Plant Biotechnology Journal

Plant Biotechnology Journal (2014) **12**, pp. 971–983 doi: 10.1111/pbi.12205

Taximin, a conserved plant-specific peptide is involved in the modulation of plant-specialized metabolism

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Received 31 January 2014;
revised 31 March 2014;
accepted 24 April 2014.
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Accession numbers: The sequences reported in this paper have been deposited in the GenBank database (accession numbers KJ028109 and KJ123822 for *Taximin* and *TB510*, respectively).

Summary

Small peptides play important roles in the signalling cascades that steer plant growth, development and defence, and often crosstalk with hormonal signalling. Thereby, they also modulate metabolism, including the production of bioactive molecules that are of high interest for human applications. Yew species (*Taxus* spp.) produce diterpenes such as the powerful anticancer agent paclitaxel, the biosynthesis of which can be stimulated by the hormone jasmonate, both in whole plants and cell suspension cultures. Here, we identified *Taximin*, as a gene encoding a hitherto unreported, plant-specific, small, cysteine-rich signalling peptide, through a transcriptome survey of jasmonate-elicited *T. baccata* suspension cells grown in two-media cultures. *Taximin* expression increased in a coordinated manner with that of paclitaxel biosynthesis genes. Tagged Taximin peptides were shown to enter the secretory system and localize to the plasma membrane. In agreement with this, the exogenous application of synthetic Taximin peptide variants could transiently modulate the biosynthesis of taxanes in *T. baccata* cell suspension cultures. Importantly, the Taximin peptide is widely conserved in the higher plant kingdom with a high degree of sequence conservation. Accordingly, *Taximin* overexpression could stimulate the production of nicotinic alkaloids in *Nicotiana tabacum* hairy root cultures in a synergistic manner with jasmonates. In contrast, no pronounced effects of *Taximin* overexpression on the specialized metabolism in *Medicago truncatula* roots were observed. This study increases our understanding of the regulation of *Taxus* diterpene biosynthesis in particular and plant metabolism in general. Ultimately, Taximin might increase the practical potential of metabolic engineering of medicinal plants.

Keywords: jasmonate, *Nicotiana*, nicotine, signal peptide, paclitaxel, *Taxus*.

Introduction

Plant-specialized metabolites play crucial roles in interactions with the environment, defence and reproduction. These small molecules are characterized by a huge variety of complex structures, many of which having important applications for humans as pharmaceuticals, nutraceuticals, agrochemicals, etc. Generally, they represent <1% of the plant dry weight, their quantity most often depending on the plant's physiological and developmental state. One important phytopharmaceutical is Taxol (paclitaxel), an anticancer agent produced by yew species (*Taxus* spp.). Paclitaxel is a complex diterpene with several rings and functional groups, and a modified lateral chain. Paclitaxel is the result of a complex biosynthetic pathway putatively composed of 19 steps, starting from isopentenyl pyrophosphate (IPP) derived from the plastidial MEP pathway (Figure 1). Many, but still not all of the genes encoding the enzymes involved in this pathway have been isolated (Croteau *et al.*, 2006). Nonetheless, the understanding about the relevant enzymes and their order of action is still incomplete. Likewise, very little is known about the genes that control the side routes, flux-limiting steps, regulatory mechanisms and transport. This lack of knowledge triggered numerous gene discovery programmes to find general mechanisms to facilitate paclitaxel production in metabolic engineering and synthetic biology platforms (Croteau *et al.*, 2006; Cusidó *et al.*, 2014).

Paclitaxel production in *Taxus* spp. plants and cell suspension cultures can be increased by elicitation, with jasmonates (JAs) being the most effective elicitors known to date. Jasmonate signalling triggers an increased expression of the paclitaxel biosynthetic pathway genes, including those that encode the first-committed enzyme, taxadiene synthase (TXS), as well as nearly all of the known hydroxylases and transferases involved in the pathway (Cusidó *et al.*, 2014; Nims *et al.*, 2006; Onrubia *et al.*, 2013). In this regard, paclitaxel synthesis obeys the regulatory rules of numerous other plant bioactive metabolite pathways, for which the corresponding biosynthetic genes are transcriptionally, rapidly and coordinately activated by JAs in so-called transcriptional regulons (De Geyter *et al.*, 2012; Pauwels *et al.*, 2009). In fact, this conserved feature of plant-specialized metabolism guaranteed success in gene discovery programmes aiming for the identification of the genes encoding the missing

