Effects of Elicitors on Tropane Alkaloids and Gene Expression in *Atropa baetica* Transgenic Hairy Roots

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Elicitation of transgenic *Atropa baetica* overexpressing the *h6h* gene with salicylic acid (SA), acetylsalicylic acid (ASA), or methyl jasmonate (MeJ) was conducted to boost tropane alkaloid yields. Scopolamine (**1**) amounts increased after treatment with ASA and MeJ, but not with SA. The highest enhancement of **1** was achieved with MeJ followed by ASA dissolved in EtOH. Transcriptomic analyses showed a direct relationship between content of **1** and gene expressions; the engineered *h6h* gene and other biosynthetic genes were stimulated. ASA dissolved in EtOH showed a high *h6h* gene expression, increasing 25-fold and 5-fold compared to controls; *tr-I* also displayed a 5-fold increase. The controls to which EtOH was added showed a 5-fold increase in *h6h* gene expression and 125-fold for *pmt*, demonstrating that EtOH also functioned as an enhancer of **1**. MeJ was the best elicitor, displaying a 25-fold increase in *h6h* expression level, not affecting the expression of the other three genes analyzed, and it appears to possibly stimulate the phenylpropanoids branch of the tropane alkaloid pathway.

The anticholinergic agents scopolamine (**1**) and hyoscyamine (**2**) are plant secondary metabolites employed in medicine as antispasmodics, for the treatment of motion sickness, in ophthalmology and anesthesia, and for cardiac and gastrointestinal diseases. Compound **1** shows more attractive pharmacological properties than **2** due to its stronger effects at lower doses and fewer side-effects; thus **1** has higher commercial value.[1] Since chemical synthesis of **1** and **2** is difficult, expensive, and not commercially exploited,[2] these compounds are extracted mainly from plants of the Solanaceae family. These metabolites are chiefly synthesized and stored in the roots, but they are also transported and accumulate in aerial parts of the plants.[3]

The induction of hairy roots following infection with *Agrobacterium rhizogenes*[4] has permitted the establishment of root cultures derived from various species of Solanaceae for the biotechnological production of medicinal compounds.[5−8] Various methods have been applied in order to enhance the productivity of secondary metabolites, and an efficient method has been the use of elicitors.[9] Elicitors are chemicals or biological factors able to induce physiological and biochemical changes in the target living organism. These include abiotic elicitors, such as metal ions and inorganic compounds, and biotic elicitors from fungi, bacteria, viruses, or herbivores, plant cell wall components, and chemicals that are released by plants at the attack site upon pathogen or herbivore attack.[10]

In a previous work, we described the overaccumulation of **1** in transgenic hairy roots of *Atropa baetica* (Solanaceae), an attractive producer system of **1**, by overexpressing the coding biosynthetic gene hyoscyamine 6β-hydroxylase (*h6h*) and also establishing a direct relationship between the *h6h* gene expression profile and the amounts of compound **1** produced.[11] The culture was able to almost totally convert hyoscyamine (**2**) into **1** with low amounts of the reaction intermediate 6β-hydroxyhyoscyamine (**3**). Jasmonic acid (JA) and its derivative methyl jasmonate (MeJ), together with salicylic acid (SA) and acetylsalicylic (ASA) acid, have been described as well-known inducers and/or enhancers of the production of plant secondary metabolites[10] and have also been reported to boost the accumulation of tropane alkaloids in hairy roots of several Solanaceae species. Thus, adventitious root cultures of *Scopolia parviflora* treated with MeJ and SA displayed higher production of **1** (700 μg g DW^{-1}) than control cultures.[12] In *A. belladonna* root cultures, treatment with SA increased the amount of tropane alkaloids released into the liquid nutrient medium, recording amounts of 5 mg/flask after 24 h, 35% of the total alkaloid content.[13] In *Brugmansia candida* hairy root cultures, the influence of SA was evaluated, and this elicitor stimulated both the accumulation of tropane alkaloids in the roots and the release of **1** into the medium: 10-fold increase after 72 h compared to control.[14]

In this study, in order to stimulate the production of tropane alkaloids and particularly compound **1**, MeJ, SA, and ASA were individually applied to *A. baetica* transgenic hairy root cultures. The expression profiles of the engineered gene *h6h* and other biosynthetic genes such as *pmt* (putrescine methyl transferase), *tr-I* (tropinone reductase I), and *tr-II* (tropinone reductase II) were evaluated in order to establish a relationship between the produced tropane alkaloids and the mRNA levels of the selected genes following elicitation.

