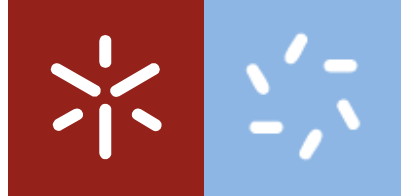


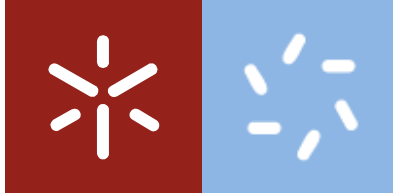


Biotechnological approaches for yield improvement of anticancer alkaloids in the plant *Catharanthus roseus*

Bernardete Sofia da Cruz Fernandes

Universidade do Minho
Escola de Ciências





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Dissertação de Mestrado

Mestrado em Biologia Molecular, Biotecnologia e Bioempreendedorismo em Plantas

Trabalho efetuado sob a orientação da

Professora Doutora Mariana Sottomayor e do

Professor Doutor Hernâni Gerós

DECLARAÇÃO

Nome: Bernardete Sofia da Cruz Fernandes

Endereço eletrónico: bernardetecruz@hotmail.com

Número do Cartão de Cidadão: 13235664

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Orientadores:

Professora Doutora Mariana Sottomayor

Professor Doutor Hernâni Gerós

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Abstract

Catharanthus roseus (L.) G. Don produces an impressive number of specialized metabolites, over 130 terpenoid indole alkaloids (TIAs), among which the anticancer vinblastine (VLB) and vincristine (VCR). VLB and VCR were the first natural drugs used in cancer therapy, still holding an important position in the treatment of cancer nowadays. VLB and VCR accumulate in very low levels in *C. roseus* leaves that remain the only source of the anticancer TIAs, since efforts to obtain their entire chemical synthesis have been unfruitful. An intense research has been carried out over the last decades in *C. roseus* to unveil the biosynthesis of TIAs pathway, however, the characterization of several biosynthetic steps is still missing, and the membrane transport mechanisms are basically uncharacterized despite their importance for TIA accumulation.

Previous studies, in our group, allowed the identification of a number of very strong candidates to important functions within the TIA pathway, including MATE1, a strong candidate to TIA vacuolar accumulation, and CYP2, a strong candidate to two of the last steps of VLB and VCR biosynthesis. These genes are strong candidates for the possible manipulation of TIA levels in order to improve their accumulation.

The work developed here includes the sub-cloning of each gene into a plant binary vector followed by the genetic transformation of *C. roseus* hypocotyls using *Agrobacterium tumefaciens*. The induction of transgenic *callus* was optimized, leading to the successful establishment of transgenic *callus* lines confirmed by performing a Gus assay. The regeneration into a whole plant was actively pursued by changing an array of conditions but remained unsuccessful. Complementarily, downregulation of MATE1 expression was carried out using Virus Inducing Silencing Gene (VIGS), by sub-cloning a specific *MATE1* fragment into a viral based plant vector followed by *Agrobacterium* mediated transformation of *C. roseus* plants. The putatively silenced plants showed significantly lower levels of the major alkaloids accumulated in *C. roseus* leaves, strongly supporting a role of MATE1 in their transmembrane transport.

Overall, the work developed gives a significant contribution to the possibility of performing future biotechnological improvements of *C. roseus* for increased levels of the anticancer VLB and VCR.

Abordagens biotecnológicas para a melhoria do rendimento dos alcalóides anticancerígenos presentes na planta *Catharanthus roseus*

Resumo

Catharanthus roseus (L.) G. Don produz um número impressionante de metabolitos secundários, mais de 130 alcalóides terpenóides indólicos (TIA), entre os quais, os anticancerígenos vinblastina (VLB) e vincristina (VCR). VLB e VCR foram os primeiros medicamentos de origem natural a serem utilizados no tratamento do cancro, mantendo ainda hoje uma posição relevante na terapia do cancro. VLB e VCR acumulam-se em níveis muito baixos nas folhas de *C. roseus*, que permanecem como a única fonte dos anticancerígenos, uma vez que, os esforços para os obter integralmente por síntese química têm sido infrutíferos. Ao longo das últimas décadas, *C. roseus* tem sido intensamente investigada com o intuito de desvendar a via de síntese dos TIA, no entanto, várias etapas ainda estão por caracterizar assim como os mecanismos de transporte membranar, apesar da sua importância para a acumulação dos TIAs.

Estudos anteriores, no nosso grupo, possibilitaram a identificação de genes candidatos a funções importantes dentro da via dos TIAs, incluindo *MATE1* um forte candidato ao transporte dos TIAs para o vacúolo, e *CYP2*, um forte candidato a intervir num dos dois últimos passos da biossíntese de VLB e VCR. Estes genes são fortes candidatos para uma eventual manipulação dos níveis de TIA, a fim de aumentar a sua acumulação.

O presente trabalho inclui a sub-clonagem de cada um dos genes num vetor binário seguido pela transformação genética dos hipocótilos de *C. roseus* usando *Agrobacterium tumefaciens*. A indução de *callus* transgênico foi otimizada, alcançando-se com sucesso linhas de *calli* transgênicas, confirmadas através da realização de um ensaio de GUS. A regeneração para obter a planta foi arduamente perseguida alterando-se uma série de condições, mas até ao momento não foi alcançada. Complementarmente, o silenciamento da expressão do gene *MATE1* foi levado a cabo usando Vírus Indutor de silenciamento do gene (VIGS), através da sub-clonagem de um fragmento específico de *MATE1* num vetor viral modificado, seguindo-se a transformação das plantas mediada por *Agrobacterium*. As plantas putativamente silenciadas apresentaram níveis significativamente mais baixos dos principais alcalóides acumulados em folhas de *C. roseus*, reforçando fortemente o papel do *MATE1* no seu transporte. No geral, o trabalho desenvolvido contribui significativamente para a possibilidade de no futuro realizarem-se melhorias biotecnológicas em *C. roseus* com vista a aumentar os níveis dos anticancerígenos VLB e VLC.

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Abbreviations

10HGO - 10-hydroxygeraniol oxidoreductase
16-OH OMT - 16-hydroxytabersonine-16-O-methyltransferase
2,4 -D - Ácido 2,4-diclorofenolacético
7DLH 7 - Deoxyloganic acid 7-hydroxylase
AFLP – Amplified Fragment length polymorphism
AVLB - Anhydrovinblastine
BA - 6-Benzylaminopurine
bp - base-pair
CaMV 35S - Cauliflower Mosaic virus promoter
CroPrx1 – *C. roseus* Peroxidase 1
D4H - Desacetoxyvindoline 4-hydroxylase
DAT - Deacetylvindoline 4-*O*-acetyltransferase
ddH₂O - Bi-distillated H₂O
DNA - Deoxyribonucleic acid
dNTPs - Deoxynucleotide
dsRNA - Double strand RNA
EDTA - Ethylenediamine tetraacetic acid
EGFP - Enhanced green fluorescent protein
EtBr - Ethidium bromide
Fwd - Forward
G10H - Geraniol-10-hydroxylase
GC% - Guanine/Cytosine content
GPPS - Geranyl diphosphate synthase
HPLC-DAD - Chromatography-Diode-Array Detection
IDI - Isopentenyl Diphosphate Isomerase
IO - Iridoid oxidase
IPAP- Internal Phloem Associated Parenchyma
kb/Kbp - kilo-base-pair
LB - Luria Bertrani
MATE - Multidrug and toxic compound extrusion
MEP - 2-C-methyl-D-erythritol 4-phosphate

mRNA - Messenger RNA
 MS - Murashige & Skoog
 MSCP - Murashige & Skoog Casein Proline
 NAA - 1-Naphthaleneacetic acid
 NMT - 16-methoxy - 2,3-dihydro - 3-hydroxytabersonine - N-methyltransferase
 OMT - 16-hydroxytabersonine 16-*O*-methyltransferase
 ON - Overnight
 P450 - Cytochrome P450 monooxygenase
 PCR – Polymerase chain reaction
 pDNA - Plamid DNA
 PGR 's – Plant Growth Regulators
 pRi - Hairy root-inducing plasmid
 Prx1 – Peroxidase 1
 PTGS - Post Transcriptional Gene Silencing
 pTi - Tumour-inducing plasmid
 Rev - Reverse
 RISC - RNA-induced silencing complex
 RNA - Ribonucleic acid
 rpm - Revolutions per minute
 RT – Room temperature
 SGD - Strictosidine β -D-glucosidase
 siRNA - Small interfering RNA
 SLS - Secologanin synthase
 STR - Strictosidine synthase
 T16H - Tabersonine 16-hydroxylase
 T3O - Tabersonine 3-oxygenase
 T3R - Tabersonine 3-reductase
 TAE buffer – Tris-acetate-EDTA buffer
 TDC - Tryptophan decarboxylase
 T-DNA - Transferred-DNA
 TDZ- Thidiazuron
 TIA - Terpenoid Indole Alkaloids
 Tm - Melting temperature
 TRV - Tobacco rattle virus

UV – Ultra-violet

v/v – volume/volume

VCR - Vincristine

VIGS – Virus Inducing Gene Silencing

VIR - Virulence

VLB – Vinblastine

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1. Introduction

1.1 The medicinal plant *Catharanthus roseus* (L.) G. Don

Catharanthus roseus (L.) G. Don commonly known as Madagascar periwinkle is a member of the Apocynaceae family that originates from Madagascar, but can nowadays be found as sub-spontaneous in many subtropical regions and also in gardens worldwide as an ornamental plant (Figure 1). This global distribution was namely caused by sailors, who took the plants on board during their travels, due to their medicinal properties. *C. roseus* is a perennial semi- shrub that grows about 80 cm high and blooms continuously year-round with pink, purple, or white flowers (Sottomayor and Barceló, 2006; van Der Heijden et al., 2004)



Figure 1 - Flowering plant of *Catharanthus roseus* cv. Little Bright Eye.

C. roseus plant has historically been used to treat a wide assortment of diseases. In several sub-tropical countries, it was used as a folk remedy for diabetes for centuries while in India, juice from the leaves was also used to treat wasp stings and other insect bites (Singh et al., 2001).

During the 1950's, researchers from the Noble research group in Canada and from the Eli Lilly Pharmaceutical company in the USA discovered that the plant contains alkaloids useful to the treatment of cancer, vinblastine (VLB) and vincristine (VCR) (Noble, 1990; Sottomayor and Barceló, 2006). These alkaloids are produced only in *C. roseus* leaves, they were the first natural drugs used in cancer therapy, and still maintain an important position in the treatment of cancer nowadays (Costa et al., 2008; Suttipanta et al., 2011)

1.2 *C. roseus* terpenoid indole alkaloids

Alkaloids are a large group of specialized metabolites of low molecular weight containing nitrogen that share a characteristic toxicity and pharmacological activity (De Luca and St Pierre, 2000; Shitan and Yazaki, 2007). *C. roseus* is known to produce an impressive number of specialized metabolites, with over 130 terpenoid indole alkaloids (TIAs) isolated and identified, some of which are valuable therapeutic compounds (St-pierre, 2007; van Der Heijden et al., 2004): VLB and VCR are widely used as drugs for cancer chemotherapy, ajmalicine is used as an anti-hypertensive agent and serpentine is used as a sedative (Figure 2) (St-pierre, 2007; van Der Heijden et al., 2004).

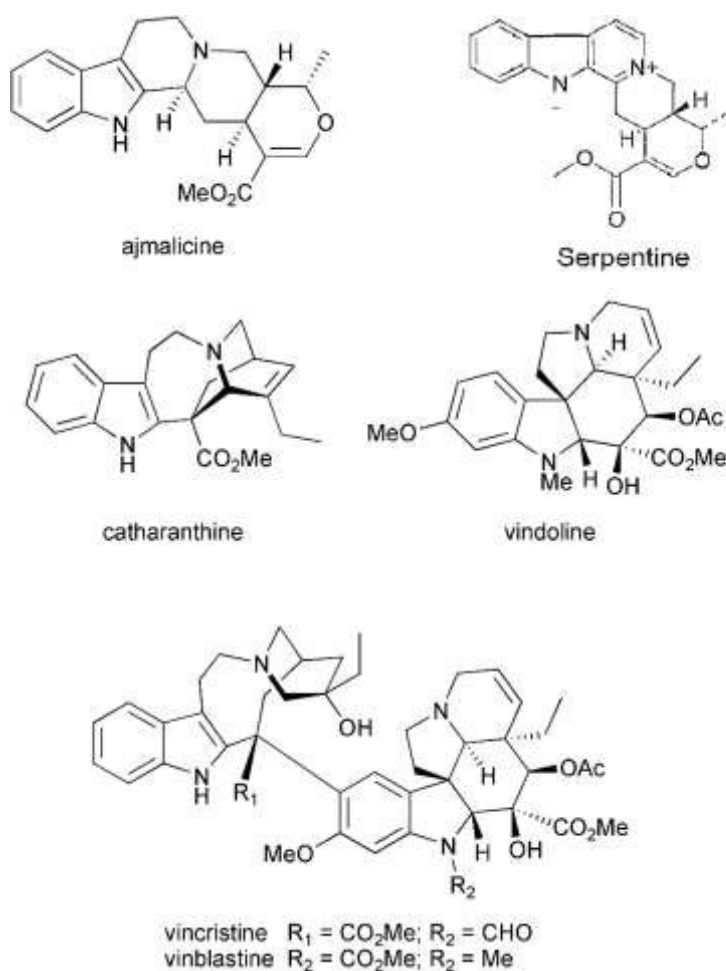


Figure 2 - Representative alkaloids of *C. roseus*. Adapted from O'Connor and Maresh, 2006.

The anticancer VLB and VCR are highly valuable anticancer drugs, but are produced in very low amounts in the plant - it takes about 500 kg of dried periwinkle leaves to isolate 1 g of VLB, and their levels depend on the physiological and developmental stage of the plant (van Der

Heijden et al., 2004). Moreover, the majority of specialized metabolites with pharmaceutical activity are only obtained from cultivated plants, and despite all attempts made, their chemical synthesis in most cases is not possible or too expensive (Zárate and Verpoorte, 2007). In fact, the highly complex structures of alkaloids VLB and VCR impair their entire chemical synthesis. The more feasible process is the semi synthesis, using the natural monomeric precursors catharanthine and vindoline, which are present in greater amount and can be extracted from *C. roseus* leaves (Roepke et al., 2010). Hence, an extensive study of the TIA biosynthetic pathway has been carried out over the last three decades, making of *C. roseus* one of the most studied plants concerning specialized metabolism. The demand for the elucidation of their biosynthesis became critical: to either increase their production in planta or to engineer these pathways in microorganisms to allow industrial production of medicinally relevant compounds (El-Sayed and Verpoorte, 2007; O'Connor and Maresh, 2006).

1.3 The terpenoid indole alkaloid (TIA) pathway

The research conducted over the last decades associated with the recent massive expansion of transcriptomic resources for *C. roseus* and the development of efficient approaches of virus-induced gene silencing led to a more accurate knowledge of genes involved in TIA production in *C. roseus*. In fact, the evolution of molecular cell biology techniques has also contributed to a major progress in deciphering the puzzling spatial organization of the TIA biosynthetic pathway (Courdavault et al., 2014). All the TIAs of *C. roseus* share a common precursor, strictosidine (O'Connor and Maresh, 2006). Strictosidine is formed by condensation of the indole precursor tryptamine with the terpenoid precursor secologanin through the catalytic action of strictosidine synthase (STR) (Figure 3). This coupling requires the combination of two metabolic pathways, the 2-*C*-methyl-D-erythritol 4-phosphate pathway (MEP) through secaloganin, and the shikimate pathway through tryptamine (Murata and Luca, 2005; van Der Heijden et al., 2004).