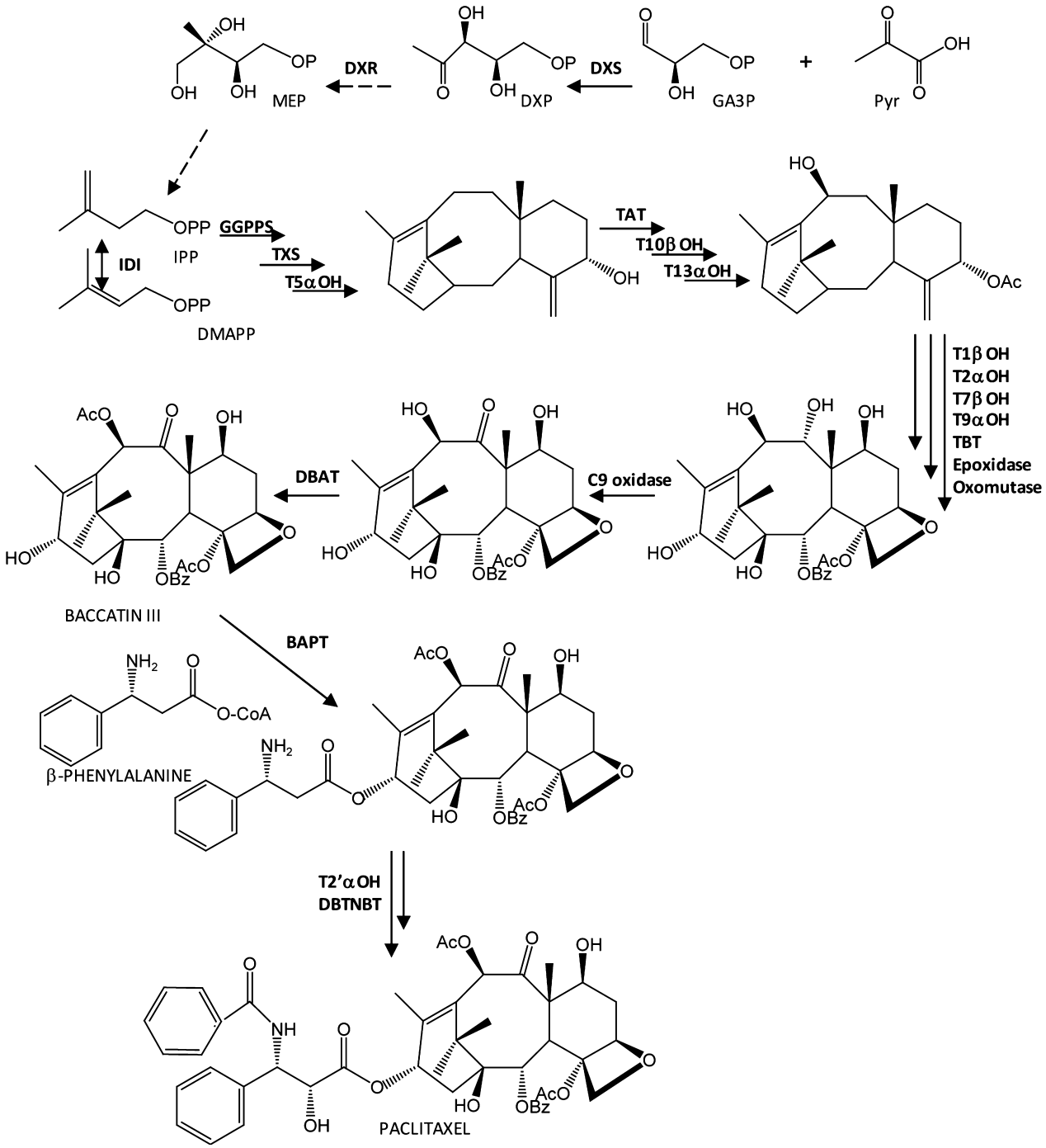

Figure 1 A concise depiction of paclitaxel biosynthesis. Pyr, pyruvate; GA3P, glyceraldehyde-3-phosphate; DXP, 1-Deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; DXS, DXP synthase; DXR, DXP reductoisomerase; IDI, IPP isomerase; GGPPS, geranylgeranyl diphosphate synthase; TXS, taxadiene synthase; T5αOH, taxane 5α-hydroxylase; TAT, taxadiene-5α-ol-*O*-acetyl transferase; T13αOH, taxane 13α-hydroxylase; T10βOH, taxane 10β-hydroxylase; T1βOH, taxane 1β-hydroxylase; T2αOH, taxane 2α-hydroxylase; T7βOH, taxane 7β-hydroxylase; T9αOH, taxane 9α-hydroxylase; TBT, taxane-2α-*O*-benzoyltransferase; DBAT, 10-deacetyl baccatin III-10-*O*-acetyltransferase; BAPT, baccatin III-3-amino, 13-phenylpropanoyltransferase; T2'OH, taxane 2'α-hydroxylase; DBTNBT, 3'-N-debenzoyl-2'-deoxytaxol-N-benzoyltransferase.

enzymatic steps from a pathway of interest, including that of paclitaxel (Croteau *et al.*, 2006; Cusidó *et al.*, 2014).

The transcription factors (TFs) or other regulatory proteins that drive the expression of the specialized metabolic pathway genes are often encoded by JA-responsive genes themselves to amplify the defence response when plants are under herbivore or pathogen attack. This is an important regulatory control point that evolved across the plant kingdom in the JA-modulated induction of specialized metabolism (De Geyter *et al.*, 2012). To date, only two JA-responsive *Taxus* TFs have been functionally characterized to some extent. TcWRKY1 from *T. chinensis* was shown to bind the promoter of the gene encoding

10-deacetylbaccatin III-10 β-*O*-acetyl transferase (DBAT) and to be a positive regulator of this gene (Li *et al.*, 2013). TcAP2 from *T. cuspidata* interacted with a JA-responsive element found in the promoters of the *Catharanthus roseus* genes from the terpenoid indole alkaloid pathway (Dai *et al.*, 2009), but its role in the regulation of paclitaxel synthesis has not been investigated yet.

Besides hormones, secretory and nonsecretory small peptides are involved in numerous aspects of plant growth, development and defence. Small signalling peptides mainly range in size between 5 and 75 amino acids (AAs), are cleavage products from precursor peptides that can be post-translationally modified and are usually perceived by membrane-bound receptors in signalling cascades that may crosstalk with hormones (Butenko *et al.*, 2009; Marshall *et al.*, 2011; Matsubayashi and Sakagami, 2006; Yamaguchi and Huffaker, 2011). Thereby, they can also (in)directly modulate or elicit plant metabolism. Small peptides, such as systemin and PIP-1 in solanaceous species and the plant elicitor peptide (Pep) in maize (*Zea mays*), have been shown to act in plant defence programmes that also involve JA signalling and/or elicit the production of terpenoid phytoalexins and volatiles (Huffaker *et al.*, 2013; Miyashita *et al.*, 2011; Sun *et al.*, 2011). No small signalling peptides have been specifically identified from *Taxus* species yet, but phytosulfokine-α (PSK-α), a 5-amino acid conserved plant signalling peptide, was tested as an elicitor in various *Taxus* cell suspension cultures, in which it considerably enhanced paclitaxel production, particularly in synergistic treatments with methyl jasmonate (MeJA) (Kim *et al.*, 2006).

In a screen for novel regulators of paclitaxel biosynthesis in cultured *T. baccata* cells, we identified a gene that we called *Taximin* and that encodes a previously unreported small signalling peptide. Functional analysis of *Taximin* indicated that this peptide-encoding gene is conserved in the higher plant kingdom and capable of modulating the synthesis of specialized metabolites in *in vitro* cultures of medicinal plants.

Results

Transcript profiling reveals candidate regulators of the coordinated transcriptional activation of paclitaxel biosynthesis

To identify regulators of taxane biosynthesis, we used complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) transcript profiling to monitor the transcriptome of suspension-cultured *T. baccata* cells and cultured first for 7 days in growth medium (GM) and subsequently 4 days in production medium (PM) supplemented with MeJA or its solvent ethanol (EtOH/mock). The use of such two-media cultures, the first for increasing biomass and the second for activating specialized metabolism, promotes a pronounced increase in paclitaxel accumulation as compared to *Taxus* cell suspensions grown in standard conditions (Cusidó *et al.*, 2002; Palazón *et al.*, 2003) and thereby creates an ideal set of samples with differential paclitaxel synthetic capacity. Across all samples, the expression of 8192 transcripts was visualized, and 858 tags with differential expression between GM and PM or between MeJA elicitation and mock treatment were identified. Several tags were identified corresponding to known paclitaxel or plastidial IPP biosynthetic pathway genes, and all show a pronounced up-regulation by MeJA elicitation and/or by the shift from GM to PM, although the effect of the latter was less pronounced in quantitative terms (Figure 2a). Overall, the comparable MeJA-induced expression patterns of the genes encoding enzymes catalysing steps in synthesis of paclitaxel or terpene precursors indicated co-regulation.

Besides possible candidates for the remaining unknown steps of the paclitaxel biosynthetic pathway (Croteau *et al.*, 2006), which include several candidate oxidases, oxomutases, epoxidases and CoA ligases, several of the differentially expressed genes corresponded to candidate regulators of taxane biosynthesis (Figure 2b). Several genes corresponding to potential regulators had a maximal transcriptional up-regulation before or concurrent with that of the paclitaxel biosynthesis genes and include genes encoding JA ZIM-domain (JAZ) repressor proteins, known elements of the core JA signalling module involved in the regulation of plant-specialized metabolism (De Geyter *et al.*, 2012; Pauwels and Goossens, 2011), as well as putative bHLH- and BSD-domain TFs and hypothetical proteins without any known domains (Figure 2b).

TB595 encodes a conserved small peptide, Taximin

Because the JAZ proteins act in a negative feedback loop and therefore are generally considered as negative regulators of the JA response (Pauwels and Goossens, 2011), we focused on the other candidate regulators in a search for potential positive drivers of paclitaxel biosynthesis. Starting from the available cDNA-AFLP tag sequences, we did not succeed in isolating the full-length (FL) cDNA sequences of the TFs. However, FL cDNA sequences with an FL open reading frame (ORF) could be obtained for two other tags, namely *TB510* and *TB595*, both corresponding to hypothetical proteins of unknown function without any known protein domain. In contrast to *TB510*, for which there was only limited sequence homology with genes from other plants, BLASTX searches indicated an extraordinary conservation of the *TB595* FL ORF sequence across the entire higher plant kingdom (Figures S1 and S2); hence, we concentrated on this gene for further functional analysis.

The *TB595* FL cDNA sequence comprised 472 nucleotides (nt) with an FL ORF of 222 nt encoding a small peptide of 73 AAs, which we denominated Taximin (Figure S1). Analysis of the Taximin gene sequence in PLAZA (Van Bel *et al.*, 2012) indicated that it is conserved across the entire higher plant kingdom, including all analysed dicots, monocots, gymnosperms, mosses and ferns (Figure 3a). ClustalW2 (Larkin *et al.*, 2007) and Seq2Logo (Thomsen and Nielsen, 2012) analyses indicated a high level of sequence conservation, both in the number and identity of AAs (Figures 3b and S2). From AA 4 on (numbering in Taximin), all homologues from all plants analysed have an identical length with a high number of conserved cysteines and prolines, and a conserved high concentration of hydrophobic AA residues. All but one (Phe at position 67) of the most conserved AAs are also conserved in the Taximin sequence (Figure 3b).

