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CaaX-prenyltransferases are essential for expression of genes involved in the early stages of monoterpenoid biosynthetic pathway in *Catharanthus roseus* cells

Vincent Courdavault[1], Martine Thiersault[1], Martine Courtois[2], Pascal Gantet[1,3], Audrey Oudin[1], Pierre Doireau[1], Benoit St-Pierre[1] and Nathalie Giglioli-Guivarc'h[1,*]
[1]*Université François-Rabelais de Tours, EA 2106 «Biomolécules et Biotechnologies Végétales», Labaratoire de Physiologie Végétale, UFR Science et Techniques, Parc de Grandmont, 37200 Tours, France; (*author for correspondence; e-mail: nathalie.guivarch@univ-tours.fr);* [2]*Université François-Rabelais de Tours, EA 2106 «Biomolécules et Biotechnologies Végétales», Laberatoire de Biologie Moléculaire et Biochimie Végétale, UFR Science Pharmaceutiques, 31 avenue Monge, 37200 Tours, France;* [3]*Present address: Université Montpellier II «Equipe analyse fonctionelle du génome du Riz», C.C. 002, 34095 Montpellier Cedex 5, France*

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Abstract

CaaX-prenyltransferases (CaaX-PTases) catalyse the covalent attachment of isoprenyl groups to conserved cysteine residues located at the C-terminal CaaX motif of a protein substrate. This post-translational modification is required for the function and/or subcellular localization of some transcription factors and components of signal transduction and membrane trafficking machinery. CaaX-PTases, including protein farnesyltransferase (PFT) and type-I protein geranylgeranyltransferase (PGGT-I), are heterodimeric enzymes composed of a common α subunit and a specific β subunit. We have established RNA interference cell lines targeting the β subunits of PFT and PGGT-I, respectively, in the *Catharanthus roseus* C20D cell line, which synthesizes monoterpenoid indole alkaloids in response to auxin depletion from the culture medium. In both types of RNAi cell lines, expression of a subset of genes involved in the early stage of monoterpenoid biosynthetic pathway (ESMB genes), including the MEP pathway, is strongly decreased. The role of CaaX-PTases in ESMB gene regulation was confirmed by using the general prenyltransferase inhibitor *s*-perillyl alcohol (SP) and the specific PFT inhibitor Manumycin A on the wild type line. Furthermore, supplementation of SP inhibited cells with monoterpenoid intermediates downstream of the steps encoded by the ESMB genes restores monoterpenoid indole alkaloids biosynthesis. We conclude that protein targets for both PFT and PGGT-I are required for the expression of ESMB genes and monoterpenoid biosynthesis in *C. roseus*, this represents a non previously described role for protein prenyltransferase in plants.

Abbreviations: ESMB genes, early stages of monoterpenoid biosynthetic pathway genes; MIA, monoterpenoid indole alkaloids; MM, maintenance medium; PFT, protein farnesyltransferase; PGGT-I, type-I protein geranylgeranyltransferase; PM, production medium; PPT, protein- prenyltransferase

Introduction

In addition to the ubiquitous isoprenoid derivatives involved in essential metabolic processes, such as prenylated proteins, sterols or phytohormones, several plant species produce a specific range of isoprenoid derived secondary metabolites. These natural compounds are generally synthesized by plant cells in response to specific developmental and environmental signals (Yang *et al.*, 1997; Wink, 1999). Isoprenoids are one of the most functionally and structurally varied group of plant metabolites playing an important role in signal transduction, cell division, growth regulation and in the exchange of signals between plants and their environment or defence against pathogens (Haralampidis *et al.*, 2001; Kessler *et al.*, 2001). The common so-called active isoprene units, isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP) are the precursors of all the isoprenoids (Bach, 1995; Chappell, 2002). In higher plants, these two intermediates are synthesized via two independent pathways, the classical mevalonate (MVA) pathway and the 2-*C*-methyl-erythritol 4-phosphate (MEP) pathway. The plastidic MEP pathway is used for the biosynthesis of various plastidic isoprenoids, such as phytol group, carotenoids, abscisic acid, gibberellins and plastoquinone (Lichtenthaler *et al.*, 1997; Lichtenthaler *et al.*, 1999; Kasahara *et al.*, 2002) (Figure 1). In contrast, the cytosolic MVA pathway leads notably to sterols, sesquiterpenes and prenylated proteins.

In Madagascar periwinkle (*Catharanthus roseus*, *C. roseus*), isoprene units derived from the plastidic MEP pathway lead to the biosynthesis of monoterpene indole alkaloids (MIA) (Lichtenthaler *et al.*, 1997; Contin *et al.*, 1998; Chahed *et al.*, 2000; Veau *et al.*, 2000). MIA are formed by the condensation of tryptamine derived from the shikimate pathway and secologanin synthesized by the monoterpenoid–secoiridoid biosynthetic pathway (Figure 1). *C. roseus* produces a wide range of MIA, including monomers such as ajmalicine and serpentine, used in hypertension treatment, and the highly valuable dimers, vincristine and vinblastine, used as antitumoral agents (van Telligen *et al.*, 1992; Levêque *et al.*, 1996).

C. roseus cell cultures are a suitable system to investigate the regulation of MIA biosynthesis. The C20D cell line, which is maintained in the presence

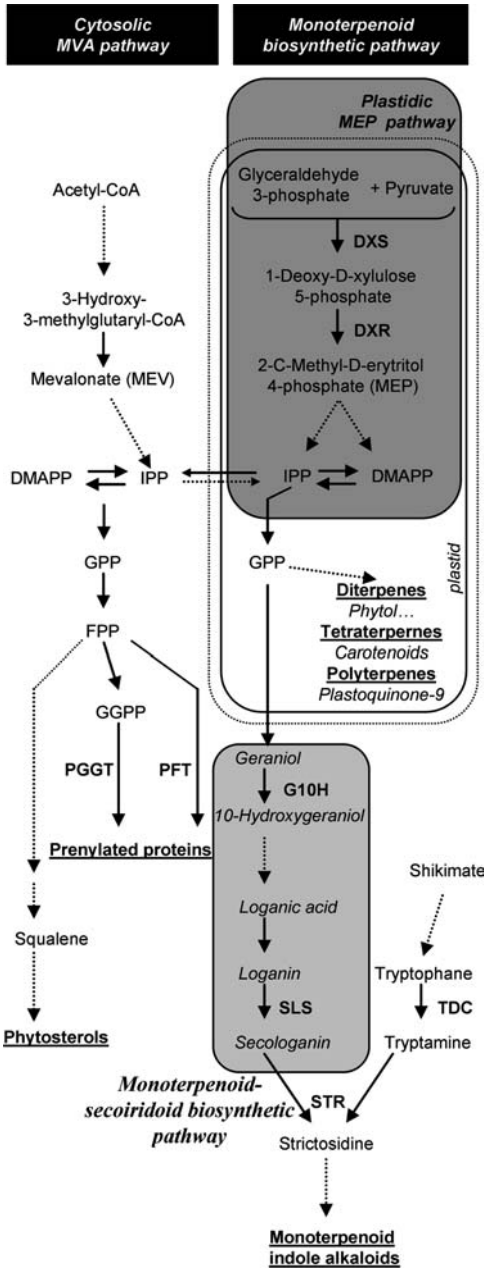

Figure 1. Biosynthesis of terpenoid compounds in *C. roseus*. Solid lines represent a single enzymatic conversion whereas dashed lines indicate multiple enzymatic conversions. Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; MEV, mevalonate; MEP, 2C-methyl-D-erythritol 4-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductase; PFT, farnesyltransferase; GGTase, type I or type II geranylgeranyltransferase; STR, strictosidine synthase; G10H, geraniol 10-hydroxylase; SLS, secologanin synthase; TDC, tryptophan decarboxylase.

of 2,4-dichlorophenoxyacetic acid (2,4-D) (maintenance medium, MM), produces the MIA ajmalicine when transferred in a 2,4-D-free medium (production medium, PM) (Mérillon *et al.*, 1986; Arvy *et al.*, 1994). This MIA biosynthesis is correlated with the expression of a specific set of MIA biosynthetic pathway genes including the first two genes of the MEP pathway, encoding 1-deoxy-D-xylulose 5-phosphate synthase (*Crdxs*) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*Crdxr*) (Veau *et al.*, 2000), and the gene encoding the first monoterpenoid–secoiridoid dedicated enzyme, geraniol 10-hydroxylase (*Crg10h*) (Papon *et al.*, in press) (Figure 1). These genes are involved in the early stages of the monoterpenoid biosynthetic pathway and are called ESMB genes.

Previous work designed to study the role of the MVA pathway in MIA biosynthesis showed, unexpectedly, that MIA biosynthesis in this cell line was completely inhibited when the MVA pathway was blocked with an inhibitor of HMG-CoA reductase (Imbault *et al.*, 1996). Since mevalonate is not a precursor for the biosynthesis of the isoprenoid moiety of MIA (Contin *et al.*, 1998), HMG-CoA reductase inhibitors might have an indirect effect on MIA biosynthesis. This class of inhibitors is also known to interfere with protein prenylation by depleting the endogenous pool of prenyl precursors (Repko and Maltese, 1989). These data lead us to investigate the potential involvement of protein prenylation events in regulation of MIA biosynthesis. In this paper, we used an RNA interference strategy to show that both CaaX-prenyltransferases (CaaX-PTases), including protein farnesyltransferase (PFT) and type-I protein geranylgeranyltransferase (PGGT-I), are required for the expression of ESMB genes.

Materials and methods

Chemicals

s-Perillyl alcohol, mevalonate and tryptamine were purchased from Sigma (L'Isle d'Abeau, France). Manumycine A GGTI 286 and GGTI 298 (Calbiochem, VWR International SAS, Parc tertiaire de la Meineau, Strasbourg, France) were dissolved in DMSO. Chaetomellic acid A was obtained from Calbiochem and dissolved in H_2O. Loganin and loganic acid were purchased from Extrasynthese (Genay, France). Secologanin was extracted and purified from young leafy shoots of *Lonicera xylosteum* L. as described by Naudascher *et al.* (1989). 1-deoxy-D-xylulose was synthesized in 3 steps according to Giner (1998) with an overall yield of 26% and dissolved in aqueous solution before addition to cell suspensions.