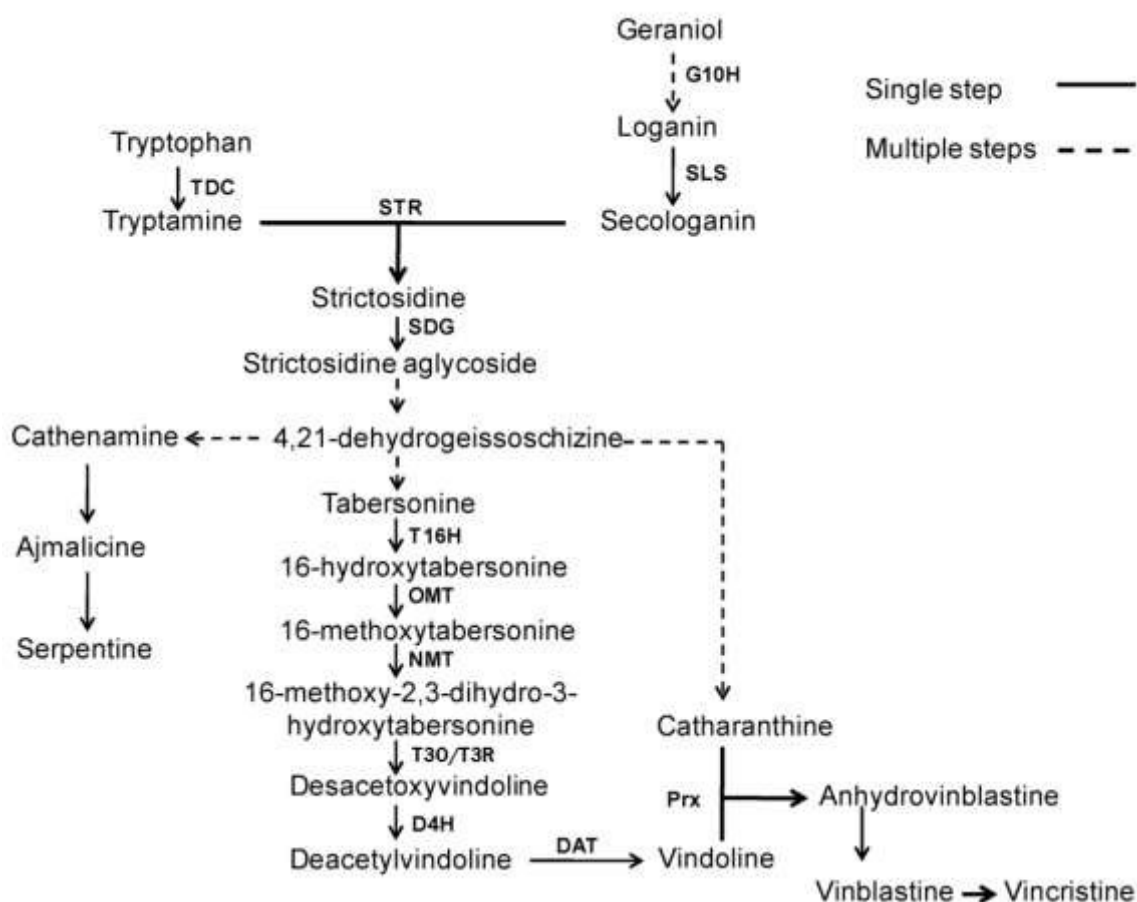


Figure 3 - The TIA biosynthetic pathway in *C. roseus*. TDC, tryptophan decarboxylase; G10H, geraniol 10-hydroxylase; SLS, secologanin synthase; STR - strictosidine synthase, SGD - strictosidine β -D-glucosidase, T16H - tabersonine 16-hydroxylase, OMT - 16-hydroxytabersonine-16-O-methyltransferase, NMT - 16-methoxy-2,3-dihydro-3-hydroxytabersonine N-methyltransferase, T30/T3R - tabersonine 3-oxygenase/ tabersonine 3-reductase D4H - desacetoxyvindoline 4-hydroxylase, DAT - deacetylvindoline 4-O-acetyltransferase, Prx1 - peroxidase 1. Adapted from Almagro, Fernández-Pérez and Pedreño, (2015).

The biosynthesis of the indole moiety via the shikimate pathway yields tryptamine, which is derived from tryptophan by the action of tryptophan decarboxylase (TDC) (De Luca et al., 1989; St-Pierre et al., 1999). Whereas the assembly of the monoterpene secoiridoid moiety requires a total of ten enzymes to convert the MEP pathway-derived monoterpenoid skeleton into secologanin (Dugé de Bernonville et al., 2015b). Recently Miettinen et al., (2014) reported the identification and characterization of the last four enzymes that fill in the missing steps of the *C. roseus* secoiridoid pathway (Figure 4).

Strictosidine is subjected to deglycosylation catalysed by strictosidine β -D-glucosidase (SGD) yielding an unstable aglycone precursor required for the assembly of the corynanthe, iboga, and aspidosperma skeletons representing different ramifications of the TIA pathway, such as ajmalicine, catharanthine, and vindoline the latter two being the monomeric precursors of VLB and

VCR (Dugé de Bernonville et al., 2015b; Liscombe and O'Connor, 2011; Murata and Luca, 2005). The sequence of reactions since the labile aglycone until the formation of catharanthine or tabersonine remain poorly characterized. Nevertheless, the 6 steps leading to the production of vindoline from the precursor tabersonine are well understood at the molecular and biochemical levels (Figures 3 and 4) (Besseau et al., 2013; Murata and Luca, 2005). The first step in the conversion of tabersonine to vindoline is the hydroxylation by the enzyme tabersonine 16-hydroxylase (T16H) (Murata and Luca, 2005; St-Pierre and De Luca, 1995). The following step is the *O*-methylation of 16-hydroxytabersonine to yield 16-methoxytabersonine by the enzyme 16-hydroxytabersonine-16-*O*-methyltransferase (16-OH OMT). This is converted into 3-hydroxy-16-methoxy-2,3-dihydroxytabersonine by tabersonine 3-oxygenase (T3O) and tabersonine 3-reductase (T3R) (Qu et al., 2015) followed by an N-methylation by the enzyme 16-methoxy - 2,3-dihydro - 3-hydroxytabersonine - N-methyltransferase (NMT), originating desacetoxyvindoline. Desacetoxyvindoline undergoes a hydroxylation by desacetoxyvindoline 4-hydroxylase (D4H) and the last step in vindoline biosynthesis is catalyzed by acetylcoenzyme A: 4-*O*-deacetylvindoline 4-*O*-acetyltransferase (DAT) (Besseau et al., 2013; Guirimand et al., 2011; Liscombe and O'Connor, 2011; Murata and Luca, 2005)

The coupling of vindoline and catharanthine form the first dimeric TIA, α -3',4'-anhydrovinblastine (AVLB), which is the precursor of all dimeric TIAs, including the anticancer VLB and VCR. This dimerization step is believed to be performed by the major class III peroxidase localized in *C. roseus* mesophyll vacuole (Sottomayor and Barceló, 2006; Sottomayor and Ros Barceló, 2003; Sottomayor et al., 1998, Costa et al. 2008). Finally, the biosynthetic steps from AVLB to the anticancer alkaloids, VCR and VLB, are not characterized. In order to identify candidate enzymes to this steps, research was conducted in our lab including the differential transcription profiling of the specialized cells of *C. roseus* leaves, the idioblasts, where the late steps of VLB biosynthesis are believed to occur Carqueijeiro, (2013).

The screening data allowed to identify a number of very strong candidate genes to important functions within the TIA pathway, namely to the bottleneck hydroxylation of AVLB into VLB, to the transmembrane transport of TIA intermediates and to transcriptional regulation of the early and late steps of the TIA pathway (Carqueijeiro, 2013).

1.4 Cytochromes P450 in the TIA pathway

Cytochrome P450 monooxygenases in plants are membrane-bound enzymes involved in a wide range of biosynthetic pathways, including in the TIA pathway (St-Pierre and De Luca, 1995).

In the TIA pathway of *C. roseus*, P450 monooxygenases have a crucial influence, until now, seven enzymes were identified and characterized: geraniol 10-hydroxylase (G10H, P450 CYP76B6), iridoid oxidase (IO, CYP76A26), 7-deoxyloganic acid 7-hydroxylase (7DLH, CYP72A224), secologanin synthase (SLS, CYP72A1), tabersonine 16-hydroxylase 1 and 2 (T16H1 and 2,) and tabersonine 3-oxygenase (T3O, CYP71D1V2) (Figure 4) (Besseau et al., 2013; Murata and Luca, 2005; Qu et al., 2015; St-Pierre and De Luca, 1995). All these P450 already identified in the *C. roseus* TIA pathway allow the prediction of a possible involvement of other P450s in several uncharacterized steps. In line with this assumption, previous studies in our laboratory revealed that several P450s are up-regulated in the TIA accumulating idioblasts. These are good candidates to the first reaction in the conversion of AVL into VLB, and also in one of the predicted reactions in the transformation of VLB into VLC, which involves two oxidative steps: the oxidation of the alkyl group to an alcohol, and then the oxidation of the alcohol group to aldehyde. If the two step hypothesis is correct, two oxydoreductases are required, one of them being a hydroxylase like a P450. Therefore, it is extremely important to study these candidate cytochrome P450s to decrypt the last steps of the TIA pathway.

1.5 Cellular and sub-cellular compartmentation of the TIA pathway in *C. roseus*

The characterization of the TIA pathway in *C. roseus* revealed a fascinating picture of cell-type specific compartmentation, involving at least four different cell types and as many subcellular compartments with inherent intercellular and intracellular metabolite translocations. Over the last decade, several tools have been applied to unveil the architecture of cellular and sub-cellular compartmentation, with the mechanisms of inter-tissue mobility of compounds remaining largely unknown (Courdavault et al., 2014).

Recent research revealed that the internal phloem associated parenchyma (IPAP) cells harbour the whole Methyl Erythritol Phosphate (MEP) pathway and the eight first reactions of the monoterpene-secoiridoid pathway, from the synthesis of geranyl diphosphate synthase (GPPS) to its conversion into the iridoid loganic acid (Figure 4). At the subcellular level, the first 10 enzymatic

steps leading to geraniol synthesis are located in the plastid, not only localized in stroma of plastids but also in stromules. (Cordavault et al., 2014; Dugé de Bernonville et al., 2015b; Miettinen et al., 2014; Salim et al., 2014)

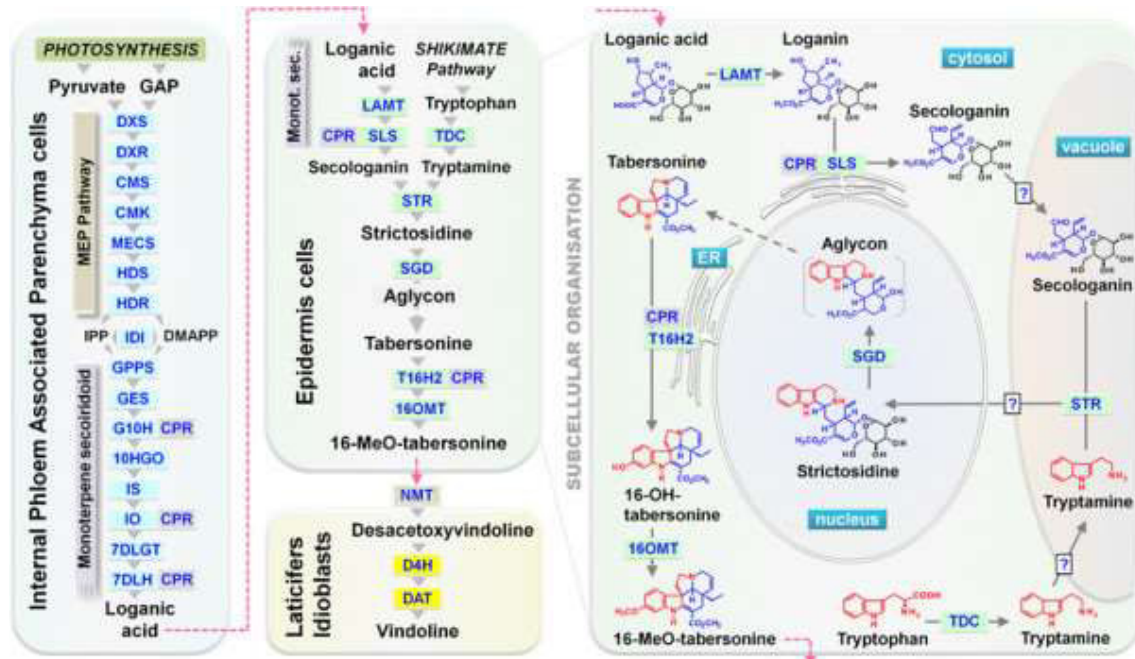


Figure 4 - Cellular and subcellular organization of the biosynthetic pathway of TIAs in *C. roseus* leaves. Known single enzymatic steps in each cell type are indicated by grey arrows and abbreviation of enzyme names. Broken grey arrows and broken pink arrows indicate unknown enzymatic steps and metabolite translocation, respectively. DXS, 1-deoxy-D-xylulose-5-phosphate (DXP) synthase; DXR, DXP reductoisomerase; CMS, 4-(cytidine 5'-diphospho)-2C-methyl-D-erythritol (CM) synthase; CMK, CM kinase; MECS, 2C-methyl-D-erythritol-2,4-cyclodiphosphate (MEC) synthase; HDS, hydroxymethylbutenyl 4-diphosphate (HD) synthase; HDR, HD reductase; IDI, isopentenyl diphosphate isomerase; GPPS, geranyl diphosphate synthase; GES, geraniol synthase; G10H (CYP76B6), geraniol 10-hydroxylase; CPR, cytochrome P450-reductase; 10HGO, 10-hydroxygeraniol oxidoreductase; IO, iridoid oxidase; IS, iridoid synthase; 7DLGT, 7-deoxyloganic acid glucosyltransferase; 7DLH, 7-deoxyloganic acid 7-hydroxylase; LAMT, loganic acid O-methyltransferase; SLS (CYP72A1), secologanin synthase; TDC, tryptophan decarboxylase; STR, strictosidine synthase; SGD, strictosidine β -glucosidase; T16H2 (CYP71D351), tabersonine 16-hydroxylase 2; 16OMT, 16-hydroxytabersonine O-methyltransferase; NMT, 16-methoxy-2,3-dihydrotabersonine N-methyltransferase; D4H, desacetoxylvindoline 4-hydroxylase; DAT, deacetylvindoline 4-O-acetyltransferase. DMAPP, dimethylallyl diphosphate; GAP, glyceraldehyde 3-phosphate; IPP, isopentenyl diphosphate (Dugé de Bernonville et al., 2015b)

Loganic acid formation is the end of the IPAP-located steps of TIA biosynthesis, whereas 7DLH mRNA still was found in IPAP cells, the transcripts of the subsequent reaction were localized in leaf epidermis (Cordavault et al., 2014). The leaf epidermis hosts a central bloc of the TIA biosynthetic pathway that begins with loganic acid, and whose subcellular compartmentalization is more complex than in IPAP cells, implicating at least four distinct organelles/compartments (Besseau et al., 2013; Guirimand et al., 2011; Cordavault et al., 2014; Dugé de Bernonville et al., 2015b). The final steps leading to secologanin synthesis occur in the cytosol of epidermal cells and to achieve the strictosidine synthesis, the precursors (secologanin and tryptamine) appear to

be internalized into the vacuole where STR catalyses their condensation. To continue the pathway, strictosidine is exported from the vacuole and internalized into the nucleus giving rise to an aglycon that in turn is thought to leave the nucleus to the cytosol and suffer successive reactions leading to 16-methoxytabersonine. Finally, the two last steps of vindoline biosynthesis were shown to occur in laticifers and idioblasts (St. Pierre et al. 1999). Laticifers and idioblasts were long identified as the sites of alkaloid accumulation in *C. roseus* by Yoder and Mahlberg (1976) (Yoder et al., 2010). Idioblasts are identified by their distinctive blue autofluorescence and by their larger size and lower chloroplast content than the surrounding mesophyll cells (De Luca and St Pierre, 2000; Guirimand et al., 2011). The strong blue fluorescence of idioblasts and also of laticifers has been credited to the TIA serpentine (Brown et al., 1984). Taking advantage of this fluorescence properties, our laboratory has isolated protoplasts from leaf idioblasts by fluorescence activated cell sorting (FACS) and performed a differential transcriptomic screening that enabled the identification of several P450 candidates as mentioned above. In conclusion, the characterized TIA biosynthetic steps are marked by a complex subcellular distribution pattern (Figure 4) including soluble cytosolic enzymes (TDC, IS, 7DLGT, LAMT, D4H and DAT), endoplasmic reticulum anchored enzymes (G10H, IO, 7DLH, SLS, T16H1 and T16H2), plastidial enzymes (MEP pathway enzymes, GPPS, GES), vacuolar enzymes (STR and PEX1) and the nuclear SGD (Dugé de Bernonville et al., 2015b).

