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Review

Functional characterisation of genes involved in pyridine alkaloid biosynthesis in tobacco

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Abstract

Although secondary metabolism in *Nicotiana tabacum* (L.) (tobacco) is rather well studied, many molecular aspects of the biosynthetic pathways and their regulation remain to be disclosed, even for prominent compounds such as nicotine and other pyridine alkaloids. To identify players in tobacco pyridine alkaloid biosynthesis a functional screen was performed, starting from a tobacco gene collection established previously by means of combined transcript profiling and metabolite analysis. First, full-length cDNA clones were isolated for 34 genes, corresponding to tobacco transcript tag sequences putatively associated with pyridine alkaloid metabolism. Full-length open reading frames were transferred to pCaMV35S-steered overexpression vectors. The effects of plant transformation with these expression cassettes on the accumulation of nicotine and other pyridine alkaloids were assessed in transgenic tobacco Bright-Yellow 2 (BY-2) cell suspensions and hairy root cultures. This screen identified potential catalysers of tobacco pyridine metabolism, amongst which a lysine decarboxylase-like gene and a GH3-like enzyme. Overexpression of the GH3-like enzyme, presumably involved in auxin homeostasis and designated NtNEG1 (*Nicotiana tabacum* Nicotine-Enhancing GH3 enzyme 1), increased nicotine levels in BY-2 hairy roots significantly. This study shows how functional genomics-based identification of genes potentially involved in biosynthetic pathways followed by systematic functional assays in plant cells can be used at large-scale to decipher plant metabolic networks at the molecular level.

Keywords: Nicotiana tabacum; Solanaceae; Tobacco; Functional genomics; Pyridine alkaloids; Nicotine; Lysine decarboxylase; GH3-like enzyme; Jasmonate; Auxin conjugates; cDNA-AFLP

Contents

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1. Introduction

Alkaloids play a prominent role among the biologically active secondary metabolites in plants. Over 12,000 alkaloids have been isolated in the course of the last two centuries (Facchini, 2001) and natural functions such as defence against herbivores have been attributed to them. Structurally they are extremely diverse, although most of them are biosynthetically derived from ubiquitous amino acid precursors. Their structural diversity determines their pharmacophoric diversity, which, in turn, explains why many alkaloids are important pharmaceuticals and constitute building blocks for semisynthetic derivatives or serve as an inspiration for fully synthetic entities. However, the absolute amounts of alkaloids in plants are often rather low, hampering their industrial exploitation. A good understanding of the biosynthetic pathways leading to alkaloids is a prerequisite to engineer their production rates.

The presence of pyridine alkaloids, such as nicotine, nornicotine, anabasine and anatabine, is characteristic for *Nicotiana* species. The first committed step in nicotine biosynthesis is the *N*-methylation of putrescine to *N*-methylputrescine (Hashimoto and Yamada, 1994) catalysed by putrescine methyltransferase (PMT). Putrescine is directly derived from ornithine or indirectly from arginine (Fig. 1). The respective reactions, including the enzymes and coding genes, are well investigated (Hashimoto and Yamada, 1994). Only recently, the gene encoding methylputrescine oxidase, a copper-containing specific diamine oxidase that catalyses the formation of *N*-methylaminobutanal, has been characterised in tobacco (Heim et al., 2007; Katoh et al., 2007).

N-Methylpyrrolinium salt, which cyclises spontaneously from *N*-methylaminobutanal (Mizusaki et al., 1972) is also a precursor for tropane alkaloids. Nicotine is formed by a condensation of *N*-methylpyrrolinium and nicotinic acid, and can be further metabolised to other alkaloids, such as nornicotine, nicotyrine and myosmine. To date, only one gene has been characterized in this pathway branch, encoding the cytochrome P450 enzyme CYP82E4 that catalyzes the demethylation of nicotine to nornicotine (Siminszky

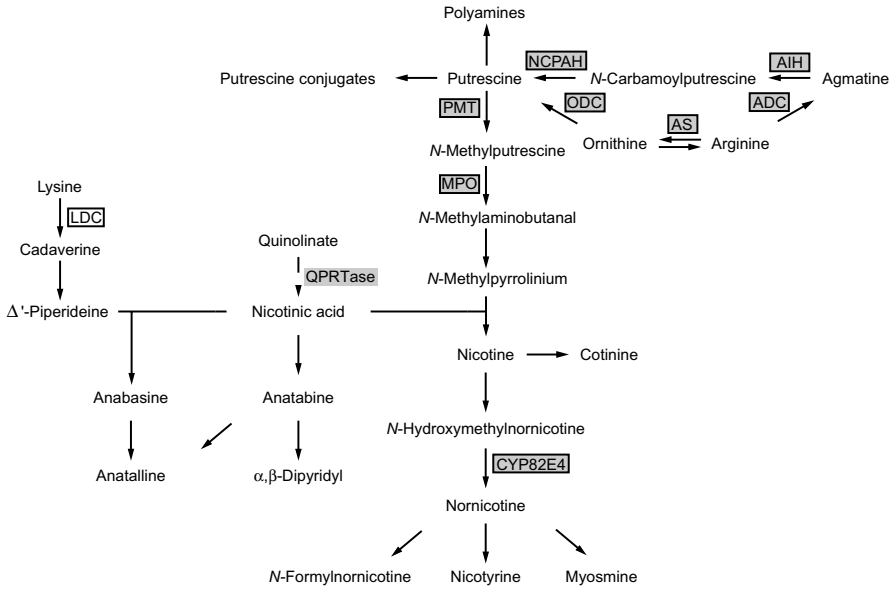

Fig. 1. Pyridine alkaloid biosynthesis in *Nicotiana tabacum*. Characterized enzymes are boxed and when the corresponding genes are known, boxes are coloured grey. Enzymes listed: ADC (arginine decarboxylase), AIH (agmatine deiminase), AS (arginase), CYP82E4 (nicotine *N*-demethylase), LDC (lysine decarboxylase), MPO (methylputrescine oxidase), NCPAH (*N*-carbamoylputrescine amidohydrolase), ODC (ornithine decarboxylase), PMT (putrescine *N*-methyltransferase), QPRT (quinolinate phosphoribosyltransferase).