Results and Discussion

In order to stimulate secondary metabolism, and thus the production of **1** in transgenic *A. baetica* hairy roots, the elicitors (SA), acetylsalicylic acid (ASA), and methyl-jasmonate (MeJ) were individually fed at three different concentrations and for different lengths of time. Elicitors were individually added to 37-day-old transgenic hairy roots, clone *h6h7*. This clone produced the maximum amounts of **1** as reported earlier.[11] The effects of the elicitor feeding on culture growth and alkaloid yields were assessed; moreover, at the molecular level, the expression profile of important tropane alkaloid biosynthetic genes was also evaluated in order to establish possible relationships between elicitation, gene activation, and compound **1** yield.

Transgenic hairy roots were elicited with different amounts of SA at various times. The different concentrations of SA and times did not generally affect culture growth compared to the control, although at 48 h there was a statistical difference between 1.0 and 0.1 mM ($p < 0.05$) (Table 1). Contrarily, in *B. candida* hairy roots the addition of SA was generally detrimental to growth, particularly after 72 h with about 40% lower fresh weight than control.[14] In *A. belladonna* hairy roots the addition of SA caused negative effects on the roots, such as tissue browning and growth retardation.[15]

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Table 1. Fresh Weight Values of *Atropa baetica* Clone *h6h7* Transgenic Hairy Roots after Elicitation with Salicylic Acid (SA), Acetylsalicylic Acid (ASA), and Methyl Jasmonate (MeJ)[a]

	time (h)			
	4	24	48	72
SA				
control	8.82 ± 0.30	9.37 ± 1.63	8.42 ± 0.39	7.91 ± 0.29
1.0 mM	8.13 ± 0.60	8.04 ± 0.79	7.34 ± 0.64*	8.67 ± 0.21
0.5 mM	9.23 ± 0.51	10.11 ± 0.73	8.92 ± 0.75	7.83 ± 0.86
0.1 mM	9.21 ± 0.54	8.36 ± 0.37	10.26 ± 0.53*	9.04 ± 0.58
ASA/EtOH				
control	8.13 ± 1.06	12.32 ± 1.25	10.45 ± 0.19*	8.16 ± 1.19
1.0 mM	9.13 ± 0.60	8.76 ± 0.66	7.77 ± 1.00	7.82 ± 0.13
0.5 mM	8.21 ± 0.88	8.71 ± 0.67	6.45 ± 0.81*	6.59 ± 0.30
0.1 mM	7.89 ± 1.46	10.38 ± 0.61	7.31 ± 0.35*	8.497 ± 0.482
ASA/H_2O				
control	6.24 ± 0.47	5.83 ± 0.45*	6.23 ± 0.74	5.17 ± 0.37*
1.0 mM	7.32 ± 0.34	7.14 ± 0.45	7.07 ± 0.54	6.91 ± 0.08
0.5 mM	8.65 ± 0.15	8.42 ± 0.08	8.77 ± 0.00	7.47 ± 0.43
0.1 mM	8.96 ± 0.11	9.90 ± 0.39*	9.37 ± 0.14	8.50 ± 0.33*
MeJ				
control	9.91 ± 1.28	11.10 ± 1.21*	7.55 ± 0.44	10.13 ± 1.15
1.0 mM	7.26 ± 0.86	7.58 ± 0.32*	9.39 ± 0.83	7.06 ± 0.56*
0.5 mM	8.19 ± 0.21	9.09 ± 0.26	10.07 ± 0.96	9.71 ± 0.56
0.1 mM	10.44 ± 0.66	9.27 ± 0.45	10.81 ± 0.78	11.42 ± 0.60*

[a] Asterisk indicates statistical difference within increasing molarities of elicitor ($p < 0.05$).

With respect to alkaloid accumulation after SA treatment, the content of **1** in both liquid medium and roots was not increased and its use appeared inhibitory since larger concentrations and longer treatments reduced total tropane alkaloid contents with respect to control to which EtOH was added (Figure 1a,b). Moreover, **2** was released into the liquid medium only slightly, unlike **1** (1300 μg/flask at 4 and 72 h) (Figures 1a and S-1 Supporting Information), with the reaction intermediate (6β-hydroxyhyoscyamine, **3**) being absent in this fraction. These results were opposite of those obtained in *B. candida* after SA elicitation, in which both compounds **1** and **2** were released into the liquid medium with amounts 6- and 10-fold larger, respectively, compared to the control.[14] Another instance of a positive effect was obtained after adding 2.0 mM SA after 72 h to *A. belladonna* transformed hairy roots, where a total alkaloid release of ca. 5000 μg was achieved.[13]