The complex subcellular compartmentation described above implies the existence of multiple steps of transmembrane transport of TIA intermediates, which requires transporter proteins poorly elucidated. So far, despite the existence of multiple intra and intercellular transports, only one transporter of the TIA pathway has been characterized to date, TPT2, that mediates the specific excretion of catharanthine at the leaf epidermis (Dugé de Bernonville et al., 2015a; Yu and De Luca, 2013). Particularly interesting is the case of transtonoplast transport, including several events along the pathway, namely the final accumulation in the vacuole, thought to be highly important in the regulation of the metabolic flux of the pathway. Meaningfully, the alkaloid nicotine was shown to be transported to the vacuole by a multidrug and toxic compound extrusion (MATE) family (Morita et al. 2009).

1.6 MATE transporters in plants

MATE transporters are H⁺ or Na⁺ antiports that take advantage of these cation gradients across the membranes to drive substrate movement (Lu et al., 2012). In plants, MATE transporters

seem to play several different physiological roles and display a high diversity including 58 different MATE transporter orthologs identified in Arabidopsis, whereas the human genome contains only 2 MATE transporters (Moriyama et al., 2008; Omote et al., 2006). This family of transporters has been shown to play a major role in the vacuolar transport of specialized metabolites in plants, namely in the vacuolar sequestration of the alkaloid nicotine through a H⁺ antiport mechanism (Morita et al. 2009, Shoji et al 2009). The likelihood of a MATE being involved in the vacuolar sequestration of another alkaloid, berberine, is very high, since it was demonstrated that a H⁺ antiport system is responsible for this process (Shitan and Yazaki, 2007; Shoji et al., 2008). Likewise, it was expectable that the alkaloids from *C. roseus* were also transported into the vacuole by a similar mechanism. In fact, our group recently demonstrated that the main TIAs from *C. roseus* leaves are accumulated in mesophyll vacuoles by a highly specific H⁺ antiport system, for which MATE transporters are chief candidates (Carqueijeiro et al., 2013). Being so, the knowledge of multidrug transporters presents in *C. roseus* will be preponderant to obtain a more reliable transporters network that may be combined with biosynthetic enzyme modules to establish effective metabolic engineering strategies to provide higher levels of plant products.

1.7 Virus Induced Gene Silencing (VIGS)

VIGS is a post transcriptional gene silencing (PTGS) method based on plant defense mechanisms against invading viruses that lead to the degradation of mRNA transcripts of a gene, or gene family. The gene to silence is targeted by a representative sequence inserted into a partially disarmed viral vector, that induces the silencing of transcript levels and the spreading through the plant, allowing the study of particular gene functions by the revealed phenotype (Liscombe and O'Connor, 2011; Lu, 2003; Senthil-kumar and Mysore, 2014). The transformed gene fragment is multiplied by the virus recruited replication machinery in the plant cell, and the transcripts are systemically spread throughout the plant. The silencing is achieved taking advantage of the viral systemic spreading in the plant and of its ability to stimulate the defense RNA silencing machinery (Figure 5) (Liscombe and O'Connor, 2011; Salim et al., 2013; Senthil-kumar and Mysore, 2014; Sung et al., 2014). The RNA-silencing process seems to be triggered by the production of double strand RNA (dsRNA) during the replication of single strand (ss) RNA virus (Matzke et al, 2001). The presence of dsRNA activates PTGs leading to dsRNA cleavage into small interfering RNA segments (siRNA) by Dicer-like enzyme-mediated cleavage. This siRNA binds to and activates the RNA-

induced silencing complex (RISC), which cleaves the viral RNA in a homology-dependent manner (Liscombe and O'Connor, 2011; Lu, 2003; Senthil-kumar and Mysore, 2014; Sung et al., 2014).

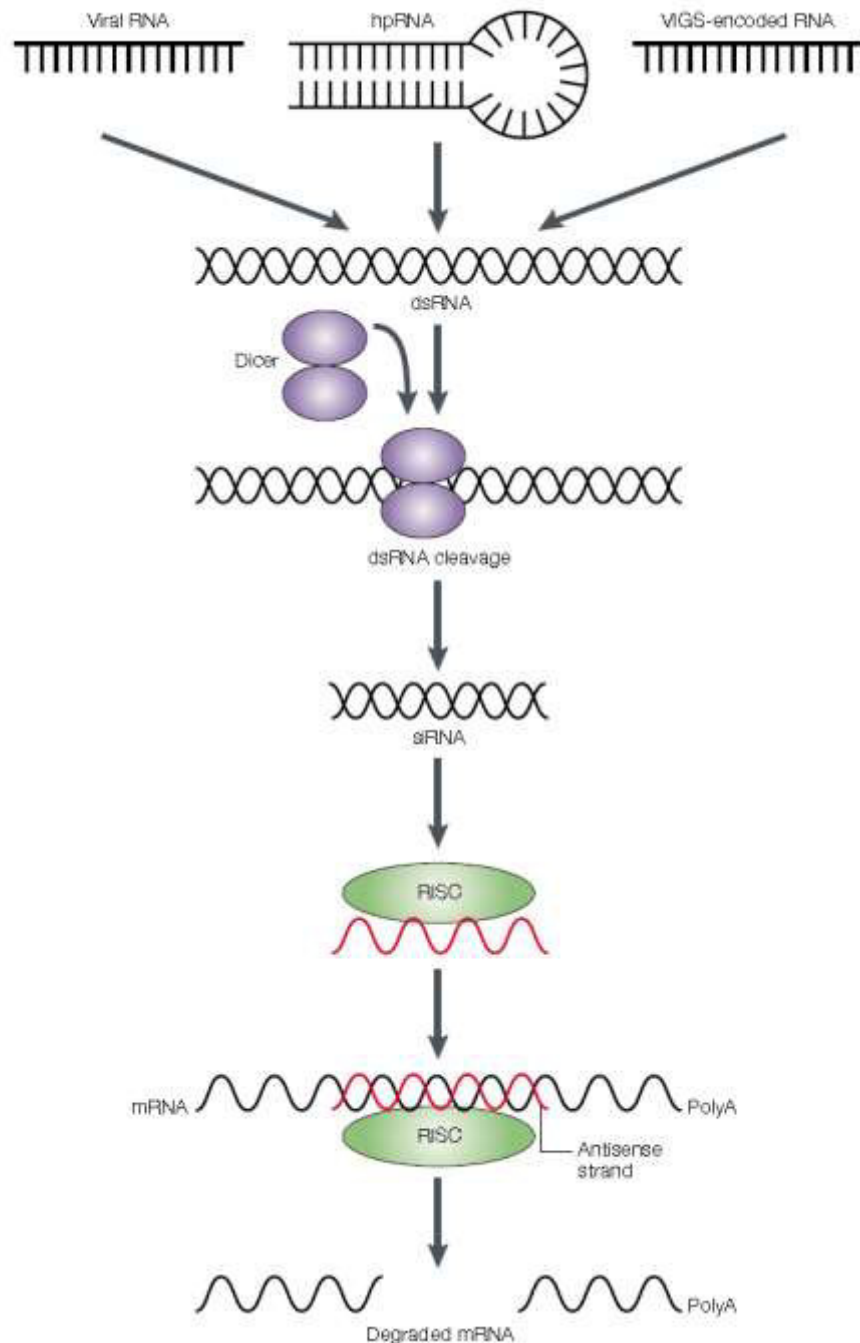


Figure 5 - The current model of RNA-mediated gene silencing in plants. This model is based on the results of *in vitro* studies of RNA-induced gene silencing, or RNA interference (RNAi), in animal extracts. RNAi is believed to operate in a similar manner in plants because small interfering RNAs (siRNAs) are found in silenced plants, and plants have homologues of the animal gene *Dicer*. Double-stranded RNAs (dsRNA) from replicating viral RNA, viral-vector-derived (VIGS, or virus-induced gene silencing) RNA or hairpin RNA (hpRNA) transcribed from a transgene, is processed by a Dicer-containing complex to generate siRNAs. An endonuclease-containing complex (called the RNAi silencing complex, RISC), is guided by the antisense strand of the siRNA to cleave specific mRNAs, so promoting their degradation (Waterhouse and Helliwell, 2003).

VIGS constitutes an efficient tool for gene function studies, gives fast results and avoids the need to produce transgenic plants, which is rather beneficial for recalcitrant species such as *C. roseus* (Dugé de Bernonville et al., 2015a). The success of the VIGS approach in functional analyses of *C. roseus* genes was first demonstrated by Liscombe and O'Connor, (2011), through the silencing of three known steps in vindoline biosynthesis (genes *NMT*, *D4H* and *DAT*). VIGS has been also successfully used to validate several candidate genes of the *C. roseus* TIA pathway selected through transcriptome resource analyses such as IS (Geu-Flores et al., 2012), CrTPT2 (Yu and De Luca, 2013), 7DLH (Salim et al., 2013) or IO (Salim et al., 2014), and candidates obtained via conventional homology-based PCR cloning, including 7DLGT (Asada et al., 2013) and a leaf-specific isoform of T16H (Besseau et al., 2013). Thus, VIGS has been used for functional validation of candidate genes and also to screen large sets of candidate genes identified being a key tool to fully clarify the TIA biosynthesis pathway.

1.8 Plant biotechnology for genetic improvement

Genetic manipulation of plants has been mainly conducted with models and with species of agronomic interest to improve specific crop traits. However, more recently, this approach has also been applied to medicinal plant species, in attempts to fully understand the specialized metabolite pathways and above all, to try to boost specialized metabolite yields of target plant species (Hansen, 1999; Zárate and Verpoorte, 2007). Genetic manipulation takes advantage of progress in plant molecular biology, in particular on the isolation and characterization of genes, together with development of DNA transfer techniques for plant cells, affording the breeding of an increasing number of genetically improved species (Pais, 2003). The process of manipulation requires an efficient gene transfer system, followed by regeneration of cells into fertile plants. Each transformation system has its advantages and applications, but also has some drawbacks (Zárate and Verpoorte, 2007). Direct transfer of foreign genetic material into plant cells is used in several transformation methods such as bombardment of plant cells with DNA-coated particles, protoplast fusion, electroporation and microinjection. When direct DNA transfer is used, transgenes tend to integrate as multiple copies, which are often fragmented and rearranged. In contrast, plant cell transformation using the bacterium *Agrobacterium tumefaciens* results mostly in single or low-copy integration of full-length T-DNA. However, *Agrobacterium*-mediated transformation is not efficient with all plant species. Especially monocotyledonous are less susceptible to *Agrobacterium*-mediated transformation, but some dicotyledonous are also recalcitrant (Van Der Fits et al., 2000).

Besides the ideal transformant should contain just a single copy of the transgene, the segregation should be mendelian and uniformly expressed from one generation to the next. Such characteristics are not easy to be found, are dependent on plant material and to some extent on the nature of the transgene (Hansen, 1999).

There is no reliable transformation process without a proper selection of transformants. In general, the gene of interest is co-inserted with a selectable marker to identify the growth of transformed cells. Selectable markers usually confer resistance to chemical agents, such as antibiotics or herbicides that inhibit various cellular functions. Scorable markers, such as GFP, are also used. Due to the increasing concerns about the use of antibiotic genes for selection, several approaches to replace selectable markers have been investigated (Hansen, 1999). The success of a transformation depends on an efficient and reliable tissue regeneration protocol for genetically transformed.

1.9 *Agrobacterium*-mediated transformation

Agrobacterium and *A. rhizogenes* are Gram negative soil bacteria belonging to the Rhizobiaceae family. These bacteria infect plants usually through a wound site, causing a plant disease known as crown gall tumors and hairy roots respectively. A stunning discovery was made four decades ago, that *Agrobacterium* has the ability to transfer a segment of its DNA, the T-DNA (transferred-DNA), to the host plant cells and to get it inserted into the plant nuclear genome. This skill involves bacteria-plant gene transfer and requires a highly specialized process involving a complex set of bacterial virulent proteins, which is not yet fully unraveled. Likewise, *A. rhizogenes* exhibits a similar gene transfer process (Hooykaas-Van Slogteren et al., 1984). The whole process is codified in a bacterial Ti (tumour inducing) plasmid, carrying the transferable T-DNA, which codifies a set of oncogenes and opine-catabolism genes, whose expression in plant cells leads to neoplastic growth of the transformed tissue and the production of opines, amino acid derivatives used almost exclusively by the bacteria as a nitrogen source. The transport of the T-DNA into the host cells and its random integration into a chromosome of plant cells is mediated by the products of the Ti plasmid virulence (vir) genes (Tzfira and Citovsky, 2006). Similarly, the T-DNA of the Ri (root inducing) plasmids possesses opine synthase genes and the rol genes, conferring the capacity to the transformed plants cells of differentiating into roots. Genes from the Ti plasmid that are

integrated in the plant chromosome are expressed at high levels in the plant (Hooykaas-Van Slogteren et al., 1984).

The manipulation of *Agrobacterium* natural functions allows the transfer of any foreign gene into plants. The Ti plasmid is genetically modified ("disarmed") by deleting the genes involved in the production of bacterial food and plant growth regulators, and inserting a gene of interest. A selectable marker, for the selection of transformed cells is also inserted. These genes generally code for proteins involved in breakdown of antibiotics, such as kanamycin. The deletion of the genes for tumor formation (disarmed Ti-Plasmid) were found to have no effect on the transfer efficiency from bacterium to plants (Swanberg and Dai, 2008). The *Agrobacterium* system is attractive because of the ease of the protocol coupled with minimal equipment costs. Moreover, transgenic plants obtained by this method often contain simple copy insertions. These advantages were a driving force to adapt this system to many different crops, including monocots. It is what has happened in the last decades, with a significant increase of reports on the successful *Agrobacterium*-mediated genetic transformation of many plant species, including, in *C. roseus* (Gelvin, 2003; Hooykaas-Van Slogteren et al., 1984).

