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Keywords alkaloids, biosynthesis, BY-2, cell culture, functional genomics, hairy roots, *Hyoscyamus muticus*, *Nicotiana tabacum*, secondary metabolism

Abstract

The aim of this work was to improve understanding of the regulation of alkaloid biosynthesis in two *Solanaceae* plants, *Nicotiana tabacum* (tobacco) and *Hyoscyamus muticus* (Egyptian henbane). In order to map the biosynthetic genes involved in the tobacco alkaloid pathway, a functional genomics-based technology was established by combining genome-based transcript profiling (cDNA-AFLP) with targeted metabolite analysis. Altogether 459 genes were found to be differentially expressed in methyl jasmonate-elicited *N. tabacum* BY-2 cells. Homology searches performed with these genes revealed that 58 % of the genes displayed similarity with genes having known functions, whereas no sequence similarity was found with 26 % of the genes, suggesting that some of them may take part in unknown steps in tobacco alkaloid biosynthesis. Alkaloids accumulated 12 hours after methyl jasmonate application, with varying kinetic patterns. For the first time the alkaloid anatalline was shown to accumulate in *Nicotiana* cell cultures, and together with anatabine they formed the main alkaloid pool. Anatalline was further characterized structurally as being present in two isomeric forms, anatalline and *trans*-2,4-di(3-pyridyl)piperidine. Contrary to the case in whole tobacco plants, nicotine was only a minor alkaloid accumulating in elicited cells, whereas the production of a precursor methylputrescine was highly induced. Based on these results, it was suggested that the limiting step in nicotine biosynthesis occurred between methylputrescine and nicotine.

Altogether 34 methyl jasmonate-modulated genes were selected for further functional testing in BY-2 cell cultures using *Agrobacterium*-mediated gene transformation. Six genes caused a lower alkaloid accumulation compared to the control when assayed in cell cultures, whereas three genes elevated the production of one or several alkaloids. One of the genes causing enhanced

alkaloid accumulation was found to possess high sequence similarity with lysine decarboxylase, a gene responsible for the conversion of lysine in early anabasine biosynthesis. However, since lysine decarboxylase activity was not shown by the corresponding protein, the exact nature of this gene requires further elucidation. The selected genes were also assayed in hairy roots, which constitutively produce alkaloids. Two highly homologous genes were found, which showed divergent effects on alkaloid biosynthesis. These genes were suggested to function in auxin homeostasis. The other gene also resulted in marked increase in nicotine accumulation.

Tropane and tobacco alkaloids share a common biosynthetic origin, and therefore it was of interest to study whether *Nicotiana* genes could have a role in the formation of tropane alkaloids in a related species *H. muticus*. It was observed that the same gene which elevated nicotine contents in *Nicotiana* showed a positive effect on tropane alkaloid intermediate in *H. muticus*, suggesting a possible conserved role of this gene in *Solanaceae* species. On the other hand, when a known tropane alkaloid pathway gene, hyoscyamine-6β-hydroxylase (*H6H*), was overexpressed in *N. tabacum* hairy roots, a 45 % conversion of hyoscyamine into scopolamine took place when hyoscyamine was supplied to the cultures. Furthermore, up to 85 % of the produced scopolamine was secreted out of the cells. Besides being able to uptake and convert a foreign substrate, an altered tobacco alkaloid production in roots was observed after hyoscyamine feeding, suggesting highly complex regulation of the production of these defence-related compounds.

In order to improve the understanding of alkaloid transport and secretion, the function of a yeast ATP-binding cassette transporter was investigated and it was shown to attribute enhanced tolerance of tropane alkaloids in *N. tabacum* cell cultures. Combined with the information of the regulation of the biosynthesis, transporters can be exploited to design novel tools to enhance the yield and diversity of alkaloids.

Häkkinen, Suvi T. A functional genomics approach to the study of alkaloid biosynthesis and metabolism in *Nicotiana tabacum* and *Hyoscyamus muticus* cell cultures [Alkaloidien biosynteesin ja metabolian tutkiminen funktionaalisella genomiikalla tupakan ja villikaalin soluviljelmissä] Espoo 2008. VTT Publications 696. 90 s. + liitt. 49 s.

Avainsanat alkaloids, biosynthesis, BY-2, cell culture, functional genomics, hairy roots, *Hyoscyamus muticus*, *Nicotiana tabacum*, secondary metabolism

Tiivistelmä

Työn pääasiallinen tavoite oli tutkia alkaloidien biosynteesiä kahdessa Solanaceae-heimon soluviljelmässä, tupakassa (*Nicotiana tabacum*) ja Egyptin villikaalissa (*Hyoscyamus muticus*). Tupakan alkaloidibiosynteesiin osallistuvien geenien kartoitus tehtiin funktionaalisen genomiikan tekniikkaa käyttäen, jossa yhdistettiin koko genomin kattava transkriptioprofilointianalyysi cDNA-AFLP ja kohdennettu metaboliittianalyysi. Nikotiinisynteesi elisitoitiin tupakan BY-2-soluviljelmissä metyylijasmonaatilla ja tuloksena löydettiin yhteensä 459 geeniä, jotka ekspressoituivat metyylijasmonaatin vaikutuksesta. Näistä geeneistä 58 %:lla oli homologia jo tunnettujen geenien kanssa, kuitenkin 26 % geeneistä osoittautui aiemmin tuntemattomiksi, ja osan näistä geeneistä oletettiin osallistuvan tupakka-alkaloidien säätelyyn. Alkaloidien muodostuminen alkoi soluissa 12 tuntia elisitoinnin jälkeen, ja eri alkaloidit noudattivat omaa kinetiikkaansa. Anatalliinin osoitettiin esiintyvän tupakan soluviljelmissä ensimmäistä kertaa, ja se muodosti anatabiinin kanssa pääalkaloidiluokan. Lisäksi anatalliini esiintyi kahdessa eri isomeria-muodossa. Toisin kuin tupakkakasvissa, elisitoiduissa soluissa nikotiinia tuotettiin vain pieniä määriä, kun taas nikotiinisynteesin välituotetta metyyliputreskiinia muodostui suuria määriä. Tämän tuloksen perusteella nikotiinin biosynteesissä rajoittava vaihe oli jokin reaktio metyyliputreskiinin ja nikotiinin välissä.

Näistä tupakasta eristetyistä geeneistä yhteensä 34 yliekspressoitiin tupakkaan agrobakteerivälitteisellä geeninsiirrolla. Alkaloidituotto oli kuudessa eri trans-geenisessä viljelmässä alhaisempaa kuin kontrolliviljelmissä, kun taas kolmella eri geenillä vaikutus viljelmän alkaloidituottoon oli positiivinen. Yhdellä näistä tupakan alkaloidituottoa lisäävistä geeneistä oli yhteneväisyyttä lysiini-dekarboksylaasin kanssa, joka toimii anabasiini-alkaloidin varhaisessa biosynteesissä. Kuitenkin lysiini-dekarboksylaasin entsyymiaktiivisuutta ei havaittu, joten tämän

geenin tarkempi kartoitus vaatii lisätutkimuksia. Valitut geenit yliekspressoitiin myös tupakan karvajuuriin, joissa alkaloidien tuottoon ei tarvita elisitaatiota. Näissä viljelmissä kaksi eri geeniä, jotka olivat hyvin samankaltaisia amino-happokoostumukseltaan, aiheuttivat erilaisia vaikutuksia alkaloidituottoon. Toinen geeneistä nosti merkittävästi nikotiinipitoisuuksia viljelmissä, ja näiden geenien toiminta kohdistuu todennäköisesti auksiiniaineenvaihduntaan.

Tropaani- ja tupakka-alkaloidit syntetisoidaan yhteisistä lähtöaineista ja näin ollen oli kiinnostavaa selvittää, voivatko tupakan geenit toimia myös sukulaiskasvin villikaalin tropaanialkaloidi-aineenvaihdunnassa. Tutkimuksissa havaittiin, että sama geeni, joka nosti nikotiinipitoisuuksia tupakan juuriviljelmissä, aiheutti villikaalin juuriviljelmissä korkeampia tropaanialkaloidien välituotteen pitoisuuksia. Näin ollen tällä geenillä saattaa olla yleisempää vaikutusta Solanaceae-heimon aineen-vaihdunnassa.

Alkaloidien kuljetusta ja erittymistä tutkittiin hiivaperäisen ABC-transportteri-geenin avulla. Tämän geenin todettiin lisäävän tropaanialkaloidien sietokykyä tupakan soluviljelmissä. Kun tropaanialkaloidireitin hyoskyamiinia konvergoiva geeni *H6H* (hyoskyamiini-6β-hydroksylaasi) yliekspressoitiin tupakan juuri-viljelmissä, 45 % viljelmiin lisätystä hyoskyamiinista muuttui skopolamiiniksi. Lisäksi suuri määrä, jopa 85 % tuotetusta skopolamiinista, erittyi soluista ulos. Sen lisäksi, että nämä tupakan karvajuuret ottivat hyvin sisäänsä ja metaboloivat alustaan lisätyn vieraan substraatin, näillä juurilla havaittiin muutoksia myös tupakka-alkaloidiaineenvaihdunnassa. Tulosten perusteella näyttää siltä, että näillä puolustusreaktioissa toimivilla aineenvaihduntatuotteilla on hyvin monimutkainen säätelyjärjestelmä.

Preface

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Espoo, October 2008

Suvi Häkkinen

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Appendices of this publication are not included in the PDF version. Please order the printed version to get the complete publication (http://www.vtt.fi/publications/index.jsp)

List of original publications

This work is based on the following publications (I–V), which are referred to in the text by their Roman numerals.

I Goossens, A., Häkkinen, S.T., Laakso, I., Seppänen-Laakso, T., Biondi, S., De Sutter, V., Lammertyn, F., Nuutila, A.M., Söderlund, H., Zabeau, M., Inzé, D. and Oksman-Caldentey, K.-M. (2003a): A functional genomics approach toward the understanding of secondary metabolism in plant cells. Proc. Natl. Acad. Sci. USA Vol. 100. Pp. 8595–8600.

II Häkkinen, S.T., Rischer, H., Laakso, I., Maaheimo, H., Seppänen-Laakso, T. and Oksman-Caldentey, K.-M. (2004): Anatalline and other methyl jasmonate inducible nicotine alkaloids from *Nicotiana tabacum* cv. BY-2 cell cultures. Planta Med. Vol. 70. Pp. 936–941.

III Häkkinen, S.T., Tilleman, S., Świątek, A., De Sutter, V., Rischer, H., Vanhoutte, I., Van Onckelen, H., Hilson, P., Inzé, D., Oksman-Caldentey, K.-M. and Goossens, A. (2007): Functional characterisation of genes involved in pyridine alkaloid biosynthesis in tobacco. Phytochemistry Vol. 68. Pp. 2773–2785.

IV Häkkinen, S.T., Moyano, E., Cusidó, R.M., Palazón, J., Piñol M.T. and Oksman-Caldentey, K.-M. (2005): Enhanced secretion of tropane alkaloids in *Nicotiana tabacum* hairy roots expressing heterologous hyoscyamine-6β-hydroxylase. J. Exp. Bot. Vol. 56. Pp. 2611–2618.

V Goossens, A., Häkkinen, S.T., Laakso, I., Oksman-Caldentey, K.-M. and Inzé, D. (2003b): Secretion of secondary metabolites by ATP-binding cassette transporters in plant cell suspension cultures. Plant Physiol. Vol. 131. Pp. 1161–1164.

The author's contribution to the appended publications

I Goossens, A., Häkkinen, S.T., Laakso, I., Seppänen-Laakso, T., Biondi, S., De Sutter, V., Lammertyn, F., Nuutila, A.M., Söderlund, H., Zabeau, M., Inzé, D. and Oksman-Caldentey, K.-M. (2003a): A functional genomics approach toward the understanding of secondary metabolism in plant cells. Proc. Natl. Acad. Sci. USA Vol. 100. Pp. 8595–8600.

Suvi Häkkinen was responsible for all the experimental work concerning cultivation of cell cultures and metabolite analyses therein, except for the polyamine analyses which were performed by Stefania Biondi. Alain Goossens was responsible for the cDNA-AFLP transcript profiling. Suvi Häkkinen was responsible for the planning of the research, experimental design, and interpretation of the results together with the rest of the research team. Writing the paper was performed together with Alain Goossens.

II Häkkinen, S.T., Rischer, H., Laakso, I., Maaheimo, H., Seppänen-Laakso, T. and Oksman-Caldentey, K.-M. (2004): Anatalline and other methyl jasmonate inducible nicotine alkaloids from *Nicotiana tabacum* cv. BY-2 cell cultures. Planta Med. Vol. 70. Pp. 936–941.

Suvi Häkkinen was responsible for all the experimental work except for the NMR and HR-MS analyses which were performed by Hannu Maaheimo and the quantitative and preparative HPLC which was performed by Heiko Rischer. Planning of the research, experimental design, interpretation of the results and writing the paper were done together with Heiko Rischer.

III Häkkinen, S.T., Tilleman, S., Świątek, A., De Sutter, V., Rischer, H., Vanhoutte, I., Van Onckelen, H., Hilson, P., Inzé, D., Oksman-Caldentey, K.-M. and Goossens, A. (2007): Functional characterisation of genes involved in pyridine alkaloid biosynthesis in tobacco. Phytochemistry Vol. 68. Pp. 2773–2785.

Suvi Häkkinen was responsible for all the experimental work except the FL-ORF cloning, RT-PCR and Q-RT-PCR, which were performed by the team of Alain Goossens. Auxin experiments were performed by Agnieszka Świątek. Suvi Häkkinen together with Alain Goossens was responsible for planning of the research, experimental design, interpretation of the results and writing the paper.

IV Häkkinen, S.T., Moyano, E., Cusidó R.M., Palazón, J., Piñol M.T. and Oksman-Caldentey, K.-M. (2005): Enhanced secretion of tropane alkaloids in *Nicotiana tabacum* hairy roots expressing heterologous hyoscyamine-6β-hydroxylase. J. Exp. Bot. Vol. 56. Pp. 2611–2618.

Suvi Häkkinen was responsible for all the experimental work except for cultivation and feeding of *Nicotiana tabacum* root cultures and tropane alkaloid analyses of *Nicotiana tabacum*, which were performed by Elisabeth Moyano and Rosa Cusidó. Suvi Häkkinen was responsible for planning of the research, experimental design, interpretation of the results and writing the paper.

V Goossens, A., Häkkinen, S.T., Laakso, I., Oksman-Caldentey, K.-M. and Inzé, D. (2003b): Secretion of secondary metabolites by ATP-binding cassette transporters in plant cell suspension cultures. Plant Physiol. Vol. 131. Pp. 1161–1164.

Suvi Häkkinen was responsible for the transformation of *Nicotiana tabacum* BY-2 cell cultures. The experimental work with yeast and feeding assays was performed by Alain Goossens. Planning of the research, experimental design, interpretation of the results and writing the paper were done together with Alain Goossens.

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
6BHH	6β-hydroxyhyoscyamine
ABC	adenosine triphosphate binding cassette
AFLP	amplified fragment length polymorphism
AS	arginase
ADC	arginine decarboxylase
BLAST	the basic local alignment search tool
cDNA	complementary DNA
DW	dry weight
EST	expressed sequence tag
FW	fresh weight
H6H	hyoscyamine-6β-hydroxylase
HCAA	hydroxycinnamic acid amide
IAA	indole acetic acid
MeJA	methyl jasmonate
MJM	methyl jasmonate-modulated
MPO	*N*-methylputrescine oxidase

MSTFA	*N*-methyl-*N*-trifluoroacetamide
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
GC	gas chromatography
GH3	growth hormone three
HPLC	high-performance liquid chromatography
MS	mass spectrometry
NMR	nuclear magnetic resonance spectroscopy
ODC	ornithine decarboxylase
ORF	open reading frame
PAL	phenylalanine ammonia lyase
PCR	polymerase chain reaction
PMT	putrescine-*N*-methyltransferase
TR I	tropinone reductase I
TR II	tropinone reductase II
T-DNA	transferred DNA

1. Introduction

Plants produce a wide variety of chemical compounds, which can be divided into two groups – primary and secondary metabolites. Primary metabolism is considered as a set of processes involved in the fundamental maintenance of life and growth, whereas secondary metabolism consists of systems having important functions for plants in survival and competing in the environment, *e.g.* in protection against ultraviolet light, as well as in various defence-related reactions. Thus, plant secondary metabolites characteristically accumulate at certain developmental stages or in specific organs. Secondary metabolites often constitute less than 1 % of the total dry weight. Plant secondary compounds are divided into five categories: polyketides, isoprenoids (*e.g.* terpenoids), alkaloids, phenylpropanoids and flavonoids (Oksman-Caldentey and Inzé 2004). Certain secondary compounds are abundant in several plant species, such as many phenolic compounds. However, in the case of alkaloids, the production is highly specific to particular families or even species. Often secondary compounds are stored inside the plant cells in vacuoles, which account for the volume of the plant cell to a varying extent depending on the physiological state of the cell. Secondary metabolites are often unique to the plant and are structurally rather complex, low molecular weight compounds.

1.1 Plant-derived alkaloids

Hitherto, more than 100 000 different secondary metabolite structures have been discovered from plants (Verpoorte et al. 2000). The best studied secondary metabolites are alkaloids, with a total number of 21 120 found in monocots, dicots and gymnosperms (Cordell et al. 2001). Over 12 000 alkaloids have been structurally characterized (Facchini et al. 2004). Alkaloids accumulate in about 20 % of all plant species and have attracted much interest owing to their strong physiological properties enabling their use as pharmaceuticals or pesticides. Due to the versatile nature of this group of compounds, alkaloids can be defined only rather loosely as ’nitrogen-containing organic substances of natural origin with a greater or lesser degree of basic character’ (Hesse 2002).