Interestingly, consulting of public expression databases such as GeneVestigator (Hruz *et al.*, 2008) and the *Medicago truncatula* Gene Expression Atlas (He *et al.*, 2009) indicated that the expression of the *Arabidopsis thaliana* and *Medicago truncatula* *Taximin* homologues was also modulated by JA elicitation and/or biotic stresses in which JA signalling is involved (Figure S3), suggesting a conserved role in JA signalling and/or plant defence processes for the Taximin peptides. Verification of *T. baccata* *Taximin* expression by quantitative real-time PCR (qRT-PCR) analysis confirmed that *Taximin* transcript levels showed a

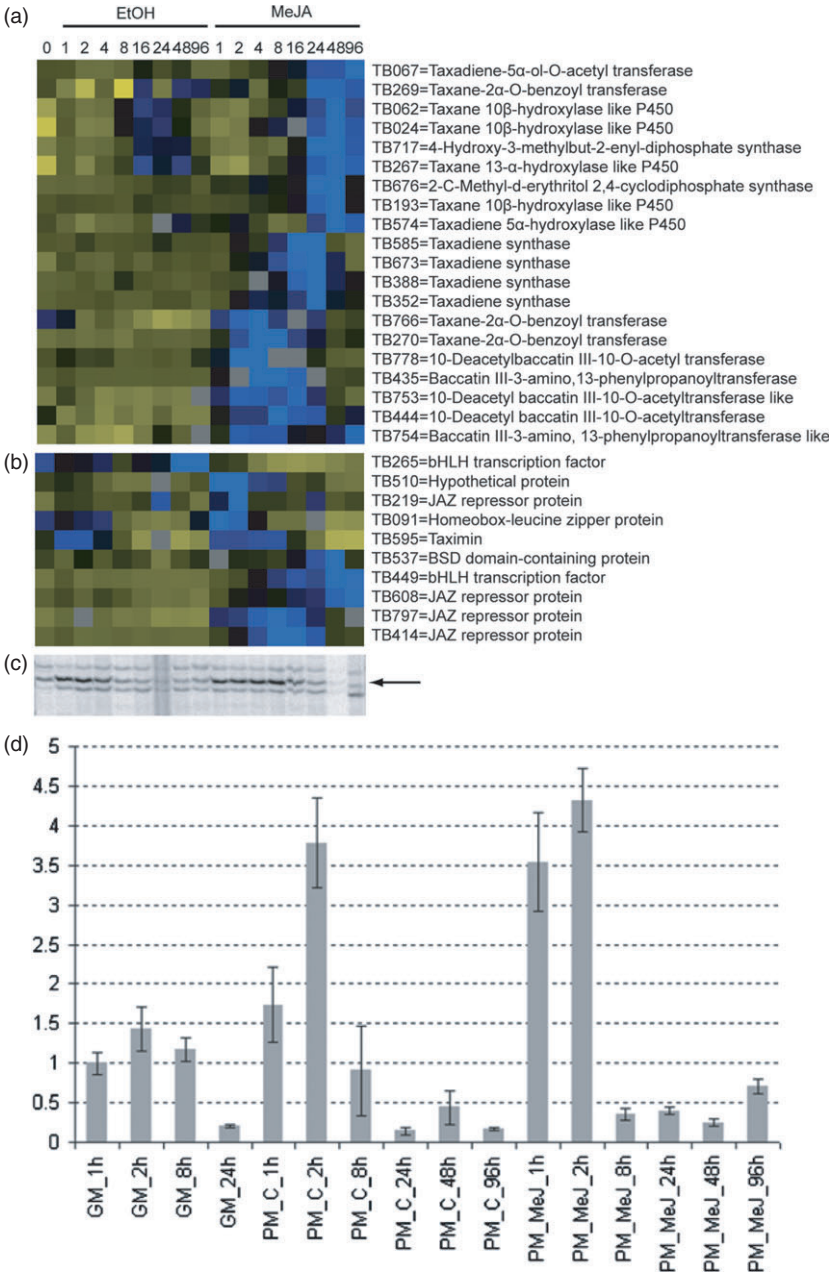

Figure 2 cDNA-AFLP-based transcript profiling of *Taxus baccata* cells. (a-b) Average linkage hierarchical clustering of differentially expressed gene tags identified by cDNA-AFLP. Treatments and time points (in h) are indicated at the top. Blue and yellow boxes reflect transcriptional activation and repression relative to the average expression level, respectively. Grey boxes correspond to missing time points. Shown are subclusters of the *T. baccata* genes encoding known enzymes catalysing paclitaxel biosynthesis (a) and known proteins involved in JA signalling and candidate regulators of paclitaxel biosynthesis (b). (c) Section of the cDNA-AFLP gel showing the expression pattern of the *TB595* tag, marked by the arrowhead on the right. (d) qRT-PCR analysis of the *TB595* gene in *Taxus baccata* cells cultured in the two-media system. *y*-axis, expression ratio relative to the normalized transcript levels in cells grown in GM for 1 h. Error bars indicate the standard error of the mean (SEM) (n = 3).

transient increase following the shift from GM to PM (Figure 2c–d), thus further suporting a potential role for Taximin as positive regulator of paclitaxel synthesis.

Taximin enters the secretory system

Analysis with SignalP v4.0 (Petersen *et al.*, 2011) predicted the existence of an N-terminal signal peptide of 27 AAs, yielding a putative mature Taximin protein of only 46 AAs (Figure 4a). In agreement with this, subcellular localization prediction tools suggested that Taximin would enter the secretory system and likely localizes to the vacuole, the plasma membrane or extracellular.

To investigate the subcellular localization of Taximin, the Venus yellow fluorescent protein (Nagai *et al.*, 2002) was fused to the C-terminus of the peptide, and the resulting construct was transiently expressed in *N. benthamiana* leaves. Confocal microscopy analysis revealed the GFP signal of the fusion protein as a thick band in the cell periphery, similar to that of 'free Venus' that localizes in the cytoplasm (Figure 4b). However, as in *N. benthamiana* leaves the vacuole pushes the cytoplasm against the plasma membrane, the fluorescence signals of both plasma membrane localized and cytosolic proteins may be similar. To discern the exact subcellular localization of the Taximin–Venus fusion protein, the cell walls of transfected *N. benthamiana* leaves were stained with propidium iodide (PI) prior to confocal microscopy analysis. In leaves infiltrated with the free Venus, separation of the GFP and PI signals was observed, whereas the signals of the PI and the Taximin–Venus fusion protein overlapped (Figure 4c). In addition, a fluorescence recovery after photobleaching (FRAP) analysis was performed. After photobleaching, the speed of fluorescence recovery in the bleached area is an estimation of