1.10 Genetic transformation of *C. roseus*

The main aim pursued with *C. roseus* transformation is the overexpression of genes encoding key components of the highly complex TIA biosynthetic pathway, expecting to achieve a higher amount of the anticancer alkaloids VLB and VCR. Bioengineering has created binary expression vectors for plants, harbouring promoters that enhance the expression of the gene target. One of the most used promoters for constitutive overexpression is the cauliflower mosaic virus 35S promoter (Goddijn et al., 1995; Pan et al., 2012). For *C. roseus* transformation exist mainly two vias: *Agrobacterium* mediated transformation and particle bombardment, both with proven efficacy. To date, most of bioengineering investigation in *C. roseus* has been made using transformed cell suspension and hairy root cultures, due to the difficulty of regenerating whole plants (Tzfira and Citovsky, 2006; Verma and Mathur, 2011). However, cell suspensions are limited by the absence of cytodifferentiation, which is necessary for the expression of all TIA pathway genes and enzymes, especially those involved in the latter steps of the pathway leading to the synthesis of vindoline. Hairy roots are also limited by the fact that vindoline and anticancer TIAs are only produced in leaves. Unfortunately, the recalcitrant nature of cultured cells and hairy roots of *C. roseus* have

hampered the regeneration into whole transgenic plants until very recently (Verma and Mathur, 2011; Wang et al., 2012; Dhandapani et al., 2008).

1.11 Regeneration of *C. roseus*

So that the process of genetic transformation of plants is successful, it needs to be followed by an efficient regeneration protocol (Tzfira and Citovsky, 2006). An efficient protocol must obtain a whole plant from cells cultures or plant portions, using a suitable plants hormone scheme that will give rise to the differentiation of cultures/explants into a whole plant (roots, stems, leaves) (Pais, 2003). Plants can be regenerated from cell cultures via two methods, somatic embryogenesis and organogenesis (Hansen, 1999). Many factors, including genotype, medium, explant type, plant growth regulators (PGRs), and culture conditions, can affect plant regeneration (Swanberg and Dai, 2008). The somatic embryogenesis regeneration through embryogenic tissues is, in general, very efficient, allowing recovery of many transformants that are, in most cases, non-chimeric, because of the assumed single cell origin of somatic embryos. This is the tissue culture regeneration strategy usually chosen for monocots, because *callus* is easily initiated from the scutellum of immature embryos after exposure to auxin. The choice of the specific auxin and its concentration is related with genotype and the species. Auxin is then reduced or withdrawn and a cytokinin is initiated to allow the generation of shoots from callus for the subsequent recovery of plants. However, the use of embryogenic tissue can have some drawbacks. First, it is very labour intensive to establish and maintain the culture. Second, the recovery of plants can be a long process, with risk of resulting in morphological abnormalities and contamination. Third, this system requires a constant source of material to initiate new embryogenic cultures. These limitations have driven the use of mature embryos from seeds as an alternate, even though their response might not be very fruitful (Hansen, 1999).

Another alternative is organogenesis, the generation of organs from a variety of tissues. Organ regeneration in plants can be broadly categorised as direct or indirect. In the first case, shoots or roots are directly induced from tissue explants, whereas indirect organogenesis involves callus formation as an intermediate prior to shoot or root induction (Perianez-Rodriguez et al., 2014). Regeneration assays demonstrated that *de novo* shoot meristem formation requires elevated levels of both auxins and cytokinins, and that these hormones have antagonistic as well as synergistic roles. Both are required for cell division and play roles in meristem establishment

and activity, but differing cytokinin to auxin ratios favour development of either root or shoot meristems. In general, increasing the ratio of cytokinin to auxin results in a shift from root to shoot organogenesis (Hill and Schaller, 2013). Explants as cotyledons, leaf fragments, hypocotyls and scutella from embryos generally have the potential to generate shoots when placed on medium containing a proper balance between cytokinin and auxin. The advantage of this system is that shoots can usually form roots quickly (Hansen, 1999).

Regeneration of *C. roseus* into whole transgenic plants is still a challenge, especially through organogenesis using vegetative tissues. Over the years, numerous concentrations of auxins, cytokinins, TZD and other PGR's as well as different cultivars and explant types, were been tested (Swanberg and Dai, 2008). *C. roseus* organogenesis was first reported in the late 1970s, however, the shoot regeneration rate was low. Much later, in Dhandapani et al. (2008), hypocotyls and cotyledons of the *C. roseus* cultivar 'little bright eye' generated whole plants via organogenesis, through balance between cytokinin and auxin, 6-benzylaminopurine (BA) and naphthaleneacetic acid (NAA) respectively. Mainly two factors, the type of plant growth regulators (combination and concentration), and the genotype specificity contributed to the final results. Still, in most of these reports, the formation of shoots and plantlets is rather infrequent and transient, besides requiring long duration. Recent research of Verma and Mathur, (2011) reported for the first time a protocol for high-frequency shoot bud regeneration from the leaves of *C. roseus*. The establishment of direct plant regeneration via adventitious shoot bud formation is crucial for application of genetic transformation techniques. Direct shoot bud organogenesis also helps in avoiding the interference of somaclonal variation in the transformation process when an intervening callus interface also becomes involved in the regeneration. However, the entire plant regeneration protocol from explants-to-whole plants took about 4 months (Verma and Mathur, 2011).

Wang et al., (2012) developed an *Agrobacterium*-mediated transformation and a stable regeneration system of *C. roseus*. In this protocol hypocotyls of *C. roseus* were used to generate an indirect organogenesis method to obtain transformed whole plants. To achieve the successful of the transformation and regeneration, it was checked several parameters, such as, *Agrobacterium* density and co-culture time, the effects of sonication and the influence of kanamycin concentration on plant regeneration. Besides obtaining the regenerated plants, this protocol also confirmed the efficiency of transformation system by biochemical assays, which until then had not been carried out (Wang et al., 2012).

Despite all the advances, the regeneration protocols accomplished for *C. roseus* still have several difficulties to be reproducible, and so, it is important to maintain the investigations in *C. roseus* regeneration.

1.12 Objectives

The main goal of this work was to obtain *C. roseus* plants and cell cultures genetically transformed and overexpressing genes presumably acting in the late steps of the TIA pathway, with the ultimate aim of obtaining an enriched source of anticancer TIAs. The genes used were: *MATE1*, whose protein product was previously identified in the vacuole proteome of *C. roseus* leaf cells and is candidate to the vacuolar accumulation of the major TIAs present in the leaves; and *CYP2*, previously identified in the TIA accumulating idioblast cells, and candidate to the late bottleneck reactions in the biosynthesis of the anticancer alkaloids VLB and VCR. A complementary goal was to investigate the functional role of *MATE1* in TIA biosynthesis, by determining the impact of its VIGS-mediated silencing in TIA levels and profile.

To achieve the first goal, the work developed involved a step-by-step framework including: i) sub-cloning of *MATE1* and *CYP2* genes into a modified binary vector pCAMBIA2301 modified, ii) optimization of a protocol of *Agrobacterium*-mediated genetic transformation of *C. roseus* hypocotyls, iii) optimization attempts of a regeneration protocol from the hypocotyls into whole plants through indirect organogenesis, and of a protocol for the production of transgenic *calli*, iv) analysis of the transformed *calli* using the GUS assay.

The second goal was achieved by: i) sub-cloning the *MATE1* gene into the viral vector pTRV2:GFP containing the tobacco rattle virus 2 genome, ii) inoculation of plants with the transformed *Agrobacterium* strain, iii) analysis and quantification of the alkaloid levels and profile present in inoculated and control plants.

2. Materials and methods

2.1 Transformation and regeneration of *Catharanthus roseus*

2.1.1 Biological material

2.1.1.1 Plant material

Seeds of *Catharanthus roseus* (L.) G. Don cv. Pacifica Cherry Red were gently offered by Panamerican Seed co. Plants were grown at 25 °C in a growth chamber, under a 16 h light and 8 h dark photoperiod, using white fluorescent light with a maximum intensity of 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

2.1.1.2 Bacterial strains and plasmid

In this work, *Escherichia coli* TOP10 was used for cloning procedures and *Agrobacterium* EHA 105 was used to perform plant transformation. Chemically competent *E. coli* cells were prepared according to a protocol adapted from Hanahan et al. (1991), described in Appendix 1, while *Agrobacterium* competent cells were chemically obtained following the protocol described in Appendix 2.

In order to generate the constructs to be used in plant transformation, *MATE1* and *CYP2* coding sequences were sub-cloned in the modified binary vector pCAMBIA2301 (pCAMBIA2301m, Figure 6). This vector harbours a GUS reporter gene and the selection marker gene NPTII (neomycin phosphotransferase gene conferring resistance to kanamycin). An intron is included in the GUS reporter sequence to ensure that glucuronidase activity occurs only in eukaryotic cells. The pCAMBIA2301m was kindly provided by Professor Pattanaik (Suttipanta et al., 2011) and it was obtained by replacing the original MCS by CaMV35S promoter-MCS-rbcS terminator cassette from pKYLX71 (<http://www.uky.edu/~ag hunt00/ky lx.html>). Unique restriction sites in the MCS are *HindIII*, *BamHI*, *PstI*, *SacI* and *XbaI*. This vector enables expression in plant systems.

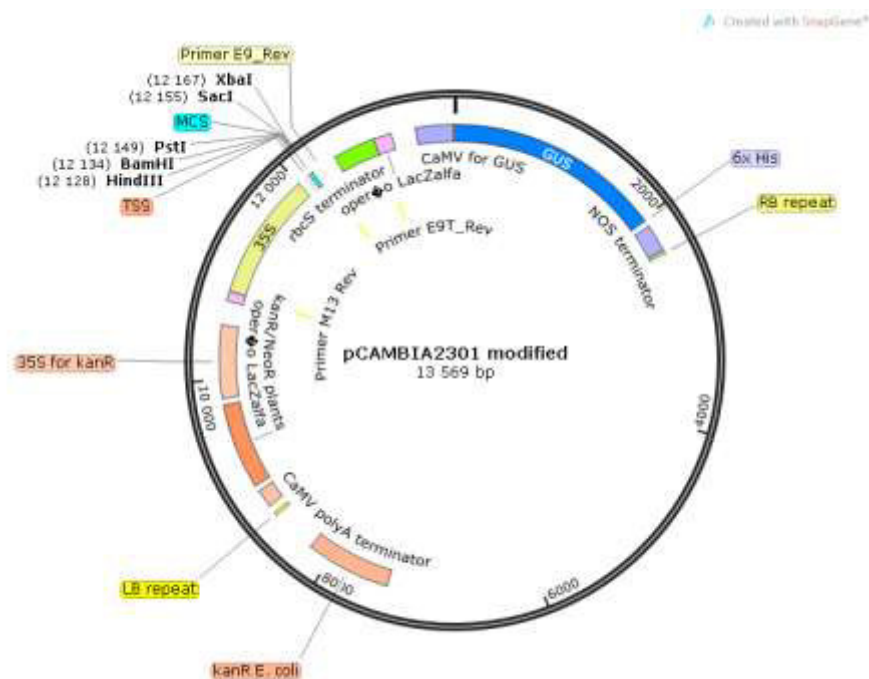


Figure 6 - Schematic representation of pCAMBIA2301m expression vector. The figure was created using the bioinformatics tool SnapGene®.

2.1.2. *MATE1* and *CYP2* genes

MATE1 (cra_locus_1952_iso_7_len_1704_ver_3, Appendix 3) and *CYP2* (cra_locus_4293_iso_2_len_2076_ver_3, Appendix 4) coding sequences were obtained from the Medicinal Plant Genomic Resource database (MPGR). These genes were firstly reported at the Bioactive Natural Products (BioNatPro) lab by Carqueijeiro (2013), following the proteomic analysis of *C. roseus* vacuoles.

2.1.3 Primer design

The restriction analyses of *MATE1* and *CYP2* coding sequences were performed with NEBcutter V2.0 (<http://tools.neb.com/NEBcutter2/>). Primers used for the amplification and directional cloning of *MATE1* and *CYP2* into pCAMBIA2301m are shown in Table 1.

Table 1 - Primers used to amplify *MATE1* and *CYP2* to clone in pCAMBIA2301m. Endonuclease restriction sites are underlined. Additional nucleotides at the 5' terminus to allow site recognition by the restriction enzyme are in red.

Primer	Sequence (5'→3')	Size (bp)
CYP2-Fwd-PstI	GCCTGCAGATGGATGTTGATATTCTTCTTCTC	33
CYP2-Rev-XbaI	GGCGTCTAGAAATCAGAGTTTAAACAGGAATGAC	32
MATE1-Fwd-SacI	CCGAGCTCATGGGTTCCAAACAAACTATG	30
MATE1-Rev-XbaI	GGCGTCTAGATTATTCATTGGACAAAGATTTGGC	35

Primer features were evaluated using OligoAnalyzer 3.1 (<http://eu.idtdna.com/analyzer/applications/oligoanalyzer/>, IDT®) (Table 2).

Table 2 - Primer features as in OligoAnalyzer 3.1.

Primer	Size (bp)	Tm (°C)	GCs (%)	Hairpin kcal/mole	Homo-dimer kcal/mole	Hetero-dimer kcal/mole
CYP2-Fwd-PstI	33	60.3	42.4	-0.21	-10.24	-6.62
CYP2-Rev-XbaI	32	59.9	43.8	-1.62	-7.31	
MATE1-Fwd-SacI	30	61.2	46.7	-0.55	-9.49	-8.91
MATE1-Rev-XbaI	35	60.8	40.0	-1.63	-7.31	

2.1.4 Polymerase Chain Reaction (PCR)

MATE1 and *CYP2* amplification was performed as described in Table 3. The PCR program consisted of one cycle at 95 °C for 3 min, 35 cycles at 95 °C for 30 s, 56 °C for 30 s, 72 °C 2.5 min and a final extension for 7 min. The reactions were performed in a T100™ thermal cycler (Bio-Rad). PCR products were ran in a 1% (w/v) agarose (Bio-rad) gel electrophoresis in 1x TAE buffer (40 mM Tris base, 10% (v/v) acetic acid and 10 mM EDTA) supplemented with 0.5 µg mL⁻¹ ethidium bromide (BioRad) to allow visualization of the DNA under UV light. The GeneRuler™ 1kb DNA Ladder (Thermo Scientific) was used as molecular marker and the electrophoretic run was performed at 80-100 V (PowerPac Basic, BioRad). The DNA bands were observed using a UV transilluminator.

Table 3 - PCR optimized setup for amplification of the *MATE1* and *CYP2*.

Reagents	Volume (μl)
ddH ₂ O	36.8
10x DreamTaq Buffer	5
10mM dNTPs	2
10μM primer FWD	2
10μM primer REV	2
cDNA template	2
<i>Dream Taq</i> DNA polymerase	0.2
Total	50

2.1.5 Purification of PCR products

PCR products were recovered and purified from the agarose gel using the GeneJET™ Gel extraction kit (Thermo Scientific), according to the manufacturer's instructions.

2.1.6 Molecular cloning

2.1.6.1 Digestion of the PCR products and of the expression vector

In order to insert *MATE1* and *CYP2* coding sequences into pCAMBIA2301m, PCR products and plasmids were digested with the appropriate restriction enzymes. The double digestion reaction conditions (Table 4) were analysed in Double Digestion web tool (<http://www.thermoscientificbio.com/webtools/doubledigest/>).

Table 4 - Double digestion reaction conditions.