Alkaloids are typically classified according to the ring system that constitutes the main part of their structure. However, some structures fit in more than one category and thus a rigid hierarchical classification is difficult to define. Of the approximately 15 classes, the pharmacologically most interesting alkaloids belong to the groups of: (1) amino alkaloids (*e.g.* ephedrine, mescaline); (2) pyridine and piperidine alkaloids (*e.g.* coniine, tobacco alkaloids); (3) tropane alkaloids (*e.g.* hyoscyamine, scopolamine, cocaine); (4) isoquinoline alkaloids (*e.g.* berberine, morphine, codeine); (5) indole alkaloids (*e.g.* physostigmine, vinblastine, vincristine); (6) quinoline alkaloids (*e.g.* quinine, camptothecin); and (7) steroidal alkaloids (*e.g.* veratramine, jervine, solasodine) (Harborne et al. 1999; Samuelsson 2004). Alkaloids are typically abundant in roots, bark, leaves and fruits, but they may also occur in every part of the plant. Despite advanced chemical structure elucidation, the biosynthesis of secondary compounds is only incompletely characterized, due to the long pathways which often involve multiple co-regulated enzymes which are still largely unknown. Dozens of genes involved in alkaloid pathways have been cloned, eight of them being directly involved in tropane and tobacco alkaloid metabolism (Sinclair et al. 2000; Wang et al. 2000; Hashimoto and Yamada 2003; Heim et al. 2007). Both tobacco and tropane alkaloids will be further discussed.

1.2 Tobacco alkaloids

Nicotine is the most abundant alkaloid in cultivated tobacco (*Nicotiana tabacum* L.), typically constituting more than 90 % of the total alkaloid pool. In addition to *Nicotiana* (Solanaceae) plants, nicotine has also been found from genera of various other families: *Asclepias* (Asclepiadaceae); *Atropa, Duboisia*, *Lycopersicon*, *Withania*, *Cestrum* (Solanaceae); *Eclipta*, *Zinnia* (Compositae); *Lycopodium* (Lycopodiaceae); *Macuna* (Leguminosae); *Sedum* and *Sempervivum* (Crassulaceae) (Hesse 2002). In *Nicotiana* species, the predominant alkaloid in the leaves, depending on the species, is either nicotine (most *Nicotiana* spp.), nornicotine (*N. tomentosiformis, N. otophora*) or rarely anabasine (*N. glauca*), although in roots nicotine dominates in almost all species (Saitoh et al. 1985; Sisson and Severson 1990). Other common tobacco alkaloid derivatives are shown in Fig. 1. Various minor tobacco alkaloids and their *N*-methyl derivatives, *e.g.* myosmine, *N*-methylmyosmine, cotinine, nicotyrine, nornicotyrine, nicotine *N*-oxide, 2,3'-bipyridyl and metanicotine are thought to arise by bacterial action or oxidation

during processing of commercial tobacco, rather than by biosynthetic action in the living tobacco plant (Leete, 1983). The biosynthesis of nicotine together with several other tobacco alkaloids takes place in plant roots, where the corresponding biosynthetic enzymes are found to be highly expressed (Dawson 1942; Mizusaki et al. 1973; Riechers and Timko 1999; Chintapakorn and Hamill 2003). Alkaloids are further transported through the xylem stream to the leaves, where they accumulate in the vacuoles (Alworth and Rapaport 1965; Saunders 1979; Baldwin 1999). In leaves alkaloids play an important role in several defence-related reactions, such as protecting the plant against herbivores and vertebrate predators, in detoxification processes, as well as in regulating growth (Wink 1998). Alkaloids can also represent an allocation of nitrogen pool that otherwise might be utilized in primary metabolism (Baldwin and Ohnmeiss 1994).

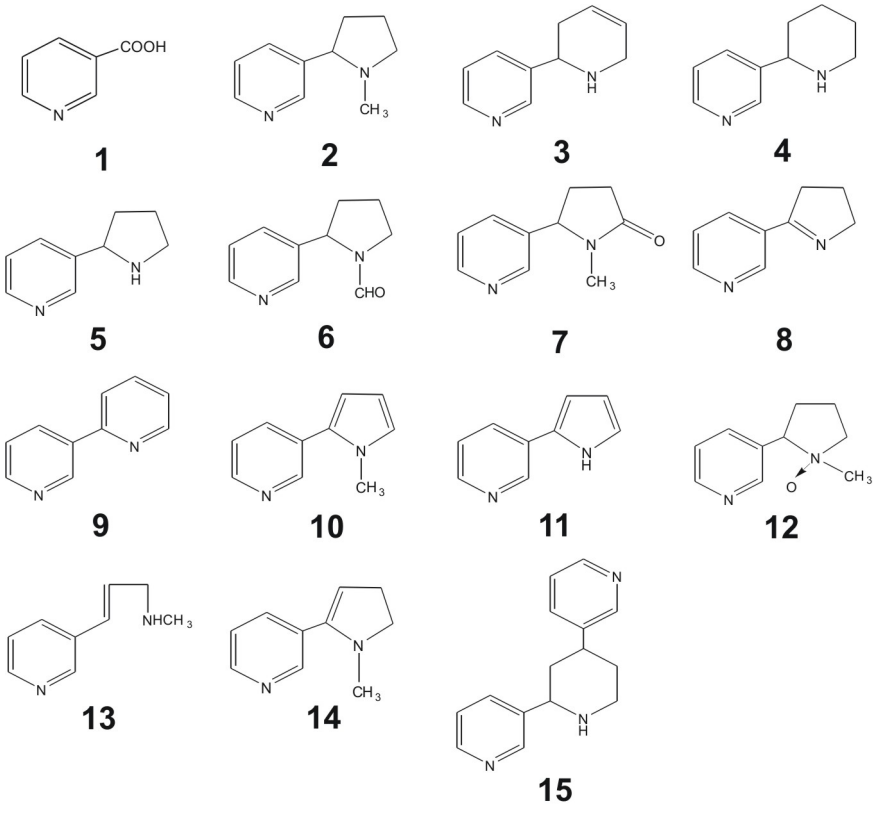

Fig. 1. Nicotine and other related alkaloids. (1) nicotinic acid, (2) nicotine, (3) anatabine, (4) anabasine, (5) nornicotine, (6) N-formylnornicotine, (7) cotinine, (8) myosmine, (9) α-β-dipyridyl, (10) nicotyrine, (11) nornicotyrine, (12) nicotine-N-oxide, (13) metanicotine, (14) N-methylmyosmine, (15) anatalline.

1.2.1 Biosynthesis of nicotine

The genetic regulation of alkaloid biosynthesis in *N. tabacum* is known to be primarily under the control of at least two independent genetic loci, *A* and *B* (Legg et al. 1969; Legg and Collins 1971). It was postulated that the *A* and *B* loci are coordinate regulators of a subset of genes encoding components of polyamine and alkaloid biosynthesis (Saunders and Bush 1979), thus the *A* and *B* loci have been referred to as *NIC1* and *NIC2*, respectively (Hibi et al. 1994). However it was shown that the *A* and *B* loci do not coordinate specifically the alkaloid biosynthesis, but rather regulate a diverse set of gene families of which only a small part (four out of a total 33 gene families) represents the alkaloid biosynthetic genes, whereas the majority of *A-B* regulated genes are involved in a variety of plant stress responses (Kidd et al. 2006).

Putrescine, a key intermediate in tobacco alkaloid biosynthesis (Fig. 2), can be synthesized directly from ornithine in a reaction catalyzed by ornithine decarboxylase (ODC, EC 4.1.1.17), or formed indirectly from arginine in a reaction sequence initiated by arginine decarboxylase (ADC, EC 4.1.1.19). Putrescine metabolism is directed to higher polyamines in one branch and to alkaloids in another branch, in a reaction catalyzed by putrescine-*N*-methyltransferase (PMT, EC 2.1.1.53). PMT is an important enzyme in the overall regulation of the alkaloid pathway (Robins et al. 1994), driving the flow of nitrogen away from polyamine biosynthesis to alkaloid biosynthesis. *N*-methylputrescine, the product of the PMT-catalyzed reaction, is further converted to *N*-methylamino butanal by a diamine oxidase DAO, or *N*-methylputrescine oxidase MPO (EC 1.4.3.6). It was first described from tobacco roots (Mizusaki et al. 1972) and has been further purified and characterised (McLauchlan et al. 1993). Recently, the gene encoding MPO has been cloned (Heim et al. 2007; Katoh et al. 2007). Interestingly, MPO has shown to catalyse the oxidation of cadaverine (Mizusaki et al. 1972; Hashimoto et al. 1990); thus it also accepts other substrates from the pathway. Spontaneous cyclisation of *N*-methylamino butanal leads to formation of the *N*-methylpyrrolinium ion, which further donates the pyrrolidine ring structure to nicotine (Leete 1983). Nicotine is formed in a condensation reaction of *N*-methylpyrrolinium and a metabolite of nicotinic acid, putatively 3,6-dihydronicotinic acid, the latter providing the pyridine ring supplied from the NAD biosynthesis pathway (Bush et al. 1999). Shoji et al. (2002) suggested that the condensation reaction between nicotinic acid and *N*-methylpyrrolinium is catalysed by an NAPDH-dependent reductase

called A622. It was shown that *A622* is jasmonate-inducible (Hibi et al. 1994; Shoji et al. 2000) and that the expression pattern of *A622* is highly similar to that of *PMT* (Shoji et al. 2002). However, the conclusive finding concerning the nature of ´nicotine synthase´ and the final step in nicotine formation has not yet been obtained. Several genes encoding enzymes involved in the upper part of the biosynthetic pathway of nicotine have been cloned, including *ADC* and *ODC* (Wang et al. 2000), *PMT* (Hibi et al. 1994; Hashimoto et al. 1998a; Riechers and Timko 1999; Winz and Baldwin 2001), *QPRT* (quinolinate phosphoribosyltransferase) (Sinclair et al. 2000), and only recently *MPO* (Heim et al. 2007; Katoh et al. 2007).

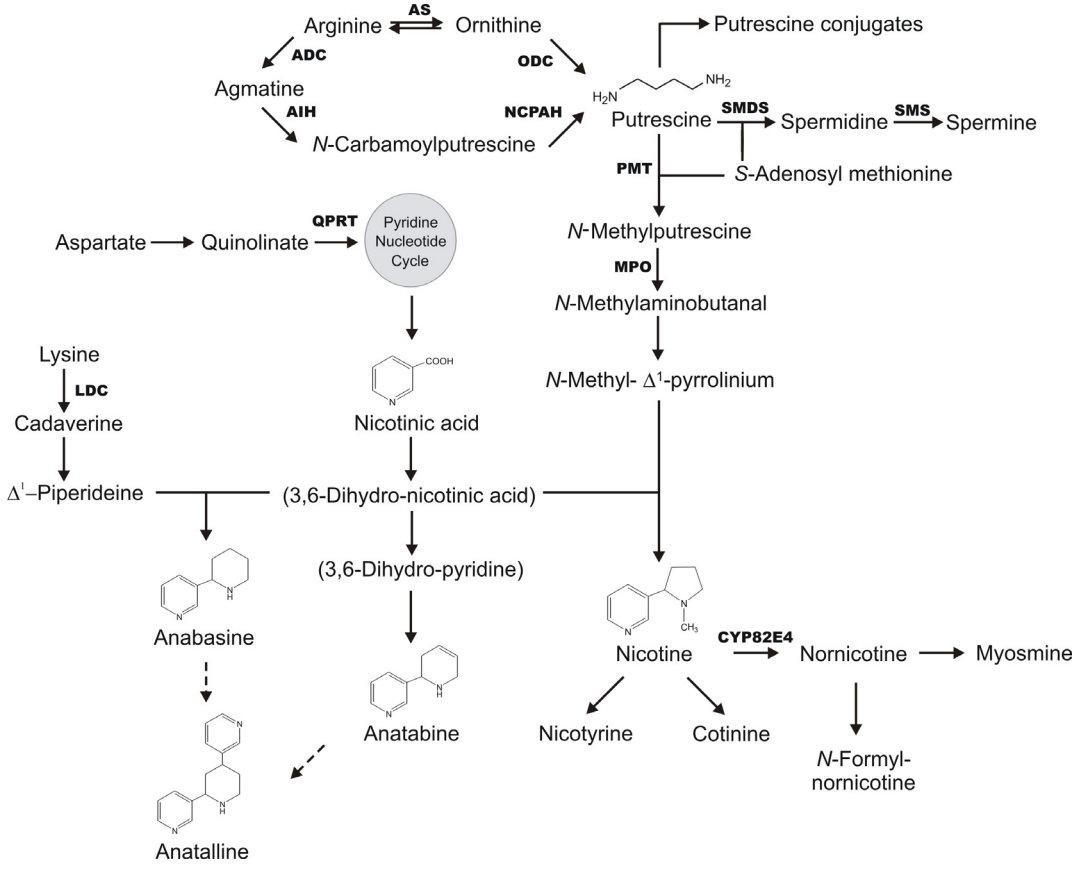

Fig. 2. Tobacco alkaloid biosynthesis. One arrow may represent more than one step. Hypothetical steps and intermediates are indicated with dashed lines and parentheses, respectively. Abbreviations: ADC, arginine decarboxylase; AS, arginase; AIH, agmatine iminohydrolase; NCPAH, N-carbamoylputrescine amidohydrolase; ODC, ornithine decarboxylase; PMT, putrescine N-methyltransferase; SMDS, spermidine synthase; SMS, spermine synthase; MPO, N-methylputrescine oxidase; QPRT, quinolinate phosphoribosyltransferase; LDC, lysine decarboxylase; CYP82E4, cytochrome P450 monooxygenase 82E4.

1.2.2 Other putrescine derivatives and polyamines

The polyamines spermidine and spermine together with the precursor putrescine occur ubiquitously in the plant kingdom. In higher plants polyamines are found in free form, conjugated to small molecules as well as bound electrostatically to negatively charged molecules. Polyamines have a significant role in plant development including growth, differentiation and response to abiotic and biotic stress (Watson and Malmberg 1996; Walters 2000). Spermidine and spermine are formed by the addition of an aminopropyl moiety by the enzymes spermidine synthase (SMDS, EC 2.5.1.16) and spermine synthase (SMS, EC 2.5.1.22), respectively (Fig. 2). This aminopropyl group is formed by the decarboxylation of *S*-adenosyl methionine (SAM). Activation of many enzymes in the polyamine biosynthetic pathway in tobacco has been reported to increase after applying methyl jasmonate (Imanishi et al. 1998; Biondi et al. 2001). In conjugated form, polyamines are mostly bound to cinnamic acids, *e.g. p*-coumaric, ferulic and caffeic acids, commonly known as hydroxycinnamic acid amides (HCAAs). Basic HCAAs typically contain the aliphatic di- and polyamines and are soluble in water, whereas neutral HCAAs, which contain aromatic amines such as tyramine, octopamine and tryptamine, are not water soluble (Facchini et al. 2002). The enzyme phenylalanine ammonia lyase, PAL (EC 4.3.1.5) plays a critical role in the synthesis of cinnamoyl putrescines, *e.g.* caffeoyl- and feruloylputrescines (Berlin and Widholm 1977; Berlin 1981). Tiburcio et al. (1985) studied the correlation between two classes of putrescine derivatives, polyamine conjugates and the alkaloids nicotine and nornicotine, and showed that in tobacco callus grown at low auxin concentration the predominant putrescine derivatives were alkaloids, whereas in high auxin the main products derived from putrescine were conjugated polyamines. They suggested that the role of bound putrescine was to serve as a pool for pyrrolidine alkaloids in the cells where alkaloid biosynthesis is active. Two genes involved in the polyamine pathway have been cloned from tobacco, *S*-adenosyl methionine decarboxylase (*SAMDC*) (Park et al. 1998) and *SMDS* (Hashimoto et al. 1998b).

1.2.3 Metabolism of other tobacco alkaloids

Nicotine is catabolised to nornicotine in a demethylation reaction. This reaction was suggested to involve cytochrome P450 (Chelvarajan et al. 1993; Imaishi et

al. 1995; Hao and Yeoman 1998), and was further elucidated recently as CYP82E4 by Siminszky et al. (2005). Nornicotine is converted to myosmine *via* a presumably irreversible reaction (Leete and Chedekel 1974). The primary metabolite of nicotine in body tissues is cotinine; approximately 70–80 % of nicotine is metabolised to cotinine and about 4 % to nicotine-*N*-oxide (Benowitz and Jacob 1999).

Anabasine is synthesized from nicotinic acid and Δ^1-piperideine deriving from lysine (Fecker et al. 1993; Bush et al. 1999; Berlin et al. 1998). Concerning the biosynthetic origin of anatabine, it was suggested that anatabine is formed by a dimerization reaction of a metabolite of nicotinic acid, 3,6-dihydronicotinic acid (Leete and Muller 1982). Although superficially similar to nicotine, anatabine and anabasine differ from nicotine in three crucial aspects: the pyrrolidine ring is replaced by a piperidine ring, there is total absence of any *N*-methylated derivatives, and they are commonly present in both (*S*)- and (*R*)-forms (Armstrong et al. 1999). Anatalline was first isolated by Kisaki and co-workers (1968) from roots of *N. tabacum* and it was found in high concentrations in methyl jasmonate-elicited BY-2 cell cultures of tobacco in the recent studies (**I**). Anatalline was shown to accumulate in two isomeric forms (**II**). The biosynthetic origin and metabolism of anatalline is still unknown.

1.3 Tropane alkaloids

Tropane alkaloids, such as atropine, hyoscyamine and scopolamine have an important role as medicinal compounds acting as parasympatolytes. The number of tropane alkaloids known from natural sources exceeds 200 (Lounasmaa and Tamminen 1993). They have been isolated from many different plant families, *e.g.* Solanaceae, Erythroxulaceae, Convolvulaceae, Proteaceae, Rhizophoraceae and Euphorbiaceae. In the Solanaceae family, common tropane alkaloid producers are *e.g. Duboisia*, *Atropa*, *Hyoscyamus, Scopolia* and *Datura* sp. L-Hyoscyamine is a levorotatory component of the racemic atropine. Scopolamine demand is estimated to be tenfold compared to that of hyoscyamine and atropine combined (Hashimoto et al. 1993). Due to the difficult synthesis of these rather complex molecules, both alkaloids are still extracted from plants.

