdered lyophilized leaf tissue (~50 mg) was extracted with 750 μL of buffer heated to 65 °C. The buffer consisted of 3:3:1 extraction buffer:lysis buffer:5% sarcosyl, and 0.1 g sodium metabisulfite per 50 mL buffer. Portions were homogenized for several minutes with a mortar and pestle, and then incubated in a water bath at 65 °C for 1 h. Next, 700 μL of chloroform:isoamyl alcohol (24:1) was added, mixed, and centrifuged at 10,000 rpm at 4 °C for 5 min. The aqueous phase was transferred to a new tube, and the DNA was precipitated with 700 μL isopropanol after gentle mixing. The tube was centrifuged for 1 m at 10,000 rpm (Marathon 13K/M centrifuge, Fisher Scientific), the supernatant was discarded, and the pellet was washed twice with 70% ethanol, washed once with 95% ethanol, and left to dry at room temperature for 0.5 h. Finally, the DNA was resolved in 60 μL of 0.5X TE buffer at 65 °C in a water bath for 0.5 h with occasional gentle mixing. The resolved DNA was transferred to a clean tube and stored at −20 °C.

From each DNA sample, 15 μL was checked for quality and quantity on 0.9% agarose gel made with 0.4X TBE and loaded with 5 μL of tracking dye. The samples were run on a 100- × 150- × 5-mm (75 mL) gel for 2 h at 50 V. The gel was stained with ethidium bromide, visualized on a ultraviolet light table, and photographed. Bands were compared with 50 ng and 500 ng λ-DNA to estimate the quantity.

Primers were constructed to amplify three gene loci for enzymes contributing to the biosynthesis of TA. The enzyme PMT is involved in the removal of putrescine from the polyamine pool because it catalyzes the N-methylation of this diamine to form N-methylputrescine (Suzuki et al. 1999). TRI is the enzyme that reduces tropinone to tropine, which is the initial divergent product leading to hyoscyamine. Alternatively, TRII catalyzes the reduction of tropinone to pseudotropine, which is the first product leading to the calystegine group of TA in *H. niger* (Nakajima and Hashimoto 1999). H6H, a 2-oxo-glutarate-dependent dioxygenase, catalyzes the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine, as well as the epoxidation of 6β-hydroxyhyoscyamine to scopolamine (Hashimoto and Yamada 1987).

Sequence information for the target genes (*pmt*, *tr1*, *h6h*) were found on NCBI Entrez Nucleotide (http://www.ncbi.nlm.nih.gov/sites/gquery). Primers for the *tr1* and *h6h* gene loci were designed to amplify intron segments by first aligning cDNA and

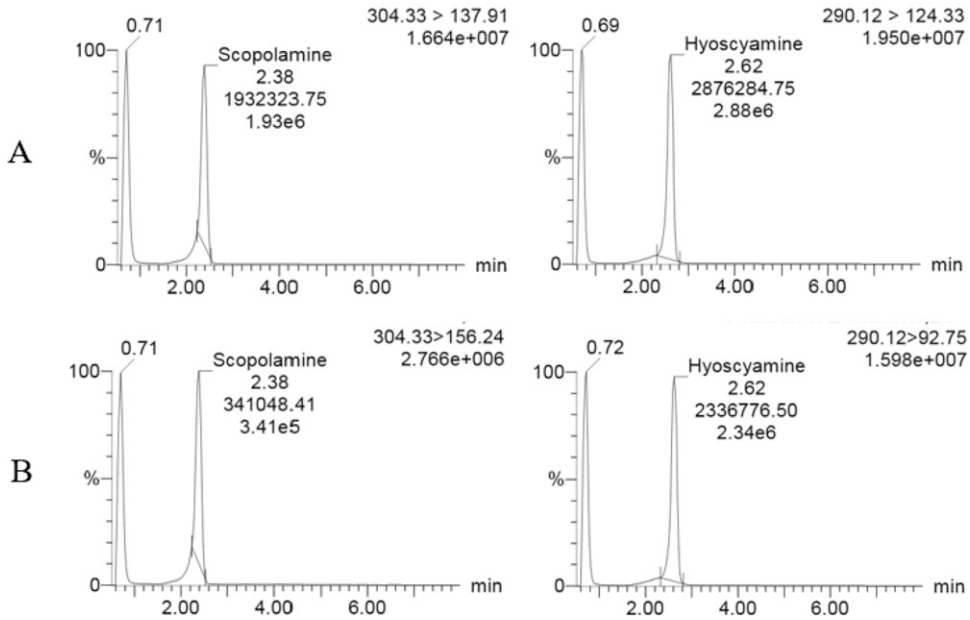


Fig. 2. Mass spectrometry multiple reaction monitor of scopolamine (left column) and hyoscyamine (right column) standards: (**A**) quantitative transition and (**B**) confirmation transition.

