Plant Biotechnology 24, 295–300 (2007)

Original Paper

A virus-induced gene silencing approach for the suppression of nicotine content in *Nicotiana benthamiana*

Mari Takizawa[1], Koichi Hori[1], Koji Inai[2], Hisabumi Takase[2], Takashi Hashimoto[2], Yuichiro Watanabe[1,*]

[1] Department of Life Sciences, the University of Tokyo, Tokyo 153-8902, Japan; [2] Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara 630-0192, Japan
*E-mail: solan@bio.c.u-tokyo.ac.jp Tel: +81-3-5454-6776 Fax: +81-3-5454-6776

Received November 8, 2006; accepted February 13, 2007 (Edited by T. Kohchi)

Abstract Virus vectors have been used to study plant gene function by transiently overexpressing specific gene products, or conversely, silencing specific endogenous gene functions. Previously, we reported the establishment of tobamovirus vectors and its applications. In this study, we observed that tobamovirus vector could drive ectopic expression of green fluorescent protein (GFP) in roots after infecting leaves, indicating successful invasion into underground tissues. Subsequently, we attempted to apply the tobamovirus vector system to suppress the expression of putrescine *N*-methyltransferase (PMT), which is the first committed key enzyme for the biosynthesis of nicotine and is active in the underground parts of plants. Two or three weeks after inoculation with the vector harboring a partial fragment of PMT cDNA, we observed a reduction in the PMT mRNA level in the root and in the nicotine content of the aerial parts of the plant *Nicotina benthamiana*. The possibilities and limitations of the virus vector for the analysis of metabolic pathways are discussed.

Key words: Nicotine, putrescine *N*-methyltransferase (PMT), tobacco, tobamovirus vector, virus-induced gene silencing (VIGS).

Plant alkaloids are one of the largest groups of natural products and pharmacologically important compounds. The *Solanaceae* plants produce a range of biologically active alkaloids including nicotine and the tropane alkaloids, such as hyoscyamine (atropine) and scopolamine (hyoscine).

Nicotine is known as a major alkaloid produced in the roots of many *Nicotiana* species. The pyridine ring of nicotine is synthesized by way of the pyridine nucleotide pathway, whereas its pyrolidine ring is derived from the diamine putrescine. Putrescine can also be metabolized to polyamines such as spermidine and spermine. The *N*-methylation of putrescine, catalyzed by putrescine *N*-methyltransferase (PMT), is the first and also the key step in the biosynthesis of nicotine and tropane alkaloids.

It is known that nicotine is synthesized exclusively in the roots of tobacco plants. After being synthesized, nicotine and other tobacco alkaloids are translocated from the roots through the xylems to the leaves, where they accumulate to high levels (Katoh et al. 2005).

The *PMT* cDNAs were cloned from tobacco (Hibi et al. 1994), *Atropa belladonna* L., *Hyoscyamus niger* L. (Suzuki et al. 1999) and *Solanum tuberosum* L. (Stenzel et al. 2006). In *Nicotiana sylvestris*, three *PMT* genes (*NsPMT1*, *NsPMT2* and *NsPMT3*) were isolated and found to encode proteins highly homologous to each other. *PMT* transcripts are expressed and accumulate exclusively in the roots of tobacco, specifically in the cortex, endodermis and xylem (Shoji et al. 2000). In tobacco, the PMT expression level is well correlated with that of nicotine accumulation in the above-ground tissues.

It has been shown that several distinct viral vectors can be used for the transient expression of foreign genes in plants, encoding for example peptides, antigen proteins (Porta et al. 2003; Fitchen et al.1995; Sugiyama et al. 1995; Turpen et al. 1995; Bendahmane et al. 1999) and antibody fragments (Franconi et al. 1999; Hendy et al. 1999; Roggero et al. 2001). This strategy provides a choice of analytical methods in basic research and applied biotechnology, since the expression level of the foreign proteins is relatively high. Rapid accumulation may also help to bypass the possible toxicity of high-level expression and RNA silencing against ectopic expression of introduced genes in plants.

Plant virus vectors harboring a segment of host gene

Abbreviations: dpi, days post inoculation; GFP, green fluorescent protein; PMT, putrescine *N*-methyltransferase; VIGS, virus-induced gene silencing.
This article can be found at http://www.jspcmb.jp/

sequence can induce silencing of the corresponding endogenous mRNAs in infected plants. This approach is termed virus-induced gene silencing (VIGS) and takes advantage of the RNA-mediated defense mechanism against invading genetic materials in plants (Baulcombe 1996). It allows transient functional knockdown of host gene expression in the relatively short term, in comparison with experiments using stable transformants expressing antisense or inverted-repeat sequences.