1.3.1 Tropane alkaloid biosynthetic pathway

Biosynthesis of tropane alkaloids (Fig. 3) starts from the amino acids arginine and ornithine, as is the case with tobacco alkaloids. Tobacco and tropane alkaloid pathways share common steps until *N*-methylpyrrolinium, which is directed towards tropinone in tropane alkaloid producing systems (Sévon et al. 2001; Humphrey and O'Hagan 2001). The role of tropinone in tropane alkaloid biosynthesis was controversial for a long time, until in 1990 Landgrebe and Leete showed it to be an intermediate in the biosynthesis of tropine (Landgrebe and Leete 1990). Tropinone is further converted into tropine and pseudotropine by the reactions catalysed by two distinct enzymes, tropinone reductase I (TRI; Nakajima et al. 1993) and tropinone reductase II (TRII; Nakajima et al. 1993; Keiner et al. 2002), respectively. Littorine, a positional isomer of hyoscyamine, is formed from tropine and phenyllactic acid, the latter deriving from phenylalanine. Recently, Li and co-workers exploited virus-induced gene silencing (VIGS) to discover the rearrangement of littorine to hyoscyamine (Li et al. 2006). It was suggested that the (*R*)-littorine is converted to hyoscyamine in a two-step process, first by formation of hyoscyamine aldehyde catalyzed by a cytochrome P450 enzyme, proceeding to the formation of hyoscyamine by an alcohol dehydrogenase-catalysed reaction.

Hyoscyamine-6β-hydroxylase (H6H; EC 1.14.11.11) catalyzes both the hydroxylation of hyoscyamine leading to 6β-hydroxyhyoscyamine and the epoxidation of the latter leading to scopolamine (Hashimoto and Yamada 1986; Matsuda et al. 1991). The hydroxylase activity of *H6H* has commonly been observed to be much higher than the epoxidase activity leading to the formation of scopolamine. Scopolamine production has been increased in hairy roots of hyoscyamine-producing plants by overexpressing *H6H* alone (Hashimoto et al. 1993; Yun et al. 1992; Jouhikainen et al. 1999), and simultaneously with *PMT* (Zhang et al. 2004). Calystegines, which are synthesized in the other branch of the tropane pathway, are polyhydroxy nortropane alkaloids, possessing strong glycosidase inhibitory activity (Asano et al. 2000). The compounds were originally found in *Calystegia sepium* (L.) (Goldmann et al. 1990), but they have also been detected in many Solanaceae and other families, even in some species which were not thought to possess the tropane alkaloid pathway, such as *Solanum tuberosum* (L.) (Dräger et al. 1995). Calystegines are formed from tropinone in a reaction catalysed by TRII (Dräger et al. 1994).

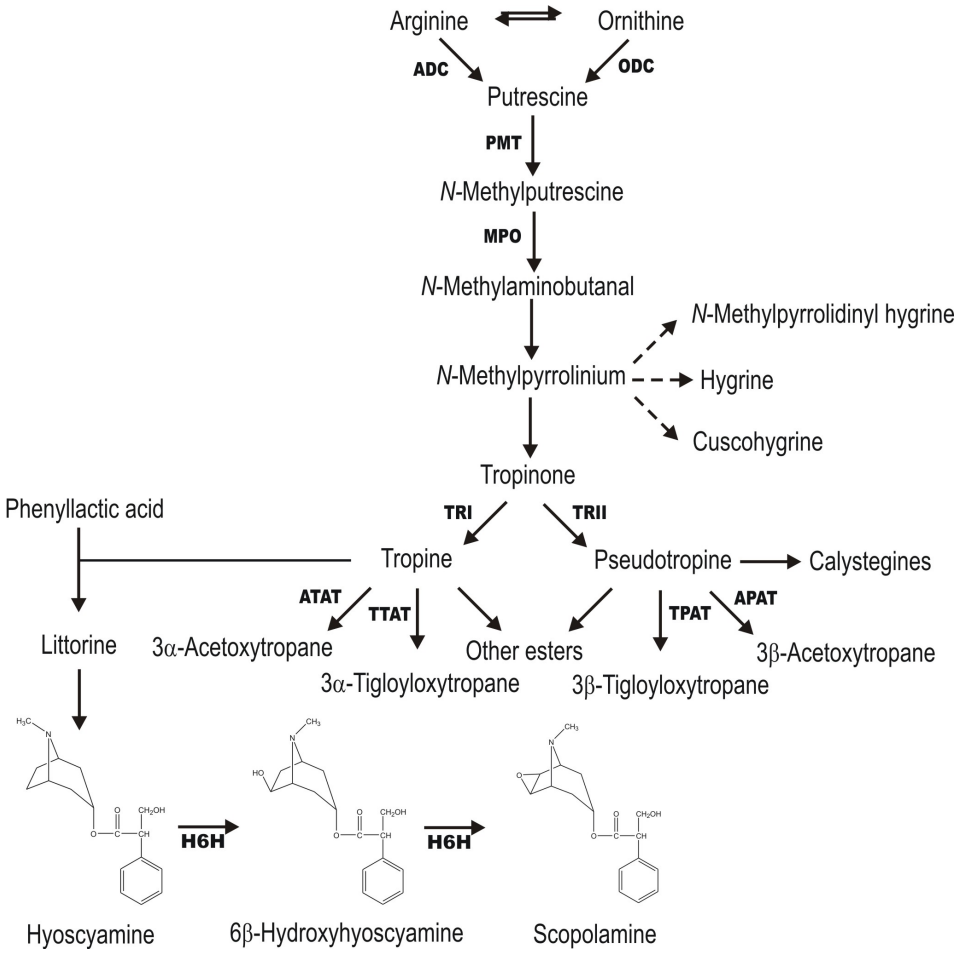

Fig 3. Tropane alkaloid pathway. One arrow may represent more than one step. Hypothetical steps are indicated with dashed lines. Abbreviations: ADC, arginine decarboxylase; ODC, ornithine decarboxylase; PMT, putrescine N-methyltransferase; MPO, N-methylputrescine oxidase; TRI, tropinone reductase I; TRII, tropinone reductase II; ATAT, acetyl-CoA:tropine acyl transferase; TTAT, tigloyl-CoA:tropine acyl transferase; TPAT, tigloyl-CoA:pseudotropine acyl transferase; APAT, acetyl-CoA:pseudotropine acyl transferase; H6H, hyoscyamine-6β-hydroxylase.

Besides tropane alkaloids and calystegines, Solanaceous plants produce other tropine- and pseudotropine-derived esters, namely 3α- and 3β-acetoxytropane (Robins et al. 1991a), and 3α- and 3β-tigloyloxytropane (Rabot et al. 1995) (Fig. 3). Two distinct acetyl-CoA-dependent acyl transferases act in transferring an acetyl group to form 3α- and 3β-acetoxytropanes from tropine and pseudotropine, respectively (Robins et al. 1991a). These compounds are present in low amounts

in *Datura* whole plants and roots (Robins et al. 1991a; Robins et al. 1994; Witte et al. 1987) and cell suspension cultures (Hiraoka and Tabata 1983), *Atropa* root and suspension cultures (Robins et al. 1994; Hartmann et al. 1986), and in *Hyoscyamus* hairy root cultures (Robins et al. 1994; Wilhelmson et al. 2006). Rabot et al. (1995) isolated and characterised a tigloyl-CoA:pseudotropine acyl transferase from transformed root cultures of *Datura stramonium* (L.). This enzyme esterifies pseudotropine with tiglic acid, forming 3β-tigloyloxytropane, and it was suggested that tigloyl-CoA and acetyl-CoA compete for the same active site of this enzyme. Interestingly, none of these esters were present in *N. tabacum* or *S. tuberosum* hairy root cultures (Robins et al. 1994).

1.4 Transporters in plant secondary metabolism

Transport of various chemical compounds across biological membranes is an essential physiological process in all living organisms. Membrane transport is involved in the uptake of nutrients, in the efflux of toxic compounds, subcellular localization of metabolites, as well as in communication between the cell and its environment. Plant cells are capable of producing a wide variety of secondary compounds, which in cytosol may be toxic to plant cells, and thus it is crucial that these compounds can be efficiently removed from the cytosol. They may be transported into the apoplastic space for further modifications, or into the vacuole, the common storage site of secondary compounds (Theodoulou 2000; Martinoia et al. 2000). The transporters are classified according to the protein family and transporter type. Phylogenetic analyses have proven to be useful for prediction of substrate specificity, as transport proteins typically cluster phylogenetically according to function (Paulsen et al. 2000).

Among all the multicellular organisms hitherto sequenced, plants have the largest representation of ABC transporter proteins encoded in their genome. Altogether 131 members of ABC proteins have been found in *Arabidopsis* (Jasinski et al. 2003). The reason for such a large representation of transporters is still unclear, but it has been suggested to have a role in adaptive functions for survival and environmental stress. The ABC superfamily contains transport systems of both uptake and efflux; the members of each of the two transporter groups generally cluster together (Saier and Paulsen 2001). Structurally, ABC transporters are characterized by one or two cytosolically oriented nucleotide

binding-folds (NBFs) linked to multiple hydrophobic transmembrane-spanning domains (TMD). The substrate crosses the membrane via the TMD domains and in some cases the substrate specificity is determined by TMD domains (Theodoulou 2000). NBFs are oriented towards the cytoplasmic side and provide the site for ATP hydrolysis. ABC transporters are classified into four main subfamilies: MDR (multidrug resistance), MRP (multidrug resistance associated protein), AOH (ABC one homolog) and PDR (pleiotropic drug resistance).

Plant alkaloids are often transported by ABC transporters in microbes and herbivores, but only a few of them have been characterized from plants (Yazaki 2006). Altogether four PDR transporters have been described in *N. tabacum*, namely *Nt*PDR1 (Sasabe et al. 2002), *Nt*PDR2 (Schenke et al. 2003), *Nt*PDR3 and *Nt*PDR4 (Ducos et al. 2005). All of them are categorized as stress responsive genes, although none have been identified as nicotine transporters. Tobacco alkaloids, which are synthesized in root tissues, are further transported to leaves, where they have a defensive role for the survival of the plant. The transporter protein responsible for this transport process has not yet been identified, but based on the studies performed with tobacco hornworm it was suggested that a P-glycoprotein mediated (MDR-like) transport is involved (Gaertner et al. 1998). In different species, the same compounds may be transported by different transporter systems. Berberine uptake in *Coptis japonica* (Thunb.) was shown to involve MDR-type ABC transporter (Sakai et al. 2002; Shitan et al. 2003), although in *Arabidopsis thaliana* (L.), which does not produce endogenous berberine, its transport is effected by another type of transporter called MATE (multi antimicrobial extrusion/ multidrug and toxin extrusion) (Li et al. 2002).

Transporters in plasma membrane might function in the secretion of endogenous metabolites having role in defence reactions in plants, as was suggested by Jasinski and co-workers (2001). A PDR5 homolog *Np*ABC1 from *N. plumbaginifolia* (L.) was localized in the plasma membrane and was shown to be strongly expressed after addition of sclareolide or the antifungal diterpene sclareol. The ABC transporter PDR5 of the yeast *Saccharomyces cerevisiae* confers resistance to several unrelated drugs, and substrates of the transport process also include plant-derived compounds, such as taxol, indole alkaloids, and flavonoids (Kolaczkowski et al. 1996; 1998). It was also shown that PDR5 is capable of accepting tropane alkaloids as substrates (**V**). Further, the effect was demonstrated

with tobacco cells, in which *PDR5*-carrying cells showed decreased cell death after administration of hyoscyamine. The plant PDR family is still poorly investigated and remains an interesting subject concerning the secretion of plant secondary metabolites and applications in biotechnological production systems using plant cells, which are often characterized by poor secretion of the produced secondary metabolites.

1.5 Discovering secondary metabolite pathways

Biosynthetic pathways of plant secondary metabolites are commonly relatively long and co-ordinately regulated by several genes encoding for enzymes with varying substrate specificities. Hitherto, only a few pathways, namely flavonoids, terpenoid indole alkaloids (*e.g.* vincristine) and isoquinoline alkaloids (*e.g.* berberine, morphine) have been well elucidated in plants (Winkel-Shirley 2001; Hashimoto and Yamada 2003). Rational engineering of complex metabolic pathways involved in the production of plant secondary metabolites has been greatly hampered by limited knowledge of the biosynthetic steps and regulatory mechanisms leading to formation of the desired compounds (Oksman-Caldentey et al. 2007). The classical approach for pathway elucidation of plant metabolites has been on the one hand at the metabolite level by using labelled precursors or on the other hand at the enzymatic level by purifying the biosynthetic enzymes from high-producing plant cells. Labelled precursor feedings have been successfully performed to develop chemical reaction schemes of the pathways. Indeed, in 1983, Leete estimated that more than 300 feeding experiments had been described involving labelled putative precursors of tobacco alkaloids only (Leete 1983). However, feeding putative precursors, *e.g.* amino acids or even more proximate precursors to cultures does not always result in elevated levels of the end products (Lockwood and Essa 1984; Hamill et al. 1990). This may be due to the complex metabolite regulation and feedback systems in plant cells, or sometimes to the high toxicity of the administered precursor (Robins et al. 1987; Chintapakorn and Hamill 2003). At the enzymatic level, pathway elucidation has been hampered by *e.g.* low levels and instability of the individual enzymes and problems in finding a substrate for activity measurements (Verpoorte 2007). The recent advances in molecular biology tools, *e.g.* expression pattern analysis and homology-based screening have superseded classical chromatographic purification and peptide sequencing

strategies. After expression in heterologous systems such as in bacteria, recombinant proteins are tested for their expected enzyme activities (Hashimoto and Yamada 2003). These researches allowed the discovery of a number of new enzymes catalysing steps in the biosynthesis of diverse alkaloids (for reviews see Hashimoto and Yamada 2003; Facchini et al. 2004). In many pathways, however, overexpression of a single enzyme does not lead to enhanced accumulation of the end product, but rather to bottlenecks due to limiting subsequent reactions (Biondi et al. 2000; Sato et al. 2001; Moyano et al. 2002). In order to perform fully successful metabolic engineering in plants, the regulation of multiple steps in parallel or manipulation of regulatory genes controlling the genetic regulation of the whole pathway is necessary.

1.5.1 Gene discovery

Functional genomics aims at determining the function of genes. As tools for this, genomics, transcriptomics, proteomics and metabolomics data are integrated by using bioinformatics approaches (Oksman-Caldentey et al. 2004). For the discovery of genes involved in a certain metabolite pathway, cell systems in which metabolism is highly activated, *e.g.* in high-producing cell lines, can be exploited. Alternatively, mutants have been used for studies of genes participating in particular pathways, as was shown by Hibi and co-workers (1994). They exploited low-nicotine producing mutants to discover genes involved in nicotine biosynthesis by differential screening, and as a result two genes were isolated: one corresponded to *PMT*, whereas the role of the other, named *A622* has still to be discovered (see section 1.2.1). The co-expression approach has often been exploited in pathway mapping based on co-regulation and co-expression of a set of genes in the same pathway (Saito et al. 2007). In order to activate secondary metabolite biosynthesis, chemical induction in the form of elicitors can be exploited. Methyl jasmonate, an octadecanoid-derived signalling molecule, was applied to *e.g.* cell cultures of *Medicago truncatula* (Gaertn.), and as a result a gene encoding for triterpene glycosyltransferase was identified by using array-based screening combined with targeted metabolite profiling (Achnine et al. 2005).

For model plants such as *Arabidopsis* and rice, for which the genome sequences are known, microarray-based strategies have been extensively used. However,

for most plants, for example various exotic medicinal plants, genomic tools based on either complete genome data or expressed sequence tags (ESTs) are not easily applicable. For such non-model plants, methods such as differential display (Yamazaki et al. 1999; Schoendorf et al. 2001; Yamazaki and Saito 2002) and cDNA-AFLP (cDNA-amplified fragment length polymorphism) (Rischer et al. 2006; **I**) have been shown to be powerful tools for gene expression studies. The methods for genome-wide expression analysis, such as cDNA-AFLP, offer an efficient tool for quantitative transcript profiling (Breyne et al. 2003). This method is applicable to any plant species, because sequence information of the genes studied is not required. In addition, it allows the identification of novel genes and distinguishes between isoforms of encoded proteins. Recent advances in functional genomics have proved to be powerful tools in pathway mapping of plant secondary compounds (see reviews by Oksman-Caldentey and Inzé 2004; Oksman-Caldentey and Saito 2005; Goossens and Rischer 2007; Saito et al. 2007).

1.5.2 Metabolite profiling of secondary compounds

Metabolic profiling has become an integral part of plant functional genomics (Fiehn et al. 2000; Oksman-Caldentey and Saito 2005). The wide variety of different chemical structures, possessing a range of physical and chemical properties, sets great challenges for analytical tools when profiling multiple metabolites in parallel. It has been estimated that within a particular species the number of metabolites lies between 5000 and 25000, which is comparable in order of magnitude to the number of genes (Trethewey 2004). Particularly, compared to primary compounds, profiling of secondary compounds is far more challenging, due to their highly divergent chemical structures and sensitivities in extraction conditions. Extraction with only a single system generally results in poor or no recovery of a number of compounds, which are retained in the plant matrix (Oksman-Caldentey et al. 2004). Currently, no single analytical technique provides the ability to profile all of the metabolome. This obstacle has been circumvented by using selective extraction and different analytical platforms. The key for understanding pathway regulation is to define intermediates and to measure flux through the pathway. By visualizing fluxes of secondary metabolite pathways integrated with transcriptomic data, a novel means for mapping pathways at the systems level from gene to metabolite can be created.