Cell growth and culture conditions

All experiments were conducted using the C20D *Catharanthus roseus* (L) G. Don (Apocynaceae) suspension cell line, which is 2,4-D-dependent and cytokinin-independent. Cells were grown in 50 ml Gamborg B5 medium (Gamborg *et al.*, 1968) containing 58 mM sucrose and 4.5 μM 2,4-D (maintenance medium, MM) or without 2,4-D (production medium, PM) as previously described (Imbault *et al.*, 1996). Cells were deep-frozen, freeze-dried and weighed, to determine dry weight, or deep-frozen in liquid nitrogen, for total RNA preparation.

Cell suspension treatments and ajmalicine determination

Addition of *s*-perillyl alcohol, dissolved in 50% ethanol, or Manumycine A to C20D cell suspensions was performed 4 days after subculture, unless other wise indicated. For supplementation of secondary metabolite precursors, 4-day old cells were pre-treated, or mock-treated, with 1 mM *s*-perillyl alcohol or 20 μM Manumycine A for 8 h before addition of the precursors. Ajmalicine content was measured on 7-day or 10-day old cells. Aliquots of 60 mg freeze-dried cells were used for alkaloid quantification according to Mérillon *et al.* (1986).

DNA and RNA extraction

Total genomic DNA was isolated from 0.15 g of freeze-dried C20D cells according to the protocol of Dellaporta (Dellaporta *et al.*, 1983). Total RNA was extracted from 3-day and 5-day old *C. roseus* cell suspensions using RNeasy Plant Minikit extraction (Qiagen, Courtaboeuf, France).

RNA gel blot hybridisation analysis

For RNA gel blot hybridisation, 20 μg of total RNA were fractionated by electrophoresis in a 1.2% (w/v) agarose gel containing 2.2 M formaldehyde according to Sambrook *et al.* (1989) and blotted onto a Nylon N+ membrane (Qbiogene, Illkirch, France). For probe preparation, full length or partial cDNA was [^{32}P]-labelled with the 'Prime-a-gene' labelling kit (Promega). Hybridisations were carried out for 15 h at 60 °C in Church buffer (0.5 M $NaPO_4$ pH 7.2, 7% (w/v) SDS, 1 mM EDTA, 1% (w/v) BSA). Washes were performed at 55°C for 45 min in 4 × SSC (1 × SSC is 0.15 M NaCl, 1.5 mM NaOAc) and 0.1% (w/v) SDS, for 45 min in 1 × SSC and 0.1% (w/v) SDS and for 30 min in 0.2 × SSC and 0.1% (w/v) SDS. Membranes were exposed to X-ray film (Fuji Xray RX) for 3 to 5 days, using amplifying screen at −80 °C.

RNA interference experiments

For RNAi experiments, a 391-bp *Crftb* partial sequence (nt 330–721) and a 348-bp *Crggt_Ib* partial sequence (nt 236–584) were amplified using FTB400-for primer (5′-CCTCTAGAATTCTCGAGTCCTTTACGGTTCTGGATGCT-3′) with FTB400-rev primer (5′-CCAAGCTTG GTACCCAGTTTGTCGTCCA ATATATTGAGA AC-3′) and GGT350-for primer (5′-CCTCT AGAATTCAGGCGCCCTCGATCGCATT-3′) with GGT350-rev primer (5′-CCAAGCTTGGTACC ACCGAACATGAAGCAGATGGC-3′), respectively. Forward primers (for) contained *Xba*I and *Eco*RI restriction sites and reverse primers (rev) contained *Hin*dIII and *Kpn*I restriction sites. The two amplified sequences were first cloned into pGEM-T vector (Promega) and then subcloned into pKANNIBAL (pKAN) vector (Wesley *et al.*, 2001) in both sense and antisense orientation between the *Eco*RI/*Kpn*I and the *Hin*dIII/*Xba*I sites, respectively, to create pKAN-ftb and pKAN-ggt_Ib. Transformation of the *C. roseus* C20D cell line was performed as described by Papon *et al.* (2004). The presence of interfering constructs was checked by PCR amplification performed on genomic DNA using a sense primer (35S) specific for CaMV 35S promoter (5′-TATCCTTCGCAA GACCCTTCC-3′) and the antisense FTB400-rev primer, for *Crftb* RNAi cell lines, the 35S and the antisens GGT350-rev primer, for *Crggt_Ib* RNAi cell lines and the 35S and an antisens primer (OCS) specific for the beginning of the *ocs* terminator (5′-AAATATCATGCGATCATAGGC-3′) for empty vector transformed cells. For analysis of *Crftb* and *Crggt_Ib* transcripts in interfered cell lines, regions of the cDNA sequences that do not overlap with the sequence used for the interfering process, were used as probes. These partial *Crftb* and *Crggt_Ib* probes were amplified by PCR using FTBB primer (5′-TGGTTGACGGGTGCTATTCC-3′) associated with F32 primer (5′-AAGTCCTTTGCCCT TTTAC-3′) and 3′GGT-for2 primer (5′-CATCTG CTTCATGTTCGGT-3′) associated with 3′UTR-GGTB primer (5′-CCGGATCCAGTTACCA-CAGCCAAAGCTTG-3′), respectively.

Protein expression in Escherichia coli

Recombinant target proteins for PFT (glutathione (GST)-CAIM) and PGGT-I (GST-CIIL) were obtained as previously described (Courdavault *et al.*, 2005). PhCaM53, the recombinant target protein of PGGT-I used for characterisation of PGGT-I activity in RNAi cell lines, was produced and partially purified according to Rodriguez-Concepcion *et al.* (1999) and Caldelari *et al.* (2001). PhCaM53 was dialysed against the PhCaM53 reaction buffer 50 mM Hepes-KOH pH 7.8, 250 mM mannitol, 5 μM $ZnCl_2$, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail (Complete EDTA-free, Roche, France), and concentrated using omega microsep 3K (Pall Gelman Sciences, Ann Arbor, USA).

Protein prenylation assay

For characterisation of PFT and PGGT-I activities in the C20D cell line and PFT activity in RNAi cell lines, protein prenylation assays were performed as previously described (Courdavault *et al.*, 2005) using 400 μg of total soluble proteins isolated from 1 g of *C. roseus* cells and 0.3 μM of GST-CAIM (for PFT activity) or GST-CIIL (for PGGT-I activity). For characterisation of PGGT-I activity in RNAi cell lines, reactions were conducted similarly except using the PhCaM53 reaction buffer and 0.2 μM of PhCaM53. At the end of the reactions, loading buffer (50 mM Tris–HCl, pH 6.8, 1% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue) was added, and 50 μg of total proteins

were separated on a 12% SDS-PAGE. Gels were stained with Coomassie blue and incubated in Amplify (Amersham) for 30 min before being subjected to fluorography for 1 week at −80 °C.

Relative quantification of CaaX-PTase activities and MIA biosynthetic gene expression

Relative quantification of CaaX-PTase activities and MIA biosynthetic gene expression was performed by gel autoradiography analysis using the Scion Image Beta 4.02 software analysis (Scion Corporation). The maximal activity of each CaaX-PTase was defined as 1.

Results

CaaX-PTase subunit genes are expressed early in cell culture cycle

Three cDNA encoding the *C. roseus* β subunit of PFT (*Crftb*, Gene bank accession number AF 389849), β subunit of PGGT-I (*Crggt_Ib*, Gene bank accession number AY 214343) (Courdavault *et al*., 2005) and α subunit of CaaX-PTases (*Crfta/ggt_Ia*, Gene bank accession number AF525030) have been cloned. The expression pattern of the corresponding genes was analysed by RNA blot hybridisation during the cell culture cycle of the C20D cell line. In presence of 2,4-D (maintenance medium, MM), RNA transcripts of *Crggt_Ib*, *Crftb* and *Crfta/ggt_Ia* were constitutively present during the cell culture cycle (Figure 2A). For *Crftb* transcripts, two bands were detected (Figure 2A); the larger one probably corresponds to partially spliced transcripts (Courdavault *et al*., 2005). *Crfta/ggt_Ia* and *Crftb* present a similar pattern of expression with increased transcript accumulation notably during the first three days. This corresponds to the phase of active cell division. The more abundant *Crggt_Ib* transcripts showed a prolonged accumulation profile. Depletion of 2,4-D from the cell culture medium (production medium, PM) is known to activate the expression of MEP pathway genes (Chahed *et al*., 2000; Veau *et al*., 2000) and *Crg10h* (Papon *et al*., in press) prior to MIA biosynthesis in C20D cell line. However, depletion of 2,4-D from the cell culture medium did not modify the expression pattern of *Crftb*, *Crggt_Ib* and *Crfta/ggt_Ia*, although, the levels of transcripts appear to be lower (Figure 2B). The expression of genes