Previously, we constructed TocJ, TogJ and LgJ vectors, which can harbor foreign gene proteins, based on the tomato mosaic tobamovirus. LgJ vectors could induce VIGS with relatively little side-effects (Hori and Watanabe 2003; Hori et al. 2004).

In this study, we first confirmed that TogJ/GFP could efficiently spread systemically and express GFP in the underground plant parts, as well as in leaves. Next we tested the feasibility of this VIGS system for the study of secondary metabolism pathways. LgJ/PMT could induce VIGS of *PMT* mRNA expressed in roots, which was followed by reduced accumulation of nicotine in the aerial plant parts.

Materials and methods

Plant Materials

Nicotiana benthamiana plants were maintained at 23°C with a 16 h photoperiod and 8 h dark period. Until 4 weeks they were grown in Jiffy 7 (Sakata) and then transferred into 10-cm hydroculture pots (Hydrocorn: Miura Horticulture) covered with Jiffy Mix (Sakata). The use of a hydroculture pot enabled us to quantitatively collect root tissues for analysis.

Cloning of the PMT gene sequence

We presumed that the *N. benthamiana* PMT (NbPMT) sequence displays close similarity to that of *Nicotiana tabacum* at the amino acid sequence level. We designed the primers based on the *N. tabacum* PMT sequence to cover about 400 bp, for the cloning of the corresponding *N. benthamiana* cDNA. The *NbPMT* sequence was amplified from cDNA of *N. benthamiana* by PCR using the described primer sets. The first round of PCR was performed with primers, PMT-F-gk (5′-aaaaagcaggctggtggtacccaattcaacacacagagaatgg-3′) and PMT-R-gs (5′-agaaagctggggtgagctccatacaactcctcctggcct-3′). As we expected, we obtained a PCR product band with a mobility consistent with that of the *N. tabacum* PMT sequence (data not shown). The plausible *NbPMT* cDNA fragment has a length of ca. 400 bp (corresponding to nt 382–890 of the *N. tabacum* cDNA, Genbank Accession No. AF126812). The second PCR was performed with primers attB1 and attB2 and the resultant fragment was subcloned in a pDONR vector (Invitrogen) with the aid of BP clonase (Invitrogen) to obtain the plasmid designated as pDONR201/NbPMT-probe. Next, we transferred the *NbPMT* fragment to pBlueGATE (Hori et al. 2004) by LR clonase (Invitrogen) to generate pBlueGATE-DONR201/NbPMT-probe, an intermediate plasmid of the LR reaction, as the template plasmid for PMT probe production.

PCR, BP and LR reactions were performed according to the manufacturer's instructions and as described previously (Hori et al. 2004).

Cloning of GFP and PMT sequences into tobamovirus vectors

TogJ/GFP, a TogJ vector harboring GFP sequence, has been described previously (Hori et al. 2004). The PMT sequence was recloned into LgJ vector (Hori et al. 2004) by way of pENTRBLUE digested with the restriction enzyme *Eag*I, using the GATEWAY System (Invitrogen), to obtain LgJ/PMT.

In vitro transcription and transcript inoculation of plants

The plasmids of TogJ, LgJ and their derivative vectors were linearized with *Mlu*I to make template DNAs for transcription, prior to run-off transcription. The *in vitro* transcription reaction was performed using 1 μg linearized template DNA, 50 units T7 RNA polymerase (Invitrogen), 1 mM ATP, 1 mM UTP, 1 mM CTP, 0.1 mM GTP and 0.52 mM m7GpppG (NEB) as a cap analogue, in 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine-$(HCl)_3$, 25 mM NaCl, and 1 mg ml^{-1} bovine serum albumin (BSA) for 60 min at 37°C.

N. benthamiana plants were inoculated with the *in vitro* transcripts as described elsewhere (Hori et al. 2004), using paint brushes and carborundum as an abrasive on the preceding dates in order to collect inoculated plants of 8 weeks old at the time of harvest.