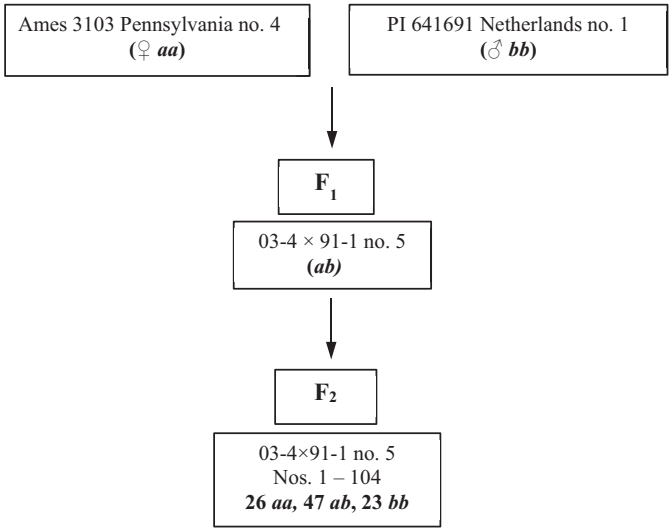


Fig. 3. Parents, $F_1$, and $F_2$ *Hyoscyamus niger* used in the experiment. The genotypes of the individuals are shown in bold. $F_1$ and $F_2$ are abbreviated with the last two digits of the accession number. The $F_2$ population genotypes segregated with 1:2:1 Mendelian distribution [$\chi^2 = 0.2292$; degrees of freedom (df) = 2; $P = 0.005$].

genomic DNA sequence information, and then by specifying primer returns from nucleotide sequences on the intron–exon borders (Supplemental Table 1). Primers for the *pmt* gene locus were designed using cDNA sequence information because the *H. niger* complete DNA sequence of this locus is unavailable. The primers were designed using Primer 3 (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Untergasser et al. 2012). The primers P5, P6, T8, and T9 were synthesized by Invitrogen Co. (Carlsbad, CA, USA). Integrated DNA Technologies Inc. (Coralville, IA, USA) manufactured all other primers. The amplified sequences were separated and distinguished by size (in bps) by gel electrophoresis and scored to identify polymorphisms.

**Statistical analysis.** Accessions were extracted and analyzed for scopolamine and hyoscyamine concentrations, and the component ratio (scopolamine:hyoscyamine) and total TA (scopolamine+hyoscyamine) were calculated. A comparison of the means with the Duncan's multiple range test groupings (Duncan 1955) was performed using SAS 9.4 software (SAS Institute Incorporated, Cary, NC, USA) to determine their chemotype (Table 1). Groupings for each variable among accessions and the variable values of the individual parental plants were used to select the parents for the breeding groups.

A statistical analysis and graphing of the parental F1 and F2 populations was performed using R (R Core Team 2021), RStudio (RStudio 2021), and the following R packages: broom (Couch et al. 2021), dplyr (Wickham et al. 2021) dunn.test (Dinno 2017), emmeans (Lenth 2021), ggplot2 (Wickham 2016), and ggrepel (Slowikowski 2021). Code and datasets are available in the Supplemental Information, Supplemental Fig. 3, and at GitHub (www.github.lunxkhiro/).

## Results and Discussion

During this study, concentrations of hyoscyamine in foliar tissues ranged from 10 to 51 $\mu g \cdot gdm^{-1}$ among the accessions, and 14 to 326 $\mu g \cdot gdm^{-1}$ scopolamine. Reciprocal crosses were performed between accessions. The root tissue hyoscyamine and scopolamine of the parental and F1 generations were not measured to avoid destructive harvest, preserving plant vitality for the F2 generation. The parental accessions selected for the monohybrid cross population were Ames 3103 ($P_1$ ♀, origin in Pennsylvania, USA) and PI 161691 ($P_2$ ♂, origin in the Netherlands). Leaf tissue hyoscyamine was 19 to 22 $\mu g \cdot gdm^{-1}$ and scopolamine was 63 to 121 $\mu g \cdot gdm^{-1}$ in the $F_1$ plants (n = 20). The parents Ames 3103 and PI 641691 had mean scopolamine and hyoscyamine values of 15.9 $\mu g \cdot gdm^{-1}$ and 52.9 $\mu g \cdot gdm^{-1}$, respectively. The $F_2$ mapping population (n = 104) contained concentrations of hyoscyamine from 1.29 to 109.99 $\mu g \cdot gdm^{-1}$ in leaf tissue and 25.33 to 488.31 $\mu g \cdot gdm^{-1}$ in root tissue. The $F_2$ scopolamine concentrations ranged from 1.25 to 99.49 $\mu g \cdot gdm^{-1}$ in foliar tissues and from 3.81 to 215.34 $\mu g \cdot gdm^{-1}$ in root tissues.