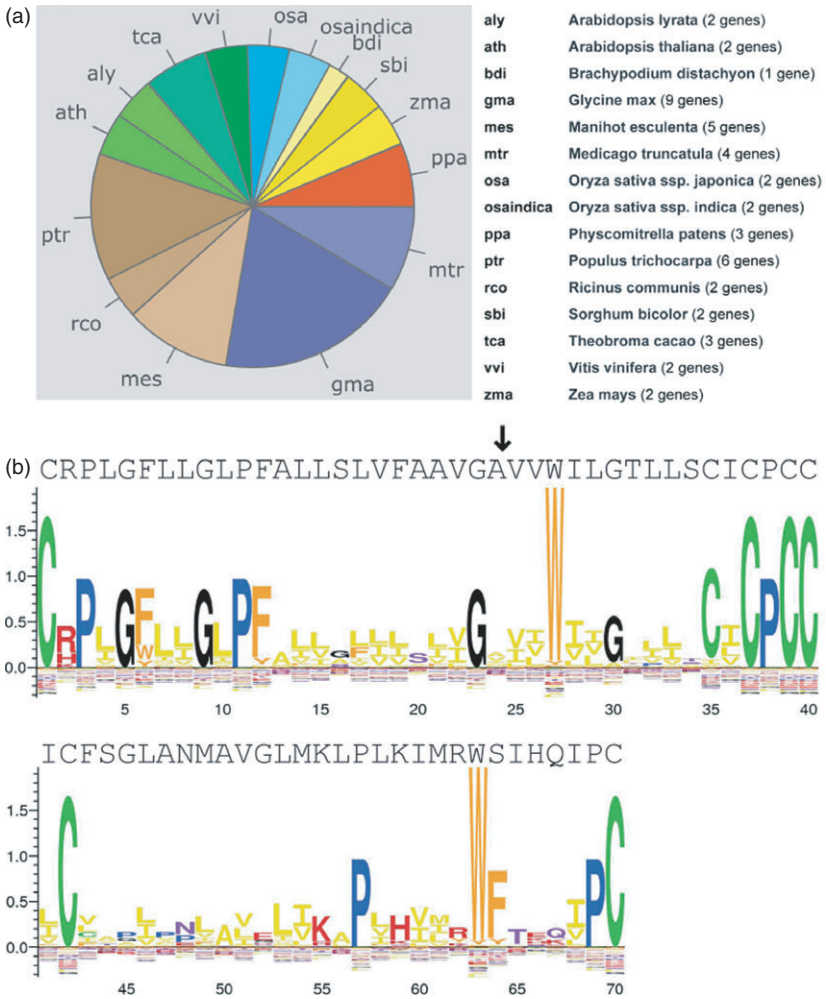

Figure 3 *Taximin* encodes a conserved small peptide. (a) Screenshot from PLAZA 2.5 showing the gene family HOM002731, to which *Taximin* belongs and which has 47 genes in 15 species. (b) Sequence logo of plant Taximin peptides. The first Taximin homologues from all plant species contained in PLAZA 2.5 complemented with those of *Picea sitchensis* and *Selaginella moellendorffii* were trimmed to equal size (from AA position 4 to 73 in Taximin) and aligned with ClustalW2 (see Figure S2). The generated multiple alignment was fed to Seq2Logo to generate a sequence logo visualizing AA conservation across the plant kingdom. The Taximin AA sequence is pasted on top of the logo in black. The end of the predicted N-terminal signal peptide of 27 AAs is marked by an arrow.

the protein mobility. Attached to a membrane, proteins are far less mobile than free cytosolic proteins, and hence, have lower diffusion and fluorescence recovery rates (Held *et al.*, 2008). Sixty seconds after photobleaching, a nearly complete fluorescence recovery was observed in cells expressing free Venus, whereas in cells expressing the Taximin–Venus fusion protein, hardly any fluorescence recovery was observed (Figure 4d), indicating a membranous nature of the Taximin–Venus fusion protein. Furthermore, *N. tabacum* Bright Yellow-2 (BY-2) cells and *M. truncatula* hairy roots stably expressing free Venus or Taximin–Venus were generated. In the BY-2 cells, the fluorescence signal of the fusion protein was predominantly localized in the plasma membrane; however, minor staining of the tonoplast was also observed (Figure 4e). In *M. truncatula* hairy roots stained with PI, the signals of the PI and the Taximin–Venus fusion protein overlapped, whereas the free Venus signal was separated from the PI signal (Figure 4f). Taken together, these data suggest the Taximin–Venus fusion protein to be localized at the plasma membrane.

Elicitation with a synthetic Taximin peptide has a transient effect on taxane synthesis

Because of the lack of efficient high-throughput transformation procedures for *Taxus* cell cultures, we investigated the role of Taximin in the regulation of *Taxus* metabolism by determining the effect of exogenous addition of Taximin peptide to *T. baccata* cells. We did not succeed in obtaining the 'wild-type' version of the Taximin peptide although, neither by chemical synthesis nor by recombinant DNA technology through heterologous expression of *Taximin* in *Escherichia coli*, *Pichia pastoris, Saccharomyces cerevisiae, N. tabacum* or *M. truncatula,* and either with or without the 27-AA signal peptide. We assumed that the extremely hydrophobic nature of the Taximin peptide was the cause. Therefore, we attempted, with success, to make a modified Taximin version, hereafter denominated HyproTaximin, in which the proline residues were substituted by hydroxyproline residues (see Experimental Procedures for details). Proline hydroxylation is a post-translational protein modification, which has been reported to occur frequently in nature with small signalling peptides (Matsubayashi, 2012).

The effect of adding HyproTaximin, alone or together with MeJA, on baccatin III and paclitaxel production in *T. baccata* cell suspensions was studied (Figure 5). In all culture conditions tested, taxane production peaked at 7 days of elicitation, dropping thereafter until day 14 to a level that was maintained till the end of the experiment (21 days). The presence of HyproTaximin in the culture increased baccatin III and paclitaxel levels 1.4- and 2.0-fold, respectively, when compared with the mock, after 7 days of culturing. As expected, MeJA addition enhanced baccatin III and paclitaxel production considerably, by 4.0- and 4.7-fold, respectively, compared with the mock-treated cells, which is in agreement with previous reports (Bentebibel

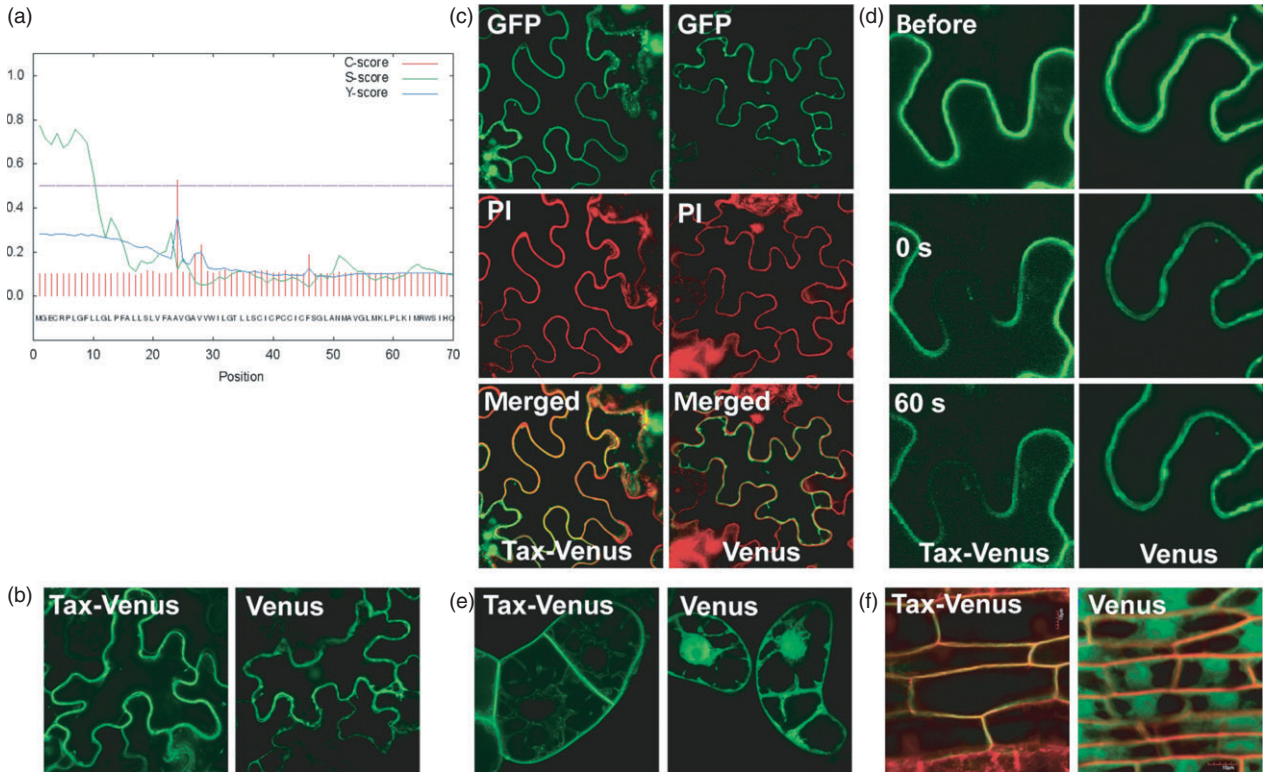

Figure 4 Taximin localizes to the plasma membrane. (a) Screenshot from Signal peptide 4.0, showing the prediction of the signal peptide of 27 AAs. (b-c) Confocal microscopy analysis of Taximin–Venus (Tax-Venus) and free Venus localization in *Nicotiana benthamiana* leaves (b) and *N. benthamiana* leaves stained with propidium iodide (PI) (c). (d) FRAP analysis in *N. benthamiana* leaves over 60 s. (e–f) Confocal microscopy analysis in BY-2 cells (e) and *Medicago truncatula* hairy roots stained with PI (f).

et al., 2005; Cusidó *et al.*, 2007; Onrubia *et al.*, 2010). The highest baccatin III and paclitaxel production levels, after 7 days of culturing, were achieved when MeJA was administered together with HyproTaximin, leading to compound levels that were 4.6- and 6.6-fold higher than in mock conditions. After day 7, however, no positive effect of HyproTaximin was observed anymore, independent of the presence of MeJA. These results suggest that HyproTaximin exerts a transient positive effect on taxane accumulation in *T. baccata* cells, which is additive with the effect of MeJA.