Double digestion for <i>MATE1</i>			Double digestion for <i>CYP2</i>		
Reagents	PCR product Volume (μl)	Plasmid Volume (μl)	Reagents	PCR product Volume (μl)	Plasmid Volume (μl)
ddH ₂ O	14.5	28	ddH ₂ O	14.4	14
10x Tango™ Buffer	2	4	10X Tango™ Buffer	2	2
<i>SacI</i>	0.65	2.6	<i>PstI</i>	0.65	1.3
<i>XbaI</i>	0.35	1.4	<i>XbaI</i>	0.35	0.7
DNA	2.5	4	DNA	2.6	2
Total	20	40	Total	20	20

The digestion reactions were incubated at 37 °C during 90 min. In order to avoid plasmid re-ligation, 1 µL of Thermosensitive Alkaline Phosphatase (FastAP, Thermo Scientific) was added to the reaction in the last 30 minutes of incubation. After this reaction time, both samples were incubated at 65 °C for 20 min to inactivate the enzymes. The products of the digestion reactions were separated in a 1 % agarose gel, and the DNA of interest was recovered using the GeneJET™ Gel extraction kit.

2.1.6.2 Ligation reaction

Ligation reactions were performed using T4 DNA ligase (Thermo Scientific), as recommended by the manufacturer (Table 5). The insert:vector ratio used was 7:1 (v:v). Ligations were incubated at room temperature (RT) for 5 h and were heat inactivated at 65 °C for 20 min.

Table 5 - Ligation reaction conditions.

<i>MATE1</i> - vector ligation		<i>CYP2</i> - vector ligation	
Reagents	Volumes (µl)	Reagents	Volume (µl)
ddH ₂ O	9	ddH ₂ O	9
10x T4 DNA ligase Buffer	2	10x T4 DNA ligase Buffer	2
T4 DNA ligase	1	T4 DNA ligase	1
pCAMBIA2301m (<i>SacI/XbaI</i>)	1	pCAMBIA2301m (<i>PstI/XbaI</i>)	1
<i>MATE1</i> (<i>SacI/XbaI</i>)	7	<i>CYP2</i> (<i>PstI/XbaI</i>)	7
Total	20	Total	20

2.1.6.3 Transformation of *E. coli*

The heat shock method was used to transform chemically competent *E. coli* TOP10 cells. For each ligation, 50-100 µL of chemically competent TOP10 cells were mixed with 5 µL of the ligation products. After an incubation of 30 min on ice, the mixture was heat-shocked at 42 °C for 45 sec and placed back on ice for 2 min. One mL of *Luria-Bertrani* (LB) medium (10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, 10 g L⁻¹ NaCl) was added to the mixture and the cells were left to recover for 1 h at 37 °C. The cell suspension was then centrifuged at 3000 rpm for 3 min at RT, and 900 µL of supernatant was removed. The cells were resuspended in the remainder volume, were plated onto LB-agar (LB with 1.5 % agar; Liofilchem) supplemented with 50 µg mL⁻¹ of kanamycin (Sigma) to select the transformants, and the plates were incubated overnight (ON) at 37 °C.

2.1.6.4 Selection of positive clones

With the intention to test a correct insertion of *MATE1* and *CYP2* into expression vector a colony PCR was performed. Single isolated colonies were picked to transfer a small amount of bacteria into a PCR tube with 10 μ L of ddH₂O, and to re-plate onto LB-agar supplemented with kanamycin 50 μ g mL⁻¹. *MATE1* and *CYP2* amplification was performed as described in Table 6. The PCR program consisted of one cycle at 95 °C for 3 min, 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C 5 min for *MATE1* and 4 min for *CYP2* and a final extension for 7 min. Positive clones were identified by gel electrophoresis as described above (section 2.1.5). Three positives colonies of each construct were picked to inoculate 5 mL of LB medium supplemented with 50 μ g mL⁻¹ kanamycin. Cultures were grown ON at 37 °C with vigorous shaking (200 rpm). Plasmid DNA was recovered by the alkaline lysis miniprep method using the GeneJET Plasmid Miniprep Kit (Thermo Scientific) according to the manufacturer's instructions. Cloning of *MATE1* and *CYP2* into pCAMBIA2301m was confirmed by restriction analysis of the miniprep DNA. Plasmid DNA was digested with *Xho*I plus *Xba*I for *MATE1*, and *Xho*I for *CYP2*. The positive clones were sent to be sequenced (STABVida). The analysis of sequence homology between the predicted sequences and the obtained sequences was performed using the multiple sequence alignment program MultiAlin (<http://multalin.toulouse.inra.fr/multalin/>).

Table 6 – Colony PCR optimized setup for amplification of the *MATE1* and *CYP*.

Reagents	Volume (μ l)
ddH ₂ O	6,7
10X DreamTaq Buffer	2,5
10mM dNTPs	0,4
10 μ M M13 FWD	0,4
10 μ M M13 REV	0,4
DNA	10
<i>Dream Taq</i> DNA polymerase	0,1
Total	25

2.1.7 Transformation and regeneration of *C. roseus* explants

2.1.7.1 Seed sterilization and germination

Seeds were sterilized with 75 % (v/v) ethanol for 1 min followed by 10 % (v/v) NaClO for 10 min. Then they were washed three times with sterile distilled water. Finally, seeds were sown in MS (Murashige&Skoog) culture medium (4.4 g L⁻¹ MS medium salt mixture, 30 g L⁻¹ sucrose with 8.5 g L⁻¹ agar) and were germinated under 16 h light and 8 h dark photoperiod at 25 °C. The hypocotyls were extracted and cut into small fragments of *ca.* 5 mm which were used in the transformation protocol.

2.1.7.2 Transformation of *Agrobacterium*

Chemically competent *Agrobacterium* cells were transformed by freeze/thaw method (Hofgen and Willmitzer, 1988). One µL of plasmid DNA (from section 2.1.6.4) was mixed with *Agrobacterium* cells, and the mixture was incubated for 5 min on ice, then was placed in liquid nitrogen for 5 min, and finally was heat-shocked at 37 °C for 5 min. One mL of LB medium was added to the mixture and the cells were left to recover for 2-3 h at 28 °C. The cell suspension was centrifuged at 4000 rpm for 3 min at RT and 900 µL of supernatant was removed. The cells were resuspended in the remainder volume, were plated onto LB-agar supplemented with 100 µg mL⁻¹ rifampicin (Duchefa) and 100 µg mL⁻¹ kanamycin (Sigma), and the plates were incubated for two days at 28 °C.

2.1.7.3 Transformation and co-cultivation of *C. roseus* explants

Agrobacterium-mediated transformation of *C. roseus* explants was performed according to Wang et al. (2012), with some modifications. One colony of each construct was picked to inoculate 5 mL of LB medium supplemented with 100 µg mL⁻¹ rifampicin (Duchefa) and 100 µg mL⁻¹ kanamycin (Sigma). The cultures were incubated ON at 28°C with shaking. Cell cultures were diluted 10 fold and the OD₆₀₀ was measured, initially to obtain a final OD of 0.8. Also was tested a final OD of 0.2-0.3. Cells were centrifuged at 2000 g for 10 min at RT and the supernatant was discarded. The cells were resuspended in liquid MS medium containing 100 µM acetosyringone (Aldrich). Cells were incubated for 2 h in the dark at 28 °C. Explants were immersed into liquid MS medium supplemented with 100 µM acetosyringone (Aldrich) in Eppendorf tubes and sonication was applied for 0, 1, 5 or 10 min. The explants were transferred into sterile plates containing the bacterial suspension and were gently shaken at 25 rpm for 30 min at RT. The explants were blot-

dried with sterile paper towels and transferred onto Petri dishes containing $\frac{1}{2}$ MS medium (2.2 g L⁻¹ MS medium salt mixture, 30 g L⁻¹ sucrose and 8.5 g L⁻¹ of agar) supplemented with 100 μ M acetosyringone (Aldrich). Explants were co-cultured for two days in the dark at 28 °C.

2.1.7.4 *C. roseus* regeneration

After co-cultivation, the explants were washed with sterile water and 250 mg L⁻¹ carbenicillin (Duchefa) or 250 mg L⁻¹ cefotaxime (Duchefa). Then, the explants were dried with sterile towels and were transferred to several *callus* induction media. The composition of MSCP1 basal medium (Wang et al., 2012) is shown in Table 7.

Table 7 - MSCP1 basal medium composition.

MSCP1 basal medium	
MS*	4.4 g L ⁻¹
Casein hydrolysate	150 mg L ⁻¹
L-proline	250 mg L ⁻¹
Sucrose	30 g L ⁻¹
2,4-D*	1 mg L ⁻¹
NAA	1 mg L ⁻¹
Zeatin*	0.1 mg L ⁻¹
Carbenicillin*	250 mg L ⁻¹
Kanamycin	40 mg L ⁻¹
Agar*	8.5 g L ⁻¹

* Item subject to optimization.

The optimization of the regeneration protocol led to several changes of the medium composition. Firstly, carbenicillin (Duchefa) concentration was increased to 500 mg L⁻¹ and 500 mg L⁻¹ cefotaxime (Duchefa) was tested to reduce *Agrobacterium* growth. Secondly, were tested different concentrations of phytohormones in order to increase *callus* generation. The agar concentration was reduced to 7.5 g L⁻¹, and MS medium salt mixture was replaced by MS medium including vitamins. Thirdly, 0.1 g L⁻¹ of myo-inositol (Sigma) was added together with the antioxidants citric acid (Sigma) and ascorbic acid (Merck) in order to control the browning of the explants. All these changes are described in Table 8. Additionally, a kanamycin resistant actively

growing callus of *Nicotiana tabacum* BY2 cell line (purchased to RIKEN) previously transformed with the empty vector pCambia2301m, was used as nurse culture: a piece of the BY2 *callus* was placed in the middle of each Petri dish and was surrounded by the hypocotyl explants. Cultures were incubated in the dark at 25 °C for *ca.* two weeks.

Table 8 - Composition of the several *media* tested in the *callus* generation

MSCP1 basal medium		A1	A2	B	C	D	E	F	G	H	I
MS (g L ⁻¹)	4.4	4.4		4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4
		MS without vitamins						MS with vitamins			
2,4-D (mg L ⁻¹)	1	1	1	1	3	3	1	3	3	1	1
Zeatin (mg L ⁻¹)	0.1	0.1	0.1	0.1	0.1	1	0.1	0.1	1	0.1	0.1
Ascorbic acid (mg L ⁻¹)	-	-	-	-	-	-	-	-	-	100	50
<i>Myo</i> -inositol (mg L ⁻¹)	-	-	-	-	-	-	-	-	-	100	100
Citric acid (mg L ⁻¹)	-	-	-	-	-	-	-	-	-	50	-
Carbenicillin (mg L ⁻¹)	250	-	500	250	250	250	250	250	250	250	250
Cefotaxime (mg L ⁻¹)	-	500	-	-	-	-	-	-	-	-	-
Agar (g L ⁻¹)	8.5	8.5	8.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5

For shoot induction, the starting *calli* were transferred to the MSCP2 basal medium (Wang et al., 2012) which composition is shown in Table 9.

Table 9 - MSCP2 basal medium composition.

MSCP2 basal medium	
MS*	4.4 g L ⁻¹
Casein hydrolysate	150 mg L ⁻¹
L-proline	250 mg L ⁻¹
Sucrose	30 g L ⁻¹
BA*	5 mg L ⁻¹
NAA*	0.5 mg L ⁻¹
Carbenicillin*	250 mg L ⁻¹
Kanamycin	70 mg L ⁻¹
Agar*	8.5 g L ⁻¹

* Item subject to optimization.

MSCP2 basal medium also underwent some changes. Firstly, carbenicillin concentration was increased to 500 mg L⁻¹ and cefotaxime 500 mg L⁻¹ was tested. Secondly *myo*-inositol and antioxidants were added, and the agar concentration was reduced. Thirdly, were tested others

phytohormone (TDZ and Zeatin) as well as the concentration of initial phytohormones were altered. All these changes are described in Table 10. The *calli* were grown under a photoperiod of 16 h light and 8 h dark at 25 °C and subcultured every 3 weeks.

Table 10 – Composition of the several media tested in the induction of shoots.

MSCP2 basal medium		1.1	1.2	2	3	4	5	6	7	8	9
MS (g L ⁻¹)	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4
	MS without vitamins			MS with vitamins							
BA (mg L ⁻¹)	5	5	5	5	-	-	-	0.5	1	-	-
NAA (mg L ⁻¹)	0.5	0.5	0,5	0.5	-	-	0.5	-	-	0.5	1
Zeatin (mg L ⁻¹)	-	-	-	-	-	-	5	-	-	-	-
TDZ (μM)	-	-	-	-	5	7.5	-	0.1	0.5	1	2.5
<i>Myo</i> -inositol (mg L ⁻¹)	-	-	-	100	-	-	-	-	-	-	-
Citric acid (mg L ⁻¹)	-	-	-	50	-	-	-	-	-	-	-
Ascorbic acid (mg L ⁻¹)	-	-	-	100	-	-	-	-	-	-	-
Carbenicilline (mg L ⁻¹)	250	500	-	250	250	250	250	250	250	250	250
Cefotaxime (mg L ⁻¹)	-	-	500	-	-	-	-	-	-	-	-
Agar (g L ⁻¹)*	8.5	8.5	8.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5

2.1.7.5 Analysis of putative transformed *calli*

In order to test if the *calli* were transformed, a nondestructive GUS staining protocol was performed using X-glcA (Duchefa) as substrate, adapted from Jefferson et al (1987). *C. roseus calli* were incubated with 200 μg mL⁻¹ X-glcA in 20 mM NaPO₄ pH 7.0 at RT until they turned blue. Transformed *calli* were washed with liquid MSCP2 medium, blot-dried, and placed on new plates with induction shoot medium containing 50 μg mL⁻¹ X-glcA.

2.2 Virus Induction Gene Silencing (VIGS)

2.2.1 Biological material

2.2.1.1 Plant Material

Plants of *C. roseus* (L.) G. Don cv. Little Bright Eye were grown at 25°C in a growth chamber, under a 16 h light and 8 h dark photoperiod, using white fluorescent light with a maximum intensity of 70 μmol m⁻² s⁻¹. Seeds were acquired from B&T World Seeds (France). For VIGS experiments plants around 9 weeks were used.

2.2.1.2 Bacterial strains and plasmids

E. coli TOP10 was used for cloning procedures and *Agrobacterium* GV3101 was used to perform plant transformation.

The plasmids used for VIGS were pTRV1 and pTRV2. *TRV1* encodes 134 and 194 kDa replicase proteins from the genomic RNA, 29 kDa movement protein, and 16 kDa cysteine-rich protein from subgenomic RNAs. *TRV2* encodes the coat protein (CP) from the genomic RNA and two nonstructural proteins from subgenomic RNAs (Figure 7A). To develop the TRV-based VIGS vector, the cDNA of *TRV1* and *TRV2* were placed between a duplicated CaMV 35S promoter (2X35S) and the nopaline synthase terminator (NOST) generating pTRV1 and pTRV2. In pTRV2, the nonessential structural genes were replaced with MCS for cloning of the target gene sequences (Figure 7B). Further, a self-cleaving ribozyme site was included at the 3' end of both vectors.