The mean scopolamine-to-hyoscyamine ratio was most significant in the Pennsylvania accession, followed by the Netherlands accession, whereas the mean TA was significantly higher in the Netherlands accession (Table 1). It should be noted that the native origins of the Maryland, Pennsylvania, and Massachusetts accessions are unknown.

**Chemotype analysis.** Accessions were extracted and analyzed for scopolamine and hyoscyamine concentrations, and the component ratio (scopolamine:hyoscyamine) and total TA (scopolamine+hyoscyamine) were calculated. Comparison of the means with Duncan's multiple range test groupings (Duncan 1955) were calculated with SAS 9.4 software (SAS Institute Inc., Cary, NC, USA) to determine their chemotype (Table 1). Groupings for each variable among accessions and the variable values of the individual parental plants were used to select the parents for the four breeding groups (Table 2).

**Polymorphism among the breeding groups.** The parents and a hybrid from each breeding group (Table 2) were

Table 2. Parents and hybrids of *Hyoscyamus niger* used in the experimental design. Leaf tissue was analyzed for scopolamine (Sc) and hyoscyamine (Hy) for each of the parental (P) individuals from each accession. The plant genotype (G) is based on the hyoscyamine 6b-hydroxylase allele. Sc and Hy are shown in micrograms per gram dry mass. The genotype is an $F_1$ offspring from line III that was selected to generate the $F_2$ segregating population.

| Line | $P_1$ ♀ | G | Sc | Hy | Sc:Hy | $P_2$ ♂ | G | Sc | Hy | Sc:Hy |
|---|---|---|---|---|---|---|---|---|---|---|
| I[i] | PI 641691-4 | *bb* | 1.1 | 0.5 | 2.2 | Ames 3102-2 | *aa* | 25.4 | 3.5 | 7.2 |
| II[i] | Ames 15563-1 | *bb* | 5.9 | 3.9 | 1.5 | Ames 3105-2 | *aa* | 7.0 | 2.1 | 3.3 |
| III[ii] | Ames 3103-4 | *aa* | 30.3 | 1.7 | 17.8 | PI 641691-1 | *bb* | 62.8 | 25.0 | 2.51 |
| $F_1$[iii] | 03-4 × 91-1-5 | *ab* | 90.2 | 19.6 | 4.6 | | | | | |
| IV[ii] | PI 596383-4 | *bb* | 14.5 | 1.5 | 9.7 | Ames 15563-1 | *bb* | 14.9 | 16.6 | 0.9 |

[i] Low × high Sc:Hy ratio parents.
[ii] High × low Sc:Hy: ratio parents.
[iii] Mean values for leaf tissue in hybrid individual number 5.

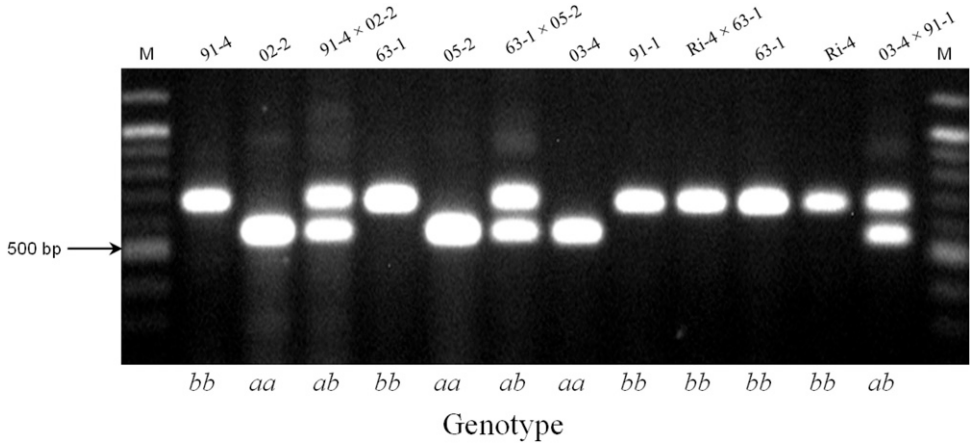


Fig. 4. The h6h H6 primer (see Supplemental Table 1) polymerase chain reaction (PCR) amplification of genomic DNA demonstrating two alleles: the expected 550-bp segment of the *h6h* sequence (*a*) and a novel 690-bp segment (*b*). The lanes are marked with the parental and $F_1$ individuals selected for the four breeding lines. The *aa* and *bb* genotypes were selected for the parental monohybrid cross from accessions Ames 3104 and PI 641691. The $F_1$ population was *ab* genotype. M lanes are 100-bp markers. Numbered lanes are the abbreviated plant line and plant number of the parents and the hybrid for each of the breeding lines.