Traditionally, mass spectrometry combined with a chromatographic method is used for metabolite analysis. Perhaps the most widely used metabolic profiling technique is GC-MS, which is nowadays routinely used for the screening of metabolite phenotypes in functional genomic studies of plants (Fiehn et al. 2000; Trethewey 2004). One of the first applications of a plant metabolic profiling approach was published by Roessner et al. (2000). In this study, GC-MS was used to discover more than 150 compounds from potato tubers, showing this profiling system to be highly reproducible and sensitive. Advantages of GC-MS technology include its low costs compared to CE-MS, LC-MS, or LC-NMR instrumentation, excellent chromatographic reproducibility and resolution, repeatable mass spectral fragmentation and few matrix effects (Kopka 2006). However, the requirement of chemical derivatisation of non-volatile compounds can increase the analysis costs and time. Other mass spectrometer-based approaches exploited in plant metabolite profiling include HPLC-MS (Yamazaki et al. 2003) and CE-MS (Sato et al. 2004). Systems incorporating time-of-flight mass analyzers (GC-TOF) (Davis et al. 1999) or Fourier transform ion cyclotron mass spectrometry (FTMS) (Aharoni et al. 2002; Hirai et al. 2004; Mungur et al. 2005) offer an attractive alternative providing higher mass fragmentation accuracies and allowing rapid analyses of complex mixtures. Another highly interesting approach for non-targeted analysis has been introduced by NMR (Choi et al. 2004; Liang et al. 2006). Following analytical measurements, metabolomics approaches require bioinformatics tools for the storage, retrieval and analysis of large datasets. For data visualization, several techniques have been exploited, such as principal components analysis (PCA) (Hirai et al. 2004; Choi et al. 2004; Rischer et al. 2006), and self-organizing maps (SOM, Kohonen networks) (Hirai et al. 2004). For metabolite profiling various chromatographic and spectroscopic databases are available, although these generally concern primary metabolites. Thus there is an urgent need to build up such a database for secondary metabolites, which can then be linked to other existing datasets.

1.6 Transgenic plant cell cultures

Plant cell cultures have been extensively exploited for various biotechnological applications as an alternative to the traditional agricultural cultivation of plants. The use of cell culture systems offers advantages to produce metabolites in a controlled environment, independent of climatic conditions and under conditions

in which the different production parameters can be optimized. Plant cell cultures can be divided in two main classes, differentiated and undifferentiated cell cultures. The former consist of *e.g.* organs like shoots, roots or embryos, whereas callus and cell suspension cultures are referred to as undifferentiated cell cultures. Since the first gene transfers in plants in 1983, achieved by four independently working groups (Bevan et al. 1983; Fraley et al. 1983; Herrera-Estrella et al. 1983; Murai et al. 1983), numerous efficient gene transfer techniques have been developed for genetic manipulation of plants. In addition to so-called direct gene transfer techniques (*e.g.* particle bombardment, electroporation, microinjection), Agrobacterium-mediated gene transfer has been the most commonly used method for gene delivery to plants, and will be discussed further in the following.

Agrobacterium (Rhizobiaceae) is a soil bacterium, which is able to deliver its own plasmid-DNA into the nuclear genome of the plant cell. The bacterium attaches into the wound site of the plant tissue and recognizes certain wound substances, *e.g.* acetosyringone, secreted by the plant (Zupan et al. 2000). As a result, the *vir* (virulence) region of the plasmid becomes activated and processing of the T-DNA (transferred DNA) for the gene transfer starts (Sheng and Citovsky 1996; Zupan et al. 2000). After successful integration of the bacterial DNA into the host plant genome the tumour formation in the wound site begins as well as the production of low molecular weight tumour substances called opines. These opines are used as a nutrient for the bacterium (Chilton et al. 1982). The host range of *Agrobacterium* is perhaps broader than that of any other plant pathogenic bacterium, although many cultivated monocotyledonous plants and legumes are not natural hosts for *Agrobacterium*. The molecular mechanism of the resistance to *Agrobacterium* is not known, although production of antimicrobial metabolites (Sahi et al. 1990), a lack of *vir* gene inducers (Usami et al. 1997), inefficient T-DNA integration (Narasimhulu et al. 1996) and *Agrobacterium*-induced programmed cell death (Hansen 2000) have all been suggested. Gene transfer in monocotyledonous plants via *Agrobacterium* has been successful with maize, rice, wheat and barley (see Nadolska-Orczyk et al. 2000).

1.6.1 Hairy root disease

Hairy root (Fig. 4) is a plant disease caused by the infection of *Agrobacterium rhizogenes* carrying Ri (root inducing) plasmid. When the bacterium infects the plant, the T-DNA of the Ri-plasmid is transferred and integrated in the nuclear genome of the host plant. As a result of the transformation, hairy roots appear at the infection site (for a review see Sevón and Oksman-Caldentey 2002). In the T-DNA there are four genetic loci, called *rol*A, *rol*B, *rol*C and *rol*D, which are responsible for the hairy root phenotype and have been shown to positively affect the secondary metabolite production in *Nicotiana* (Palazón et al. 1998) and in *Atropa* (Bonhomme et al. 2000). Hairy roots are able to grow without auxins, and certain *Aux* genes from *Agrobacterium* have been shown to provide transformed cells with an additional source of auxin (Morris 1986; Chriqui et al. 1996). Moyano and co-workers (1999) proposed that auxin-responsible genes in *A. rhizogenes* affect the root morphology and further alkaloid biosynthesis, resulting in lower production of alkaloids in roots carrying the *Aux1* gene. Hairy roots characteristically lack geotropism and have a high degree of lateral branching (David et al. 1984). In addition, hairy root cultures have demonstrated their ability to rapidly produce biomass as well as high contents of tropane alkaloids (Jouhikainen et al. 1999). Unlike crown gall tumours, hairy roots are capable of spontaneously regenerating into plants (Oksman-Caldentey et al. 1991).

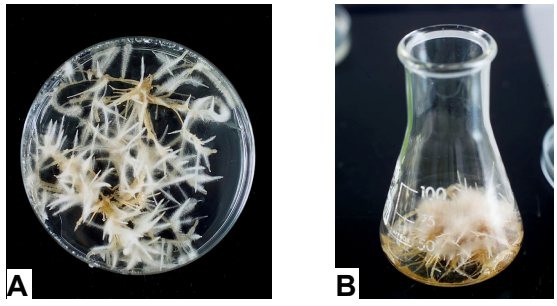

Fig. 4. Hairy roots (Hyoscyamus muticus L.) grown on solid (A) and in liquid (B) medium.

1.6.2 Crown gall disease

Crown gall disease is caused by *A. tumefaciens* and characteristically this tumour tissue differs from non-transformed tissue in its ability to grow in the absence of hormones, such as auxins and cytokinins. Crown gall tumours result from overproduction of the phytohormones specified by *A. tumefaciens* T-DNA genes located in Ti (tumour inducing) plasmid (Gelvin 1990). In addition, tumour tissues produce opines, which can also serve as markers of the transformed nature of the cells, since non-transformed plant cells do not synthesize opines. However, the opine production tends to fade during subculturing (Moyano et al. 1999). When all the genes within the T-DNA region are removed, *Agrobacterium* is still capable of transforming this DNA, although the resulting ´disarmed´ Ti plasmid and the host bacterium are no longer oncogenic and thus the tumour formation is prevented (Hellens et al. 2000). Modern Ti plasmid vectors used in plant transformation are binary vectors, T-DNA and *vir* regions residing on separate plasmids (Hoekema et al. 1983).

1.6.3 Hairy roots as producers of secondary metabolites

Hairy roots are able to accumulate the same alkaloids as the parent plant, even in higher quantities than the intact plants or undifferentiated cell cultures (see reviews by Sevón and Oksman-Caldentey 2002; Rao and Ravishankar 2002). In addition, hairy roots gain biomass rather rapidly and have simple cultivation medium requirements, being able to grow without phytohormones. They show high genetic stability as well as more stable metabolic production than that of undifferentiated cell cultures. Thus, hairy roots offer an attractive alternative for the production of various high-value secondary compounds, such as the recently reported production of camptothecin (*Camptotheca acuminata*), morphine derivatives (*Papaver somniferum*), and ajmalicine (*Rauvolfia micrantha*) (Guillon et al. 2006). Hairy roots of several Solanaceae species have been successfully used for the production of tropane alkaloids (Sevón and Oksman-Caldentey 2002) and tobacco alkaloids (Parr and Hamill 1987; Hamill et al. 1990).

2. The aims of the present study

The main aim of this work was to improve understanding of the regulation and biosynthesis of tobacco alkaloids using a functional genomics approach. This was achieved by investigating *Nicotiana tabacum* BY-2 cell culture as a model system. Functional analysis of some of the discovered genes was performed with stably transformed *N. tabacum* BY-2 cell suspension cultures and in hairy root cultures, using targeted metabolite profiling. In addition, the selected *Nicotiana* genes were tested in the related species *Hyoscyamus muticus*, in order to study the role of these genes in tropane alkaloid biosynthesis.

Secondly, the aim was to improve understanding of the mechanisms of alkaloid accumulation and secretion in two Solanaceae species, *N. tabacum* and *H. muticus*. This was achieved by overexpressing an ATP-binding cassette transporter gene in *N. tabacum* cell cultures and on the other hand by studying the effect of overexpressed hyoscyamine-6β-hydroxylase (*H6H*) gene on hyoscyamine bioconversion and secretion in *N. tabacum* and *H. muticus* hairy root cultures.

3. Materials and methods

Materials and analytical methods are presented below. More detailed information is also available in papers I–V.

3.1 Plant material and micro-organisms

Seeds of *Nicotiana tabacum* cv. Bright Yellow 2 were kindly provided by Prof. Nagata (University of Tokyo). The plants initiated from these seeds were cultivated in the greenhouse at VTT and the leaves were used for alkaloid analyses (**II**) and as a starting material for the initiation of hairy roots (**III**).

N. tabacum BY-2 cell suspension culture was received from University of Gent. The culture was established by Prof. Nagata in the University of Tokyo, as described in Nagata et al. (2004). The cell suspension culture was maintained as described in (Nagata and Kumagai 1999). BY-2 cell culture was used for the studies of secondary metabolite regulation (**I**), anatalline isolation (**II**), functional characterization of the genes isolated from BY-2 (**III**), and for transportation of secondary compounds (**V**).

Agrobacterium tumefaciens LBA4404 pBBR1MCS-5 carrying the Gateway™ (Invitrogen) overexpression vector pK7WGD2 with *Sc*PDR5-W303 and *Sc*PDR5-US50 (**V**) was used for transformation of BY-2 cell cultures. For studying the functional properties of the genes isolated from BY-2, FL-ORF amplicons from *N. tabacum* BY-2 (**III**, Table 1) were introduced into Gateway™ (Invitrogen) overexpression vector pK7WG2D and subsequently introduced into *A. tumefaciens* LBA4404 pBBR1MCS-5 and *A. rhizogenes* LBA9402. *A. rhizogenes* LBA9402 carrying pLAL21 (35S-*H6H*) was used for transformation of *Nicotiana* and *Hyoscyamus* hairy roots (**IV**). *A. tumefaciens* was maintained in LB medium with rifampicin (25 ppm), gentamycin (25 ppm), streptomycin (25 ppm) and spectinomycin (100 ppm) for bacterial selection. *A. rhizogenes* was maintained in YMB medium with rifampicin (100 ppm) (control) and additionally streptomycin (25 ppm) and spectinomycin (100 ppm) (pK7WG2D-carrying lines) as bacterial selection agents. The cultures were grown on solid or in liquid medium for 48–72 hours (+28 °C) before the use.

3.2 Establishment of transgenic cultures

3.2.1 Transgenic BY-2 cell cultures

Transformation of BY-2 cultures (**III**, **V**) was performed by using two-day *A. tumefaciens* liquid culture. Seven days old BY-2 cell suspension culture was subcultured with a ratio of 1:10 three days prior to co-cultivation with *Agrobacterium*. After three days of co-cultivation in room temperature, the cell mixture was plated on solid medium containing kanamycin (50 ppm) for selection of transformed cells, and vancomycin (500 ppm) and carbenicillin (500 ppm) for elimination of bacteria. The first transformed calli appeared 14–21 days after infection and were routinely subcultured on the selective medium.

3.2.2 Transgenic hairy root cultures

The hairy roots of *Hyoscyamus muticus* L. strain Cairo (Egyptian henbane) (**IV**) were transformed as described by Jouhikainen et al. (1999), and were maintained as described by Oksman-Caldentey et al. (1991). Transformed hairy roots of *N. tabacum* (**III**, **IV**) were initiated by infecting the surface-sterilized leaves with *Agrobacterium rhizogenes*. Leaves were collected from plants grown in the greenhouse. Surface sterilization was performed by 10 min treatment in 0.6 % (v/v) sodium hypochlorite, with added Tween 80, after dipping the leaves in 70 % ethanol. Subsequently, the leaves were washed four times with sterile water and surface dried before the infection. Leaves were cut into ca. 1 cm^2 pieces and placed mid-rib upwards on petri dishes containing hormone-free cultivation medium. Infection was performed by wounding the mid-ribs of the leaves with a sterile needle containing two-day *Agrobacterium* from solid medium. After 48 hours the leaves were transferred to solid medium containing cultivation medium and 500 ppm cefotaxime for elimination of excess bacterium.

3.2.3 Cultivation of hairy roots

Hairy root cultures were cultivated in shake flasks in 20 ml liquid medium for 28 days unless described otherwise. When cultivated in 6-well plates (Nunclon™, Ø 40 mm), each well was inoculated with one 10–20 mm long root and 6 ml

liquid growth medium. Subsequently, the plate was sealed with parafilm. Each 6-well plate contained only one gene construct and the growth was measured after 21 days.

3.2.4 Polymerase chain reaction

The presence of the transgene originating from cDNA-AFLP tags (**I**) was verified with PCR in the following way. Genomic DNA was extracted with the acetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980). A complete PCR mixture contained 100 ng total DNA, 10 pmol of each oliginucleotide primer, 200 µM dNTPs, 1.2 U Taq, and buffer supplied by the enzyme manufacturer (Promega, UK) in a total volume of 50 µl. The oligonucleotide primers used to amplify the ATTB1 and ATTB2 regions of the Gateway vector were 5'-GGGGACAAG-TTTGTACAAAAAAGCAGGC-3' and 3'-GGGGACCACTTTGTACAAGAAAGCTGGG-5', respectively. PCR amplification conditions were as follows: initial denaturation at 95 °C for 1 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 1 min, and extension at 72 °C for 1.5 min, followed by the final extension at 72 °C for 7 min. The presence of *rol*B-gene was verified as described in Sevón et al. (1995), and the absence of *virD1*-gene, indicating the *Agrobacterium* removal, was checked with the primers reported by (Hamill et al. 1991). The PCR reaction mixture for *virD1* was similar to that described for the ATTB site, and the PCR amplification was performed as follows: initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 1 min, and extension at 72 °C for 1.5 min, followed by the final extension at 72 °C for 7 min.

3.3 Elicitation of BY-2 cell cultures

Elicitation of BY-2 cell cultures with methyl jasmonate (MeJA) was performed as described in (**I**) and (**II**). Before applying MeJA at a final concentration of 50 µM, the cell cultures were washed and cultivated in hormone-free medium to avoid the inhibitory effects of auxin on tobacco alkaloid accumulation. When testing the transgenic cell cultures (**III**), three individual cell culture clones of each construct were established. The experiment was started when the cultures were six days old. In order to speed up the functional testing, some of the genes

were tested by pooling the three individual culture clones before elicitation. Excess medium was removed by filtering (Miracloth), and the culture was washed with 100 ml of hormone-free medium, and finally the culture was accurately weighed to a known density in the same medium. The resulting cell slurry was divided into 100 ml shake flasks in order to minimize potential flask-to-flask variation. After MeJA addition, the shake flasks were cultivated at +28°C in the dark. Samples were taken between 1 h and 98 h (**I**), or at 48 h (**II**, **III**) after elicitation, unless otherwise described.

3.4 Sampling

The samples from cell suspension and hairy root cultures were taken by filtering them under suction followed by freeze drying (2–3 days, 50 mbar). In BY-2 elicitation experiments, shake flasks containing the same transgenic line were pooled together before freezing and drying.

3.5 Analytical methods

3.5.1 cDNA-AFLP

Transcript profiling of BY-2 cell cultures was performed by the cDNA-AFLP method as described in (**I**) and (Breyne et al. 2002; Breyne et al. 2003). For RNA extraction and cDNA synthesis, samples were digested first with *Mse*I, followed by *Bst*YI, or *vice versa*. After selective preamplification, the screening of messengers was performed by using two or three additional selective nucleotides, resulting in a total of 160 primer combinations. Quantitative data analysis was performed by AFLP-Quantapro Software (Keygene, The Netherlands). For characterization of the cDNA-AFLP fragments the sequences were compared against nucleotide and protein sequences by BLAST sequence alignments.

3.5.2 Tobacco alkaloids

Tobacco alkaloids were analysed as described by HPLC (**II**, **III**) and GC-MS (**I**–**IV**) from freeze dried plant material. Alkaloids were extracted with

dichloromethane from basic solution. The internal standard 2,4'-dipyridyl (TCI) was added before extraction. (±)-Nicotine (Sigma), (±)-Anabasine (Sigma), (*R*,*S*)-Anatabine (TRC), Anatalline (VTT), and *trans*-Anatalline (VTT) were used as reference compounds.

3.5.3 Tropane alkaloids

Tropane alkaloids were analysed by GC-MS as described in (**IV**). The lipids were removed with petroleum ether and the alkaloids were subsequently extracted with dichloromethane from basic solution. Homatropine (Sigma) was used as an internal standard. Samples were further derivatized with MSTFA (Pierce) before GC-MS analyses. Hyoscyamine (Merck), scopolamine-HBr (Sigma), tropine (Sigma) and tropinone (Sigma) were used as reference compounds. Other alkaloids and alkaloid derivatives were identified on the basis of the GC-MS spectral data (Hartmann et al. 1986; Witte et al. 1987).