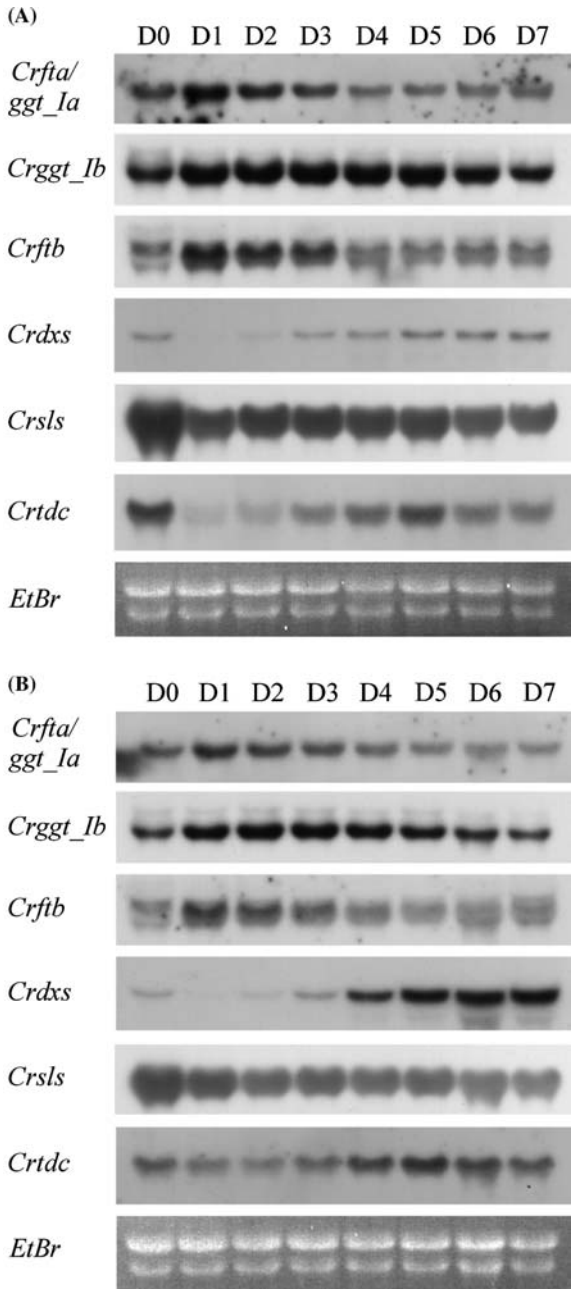

Figure 2. RNA gel blot analysis of *Crfta/ggt_Ia*, *Crggt_Ib*, *Crftb*, *Crdxs*, *Crsls* and *Crtdc* transcript levels. Total RNA was extracted from *C. roseus* C20D cell line at various days after subculture and subjected to Northern blot analysis using *Crfta/ggt_Ia*, *Crggt_Ib*, *Crftb*, *Crdxs*, *Crsls* and *Crtdc* cDNA probes. Equal loading of RNA samples was confirmed by ethidium bromide (EtBr) staining. Cells were cultured either in MM (A) or in PM (B). This experiment is representative of three independent northern blot analyses.

encoding the CaaX-PTase subunits was also compared with that of MIA biosynthetic pathway genes: *Crdxs* that reflects expression of other ESMB genes (Veau *et al.*, 2000), tryptophan decarboxylase (*Crtdc*), and secologanin synthase (*Crsls*). In the presence of 2,4-D, *Crdxs* was weakly expressed during the cell culture cycle except for days 1 and 2 where transcripts were barely detectable. *Crtdc* showed a biphasic expression pattern with maxima at day 0 and day 5 (Figure 2A). *Crsls* is constitutively expressed throughout the cell culture cycle except for a transient increase on day 0. 2,4-D depletion from the cell culture medium did not affect the expression pattern of these genes, but induced an increase of the level of *Crdxs* transcripts, most notably during the last 4 days of the cell culture (Figure 2B), as previously described (Veau *et al.*, 2000). Thus, *Crftb*, *Crggt_Ib* and *Crfta*/*ggt_Ia* were expressed early during the cell culture cycle and at least one day before ESMB genes.

CaaX-PTase activities reach maxima in the late log phase

Measurements of PFT and PGGT-I activities were performed by *in vitro* prenylation assays using specific recombinant protein substrates, glutathione (GST)-CAIM and GST-CIIL, respectively. In MM, PFT activity was present at each day of the cell culture cycle but was most abundant on days 4 and 5 (Figure 3A). As observed for PFT transcript accumulation (Figure 2), depletion of 2,4-D from the cell culture medium (PM) did not modify the pattern of PFT activity. In MM, PGGT-I was also active during the entire cell culture cycle, but activity was highest from day 3 to day 7 (Figure 3B). In contrast, depletion of 2,4-D (PM) led to a decrease of PGGT-I activity from day 3 to the end of the culture cycle (Figure 3B). Therefore in PM, both PFT and PGGT-I presented a peak of activity at days 4–5 which corresponds to the beginning of ESMB gene expression and MIA biosynthesis.

Specific silencing of target protein prenyltransferase β-subunit gene in Crftb and Crggt_Ib RNAi cell lines

A gene silencing approach was developed to investigate a potential role for CaaX-PTases in MIA biosynthesis. Genomic DNA hybridisation analysis indicated that *Crftb* and *Crggt_Ib* (Courdavault *et al.*, 2005) were encoded by a single copy gene per haploid genome as in other plant species (Yang *et al.*, 1993). Thus, a gene silencing strategy should have a gene-specific effect. However, in order to avoid cross-hybridi-

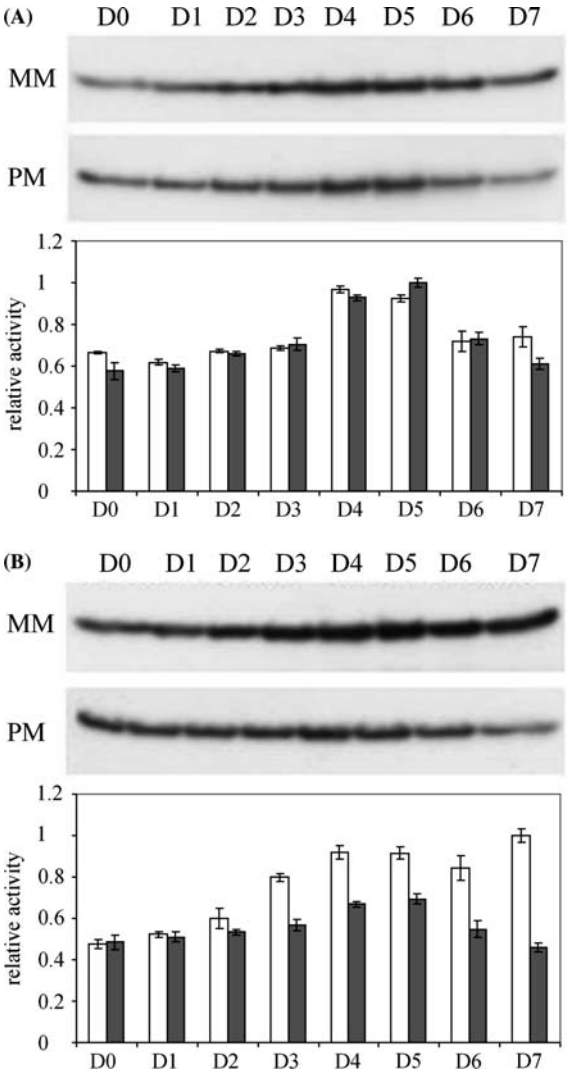

Figure 3. CaaX-PTases activities in C20D cell line. Total soluble proteins were isolated from the C20D cell line at each day of cell culture cycle and assayed for CaaX-PTase activities. PFT (A) and PGGT-I (B) activities, in maintenance medium (MM) and in production medium (PM) grown cells, were assayed by *in vitro* prenylation of GST-CAIM and GST-CIIL target protein, respectively, with appropriate [^{3}H] prenyl donor. After incubation, the reaction mixtures were separated on a 12% SDS-PAGE and subjected to autoradiography. Histograms showing the relative activity of PFT (A) and PGGT-I (B) are the mean of three replications whereas each autoradiogram shown in (A) and (B) was produced from one representative experiment. White and grey bars represent CaaX-PTase activities in MM and PM respectively.

sation between *Crftb* RNAi and *Crggt_Ib* transcripts, and *vice versa*, regions of low identity were cloned into the dsRNA generating pKANNIBAL (pKAN) vector for the subsequent generation of RNAi suspension cell lines. Any 21-bp subfragment of the selected region will have less than 70% identity with the paralogous gene transcript. For transformation, C20D cells were co-bombarded with one of the three different constructs: pKAN, pKAN-ftb or pKAN-ggt_Ib, and with the pGL2 vector carrying a hygromycin resistance gene. Transformed cell lines were first selected for hygromycin resistance on solid media and cell suspensions were subsequently selected for apparently normal growth characteristics in liquid maintenance media. The presence of the interfering construct was confirmed by PCR amplification in all selected transgenic cell lines (Figure 4B, D and F), except in lines G2 and G18 (Figure 4B). All transformed cell lines showed a normal level of *Crtdc* transcripts (Figure 4A, C and E). Furthermore, these cell lines were analysed for *Crftb* and *Crggt_Ib* expression. Compared with the untransformed C20D cell line, a large decrease in *Crggt_Ib* transcript levels was observed in the *Crggt_Ib* RNAi cell lines G4, G5, G13, G23, G24, and G27 (Figure 4A and E). In addition, a slight decrease in *Crggt-Ib* transcripts was also observed in less silenced lines (G16 and G31). In most cases, the level of *Crftb* transcripts did not change, except for some expected inter line variations (Figure 4A). As expected, *Crftb* RNAi had the opposite effect. The level of *Crftb* transcripts was drastically lower in most *Crftb* RNAi cell lines except in lines F4, F31 and F34. Levels of *Crggt_Ib* mRNA remained normal in most cell lines except for a slightly lower level in lines F26, F28 and F35, which also could result from inter line variations (Figure 4C). In contrast, in empty vector transformed cell lines, the level of *Crftb, Crggt_Ib* and *Crtdc* transcripts was not affected (Figure 4E). These results suggest potentially that no cross-hybridisation occurs between RNAi of both CaaX-PTase β subunits. Six *Crftb* RNAi and six *Crggt_Ib* RNAi cell lines were selected for further characterization.