examined for DNA polymorphisms at the loci *pmt* (primers P1, P4, P6), *tr1* (primers T2, T7), and *h6h* (primers H1, H3, H4, H6). These gene loci were selected for three committed steps in TA biosynthesis: putrescine n-methyltransferase, which catalyzes the *S*-adenosylmethionine dependent *N*-methylation of putresine, is the first committed and rate-limiting step to tropane alkaloid biosynthesis (Geng et al. 2018); tropinone reductase I (TRI), a NADPH-dependent short-chain dehydrogenase that constitutes a branch point in tropane alkaloid biosynthesis, is the enzyme that reduces tropinone to tropine, which is the divergent product leading to hyoscyamine; H6H, a 2-oxoglutarate dependent dioxygenase, catalyzes the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine as well as the epoxidation of 6β-hydroxyhyoscyamine to scopolamine (Hashimoto and Yamada 1987) (Fig. 1).

The expected amplicon for primer P1 (Supplemental Fig. 1.1A) was visible in 10 of the 12 samples. A stronger band was found in nine samples near the 1500 bp marker. Several other weak bands were observed in most samples. Primer P4 (Supplemental Fig. 1.1B) amplified a strong signal near 1100 bps, and a very weak band can be seen near the expected size of 455 bps. *Pmt* primer P6 amplified a product much larger than expected near 1400 bps (Supplemental Fig. 1.1C). However, the primer was designed using a cDNA template, and the product seen likely contains intron segments. The strong signal is visible in eight samples, with two additional samples exhibiting a weaker signal.

The *tr1* gene locus was amplified by primer T2 in all samples (Supplemental Fig. 2D). In addition to the expected size, the $P_1$ of breeding group I and both parents and hybrid of breeding group IV showed a dense band of approximately 400 bps. This curious result may indicate that polymorphisms at some sites bordered with a sequence homologous to the primer complement. Primer T7 amplified a 695-bp fragment, but there were also faint bands of various sizes for all the samples, indicating the occurrence of nonspecific annealing.

Primers H1, H3, and H4 did not show any prominent polymorphism at the loci (Supplemental Fig. 1.2F, G, and H), and each amplified the expected fragment from the *h6h* gene locus. H1 and H3 showed multiple bands of nonspecific annealing; primer H4 was specific and amplified a single fragment of the anticipated size. The marker H6 (Supplemental Fig. 1.2I) reveals two distinct alleles at the *h6h* locus. One was the expected size of 550 bps and the other was 690 bps, herein referred to as *a* and *b,* respectively (Fig. 4). Parent 91-4 (lane 1) is homozygous for allele *b,* and parent 02-2 (lane 2) is homozygous for *a*. Their hybrid in lane 3 (IF1–5) is the heterozygote *ab*. Breeding group II is the same. In group III, the female carries the *aa* genotype and the male carries the *bb* genotype. In breeding group IV, both the parents and hybrid are the *bb* genotype. All parents of North American populations are the homozygous *aa* genotype (accessions 02, 03, and 05). The other accessions (63, 91, and Ri), which are of European origin, are *bb* genotypes.

Among breeding groups I, II, and III, the parents were diallelic homozygotes, and their hybrids were heterozygous. Breeding group IV parents were monoallelic homozygotes, and the hybrid was homozygous. Breeding group III was selected for further study (Table 2, Fig. 3) based the ratio of scopolamine to hyoscyamine. Breeding group III was a cross of a high ratio with a low ratio of parental lines.

**Segregation analysis.** The $F_2$ population distribution of *aa*, *ab*, and *bb* genotypes were 26, 47, and 23, respectively. The chi-square correlation to Mendelian distribution indicated $\chi^2 = 0.2292$, with 2 degrees of freedom and $P = 0.005$. The segregation followed Mendelian segregation.