Northern blot analysis

Total RNA was purified as previously described (Shirzadegan et al. 1991). Total RNAs (1 μg for virus and 20 μg for PMT detection) were blotted onto Hybond N+ membranes and detected as previously described (Hori and Watanabe 2003). The tobacco mild green mosaic virus (TMGMV) probe was used to detect and confirm vector integrity as described elsewhere (Hori and Watanabe 2003). DIG-labeled PMT antisense probe was synthesized from pBlueGATE-DONR201/NbPMT-probe template DNA using a DIG-RNA labeling kit according to the manufacturer's recommendations. Blotted Hybond N+ (Amersham Bioscience) membranes were hybridized with probes using blocking reagent (Roche) under high stringency conditions at 68°C overnight, according to the manufacturer's recommendations. Membranes were washed twice with 0.2×SSC–0.1% SDS for 30 min each at 68°C. The hybridization signals were detected using anti-digoxigenin-AP Fab fragments (Roche) and a CDP-STAR (Amersham Bioscience) using Hyperfilm ECL film (Amersham Pharmacia Biotech).

Western blot analysis

Protein samples were separated on SDS-10% (for GFP analysis) or 15% (for coat protein analysis) polyacrylamide gels and transferred to PVDF membranes (imunobolin-P, Millipore). Blotted membranes were probed with rabbit anti-GFP (Santa Cruz Biotechnology) or rabbit anti-tomato mosaic tobamovirus (ToMV) coat protein (CP) (Saito et al. 1987) to confirm virus propagation. Alkaline phosphate-conjugated goat

anti-rabbit IgG (Cell Signaling) was used as secondary antibody, and the reactions were visualized with BCIP/NBT phosphate substrate (KPL). Protein concentrations were determined by the Bradford assay using a commercial kit (Bio-Rad Laboratories).

Nicotine analysis

The whole above-ground parts of the plants were blotted to remove extra water before lyophilization. Total tissues were lyophilized and subjected to analysis. Lyophilized samples were homogenized, and soaked in 3 ml of 0.1 N H_2SO_4. The homogenate was sonicated for 20 min and centrifuged at 13000 g for 10 min. The supernatant (1 ml) was mixed with 0.1 ml of 28% NH_4OH. The mixture was centrifuged at 13000 g for 5 min, and the supernatant was loaded onto an Extrelut-1 column (Merck) and eluted with 6 ml of chloroform. The chloroform extract was dried at 37°C. The dry residues were dissolved in ethanol containing 0.1% (v/v) dodecane and analyzed by gas-liquid chromatography (model GC-14A; Shimazu) equipped with an Rtx-5Amine capillary column (Restek). The column temperature was held at 100°C for 10 min, then it was increased from 100°C to 300°C at a rate of $30°C \cdot min^{-1}$.

Results

Expression of GFP by TogJ/GFP in roots

It was visually demonstrated that the TogJ/GFP vector could efficiently spread into roots of *N. benthamiana*, by the GFP-specific fluorescence levels which were above the background yellow autofluorescence in the root tissues. By 8 days after inoculation of one of the above-ground leaves with TogJ/GFP, we could detect strong GFP fluorescence in the roots and upper leaves (Figure 1).

Western blot analysis using anti-GFP and anti-ToMV CP antibodies confirmed that the vector could spread and express exogenous genes in root tissues as well as the inoculated or the upper leaves. (Figure 2). The level of GFP expression was almost the same as that in the aerial parts of the plants (data not shown). In addition, Northern blot analysis revealed that TogJ could stably maintain the GFP sequence during the propagation into root tissues, without detectable loss or alteration of the inserted sequence (data not shown).

Nicotiana benthamiana Putrescine N-methyltransferase (PMT)

Putrescine *N*-methyltransferase (PMT) catalyzes the first committed step of nicotine biosynthesis in tobacco. We adopted *N. benthamiana* plants for the purpose of suppressing nicotine content by inducing RNA silencing of PMT. Until now, *PMT* mRNA sequences were not isolated and reported in *N. benthamiana*. We designed PCR primers from *N. tabacum* to amplify ca. 400 bp of the *N. benthamiana PMT* sequence, assuming that the *PMT* sequences of both plant species show considerable homology. As expected, we could obtain a band of which the sequence showed extensive homology to *N. tabacum PMT* over the 400 bp.