3.5.4 Polyamines

Polyamines were extracted from frozen samples as described by Biondi et al. (2001) (**I**). Briefly, free polyamines were extracted with perchloric acid. After washing with perchloric acid, the resulting pellets were resuspended in perchloric acid. Subsequently, perchloric acid-soluble and -insoluble conjugates were recovered after acid hydrolysis of the supernatant and washed pellets, respectively. Aliquots of the supernatants, and the hydrolysed supernatants and pellets were made alkaline with disodium carbonate and after dansylation the amines were extracted with toluene. Samples were analysed by HPLC as described in Scaramagli et al. (1999).

3.5.5 Phenolic compounds

Phenylpropanoids were extracted and analysed by GC-MS as described in (**I**). Additionally, phenolic acids were extracted using the method described by Maillard and Berset (1995) and subsequently analysed by HPLC (Nuutila et al. 2002).

4. Results and discussion

Genetic knowledge of biosynthetic pathways is of crucial importance in order to improve the low product yields of various secondary metabolites in plant cells. This study is part of a larger collaboration project related to plant metabolic engineering in which a Solucel® technology platform has been developed (Fig. 5).

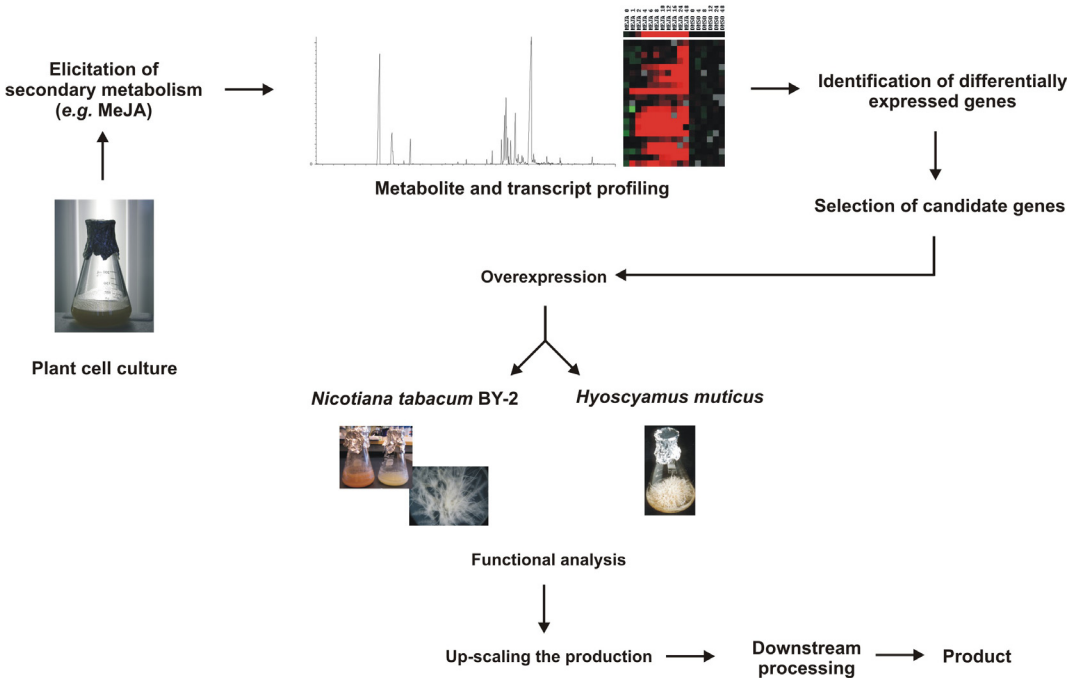

Fig. 5. Scheme of the study platform.

4.1 *N. tabacum* BY-2 cell culture as a model system for the discovery of secondary metabolite genes (I)

N. tabacum cv. 'Bright Yellow-2' (referred to as BY-2) was used when establishing the technology platform for genetic control of the tobacco alkaloid metabolism. BY-2 cell suspension culture (Nagata et al. 2004) was a desirable choice for this study, as it is a rapidly multiplying culture, cells propagating 100-fold in seven days (Nagata and Kumagai 1999). Moreover, tobacco alkaloid biosynthesis in BY-2 can be induced by applying methyl jasmonate (MeJA) as

an elicitor (Imanishi et al. 1998), forming a suitable inducible system for the cDNA-AFLP (cDNA-amplified fragment length polymorphism) technology we applied, which is based on differential regulation of transcripts. Under the conditions in which the desired metabolites are elicited, the genome-wide transcript profiling methods, such as cDNA-AFLP, can be used to identify genes the expression of which correlates with the accumulation of the desired metabolites. Because no prior sequence information of the genes studied is needed, this system is applicable to any plant and to any metabolite of interest.

As the pathway of tobacco alkaloids is rather short, including several genes already known (Fig. 2), this system was suitable for testing the technology described. By using cDNA-AFLP-based transcript profiling, a total of 459 unique MJM (methyl jasmonate-modulated) gene tags were isolated. Homology searches performed with these tags revealed that 58 % of the tags displayed similarity with genes having known functions, although no homology was found for 26 % of the tags (Fig. 6). All but two of the genes known to be involved in tobacco alkaloid biosynthesis were found in a single experiment, and they were shown to cluster together with various novel genes or genes encoding proteins with unknown functions. These novel genes were potential candidate genes to fill in the gaps in the tobacco alkaloid pathway. Two genes from the nicotine pathway which were not picked up by the cDNA-AFLP system applied were *PMT* and the recently characterized *MPO*. As cDNA-AFLP allows the coverage of more than 80 % of the transcripts (Breyne et al. 2003), the remaining 20 % of the genes either do not possess the restriction sites of the enzymes applied, the sequence tags are too short, or they derive from 3'-untranslated ends not allowing unambiguous identification. Further analysis with RT-PCR revealed the presence and MeJA-induced expression of *PMT*, although despite several putative oxidases found in the MJM gene set, we have not identified a gene similar to *MPO* described by Heim and co-workers (2007). In addition to genes involved in alkaloid biosynthesis, several genes known to be involved in the formation of other secondary compounds, *e.g.* phenolics, were shown to be modulated by methyl jasmonate (**I**, Supp. Fig. 4).

Further studies of these MJM genes included the search for transcriptional regulators of tobacco alkaloid biosynthesis using protoplast-based transient expression assay (De Sutter et al. 2005) and screening of both regulatory and structural genes in stably transformed tobacco cells (**III**).

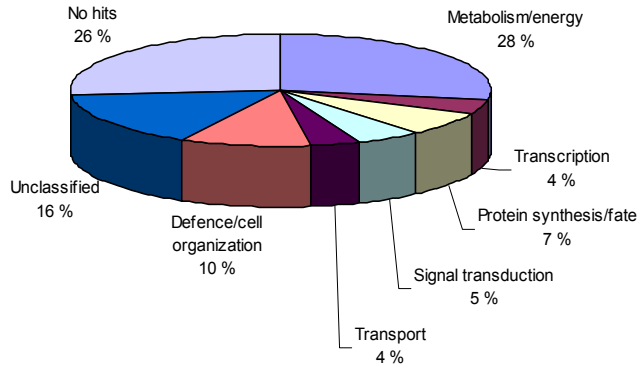

Fig. 6. Categorization of the MJM genes from N. tabacum BY-2.

4.1.1 Jasmonate-mediated elicitation of tobacco alkaloids and polyamines (I)

Elicitation with MeJA induced the accumulation of four tobacco alkaloids in BY-2 cell culture. Nicotine, which in most tobacco plants is the most abundant alkaloid, was only a minor alkaloid, together with anabasine, accumulating in elicited BY-2 cells. The accumulation of alkaloids was followed for up to 98 h, and they were shown to reach their maximum levels at that point. However, long MeJA exposure causes gradual decrease in the number of productive cells, and after 98 h the biomass in the elicited cells was only 40 % of that of the non-treated cells (**I**, Fig. 1a). Since it was observed that all the studied alkaloids began to accumulate well before 48 h, this was considered to be a suitable and long enough time to observe the changes in the accumulation pattern in further experiments (**III**). After 48 h elicitation, the percentages of the total alkaloid contents for individual alkaloids were on average: nicotine (< 1 %), anabasine (1 %), anatabine (76 %) and the two anatalline isomers (22 %), measured from three separate experiments.

Accumulation patterns of individual alkaloids followed differential kinetics (Fig. 7). Nicotine and anatabine was shown to accumulate 12 h after elicitation, whereas anabasine and anatalline appeared only after 24 h. Interestingly, it has been speculated by two previous authors whether the last step in the biosynthesis of both nicotine and anatabine could be catalyzed by the same enzyme (Friesen and Leete 1990; Chintapakorn and Hamill 2003). The accumulation kinetics

observed in our studies support this hypothesis, although the final steps in the formation of these alkaloids still need further elucidation. The pattern of nicotine accumulation is in accordance with the results obtained by Imanishi et al. (1998), however the quantitative nicotine values measured at 36 h were much lower in our studies. A comprehensive explanation for this phenomenon was not found; however it could partly be caused by the differences in the experimental conditions, *e.g.* omitting benzylaminopurine treatment before MeJA application in this study. Furthermore, since the origin of the BY-2 cell line dates back twenty years and it has been delivered to several laboratories, modifications in the physiology of this cell line are apparent.

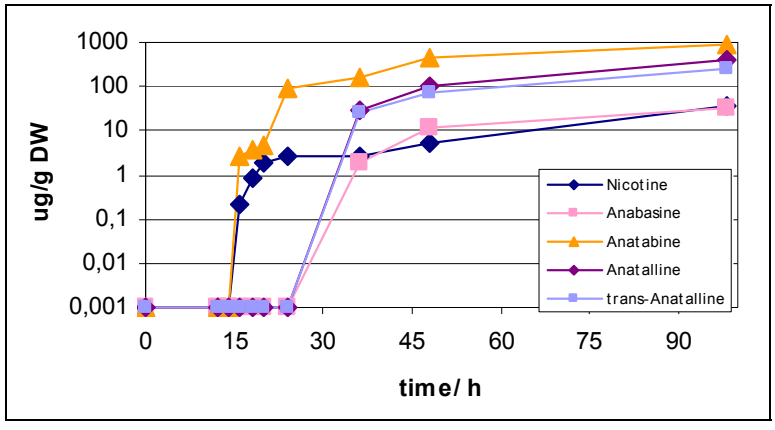

Fig. 7. Alkaloid accumulation in MeJA-elicited N. tabacum BY-2 cells.

Elicitation with MeJA also caused a marked accumulation of free and soluble conjugated methyl putrescine (mPut) (**I**, Fig 1C). The level of free mPut started to increase shortly after the onset of MeJA treatment, reaching over 50-fold in 48 h compared to that of the control cells. A similar pattern of mPut accumulation was observed earlier by Imanishi et al. (1998). As well as in the free form, mPut also appeared covalently bound to small molecular weight compounds, mainly hydroxycinnamic acids (so-called soluble conjugates) in elicited cells (**I**, Fig 1C). The physiological function of most hydroxycinnamic acid amides (HCAAs) is still unknown, although they have been implicated in several growth and developmental processes (Facchini et al. 2002). It has been suggested earlier that these HCAAs in elicited tissues may function in detoxifying cells from excess phenolics, and conjugation may also help to regulate the size of the free amine pool (Biondi et al. 2001).

In BY-2 cells MeJA treatment resulted in a slightly enhanced accumulation of conjugated acid-insoluble mPut, although the levels of other conjugated polyamines were not altered. The accumulation of insoluble conjugated amines represents mainly compounds associated with cell walls and proteins. The accumulation of wall-bound amides of hydroxycinnamic acids (*e.g.* coumarouyl- and feruloyltyramine) is known to be stimulated by elicitors, *e.g.* MeJA (Biondi et al. 2001). Low levels of insoluble conjugates were also observed in non-elicited cells with increasing cultivation time. Recently, Gális et al. (2006) described a novel MYB transcription factor from tobacco, which was shown to be involved in the MeJA signal transduction and inducing the biosynthesis of phenylpropanoid-putrescine conjugates both at genetic and metabolite levels. This *Nt*MYBJS1 transcription factor was induced in the temporal expression pattern with other phenylpropanoid-related genes, such as phenylalanine ammonia lyase (*PAL*) and 4-coumarate:coenzymeA ligase (*4CL*).

Since MeJA treatment resulted in a strong induction of mPut accumulation, whereas nicotine represented only a minor compound in the total alkaloid pool, it is presumed that the limiting step in nicotine biosynthesis is either in the conversion catalysed by *N*-methylputrescine oxidase (MPO) or later on the conversion of the *N*-methylaminobutanal into *N*-methylpyrrolinium (Fig. 2). The MPO activity increases in conditions which promote nicotine biosynthesis in *Nicotiana* plants (Mizusaki et al. 1972; Saunders and Bush 1979), and it was shown that as in other known tobacco alkaloid biosynthetic enzymes, *MPO* is also jasmonate inducible (Heim et al. 2007). Therefore, a putative oxidase from the MJM gene set, annotated as T361 (**III**, Table 1) and displaying co-induction with other early-induced MJM genes, was subjected to functional testing. However, no effects on alkaloid accumulation were observed with the lines carrying this gene. Only recently a gene corresponding to *MPO* in tobacco was cloned (Heim et al. 2007), but no similar sequence was found in the MJM gene set described here. Both anatabine and nicotine compete for nicotinic acid, but apparently nicotine biosynthesis is limited by the supply of the other substrate required for nicotine condensation, *N*-methylpyrrolinium. This was also suggested by the studies of Friesen et al. (1992) based on feeding experiments with *N*-methylpyrrolinium and nicotinic acid. Feedback loops of enzymatic activity cannot be excluded, *e.g.* the high accumulation of mPut resulting in lowered activity of MPO, as was reported by Robins et al. (1991b) in *Datura* hairy root cultures. Characterization of the final step in nicotine formation and the nature of the putative 'nicotine synthase' (see section 1.2.1) remain to be resolved.

4.1.2 Anatalline accumulates in two isomeric forms in *N. tabacum* BY-2 cell suspension cultures after elicitation (II)

In addition to the widely distributed alkaloids in *Nicotiana* species, anatalline (Fig. 8) was found for the first time to be accumulated in *Nicotiana* cell cultures. Anatalline was further isolated and characterized structurally (**II**) and it was found always to be present in two isomeric forms, anatalline and *trans*-anatalline (*trans*-2,4-di(3-pyridyl)piperidine). In order to study whether anatalline isomerisation could take place during sample extraction, the isolated pure anatalline isomer was subjected to extraction conditions. It was confirmed that both anatalline and *trans*-anatalline are synthesized in the cells, and that the extraction itself does not affect the ratios of the anatalline isomers (unpublished results). Other pyridine derivatives from *N. tabacum* plants have also been shown to be naturally present as diastereomers (Wei et al. 2005). As suggested by Kisaki et al. (1968), based on two asymmetric carbons in anatalline structure, altogether four stereoisomers should exist. However, it is possible that the remaining two isomers are enantiomeric forms of anatalline and *trans*-anatalline and thus could only be identified by *e.g*. chiral separation.

Anatalline has been described from *N. tabacum* roots (Kisaki et al. 1968), although neither of these isomers were found in tobacco leaves of greenhouse-grown *N. tabacum* SR1 or BY-2 in MS analysis (**II**, Table 3). Moreover, anatalline was reported as a trace compound in *N. tabacum* L. cv. 'Ibusuki' (Miyano et al. 1981), and recently from cigarette smoke (Geng et al. 2007). Thus it is apparent that anatalline contents vary according to the cultivation conditions and developmental stage of the plant. For example, field-grown tobacco displayed significantly higher alkaloid levels compared to those of greenhouse-cultivated plants (Sisson and Severson 1990). Cultivar and culturing conditions also affected the anatalline fraction in hairy roots. In *N. tabacum* BY-2 roots the anatalline isomer represented approximately 3 % of the total alkaloids (**III**), whereas in *N. tabacum* cv. Xanthi the levels of anatalline were higher, approximately 10 % (**IV**). In MeJA-treated cells, the content of anatalline isomer represented 6–14 % of the total alkaloid pool (after 48 h elicitation). The kinetic pattern of anatalline followed that of anabasine (**I**, Fig. 1b).

The biosynthetic pathway of anatalline is still unknown. It is suggested that anatalline is synthesized from nicotinic acid, either *via* anabasine or anatabine.

Previously, based on bioconversion studies performed with methylated anabasine and anatabine, it was suggested that wheras tobacco cells probably do not possess the capacity to degrade anabasine, anatabine may be subjected to further metabolism (Bartholomeusz et al. 2005). However, degradation products of anatabine were not identified in the cited study, and the potential anatalline formation as a result of anatabine metabolism remains unresolved.

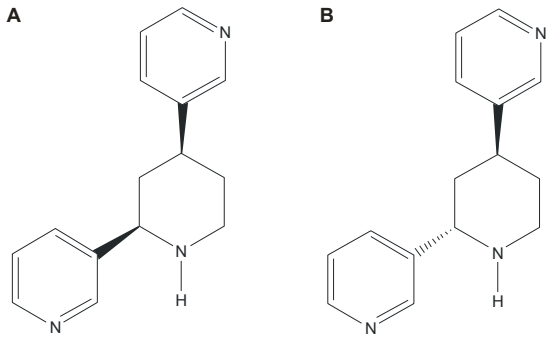

Fig. 8. Anatalline (A) and trans-2,4-di(3-pyridyl)piperidine (B).

In order to determine whether feeding of known tobacco alkaloid precursors would affect anatalline accumulation, *L*-lysine, cadaverine and nicotinic acid were fed to MeJA-treated and non-treated cell cultures. When fed to non-elicited cells, alkaloid accumulation was unaffected, indicating that either precursors were not taken up efficiently from the culture medium, or that the precursors were taken up but possibly differently metabolised when the biosynthetic machinery for alkaloid production was not activated. Since the uptake pattern of the fed precursors was not monitored, it is not known to what extent the precursors were responsible for the observed effects. However, in elicited cells, feeding did cause decreased accumulation of metabolites, suggesting that precursors were taken up by the cells at least to some extent and thus either caused inhibition of endogenous alkaloid production or resulted in leakage of the metabolites to the culture medium.