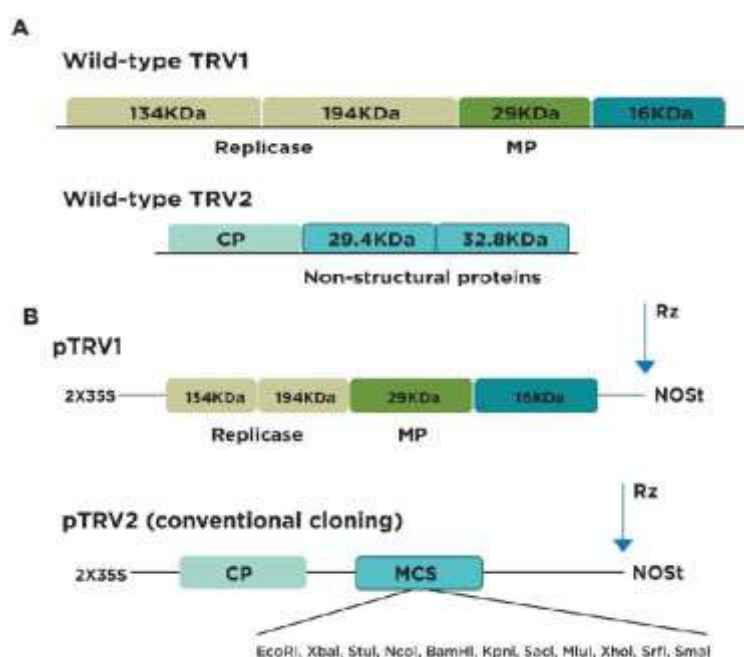


Figure 7 - Schematic representation of TRV1 and TRV2 (A), and of pTRV1 and pTRV2 vectors (B). Adapted from Senthil-Kumar and Mysore (2014).

In this work, the vector used to clone the target gene was a modified pTRV2 vector, pTRV2:GFP, generated by fusing the enhanced green fluorescent protein (EGFP) coding sequence to the 3' terminus of the CP sequence in the pTRV2 plasmid. This vector was developed and kindly provided by Professor Nan Ma (Tian et al., 2013) (Figure 8). pTRV1 was kindly offered by Professor Daniele Werck from the Institute de Biologie Moléculaire des Plantes, France.

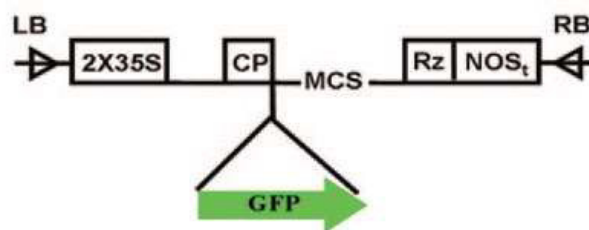


Figure 8 - Schematic representation of pTRV2:GFP vector. Adapted from Tiang et al. (2013).

2.2.2 Primer design and cloning

For the silencing of the *MATE1* gene, a fragment with 412 bp of this gene was selected and cloned into the modified pTRV2 vector. This appropriate gene fragment is localized in the coding sequence of *MATE1* (Appendix 5). The homology with other genes of the same family was analysed using the blast tool available at the ORCAE database (<http://bioinformatics.psb.ugent.be/orcae/overview/Catro>). Primers to clone the chosen fragment were manually designed including endonuclease restriction sites to allow directional cloning (Table 11). The restriction enzymes were chosen because they cut only once at the MCS and do not cut the *MATE1* fragment. Primer parameters were evaluated using the OligoAnalyzer tool.

Table 11 - Primers used to amplify the *MATE1* fragment to clone into modified pTRV2:GFP. Endonuclease restriction sites are underlined. Additional nucleotides at the 5' terminus to allow site recognition by the restriction enzyme are in red.

Primer	Sequence (5'→3')	Size (bp)	GC%	Tm (°C)	Self-dimer (kcal/mo)	Hairpin	Hetero-dimer kcal/mole
MATE_1_VIGS_Ec oRI_CDS_FWD_2	CGCCGAATTCATGGGTCCAAACAAACTA	30	43.3	61.2	-8.51	-0.55	-7.18
MATE_1_VIGS_C DS_XhoI_REV_1	GGCTCGAGGTCCACATAGTGTTCCTG	29	55.2	63.5	-9.96	-2.3	

2.2.3 PCR

MATE1 fragment amplification was performed as described in Table 12. The PCR program consisted of one cycle at 95 °C for 3 min, 35 cycles at 95 °C for 30 s, 56 °C for 30 s, 72 °C for 75 s and a final extension of 7 min. PCR products were ran in a 2 % agarose gel, recovered and

purified using the GeneJET™ Gel extraction kit (Thermo Scientific) following the manufacturer's instructions.

Table 12 - PCR optimized setup for amplification of the MATE1 fragment

Reagents	Volume (μl)
ddH ₂ O	45.5
10X Pfu Buffer + MgSO ₄	6.25
10mM dNTPs	2.5
10 μM primer FWD	2.5
10 μM primer REV	2.5
cDNA	2.5
<i>Pfu</i> DNA polymerase	0.75
Total	50

2.2.4 Molecular cloning

2.2.4.1 Digestion and ligation of the PCR products and of the expression vector

In order to insert the cloning fragment of *MATE1* into pTRV2:GFP vector, the purified PCR product of *MATE1* fragment and the vector were double digested with *EcoRI* and *XhoI* restriction enzymes, following the manufacturer's instructions. The digestion reactions were composed as described in the Table 13 and were incubated at 37 °C during 3 h.

Table 13 - Double digestion reaction conditions.

MATE1 digestion		pTRV2:GFP digestion	
Reagents	Volume (μl)	Reagents	Volume (μl)
ddH ₂ O	8	ddH ₂ O	23
10X Tango™ Buffer	8	10X Tango™ Buffer	8
<i>EcoRI</i>	2	<i>PstI</i>	2
<i>XhoI</i>	2	<i>XbaI</i>	2
DNA	20	DNA	5
Total	40	Total	40

To avoid plasmid re-ligation, 1 μL of FAST AP (Thermo Scientific) was added to the reaction in the last hour of incubation. After this reaction time, enzymes were inactivated by adding 20 mM EDTA pH 8.0. The products of digestion were purified using the GeneJET™ Gel extraction kit, according to the manufacturer's instructions. Ligation of *MATE1* fragment into pTRV2:GFP was

performed with T4 DNA ligase (Table 14). Ligations were incubated at RT for 2 h 30 min and finally were inactivated at 65 °C for 20 min.

Table 14 - Ligation reaction of MATE1 into pTRV2:GFP vector, using a 8:1 molar ratio.

MATE1-pTRV2:GFP ligation	
Reagents	Volume (μl)
ddH ₂ O	3.5
10x T4 DNA ligase Buffer	1
T4 DNA ligase	1
pTRV2:GFP	0.5
MATE1	4
Total	10

2.2.4.2 Transformation of *E. coli*

Chemically competent *E. coli* TOP10 cells were transformed as described in section 2.1.6.3. The transformed cells were plated onto LB-agar supplemented with 50 μg mL⁻¹ of kanamycin and the plates were incubated ON at 37 °C.

2.2.4.3 Selection of positive clones

Three colonies of MATE1-pTRV2:GFP were picked and grown in 5 mL of LB medium supplemented with 50 μg mL⁻¹ of kanamycin. Cultures were incubated ON at 37 °C with vigorous shaking (200 rpm). Plasmid DNA was recovered by the alkaline lysis miniprep method using the GeneJET Plasmid Miniprep Kit, according to manufacturer instructions. Cloning of the *MATE1* fragment into pTRV2:GFP vector was confirmed by restriction analysis with *EcoRI* and *XhoI* (Table 15). The reactions were promptly incubated at 37 °C for 1 h 30 min, following an incubation at 65 °C for 20 min to inactivate the enzymes.

Table 15 - Double digestion reaction conditions.

MATE1-pTRV2:GFP digestion	
Reagents	Volume (μl)
ddH ₂ O	13
10X Tango™ Buffer	4
<i>EcoRI</i>	1
<i>XhoI</i>	1
DNA	1
Total	20

The digested products were ran in a 2 % (w/v) agarose gel prepared with 1x TAE buffer.

The positive clones were sent to be sequenced (STABVida). The analysis of sequence homology between the predicted sequences and the obtained sequences was performed using the multiple sequence alignment program MultiAlin (<http://multalin.toulouse.inra.fr/multalin/>).

2.2.5 Virus Inducing Gene Silencing in *C. roseus*

The VIGS protocol covers a range of steps listed in the scheme below (Figure 9).

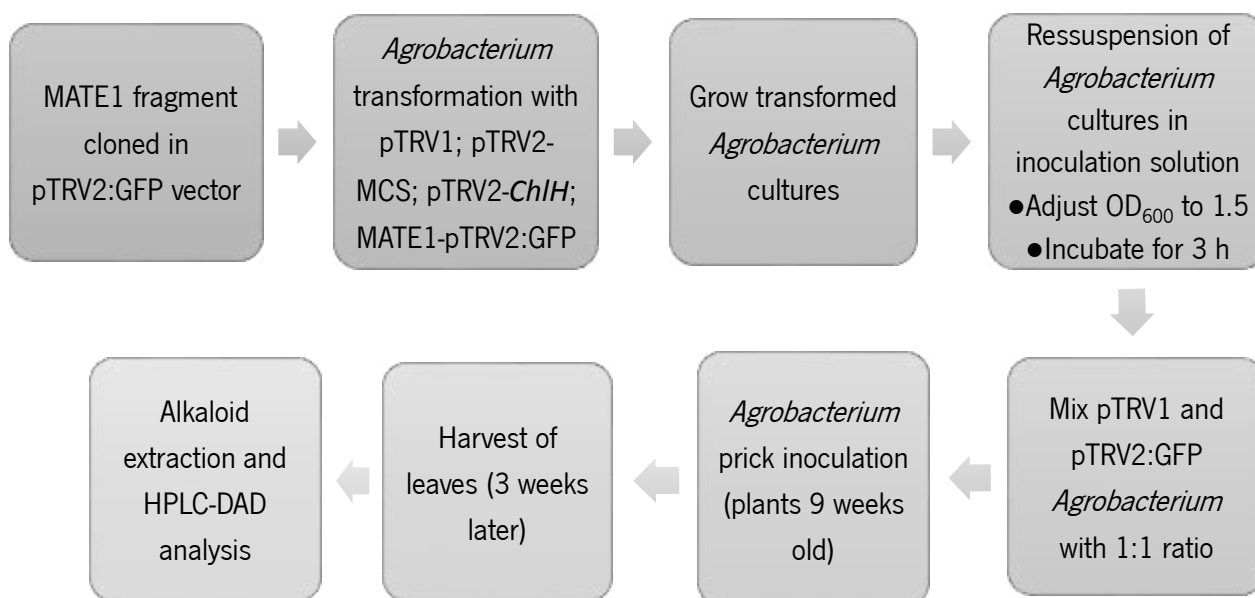


Figure 9 - Scheme of the VIGS protocol.

2.2.5.1 Transformation of *Agrobacterium*

Chemically competent *Agrobacterium* cells were transformed as described in section 2.1.7.2. *Agrobacterium* cells were transformed with the following constructs: MATE1-pTRV2:GFP, pTRV2-*ChlH* (magnesium chelatase subunit I, positive control), pTRV2-MCS (Multiple cloning site, negative control) and pTRV1 vector. Transformed cells were plated onto LB-agar supplemented with 50 µg mL⁻¹ kanamycin and incubated for two days at 28 °C. Single colonies of each construct were picked and grown in 5 mL of LB medium supplemented with 50 µg mL⁻¹ kanamycin, 25 µg mL⁻¹ gentamicin, 10 mM MES and 20 µM acetosyringone. The cultures were incubated at 28 °C in a shaker at 250 rpm ON.

2.2.5.2 *Agrobacterium* prick inoculation

Cell cultures with OD₆₀₀ of 0.6 were centrifuged at 5000 g for 5 min at RT. The supernatant was discarded and the pellet was resuspended in 5 mL of inoculation solution (10 mM MES, 200 µM acetosyringone, 10 mM MgCl₂). The cells were centrifuged again at 5000 g at RT for 5 min and were resuspended with infiltration buffer to adjust the OD₆₀₀ to 1.5. Cell suspensions were incubated at 28 °C with 100 rpm shaking for 3 h. For each construct ten 9 week old plants were transformed (Table 16).

Table 16 – Plant numbers labelled according to the respective construct.

Trays	Constructs
1-10	pTRV2- <i>ChlH</i>
11-20	pTRV2-MCS
21-30	pTRV2:GFP-MATE1
31-40	mock

For further analysis of the alkaloid levels, one or two leaves of each seedling were harvested before inoculation. Right before inoculation, cells harbouring each pTRV2 construct were mixed 1:1 v/v with pTRV1 transformed cells, and used to inoculate the plants. Plants were inoculated using a 1 mL syringe needle containing *Agrobacterium* mixture. Each plant was punctured in two different regions, one at the stem below the true leaves and one at the apical meristem, and a drop was left at the point of entry (Figure 10). To avoid cross contamination, gloves were switched after each different treatment.



Figure 10 - *C. roseus* 9 week old plant used in the VIGS protocol. Red dots mark points of *Agrobacterium* prick inoculation.

2.2.5.3 Alkaloid extraction

Leaf harvesting was performed 3 weeks after the inoculation as soon as pTRV2-*ChlH* inoculated plants showed bleached leaves. The leaves were harvested separately, packaged in Eppendorf tubes and flash-frozen in liquid nitrogen.

Samples to be used for alkaloid extraction were lyophilized. Then, it was added 8.75 μg of the alkaloid papaverine in each sample as an internal standard. For the extraction of alkaloids, lyophilized leaves were ground to a fine powder with a glass rod, 1 mL of MeOH was added and samples were mixed in the vortex for 10 minutes. Samples were sonicated (Ultrasonic cleaner, VWR) for 30 minutes, centrifuged at 13000 rpm for 15 min and, filtered through a 0.2 μm syringe filter. Finally, samples were analyzed by High Performance Liquid Chromatography-Diode-Array Detection (HPLC-DAD). The analyses were performed on a Phenomenex Gemini® C18 column (150 x 4.6 mm; 3 μm particle size) using methanol (A) and 25 mM ammonium acetate pH 10.0 (B), at a flow rate of 0.5 mL min⁻¹, with the following gradient: 40 % B at 0 min, 38 % B at 6 min, 38 % B at 35 min, 25 % B at 40 min, 10 % B at 45 min, 40 % B at 50 min, and 40 % B at 55 min. Alkaloids were detected at 260 nm. The HPLC-DAD system (Jasco Corporation, Japan) was composed by a low-pressure quaternary gradient unit with an in-line degasser (model PU-2089 Plus), a UV-Vis detector (model UV-2070 Plus) and a manual injector (Rheodyne, model 7725i). The system control and data analysis was processed on a Jasco ChromPass Chromatography Data

software (version 1.8.6.1). Quantification of alkaloids was achieved using calibration curves obtained with external standards, and normalization was performed using papaverine as internal standard.

3. Results

3.1 Optimization of engineering strategies of *C. roseus* for overexpression of MATE1 and CYP2

In order to perform optimization of a methodology of transformation and cultivation/regeneration of *C. roseus* to obtain genetically engineered plants for improved yields of the anticancer TIAs, the genes *MATE1* and *CYP2* were used. *MATE1* was previously identified in the vacuole proteome of *C. roseus* leaf cells and is a candidate to the vacuolar accumulation of the major TIAs present in the leaves. *CYP 2* was previously shown to be highly expressed in the TIA accumulating idioblast cells, and is a candidate to the late bottleneck reactions in the biosynthesis of the anticancer alkaloids VLB and VCR.

3.1.1 Sub-cloning of *C. roseus* *MATE1* and *CYP2* into a plant binary vector

MATE1 and *CYP2* genes had previously been cloned in an entry vector and, to carry out their overexpression in *C. roseus*, it was decided to perform its sub-cloning in the plant expression vector pCAMBIA2301m. For this, the full coding sequences of *MATE1* and *CYP2* were amplified using primers that added the appropriate restriction sites to allow directional sub-cloning into the vector. The sequences were successfully amplified using the primers described in Table 1 and the conditions described in section 2.1.4 and 2.1.5 of Material and Methods (Figure 11).