Regarding the alkaloid content detected in hairy roots, SA did not affect the alkaloid profile, with compound **2** being the minor alkaloid and **1** and **3** the more abundant ones (Figures 1b and S2a,b, Supporting Information). Again, as it occurred in the liquid medium fraction, the different treatments displayed a lower alkaloid accumulation with respect to control except for the intermediate (compound **3**) at 4 and 48 h (Figure S2a, Supporting Information). The best SA treatment accounted only for 39.5% of **1** and 63% of **3** compared to the amounts detected in the control, with a clear negative effect with time and increased concentration of the elicitor. These inhibitory results were similar to those reported in *A. belladonna* root cultures, in which at 0.2 mM of SA treatment some negative effects on alkaloid production were observed, more clearly at a higher concentration (2.0 mM for 7 days treatment), the total alkaloid amounts decreasing 90% compared to control.[13] Surprisingly, here the ethanol used to dissolve the elicitor activated the production of **1** (8400 μg g DW^{-1}), with an increased amount of 14.6-fold compared to plants,[16] which indicates the stimulating role of this solvent in this culture system. Elicitation with ASA dissolved in either water or ethanol was also carried out in a similar fashion. In some instances, ASA did affect the growth of the hairy root cultures, and this was more evident when dissolved in water (Table 2).

Regarding compound **1** production after culture elicitation with ASA dissolved in either ethanol or water, this alkaloid was the only compound detected in the liquid medium (Figures 2a, 3a). Moreover, with ASA/EtOH the best result was achieved with 0.1 mM ASA after 24 h, reaching a total amount of **1** of 6850 μg g DW^{-1} (1100 μg in liquid medium plus 5750 μg in the roots) (Figure 2a,b), 11.4-fold higher compared to intact plant.[16] This effect was in accordance with those obtained after elicitation of *Catharanthus roseus* tumor suspension cultures, in which an increase of 505% total alkaloids was observed.[17] Likewise, a stimulating effect was also obtained by feeding ASA/H_2O 0.5 mM after 24 h with total amounts of 5360 μg g DW^{-1} of compound **1** (1360 in liquid medium plus 4000 in the roots), 8.75-fold larger compared to the native plant (Figures 3b and 4b).[16] Regardless of time, the highest ASA/H_2O treatment (1.0 mM) displayed an inhibitory effect, showing lower production of **1** compared to control, 2500 μg g DW^{-1} (Figure 3a,b).

In the ethanol-dissolved ASA treatment, ethanol fed to control cultures acted as a clear elicitor, stimulating the production of **1** with amounts that were equal to those observed with ASA/H_2O (6540 μg g DW^{-1}), 10.9-fold compared to mature plants[16] (Figures 2a,b and 3a,b). With respect to compound **2**, which was detected only in the roots, the best production was recorded with ASA/EtOH (0.5 mM 4 h) with amounts close to 1200 μg g DW^{-1} (Figure S3a, Supporting Information). On the other hand, 0.5 mM 4 h ASA/H_2O produced the best amounts of **2**, with a yield near 1000 μg g DW^{-1} (Figure S3b, Supporting Information). Contrarily, 1.0 mM ASA/H_2O induced a clear reduction of compound **2** content with only 150 and 100 μg g DW^{-1} after 24 and 72 h, respectively (Figure S3a,b, Supporting Information).

On the other hand, compound **3** production was highest (1460 μg g DW^{-1}) with the ASA/H_2O treatment (0.5 mM 4 h), similar to the production of ASA/EtOH feeding (0.1 mM, 24 h), although the control showed larger values (Figure S4a,b, Supporting Information). Furthermore, as it occurred with **1**, treatment with ASA dissolved in water produced an initial stimulation during the first hours, followed by a drop after 24 h of treatment. The accumulation of the intermediate of the reaction catalyzed by the enzyme H6H (hyoscyamine 6β-hydroxylase) also confirms the activation of the overexpressed *h6h* gene to produce both the intermediate (compound **3**) and the final product **1** in our transgenic *A. baetica*-elicited hairy roots.

Taking into account that the stimulation of the elicitor ASA on compound **1** yield was higher when it was dissolved in EtOH than in water, the effect of the autoclaving on the stability of the ASA solution was evaluated. Before and after autoclaving the 0.5 M solution of ASA, ^{1}H NMR analyses were conducted to determine its stability. It was determined that ASA converted to SA in the proportion 1:3 (Figure S5a,b, Supporting Information), which explains the obtained lower yield of compound **1** following elicitation with the aqueous ASA solution compared to the ethanolic solution. Moreover, the conversion of ASA into SA would also explain the lack of a synergistic effect of ASA plus ethanol when compared with ASA aqueous solution, which had suffered a breakdown into SA.