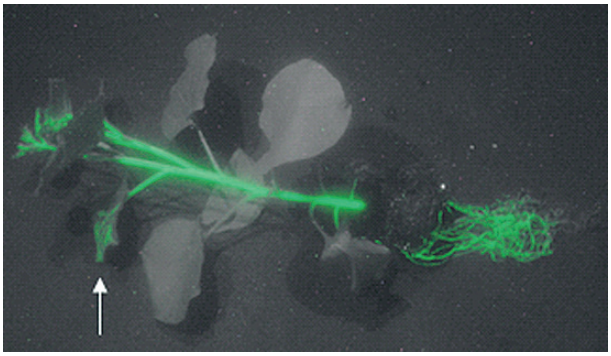

Figure 1. Systemic infection of *N. benthamiana* with TogJ/GFP. At 8 days post-inoculation (dpi), GFP was observed in the upper leaves and root. White arrow indicates the inoculated leaf.

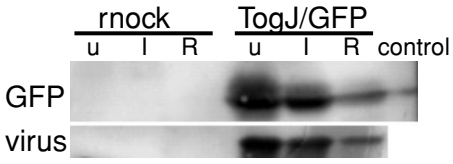

Figure 2. GFP expression in roots. Western blot analysis using anti-GFP and anti-ToMV-CP antibodies confirmed that the vector could spread and express exogenous genes in root tissues. *N. benthamiana* was inoculated with TogJ/GFP or water (mock), and 7 days after, the inoculated leaf (I), the upper leaf (U) and root (R) were collected. 2.5 ng of crude extracts (root) or 30 ng of crude extracts (leaf) and control (GFP 5 pg) were subjected to SDS-PAGE (GFP 10% SDS, CP 15% SDS) and immunoblot analysis with antibodies against GFP and ToMV CP.

The LgJ/PMT vector could elicit VIGS in roots by infecting the leaves

Previously we have shown that silencing of phytoene desaturase (PDS) induced a bleached phenotype, reflecting the inhibition of carotenoid synthesis in leaves (Hori et al. 2004). The results were readily visible in the external appearance of the infected plants, and subsequent biochemical analysis confirmed that VIGS was induced by the infection.

In the previous study on suppression of PDS, we judged that insertion of more than 200 nt should be used for efficient induction of VIGS using LgJ vectors (Hori et al. 2004). Thus, we inserted a fragment of the partial *PMT* cDNA sequence of 421 bp into the tobamovirus vector LgJ, using the GATEWAY *in vitro* recombination system, to create LgJ/PMT.

N. benthamiana plants were infected with LgJ/PMT so that they grew to be 8-week-old plants at the time of harvest for the subsequent analysis. After several rounds of infection and analysis we empirically learned that the plants of 8-weeks old were a suitable and reliable source for the analysis of nicotine content before senescence.