et al., 2005; Fig. 1). Yet, the biosynthetic routes leading to anabasine, anatabine and anatalline are even less clear. Anatabine (Leete and Slattery, 1976) and the pyridine ring of anabasine (Bush et al., 1993) are derived from nicotinic acid, whereas the piperidine ring of anabasine is derived from lysine via Δ^1–piperideine (Leete, 1980). Anatalline is usually a minor alkaloid in tobacco roots but has recently been found as a major constituent in elicited Bright-Yellow 2 (BY-2) cell cultures (Häkkinen et al., 2004). However, none of the enzymes involved in the later parts of the biosynthesis have been identified so far, and virtually nothing is known about the transcriptional control of tobacco pyridine alkaloid biosynthesis, in contrast to the relatively well studied transcriptional regulation of, for instance, the terpenoid indole alkaloid pathway in *Catharanthus roseus* (Memelink et al., 2001; Goossens and Rischer, 2007).

Here, we present the continuation of a functional genomics effort to unravel the regulation of plant secondary metabolism, using tobacco pyridine alkaloid biosynthesis as a model system. This research was launched with a comprehensive transcript profiling study of nicotine alkaloid-producing tobacco BY-2 cells that established a substantial gene collection of tobacco genes potentially involved in alkaloid biosynthesis (Goossens et al., 2003a). In a first follow-up study, we screened for transcriptional regulators of tobacco alkaloid biosynthesis using a protoplast-based transient expression assay and identified two AP2-domain transcription factors that stimulate *Putrescine N-Methyl Transferase* (*PMT*) gene expression (De Sutter et al., 2005). Here, a second follow-up study is presented, consisting of a screen based on constitutive overexpression of genes in stably transformed tobacco BY-2 cell suspension or hairy root cultures. Two genes were identified, encoding putative enzymes that might be either directly or indirectly involved in nicotine alkaloid biosynthesis.

Fig. 2. Average linkage hierarchical clustering of BY-2 MJM expression patterns. Known or potential regulators (upper panel) or enzymes and transporters (lower panel) involved in tobacco pyridine alkaloid biosynthesis are shown. Treatments and time points (h) are indicated at the top. Red and green boxes reflect transcriptional activation and repression by methyl jasmonate (MeJA), respectively, relative to the average expression level in mock (DMSO) treated cells. Gray boxes correspond to missing time points.

2. Results and discussion

2.1. Selection of genes potentially involved in tobacco pyridine alkaloid biosynthesis

In previous research, combined targeted metabolite analysis and cDNA-AFLP transcript profiling was performed on BY-2 cultured cells elicited with the signalling molecule methyl jasmonate (MeJA) (Goossens et al., 2003a). A set of 459 MeJA-modulated (MJM) cDNA-AFLP tags was identified that matched the observed MeJA-induced shifts

Table 1
Overview of the tobacco BY-2 MJM genes introduced in the functional analysis pipeline

MJM[a]	EMBL[b]	Annotation	Cloning[b]
C1	CQ808705	Putative reductase	C
C18	AJ966359	RNA-binding-like protein	C
C112	CQ808719	Putative protein	C
C127	CQ808735	GH3-like protein	C
C165	CQ808761	Putative ion channel protein	R
C171	AM851007	Putative hydrolase	P
C175	CQ808768	GH3-like protein	C
C228	AF321137	ADC	E
C308	AF233849	ODC	E
C360	CQ808877	Putative protein	C
C365	AM851008	Putative protein	P
C406	AJ966360	RNA-binding-like protein	P
C468	AM851009	Sulfate transporter like protein	P
C476	CQ808961	MAPK kinase	R
C477	AM851010	Putative zinc transporter	C
MAP2	CQ808981	Putative protein	C
MC118	AM851011	Putative reductase	C
MC126	AM779762	Putative protein	P
MC204	CQ809012	Putative protein	R
MC212	AB038494	QPRT	E
MC304	AJ 966361	Putative protein	P
MC307	AJ 966362	Putative protein	P
MT101	CQ809052	GTP-binding-like protein	R
MT401	CQ809143	Glutathione S-transferase	C
T21	CQ809162	Cyclophilin	C
T36	AM851012	Putative esterase	C
T114	AM851013	Putative cinnamyl alcohol dehydrogenase	P
T172	CQ809147	Protein phosphatase 2C	C
T221	AM851014	Putative strictosidine synthase	P
T323	CQ809206	Putative endo-1,4-β-glucanase	C
T361	AM851015	Putative amine oxidase	C
T407	AM851016	Putative protein	P
T440	AM851017	Berberine bridge enzyme like protein	P
T464	CQ809292	Epimerase-like protein	C

[a] Tag code from Goossens et al. (2003a).
[b] Cloning method: C, colony hybridisation screening of the cDNA-library; E, sequence available from public databases; P, PCR-based screening of the cDNA-library; R, RACE-PCR.

in biosynthesis of tobacco pyridine alkaloids and other metabolites. This gene inventory contained most of the genes known so far to be involved in the biosynthesis of alkaloids in *Nicotiana* species. All of them displayed co-induction in the elicited tobacco BY-2 cells and clustered together with novel genes or genes encoding proteins with unknown functions. These genes represent candidates potentially coding for missing links in tobacco alkaloid biosynthesis. Indeed, in a previous screen performed by transient expression assays in tobacco protoplasts, two tobacco AP2-domain transcription factors were found in this collection, MAP3 (designated NtORC1) and C330 (designated NtJAP1), that positively regulate the *PMT* promoter (De Sutter et al., 2005). For this rationally designed screen, genes were selected (see Table 2 in De Sutter et al., 2005) and cloned that contained cDNA-AFLP tags fitting two criteria. First, their expression had to be induced within, at most, 6 h following MeJA elicitation, corresponding to the early co-induction of nicotine biosynthesis (within ca. 2 h) or phenylpropanoid biosynthesis (within 4 h) genes (Goossens et al., 2003a). Second, the genes had to code for proteins of unknown function or whose sequence suggested they might be involved in signal transduction pathways. Here, in this new screen, our candidate gene list was extended to 34 genes, by adding genes containing cDNA-AFLP tags that had a similar expression pattern and that mainly coded for putative enzymes (Fig. 2, Table 1). This set also contained genes encoding known enzymes of the nicotine biosynthetic pathway, such as arginine decarboxylase (ADC, tag C228), ornithine decarboxylase (ODC, tag C308) and quinolinate phosphoribosyltransferase (QPRT, tag MC212).