Down regulation of target CaaX-PTase activities in Crftb and Crggt_Ib RNAi cell lines

To measure residual CaaX-PTase activities in RNAi cell lines, *in vitro* prenylation assays for

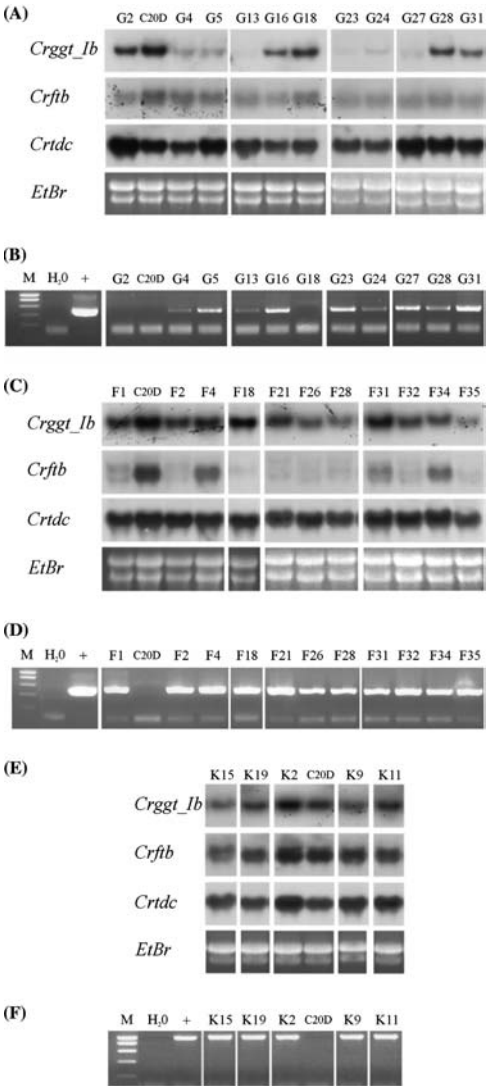

Figure 4. Characterization of *Crggt_Ib* RNAi, *Crftb* RNAi and empty vector transformed cell lines. *Crggt_Ib* RNAi, *Crftb* RNAi and empty vector-transformed cell lines were labelled with alphanumerical code starting with G, F or K, respectively. (A, C and E) Total RNA was extracted from 3-day old *Crggt_Ib* RNAi (A), *Crftb* RNAi (C) and empty vector transformed cell lines (E) cultured in MM, and subjected to northern blot analysis using *Crggt_Ib, Crftb*, and *Crtdc* cDNA probes. Equal loading of RNA samples in each lane was confirmed by ethidium bromide (EtBr) staining. (B, D and F) Presence of the interfering constructs was confirmed by PCR performed on DNA extracted from *Crggt_Ib* RNAi (B), *Crftb* RNAi (D), empty vector transformed and C20D (F) cell line. (H_2O) represents the water control with no template, (+) represents the PCR control with corresponding plasmid pKAN-ggt_Ib (B), pKAN-ftb (D) and pKAN (F) as template. The upper band correspond to the amplification product and the lower band to primer dimers. (M) DNA molecular weight standards.

PFT activity were carried out using GST-CAIM, and for PGGT-I activity using the *Petunia hybrida* Calmodulin53 (PhCaM53), which has been described to be a more affine protein substrate (Rodriguez-Concepcion *et al*., 1999, Caldelari *et al*., 2001). PFT and PGGT-I activities were assayed in soluble protein extracts prepared from C20D cells and transformed cell lines. The PFT substrate GST-CAIM was farnesylated by protein extracts prepared from C20D cells, empty vector cell lines and *Crggt_Ib* RNAi cell lines but only to a minor degree by protein extracts prepared from *Crftb* RNAi cell lines (Figure 5A and B). In contrast, the PGGT-I substrate PhCaM53 was geranylgeranylated by protein extracts prepared from C20D cells, empty vector cell lines and *Crftb* RNAi cell lines but to a lesser extend by protein extracts prepared from *Crggt_Ib* RNAi cell lines (Figure 5A and B). Therefore, in most RNAi cell lines, decreased levels of CaaX-PTases β-subunit transcript lead to a large inhibition of the corresponding CaaX-PTase activities. In some RNAi cell lines, for examples F2 and G27, the level of enzyme activity at day 5 (Figure 5) appears to be higher than expected based on the level of transcript at day 3 (Figure 4). It is possible that in the early phase of cell culture before day 3, the level of transcript in line F2 and G27 had been slightly higher. In C20D cell line and *in planta*, we indeed observed a time shift between the peak of transcript and enzyme activity accumulation. In the C20D cell line, the peak of PFT and PGGT-I activities were delayed compared with the peaks of CaaX-PTase transcript accumulation (Figures 2A and 3). *In planta*, the level of PFT and PGGT-I activities in mature leaf is higher than expected based on the residual level of transcript, which declined rapidly following leaf maturation (Courdavault *et al*., 2005).

Nevertheless, these results suggest that most *Crftb* RNAi and *Crggt_Ib* RNAi cell lines were efficiently silenced for the targeted CaaX-PTase activity without affecting the activity of the other CaaX-PTase.

Effect of reduced CaaX-PTase activities on MIA biosynthetic genes expression

To determine whether the down regulation of CaaX-PTase activities influences the expression of MIA biosynthetic pathway genes and MIA biosynthesis, the selected RNAi cell lines were cultured in PM for 5 and 7 days, respectively. Both empty vector transformed cell lines and the C20D cell line were used as controls. Two sets of genes were selected for northern blot analysis: the first one corresponded to ESMB genes including *Crdxs*, *Crdxr* and *Crg10h*. The second set of selected genes encodes enzymes involved in the

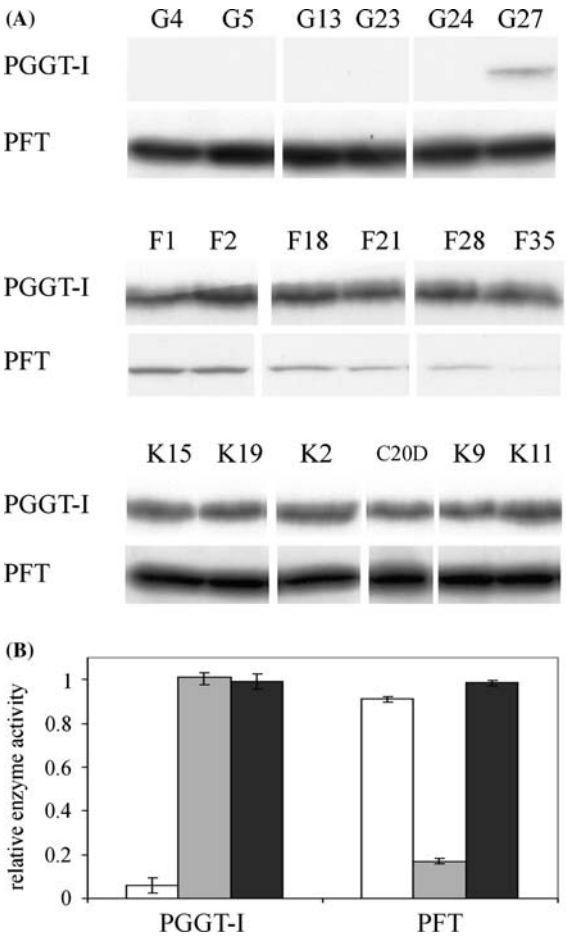

Figure 5. PGGT-I and PFT activities in selected *Crggt_Ib* RNAi, *Crftb* RNAi cell lines and empty vector transformed cell lines. (A) Total soluble proteins were isolated from 5-day old C20D or transformed cell lines cultured in PM. PGGT-I and PFT activities were assayed by *in vitro* prenylation of PhCaM53 and GST-CAIM target protein, respectively, with appropriate [^{3}H] prenyl donor. After incubation, the reaction mixtures were separated on a 12% SDS-PAGE and subjected to autoradiography. Autoradiograms shown are representative of two experiments. (B) Relative PGGT-I and PFT activities in selected *Crggt_Ib* RNAi (white bars), *Crftb* RNAi (light grey bars) and empty vector transformed cell lines (dark grey bars) compared with the C20D cell line (defined as 1 for each CaaX-PTase activity). For identification of cell line labels, see the legend of figure 4.

central stage of MIA biosynthesis including *Crsls*, *Crstr* and *Crtdc* (Figure 1).

In the C20D and empty vector transformed cell lines grown in PM, the transcripts of both sets of genes were present at a high level (Figure 6A). Moreover, in empty vector transformed cell lines, the level of MIA reached 80% of the MIA level in the C20D cell line and showed increased inter cell line variability (Figure 6D). However, no alteration in the level of expression of ESMB genes was observed in empty vector transformed cell lines. In cell lines silenced either for *Crftb* or *Crggt_Ib*, the expression of ESMB genes was decreased compared with empty vector transformed cell lines (Figure 6A), while the level of *Crsls*, *Crstr* and *Crtdc* transcripts was not reduced (Figure 6C).