Feeding precursors of alkaloid metabolism is known to result in versatile effects on the activities of enzymes participating in the steps of alkaloid pathways, and it is possible that high accumulation of intermediates resulted in lowered activities of one or more enzymes in the alkaloid metabolism (Robins et al. 1991b; Friesen et al. 1992). It was further suggested by Friesen et al. (1992),

based on the examination of nicotinic acid supplementation, that nicotinic acid inhibits the demethylation reaction of nicotine leading to nornicotine, resulting in reduced levels of both nicotine and nornicotine in *Nicotiana* root cultures. The levels of anatabine and anabasine were elevated as a result of feeding with nicotinic acid and cadaverine, respectively (Friesen et al. 1992; Walton et al. 1988). However, these studies were performed with differentiated cells, in which alkaloids are produced constitutively. Possibly a more favourable approach for unravelling the metabolic origin of anatalline would be a combination of using labelled substrates allowing the information of carbon integration, as well as a production system in which elicitation is not required, such as hairy roots.

4.1.3 Jasmonate-mediated elicitation of phenylpropanoids (I)

During the MeJA treatment, the colour of the elicited BY-2 cell culture changed from typical yellow into reddish orange (Fig. 9). Since secondary metabolites, such as certain phenolic compounds, are involved in plant pigmentation, the elicited cultures were examined for their content of phenylpropanoids. The key enzyme acting in the phenylpropanoid pathway and regulating the overall flux of major phenylpropanoids is phenylalanine ammonia lyase (PAL), which connects the primary and secondary metabolism (Bate et al. 1994). It is known that the activity of PAL can be induced by wounding or elicitor treatment (Nagai et al. 1988; Hahlbrock and Scheel 1989). Indeed, in this study MeJA caused the induction not only of *PAL*, but also of chalcone synthase (*CHS*) further down in the pathway, and several other genes possessing similarities with the known genes involved in the phenylpropanoid metabolism (**I**, Supp. Fig. 4).

Fig. 9. BY-2 cell culture elicited with MeJA 48 h (left) and non-elicited 48 h (right).

When samples of elicited and non-treated BY-2 cells were analysed, no major changes in the common flavonoids, coumarins, lignans or anthocyanins were observed (**I**). Since *CHS* was also upregulated after elicitation, the presence of common compounds contributing to yellow pigmentation in plants, *e.g*. chalcone and tetrahydroxychalcone, was examined but neither were found in the samples. However, various trimethylsilyl derivatives of trace compounds suggested certain phenylpropanoids or their derivatives to be present in low quantities in these samples. Thus, further examination of putative cell wall-bound phenolic acids and flavonoids was performed by HPLC in methanolic extracts after alkaline hydrolysis. The amounts of caffeic acid and 4-coumaric acid were 3-fold and 2-fold in elicited cultures, respectively, whereas the contents of ferulic acid were slightly decreased (Table 1, unpublished results). Interestingly, it was recently reported by Gális and co-workers (2006) that the MeJA inducible MYB transcription factor *Nt*MYBJS1 positively regulates several phenylpropanoid-related genes in BY-2, and further causes the accumulation of hydroxycinnamoyl-polyamine conjugates, *e.g.* caffeoyl-, feruloyl-, and coumaroylputrescines. Indeed, the results of increased amounts of these hydroxycinnamic acid derivatives shown in Table 1 are well supported by the findings of Gális et al. (2006), since it is likely that the linkage between these phenolic acids and putrescine is broken during the alkaline hydrolysis of the samples (Maillard and Berset 1995; Clayden et al. 2001).

Table 1. Accumulation of phenolic acids (μg/g DW) in BY-2 cell cultures in methanolic extracts after alkaline hydrolysis.

	Caffeic acid	***4-Coumaric acid***	***Ferulic acid***
Control	67.8	9.8	33.2
MeJA 98 h	205.9	19.2	26.0

Although no specific compound causing this colour change as a result of MeJA application has hitherto been identified, it is possibly caused by the accumulation of several phenylpropanoid-like compounds appearing in low quantities. This assumption is based on the fact that MeJA treatment caused the alteration of transcription levels of several phenylpropanoid metabolism-related genes, although no major metabolite was found to be solely responsible for this colour formation, which according to Matsuoka and Galis (2006) is localized in the vacuole. The role of putative bound chalcone-related compounds in elicited cells

may also not be excluded, based on the colour change towards more red in more alkaline pH, and the accumulation behaviour of bound phenolics described above.

4.2 Functional analysis of *Nicotiana tabacum* genes involved in tobacco alkaloid metabolism

The selected genes were tested for their function by overexpressing them in BY-2 cells and hairy root cultures. Their heterologous expression was studied in *H. muticus* hairy roots as described in section 4.2.3. *Nicotiana* and *Hyoscyamus* utilise parts of the same biosynthesis pathway for tobacco and tropane alkaloids, respectively.

4.2.1 Screening of genes in tobacco cell suspension cultures (III)

The selected genes from the MJM data set were introduced to BY-2 cell cultures and subjected to the targeted metabolite profiling in order to screen the candidate genes involved in alkaloid metabolism. A total of 34 genes originating from the MeJA-modulated set of cDNA-AFLP tags of *N. tabacum* BY-2 (**I**) were selected for functional testing. These genes consisted of already known genes in the alkaloid pathway, *ADC*, *ODC* and *QPRT*, as well as novel genes or genes encoding for proteins with unknown functions (**III**, Table 1). From altogether 459 MeJA-modulated (MJM) gene tags, these 34 genes were selected satisfying the following criteria: their expression was induced within 6 h after MeJA elicitation, and they coded for putative enzymes or proteins of unknown function, or on the basis of their sequence should be involved in signal transduction.

The selected genes were stably transformed in BY-2 cells *via Agrobacterium tumefaciens* and the changes in metabolite accumulation were compared against the control (carrying 35*S*-GUS) lines, both after elicitation with MeJA as well as without elicitation. In order to obtain better insight into the variability of the system applied, the importance of different experimental factors was examined. The analytical variation associated with the extraction and instrumentation was calculated from replicate analytical extracts (n = 6), giving a coefficient of variation (CV) value less than 10 %, depending on the compound. The variation between cultivation flasks gave CV less than 12 % (n = 6), representing intra-

experimental and analytical variation, indicating that the elicitation protocol applied here was acceptable and did not substantially increase the variation in the experimental setup. Day-to-day variation was determined with one control line which was assayed in five separate experiments, giving a mean CV of 38 %, depending on the alkaloid (Table 2, a–e). This was somewhat lower than the variation between individual clones assayed separately (mean CV 49 %) (Table 2, f–j). It is interesting to note that the biological variability in current research fits well to that previously reported (approximately 50 %) in *Medicaco truncatula* cell cultures by Broeckling and co-workers (2004). Consequently, the temporal effects together with clone-to-clone variation were substantial factors affecting variance in the experimental system applied. On this account, in each experiment a control line was generated and monitored in parallel with the transgenic lines tested. The results of the control culture in each experiment were referred against the overall variability of control cultures, and the experiment was considered valid when the control cultures gave results fitting within these limits.

Table 2. Variability in BY-2 control cultures. Alkaloid contents (mg/g DW) were measured in cell suspension cultures 48 h after elicitation with MeJA. Samples a–e represent the same clone (temporal variation), samples f–j represent individual clones (temporal and clonal variation) tested in separate experiments. Each result is an average of duplicate analytical extractions.

	Anatabine	***Anabasine***	***Nicotine***	***Anatalline***	
BY-2 GUS a	4.33	0.21	0.09	0.31	
BY-2 GUS b	4.63	0.30	0.11	0.44	
BY-2 GUS c	5.65	0.27	0.08	0.45	
BY-2 GUS d	2.27	0.07	0.01	0.29	
BY-2 GUS e	6.09	0.30	0.13	0.54	
Mean	**4.59**	**0.23**	**0.08**	**0.41**	
SD	**1.48**	**0.10**	**0.04**	**0.10**	
CV%	**32**	**42**	**51**	**26**	**mean = 38**
BY-2 GUS f	5.34	0.36	0.17	0.13	
BY-2 GUS g	5.95	0.39	0.14	0.12	
BY-2 GUS h	4.93	0.36	0.20	0.34	
BY-2 GUS i	1.81	0.05	0.06	0.16	
BY-2 GUS j	3.12	0.15	0.09	0.38	
Mean	**4.23**	**0.26**	**0.13**	**0.23**	
SD	**1.72**	**0.15**	**0.06**	**0.12**	
CV%	**41**	**58**	**43**	**54**	**mean = 49**

For each transgene, three individual clones were assayed and the alkaloid accumulation of each clone was examined and compared to that of the experimental control. Taking into account the variation displayed by the control cultures, a threefold difference against the control was considered significant. Of the 34 genes stably overexpressed in BY-2 cell suspension cultures, six lines carrying different gene constructs were shown to accumulate lower levels of alkaloids compared to the control cultures (**III**, Table 2). These genes included the three known genes *ADC*, *ODC* and *QPRT*, which all showed repressed accumulation of several alkaloids. Transgenic tobacco lines transformed with p35*S*-driven overexpression have earlier been shown to undergo co-suppression-mediated gene silencing (Niebel et al. 1995). Except for *QPRT*, of which the mRNA steady-state levels were shown to be elevated (**III**, Fig. 3), reduced accumulation of alkaloids could have resulted from silencing of corresponding endogenous genes or mutations within the 35*S*-promoter. Besides the transcriptional level, in the case of enzymes participating in the early biosynthetic steps, measuring the levels of more proximate intermediates would have given better insight into the functional role of ODC and ADC. As has been reported earlier, overexpression of yeast *ODC* in tobacco roots led to enhanced putrescine levels, although apparently some other enzyme than ODC was a limiting step in nicotine formation (Hamill et al. 1990). On the other hand, cells may possess coping mechanisms when the intracellular concentration of toxic metabolites becomes too high, resulting in *e.g.* further modification of the compounds to less toxic forms or balancing the intermediate supply (Chintapakorn and Hamill 2003). No major additional peaks were observed in the metabolite profiles obtained with HPLC, but the possibility of the formation of alkaloid derivatives other than those extracted in alkaloid fraction and detected with the UV detector cannot be excluded. Whether active secretion of the metabolites outside the cells took place is not known, since in these analyses the alkaloid levels in the culture medium were not routinely performed.

Three overexpression lines, carrying either genes annotated as unknown proteins (*MAP2* and *MC126*) or glutathione-*S*-transferase-like protein (*MT401*), showed increased accumulation of alkaloids, especially nicotine (**III**, Table 2). In addition to nicotine, enhanced levels of other alkaloids were also observed with the gene *MC126*, and thus the functional role of this gene was further assessed. Significantly higher levels of nicotine, anabasine and anatabine compared to control cultures were displayed by lines overexpressing *MC126* (**III**, Table 3).

High sequence similarity was found between MC126 and two members of the family consisting of putative lysine decarboxylases (LDC) (**III**, Fig. 4), although further studies are needed to assign the functional role of *MC126*. An alternative function site of *MC126* may lie in phenylpropanoid conjugates, which have shown to be accumulated in MeJA-elicited cell cultures (Gális et al. 2006), and which are synthesized in higher amounts in *LDC*-overexpressing cells (Berlin et al. 1998). The presence of these compounds in elicited BY-2 cell cultures is probable (see section 4.1.3).

4.2.2 Screening of genes in tobacco hairy root cultures (III)

In order to test the functional role of the selected genes in differentiated systems, in which alkaloids are produced constitutively, transformed hairy root cultures of *N. tabacum* were initiated. Altogether eight constructs were overexpressed in hairy roots and further subjected to metabolite analysis. In order to obtain information about clone-to-clone variation in the metabolite production, five individual control root clones were analysed, two of them in separate cultivation passages, and subsequently the variations in each alkaloid were determined, with a mean CV of 42 %. When different gene constructs were tested in hairy roots, the above-mentioned limits were set to define considerable differences against controls. In this way we could circumvent the day-to-day variations when screening multiple root lines, at the same time obtaining better insight into the clonal differences in the production levels.

Two of the constructs tested showed altered alkaloid production levels compared to the control cultures, namely *C127* and *C175*, both annotated as GH3-like proteins based on the BLAST searches of the sequences. These genes encode for enzymes having 89 % amino acid similarities, but their effects on alkaloid metabolism were divergent (**III**, Table 4). In the case of *C127*, a clear positive effect on nicotine biosynthesis was observed (Fig. 10). For anatalline, the positive effect was moderate, while the other alkaloids were not effected.

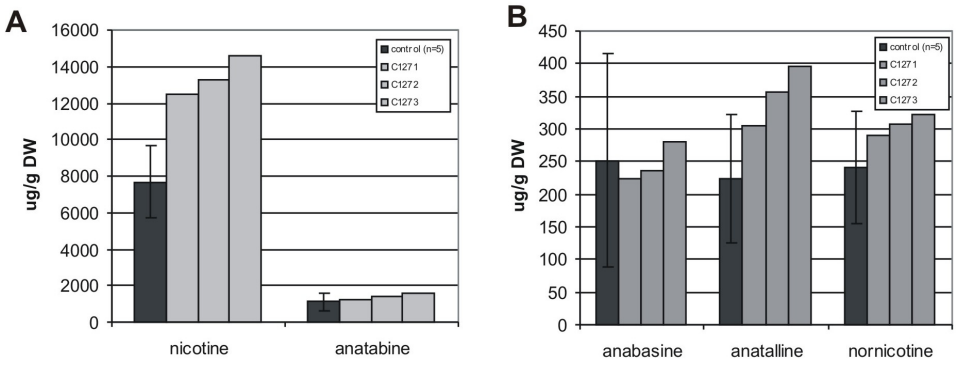

Fig. 10. Alkaloid accumulation in N. tabacum BY-2 control hairy roots and three C127-carrying hairy root clones.

The closest homologies for *C127* were found to be two *Arabidopsis* GH3 (growth hormone 3) proteins, coding for WES1 and DFL1, both exhibiting activity towards auxin indole acetic acid (IAA) (Staswick et al. 2002). On this account, and since *C127* was upregulated by MeJA, the IAA levels of MeJA-treated BY-2 cells were tested. Higher levels of conjugated IAA in elicited cells versus control cells suggested inactive auxin to play a role in MeJA-mediated stress response. Furthermore, the expression of *C127* was higher in roots than in leaves (**III**, Fig. 7), the former being the site of the nicotine biosynthesis. In hairy roots carrying the *C175* construct, contrary to *C127*, reduced levels of anatabine and nornicotine were observed. The cause of the differential effect on nicotine biosynthesis was considered to be *e.g.* different adenylation activities or substrate specifities, as has been observed before with two closely related (90 % amino acid identity) enzymes WES1 and DFL1 (Staswick et al. 2002). Indeed, two other enzymes in the nicotine biosynthetic pathway, PMT and spermidine synthase (Fig. 2) share highly homologous amino acid sequences, although they catalyze different reactions with high specificity for substrate (Hibi et al. 1994). Since the experimental setup described here was designed to screen a large number of candidate genes involved in tobacco alkaloid metabolism, which imposes limitations on the extent of the experiment, a clear drawback was the low number of individual clones tested, especially in the case of *C175*. High variations between individual hairy root clones in respect of alkaloid production have been reported (Sevón et al. 1998; Jouhikainen et al. 1999), and for this reason statistical analyses of several clones should be performed in order to obtain a more clear insight into the gene-to-metabolite interaction. Hairy roots

offer a good choice for studying constitutive alkaloid biosynthesis since they are the actual site of alkaloid biosynthesis. However, the slow growth rate of hairy roots, with maximum alkaloid accumulation taking place after several weeks' cultivation, sets a challenge for their use in high-throughput systems.

4.2.3 Screening of genes in *Hyoscyamus muticus* hairy root cultures

Since *Nicotiana* and *Hyoscyamus*, both belonging to the family Solanaceae, share the upper pathway for alkaloid biosynthesis in common, the aim was to test the selected *Nicotiana* genes found in the cDNA-AFLP analysis using the so-called combinatorial biochemistry approach (unpublished results). Altogether 11 genes from the MJM data set (**III**) were introduced into *Hyoscyamus muticus* hairy roots and the tropane alkaloids were identified by GC-MS based on the mass fragmentation pattern and retention order (Hartmann et al. 1986; Witte et al. 1987). A total of 14 alkaloids and derivatives were identified, along with three unidentified alkaloid compounds, UI1-UI3 (Table 3), and their amounts were calculated using homatropine as an internal standard. Tropinone, hygrine and alkaloid414 were excluded from the results because of their high variation in replicate sample extracts. For tropine and pseudotropine the extraction method used here gave incomplete recovery (approximately 20 %). Similar results were reported earlier by Hartmann et al. (1986). In alkaloid extract, several minor peaks which could not be identified were detected. Accumulation of three of these unidentified compounds (UI1-UI3) was followed. It was suggested that on the basis of their main fragments 82 and 84, UI1 and UI2 could be derivatives of tropine and *N*-methylpyrrolidinyl, respectively. On the other hand, the mass fragmentation of UI3 closely resembled that of hyoscyamine. The biosynthetic origin of alkaloid414 is unknown, whereas apoatropine is possibly a dehydration product of atropine formed during the analytical procedure (Brochmann-Hanssen and Svendsen 1962). The positions of the detected compounds in the tropane alkaloid pathway is presented in Fig. 3.

Table 3. Tropane alkaloids and alkaloid derivatives analysed by GC-MS.