2.2. Gateway-based FL-ORF cloning pipeline

For overexpression experiments, it is necessary to first isolate full-length open reading frames (FL-ORFs). As the isolation of FL-ORFs based on cDNA-AFLP sequences was a limiting step in our discovery platform, we applied three different methods in parallel to streamline the procedure (see Section 4; Table 1). The first method consisted of a classical RACE–PCR protocol with primers designed from the cDNA-AFLP tag sequences. The other two methods were based on the screening of a custom-made high-quality cDNA library synthesized with mRNA extracted from MeJA-elicited BY-2 cells. On the one hand, the library was screened by classical colony hybridization with cDNA-AFLP tags as radiolabeled probes. On the other hand, a library subset was formatted into an arrayed cDNA clone collection and screened by PCR (see Section 4). Combining these methods, the FL-ORFs of the 'enzyme gene set' could be isolated as a Gateway entry clone (Table 1). The FL-ORFs of the 'regulator genes' had previously been captured as Gateway entry clones (De Sutter et al., 2005). For this new screen, all 34 ORFs (regulator and enzyme sets) were subcloned in the binary plant expression vector pK7WG2D to assess the gain-of-function effect of the cognate proteins on alkaloid accumulation in transgenic tobacco cultures. These constructs were introduced into *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* for tobacco cell suspension and hairy root transformation, respectively.

2.3. Functional analysis screen in BY-2 cell suspension cultures

Alkaloid production in BY-2 cell cultures is optimally determined after 48 h of elicitation with MeJA (Häkkinen et al., 2004). In general, the accumulation levels of the pyridine alkaloids measured closely resembled those previously described in elicited BY-2 cell cultures (Häkkinen et al., 2004): anatabine, being the most abundant alkaloid, followed by anatalline and anabasine, and with nicotine accumulating to only 1–4% of that of the major alkaloid anatabine. Non-elicited cultures had no more than trace amounts of alkaloids.

Of the 34 constructs tested in stable transformed BY-2 cells, nine caused an altered accumulation of one or more alkaloids, compared to the control (Table 2). Six constructs repressed alkaloid accumulation. Unexpectedly, three of

Table 2
Effects of tobacco BY-2 MJM transgenes in BY-2 cell suspension cultures after elicitation

MJM[a]	EMBL[b]	Annotation	#C[c]	NIC	TAB	BAS	TAL[d]
C127	CQ808735	GH3-like protein	3[e]	o	−	o	o
C165	CQ808761	Putative ion channel protein	2[e]	−	o	o	o
C228	AF321137	ADC	3	o	−	−	−
C308	AF233849	ODC	3	−	−	−	−
MAP2	CQ808981	Putative protein	3	+	o	o	o
MC126	AM779762	Putative protein	3	+	o	+	+
MC212	AB038494	QPRT	2	−	−	−	−
MT401	CQ809143	Glutathione S-transferase	3[e]	+	o	o	o
T464	CQ809292	Epimerase-like protein	3	o	o	o	−

[a] Tag code from Goossens et al. (2003a).
[b] EMBL accession number.
[c] The number of clones tested.
[d] The alkaloid concentration in 10 independent *GUS* lines was determined and the mean used as reference value. The CV% of the individual alkaloids of these control cultures was calculated. A 3-fold difference in alkaloid levels in the transgenic lines compared to the control value was judged considerable. NIC, nicotine; BAS, anabasine; TAB, anatabine; TAL, anatalline; +, positive effect; −, negative effect; o, no effect.
[e] The effects of the *C127*, *C165* and *MT401* transgene cassettes on alkaloid accumulation was only observed in one out of three clones tested.

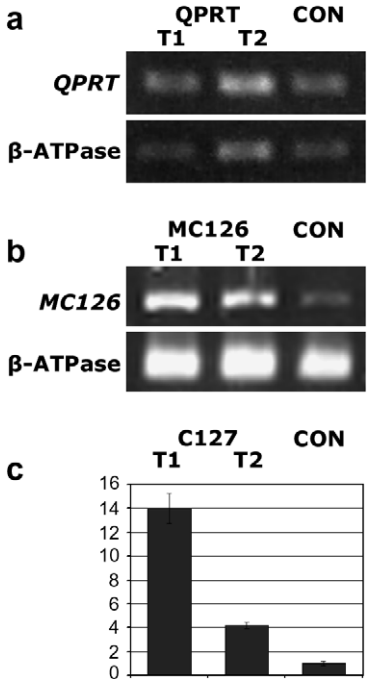

Fig. 3. Verification of transgene overexpression. RT-PCR analysis for QPRT (a), and MC126 (b) expression in transgenic BY-2 cells and Q-RT-PCR analysis for C127 expression in transgenic BY-2 hairy roots (c). Transgene expression levels from two independent transformants (T1 and T2) were compared with the expression levels of the corresponding endogenes in control (CON) BY-2 cells transformed with the GUS overexpression cassette (a, b) and BY-2 hairy roots transformed with the LBA9402 plasmid (c), respectively. Numbers in the Y-axis represent fold induction as compared to the control line. In all cases β-ATPase (U96496) was used as the reference gene.