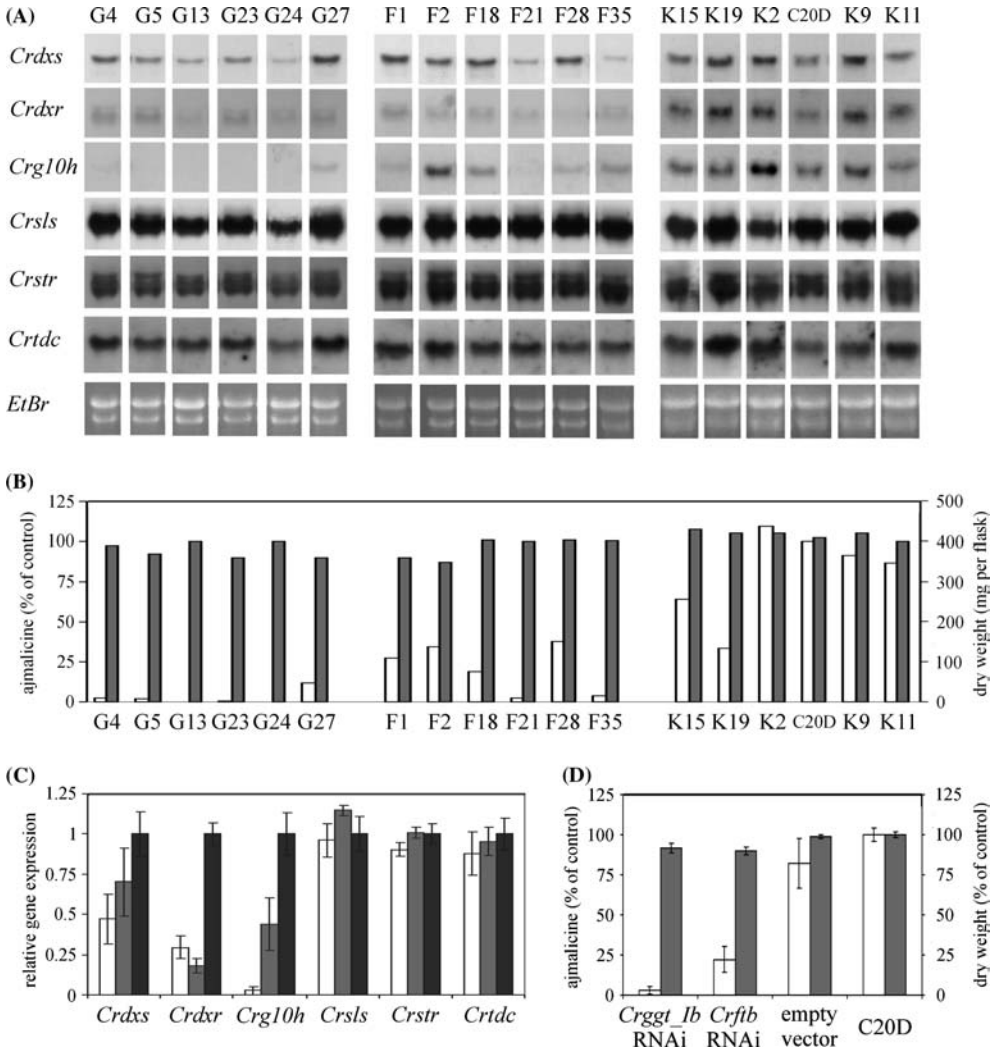

Figure 6. Expression of genes involved in MIA biosynthetic pathway and ajmalicine content in *Crggt_Ib* RNAi, *Crftb* RNAi and empty vector transformed cell lines. (A) RNA gel blot analysis of *Crdxs*, *Crdxr*, *Crg10h*, *Crsls*, *Crstr* and *Crtdc* transcript levels in *Crggt_Ib* RNAi, *Crftb* RNAi and empty vector transformed cells lines. Total RNA was prepared from 5-day old transformed cells lines cultured in PM. Equal loading of RNA samples in each lane was confirmed by ethidium bromide (EtBr) staining. (B) Ajmalicine content (white bars) and total dry weight (grey bars) per flask of 50 mL of cell suspension were estimated in 7-day old culture. (C) Relative expression of each MIA biosynthetic pathway gene in *Crggt_Ib* RNAi (white bars) and *Crftb* RNAi cell lines (light grey bars) compared with empty vector transformed cell lines (dark grey bars). Expression in empty vector cell lines is defined as 1. (D) Mean ajmalicine content (white bars) and dry weight (grey bars) in *Crggt_Ib* RNAi, *Crftb* RNAi and empty vector transformed cell lines shown in panel B compared with the C20D cell line (control). In this case, ajmalicine content and dry weight in C20D is the mean of 3 cultures cultivated independently whereas in (A) and (B), MIA biosynthetic gene expression, ajmalicine content and dry weight are obtained from one representative culture. For identification of cell line labels, see the legend of figure 4.

Indeed, expression of *Crdxr* and *Crg10h* was markedly inhibited when either PFT or PGGT-I activity are down regulated (Figure 6A and C). For *Crdxs*, silencing of either CaaX-PTase activity had a modest inhibitory effect, which may be unequivocal only in *Crggt_Ib* RNAi cell lines (Figure 6A and C). In addition, cell lines silenced either for *Crftb* or *Crggt_Ib* showed decrease in MIA biosynthesis relative to the empty vector transformed cell lines (Figure 6B and D) that is correlated with down-regulation of ESMB gene expression (Figure 6C). In most cases, residual MIA biosynthesis is linked to the level of ESMB gene expression.

These results suggest that both PFT and PGGT-I activities are required for ESMB gene expression and, thus, for proper monoterpenoid and MIA biosynthesis.

Protein prenyltransferase inhibitors block MIA biosynthesis

In the cell lines transformed with RNAi constructs driven by the CaMV 35S promoter, we cannot exclude the possibility that constitutive inhibition of CaaX-PTase activities may have pleiotropic effects, notably on cell division (Qian *et al*., 1996), which could indirectly interfere with ESMB gene expression. For example, the final dry weight of *Crftb* and *Crggt_Ib* RNAi cell lines was 10 to 20 % lower than the final dry weight of the C20D and empty vector transformed cell lines (Figure 6B). To confirm whether CaaX-PTases have a role in the regulation of MIA biosynthesis, we used an inhibitor-based strategy to block CaaX-PTase activities. The protein prenyltransferase inhibitor *s*-perillyl alcohol (SP) was tested (Crowell *et al*., 1994; Gelb *et al*., 1995). The cells were first treated at each day of the cell culture cycle. Treatment with 1 mM SP on any of the first three days after subculture drastically decreased cell growth (Figure 7A), apparently due to extensive cell death (data not shown). This may result from the requirement of both farnesylated and geranylgeranylated CaaX-proteins during the active growth phase, since silencing of either activity had milder effects (Figure 6). SP toxicity may also result from the inhibition of type-II protein geranylgeranyltransferase (PGGT-II) activity or ubiquinone synthesis (Ren and Gould, 1994; Ren *et al*., 1997). These side effects are unlikely to be induced in

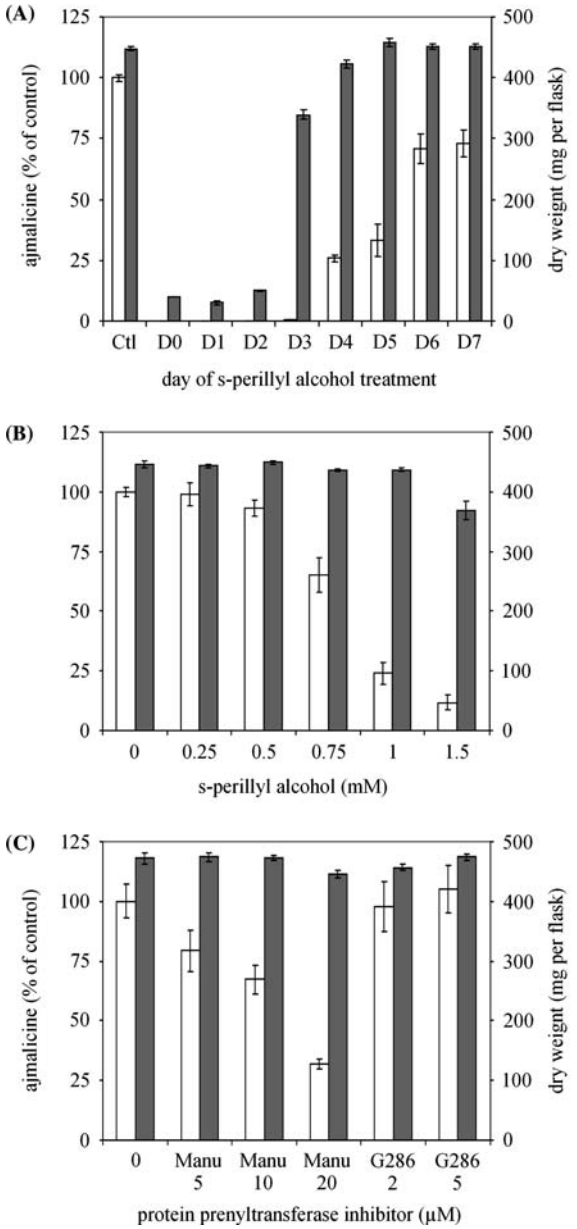

Figure 7. Effect of protein prenyltransferase inhibitors on ajmalicine accumulation and cell growth in C20D cell line. (A) Time course of the effect of *s*-perillyl alcohol (SP). (B) Effect of SP concentration, and (C) effect of Manumycine A and GGTI 286 (G286) treatments. (A) At day 0, 5 mL of 7-day old cells were transferred to 45 mL of fresh PM. SP (1 mM) was added to cell suspension from day 0 (D0) to day 7 (D7). As control, a cell culture was mock-treated with SP (Ctl). (B) and (C) 4 days after subculture into fresh PM, cell suspensions were supplemented with various concentrations of SP, Manumycine A or GGTI 286. Ajmalicine accumulation (white bars) and total dry weight (grey bars) of 50 mL of suspension cells (a flask) were measured at three days after the end of the culture (D10) (A) or at day 7 of the culture (B) and (C). Each bar represents the mean (±SE) of at least three replicates.

RNAi cell lines, which might explain that constitutive silencing of either CaaX-PTase only had a small effect on cell growth. In contrast, in cells treated at day 3 with SP, the cell dry weight was only 20% lower than in control lines; yet accumulation of MIA was 98% lower. This reduction in cell growth compares with the effect of either RNAi constructions on cell dry weight. In addition, SP treatment at day 4 or 5 had marginal or no effect on cell dry weight (Figure 7A) yet resulted in a 60–70% decrease in MIA accumulation (Figure 7A). At day 4 and 5, cell growth ceased and ajmalicine started to accumulate (supplemental data S1) (Arvy *et al*., 1994). Thus, our results indicate that CaaX-PTases are essential for MIA biosynthesis even after the end of cell growth. Based on these results, subsequent inhibitor treatments were performed at day 4.

Increasing concentrations of SP produced a dose-dependent decrease in ajmalicine accumulation (Figure 7B). For example, with 1 mM SP, ajmalicine accumulation was reduced by 80% without changes in the dry weight (Figure 7B) and DNA content of the cell cultures (data not shown). However, 1.5 mM SP reduced cell growth. Treatments with specific inhibitors of PFT or PGGT-I were also performed at day 4. Inhibition of PFT activity by Manumycin A, a competitive inhibitor of FPP (Hara *et al*., 1993; Tamanoi, 1993), led to a dose-dependent decrease of ajmalicine accumulation (Figure 7C). Specifically, Manumycine A at 20 μM resulted in a 70% decreased in ajmalicine accumulation without changes in the total culture dry weight (Figure 7C). Similar results were also obtained using chaetomellic acid A (data not shown), another competitive inhibitor of PFT with respect to FPP (Gibbs *et al*., 1993). Surprisingly, peptidomimetic inhibitors of PGGT-I, GGTI 286 (Figure 7C) and GGTI 298 (data not shown) had no effect on ajmalicine accumulation. However, addition of GGTI-286 to the C20D cell culture did not inhibit the level of PGGT-I activity (Figure 8). It is possible that these peptidomimetic inhibitors were not taken up by cells or deesterified for proper action. In contrast, SP abolished both CaaX-PTase activities while Manumycine A inhibits specifically PFT activity (Figure 8). In animals, SP has been shown to undergo metabolical conversion into more potent inhibitors of CaaX-PTases, such as perillic acid methyl ester. It is not known whether SP was metabolized in the plant cells, but inhibitory concentrations of SP paralleled more closely the K_i of SP, about 1 mM, than the K_i of perillic acid methyl ester, estimated at 10 μM (Gelb *et al*., 1995).