MeJ was employed in an attempt to further boost compound **1** content in transgenic *A. baetica* hairy roots. Regarding fresh weight, none of the treatments or concentrations evaluated significantly affected the growth of hairy roots, except at 24 h between control and 1.0 mM or 72 h between 1.0 and 0.1 mM ($p < 0.05$) (Table 2). MeJ feeding (0.1 mM, 4 h) resulted in the highest accumulation of **1** with a total amount of 9500 μg g DW^{-1} (3500 in liquid medium and 6000 in hairy roots) (Figure 4a,b), representing a 15.8-fold increase compared to plants.[16]

Similar stimulating effects on tropane alkaloid production were described in *S. parviflora* hairy roots, in which the addition of MeJ boosted tropane alkaloid yield, as well as the release of **1** into the liquid medium.[12] In *Hyoscyamus niger* hairy root cultures the addition of MeJ augmented compound **1** production and stimulated the activation of biosynthetic genes such as *h6h*.[18] In our system

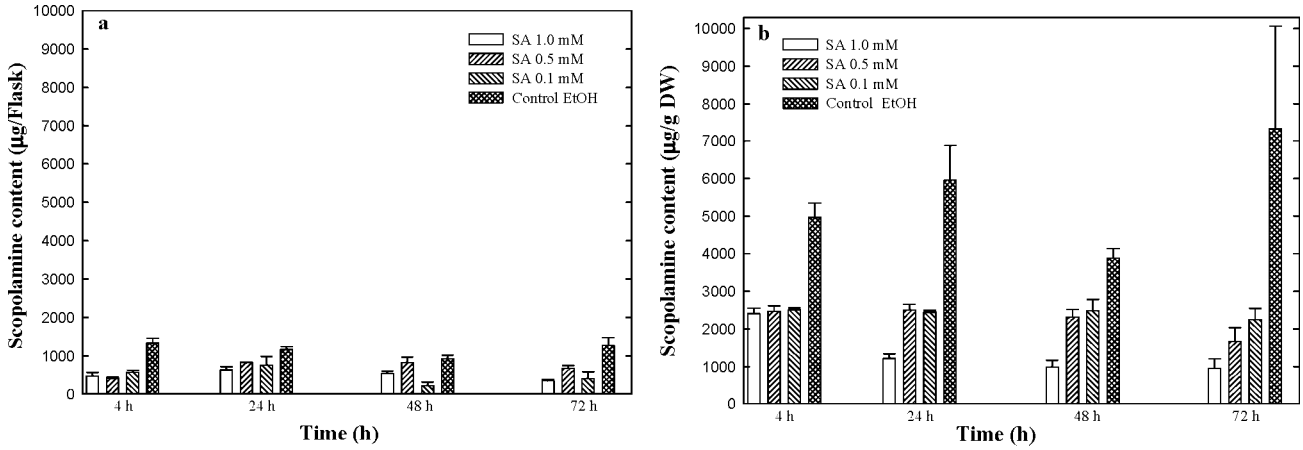

Figure 1. Scopolamine content in liquid nutrient medium (a) and in hairy roots (b) after elicitation of *Atropa baetica* transgenic hairy root cultures with SA. Each value is the mean of three replicates ± standard error.

Table 2. Primer Pairs Employed for the PCR Amplification of the Tropane Alkaloid Biosynthetic Genes *h6h*, *pmt*, *tr-I*, and *tr-II*

	primer pairs
h6h	forward: 5′-GAGACATTTGATGGCTACTTTT-3′
	reverse: 5′-TGCTTAAGACATTGATTTTATATGGC-3′
pmt	forward: 5′-ATTGTTCATCTCCCACTTGG-3′
	reverse: 5′-TCTTTTGCTGGACCAATAGG-3′
tr-I	forward: 5′-GCTTCCAAAGCTGCAATAAATC-3′
	reverse: 5′- TGAATCCACCATCAGCCC-3′
tr-II	forward: 5′-ATGGACCAACTGACAAGATGC-3′
	reverse: 5′- CACCATTAGCCATAAATCCACC-3′

the amounts of **1** were similar to those obtained by Zhang et al.[19] when *h6h* was overexpressed in *H. niger*, but were lower than *H. niger* transgenic lines when both *h6h* and *pmt* were overexpressed,[19] suggesting that the transgenic lines that carried both genes forced the metabolic flux to accumulate much more **1** than those presented here. Compounds **2** and **3** were not recorded in the hairy roots or in the liquid media. Likely, this could be due to the high activation of the *h6h* gene by MeJ, resulting also in the largest increase in **1**.