these repressing constructs corresponded to known genes from the pyridine alkaloid pathway, namely *ADC*, *ODC*, and *QPRT*. Co-suppression-mediated gene silencing effects have been reported repeatedly in transgene tobacco lines transformed with p35S-driven overexpression constructs (Niebel et al., 1995), which might explain these effects in the lines transformed with the *ADC*, *ODC*, and *QPRT* overexpression cassettes. However, reverse transcription PCR (RT-PCR) based expression analysis indicated that at least for the *QPRT* lines gene silencing did not seem to be the cause for the observed effects (Fig. 3a). Steady-state mRNA levels of nicotine biosynthesis genes were not assessed in the *ADC* or *ODC* transgenic lines to further verify this assumption. Alternatively, control mechanisms might exist in tobacco cells to manage accumulation of toxic pyridine alkaloids or intermediates thereof (Goossens et al., 2003a). For instance, down-regulation of *PMT* expression with antisense technology in transgenic roots of the high-alkaloid-producing variety *Nicotiana tabacum* cv. NC95 not only led to decreased nicotine levels, but, unexpectedly, also to elevated levels of anatabine, presumably to cope with the relative oversupply of nicotinic acid in transgene *PMT*-silenced tobacco cells (Chintapakorn and Hamill, 2003).

Transformation with three overexpression constructs, corresponding to tags MC126, MAP2 and MT401, increased alkaloid production. In all cases, the effect was directed towards nicotine accumulation. An additional marked increase in the levels of anatalline and anabasine was observed only in the lines carrying the *MC126* overexpression construct (Table 2). Since this latter observation

Table 3
Effects of *MC126* overexpression on alkaloid accumulation of independent transformed tobacco BY-2 cell suspension cultures after elicitation

	Anatabine	Anabasine	Anatalline	Nicotine[a]
MC126-C1	10.88	0.88	1.39	0.73
MC126-C2	8.96	0.78	0.37	0.74
MC126-C5	5.59	0.24	0.19	0.44
MC126-C10	7.55	0.47	0.37	0.51
MC126-C14	3.36	0.31	0.31	0.30
MC126-C19	6.79	0.69	0.42	0.33
Mean				
MC126	7.19 (±2.62)	0.56 (±0.26)	0.51 (±0.44)	0.51 (±0.19)
GUS[b]	4.41 (±1.52)	0.25 (±0.12)	0.29 (±0.13)	0.11 (±0.05)
One-way ANOVA	$F(1,14) = 7.33$	$F(1,14) = 11.04$	NS[c]	$F(1,14) = 40.10$
	$p < 0.05$	$p < 0.01$		$p < 0.001$

[a] All values are indicated in mg/g dry weight and represent the values measured after three passages of subculturing.
[b] The alkaloid concentration in 10 independent *GUS* lines was determined and the mean used as reference value. The standard deviation is indicated between parentheses.
[c] Not significant.

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NtMC126          183-HGLVDAVVRCKSSERTAVVALPGGIGTLDEIFEIMALIQLERIGSQLPVPFLLMN-237
At1g50575        184-HGLVDAVIRNNVSEKTAIIALPGGIGTLDEMPEILALIQLERIGSALPVPFIVMN-238
Os03g0587100     213-HGLVDAAVRNCPTDRTAIIALPGGIGTLDELFEMMALIQLERIGSTLPVPFLLLN-267
Mlctf            105-H------------ADAFIVLPGGLGTLDELFEAWTAGYIG----MHRKPIVMLD-142
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Fig. 4. Protein sequence alignment of NtMC126 with putative lysine decarboxylases. ClustalW (http://www.ebi.ac.uk/clustalw/) generated multiple sequence alignment, centred around the conserved PGGXGTXXE motif, of the amino acid sequences of NtMC126 and the closest *Arabidopsis* (At1g50575) and rice (Os03g0587100) homologues and a representative member (Mlctf/AAA62920) of the PFAM03641 family of putative LDCs (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi).

pointed towards an overall activation of the pyridine alkaloid pathway (Fig. 1), the effects of the *MC126* gene were investigated more thoroughly. Altogether six clones carrying the *MC126* overexpression construct were generated and analysed. Overexpression of the transgene in these lines was confirmed with RT-PCR (Fig. 3b). The overall alkaloid levels were higher in all lines carrying the *MC126* overexpression cassette, as compared to those in the control lines. In particular, a significant increment in nicotine accumulation was measured (Table 3). Subsequently, the alkaloid production of two of the best producing clones was scored during several cultivation passages. Over time, the production decreased in the high-producing clones to the same level as that of the control lines (data not shown), indicating that the higher alkaloid production levels could not be maintained during further subculturing.

2.4. Potential function of MC126

BLAST searches matched *MC126* to a gene coding for a putative protein with unknown function that had strong sequence similarity with annotated genes of *Oryza sativa* (rice) and *Arabidopsis thaliana* (Fig. 4). The corresponding peptide sequences contained the PFAM03641 domain that defined a family including proteins annotated as putative lysine decarboxylases (LDC), although evidence for this enzymatic activity is not clear (http://www.sanger.ac.uk//cgi-bin/Pfam/). The members of this family share a highly conserved motif PGGXGTXXE that is probably functionally important (Fig. 4). So far, we have not been able to assign a function to the *MC126* gene product. Recombinant HIS-tagged purified MC126 protein did not exhibit LDC activity in the experiments conducted yet (T. Okada, K. Saito, and A.G., unpublished results). Additional biochemical assays will be required to assess the exact functionality of the MC126 protein in polyamine and pyridine alkaloid biosynthesis in *N. tabacum* considering, for example, that ODC from *Nicotiana glutinosa* is capable of decarboxylating both L-ornithine and L-lysine, and that the balance between ODC and LDC activity depends in part on the pH of the reaction buffer (Lee and Cho, 2001). The outcome of these experiments could also be indicative of whether *MC126* overexpression might affect the accumulation of phenylpropanoid-polyamine conjugates. These compounds are also detected in MeJA-elicited cell cultures (Gális et al., 2006), and their biosynthesis can be stimulated by overexpression of a heterologous bacterial LDC (Berlin et al., 1998).