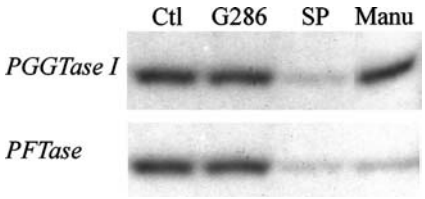

Figure 8. Analysis of the effect of *s*-perillyl alcohol (SP), Manumycine A and GGTI 286 treatments on PGGT-I and PFT activities in the C20D cell line. Four days after subculture into fresh PM, cell suspensions were supplemented with 1 mM SP, 10 μM Manumycine A or 5 μM GGTI 286. Total soluble proteins were isolated at day 5 and PGGT-I and PFT activities were assayed by *in vitro* prenylation of GST-CAIM and GST-CIIL target protein, respectively, with appropriate [^{3}H] prenyl donor. After incubation, the reaction mixtures were separated on a 12% SDS-PAGE and subjected to autoradiography.

Protein prenyltransferase inhibitors decrease ESMB biosynthetic pathway gene expression

To evaluate the requirement of CaaX-PTases in the expression of MIA pathway genes, the expression of *Crdxs*, *Crdxr*, *Crg10h*, *Crsls*, *Crtdc* and *Crstr* was studied in 5-day old cells pre-treated on day 4 with increasing concentrations of SP or Manumycine A. As previously shown for *Crdxs* and *Crdxr* (Veau *et al*., 2000), *Crg10h* genes is up regulated by 2,4-D suppression (Papon *et al*., in press; this study Figure 9). In contrast, 2,4-D suppression had no effect on *Crsls*, *Crstr* and *Crtdc* expression. Interestingly, the addition of SP or Manumycine A decreased, in a dose-dependent manner, the accumulation of *Crdxs, Crdxr* and *Crg10h* transcripts. At 1 mM, SP completely abolished the effect of 2,4-D suppression on the level of ESMB transcripts, but did not affect the level of *Crsls*, *Crstr* and *Crtdc* transcripts (Figure 9). As previously shown in RNAi cell lines, *Crdxs* is less sensitive to the general CaaX-PTase inhibitor, SP, than *Crdxr* and *Crg10h*. Furthermore, inhibition of PFT by Manumycine A had an inhibitory effect on *Crdxs*, *Crdxr* and *Crg10h* expression. Note that low concentrations of Manumycine A (5 and 10 μM) slightly enhanced accumulation of *Crsls*, *Crstr* and *Crtdc* transcripts.

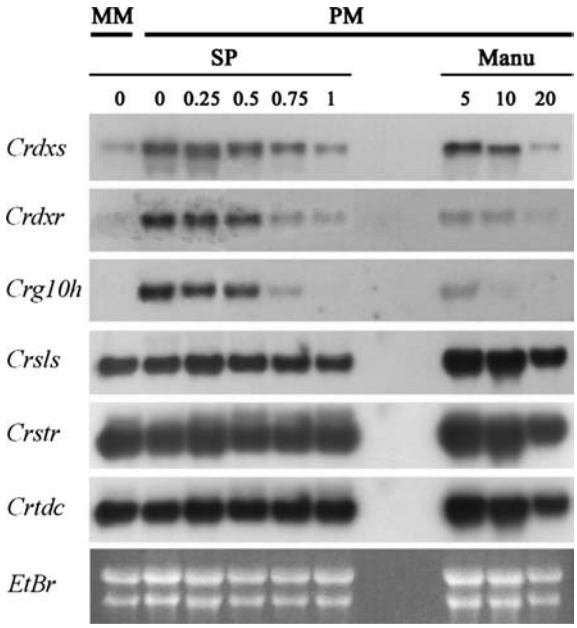

Figure 9. Expression of *Crdxs*, *Crdxr*, *Crg10h*, *Crsls*, *Crstr* and *Crtdc* genes in *s*-perillyl alcohol (SP) and Manumycine A (Manu) treated C20D cells. Cells grown in production medium (PM) were supplemented 4 days after subculture with increasing concentrations of SP (0 to 1 mM) or Manu (5–20 μM). At day 5, total RNA was prepared from treated cells and from 5-day old C20D cells cultured in maintenance medium (MM), and subjected to northern blot analysis. Equal loading of RNA samples in each lane was confirmed by ethidium bromide (EtBr) staining.

Together, these results confirm the requirement of CaaX-PTase activities in ESMB genes expression as described for RNAi cell lines.

Exogenous supply of monoterpenoids restores MIA accumulation in the presence of protein prenyltransferase inhibitors

In order to verify that a single branch of the MIA biosynthetic pathway is dependent on CaaX-PTase activities, we attempted to restore ajmalicine accumulation in C20D cells pre-treated with SP or Manumycine A, by supplementation with various terpenoid and indole precursors. Addition of intermediates of the monoterpenoid-secoiridoid biosynthetic pathway, such as loganin, loganic acid or secologanin, restored ajmalicine accumulation in SP pre-treated cells. Notably, the addition of 500 μM of one of these compounds completely restored ajmalicine accumulation (Table 1). In a similar fashion, only supplementation of Manumycine A pre-treated C20D cells with secologanin, restored ajmalicine accumulation. Supplementation of control cells with monoterpenoid–secoiridoid intermediates also increased ajmalicine accumulation (Table 1) suggesting that the monoterpenoid biosynthetic pathway is limiting for MIA biosynthesis. In contrast, the addition of exogenous deoxyxylulose, the unphosphorylated form of the product of the first step of the MEP pathway, increased ajmalicine accumulation by approximately 36% in control cells but did not restore ajmalicine accumulation in SP or Manumycine A pre-treated cells (Table 1). Addition of tryptamine, the MIA indole precursor, or mevalonic acid, a MVA pathway intermediate did not increase ajmalicine accumulation in control, SP and Manumycine A pre-treated cells (Table 1). The addition of geraniol, the substrate of *Crg10H*, was found to be toxic (data not shown). Together, these results suggest that proteins prenylated by CaaX-PTases are essential for the activity of part of the MEP and monoterpenoid–secoiridoid biosynthetic pathways, which appears to include steps between 1-deoxy-D-xylulose 5-phosphate and loganic acid biosynthesis.

Discussion

The role of prenylation in plant cell growth and development

The covalent modification of proteins by prenyl moiety addition, the so-called protein prenylation, is a common posttranslational event in animal, yeast and plant cells. Two distinct isoprenoid intermediates, either the 15-carbon farnesyl or 20-carbon geranylgeranyl, can be attached by thioether linkage to conserved cysteine residues at the carboxyl terminus of target proteins promoting membrane–protein or protein–protein interactions (Casey *et al.*, 1989; Farnsworth *et al.*, 1990, Glomset *et al.*, 1990; Casey, 1995). Three distinct protein prenyltransferases can be classified in two functional classes: CaaX-PTases and Rab geranylgeranyltransferase. In the first class, PFT (E.C. 2.5.1.58) catalyzes the transfer of farnesyl moiety from farnesyl diphosphate (FPP, Figure 1) to a protein substrate bearing a tetrapeptide CaaX motif at the carboxyl terminus where "C" is cysteine, "a" is an aliphatic amino acid and, "X" is preferentially serine, methionine, cysteine,

Table 1. Effect of secondary metabolite precursor supply on ajmalicine accumulation of C20D cells pre-treated with protein-prenyltransferase inhibitors.

Treatment		PM		PM + SP		PM + Manu	
precursors	MM	ajmalicine (% of control)	dry weight (mg) [a]	ajmalicine (% of control)	dry weight (mg) [a]	ajmalicine (% of control)	dry weight (mg) [a]
None		100 ± 9.5	442 ± 12	30.2 ± 8.8	430 ± 6	31.8 ± 1.9	445 ± 3
mevalonate	1	98.3 ± 4.5	437 ± 8	11.1 ± 5.9	401 ± 15	10.1 ± 6.4	464 ± 2
tryptamine	0.025	87 ± 2.8	460 ± 9	10.7 ± 4.6	420 ± 17	23.1 ± 0.1	417 ± 14
deoxyxylulose	2	135.8 ± 12	442 ± 6	28.23 ± 3.0	430 ± 7	33.7 ± 7.3	440 ± 3
loganic acid	0.4	102.0 ± 2.8	445 ± 7	90.2 ± 2.5	408 ± 6	Nd	Nd
	0.5	129.3 ± 6.8	450 ± 3	99.2 ± 5.0	414 ± 7	Nd	Nd
loganin	0.4	144.0 ± 4.7	451 ± 4	114.2 ± 4.7	420 ± 6	Nd	Nd
	0.5	155.0 ± 11	446 ± 5	135.8 ± 6.2	428 ± 7	Nd	Nd
secologanine	0.4	155.7 ± 13	432 ± 5	120.9 ± 19	423 ± 4	Nd	Nd
	0.5	156.4 ± 28	411 ± 2	149.0 ± 4.6	424 ± 9	125 ± 7	436 ± 7

C20D cells were grown in production medium without protein-prenyltransferase inhibitor (PM) or with *s*-perillyl alcohol at 1 mM (PM + SP) or Manumycine A at 20 μM (PM + Manu) added at day 4. Exogenous metabolites were added 8 h after protein-prenyltransferase inhibitor supply. Ajmalicine content and dry weight are measured at day 7 after subculture. Nd, not determined. Values are the average of at least three different experiments. [a]dry weight was expressed as mg/50 mL of cell suspension (one flask).

alanine or glutamine. PGGT-I (E.C. 2.5.1.59) catalyzes the transfer of a geranylgeranyl moiety from geranylgeranyl diphosphate (GGPP, Figure 1) to a protein substrate with, preferentially, a CaaL motif. PFT and PGGT-I are heterodimeric Zn^{2+} metalloenzymes consisting of a common α subunit and a distinct β subunit which determine isoprenoid and protein substrate preferences. The Rab geranylgeranyltransferase are also referred to type II protein geranylgeranyltransferase (E.C. 2.5.1.60) and recognized Rab protein with a XXCC, CCXX or CXC carboxyl terminus consensus motif where the two cysteine residues are geranylgeranylated.