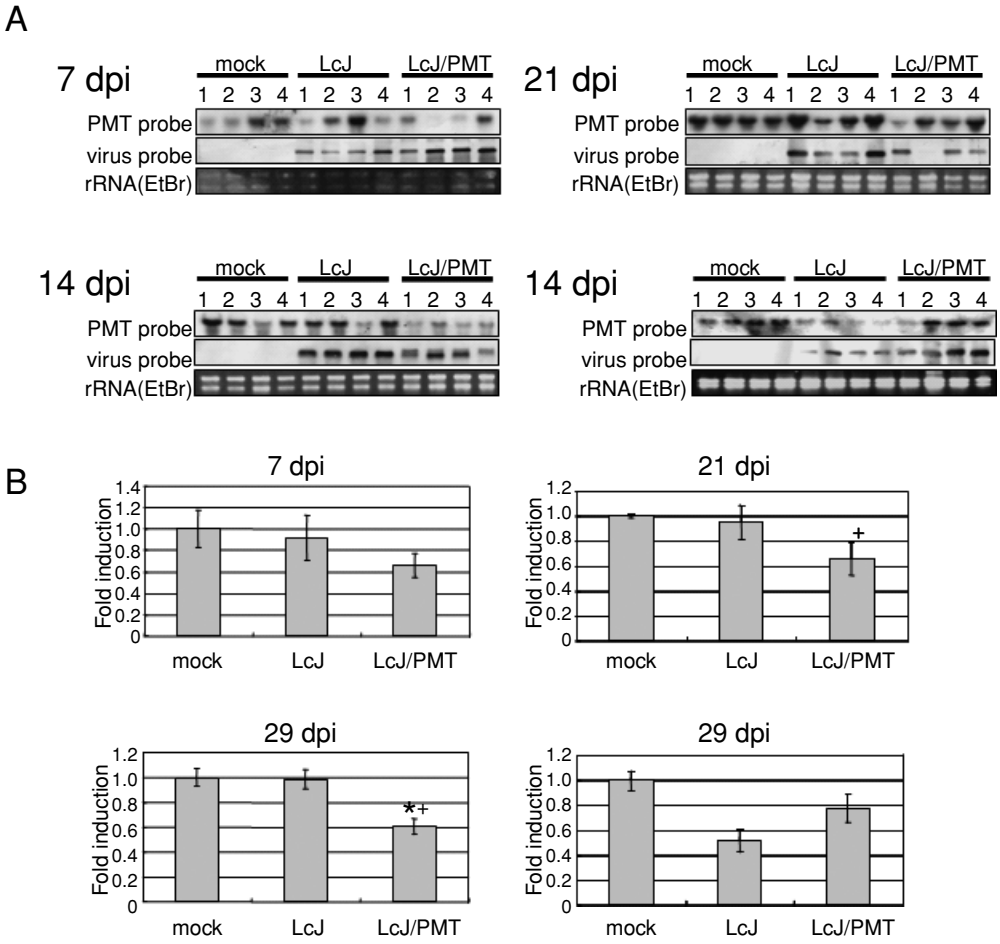

Figure 3. Effect of LgJ/PMT on PMT mRNA expression. (A) Total RNA of the whole root at the indicated time was subjected to Northern blot analysis. Each lane represents biological replicate for the indicated treated plant. (B) Quantification of data shown in A. The average signal for each plotted gene was graphed for each sample. Fold-induction represents the relative value between the expression of the mock inoculated plants and the LcJ or LgJ/PMT inoculated lines. The vertical lines represent the standard error for the four biological replicates. Asterisks and crosses denote values that are significantly different from the control or empty virus LcJ, respectively as evaluated by Student's *t*-test.

Subsequently, we estimated the level of PMT suppression using 8-week-old plants at harvest by inoculating with vectors at various times prior to the harvest.

During a series of nicotine analyses, we noticed that infection of empty vector LcJ itself elevated the level of *PMT* mRNA slightly, when compared with non-infected *N. benthamiana* plants of the same age. This resulted possibly from some defense mechanism. Thereafter, we decided to evaluate the effects of suppression by comparison with both empty virus vector LcJ or mock inoculated plants as controls.

To evaluate the suppression level over time after vector infection, Northern blot analysis was performed to check the levels of *PMT* mRNA in the roots of the plants infected with LcJ or with LcJ/PMT, at various times.

It was revealed that the *PMT* mRNA was suppressed 14 days to 21 days after inoculation (Figure 3). After 28 days, we observed increased levels of PMT mRNA relative to those at 14 days, possibly due to a recovery process.

These results indicated that by inoculating one leaf, LgJ/PMT could spread into the root tissues and induce silencing of endogenous *PMT* mRNA expressed in the roots.

Nicotine content in leaves was reduced by silencing the PMT mRNA expression in roots

Nicotine content was measured in the leaves of the plants. The results showed that, in PMT-silenced plants, the concentration of nicotine in the leaves 21 to 28 days after inoculation were reduced in comparison with that in the plants infected with empty virus vector LcJ. This observation showed that though there is a time lag between the decrease of mRNA accumulation and nicotine content, nicotine concentration well reflected the results of Northern blot analysis of *PMT* mRNA suppression shown in the previous section.

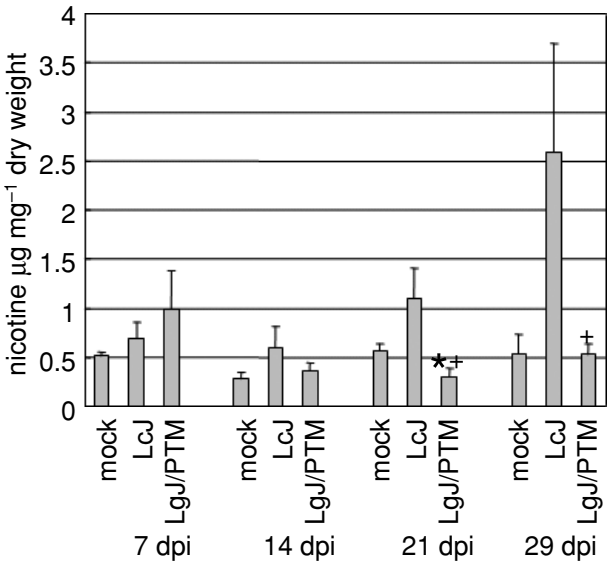

Figure 4. Nicotine content in roots. The concentration of nicotine in the leaves 21 to 28 days after inoculation was reduced in comparison with that in the plants infected with empty vector LcJ. The vertical lines represent the standard error for the four biological replicates. Aseterisks and crosses denote values that are significantly different from the mock or LcJ, respectively as evaluated by Student's *t*-test.