**Multiple sequence alignment and annotation of *h6h* and *b* genotypes.** Samples of DNA from the $F_2$ population possessing the *aa* and *bb* genotypes (three of each) were amplified with primer set H6, separated on agarose gel, and extracted from the gel with the Monarch DNA Gel Extraction Kit (New England Biolabs Inc., Ipswich, MA, USA). The purified amplicons were Sanger-sequenced (Eurofin Genomics, Louisville, KY, USA). A consensus sequence was constructed for each genotype using Genome Workbench (Kuznetsov and Bollin 2020, 2021) and compared with the DNA consensus sequence of the published *H. niger* hyoscyamine 6β-hydroxylase sequence (Kanegae et al. 1994, Kanegae et al. 1994). The sequences of the *aa* genotype were identical to those of the *h6h* gene DNA, producing the expected 550-bp amplicon. The three *aa* homozygous allele sequences contained several single nucleotide polymorphisms

(data not shown). The *bb* genotype consensus sequence was 633 bp after trimming. The amplicon overlapped exons 3 and 4 of the *h6h* gene, with a segment in the intervening intron comprising 106 bp not present in the expected nucleotide sequence (Fig. 5).

**Correlation of genotype to chemotype.** The least-square means of the genotypes hyoscyamine and scopolamine concentrations were calculated with means using R (Lenth 2021). The contrast function was used to compare hyoscyamine and scopolamine concentrations in leaf and root tissues between the *aa* vs. *ab*, *aa* vs. *bb*, and *aa* vs. *ab* + *bb* genotypes (Table 3). The *bb* genotype showed a significantly higher concentration of scopolamine in root tissue than the *aa* genotype ($P = 0.0494$). The hyoscyamine content in root tissue was similar, but only marginally significant ($P = 0.0665$) (Wasserstein and Lazar 2016); however, the contrast of *aa* vs. *ab* + *bb* was significantly greater among the individuals possessing the *b* allele ($P = 0.0436$). No significant differences between the genotypes were observed in foliar tissue hyoscyamine and scopolamine.

To some degree, the concentrations of hyoscyamine and scopolamine in leaf tissue followed the same pattern as the instances described for the root tissue. This is supported by the median, quadrants, and outliers shown in Fig. 6. The median and quadrant distributions of hyoscyamine and scopolamine are increased in the $F_2$ population, corresponding to the presence of the *b* allele. Furthermore, outliers with exceptionally high concentrations of hyoscyamine and scopolamine were more pronounced with the presence of the allele *b* (Table 4).

Specific outliers in the plant population with increased levels of hyoscyamine, scopolamine, or both are candidates for selection in breeding (Fig. 6, Table 4). $F_2$ plants numbered 50 and 52 possessing the *bb* genotype have increased levels of hyoscyamine in leaf tissue (102.19 and 109.99 $\mu g \cdot gdm^{-1}$, respectively), which is almost 4.5-times the mean of the population. Plant 50 had 88.87 $\mu g \cdot gdm^{-1}$ scopolamine in leaf tissue. However, at 99.48 $\mu g \cdot gdm^{-1}$, plant 79 (*ab* genotype) contained the most leaf tissue scopolamine in the population, which is 4.4-times more than the population mean. Plant 26 contained 488.31 $\mu g \cdot gdm^{-1}$ hyoscyamine in the root tissue, and plant 4 had 215.34 $\mu g \cdot gdm^{-1}$ scopolamine in the root tissue. Both plants were *ab* genotypes.

Table 3. $F_2$ population least-square means and orthogonal contrasts of tropane alkaloids scopolamine and hyoscyamine content between genotypes in *Hyoscyamus niger*. The F2 population was generated from a cross between the *aa* and *bb* genotypes of the hyoscyamine 6β-hydroxylase gene.