2.5. Functional analysis screen in BY-2 hairy root cultures

Constitutive overproduction of alkaloids in BY-2 suspension cells may be detrimental to cell viability (De Sutter et al., 2005; Goossens et al., 2003b). Therefore, a functional screening was initiated in BY-2 hairy roots that, in contrast to BY-2 suspension cells, produce pyridine alkaloids constitutively. Unfortunately, despite several attempts, no transgenic hairy roots carrying the *MC126* overexpression constructs could be obtained. Yet, for four constructs of the enzyme set and four constructs of the regulator set, at least two independent transgenic hairy root lines could be established thus far (Table 4).

Maximum alkaloid accumulation in transformed hairy roots is usually observed after 28 days of cultivation (Jouhikainen et al., 1999); therefore this point was chosen for

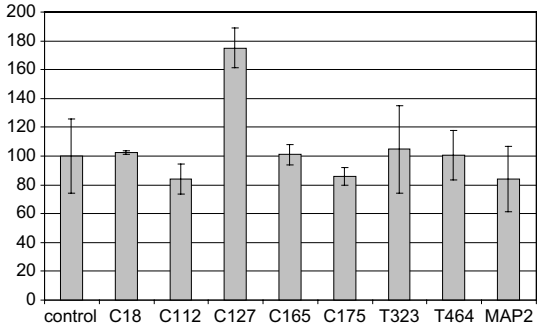

Fig. 5. Comparison of nicotine accumulation in *N. tabacum* BY-2 control and transgenic hairy root cultures. Values are indicated as % relative to the control line. Error bars represent standard deviation of individual clones (the number of clones analysed is presented in Table 3). Nicotine accumulation in *NtC127* is significantly increased (One-way ANOVA: $F(1,10) = 22.80$, $p = 0.001$).

Table 4
Effects of tobacco BY-2 MJM transgenes in BY-2 hairy roots cultures

MJM[a]	EMBL[b]	Annotation	#C	NIC	TAB	BAS	TAL	NOR[c]
C18	AJ966359	RNA-binding-like protein	2	o	o	o	o	o
C112	CQ808719	Putative protein	6	o	o	o	o	o
C127	CQ808735	GH3-like protein	3	+	o	o	+	o
C165	CQ808761	Putative ion channel protein	2	o	o	o	o	o
C175	CQ808768	GH3-like protein	2	o	−	o	o	−
T323	CQ809206	Putative endo-1,4-β-glucanase	6	o	o	o	o	o
T464	CQ809292	Epimerase-like protein	2	o	o	o	o	o
MAP2	CQ808981	Putative protein	6	o	o	o	o	o

[a] Tag code from Goossens et al. (2003a).
[b] EMBL accession number.
[c] Alkaloid levels produced in the transgenic cultures were compared to the mean alkaloid concentration of five independent transgenic hairy root lines carrying the *A. rhizogenes* LBA9402 plasmid. The CV% of the individual alkaloids of these control cultures was calculated, and used as a criterion to determine the significance of differences in alkaloid accumulation levels in transgenic cultures. #C, number of clones tested; NIC, nicotine; TAB, anatabine; BAS, anabasine; TAL, anatalline; NOR, nornicotine; +, positive effect; −, negative effect; o, no effect.

sample collection for alkaloid profiling. In only one of the eight series of lines transformed with a particular overexpression construct, a clear positive effect on pyridine alkaloid biosynthesis was exhibited (Table 4). Overexpression of *C127* almost doubled nicotine (Fig. 5) and anatalline accumulation levels in all three generated *C127* lines, with a mean of 1.9-fold and 1.8-fold, respectively. Quantitative real-time PCR (Q-RT-PCR) analysis confirmed that the higher alkaloid levels correlated with higher *C127* expression levels (Fig. 3c). The production of alkaloids in all the three C127 lines remained significantly higher compared to the control lines in the three subsequent transfer passages; even though there was a tendency to a small decrease.

In tobacco hairy roots carrying the *C175* overexpression construct, a small reduction of anatabine and nornicotine levels was observed, whereas the accumulation of nicotine, anabasine and anatalline was not affected (Table 4). Overall, these results should be interpreted with care because