	T_{ret}	***Compound***
1	4.25	tropinone
2	4.68	hygrine
3	5.06	tropine
4	5.45	pseudotropine
5	5.65	3α-acetoxytropane
6	5.77	3β-acetoxytropane
7	6.98	UI1
8	8.53	cuscohygrine
9	9.14	*N*-methyl-pyrrolidinyl hygrine
10	11.24	apoatropine
11	11.34	UI2
12	11.68	IS (homatropine)
13	12.43	hyoscyamine + littorine
14	12.57	UI3
15	13.15	6β-hydroxyhyoscyamine
16	13.24	scopolamine
17	13.41	6β-hydroxyhyoscyamine isomer
18	22.11	alkaloid414

Hairy roots were cultivated in shake flasks and the tropane alkaloid accumulation was measured after 28-day cultivation from roots carrying eight different gene constructs. In addition, three constructs were tested in multiwell cultivations (see section 4.2.4). Of each construct, two to seven individual clones and altogether eight control clones (four carrying the backbone vector LBA9402 only, and four also carrying 35*S*-GUS) were established. Alkaloid contents of individual transgenic clones were compared against the variability displayed by the controls, and the percentage change was calculated against the control mean (Table 4, unpublished results). In addition, statistically significant differences

were determined using analysis of variance (ANOVA) or Welch-ANOVA (see Table 4 legend). Several transgenic root clones carrying different gene constructs showed changes in the accumulation of alkaloid compounds compared to controls, although none of the genes tested could be confirmed to have a specific role in the tropane alkaloid pathway. However, it is difficult to draw conclusions concerning the functions of individual genes solely on the basis of metabolite accumulation. With increasing numbers of individual clones tested it would be possible to perform *e.g.* cluster analysis to aid the interpretation of the results. However, it is highly interesting to screen the effects of heterologous genes in related organisms, leading to identification of genes or regulators with a putatively broader function in secondary metabolite biochemistry.

In these studies using targeted chemical analysis, no major alkaloid compounds which could be novel products deriving from combinatorial biosynthesis were found, although such results have been reported earlier. One study performed with somatic hybrids of two *Solanum* species resulted in the formation of a novel glycoalkaloid which was not naturally present in either of the parental plants (Laurila et al. 1996). Another example was given by Frick and Kutchan (1999), who reported broadened substrate specificities of different recombinant *O*-methyltransferases of *Thalictrum tuberosum*. In the future, applications of non-targeted metabolite profiling would offer novel tools to study the formation of new compounds.

Table 4. Functional analysis of H. muticus hairy roots. Annotation of the constructs is given in paper III (Table 1). Abbreviations: 3α-acetoxytropane (3α-acet.); 3β-acetoxytropane (3β-acet.); N-methylpyrrolidinyl hygrine (N-met-pyrr.); pseudotropine (pseudotr.); hyoscyamine (hyos.); 6β-hydroxyhyoscyamine (6BHH); unidentified compounds (UI1 and UI3).

Construct (nr. clones tested)	***Comp. altered***	***Nr. clones affected***	***Change %***	***Comp. altered***	***Nr. clones affected***	***Change %***	***Comp. altered***	***Nr. clones affected***	***Change %***	***Comp. altered***	***Nr. clones affected***	***Change %***
C360 (n = 7)	3α-acet.	1	+48	3β-acet..	1	+65	*N*-met-pyrr	7	-69…90*[1]	hyos.	1	+40
C476 (n = 4)	pseudotr.	4	+14…69	UI1	2	+43…55	hyos.	1	+47	6BHH	1	+43
MC204 (n = 5)	pseudotr.	5	+30…58	3β-acet.	5	tr***[2]	UI3	3	+23…34			
T21 (n = 5)	pseudotr.	3	+22…50	3β-acet.	2	tr	hyos.	3	-38…42	6BHH	3	-57…66
T172 (n = 2)	pseudotr.	2	+26…31	3β-acet.	2	tr	hyos.	2	-45…47	6BHH	2	-60…62***[2]
MT401 (n = 3)	pseudotr.	2	+11…35	*N*-met-pyrr.	3	-79…94**[1]	hyos.	3	-36…77	6BHH	3	-54…84*[1]
MT101 (n = 5)	pseudotr.	3	-28…51***[2]	3β-acet.	1	+56	*N*-met-pyrr.	5	-81…94***[2]	6BHH	4	-51…54
T464 (n = 2)	pseudotr.	2	+73	3β-acet.	2	-96…97	hyos.	2	-48…60	UI3	2	-48…50*[1]

tr: only traces found in the samples

Significance level indicated with asterix: * ($p < 0.05$); ** ($p < 0.01$), *** ($p < 0.001$). [1] One-way ANOVA. [2] Welch-ANOVA (more tolerant of differences in group variances than ANOVA and used when the Levene statistic for the homogeneity of variances indicated that the group variances did not fulfil the requirements of ANOVA)

4.2.4 Screening of genes using multi-well plates

In order to down-scale the cultivation platform, three *Nicotiana* genes introduced into *H. muticus* hairy roots were screened after cultivation in 6-well plates (unpublished results). As a result, four individual clones of three gene constructs: *MC304* and *C112* (annotated as putative proteins), and *C127* (annotated as GH3-like protein or *Nt*NEG1, **III**) were tested. With gene *C112* a statistically significant reduction in the production of *N*-methylpyrrolidinyl hygrine was observed (Fig. 11B). Interestingly, the gene *C127*, which caused elevated nicotine production in *Nicotiana* hairy roots, resulted in induced biosynthesis of the alkaloid intermediate 3β-acetoxytropane in *H. muticus* (Fig. 11A).

Several unique features make the cultivation of hairy roots in multiwell format very attractive. They are space-saving and can easily be handled because they are available pre-sterilized and can be stacked during cultivation. Moreover, it is possible to grow a number of independant roots in parallel under uniform environmental conditions. In addition, the cultivation time can be reduced. In this study the time required for the consumption of all the medium was 21 days, which is one fourth shorter than the time normally used for hairy roots tested here to reach the maximum alkaloid production. The biomass yield per well ranged from 50 to 100 mg dry weight, which is sufficient for replicate sample extracts for the analytical systems employed here. Surprisingly few studies concerning the cultivation of plant cells in multiwells have been published, although cell suspension cultures grown in multiwells have been reported to yield biomass and secondary metabolite production levels similar to those obtained in flask cultures (Srinivasan et al. 1997). However, in this study performed with hairy roots, the variation of biomass yield between individual wells was higher (CV 14 %, n = 12) than that of flasks (CV 7 %, n = 10). Recently, studies of camptothecin (Sirikantaramas et al. 2007) and artemisin (Weathers et al. 2005) production using multiwell cultures of hairy roots have been published.

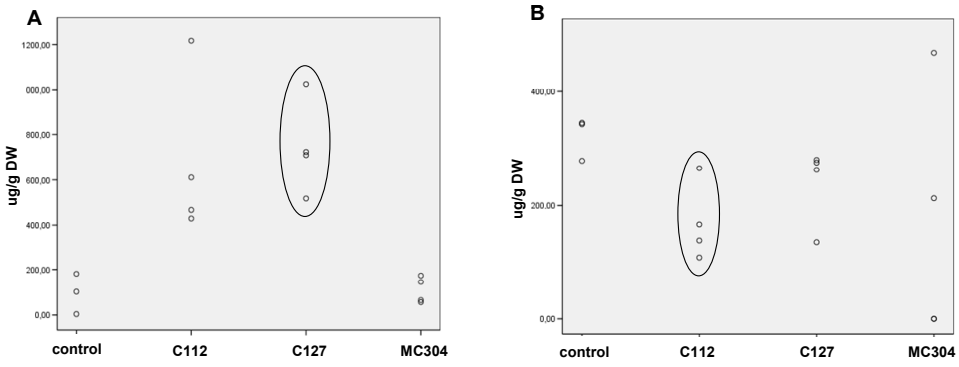

Fig. 11. Accumulation of alkaloids in H. muticus hairy root cultures cultivated in multiwell plates. A) 3β-acetoxytropane; B) N-methylpyrrolidinyl hygrine. Dots represent individual clones. Circles represent a significant difference compared to the control (in Fig. A, $p < 0.01$; in Fig. B, $p < 0.05$).

4.3 The effect of *H6H* gene on bioconversion of hyoscyamine in *N. tabacum* and *H. muticus* hairy roots (IV)

The production of scopolamine, a valuable anticholinergic drug (see section 1.3), can be enhanced in hairy roots of various Solanaceae species by overexpressing the hyoscyamine-6β-hydroxylase (*H6H*) gene (Yun et al. 1992; Jouhikainen et al. 1999; Zhang et al. 2004). *Nicotiana* species produce alkaloids from the same biosynthetic origin as tropane alkaloid-producing species, although the pathway from *N*-methylpyrrolinium is directed towards nicotine (Fig. 2 and 3) and the tropane alkaloid biosynthetic genes are lacking in *Nicotiana*. In this work the uptake of hyoscyamine as well as bioconversion of hyoscyamine to scopolamine was followed with *N. tabacum* and *Hyoscyamus muticus* hairy roots carrying heterologous hyoscyamine-6β-hydroxylase (*H6H*). Altogether ten individual *N. tabacum* hairy root clones were established and the roots showed good uptake characteristics for hyoscyamine, on average 95 % and 94 % of hyoscyamine added to the medium was taken up at concentrations of 100 mg/l and 200 mg/l, respectively (**IV**, Table 1). Three out of ten clones correctly expressed *H6H*, and the best bioconversion rate was obtained with 100 mg/l hyoscyamine addition resulting in hyoscyamine to scopolamine conversion of 45 % (**IV**, Table 2). Hyoscyamine addition at 200 mg/l led to reduced biomass production as well as lowered hyoscyamine-scopolamine conversion compared to that of 100 mg/l hyoscyamine addition (**IV**, Table 2).

In *N. tabacum* hairy roots the produced scopolamine was efficiently secreted out of the cells. Up to 85 % of the total scopolamine produced was secreted into the culture medium, compared to only 12 % for *H. muticus* (Fig. 12). This is noteworthy, since as observed in the current study, tropane alkaloids are commonly retained in the root tissues in hairy roots of endogenous tropane alkaloid producers (*e.g.* Hashimoto et al. 1993; Jouhikainen et al. 1999). In another study, tobacco cells overexpressing terpenoid indole alkaloid genes secreted the products into the medium, whereas in the endogenous producer *Catharanthus roseus* the alkaloids are stored inside the cell vacuoles (Hallard et al. 1997). Earlier it was reported that in *Nicotiana* roots the promoter of the *H. niger H6H* gene was only expressed in meristem cells, whereas in *Hyoscyamus* expression was also demonstrated in the pericycle (Kanegae et al. 1994). Thus, it was suggested that scopolamine-producing plants possess a regulatory mechanism for the cell-specific expression of *H6H*. Further experiments performed with the immobilized cell cultures deriving from hairy roots described here showed that a marked secretion of scopolamine (74–81 %) also took place in bioreactor cultivations of these undifferentiated cells (Moyano et al. 2007). The transport mechanism of scopolamine in *Hyoscyamus* and *Nicotiana* is not known, although it is likely that different transporter proteins are involved in the two species because scopolamine is an endogenous product in the former but a foreign metabolite in the latter. Earlier it has been reported that berberine is transported by an MDR-type transporter in *Coptis japonica* (Shitan et al. 2003), for which berberine is an endogenous metabolite, whereas in *Arabidopsis* another type of transporter MATE is involved (Li et al. 2002).

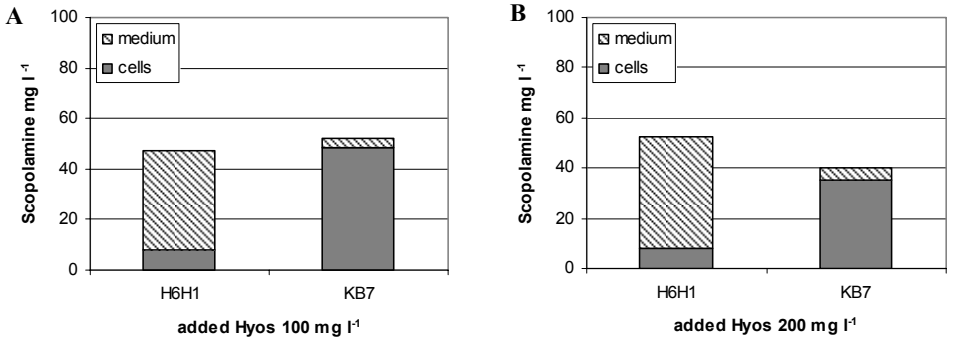

Fig 12. Scopolamine distribution in cells and in medium in N. tabacum (H6H1) and H. muticus (KB7) hairy roots after hyoscyamine feeding at 100 mg l^{-1} (A) and 200 mg l^{-1} (B).

With the *H6H*-carrying *H. muticus* hairy roots studied here, only 15 % of the added hyoscyamine was converted to scopolamine (**IV**, Table 3). Hyoscyamine feeding did not have major effects on the ratios of the accumulated alkaloids; only a slight reduction of the proportion of scopolamine was observed with the higher feeding amount (Table 5). When 100 mg/l hyoscyamine was added to the culture medium, it was efficiently taken up and accumulated in cells as hyoscyamine, 6β-hydroxyhyoscyamine (6BHH) and scopolamine in hairy roots KB7 carrying *H6H*. However, when the amount of added hyoscyamine was doubled to 200 mg/l, the total alkaloid pool did not show a linear increase in KB7 but remained rather stable. As suggested earlier by Hashimoto and Yamada (1983), these results possibly indicate that the added hyoscyamine resulted in lowered endogenous hyoscyamine production, or that hyoscyamine was further degraded and modified when taken up by the cells. In the current study, hyoscyamine-producing *H. muticus* roots were used as a reference to *N. tabacum* roots. However, it should to be noted that clone-to-clone variation in metabolic behaviour in transgenic hairy roots may be substantial (Sevón et al. 1998; Jouhikainen et al. 1999). These results however provide additional insights concerning the divergent role of H6H in different Solanaceae species, as varying hydroxylase and epoxidase activities of H6H have been reported in endogenous and non-hyoscyamine producers (Yun et al. 1993; Rocha et al. 2002).

Table 5. Total (medium and root associated) hyoscyamine (Hyos), 6β-hydroxyhyoscyamine (6BHH) and scopolamine (Scop) content and percentage of each alkaloid in control and 35S-h6h carrying (KB7) H. muticus hairy roots supplemented with 100 mg l^{-1} or 200 mg l^{-1} hyoscyamine.

	mg l^{-1}				***% of Total***		
	Hyos	*6BHH*	*Scop*	***Total***	*Hyos*	*6BHH*	*Scop*
Control	77	2	0	79	97	3	0
Control+100 mg l^{-1} Hyos	135	1	0	136	99	1	0
Control+200 mg l^{-1} Hyos	218	3	0	221	99	1	0
KB7	124	38	37	199	62	19	19
KB7+100 mg l^{-1} Hyos	185	66	52	303	61	22	17
KB7+200 mg l^{-1} Hyos	203	73	40	316	64	23	13

4.4 Hyoscyamine addition causes altered tobacco alkaloid production in *N. tabacum* hairy roots (IV)

When hyoscyamine was fed to *N. tabacum* hairy root cultures, the roots not only metabolized the hyoscyamine but also produced tobacco alkaloids in varying amounts. Addition of 200 mg/l hyoscyamine caused increased accumulation of total alkaloids in three out of four root clones tested, mainly contributing to the increment in nicotine levels (**IV**, Table 4). Earlier, elevated nicotine levels have been reported in leaves of *N. tabacum* transformed with two genes of the tropane alkaloid pathway, *H6H* and *TRI* (Rocha et al. 2002). This phenomenon is not completely understood. It has been suggested that metabolic engineering of the pathway as such may result in perturbation of the overall biosynthetic activity (Rocha et al. 2002). However, this hypothesis was not supported by the current study, when seven *H6H*-transformed root cultures as such did not show significantly higher tobacco alkaloid production (**IV**, Table 4). Rather, based on these results, elevated tobacco alkaloid production is due to the application of hyoscyamine to *N. tabacum* as a foreign substrate. This hypothesis requires for its verification more detailed information of the action of hyoscyamine as a putative elicitor in non-hyoscyamine producing *Solanaceae*, as well as better understanding of the factors affecting the regulation of tobacco alkaloid biosynthesis.

4.5 The yeast ABC transporter enhances the tolerance of tropane alkaloids in plant cell cultures (V)

The tolerance of yeast *Saccharamyces cerevisiae* lines deficient in ABC (ATP Binding Cassette) -type transporters for tropane alkaloids was assayed, and enhanced sensitivity was associated with lines deficient in *PDR5*-type transporter. Although earlier it had been shown that PDR5 has a broad substrate specificity (see review by Bauer et al. 1999) and accepts various plant-derived substances as substrates (Kolaczkowski et al. 1996; 1998), it was shown in this study for the first time that the PDR5 substrate range includes the tropane alkaloids hyoscyamine and scopolamine. Altogether six ABC-type transporters were subjected to testing in three isogenic yeast lines, US50-18C, BY4741 and W303-1A. Other strains than the *pdr5* mutant did not differentiate from wild type strains in their sensitivity towards the plant-derived metabolites hyoscyamine and scopolamine (**V**, Fig. 1A-C). For *pdr5* mutant strains the concentrations 50 and 100 mM scopolamine

and 20 and 50 mM hyoscyamine (in strains US50-18C/BY4741 and W303-1A, respectively) were lethal. However, the activity of PDR5 towards nicotine was not clear, since only one out of three *pdr5* yeast lines tested showed a weak effect at a nicotine concentration of 12.5 mM (**V**, Fig. 1A).

In order to further test whether yeast *PDR5* could have a similar function in plant cells, it was overexpressed in *N. tabacum* BY-2 cells. The *PDR5*-coding sequence from two yeast lines US50-18C and W303-1A, having distinct tolerances to the alkaloids tested, was inserted into the Gateway plant transformation vector. The control cells tolerated hyoscyamine up to 10 mM concentration, 50 mM being lethal after 24 h incubation. The highest tolerance for hyoscyamine was obtained with the lines expressing *PDR5* from yeast line W303-1A (referred to as W303). In the presence of 30 mM hyoscyamine, W303 lines showed 35 % decrease in cell death (measured as fold increase after 24 h incubation) compared to the control lines (referred to as GUS) (Fig. 13).