| Root tissue | | | | |
|---|---|---|---|---|
| Scopolamine | | | | |
| Genotype | LS means[i] | *SE* | Lower CL[ii] | Upper CL[iii] |
| *aa* | 32.1 | 6.80 | 18.6 | 45.6 |
| *ab* | 37.2 | 5.22 | 26.8 | 47.6 |
| *bb* | 53.3 | 8.17 | 37.0 | 69.5 |
| Contrasts | Estimate | *SE* | t ratio | *P* value |
| *aa* vs. *ab* | −5.09 | 8.57 | −0.594 | 0.5440 |
| *aa* vs. *bb* | −21.18 | 10.62 | −1.994 | 0.0494* |
| *aa* vs. *ab*+*bb* | −13.14 | 8.35 | −1.574 | 0.1192 |
| Hyoscyamine | | | | |
| Genotype | LS means | *SE* | Lower CL | Upper CL |
| *aa* | 98.3 | 16.5 | 65.5 | 131 |
| *ab* | 133.4 | 12.7 | 108.2 | 159 |
| *bb* | 146.3 | 19.8 | 106.8 | 186 |
| Contrasts | Estimate | *SE* | t ratio | *P* value |
| *aa* vs. *ab* | −35.1 | 20.8 | −1.685 | 0.957 |
| *aa* vs. *bb* | −48.0 | 25.8 | −1.859 | 0.0665† |
| *aa* vs. *ab*+*bb* | −41.5 | 20.3 | −2.048 | 0.0436* |
| Leaf tissue | | | | |
| Scopolamine | | | | |
| Genotype | LS means | *SE* | Lower CL | Upper CL |
| *aa* | 20.0 | 3.81 | 12.4 | 27.6 |
| *ab* | 20.9 | 2.93 | 15.1 | 26.7 |
| *bb* | 26.3 | 4.58 | 17.2 | 35.5 |
| Contrasts | Estimate | *SE* | t ratio | *P* value |
| *aa* vs. *ab* | −0.916 | 4.81 | −0.190 | 0.8494 |
| *aa* vs. *bb* | −6.332 | 5.96 | −1.062 | 0.2912 |
| *aa* vs. *ab*+*bb* | −3.624 | 4.68 | −0.774 | 0.4413 |
| Hyoscyamine | | | | |
| Genotype | LS means | *SE* | Lower CL | Upper CL |
| *aa* | 17.8 | 4.16 | 9.5 | 26.0 |
| *ab* | 21.2 | 3.20 | 14.9 | 27.6 |
| *bb* | 29.8 | 5.00 | 19.9 | 39.8 |
| Contrasts | Estimate | *SE* | t ratio | *P* value |
| *aa* vs. *ab* | −3.45 | 5.25 | −0.657 | 0.5131 |
| *aa* vs. *bb* | −12.05 | 6.51 | −1.852 | 0.0665† |
| *aa* vs. *ab*+*bb* | −7.75 | 5.11 | −1.516 | 0.1332 |

[i] Least-square (LS) means, degrees of freedom (df) = 85.
[ii] Lower confidence limit (CL).
[iii] Upper confidence limit.
* Significant at $P < 0.05$.
† Marginally significant (Wasserstein and Lazar 2016) at $P < 0.067$.

## Conclusions

The *h6h b* allele is a marker for plants that produce more significant amounts of TA, most notably in root tissues. With the presence of the variant allele, elite individuals possessing exceptionally high concentrations of TA were seen in the population. These individuals are both homozygous and heterozygous, presumably demonstrating partial dominance of *b* allele. A marker easily detected with the primer H6 can identify these plants.

Among the tropane alkaloid-producing plants, the H6H enzyme varies between species, and the enzyme in *H. niger* is known to be the most active in the completion of biochemical pathway from hyoscyamine to scopolamine. Lan et al. (2018) compared the scopolamine in *Scopolia lurida* engineered root cultures with the *Scopolia lurida* and *H. niger h6h* genes and found that the *Hn* H6H overexpressed root cultures contained more scopolamine. The scopolamine-to-hyoscyamine ratio can be increased in hairy roots of hyoscyamine-rich plants by overexpressing *h6h* (Hashimoto et al. 1993). Yun et al. (1992) reported the successful introduction of the *h6h* gene from *H. niger* into related species *Atropa belladonna*, a typically hyoscyamine-rich plant, resulting in an almost complete conversion of hyoscyamine to scopolamine. Later, Jouhikainen et al. (1999), while enhancing the production of scopolamine in transgenic