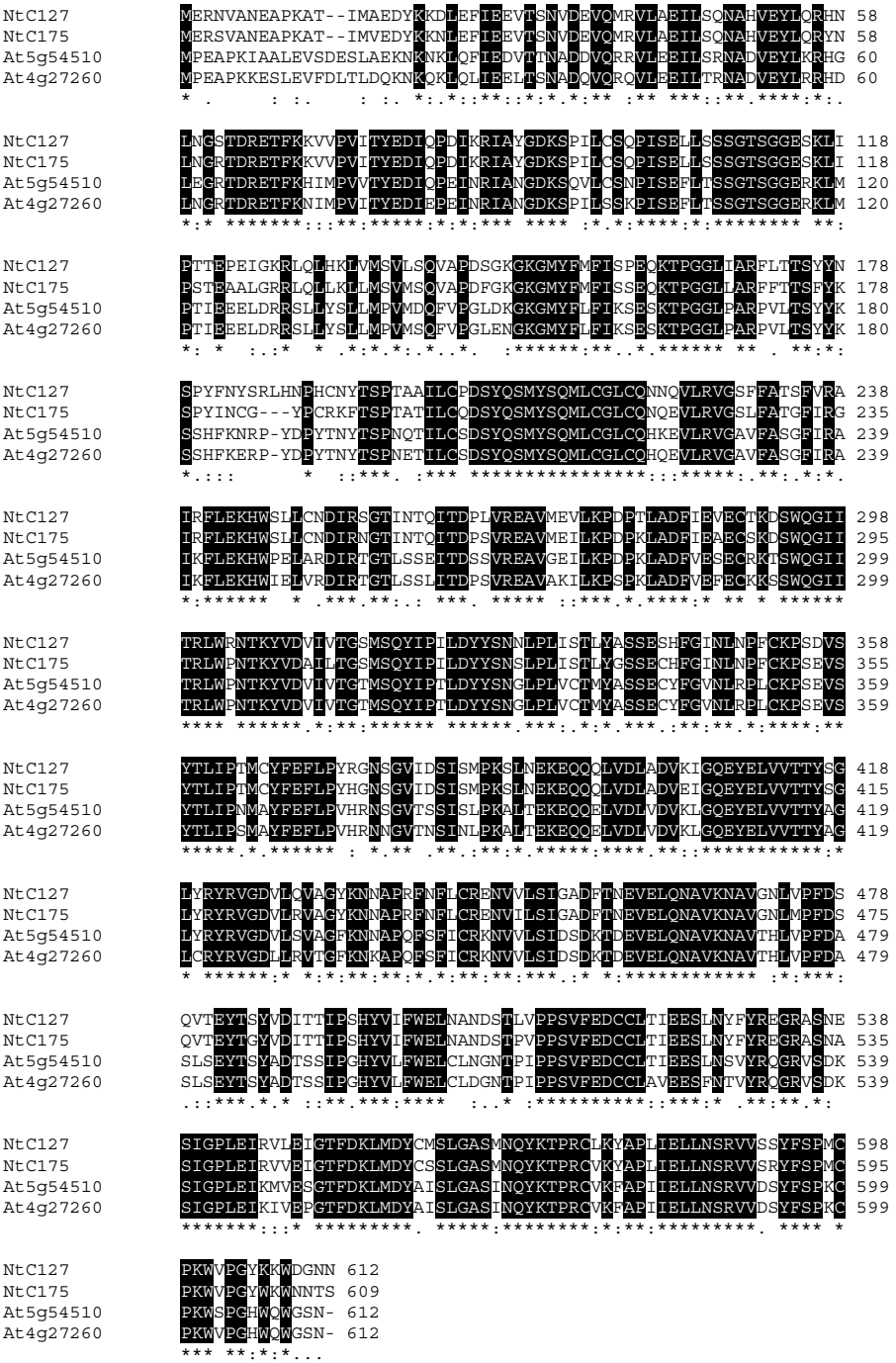

Fig. 6. Protein sequence alignment of tobacco and *Arabidopsis* GH3-like adenyltransferases. ClustalW (http://www.ebi.ac.uk/clustalw/) generated multiple sequence alignment of the amino acid sequences of NtC127, NtC175 and the closest *Arabidopsis* homologues (At4g27260 and At5g54510).

some of the constructs had low number of independent clones or overexpression was not as pronounced as in the *C127* lines, including e.g. for the *C175* lines (that show an overexpression level of ca. 2- to 4-fold, data not shown). It has been observed before that variability in alkaloid production can occur among transgenic *in vitro*-cultured lines carrying the same transformation construct and that, therefore, usually a certain number of transgenic lines need to be tested in order to find high alkaloid producers (Jouhikainen et al., 1999). However, the stability of alkaloid production in hairy roots is generally higher than that of undifferentiated cell cultures (Flores et al., 1987; Sevón et al., 1998). Indeed, given that particularly nicotine levels varied only slightly between independent hairy root lines and across cultivation passages (ranging on average to approximately 7.7 mg/g DW), the marked increase in nicotine levels in all three *C127* lines (Fig. 5) to more than 12.5 mg/g DW for the lowest producing *C127* line, prompted us to examine the potential role of *C127* gene product.

2.6. Potential functions of C127 and C175

BLAST analysis matched C127 to GH3-like enzymes, with the *Arabidopsis* GH3s At5g54510 and At4g27260 as closest homologues (with 67% and 66% identity at the amino acid level, respectively, Fig. 6). Interestingly, At4g27620 codes for WES1, the GH3 enzyme that links auxin-mediated growth regulation with stress adaptation response in *Arabidopsis* (Park et al., 2007). A *wes1-D* enhancer trap line exhibited reduced growth but increased resistance to both abiotic and biotic stresses, and upregulation of various stress-responsive genes (Park et al., 2007). Mutant *wes1-D* leaves displayed a 7.2-fold increase in indole-3-acetic acid (IAA)-Asp and a slight (1.8-fold) decrease in free IAA content when compared to wild type leaves (Park et al., 2007). In another *Arabidopsis* GH3 activation-tagging mutant, the *dfl1-D* mutant in which *At5g54510* is overexpressed, IAA conjugate levels were elevated ca. 4-fold, whereas no significant differences in free IAA levels could be detected (Staswick et al., 2005). Both WES1 and DFL1 exhibit activity on IAA in an assay for adenylation (Staswick et al., 2002).

Auxins are known to down-regulate expression of genes involved in nicotine biosynthesis, both *in planta* and in *in vitro* cultures (Hibi et al., 1994). Therefore, and because MeJA rapidly increased *C127* expression in BY-2 cells (Goossens et al., 2003a; Fig. 2), we postulated that elicitation of nicotine biosynthesis by MeJA would be mediated, at least partly, by altered auxin homeostasis. Hence, we compared IAA levels in mock versus MeJA-treated tobacco BY-2 cells: free IAA did not differ significantly between the two samples (8.32 ± 0.76 vs. 11.22 ± 0.94 pmol/g fresh weight, respectively), but IAA conjugates were 2.6-fold higher in MeJA-treated cells (15.26 ± 0.82 vs. 39.29 ± 6.40 pmol/g fresh weight, respectively), indicating that jasmonate elicitation and inactive auxin content might be correlated. In agreement with a positive role of *C127* in the regulation of nicotine biosynthesis is the observation