Taximin acts synergistically with MeJA to stimulate nicotine synthesis in tobacco hairy roots

Considering the conserved nature of the Taximin peptide, we also investigated its possible effect on the specialized metabolism of other plant species, more specifically by overexpression in transformed hairy root cultures, a widely used system for the production of valuable plant bioactive metabolites (Georgiev *et al.*, 2007, 2012). *Nicotiana tabacum* is an appropriate model plant for studying the functionality of foreign genes, particularly in hairy root cultures, which can be easily generated and produce high levels of alkaloids. Therefore, *N. tabacum* leaf discs were infected with *A. rhizogenes* LBA9402 carrying the plasmid pK7WG2D with the *Taximin* gene (TAX^{OE}) or GUS (Control) under the control of the CaMV 35S promoter. Hairy roots appeared after 2–4 weeks, and *Taximin* expression was verified by (q)RT-PCR (Figure S4). Based on this, three control and seven TAX^{OE} root lines were selected for further analysis. Some morphological traits, such as length of secondary roots or branching, appeared slightly altered in TAX^{OE} lines (Figure 6a), but overall, no substantial differences in growth capacity, measured as fresh and dry weight, were observed between control and TAX^{OE} root lines cultured in standard conditions (data not shown). Nicotine was by far the main alkaloid found in the generated tobacco hairy roots, followed by anatabine and then anabasine, whereas nornicotine was only detected in trace amounts. This, as well as the observed extent of variability in the alkaloid levels of individual lines carrying the same construct, was in line with previous reports (Häkkinen *et al.*, 2005; Palazón *et al.*, 1998). Furthermore, no statistically significant differences in alkaloid production were detected between control and TAX^{OE} lines, when cultured in standard (mock) conditions (Figure 6b and Table S1).

When elicited with MeJA, however, TAX^{OE} lines responded with a stronger increase in alkaloid production (Figure 6b and Table S1). The nicotine levels found in the elicited TAX^{OE} roots ranged from 4.5 to 9.7 mg/g of dry weight (DW), the overall average production being 6.3 mg/L, while levels in the elicited control roots ranged between 2.7 and 4 mg/g of DW, with an average of 3.5 mg/g of DW. Thus, the improvement in nicotine production in response to elicitation was significantly higher in the TAX^{OE} than in the control lines (3.4-fold versus 1.9-fold, on average). Similarly, prior to elicitation, the anabasine and anatabine contents showed no obvious differences between control and TAX^{OE} lines, but following MeJA elicitation, their production was enhanced more in the TAX^{OE} than in the control lines (2.8- and 3.6-fold versus 2.0- and 1.8-fold, respectively) (Figure 6b and Table S1).

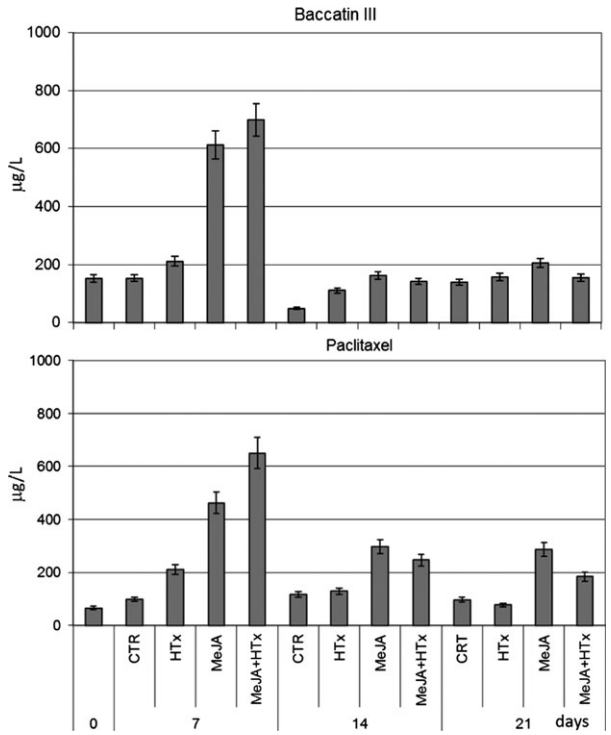

Figure 5 Elicitation of *Taxus baccata* cell cultures with the HyproTaximin peptide. Paclitaxel and baccatin III contents (expressed as µg/L) in *T. baccata* suspension cells mock-treated (CTR) or elicited with 10 µM HyproTaximin (HTx), 100 µM MeJA or both during 21 days of culture. Error bars indicate the SEM (n = 3).

Methyl jasmonate elicitation also modulates biomass production and morphological traits of tobacco hairy roots (Figure 6a). For instance, after 8 days of culturing, MeJA elicitation reduced fresh and dry weight on average by 70% and 75%, respectively, compared with mock-grown hairy roots (Table 1). Three of the TAX^{OE} lines (7, 9 and 11) also turned out to be hypersensitive to this MeJA effect, as fresh weight was decreased by ca 55% in these lines after MeJA elicitation (Table 1).

Expression analysis by qRT-PCR indicated that the synergistic effect between *Taximin* overexpression and MeJA elicitation on alkaloid production was not caused by an increased expression of alkaloid synthesis genes (Figure S4). Transcript levels of *PMT* and *QPRT*, corresponding to committed enzymes in the pyrrolinium and pyridine branches of tobacco alkaloid biosynthesis, respectively, clearly increased by MeJA elicitation in all lines, but without any significant differences between control and TAX^{OE} lines (Figure S4). Similarly, there was no correlation between the *PMT*/*QPRT* transcript levels and alkaloid levels in the elicited TAX^{OE} lines, suggesting that Taximin does not act on a transcriptional level in the regulation of specialized metabolism.

Taximin overexpression has only a minor effect on triterpene saponin accumulation in *M. truncatula* hairy roots

Another convenient plant model system for the study of the regulation of specialized metabolism is *M. truncatula*, a legume species from which fast-growing hairy root cultures producing triterpene saponins and flavonoids can be generated easily (Georgiev *et al.*, 2007, 2012; Gholami *et al.*, 2014; Guillon *et al.*, 2006a,b; Pollier *et al.*, 2011, 2013). Therefore, we made *M. truncatula* hairy roots stably overexpressing *Taximin* (TAX^{OE} roots) to further assess its role and utility. *Taximin* expression was confirmed by qRT-PCR (Figure S5), and untargeted metabolite profiling was carried out using liquid chromatography electron spray ionization Fourier transform ion cyclotron resonance mass spectrometry (LC-ESI FT-ICR MS) (Pollier and Goossens, 2013; Pollier *et al.*, 2011). Using this technique, compounds of medium polarity, including saponins and phenolic compounds such as flavonoids, can be profiled. Comparative analysis of the root extracts of control and TAX^{OE} lines yielded a total of 5129 *m/z* peaks. A Student's *t*-test with Welch correction was used to pinpoint significantly changed *m/z* peaks; however, only a single significantly differential peak, decreasing 2.6-fold and corresponding to a yet unknown compound, was observed (false discovery rate (FDR) $\leq$0.01), indicating that there was essentially no effect of *Taximin* overexpression on the metabolism of *M. truncatula* hairy roots cultured in standard conditions, similar to the transformed tobacco roots.