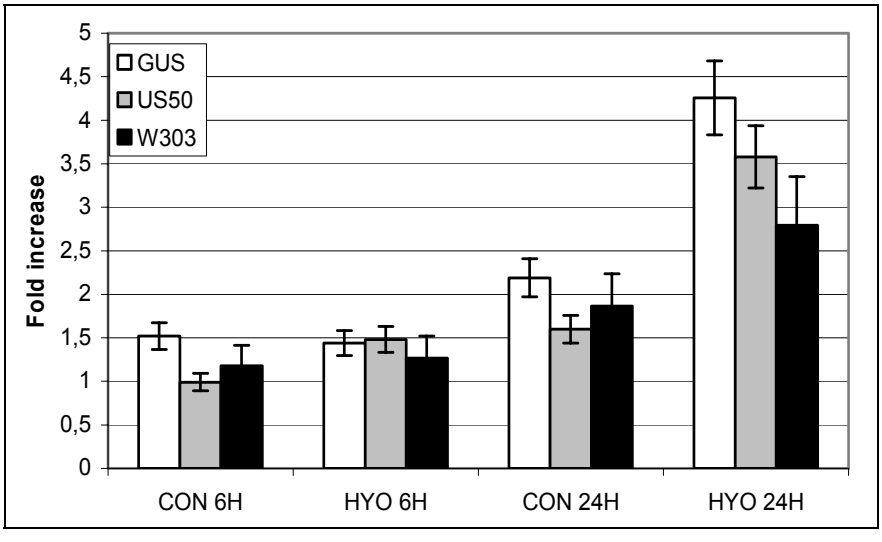

Fig. 13. Hyoscyamine- induced cell death (indicated as fold increase in OD_{600} relative to the value at the start of the experiment) in BY-2 control cells (GUS) and in cells carrying pK7WGD2-ScPDR5-US50 (US50) and pK7WGD2-ScPDR5-W303 (W303). Control (CON); hyoscyamine added at 30 mM (HYO).

Tolerance to nicotine followed a distinct pattern compared to that of hyoscyamine. A concentration of 10 mM already caused complete growth arrest in control cells, and the tolerance did not improve when *PDR5* was overexpressed. The

transporter protein responsible for the transport of tobacco alkaloids from root tissues to leaves has not yet been identified, but it has been suggested that this transport process requires the function of both plasma membrane transporters in roots and in leaves, and in addition a vacuolar transporter in mesophyll cells (Yazaki 2006). Gaertner et al. (1998) proposed that nicotine transport might involve an MDR-like transporter, which was activated in the tobacco hornworm, *Manduca sexta*. This MDR-like transporter, which was shown to secrete nicotine from the tissues of insects, was inhibited by atropine (racemic hyoscyamine). Although the broad substrate specificity of ABC transporters has generally been accepted, when it comes to translocation of endogenous substrates and functions in cell signalling, the substrate range of ABC transporters has been reported to be narrow (Yazaki 2006, and references therein). Hitherto, four PDR transporters have been described in *Nicotiana* (Sasabe et al. 2002; Schenke et al. 2003; Ducos et al. 2005). Localized in the plasma membrane, they have been shown to play a role in pathogen resistance and one of them responds to iron deficiency. However, activity towards nicotine has not been reported.

In order to discover the functions of transporter proteins in plant systems, subcellular localization as well as information concerning the regulation of gene and protein expression should be included. In addition, it is possible that the expression of *PDR5* genes tested in this study also follows an organ specific pattern as has been reported earlier with *Arabidopsis thaliana* transporters displaying distinct expression patterns in regard to qualitative differences in organ expression and quantitative differences in expression within one organ (van den Brûle and Smart 2002). Interestingly, the cDNA-AFLP analysis of tobacco cells performed in this study (**I**) led to identification of several putative transporter genes, which might be involved in nicotine transport. Recently, it has been shown that one MATE-family (multidrug and toxin extrusion) transporter from this MJM gene set was induced by MeJA in a similar manner to the nicotine biosynthetic genes and it was suggested to be specific for alkaloids (unpublished results). When considering the metabolic engineering of plant systems for enhanced production of plant-derived compounds or other applications, the information obtained concerning the accumulation and transport mechanisms of these compounds will be of great value. For example, overexpressing an ABC transporter with broad substrate specificity in plants has been shown to be a potential instrument when applied to phytoremediation (Yazaki et al. 2006).

5. Conclusions

Plant cell cultures combined with knowledge in genetic engineering have been exploited with the aim of improving the production yields of high-value secondary metabolites, which are often produced in very low quantities in plants (less than 1 % dry weight). However, lack of understanding of the regulation of the production of plant-based compounds is still a major problem encountered in metabolic engineering of secondary metabolite pathways, and more detailed information about the flux and control mechanisms of the whole biosynthetic machinery is needed. In the current study, using *Nicotiana tabacum* cell cultures as a model system, we exploited genome-wide transcript profiling combined with targeted metabolite analysis for investigating the secondary metabolite pathway of tobacco. This approach proved to be a powerful tool in gene discovery, resulting in a set of almost 500 known and novel genes, several of them taking part in tobacco alkaloid biosynthesis. Using stably transformed *N. tabacum* cells, the functional analysis screen of some of these genes revealed the presence of two putative enzymes, which probably either directly or indirectly participate in tobacco alkaloid biosynthesis. The gene *MC126* showed homology with lysine decarboxylase (LDC) and altered overall tobacco alkaloid biosynthesis in elicited BY-2 cells. The gene *C127* was designated as a GH3-like protein and resulted in highly elevated nicotine levels in *N. tabacum* hairy roots. Further proof of the potential of this approach was shown recently with the studies performed with a medicinal plant *Catharanthus roseus*, in which gene expression and metabolite profiles were combined with correlation analysis, leading to discovery of novel gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis (Rischer et al. 2006).

Secondary metabolites usually accumulate inside plant cells in vacuoles, making their downstream processing more demanding. Therefore, knowledge gained from cellular transport systems can provide valuable information concerning the metabolic engineering of plants for large-scale production systems. In the current study, the potential of ABC-type transporters was shown by using a yeast *PDR5* transporter, which exhibited an enhanced tolerance for tropane alkaloids, broadening the substrate range of this type of transporters. In addition, further insight was obtained into the cellular responses of tobacco and tropane alkaloids, two alkaloid groups partly sharing a common biosynthetic pathway. When the

gene responsible for conversion of hyoscyamine into scopolamine (*H6H*) was introduced into *Nicotiana*, which does not endogenously produce either of these alkaloids, high amounts of scopolamine were secreted from the cells when hyoscyamine was added to the medium. Due to its ability to take up and convert exogenously applied hyoscyamine, *Nicotiana* could be of interest when designing a candidate production host for tropane alkaloids or possibly for other secondary compounds which are currently still being extracted from whole plants for commercial production. Combined with the information of the regulation of the biosynthesis, transporters can be utilized to design systems in which compartmentalization of biosynthetic steps takes place, and finally optimally leading to efflux of desired compounds through the plasma membrane. Identification and exploitation of transporters will provide novel tools to enhance the yield and diversity of alkaloid biosynthesis, and represent an important asset for producing alkaloids on a large scale.

In addition to multiple differentially regulated genes, the gene discovery platform applied in the current study led to the identification of a novel alkaloid in *N. tabacum* cell cultures. Anatalline, consisting of three heteroring structures, was shown to be the second largest constituent in the alkaloid pool in methyl jasmonate-elicited cells. The biosynthesis of anatalline was speculated, possibly involving the metabolism of either anabasine or anatabine. Despite the fact that precursor feeding has been extensively used in the past to discover the metabolic steps in plant systems, it has often shown its limitations in systems consisting of highly complex regulation networks, as was also seen in the current research. Thus, new methods are required in pathway mapping of secondary compounds, such as functional genomics-based approaches. A more complete understanding of the metabolite networks will be possible by using systems biology for integrating genomics, transcriptomics or proteomics with metabolomics, and may optimally also result in the discovery of completely new pathways (Oksman-Caldentey and Inzé 2004).

The challenge for the post-genomic era is to assign functions for the genes discovered. Besides determining the specific function of the gene, *e.g.* the activity associated with the gene product, the overall role of the gene in the context of the whole organism also comes into question. This was also seen in the current research, when a known gene from tropane alkaloid pathway, *H6H*, was overexpressed in tobacco, resulting in alterations in the accumulation of

tobacco alkaloids. It is apparent that non-targeted profiling and metabolomics, aiming at a more complete understanding of the whole organism, will allow wider knowledge of hitherto invisible or unpredicted metabolic events.

The targeted chemical analysis used here was a rational choice considering the aim to discover the tobacco alkaloid pathway. However, when dealing with jasmonates, which are known to elicit a wide range of stress-related responses in plant cells, a non-targeted analytical approach would provide better insight into the system in which metabolic engineering of unknown genes is being applied. Unlike in many plant cell cultures, in which cell division is a rather slow process, BY-2 cell line possesses advantages due to its high multiplication rate. However, for functional analysis a system in which constitutive production takes place, such as hairy roots, will offer better insight into the effects of the target gene, since secondary metabolism is often linked to cellular differentiation. Furthermore, when performing large screens, advanced methods for high-throughput testing are required, including automatization and down-scaling of the analytics.

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Title

A functional genomics approach to the study of alkaloid biosynthesis and metabolism in *Nicotiana tabacum* and *Hyoscyamus muticus* cell cultures

Abstract

The aim of this work was to improve understanding of the regulation of alkaloid biosynthesis in two *Solanaceae* plants, *Nicotiana tabacum* (tobacco) and *Hyoscyamus muticus* (Egyptian henbane). In order to map the biosynthetic genes involved in the tobacco alkaloid pathway, a functional genomics-based technology was established by combining genome-based transcript profiling (cDNA-AFLP) with targeted metabolite analysis. Altogether 459 genes were found to be differentially expressed in methyl jasmonate-elicited *N. tabacum* BY-2 cells. Homology searches performed with these genes revealed that 58 % of the genes displayed similarity with genes having known functions, whereas no sequence similarity was found with 26 % of the genes, suggesting that some of them may take part in unknown steps in tobacco alkaloid biosynthesis. Alkaloids accumulated 12 hours after methyl jasmonate application, with varying kinetic patterns. For the first time the alkaloid anatalline was shown to accumulate in *Nicotiana* cell cultures, and together with anatabine they formed the main alkaloid pool. Anatalline was further characterized structurally as being present in two isomeric forms, anatalline and *trans*-2,4-di(3-pyridyl)piperidine. Contrary to the case in whole tobacco plants, nicotine was only a minor alkaloid accumulating in elicited cells, whereas the production of a precursor methylputrescine was highly induced. Based on these results, it was suggested that the limiting step in nicotine biosynthesis occurred between methylputrescine and nicotine.

Altogether 34 methyl jasmonate-modulated genes were selected for further functional testing in BY-2 cell cultures using *Agrobacterium*-mediated gene transformation. Six genes caused a lower alkaloid accumulation compared to the control when assayed in cell cultures, whereas three genes elevated the production of one or several alkaloids. One of the genes causing enhanced alkaloid accumulation was found to possess high sequence similarity with lysine decarboxylase, a gene responsible for the conversion of lysine in early anabasine biosynthesis. However, since lysine decarboxylase activity was not shown by the corresponding protein, the exact nature of this gene requires further elucidation. The selected genes were also assayed in hairy roots, which constitutively produce alkaloids. Two highly homologous genes were found, which showed divergent effects on alkaloid biosynthesis. These genes were suggested to function in auxin homeostasis. The other gene also resulted in marked increase in nicotine accumulation.

Tropane and tobacco alkaloids share a common biosynthetic origin, and therefore it was of interest to study whether *Nicotiana* genes could have a role in the formation of tropane alkaloids in a related species *H. muticus*. It was observed that the same gene which elevated nicotine contents in *Nicotiana* showed a positive effect on tropane alkaloid intermediate in *H. muticus*, suggesting a possible conserved role of this gene in *Solanaceae* species. On the other hand, when a known tropane alkaloid pathway gene, hyoscyamine-6β-hydroxylase (*H6H*), was overexpressed in *N. tabacum* hairy roots, a 45 % conversion of hyoscyamine into scopolamine took place when hyoscyamine was supplied to the cultures. Furthermore, up to 85 % of the produced scopolamine was secreted out of the cells. Besides being able to uptake and convert a foreign substrate, an altered tobacco alkaloid production in roots was observed after hyoscyamine feeding, suggesting highly complex regulation of the production of these defence-related compounds.

In order to improve the understanding of alkaloid transport and secretion, the function of a yeast ATP-binding cassette transporter was investigated and it was shown to attribute enhanced tolerance of tropane alkaloids in *N. tabacum* cell cultures. Combined with the information of the regulation of the biosynthesis, transporters can be exploited to design novel tools to enhance the yield and diversity of alkaloids.

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Nimeke

Alkaloidien biosynteesin ja metabolian tutkiminen funktionaalisella genomiikalla tupakan ja villikaalin soluviljelmissä

Tiivistelmä

Työn pääasiallinen tavoite oli tutkia alkaloidien biosynteesiä kahdessa Solanaceae-heimon soluviljelmässä, tupakassa (*Nicotiana tabacum*) ja Egyptin villikaalissa (*Hyoscyamus muticus*). Tupakan alkaloidibiosynteesiin osallistuvien geenien kartoitus tehtiin funktionaalisen genomiikan tekniikkaa käyttäen, jossa yhdistettiin koko genomin kattava transkriptioprofilointianalyysi cDNA-AFLP ja kohdennettu metaboliittianalyysi. Nikotiinisynteesi elisitoitiin tupakan BY-2-soluviljelmissä metyylijasmonaatilla ja tuloksena löydettiin yhteensä 459 geeniä, jotka ekspressoituivat metyylijasmonaatin vaikutuksesta. Näistä geeneistä 58 %:lla oli homologia jo tunnettujen geenien kanssa, kuitenkin 26 % geeneistä osoittautui aiemmin tuntemattomiksi, ja osan näistä geeneistä oletettiin osallistuvan tupakka-alkaloidien säätelyyn. Alkaloidien muodostuminen alkoi soluissa 12 tuntia elisitoinnin jälkeen, ja eri alkaloidit noudattivat omaa kinetiikkaansa. Anatalliinin osoitettiin esiintyvän tupakan soluviljelmissä ensimmäistä kertaa, ja se muodosti anatabiinin kanssa pääalkaloidiluokan. Lisäksi anatalliini esiintyi kahdessa eri isomeriamuodossa. Toisin kuin tupakkakasvissa, elisitoiduissa soluissa nikotiinia tuotettiin vain pieniä määriä, kun taas nikotiinisynteesin välituotetta metyyliputreskiinia muodostui suuria määriä. Tämän tuloksen perusteella nikotiinin biosynteesissä rajoittava vaihe oli jokin reaktio metyyliputreskiinin ja nikotiinin välissä.

Näistä tupakasta eristetyistä geeneistä yhteensä 34 yliekspressoitiin tupakkaan agrobakteerivälitteisellä geeninsiirrolla. Alkaloidituotto oli kuudessa eri transgeenisessä viljelmässä alhaisempaa kuin kontrolliviljelmissä, kun taas kolmella eri geenillä vaikutus viljelmän alkaloidituottoon oli positiivinen. Yhdellä näistä tupakan alkaloidituottoa lisäävistä geeneistä oli yhteneväisyyttä lysiini-dekarboksylaasin kanssa, joka toimii anabasiini-alkaloidin varhaisessa biosynteesissä. Kuitenkin lysiini-dekarboksylaasin entsyymiaktiivisuutta ei havaittu, joten tämän geenin tarkempi kartoitus vaatii lisätutkimuksia. Valitut geenit yliekspressoitiin myös tupakan karvajuuriin, joissa alkaloidien tuottoon ei tarvita elisitaatiota. Näissä viljelmissä kaksi eri geeniä, jotka olivat hyvin samankaltaisia aminohappokoostumukseltaan, aiheuttivat erilaisia vaikutuksia alkaloidituottoon. Toinen geeneistä nosti merkittävästi nikotiinipitoisuuksia viljelmissä, ja näiden geenien toiminta kohdistuu todennäköisesti auksiiniaineenvaihduntaan.

Tropaani- ja tupakka-alkaloidit syntetisoidaan yhteisistä lähtöaineista ja näin ollen oli kiinnostavaa selvittää, voivatko tupakan geenit toimia myös sukulaiskasvin villikaalin tropaanialkaloidi-aineenvaihdunnassa. Tutkimuksissa havaittiin, että sama geeni, joka nosti nikotiinipitoisuuksia tupakan juuriviljelmissä, aiheutti villikaalin juuriviljelmissä korkeampia tropaanialkaloidien välituotteen pitoisuuksia. Näin ollen tällä geenillä saattaa olla yleisempää vaikutusta Solanaceae-heimon aineenvaihdunnassa.

Alkaloidien kuljetusta ja erittymistä tutkittiin hiivaperäisen ABC-transportterigeenin avulla. Tämän geenin todettiin lisäävän tropaanialkaloidien sietokykyä tupakan soluviljelmissä. Kun tropaanialkaloidireitin hyoskyamiinia konvergoiva geeni *H6H* (hyoskyamiini-6β-hydroksylaasi) yliekspressoitiin tupakan juuriviljelmissä, 45 % viljelmiin lisätystä hyoskyamiinista muuttui skopolamiiniksi. Lisäksi suuri määrä, jopa 85 % tuotetusta skopolamiinista, erittyi soluista ulos. Sen lisäksi, että nämä tupakan karvajuuret ottivat hyvin sisäänsä ja metaboloivat alustaan lisätyn vieraan substraatin, näillä juurilla havaittiin muutoksia myös tupakka-alkaloidiaineenvaihdunnassa. Tulosten perusteella näyttää siltä, että näillä puolustusreaktioissa toimivilla aineenvaihduntatuotteilla on hyvin monimutkainen säätelyjärjestelmä.

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