A number of physiological functions of CaaX-PTases have been characterized in plants including the initiation of cell division (Qian *et al*., 1996), the regulation of cellular differentiation in the meristem, meristem maintenance in addition to their role in the regulation of flower development (Yalovsky *et al*., 2000b; Ziegelhoffer *et al*., 2000), and abscisic acid signalling (Cutler *et al*., 1996; Pei *et al*., 1998). Protein prenyltransferases are also important, but not essential, for plants and plant cells growth, as observed in organisms altered in PFT and/or PGGT-I activity. Indeed, the *Arabidopsis thaliana era1* mutants (enhanced response to abscisic acid) lacking the PFT β subunit (Cutler *et al*., 1996; Pei *et al*., 1998) are viable. In addition, the lack of both PFT and PGGT-I activities in the recently characterized *Arabidopsis* mutant of CaaX-PTase α subunit, *pluripetala* (*plp*) is not lethal under ideal growth conditions albeit an important decreased growth rate was noted (Running *et al*., 2004). Up to now, no plant mutants of PGGT-I β subunit have been described although potential *Arabidopsis thaliana* T-DNA insertion mutants are present in various libraries (our personal observations). We show here that down regulation of PGGT-I activity can be generated by interference RNA in *C. roseus* cells (Figures 4 and 5). This deficiency is not lethal but leads only to slightly decreased growth comparable to those observed in the *C. roseus* silenced cell lines for PFT β subunit (Figure 6). It could potentially suggest that the important decreased growth rate of *plp* is not solely due to the deficiency of PGGT-I activity but rather a consequence of both CaaX-PTase activities inhibition.

CaaX-PTases are required for plant cell metabolic differentiation

Plant cell lines may undergo metabolic pathway expression triggered by exogenous environmental signals or endogenous developmental and hormonal signals. In *C. roseus* C20D cell line, 2,4-D depletion from the cell culture medium initiate MIA biosynthesis and the expression of several MIA biosynthetic pathway genes, the so called ESMB genes, whereas presence of 2,4-D repress expression of these genes and the MIA biosynthe-

sis (Chahed *et al*., 2000; Veau *et al*., 2000; Papon *et al*., in press). In contrast, 2,4-D does not influence the expression of other MIA biosynthetic genes including those coding for SLS, the terminal step of the monoterpenoid-secoiridoid biosynthetic pathway, TDC, which supplies indole precursors for MIA biosynthesis, and STR, which catalyzes MIA precursor condensation (this study).

Genetic and pharmacological results presented here, show that, in C20D cell line, functional CaaX-PTases are required for the expression of ESMB genes but not for *Crsls*, *Crtdc* and *Crstr* (Figures 6A and 9). This requirement correlate with the inhibitory effect of 2,4-D on gene expression. This could suggests that PFT and PGGT-I dependent prenylation are both required, by their protein substrates, to the cascade of events leading to an efficient transcriptional activation of ESMB genes following 2,4-D depletion. In particular, prenylated proteins are involved during late growth phase as suggested by kinetics of SP and/or Manumycine A treatments. Interestingly, it was recently shown that ESMB genes are expressed specifically in internal phloem parenchyma cells of young aerial parts in *C. roseus* (Burlat *et al*., 2004), while *Crsls*, *Crtdc* and *Crstr* are expressed in epidermal cells (St-Pierre *et al*., 1999; Irmler *et al*., 2000). Such specific tissue expression in phloem parenchyma potentially involves coordinated regulatory processes, which may include the CaaX-PTase activities inferred in this work. Indeed, in differentiated cells, the effect of SP in hairy root cultures of *C. roseus* confirm that, in organized tissues, CaaX-PTases are also essential for ESMB genes regulation (Courdavault *et al*., 2005).

The effect of *era1* and *plp Arabidopsis* mutants on MEP pathway expression has not been studied. The synthesis of chlorophyll is not blocked in these mutants indicating that the MEP pathway derived phytol side chain is synthesized. This raise the question whether requirement of CaaX-PTases for MEP pathway expression in *C. roseus*, has a broader signification. In monocots and dicots, it has been suggested that the MEP pathway *dxs* genes are differentially regulated to cope with distinct functions. Indeed, class-I and class-II *dxs* genes were suggested to be dedicated to housekeeping and secondary metabolic functions, respectively (Walter *et al*., 2002). Therefore, lack of CaaX-PTase activities in *Arabidopsis* mutants might not interfere with housekeeping functions of the MEP pathway allowing phytol biosynthesis. In *C. roseus* RNAi cell lines, the residual level of expression of the *Crdxs* (class-II) and *Crdxr* (Figure 6A) could be related to housekeeping functions that not require prenylated protein.

CaaX-PTase potential substrates and their role in ESMB gene expression regulation

In plants, only few proteins have been shown to be prenylated *in vivo* and their localization and proper function dependant of their prenylation status. These proteins include notably the transcriptional factors APETALA1 (Yalovsky *et al*., 2000a) and the Ca^{2+}-binding calmodulin PhCaM53 (Rodriguez-concepcion *et al*., 1999, 2000), which function in meristems organization and Ca^{2+} signalling, respectively. Moreover, several other proteins involved in various signal transduction pathways may be modified by prenylation according to their C-terminus amino acid sequence such as heterotrimeric G protein (Iwasaki *et al*., 2003; Ullah *et al*., 2003; Pandey and Assmann, 2004), and plant specific GTPase proteins, called ROP (Zheng and Yang, 2000; Tao *et al*., 2002). Such kind of proteins could be part of the regulatory process following 2,4-D depletion that allows the MIA biosynthesis and ESMB gene expression in the C20D cell line. Lack of CaaX-PTase activities could therefore interfere with this process by alteration of the function of their protein substrates, and thus could induce the down-regulation of ESMB gene expression. Further investigations will be necessary to identify CaaX-PTase protein substrates linked to ESMB gene expression.

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References

Arvy, M.P., Imbault, N., Naudascher, F., Thiersault, M. and Doireau, P. 1994. 2,4-D and alkaloid accumulation in periwinkle cell suspensions. Biochimie 76: 410–416.

Bach, T.J. 1995. Some new aspects of isoprenoid biosynthesis in plants. Lipids 30: 191–202.

Burlat, V., Oudin, A., Courtois, M., Rideau, M. and St-Pierre, B. 2004. Co-expression of three MEP pathway genes and geraniol 10-hydroxylase in internal phloem parenchyma of *Catharanthus roseus* implicates multicellular translocation of intermediates during the biosynthesis of monoterpene indole alkaloids and isoprenoid-derived primary metabolites. Plant J. 38: 131–141.

Caldelari, D., Sternberg, H., Rodriguez-Concepcion, M., Gruissem, W. and Yalovsky, S. 2001. Efficient prenylation by a plant geranylgeranyltransferase-I requires a functional CaaL box motif and a proximal polybasic domain. Plant Physiol. 126: 1416–1429.

Casey, P.J. 1995. Protein lipidation in cell signalling. Science 268: 221–225.

Casey, P.J., Solski, P.A., Der, C.J. and Buss, J.E. 1989. p21ras is modified by a farnesyl isoprenoid. Proc. Natl. Acad. Sci. USA 86: 8323–8327.

Chahed, K., Oudin, A., Guivarc'h, N., Hamdi, S., Chénieux, J.-C., Rideau, M. and Clastre, M. 2000. 1-Deoxy-D-xylulose 5-phosphate synthase from periwinkle: cDNA identification and induced gene expression in terpenoid indole alkaloid-producing cells. Plant Physiol. Biochem. 38: 559–566.

Chappell, J.. 2002. The genetics and molecular genetics of terpene and sterol origami. Curr. Opin. Plant Biol. 5: 151–157.

Contin, A., Heijden, R., Lefeber, A.W.M. and Verpoort, R. 1998. The iridoid glucoside secologanin is derived from the novel triose phosphate/pyruvate pathway in a *Catharanthus roseus* cell culture. FEBS Lett. 437: 413–416.

Courdavault, V., Burlat, V. and St-Pierre, B. Giglioli-Guivarc'h, N. 2005. Characterisation of CaaX-prenyltransferases in *Catharanthus roseus*: relationships with the expression of genes involved in the early stages of monoterpenoid biosynthetic pathway. Plant Sci. 168: 1097–1107.

Crowell, P.L., Ren, Z., Lin, S., Vedejs, E. and Gould, M.N. 1994. Structure-activity relationships among monoterpene inhibitors of protein isoprenylation and cell proliferation. Biochem. Pharmacol. 47: 1405–1415.

Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S. and McCourt, P. 1996. A protein farnesyltransferase involved in abscisic acid signal transduction in *Arabidopsis*. Science 273: 1239–1241.

Dellaporta, S.L., Wood, J. and Hicks, J.B. 1983. A plant DNA minipreparation, Version II. Plant Mol. Biol. Rep. 1: 19–21.

Farnsworth, C.C., Gelb, M.H. and Glomset, J.A. 1990. Identification of geranylgeranyl-modified proteins in HeLa cells. Science 247: 320–322.

Gamborg, O.L., Miller, R.R.A. and Ojima, K. 1968. Nutriment requirements of suspension cultures of soybean root cells. Exp. Cells Res. 50: 151–158.