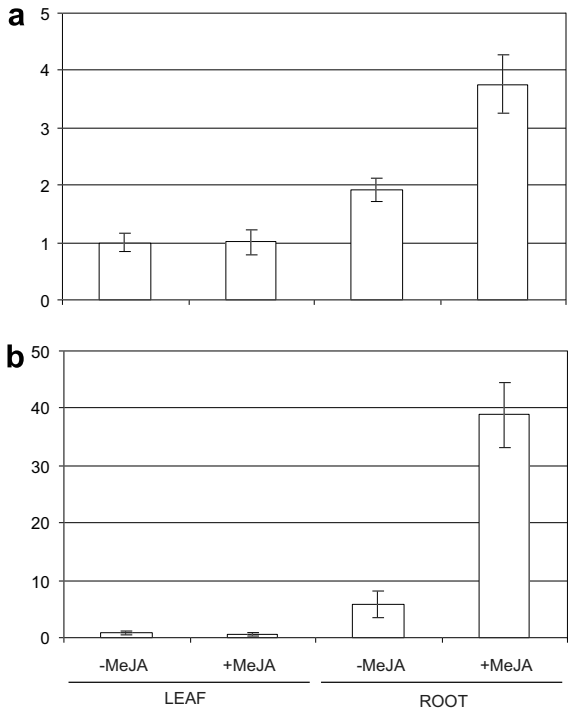

Fig. 7. Expression analysis of *NtC127* in tobacco seedlings. Q-RT-PCR analysis for *NtC127* (a) and *PMT* (b) expression in roots and leaves of *N. tabacum* SR1 seedlings treated or not with MeJA for 2 h. Samples and treatments are indicated at the bottom. Numbers on the left indicate the normalised expression ratio using β-ATPase (U96496) as the reference gene and the leaf minus MeJA sample as the reference sample.

that *C127* expression levels are ca. 2-fold higher in roots, the exclusive production site of nicotine in tobacco plants, than in leaves that are incapable of synthesising nicotine, but serve as its storage site (Fig. 7). Given these converging evidences, we renamed C127 as Nicotine-Enhancing GH3 enzyme 1, NtNEG1. Preliminary data from overexpression of *NtNEG1* in transgenic hairy roots of *Hyoscyamus muticus* (L.) suggest that NtNEG1 can also stimulate flux through the pseudotropine branch of the tropane alkaloid pathway that shares precursors with the nicotine biosynthetic pathway, pointing to a conserved role of NtNEG1 in regulating alkaloid biosynthesis in Solanaceae species (A.G., S.T.H. and K.-M.O.-C., unpublished results).

In the *C175* lines nicotine biosynthesis was not altered whereas accumulation of some of the other pyridine alkaloids was slightly repressed (Table 4). *C175* encodes a GH3-like enzyme with 89% and 66% amino acid identity to NtNEG1 and to either AtWES1 or AtDFL1, respectively (Fig. 6); therefore, it was designated as NEG-Like GH3 enzyme 1, NtNLG1. The divergent effects on alkaloid biosynthesis of NtNEG1 and NtNLG1 might reflect different adenylation activities or substrate specificities; for instance, despite their close relation (90% amino acid identity), both WES1 and DFL1 can adenylate IAA, but only WES1 can additionally adenylate salicylic acid (Staswick et al., 2002), another known antagonist of jasmonate responses. Remarkably also, the mode-of-action of NtNEG1 appears

opposite to that of two other members of the GH3 family that have recently been identified in tobacco, namely JAR4 and JAR6 (Kang et al., 2006; Wang et al., 2007). JAR4 and JAR6 conjugate jasmonic acid to amino acids, such as Ile, Val and Leu to mediate defence responses in tobacco. Whereas silencing of the two *JAR* genes significantly reduced levels of trypsin proteinase inhibitors in tobacco leaves, nicotine levels remained normal, in contrast to the clear effect of *NtNEG1* overexpression that resulted in a net stimulation of nicotine biosynthesis.

3. Conclusions

Here, we have continued exploring a select set of new genes that are potentially involved in pyridine alkaloid metabolism and that we had previously isolated from *N. tabacum* via a combined transcript and metabolite profiling effort (Goossens et al., 2003a). The functions of the selected genes were analysed by a gain-of-function approach in transgenic BY-2 cell or hairy root cultures that had served as the original source of the genes, too. This functional genomics-based screen pinpointed several novel, potentially powerful, catalysers of tobacco pyridine alkaloid biosynthesis. Further in-depth research will allow clarifying the exact mode-of-action of these potential catalysers and the generic nature of their activity with regard to the regulation of alkaloid metabolism (or perhaps even plant secondary metabolism in general). Undoubtedly our insight into plant secondary metabolism will further benefit from similar large-scale screens being (or to be) set up in the research community and from the new technologies and data handling methods that are developed in the rapidly expanding field of plant metabolomics and functional genomics.

4. Experimental

4.1. Gateway-based FL-ORF cloning

For the cloning of tobacco genes, FL-ORFs were isolated either via RACE technology (Invitrogen, Carlsbad, CA) or by screening of a BY-2 cDNA library. This custom-made library (Invitrogen) was derived from tobacco BY-2 cells harvested at different time points following MeJA elicitation. This library was screened either via classical colony filter hybridization with cDNA-AFLP tag sequences as probes or via PCR. Both for RACE-PCR and the PCR screening of the cDNA library, primers were designed based on the sequence of cDNA-AFLP tags with the PRIMER3 software (Rozen and Skaletsky, 2000).

For the PCR screening of the library, a library subset was formatted into an arrayed cDNA clone collection prior to PCR. This latter method is similar to the combinatorial analysis of large-scale BAC libraries. Approximately 25,000 colonies were randomly picked and transferred with a PBA Flexys workstation (Genomic Solutions, Ann Arbor, MI, USA) into 72 384-well microtitre plates. DNA was prepared (standard alkaline lysis protocol; Nucleobond Plasmid Purification, Clontech, CA, USA) for each pool of 384 clones in a single plate; then six DNA superpools were created each containing 12 plate pools (4608 clones). PCRs were performed with Silverstar DNA polymerase (Eurogentec, Belgium). Steps included: one cycle at 94 °C for 2 min, followed by 40 cycles at 94 °C for 15 s, at 55 °C for 20 s, at 72 °C for 30 s, and ended by one cycle at 72 °C for 2 min. A positive superpool was deconvoluted in single pool hit(s) by 12 additional plate pool PCRs. A positive pool was deconvoluted in single positive clone(s) through a tri-dimensional subpooling scheme consisting of 24 additional PCRs: 12 PCRs corresponded to 32 clones in successive pairs of columns in the 384-well plate; eight PCRs to 48 clones in successive pairs of rows in the plate; and four PCRs to 96 clones in each of the four quadrants of the plate. A PHP4 web-based application running a MySQL3 database was built to store and visualize cDNA-AFLP tag data and combinatorial screening results and to assist in deconvoluting of row/column/quadrant hits from 384-well plates into single clone positions.