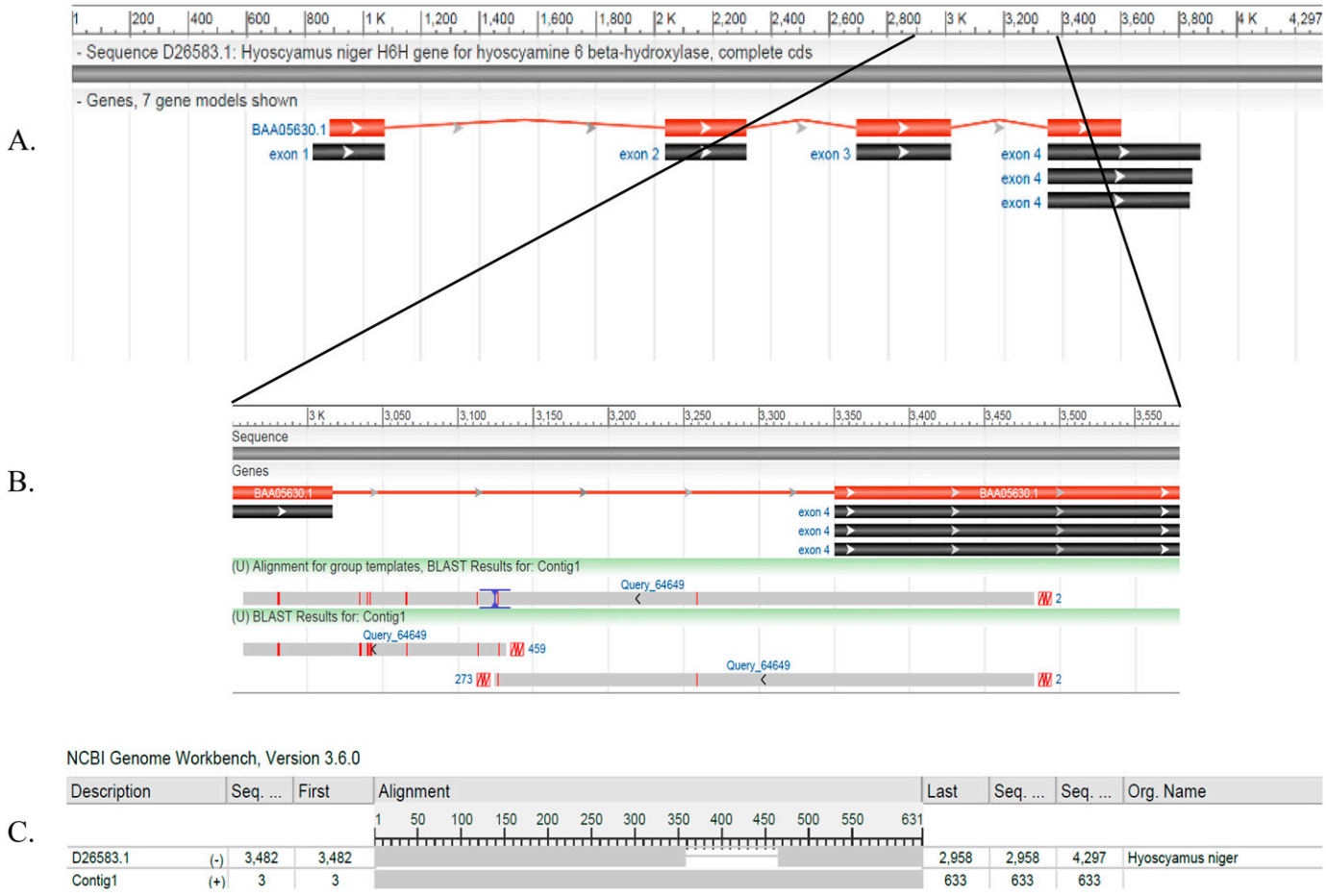


Fig. 5. Sequence alignment of the *b* allele to *H. niger h6h* gene coding sequence (NCBI 2021). (**A**) Annotated gene showing sequence size (4297 bp), introns, and exons. (**B**) Sequence alignment of the *h6h b* amplicon to *h6h* gene. (**C**) Alignment of the consensus *bb* genotype (shown here as Contig1) to the *h6h* gene sequence. The consensus sequence contains a 106-bp segment that is not present in the *h6h* gene. The *h6h* gene and *bb* amplicon sequences may be viewed in Supplemental Fig. 2.

root cultures, introduced the 35S-*h6h* transgene that codes H6H into *Hyoscyamus muticus* with promising results (up to 10 mg·gdm$^{-1}$ in hairy root). Zárate et al. (2006) observed that transgenic *Atropa baetica* overexpressing the gene encoding H6H showed a nine-fold increase in scopolamine in both the plant and hairy root cultures derived from the plants. Since that introduction, many in planta and in vitro metabolically engineered organisms with the *H. niger* construct for the *h6h* gene have been explored (Cardilloa et al. 2017; Dehghan et al. 2017; Häkkinen et al. 2005; Lan et al. 2018; Palazón et al. 2003; Xia et al. 2016).

Substituting the more active form of the gene for H6H identified here would further increase the transformation of hyoscyamine to the more valued scopolamine in transgenic plants and other heterologous systems, such as *Atropa belladonna*. *Atropa belladonna* is known to produce very high concentrations of hyoscyamine. Introduction of this superior

Table 4. Tabulation of hyoscyamine and scopolamine concentration (μg·gdm$^{-1}$) outliers found in the $F_2$ population in leaf and root tissue among genotypes. These outliers contained concentrations of hyoscyamine and scopolamine ≥1.5-times the third interquartile concentration found in each genotype population.

| | Hyoscyamine | | | Scopolamine | | |
|---|---|---|---|---|---|---|
| Leaf | 20[i] (84.9)[ii] | 26 (79.1) | 52 (110.0) | 20 (76.4) | 79 (99.5) | 50 (88.9) |
| | 44 (56.3) | 79 (62.2) | 50 (102.2) | 44 (43.7) | 36 (69.8) | 59 (76.2) |
| | | 87 (61.2) | 39 (75.0) | | | |
| Root | 38 (249.2) | 26 (494.8) | | 38 (109.9) | 4 (205.3) | |
| | | | | 11 (108.4) | 35 (111.0) | |
| | | | | | 14 (106.1) | |
| | *aa* | *ab* | *bb* | *aa* | *ab* | *bb* |
| | Genotype | | | | | |

[i] The F2 individual shown in Fig. 6.
[ii] Micrograms per gram dry mass.