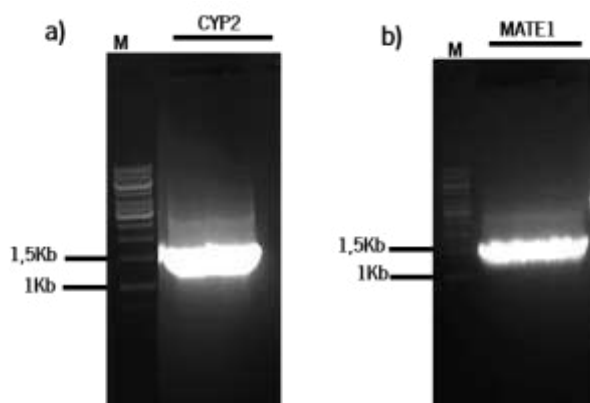


Figure 11 - Agarose gel electrophoresis of PCR amplified *CYP2* and *MATE1* for sub-cloning into pCAMBIA2301m. a) *CYP2*, b) *MATE1*. M, Molecular weight marker, GeneRuler™ 1kb DNA Ladder (Thermo Scientific). The observed bands seem to possess the predicted size, near 1.5 kbp.

The *CYP2* and *MATE1* PCR products with expected band size were purified. Both the purified products and the plasmid suffered a restriction and subsequent ligation. *E. coli* TOP 10 cells were transformed with the constructs CYP2-pCAMBIA2301m and MATE1-pCAMBIA2301m and colony PCR screening was performed to identify positive clones. The reaction was set with the universal primers M13, which amplify the complete insert to yield a 3.5 kb band, different from the 2 kb band of an empty vector, enabling to detect the positives colonies (Figure 12).

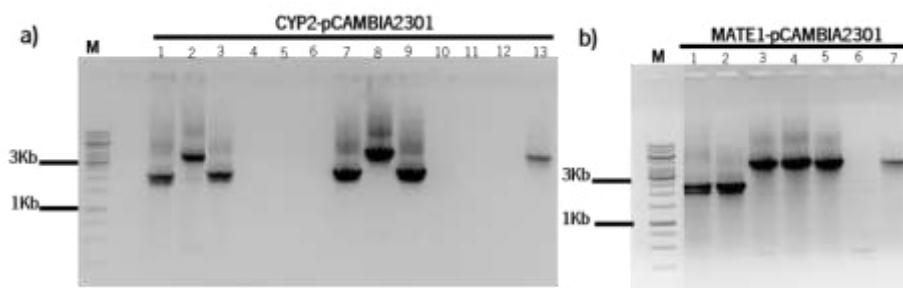


Figure 12 - Agarose gel electrophoresis of the colony PCR screening after transformation with a) CYP2-pCAMBIA2301m and b) MATE1-pCAMBIA2301m. M, Molecular weight marker, GeneRuler™ 1kb DNA Ladder (Thermo Scientific). Positives colonies: a) 2, 8, 13 and b) 3, 4, 5, 7.

The DNA of the positive clones was recovered by the alkaline lysis miniprep method from *E. coli* cells. Before sending to sequencing, a restriction test was performed. As expected, the restriction of CYP2-pCAMBIA2301m with *Xho*I gave four bands, corresponding to the 3 *Xho*I restriction sites present in pCAMBIA2301m and one in the sequence of *CYP2* (Figure 13 a). The restriction of MATE1-pCAMBIA2301m with *Xho*I and *Xba*I gave also four bands, corresponding to the 3 restriction sites of *Xho*I in pCAMBIA2301m and one restriction site of *Xba*I in *MATE1* (Figure 13 b).

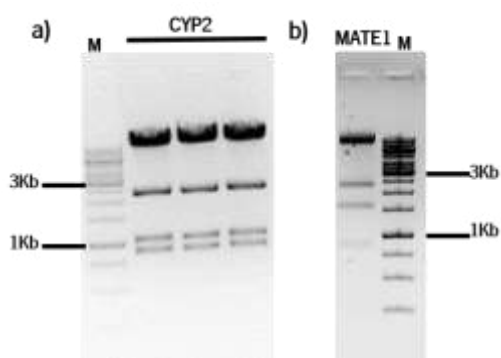


Figure 13 - Agarose gel electrophoresis of the restriction analysis of CYP2-pCAMBIA2301m and MATE1-pCAMBIA2301m selected clones: a) All the represented clones are positive as indicated by the presence of four band with 870 pb, 1043 pb, 2349 pb, and 9299 pb corresponding to the 3 restriction sites of *Xho*I in the plasmid and one restriction site in *CYP2*. b) The clone represented is positive, as indicated by the presence of four bands with, 870 pb, 1528 pb, 2349 pb, 10314 pb, corresponding to the 3 restriction sites of *Xho*I in the plasmid and one restriction site of *Xba*I in *MATE1*. M, Molecular weight marker, GeneRuler™ 1kb DNA Ladder (Thermo Scientific).

The results of sequencing were aligned with the nucleotide sequence of *MATE1* retrieved from the *C. roseus* transcriptomic database Medicinal Plant Genomics Resource (MPGR, <http://medicinalplantgenomics.msu.edu/>) with the gene ID cra_locus_1952, and a perfect alignment was obtained, as shown in Figure 14. The same was done with the results of *CYP2* sequencing, and the alignment with the corresponding MPGR gene ID cra_locus_4293 also showed to be perfect (Figure 15).

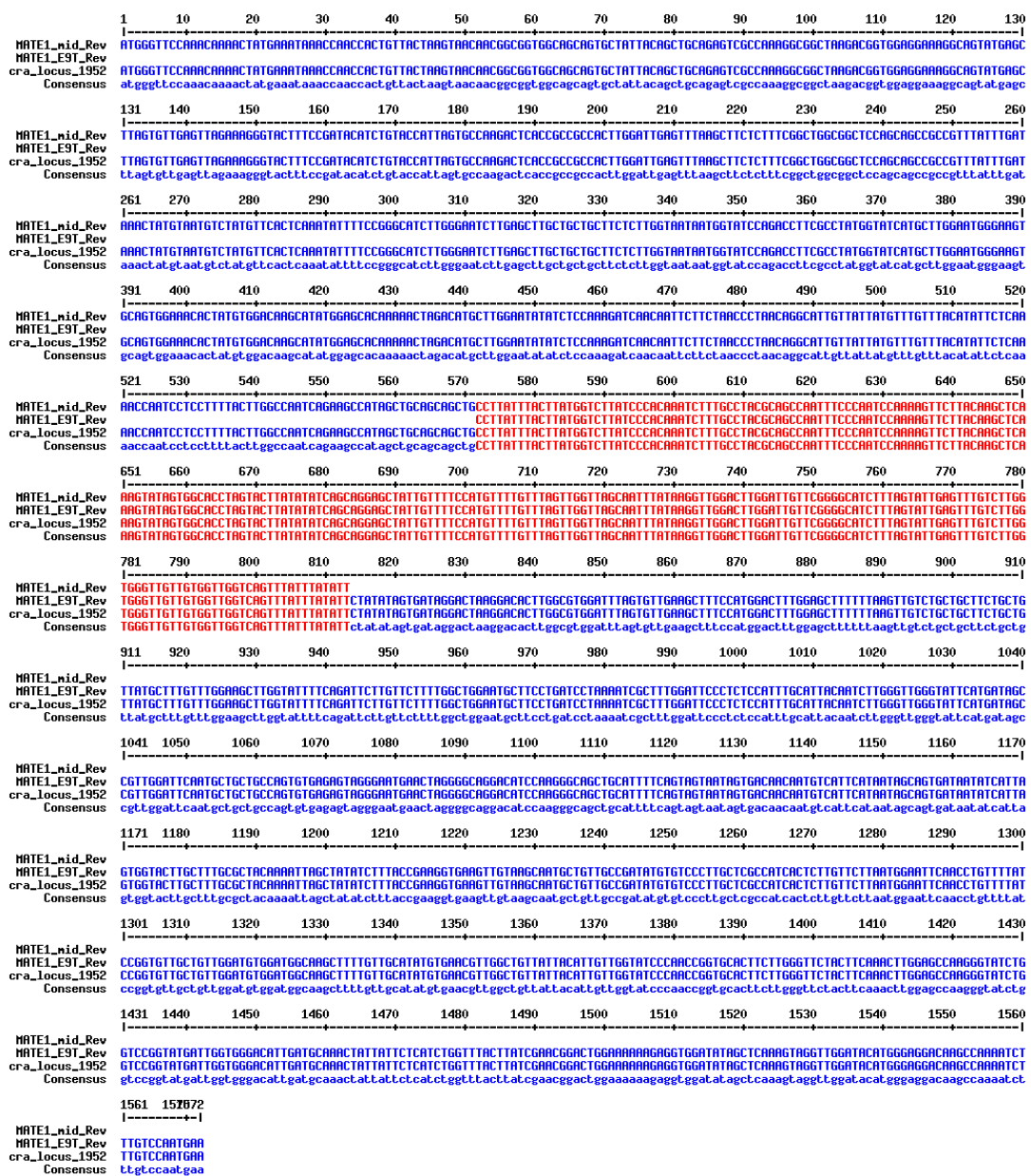


Figure 14 - Alignment between the nucleotide sequence of *MATE1* with the MPGR gene ID cra_locus_1952 and the sequencing of *MATE1* cloned in pCAMBIA2301m.

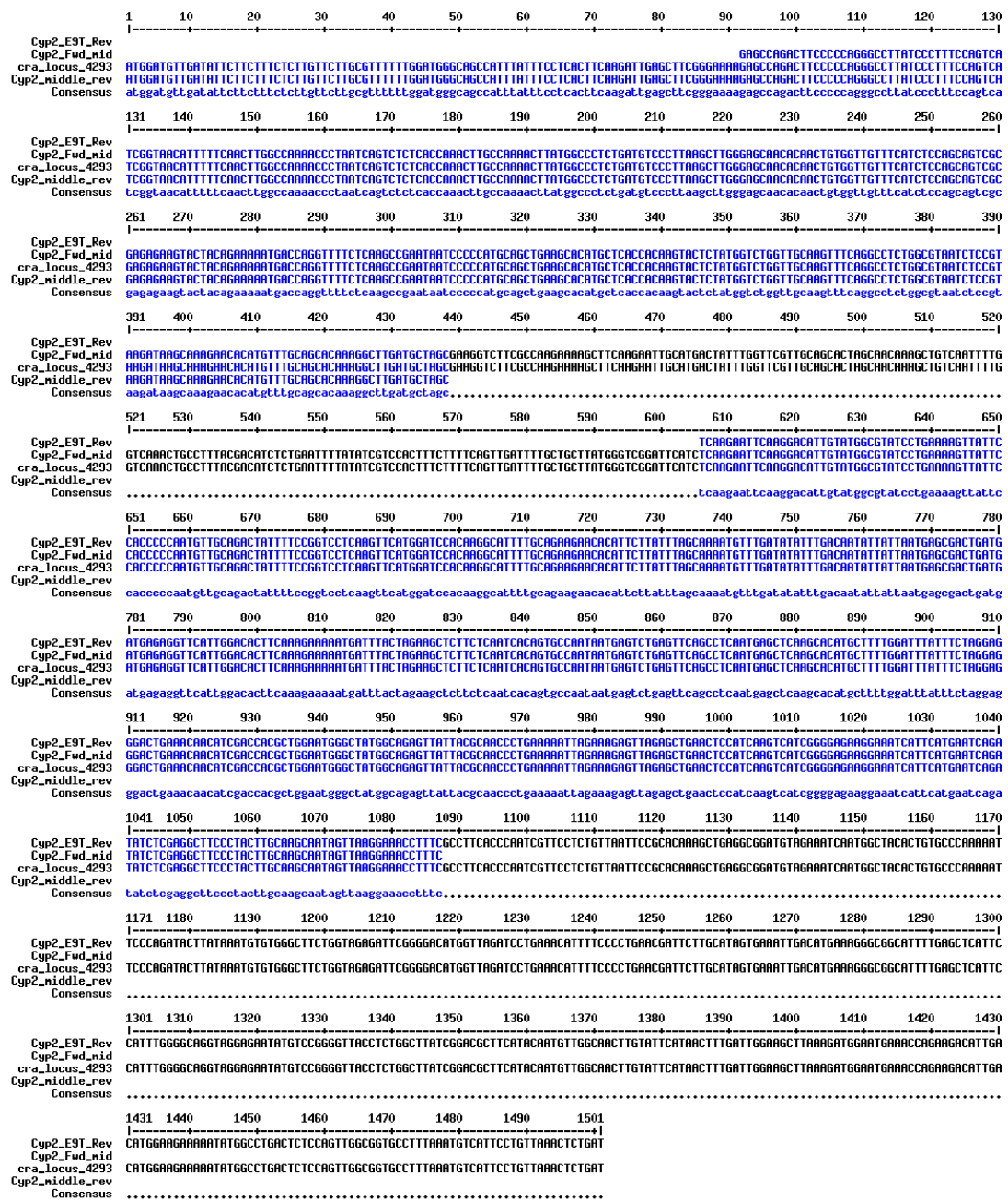


Figure 15 - Alignment between the nucleotide sequence of *CYP2* with the MPGR gene ID *cra_locus_4293* and the sequence of *CYP2* cloned in pCambia2301m.

3.1.2 Transformation of *C. roseus* hypocotyl explants

Seeds of *C. roseus* were germinated on MS medium and the hypocotyls of 8 days old germinated seedlings, with about 5 mm, were excised and were used as plant source material in all attempts of transformation and regeneration of *C. roseus*. The figure 16 below shows the process to obtain the explants.

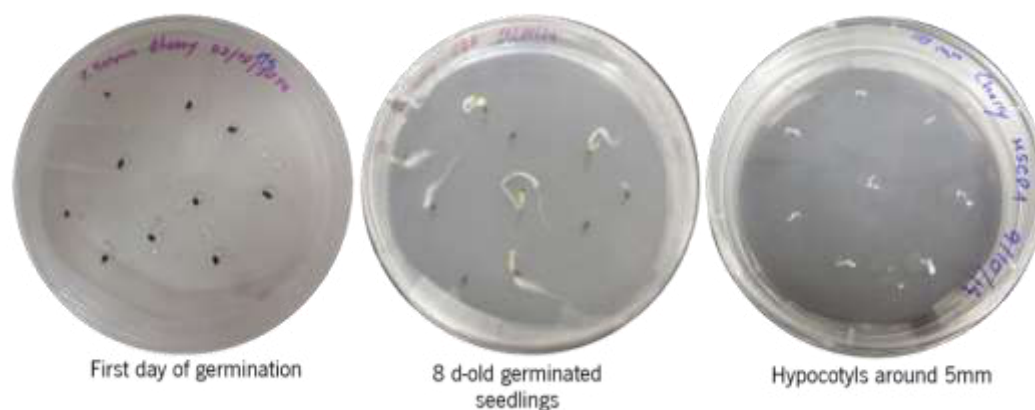


Figure 16 - Steps to obtain the explants used in transformation protocols.