Based on the FL cDNA clone sequences, FL-ORF amplicons were generated and transferred to the expression constructs derived from pK7WG2D as described (De Sutter et al., 2005), and subsequently introduced into *A. tumefaciens* strain LBA4404 pBBR1MCS-5 (van der Fits et al., 2000) or *A. rhizogenes* strain LBA9402 for generation of transgenic *N. tabacum* BY-2 cell suspension and hairy root cultures, respectively.

4.2. Maintenance and transformation of BY-2 cell suspension cultures

The *N. tabacum* BY-2 cell suspension culture was maintained as described (Nagata and Kumagai, 1999). Gene constructs were transferred to BY-2 cultures by *A. tumefaciens*-mediated transformation according to the protocol from De Sutter et al. (2005). Transformed colonies appeared approximately within 14–21 days on the plates. The colonies were picked and transferred to fresh plates, and their transgenic nature was confirmed by PCR. Confirmed transformed calli were subsequently suspended in liquid medium containing 50 ppm kanamycin (Duchefa, The Netherlands) to keep selection pressure. Altogether three independent transgenic lines for each construct were selected for the initial functional screening. The cultures were elicited as described by Häkkinen et al. (2004).

4.3. Generation and transformation of N. tabacum BY-2 hairy root cultures

Generation of transformed tobacco hairy roots was initiated by infecting surface-sterilized *N. tabacum* cv. BY-2 leaves with a 2-day-old *A. rhizogenes* LBA9402 culture. Leaves were infected by wounding the mid-ribs with a sterile needle inoculated with the desired *Agrobacterium* strain.

After 48 h, the leaves were transferred to solid modified Gamborg B5 medium supplemented with 500 ppm cefotaxime (Duchefa, The Netherlands) to eliminate *Agrobacterium*. The transgenic nature of the roots and the absence of *Agrobacterium* were confirmed by PCR. For alkaloid accumulation analysis, hairy roots were inoculated in 20 ml medium in 100 ml shake flasks and cultivated in a rotary shaker (70 rpm, 24 °C) in modified Gamborg B5 medium without casein (Jouhikainen et al., 1999) for 28 days.

4.4. Alkaloid measurements

Alkaloids of the transformed BY-2 suspension cultures and hairy root cultures were analysed by HPLC (Häkkinen et al., 2004) and by GC–MS (Häkkinen et al., 2005), respectively. In each analytical experiment a control BY-2 line transformed with the *GUS* gene was carried along as a control. Analysis of variance (ANOVA) was conducted using SPSS 15.0.1 (SPSS Inc., Chicago, Illinois, USA).

4.5. RT-PCR and Q-RT-PCR expression analysis

Hundred mg of either transformed BY-2 cells and roots, or *N. tabacum* SR1 seedling leaves and roots were used for RNA extraction following the ConcertTM Plant RNA Reagent protocol (Invitrogen). Single-stranded cDNA was prepared from this total RNA with SuperscriptII RT-polymerase (Invitrogen). Transgene overexpression in transformed BY-2 cell and root lines was verified by RT-PCR and Q-RT-PCR, respectively. RT-PCR products were visualized on SYBR safe (Invitrogen) stained agarose gels. Q-RT-PCR reactions with BY-2 root and SR1 seedling material were run on a LightCycler 480 instrument with the LightCycler 480 SYBR Green I Master kit (Roche Applied Science, Mannheim, Germany). ΔCT relative quantification with gene normalization was performed with the qBASE program (medgen.ugent.be/qbase). Primers were constructed based on sequence data from *QPRT* (AB038494), *MC126* (AM779762), *C127* (CQ808735) and *PMT* (AF126812). *β-ATPase* (U96496) was used as the reference gene (Reed and Jelesko, 2004). Primers used were: *QPRT*-forward (fw): 5′-ATACGGAGGGCTTCAGGAAATG-3′, *QPRT*-reverse (rev): 5′-GTCAAGTGCTTTCACGGAATGC-3′, *MC126*-fw: 5′-AAAATGGGGTTTGGTGCAG-3′, *MC 126*-rev: 5′-GTCAAGGTAAACTTCTACATTCT-3′, *C127*-fw: 5′-TCAACTACAGTCGTCTTCATAACC-3′, *C127*-rev: 5′-AGTGCTTCTCCAGGAATCGG-3′, *PMT*-fw: 5′-TGGATGGAGCAATTCAACA-3′, *PMT*-rev: 5′-AACCAATTCCTCCGCCGATG-3′, *β-ATPase*-fw: 5′-CCATCAACACCACCGAAGTCC-3′ and *β-ATPase*-rev: 5′-GATGACCTGGCACACCTTCC-3′.

4.6. Auxin quantification

Samples of BY-2 cell suspension cultures were processed and analyzed as described (Prinsen et al., 1998). Briefly, frozen cells were ground in liquid nitrogen and extracted overnight in 80% MeOH at −20 °C. For recovery calculations 69 pmol of $^{13}C_6$-IAA (Cambridge Isotope Laboratories Inc., Andover, Massachusetts, USA) was added to the samples. After centrifugation (20,000*g*, 15 min, 4 °C), the supernatant was collected and passed through a C18 cartridge (Varian, Harbor City, CA). Methanol was removed by drying under nitrogen stream, the remaining water phase divided in two. The first part was processed directly for free IAA analysis; the second was subjected to alkaline hydrolysis to release conjugated IAA. Samples were suspended in 0.05 M HCl and passed through a C18 cartridge. Bound fraction was eluted with diethyl ether. Samples were methylated by ethereal diazomethane, analysed by microLC-(ES+)MS/MS in SRM mode, and quantified with Masslynx software (Waters, Zellik, Belgium) based on the principle of isotope dilution.

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