In our transgenic culture MeJ acted as the best elicitor, inducing the highest amounts of **1** among the elicitors assayed. It is important to note that the amounts of **1** released into the liquid medium were the largest (Figure 4a), indicating that MeJ was also able to liberate the excess of **1**, suggesting that the liquid medium was also functioning as an active storage compartment and not simply as a nutrient source.[20]

After analysis of tropane alkaloid contents in transgenic *A. baetica* hairy root cultures following treatments with three different elicitors, the mRNA expression profiles were semiquantitatively assessed for the overexpressed *h6h* gene and for three other tropane alkaloid biosynthetic genes: *pmt* (putrescine methyl transferase), which catalyzes the bioconversion of putrescine into *N*-methylputrescine, and *tr-I* and *tr-II* (tropinone reductase I and II), which are responsible of the enantioselective reduction of tropinone into tropine or pseudotropine, respectively.

Metabolically *pmt* and *tr-I* direct the biosynthetic flow toward compound **1**, via tropinone, while *tr-II* converts tropinone to pseudotropine and thus pushes the metabolic flow away from **1** (Figure 5). The findings of these analyses allowed us to establish correlations between compound **1** production and the expression levels of the selected genes.

Elicitation with ASA/H_2O, despite partial hydrolysis of ASA to SA, stimulated the expression of the manipulated *h6h* gene, exhibiting a 5-fold increase in the mRNA level compared to control, and *pmt* and *tr-I* were also overexpressed 5- and 125-fold, respectively. On the other hand, *tr-II* was not affected by ASA/H_2O treatment and displayed an expression pattern similar to the control (Figure S6, Supporting Information).

These results are opposite to those obtained in hairy root culture of *S. parviflora* fed with Gram-negative and Gram-positive bacteria extracts, displaying a subsequent boost of compound **1** content, but with a down-regulation of the *h6h* gene and overexpression of *pmt*.[21] Here, the higher expression level of the different genes directing the pathway toward **1** confirms that the ASA elicitor is capable of incrementing its production, affecting not only the overexpressed *h6h* gene but also those native genes of the pathway that were not manipulated.

The ASA/EtOH treatment showed a high *h6h* gene expression, increasing 5-fold compared to ethanol control, but 25-fold compared to water control (Figure S6, Supporting Information). The other genes (*tr-I* and *tr-II*) were almost not affected, although *pmt* expression level was much lower in the ASA/EtOH. Again, it appears that the overexpression of the *h6h* gene was enough to increase the production of **1** in transgenic *A. baetica* hairy root cultures.

Surprisingly, control cultures to which EtOH was added induced a clear boost of **1**. It was recorded that *h6h* gene expression increased 5-fold, whereas *pmt* increased 125-fold compared to control fed with water (Figure S6, Supporting Information). The effect of ethanol on gene expression level could be due to the activation of several heat-shock protein genes as reported in *Escherichia coli*[22] or in *Glycine max*, where ethanol stimulated the expression of heat-shock protein related genes.[23]

The MeJ-treated samples displayed an increase only in *h6h* gene expression, 25-fold compared to control (Figure S6, Supporting Information), and did not affect the other genes analyzed. This result is opposite to those observed in MeJ-elicited *H. niger* hairy roots, where the expression level of the *h6h* and *pmt* genes was stimulated with a parallel boost in compound **1**.[18] This positive effect was also observed in MeJ-treated adventitious hairy cultures of *S. parviflora*, which displayed an increase in compound **1** and up-regulation of both *h6h* and *pmt* genes,[12] and in *Datura stramonium*, where MeJ also activated *pmt* gene expression.[24]

The overexpression of *h6h* but not *pmt*, *trI*, and *trII* after MeJ feeding in *A. baetica* does not seem to be enough to explain the high yield of **1**. This could be due to different factors, such as the activation of different signaling pathways and/or the cross-talking between different signaling pathways, or the direct activation of other genes,[25,26] as it is known that MeJ activates the expression of several genes acting through different transcriptional factors, such as ORCA3, whose overexpression in *C. roseus* suspension cultures resulted in the enhanced expression of five genes of the terpenoid indole alkaloid pathway.[27,28]