As *Taximin* acted synergistically with MeJA in tobacco roots, we also treated the *M. truncatula* root lines with MeJA prior to harvesting. Comparative analysis of the root extracts of MeJA-elicited control and TAX^{OE} roots yielded a total of 9429 *m/z* peaks, of which 56 were significantly changed (FDR $\leq$0.01). These 56 peaks were distributed over 10 peak groups, indicating that peaks corresponding to ten metabolites were significantly changed. One of the ten metabolites, of unknown identity, increased slightly (2.2-fold) in the TAX^{OE} roots, whereas the other nine all decreased (1.2- to 2.3-fold). Eight of the latter metabolites were low abundant, unknown compounds, whereas the 9th corresponded to 3-Glc-malonyl-28-Glc-medicagenic acid (Figure 7). Also without MeJA treatment, this compound seemed to be slightly lowered (1.7-fold) in TAX^{OE} roots, however, not significantly. Levels of other saponins, such as 3-Glc-28-Glc-medicagenic acid and soyasaponin I, the most abundant saponins in *M. truncatula* hairy roots (Pollier *et al.*, 2011), were not significantly altered. Hence, despite some minimal significant effects, there was no broad effect of *Taximin* overexpression in MeJA-treated *M. truncatula* hairy roots.

Discussion

Since the pioneering study of Jennewein *et al.* (2004), several transcriptome studies of (Me)JA-elicited *Taxus* cells or tissues have been undertaken (Croteau *et al.*, 2006; Cusidó *et al.*, 2014). Many of these have the potential to reveal the missing steps in the paclitaxel biosynthetic pathway, as well as to lead to the discovery of regulators of taxane synthesis in particular or *Taxus* metabolism in general. Here, we carried out a cDNA-AFLP-based transcript profiling of MeJA-elicited *T. baccata* suspension cells grown in a two-media culture system designed to promote paclitaxel accumulation (Palazón *et al.*, 2003). This analysis led to the identification of the *Taximin* gene, the expression of which is modulated coordinately with that of known paclitaxel synthesis genes and encoding a novel small cysteine-rich peptide (CRP) that is extremely conserved in the plant kingdom and with a hitherto unknown role. Before this study, no small peptides had been specifically identified from *Taxus* species yet. In one study although, the conserved 5-AA peptide PSK-α, a growth-promoting peptide hormone (Butenko

Figure 6 *Taximin* overexpressing *Nicotiana tabacum* hairy roots accumulate higher alkaloid levels. (a) Differences in morphological traits in *Taximin* overexpressing (TAX) and control (CTR) hairy root lines. (b) Alkaloid accumulation in different CTR and TAX lines after mock (EtOH) and MeJA treatment. Green, Nicotine; yellow, Anabasine; orange, Anatabine. Error bars indicate the SEM (n = 3–6).

et al., 2009; Matsubayashi and Sakagami, 2006), was found to act synergistically with MeJA in the elicitation of paclitaxel production in various *Taxus* cell suspension cultures (Kim *et al.*, 2006). This observation highlighted the potential of small peptide hormones for the biotechnological production of high-value plant molecules. In the same study, another well-known small plant signalling peptide, the Solanaceae-specific systemin with a reported role in defence and JA signalling (Sun *et al.*, 2011), was tested for its effects on *Taxus* metabolism, but was found ineffective.

Taximin corresponds to a peptide of 73 AAs, with a predicted N-terminal signal peptide of 27 AAs. Correspondingly, GFP-tagged Taximin proteins were found to enter the secretory system and accumulate in the plasma membrane. Analysis of the predicted mature Taximin peptide sequence of 46 AAs indicated a remarkably high level of sequence conservation across the entire higher plant kingdom, including angiosperms, gymnosperms, mosses and ferns, both in the identity of the AAs and the length of the peptide. Particularly striking were the high number of conserved cysteines (6), prolines (3) and hydrophobic AA residues (24). Taximin can be considered as a CRP, despite its high hydrophobicity (CRPs are usually cationic; Marshall *et al.*, 2011), as it has <160 AAs, a conserved N-terminal secretory signal peptide and between 4 and 16 C-terminal cysteines (Marshall *et al.*, 2011). CRPs are one of the most prominent small peptide classes found in plants, and several members are already known to be implicated in a wide range of cellular signalling processes, including plant development, reproduction, defence and plant–microbe symbiosis (Marshall *et al.*, 2011). In defence, CRPs rather tend to act as antimicrobial peptides or 'defensins', conferring a broad spectrum of resistance *in planta*, whereas in development and reproduction, CRPs play diverse roles, but usually act as peptide hormones perceived by receptors and trigger signalling cascades (Marshall *et al.*, 2011). In this study, we further assessed

Table 1 Fresh (FW) and Dry weight (DW) expressed in g of tobacco hairy roots at the end of the 8-day culture period. Values are the average ±SE (n = 4–6)

Root line	FW		DW	
	EtOH	MeJA	EtOH	MeJA
CTR1	1.377 ± 0.125	0.864 ± 0.142	0.095 ± 0.008	0.067 ± 0.003
CTR2	1.770 ± 0.113	1.194 ± 0.119	0.127 ± 0.005	0.099 ± 0.004
CTR11	1.733 ± 0.098	1.131 ± 0.168	0.125 ± 0.006	0.094 ± 0.006
TAX1	1.311 ± 0.121	0.932 ± 0.122	0.104 ± 0.005	0.077 ± 0.003
TAX6	1.514 ± 0.107	1.087 ± 0.137	0.109 ± 0.008	0.086 ± 0.007
TAX7	2.117 ± 0.135	0.944 ± 0.102	0.145 ± 0.015	0.076 ± 0.004
TAX9	1.939 ± 0.128	0.994 ± 0.089	0.141 ± 0.011	0.086 ± 0.005
TAX10	1.431 ± 0.099	1.095 ± 0.097	0.110 ± 0.008	0.083 ± 0.007
TAX11	1.818 ± 0.111	0.878 ± 0.120	0.130 ± 0.008	0.072 ± 0.004
TAX12	2.730 ± 0.147	1.754 ± 0.154	0.168 ± 0.010	0.123 ± 0.008

the possible functionality of Taximin, both in *Taxus* and in other plant species.

The hydrophobic nature of Taximin seriously hampered functional analysis. In our hands, it was impossible to produce the Taximin peptide for elicitation experiments, neither by chemical synthesis, nor by recombinant DNA technology in several microbial or plant cells. As proline hydroxylation is a post-translational protein modification that has been reported to occur frequently in small signalling peptides (Matsubayashi, 2012), we attempted, with success, to produce a modified, less hydrophobic and more cationic Taximin version, HyproTaximin, by substituting two of the prolines with hydroxyproline residues. When applied to *T. baccata* cells, HyproTaximin had a transient beneficial effect on taxane production, in particular of paclitaxel and baccatin III, but at the end of the 21-day culturing period, no significant differences in taxane levels with mock-treated cells were detected. No synergistic effects with MeJA elicitation were observed either. Further research will be required to determine whether and how the predicted 46-AA Taximin peptide is further processed *in planta*, and whether a more bioactive form of the peptide can be produced in the future. Indeed, many peptide hormones, including PSK-α and systemin, are first produced as larger 'propeptides' that undergo substantial proteolytical processing, besides cleavage of the N-terminal signal peptide, to a smaller, bioactive form (Butenko *et al.*, 2009; Matsubayashi, 2012; Matsubayashi and Sakagami, 2006; Sun *et al.*, 2011). Furthermore, many peptide hormones undergo further post-translational modifications, namely tyrosine sulphation, proline hydroxylation and hydroxyproline arabinosylation, which may modulate their functionality (Matsubayashi, 2012).