Gelb, M.H., Tamanoi, F., Yokoyama, K., Ghomashchi, F., Esson, K. and Gould, M.N. 1995. The inhibition of protein prenyltransferases by oxygenated metabolites of limonene and perillyl alcohol. Cancer Lett. 91: 169–175.

Gibbs, J.B., Pompliano, D.L., Mosser, S.D., Rands, E., Lingham, R.B., Singh, S.B., Scolnick, E.M., Kohl, N.E. and Oliff, A. 1993. Selective inhibition of farnesyl-protein transferase blocks ras processing in vivo. J. Biol. Chem. 268: 7617–7620.

Giner, J.L. 1998. New and efficient synthetic routes to 1-Deoxy-D-xylulose. Tetrahedron Lett. 39: 2479–2482.

Glomset, J.A., Gelb, M.H. and Farnsworth, C.C. 1990. Prenyl proteins in eucaryotic cells: a new type of membrane anchor. Trends Biochem. Sci. 15: 139–142.

Hara, M., Akasaka, K., Akinaga, S., Okabe, M., Nakano, H., Gomez, R., Wood, D., Uh, M. and Tamanoi, F. 1993. Identification of Ras farnesyltransferase inhibitors by microbial screening. Proc. Natl. Acad. Sci. USA 90: 2281–2285.

Haralampidis, K., Bryan, G., Qi, X., Papadopoulou, K., Bakht, S., Melton, R. and Osbourn, A. 2001. A new class of oxidosqualene cyclases directs synthesis of antimicrobial phytoprotectants in monocots. Proc. Natl. Acad. Sci. USA 98: 13431–13436.

Imbault, N., Thiersault, M, Dupéron, P., Benabdelmouna, A. and Doireau, P. 1996. Pravastatine: a tool for investigating the availability of mevalonate metabolites for primary and secondary metabolism in *Catharanthus roseus* cell suspensions. Physiol. Plant 98: 803–809.

Irmler, S, Schroder, G., St-Pierre, B., Crouch, N.P., Hotze, M., Schmidt, J., Strack, D., Matern, U. and Schroder, J. 2000. Indole alkaloid biosynthesis in *Catharanthus roseus*: new enzyme activities and identification of cytochrome P450 CYP72A1 as secologanin synthase. Plant J. 24: 797–804.

Iwasaki, Y., Fujisawa, Y. and Kato, H. 2003. Function of Heterotrimeric G Protein in Gibberellin Signalling. J. Plant Growth Regul. 22: 126–133.

Kasahara, H., Hanada, A., Kuzuyama, T., Takagi, M., Kamiya, Y. and Yamaguchi, S. 2002. Contribution of the mevalonate and methylerythritol phosphate pathways to the biosynthesis of gibberellins in *Arabidopsis*. J. Biol. Chem. 277: 45188–45194.

Kessler, A. and Baldwin, I.T. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. Science 291: 2141–2144.

Levêque, D., Wihlm, J. and Jehl, F. 1996. Pharmacology of *Catharanthus* alkaloids. Bull. Cancer 83: 176–186.

Lichtenthaler, H.K., Schwender, J., Disch, A. and Rhomer, M. 1997. Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a so far unexpected novel pathway. FEBS Lett. 400: 271–274.

Lichtenthaler, H.K. 1999. The 1-deoxy-D-xylulose 5-phosphate pathway of isoprenoid biosynthesis in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 47–65.

Mérillon, J.M., Ouelhazi, I., Doireau, P., Guillot, A., Chenieux, J.C. and Rideau, M. 1986. Indol alkaloid accumulation and tryptophan decarboxylase activity in *Catharanthus roseus* cells cultured in three different media. Plant Cell Rep. 5: 23–26.

Naudascher, F., Doireau, P., Guillot, A., Viel, C. and Thiersault, M. 1989. Time-course studies on the use of secologanin by *Catharanthus roseus* cells cultured in vitro. J. Plant Physiol. 134: 608–612.

Pandey, S. and Assmann, S.M. 2004. The *Arabidopsis* putative G protein-coupled receptor GCR1 interacts with the G protein α subunit GPA1 and regulates abscisic acid signalling. Plant Cell 16: 1616–1632.

Papon N., Bremer J., Vansiri A., Andreu F., Rideau M. and Crèche J. Cytokinin and ethylene control indole alkaloid production at the level of the MEP/terpenoid pathway in *Catharanthus roseus* suspension cells. Planta Med. In press.

Papon, N., Vansiri, A., Gantet, P., Chénieux, J.C., Rideau, M. and Crèche, J. 2004. Histidine-containing phosphotransfer domain extinction by RNA interference turns off a cytokinin signalling circuitry in *Catharanthus roseus* suspension cells. FEBS Lett. 558: 85–88.

Pei, Z.M., Ghassemian, M., Kwak, C.M., Mc Court, P. and Schoerder, J.I. 1998. Role of farnesyltransferase in ABA regulation of guard cell anion channels and plant water loss. Science 282: 287–290.

Qian, D., Zhou, G., Ju, R., Cramer, C.L. and Yang, Z. 1996. Protein farnesyltransferase in plants: molecular characterization and involvement in cell cycle control. Plant Cell 8: 2381–2394.

Ren, Z., Elson, C.E. and Gould, M.N. 1997. Inhibition of type I and type II geranylgeranyl-protein transferases by the monoterpene perillyl alcohol in NIH3T3 cells. Biochem. Pharmacol. 54: 113–120.

Ren, Z. and Gould, M.N. 1994. Inhibition of ubiquinone and cholesterol synthesis by the monoterpene perillyl alcohol. Cancer Lett. 76: 185–90.

Repko, E.M. and Maltese, W.A. 1989. Post-translational isoprenylation of cellular proteins is altered in response to mevalonate availability. J. Biol. Chem. 264: 9945–9952.

Rodriguez-Concepcion, M., Yalovsky, S., Zik, M., Fromm, H. and Gruissem, W. 1999. The prenylation status of a novel plant calmodulin directs plasma membrane or nuclear localization of the protein. EMBO J. 18: 1996–2007.

Rodriguez-Concepcion, M., Toledo-Ortiz, G., Yalovsky, S., Caldelari, D. and Gruissem, W. 2000. Carboxyl-methylation of prenylated calmodulin CaM53 is required for efficient plasma membrane targeting of the protein. Plant J. 24: 775–784.

Running, M.P., Lavy, M., Sternberg, H., Galichet, A., Gruissem, W., Hake, S., Ori, N. and Yalosky, S. 2004. Enlarged meristems and delayed growth in *plp* mutants result from lack of CaaX prenyltransferases. Proc. Natl. Acad. Sci. USA 101: 7815–7820.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

St-Pierre, B., Vazquez-Flota, F.A. and De Luca, V. 1999. Multicellular compartimentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate. Plant Cell 11: 887–900.

Tamanoi, F. 1993. Inhibitors of Ras farnesyltransferases. Trends Biochem Sci 8: 349–353.

Tao, L.Z., Cheung, A.Y. and Wu, H.M. 2002. Plant Rac-Like GTPases are activated by auxin and mediate auxin-responsive gene expression. Plant Cell 14: 2745–2760.

Ullah, H., Chen, J.G., Temple, B., Boyes, D.C., Alonso, J.M., Davis, K.R., Ecker, J.R. and Jones, A.M. 2003. The beta-subunit of the *Arabidopsis* G protein negatively regulates auxin-induced cell division and affects multiple developmental processes. Plant Cell 15: 393–409.

Van Telligen, O., Sips, J.H.M., Beijnen, J.H., Bult, A. and Nooijen, W.J. 1992. Pharmacology, bio-analysis and pharmacokinetics in the Vinca alkaloids and semi-synthetic derivatives. Anticancer Res. 12: 1699–1716.

Veau, B., Courtois, M., Oudin, A., Chénieux, J.C., Rideau, M. and Clastre, M. 2000. Cloning and expression of cDNAs encoding two enzymes of the MEP pathway in *Catharanthus roseus*. Biochim. Biophys. Acta 1517: 159–163.

Walter, M.H., Hans, J. and Strack, D. 2002. Two distantly related genes encoding 1-deoxy-d-xylulose 5-phosphate synthases: differential regulation in shoots and apocarotenoid-accumulating mycorrhizal roots. Plant J. 31: 243–254.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M.B., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., Stoutjesdijk, P.A., Robinson, S.P., Gleave, A.P., Green, A.G. and Waterhouse, P.M. 2001. Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J. 27: 581–590.

Wink M. 1999. Introduction: biochemistry, role and biotechnology of secondary metabolites. In: M. Wink (Ed), Biochemistry of plant secondary metabolism Vol. 2, Sheffield Academic Press and CRC Press, Annual Plant Reviews, pp. 1–16.

Yalovsky, S., Rodriguez-Concepcion, M., Bracha, K., Toledo-Ortiz, G. and Gruissem, W. 2000a. Prenylation of the floral transcription factor APETALA1 modulates its function. Plant Cell 12: 1257–1266.

Yalovsky, S., Kulukiam, A., Rodriguez-Concepcion, M., Young, C.A. and Gruissem, W. 2000b. Functional requirement of plant farnesyltransferase during development in *Arabidopsis*. Plant Cell 12: 1267–1278.

Yang, Y., Shah, J. and Klessing, D.F. 1997. Signal perception and transduction in plant defense responses. Genes Dev. 11: 1621–1639.

Yang, Z., Cramer, C.L. and Watson, J.. 1993. Protein farnesyltransferase in plants. Molecular cloning and expression of a homolog of the beta subunit from the garden Pea. Plant Physiol. 101: 667–674.

Zheng, Z.L. and Yang, Z. 2000. The Rop GTPase: an emerging signalling switch in plants. Plant Mol. Biol. 44: 1–9.

Zhang, F.L. and Casey, P.J. 1996. Protein prenylation: molecular mechanisms and functional consequences. Annu. Rev. Biochem. 65: 241–269.

Ziegelhoffer, E.C., Medrano, L.J. and Meyerowitz, E.M. 2000. Cloning of the *Arabidopsis* WIGGUM gene identifies a role for farnesylation in meristem development. Proc. Natl. Acad. Sci. USA 97: 7633–7638.