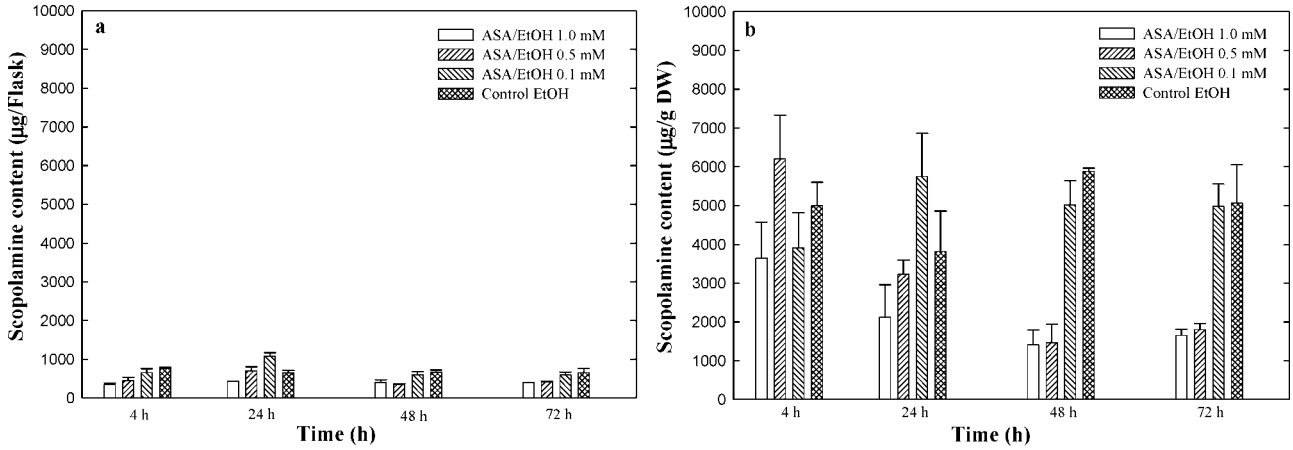

Figure 2. Scopolamine content in liquid nutrient medium (a) and in hairy roots (b) after elicitation of *Atropa baetica* transgenic hairy root cultures with ASA dissolved in EtOH. Each value is the mean of three replicates ± standard error.

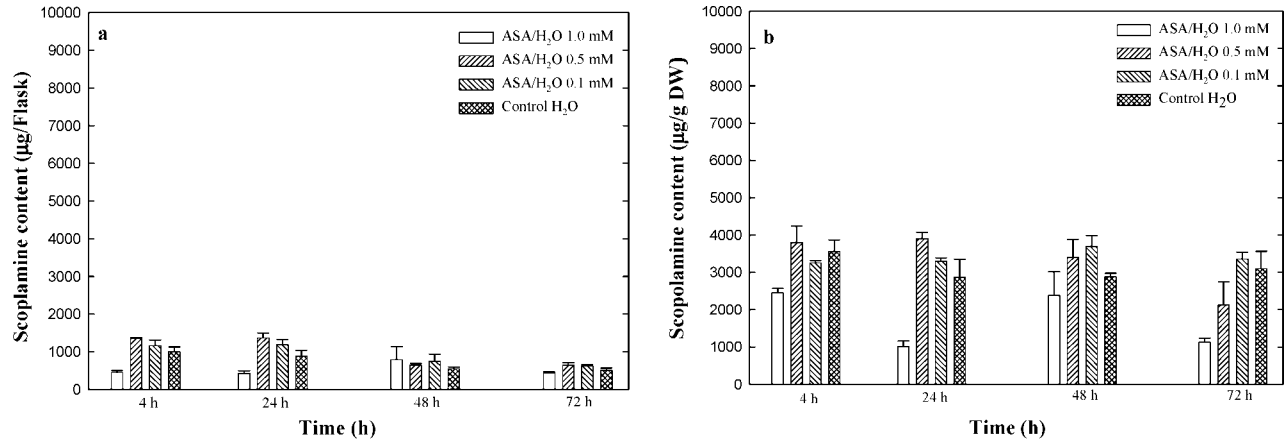

Figure 3. Scopolamine content in liquid nutrient medium (a) and in hairy roots (b) after elicitation of *Atropa baetica* transgenic hairy root cultures with ASA dissolved in water. Each value is the mean of three replicates ± standard error.