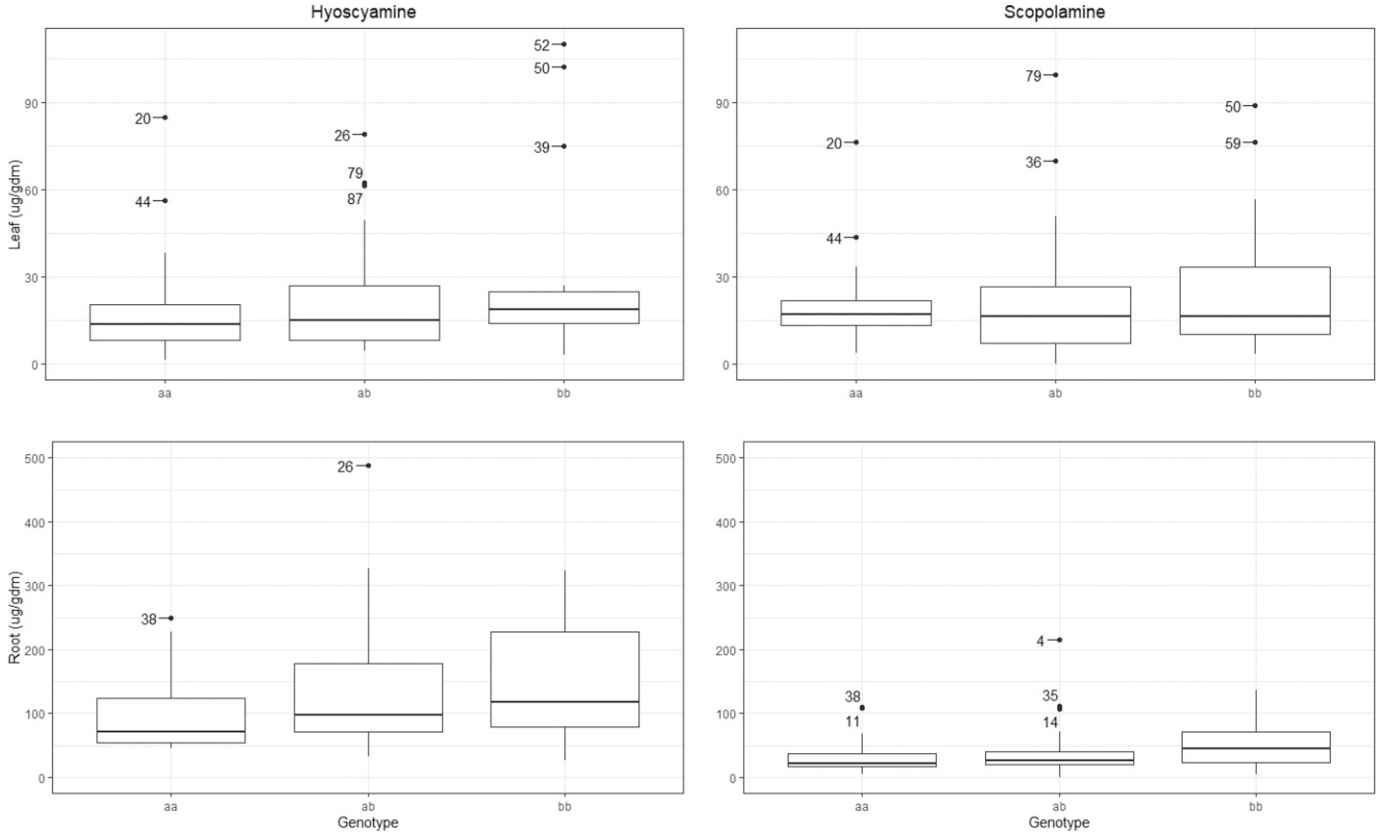


Fig. 6. Boxplot of hyoscyamine (Hy) and scopolamine (Sc) contents in leaf and root tissue of *Hyoscyamus niger* L. The median and quadrants show greater concentrations of Hy and Sc in leaf and root tissues of plants with the *b* allele present. In each plot, the outliers are highly increased in Hy and Sc concentrations with the presence of the *b* allele.

*h6h* and selecting for hairy root cultures could result in elite lines.

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