In an alternative approach to assess the role of Taximin in the regulation of plant metabolism, we overexpressed the *Taximin* gene in hairy root cultures of two well-known medicinal plant model systems, *N. tabacum* and *M. truncatula*. No pronounced effects of the heterologous expression of *Taximin* on the specialized metabolism in *M. truncatula* roots were observed, either in the absence or presence of MeJA. In contrast, a positive synergistic effect between *Taximin* overexpression and MeJA elicitation on alkaloid biosynthesis in tobacco hairy roots was detected, suggesting that Taximin indeed may have a broader role in the regulation of plant metabolism and that it may be a useful novel tool for the engineering of specialized metabolite production, at least in certain plant species. The range of plant species and metabolite classes on which Taximin may have an effect will be the scope for further research. Similarly, endogenous *Taximin* homologues may result more efficient or have other specificities because of particular sequence properties. For instance, *T. baccata* Taximin has a serine residue at position 67, whereas most Taximin homologues in the plant kingdom have a conserved hydrophobic phenylalanine or tyrosine residue at this position (Figure 3b).

The positive effect of *Taximin* overexpression on alkaloid accumulation in tobacco hairy roots was not accompanied by increased transcript levels of alkaloid biosynthesis genes, indicating that Taximin does not act as a transactivator of the genes involved in specialized metabolic pathways. This, together with the observed effects of *Taximin* overexpression on tobacco root morphology and growth, might suggest that Taximin is not a direct regulator of plant-specialized metabolism, in contrast to the phytohormone JA (De Geyter *et al.*, 2012; Pauwels *et al.*, 2009). More in-depth functional analysis will be conducted in the future to determine whether Taximin acts as a direct endogenous peptide elicitor of specialized metabolism, such as systemin and Pep (Yamaguchi and Huffaker, 2011), or rather as a development-regulating peptide hormone, such as many CRPs, with additional, indirect effects on plant metabolism. For that, model plants, such as *A. thaliana* and *M. truncatula* with established toolkits, profiling and transformation platforms, gain- and loss-of-function plant collections and a full view on the genome sequence and the number of endogenous *Taximin* homologues present, will likely be more effective. Similarly, mining of the genomes of such model species should on the long term also allow identifying the Taximin receptor and mapping the downstream signalling cascades. Ultimately, this may also allow establishing the utility of Taximin-like peptides in biotechnological programmes for the plant-based production of high-value metabolites, such as paclitaxel.

Experimental procedures

Generation, maintenance and elicitation of *T. baccata* cell suspension cultures

Taxus baccata cell suspension cultures were established from stem-derived calli, cultured and elicited for taxane production as described previously (Onrubia *et al.*, 2010). In brief, two different media were used to improve the productivity of the *T. baccata* cell suspension cultures. First a GM, consisting of Gamborg's B5 supplemented with 2× B5 vitamins, 0.5% sucrose, 0.5% fructose, 2 mg/L NAA, 0.1 mg/L BAP and 0.5 mg/L GA3, was used till sufficient biomass was generated, after which cells were transferred to the PM, consisting of Gamborg's B5 supplemented with 2× B5 vitamins, 3% sucrose, 0.1 mg/L of kinetin and 2 mg/L picloram.

Methyl jasmonate (dissolved in ethanol) (Sigma–Aldrich, St. Louis, MO) and HyproTaximin (dissolved in dimethyl sulphoxide) were added in the second stage, both separately and together, after filter-sterilizing (0.22-μm sterile PES filters; Millipore, Billerica, MA) to give final concentrations of 100 and 10 μM, respectively. The cell suspensions were immediately shortly sonicated in an ultrasonic (US) bath to facilitate the entrance of MeJA and HyproTaximin into the producer cells. The cell cultures were maintained in these conditions for 21 days, and three flasks were harvested from each treatment for analysis at different

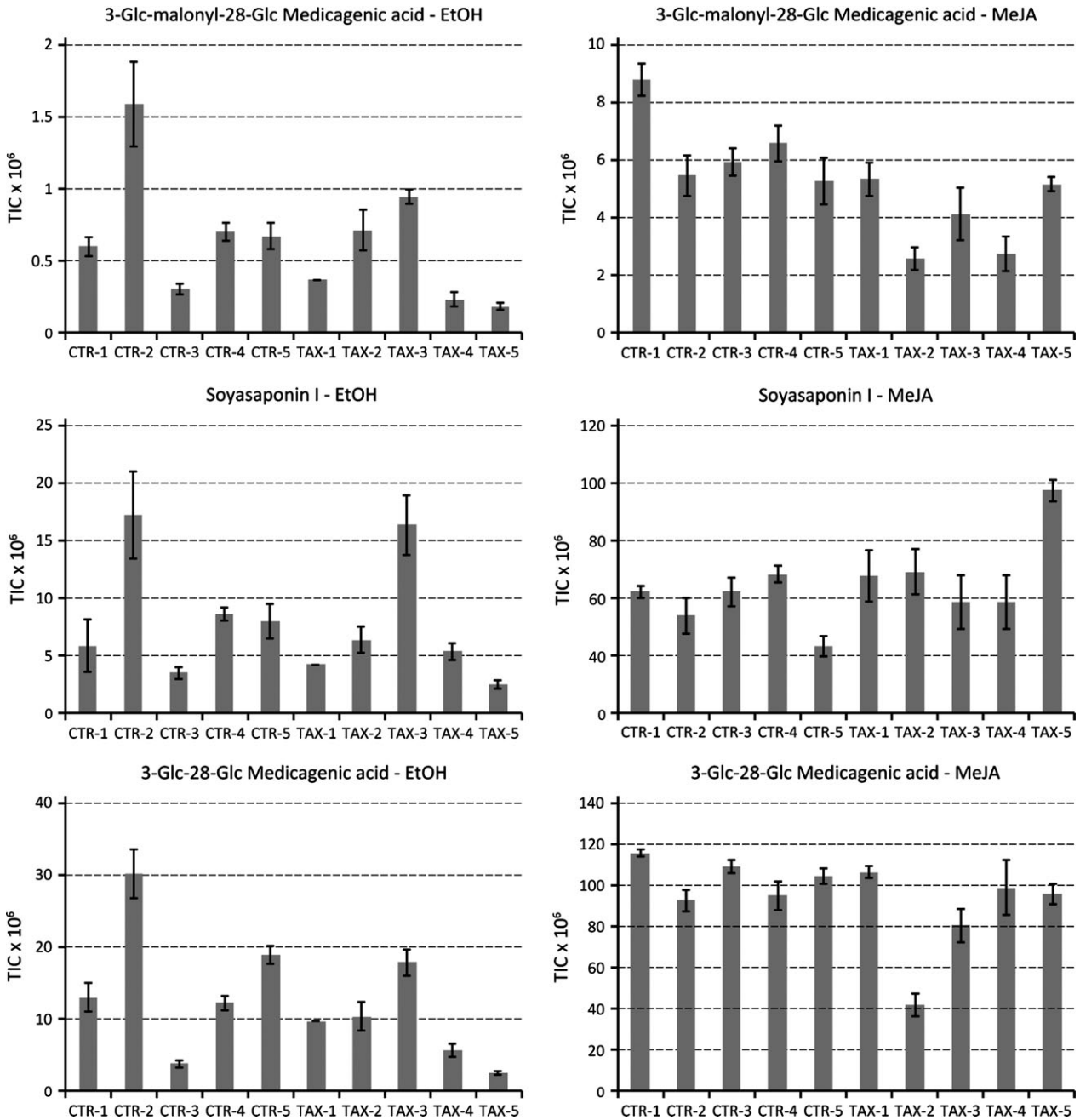

Figure 7 Metabolite profiling of *Medicago truncatula* hairy roots overexpressing *Taximin*. Average total ion current (TIC) of the peaks corresponding to 3-Glc-malonyl-28-Glc-medicagenic acid (top), soyasaponin I (middle) and 3-Glc-28-Glc-medicagenic acid (bottom) in control (CTR) and *Taximin* overexpressing (TAX) lines treated with ethanol (EtOH, left) or MeJA (right). Error bars indicate SEM (n = 5).

times after elicitation. For US exposure, the cultures were sonicated with low-frequency US (40 kHz) for 1 min in an ultrasonic bath (Branson B5200-4 Ultrasonic Bath, Branson Ultrasonics, L'Hospitalet de Llobregat, Spain).