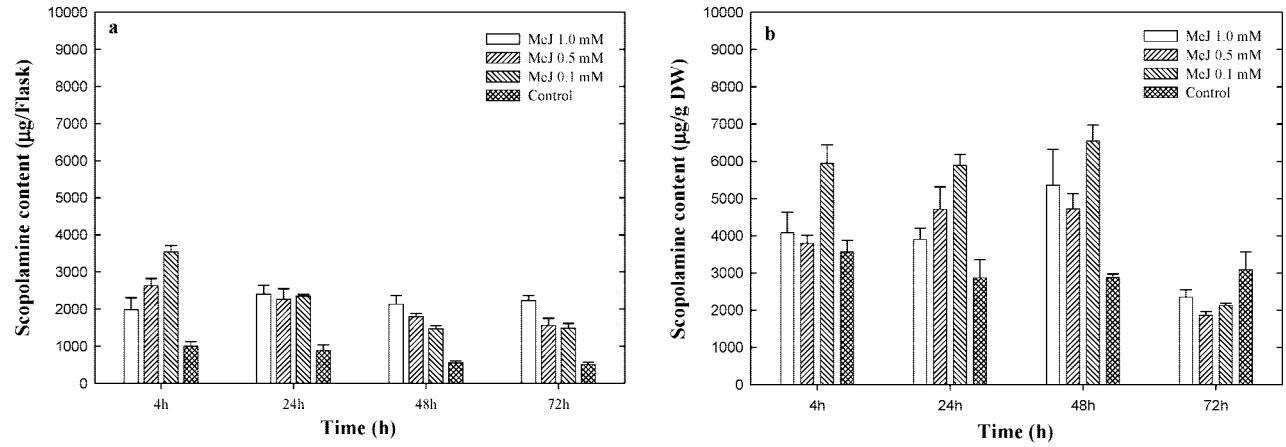

Figure 4. Scopolamine content in liquid nutrient medium (a) and in hairy roots (b) after elicitation of *Atropa baetica* transgenic hairy root cultures with MeJ. Each value is the mean of three replicates ± standard error.

According to the available information, on the basis of radiolabeled tropane alkaloid precursor feeding experiments, it has been reported that for the phenylpropanoid branch of the pathway (Figure 5) phenylalanine as the initial amino acid and phenyllactate are obligatory intermediates in the biosynthesis of tropic acid, but not cinnamic acid and 3-hydroxy-3-phenylpropionic acid.[29−31] Furthermore, it has also been shown that MeJ increased the accumulation of tropane alkaloids in *D. stramonium* root cultures by incrementing tropine synthesis with a parallel decline in phenyllactate, which originates from phenylalanine[32] (Figure 5). All these data suggest a likely positive effect of MeJ in the stimulation of genes of the phenylpropanoid branch of the pathway, inducing the overaccumulation of **1** in *A. baetica.* Moreover, it could also be possible that together with the *h6h* gene expression increase, and the suggested phenylpropanoid branch genes activation, a cytochrome P450 identified from *H. niger*[33] (CYP80F1), which catalyzes the oxidation of (*R*)-littorine with rearrangement to form hyoscyamine, might have also been stimulated by MeJ and would contribute to the largest enhancement of **1** in *A. baetica* transgenic hairy roots.

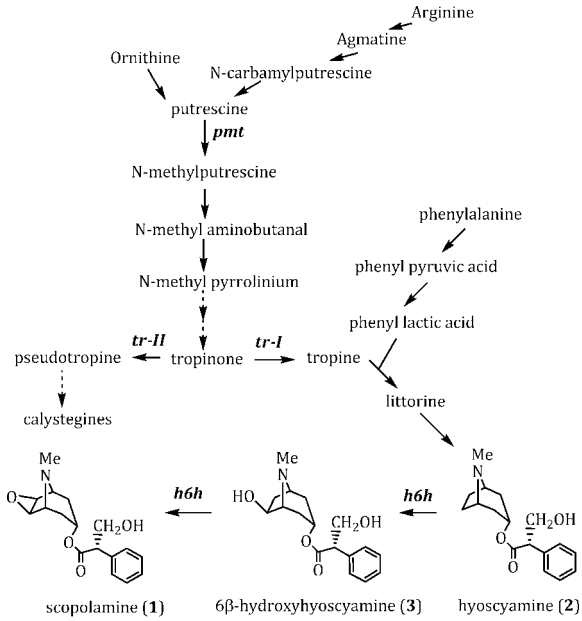

Figure 5. Scheme of the main biosynthetic tropane alkaloid pathway including the phenylpropanoid branch. *pmt* = putrescine methyl transferase, *trI-II* = tropinone reductase I and II, *h6h* = hyoscyamine 6β-hydroxylase.