Discussion

Plant viruses have been used as a molecular tool for functional analysis of specific genes in reverse-genetic approaches, because a relatively short amount of time is needed for establishment of transgenic plants expressing antisense sequences; thus, they are both labor- and time-saving techniques. When the virus infects plant tissues and spreads systemically throughout the tissues, the corresponding endogenous gene transcripts, which are homologous to the insert fragment in the VIGS-vector, are targeted and degraded by post-transcriptional gene silencing (PTGS). Several RNA viruses with different properties and host ranges have been developed and utilized as VIGS vectors. Functional analysis of plant genes using VIGS has been commonly tested in the susceptible *N. benthamiana* plant as a model system.

We previously reported suppression of PDS mRNA in *N. benthamiana* plants infected with LgJ/PDS, using the VIGS approach. A tobamovirus vector, termed LgJ, based on an attenuated strain was constructed and functioned quite well for the PDS suppression in systemically infected leaves as well as inoculated leaves (Hori et al. 2004). In this present study, we wished to address whether the LgJ vector could spread into the underground parts of the plants, and express foreign genes or suppress the expression of endogenous genes.

Nicotine is known as an insecticidal alkaloid produced in the root of *Nicotiana* species from ornithine and/or arginine by way of putrescine (Leete 1990). Putrescine is converted to *N*-methylputrescine in plants that produce nicotine or tropane alkaloids; it is also known to be converted to polyamines, or conjugated with cinnamic acid derivatives or fatty acids. Putrescine *N*-methyltransferase (i.e., PMT) is engaged in the commitment of putrescine for the alkaloid biosynthesis, and is the key enzyme, performing the first committed step. We assumed that PMT represented a good target for testing the VIGS approach, as the change in metabolite composition could be tested.

During the course of analysis, we found that the nicotine content in the plants varied depending on the age and the mechanical damages suffered. After refinement of the experimental procedures, we could observe the suppression of *PMT* mRNA accumulation in roots and the reduced nicotine content in the above-ground tissues, after LgJ/PMT infection of above-ground leaves. We performed a series of experiments with LgJ/PMT-inoculated *N. benthamiana* plants 7-, 14-, 21- or 28-days post-inoculation. The most effective reduction in the levels of *PMT* mRNA was observed in 14 dpi plant roots; although similarly reduced mRNA levels were observed with 21 dpi roots. Quantification of nicotine content in the above-ground tissues revealed that 21-dpi plants displayed the greatest differences compared to both mock or LcJ inoculated plants. It is quite likely that *PMT* mRNA levels were first reduced by VIGS and that after a time lag, reduction in the levels of nicotine, which is produced by PMT activity in roots and transported to the above-ground tissues, then followed.

Accumulation of *PMT* mRNA at 29 dpi possibly showed some intermingled effects. It has been reported that the RNA silencing mechanism acts quite well at the early stages of infection, but that over time after infection, the virus can resume active multiplication by producing so-called suppressor molecules. Consistent with this notion, we observed a reversion of the VIGS-induced bleaching phenotype by LgJ/PDS (Hori et al. 2004) in the PDS suppression study.

It was previously reported that overexpression of PMT increased the nicotine content in *N. sylvestris*, whereas suppression of endogenous PMT activity severely decreased the nicotine content and induced abnormal morphologies (Sato et al. 2001). This shows that molecular engineering of secondary metabolites has the potential to increase productivity and improve product composition.

Metabolomics is the global study of metabolite profiles in a system (cell, tissue or organism) under a given set of conditions. Metabolites are results of the interaction of the organism's genome with its environment in an integrated manner. They are not merely the end product of gene expression. The large number of reactions catalyzed by enzymes active in secondary metabolism provides the opportunity to select target enzymes for metabolic engineering. Several

efficient techniques are currently available for the suppression of endogenous gene expression. These techniques should open up the field for the metabolic engineering of medicinal plants, and we assume that VIGS could be one of the choices for such purposes. However, it should be noted that caution is necessary in making the experimental plans, as to what to analyze and when to check the resultant compounds/phenomena, because there are time lags between the inoculation, the silencing and the changes.

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