Expression analysis

Total RNA from *T. baccata* cells and *N. tabacum* hairy roots was isolated with the RNeasy (Qiagen, Hilden, Germany) and ARNzol (REAL, Valencia, Spain) kits, respectively. cDNA-AFLP-based transcript profiling of *T. baccata* samples was performed as described (Rischer *et al.*, 2006; Vuylsteke *et al.*, 2007). For qRT-PCR, cDNA was prepared from total RNA with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). qRT-PCR was performed using the SYBR Green PCR Mastermix (Roche Applied Science, Mannheim, Germany) in a 384-well platform system (LightCycler® 480 Instrument; Roche Applied Science). Gene-specific primers (http://bioinfo.ut.ee/primer3-0.4.0/) were designed with Primer3 software version 0.4.0 (Table S2). Expression levels were normalized to those of the *18S* from *T. baccata* (Onrubia *et al.*, 2013) or the β-*ATPase* from *N. tabacum* (Häkkinen *et al.*, 2007).

Cloning of *T. baccata* sequences

The *Taximin* FL cDNA sequence was obtained by Rapid Amplification of cDNA Ends (RACE) (Gibco BRL-Invitrogen, Carlsbad).

The *Taximin* FL ORF was PCR-amplified as described (Underwood *et al.*, 2006), by Gateway™ recombination cloned into the entry vector pDONR221, sequence-verified and transferred to the pK7WG2D vector (Karimi *et al.*, 2002) for overexpression in hairy roots. For subcellular localization, the Venus yellow fluorescent protein (Nagai *et al.*, 2002) was fused to the C-terminus of the Taximin peptide via PCR amplification, and the resulting PCR product was Gateway™ recombined into the entry vector pDONR221 and from there in the expression vector pK7WG2 (Karimi *et al.*, 2002).

Generation, maintenance and elicitation of *N. tabacum* cell and hairy root cultures

BY-2 tobacco (*N. tabacum* L.) cell suspensions were maintained and transformed as described (De Sutter *et al.*, 2005). Transgenic hairy roots were obtained from *N. tabacum* cv Xanthi plantlets, maintained *in vitro* on MS medium, supplemented with 30 g/L sucrose and pH 5.7 with a photoperiod of 16-h light and 8-h darkness at 25 °C and subcultured monthly. Tobacco leaves were infected by wounding the midribs with a sterile needle inoculated with *Agrobacterium rhizogenes* LBA9402 strains carrying the pK7WG2D-Taximin vector. Hairy roots appeared 2–4 weeks after infection were excised and cultured individually on MS medium supplemented with 30 g/L sucrose and 500 mg/L cefotaxime to eliminate the bacteria and 100 mg/L kanamycin for transformant selection. The generated hairy root lines were kept in the dark at 25 °C and routinely subcultured every 3–4 weeks in half-strength Gamborg's B5 salts and full-strength Gamborg's vitamins (B5/2) solid medium. For the analysis, 2.5 g of roots was inoculated for 8 days in plates with B5/2 solid medium, supplemented with 100 μM MeJA or mock-treated with its solvent EtOH.

Generation, maintenance and elicitation of *M. truncatula* hairy root cultures

Generation of *M. truncatula* (ecotype Jemalong J5) hairy roots via *A. rhizogenes*-mediated transformation and maintenance of the hairy roots was carried out as described (Pollier *et al.*, 2011). Prior to elicitation, the hairy roots were grown for 21 days in liquid MS medium (pH 5.8) supplemented with vitamins and 1% (w/v) sucrose. Elicitation was performed by adding MeJA to a final concentration of 100 μM or an equivalent amount of EtOH as a mock. Five biological repeats of five independent transgenic hairy root lines were treated and harvested after 48 h.

Metabolite profiling

Taxanes were extracted, identified and quantified as described (Onrubia *et al.*, 2013).

Tobacco alkaloids were extracted as reported by Sheng *et al.* (2005), and the alkaloid contents of the samples were determined using a Flame Ionization Detector (FID) Gas Chromatography System (Hewlett-Packard HP5890 Series II Plus). The analyses were performed on an RTX-35 amine column (30 m, 0.25 mm i.d. × 0.5 μm d.f.) in split mode, with 1 mL/min of helium flux and a programmed oven temperature of 60–240 °C, increasing by 10 °C/min. Injector and detector temperatures were kept at 270 °C. The nicotine alkaloids were identified by comparing retention times with the standards: nicotine, nornicotine (LGC Promochem, Barcelona, Spain), anabasine (Chromadex, Irvine, CA) and anatabine (TransMIT, Marburg, Germany).

Medicago truncatula hairy roots were harvested and extracted as reported (Pollier *et al.*, 2011), and LC-ESI-FT-ICR-MS analysis was carried out as described (Pollier and Goossens, 2013). Chromatograms were integrated and aligned with the XCMS package (Smith *et al.*, 2006) in R version 2.6.1. using the following parameter values: xcmsSet (fwhm = 10, max = 300, snthresh = 2, mzdiff = 0.1), group (bw = 8, max = 300, mzwid = 0.1), rector (method = loess, family = symmetric). A second grouping was carried out with the same parameter values.

Localization in *N. benthamiana* leaves

The constructs for localization and a 35S:p19 construct to suppress gene silencing (Voinnet *et al.*, 2003) were individually transformed into the *Agrobacterium tumefaciens* strain C58C1, carrying the pMP90 helper plasmid. The resulting strains were used to infiltrate the abaxial side of fully expanded leaves of 3- to 4-week-old *N. benthamiana* plants grown at 25 °C under a 16/8-h light/dark regime (Boruc *et al.*, 2010). The infiltrated plants were incubated under normal growth conditions for 3 days prior to confocal microscopy analysis with an Olympus FluoView FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan), equipped with a 63x water-immersion objective (numerical aperture of 1.2).

Chemical synthesis of the Taximin peptide

The NH2.VVWILGTLLSCICP*CCICFSGLANMAVGLMKLP*LKIMRW SIHQIPC.OH peptide (P* indicates hydroxyproline) was synthesized using the Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry on a 433A peptide synthesizer (Applied Biosystems, Framingham, MA). The crude peptide was rinsed 5 times with 50% acetonitrile in water. The purity was estimated to be 30% (based on MALDI-MS analysis).

Acknowledgements

We thank Tessa Moses, Peter Marhavý and Sophie Carbonelle for technical advice and assistance. Miriam Onrubia is indebted to the European Union-Human Resources and Mobility for an Early Stage Training grant (MEST-CT-2004-514632) and the 'Agència de Gestió d'Ajuts Universitaris i de Recerca' (AGAUR) for a 'BEDGR2010' grant. Jacob Pollier is a postdoctoral fellow of the Research Foundation Flanders (FWO). This research has been supported by grants from the Spanish MEC (BIO2008-01210; PCI2006-A7-0535; BIO2011-29856-C02-01) and the Catalan Government (2009SGR1217).

Conflicts of interest

No conflicts of interest are to be declared.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 The FL cDNA sequence of *Taximin*.
Figure S2 Comparison of the AA sequence of Taximin with those of the first homologues of 16 other plant species.
Figure S3 Expression of *Taximin* homologues from model plants.
Figure S4 PCR analysis of transgenic *Nicotiana tabacum* hairy roots.
Figure S5 qRT-PCR analysis of transgenic *Medicago truncatula* hairy roots.
Table S1 Average alkaloid accumulation in control (CTR) and *Taximin* overexpressing (TAX) *N. tabacum* hairy root lines after mock (EtOH) and MeJA treatment.
Table S2 Primers used for qRT-PCR.