Experimental Section

Plant Material. *Atropa baetica* transgenic hairy roots, transformed with *Agrobacterium rhizogenes* LBA 1334, were established as described earlier.[11] Experimental cultures were initiated by the inoculation of ca. 0.75 g (fresh weight) of clone *h6h*7 of transgenic hairy roots cultured in 250 mL Erlenmeyer culture flasks containing 50 mL of sterile MS[34] liquid medium with 3% sucrose, which were placed on an orbital shaker at 95 rpm in the dark at 25 ± 2 °C.

Preparation of Stock Solutions. A solution of 1 M salicylic acid (SA) (Fluka, Germany) was prepared by dissolving the compound in an adequate volume of ethanol of analytical grade (Merck, Darmstadt, Germany). Acetylsalicylic acid (ASA) (Acofarma, Spain) was also dissolved in ethanol to a final concentration of 0.5 M. ASA was also dissolved in distilled water at the same concentration and autoclaved at 121 °C for 20 min. Methyl jasmonate oily solution (MeJ) purchased from Aldrich (Switzerland) was used directly without diluting or concentrating. All solutions, except the ASA dissolved in distilled water, were filter sterilized through a nylon membrane of 0.22 μm pore size (Schleicher & Schuell, UK).

Addition of Elicitors. Sterilized solutions were individually added to 37-day-old transgenic hairy root cultures to a final concentration of 0.1, 0.5, and 1 mM. The control cultures were carried out by the addition of the same volume of either ethanol (analytical grade) or water, except for MeJ controls, which did not receive any addition, as the volume of MeJ oily solution added ranged from ca. 1.0 to 11.0 μL. All experimental cultures were conducted in triplicate, and the elicitation effect was measured at 4, 24, 48, and 72 h after feeding.

Extraction and Analysis of Tropane Alkaloids Present in the Roots and Liquid Nutrient Medium. Liquid medium and hairy roots were separated by vacuum filtration. The liquid medium sample was liquid−liquid extracted as reported earlier.[8] After lyophillization of the hairy roots, tropane alkaloids were extracted following a modification of the method reported by Kamada[5] and employed previously.[8,16,35] For quantitative determination of the major tropane alkaloids (**1**−**3**), the samples were analyzed by HPLC as reported[16] but employing a Jasco system equipped with a PU-980 pump, a MD-2010 Plus photodiode array detector, and a Rheodyne 7725i manual injector, fitted with a Waters Symmetry C-18 reversed-phase column (150 × 4.6 mm), packed with 3.5 μm particles. The mobile phase consisted of a mixture of 50 mM potassium dihydrogen phosphate (pH 3.0) and acetonitrile (78/22) at a flow rate of 1 mL/min, and detection was conducted at 215 nm.

RNA Extraction and Semiquantitative Gene Expression Studies using RT-PCR. RNA was extracted using an RNeasy Plant Mini Kit following the instructions provided (Quiagen, Hilden, Germany). RNA amounts were quantified following spectrophotometric measurements and kept at −20 °C until further analysis. RT-PCR (retrotranscriptase−polymerase chain reaction) was performed to quantify the level of expression of the genes *h6h*, *pmt*, *trI*, and *trII* of the different samples studied.

Prior to retrotranscriptase analysis (RT), RNA samples were treated with DNase using a commercial DNase kit (Ambion, Austin, TX). RT was performed as previously described with DNA-free RNA samples by taking an aliquot of 1 μg, which was serially diluted down 5×.[11] All dilutions, including the 1× sample (1 μg), were initially incubated at 85 °C for 3 min, followed by the addition of RT incubation buffer and appropriate amount of enzyme, then incubated at 44 °C for 1 h and finally at 92 °C for 10 min to inactivate the retrotranscriptase enzyme (Ambion). The cDNA product of these reactions was then used to amplify the specific *h6h*, *pmt, trI*, and *trII* cDNAs, by incubation at 94 °C (3 min), followed by 30 cycles of 94 °C (45 s), 55 °C (30 s), and 72 °C (1 min) and a final elongation step of 72 °C (7 min). All reactions were carried out in a total volume of 25 μL containing, 0.25 mM dNTP, 0.5 μM of primers (Table 2), 0.1 μg of cDNA, and 1 unit of Taq polymerase plus 2.5 μL of a 10× reaction buffer (GeneScript, CA).

Statistical Analysis. For statistical comparisons of the fresh weight data and tropane alkaloid contents following elicitation, the mean values of the three replicates were calculated. These were assessed by performing a Student's *t* test and ANOVA analysis for establishing possible differences among and within the different elicitors employed, as well as the varied concentrations of elicitor and length of the treatments.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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