The constructs, MATE1-pCAMBIA2301m, CYP2-PCAMBIA2301m and the empty vector pCAMBIA2301m were used to transform *Agrobacterium*. The empty binary vector was used as a negative control. The transformed colonies were used to infect *C. roseus* hypocotyls through co-culture. The parameters for co-culture were initially based on the experiments from Wang et al., (2012), and they were optimized along the thesis. The *Agrobacterium* culture was initially grown to a final OD of 0.8, but throughout the work, the OD was decreased first to 0.4 and then was to a maximum of 0.3, in order to avoid difficulties in controlling overgrowth of the bacteria during co-culture and contamination after co-culture. In an attempt to understand the influence of sonication in the transformation frequency, the hypocotyls were subjected to 1, 5 or 10 minutes of sonication. Acetosyringone was added to the hypocotyls before and during the transformation process due to its role in plant-pathogen recognition, especially due to its important role in the unique interaction of the genus *Agrobacterium* with plant cells (Hooykaas, 1989). The co-culture was performed for two days and was followed by the process of regeneration or establishment of transgenic *calli* cultures.

3.1.3 Generation of transgenic *calli*

With the aim of obtaining stable transgenic *calli* and, above all, transgenic plants, the protocol described by Wang et al. (2012) was initially followed with minor adjustments. Due to the long periods of time involved in the growth and establishment of the *in vitro* cultures, the first attempts of transformation and cultivation/regeneration were performed with the empty vector, in the very beginning of work and in parallel with the sub-cloning of *MATE1* and *CYP2*.

The first medium used, MSCP1 (Table 7) included the auxins 2,4-D and NAA at 1 mg L^{-1} and the cytokinin zeatin at 0.1 mg L^{-1} , and should promote the dedifferentiation of the hypocotyls and the consequent formation of *callus*, in a timeline of 10 days. After being placed in MSCP1 medium, all explants showed an excessive proliferation of *Agrobacterium* and absence of regenerative signals as can be seen in Figure 17.



Figure 17 - Hypocotyls on *callus* induction medium surrounded by a clear overgrowth of *Agrobacterium*.

In order to combat the excessive proliferation of *Agrobacterium*, the concentration of carbenicillin was increased up to 500 mg L^{-1} and the antibiotic cefotaxime was also tested (Table 8, A1 and A2). The explants presented an effective reduction of *Agrobacterium* proliferation, but had only very faint signs of *callus* proliferation and had a general browning (Figure 18).

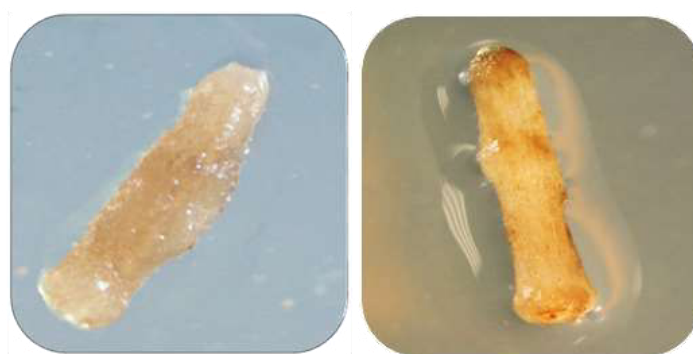


Figure 18 – Hypocotyls on *callus* induction medium: on the left a hypocotyl after 14 days on A1 medium with 500 mg L^{-1} of carbenicillin, on the right a hypocotyl after 10 days on A2 medium with 500 mg L^{-1} cefotaxime. Both hypocotyls show some signs of *callus* induction and a brownish coloration.

Since the concentration of antibiotic was clearly too high, it was decided to reduce its concentration to the initial concentration (250 mg L^{-1}) and reduce instead the final OD of the

Agrobacterium culture as a measure to curb the proliferation of bacteria. As cefotaxime showed no signs of being an effective alternative to carbenicillin, it was set aside, and the experiments advanced only with carbenicillin.

To find an alternative to basal MCSP1 medium, a series of different hormone balances were tested in non-transformed hypocotyls, also adding *myo*-inositol and the antioxidants ascorbic and citric acids (Table 8). Furthermore, the concentration of agar was decreased and removing MS vitamins was also tested. The explants were also no longer under photoperiod and were incubated in the darkness.

The results showed that changing the hormone concentrations as represented in Table 8, media B to G, did not increase *callus* induction (Figure 19 B to G). With the introduction of *myo*-inositol and antioxidants as represented in Table 8, medium H and I, it was possible to observe the clear appearance of *callus* growth (Figure 19H and I) Thus, the investigation continued only with the medium I.



Figure 19 - Hypocotyls after 9 days on callus induction media B to I, as detailed in table 7. All are non-transformed control hypocotyls.

In an attempt to increase the de-differentiation and growth of *callus*, a nurse culture procedure was employed, which consists in putting a piece of actively growing callus in the middle of each Petri dish to increase cell proliferation signals, while the explants were placed surrounding the *callus* (Figure 20). The culture used as nurse was a commercially established *callus* line of *C. roseus* resistant to kanamycin and therefore susceptible to be used under selection conditions. With the introduction of this technique there was an increase in the generation and growth of *callus*.



Figure 20 - Application of the nurse culture procedure. In the middle of the Petri dish there is a portion of actively growing *callus* surrounded by hypocotyls.

With this optimization process, it was possible to establish an effective method to generate *callus* after around 12 days on induction medium I, consisting of 2.4-D and NAA at 1 mg L⁻¹, zeatin at 0.1 mg L⁻¹, *myo*-inositol at 100 mg L⁻¹, ascorbic acid 50 mg L⁻¹. Figure 21 shows the generation of *callus* in explants transformed with each construct, with or without sonication and of a non-transformed control in the presence and absence of kanamycin. It is obvious that kanamycin clearly slows down *callus* proliferation. Otherwise, there were not significant differences in the generation

of *callus* between explants transformed with each construct or submitted to sonication during the transformation procedure.

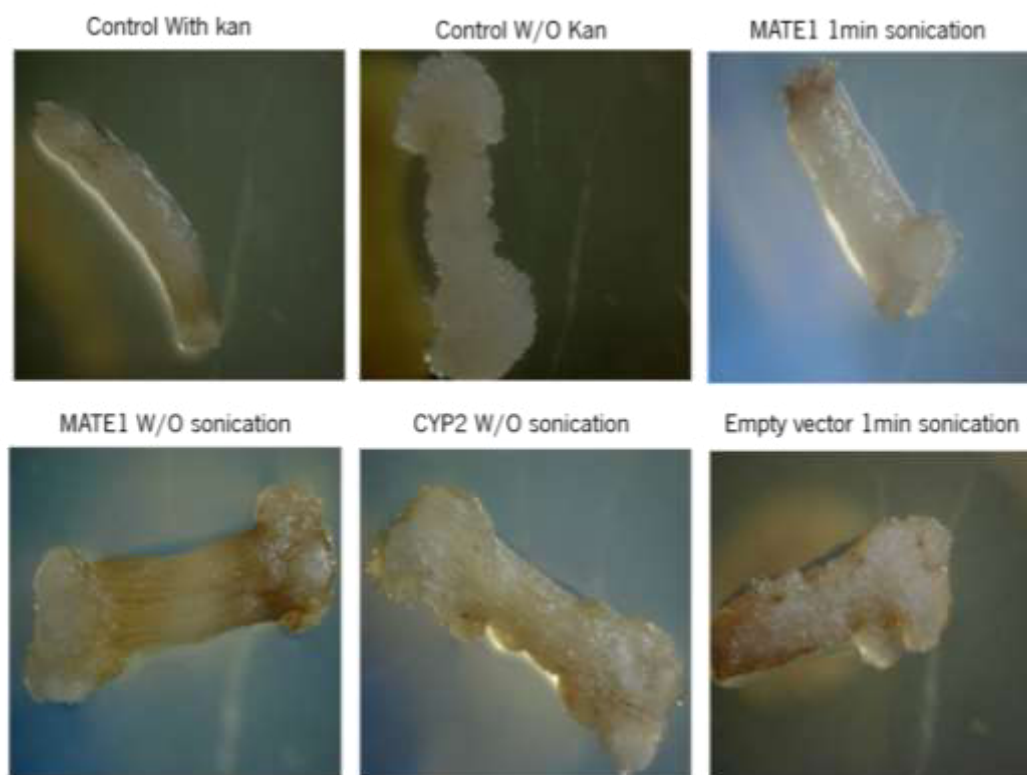


Figure 21 - *Callus* proliferation in hypocotyls submitted to transformation using different constructs and procedures, together with non-transformed controls, after 12 days in medium I using a nurse culture.

3.1.4 Induction of Shoots

An efficient regeneration system is a basic prerequisite for the genetic improvement of plants, therefore, after callus induction, the regeneration protocol of Wang et al. (2012) was used as a starting point to induce organogenesis. The MSCP2 medium for shoot induction was tested (Table 9) and as found above before for MSCP1 it did not provide the expected results, since no organogenesis events were observed. Therefore, alternative media were tried, based on the work of Cardoso (2009) and Dhandapani (2008). The hormone concentrations were altered and new hormones were introduced, including TDZ and zeatin (Table 10). All media tested, until now, did not provide the expected effects, with the more evident results being the increase of *callus* proliferation, which continues steadily upon sub-culturing in induction shoot medium every 3 weeks (Figure 22). Therefore, several transgenic *callus* lines overexpressing MATE1 and CYP2 were generated, but it was not possible to regenerate transgenic plants.



Figure 22 - *Calli* in shoot induction medium showing the proliferation of *calli* upon successive subcultures along *ca* 15 weeks.

3.1.5 Analysis of GUS expression in transgenic *calli*

The beta-glucuronidase gene (GUS) is a reporter gene inserted in pCAMBIA2301m, enabling the analysis of transgene expression in transformed plant cells by using the GUS assay. So, to confirm the transformation of *callus* cells, a non-destructive histochemical analysis of GUS activity was carried out in putative kanamycin resistant transgenic *calli* and in non-transformed *calli*. A positive GUS blue coloration was visibly detected in *callus* cells that had been transformed with any of the pCAMBIA2301m. The results of the GUS assay are represented in figures 23, 24, 25 and it is possible to see blue stains all over the *callus* for all transformations performed.

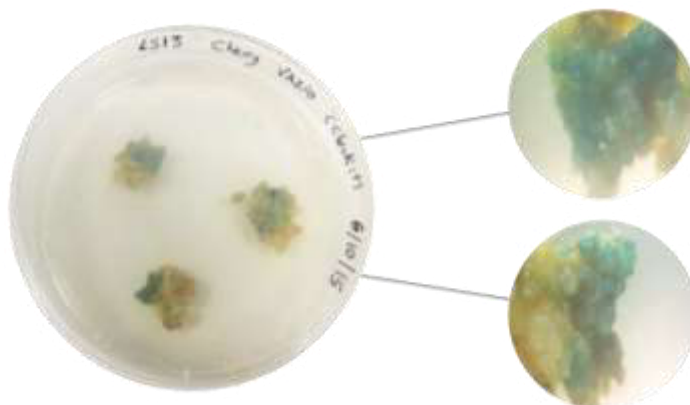


Figure 23 - Histochemical detection of GUS activity in *callus* transformed with the empty pCAMBIA2301m vector.

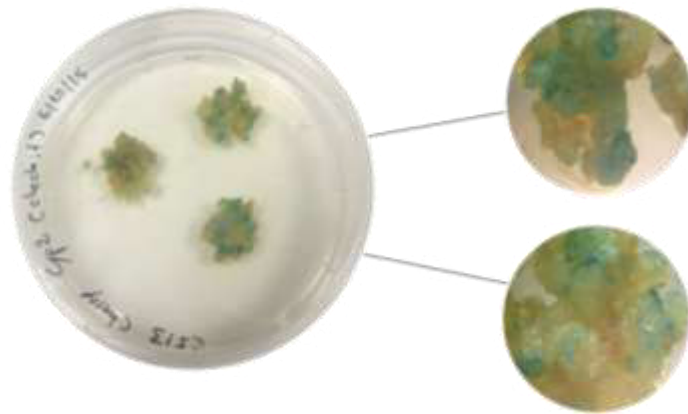


Figure 24 - Histochemical detection of GUS activity in *callus* transformed with CYP2-pCAMBIA2301m.

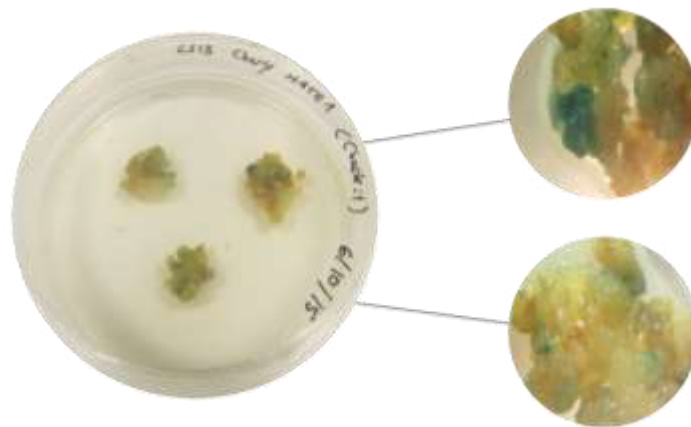


Figure 25 - Histochemical detection of GUS activity in *callus* transformed with MATE-pCAMBIA2301m.

3.2 Virus-inducing Gene silencing of *MATE 1* in *C. roseus* plants

Virus-induced gene silencing (VIGS) is an efficient and effective technique for gene functional characterization in diverse plant systems, including in *C. roseus* (Liscombe and O'Connor, 2011). The analysis is based on the silencing of a target gene by transforming a differentiated plant with a fragment of the target gene inserted within a viral genome, which will induce the silencing machinery of plant cells, at the same time it will mediate systemic spreading of its expression and therefore of silencing. In the current work, VIGS was used to try to achieve the silencing of the *MATE1* gene.

3.2.1 Molecular sub-cloning of a *MATE1* fragment into pTRV2:GFP

To carry out the silencing of *MATE1* using VIGS, the full nucleotide sequence was analysed and a specific fragment was selected. The fragment have 413 nucleotides, near to the recommended size between 200–400 nucleotides and it was selected from a coding of the gene. This region do not share homology with other genes in particular genes of the same family neither produce similar 21 nucleotides siRNAs that can match with other genes (Senthil-kumar and Mysore, 2014). The fragment selected was cloned into pTRV2:GFP, an *Agrobacterium* plant vector including a modified virus vector. To proceed with cloning, the full coding sequence of *MATE1* was used as template to amplify the *MATE1* fragment using primers that added the appropriate restriction sites to allow directional cloning into the vector. The amplification product are shown in Figure 26.

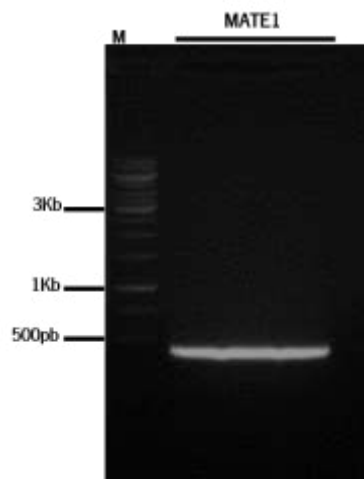


Figure 26 - Agarose gel electrophoresis of the PCR amplified *MATE1* fragment with 413pb for sub-cloning into pTRV2:GFP. M, Molecular weight marker, GeneRuler™ 1kb DNA Ladder (Thermo Scientific).

The PCR amplification product of the *MATE1* fragment presented a band slightly lower than 500 pb, compatible with the expected 413 bp length of the fragment. Both the purified product and the plasmid suffered a restriction and subsequent ligation. *E. coli* TOP 10 cells were transformed with the construct MATE1-pTRV2:GFP. The DNA of 4 selected clones was submitted to restriction analysis using *EcoRI* and *XhoI*, the enzymes used for cloning the fragment of *MATE1* into pTRV2:GFP, to detect the presence of excised *MATE1* fragment.

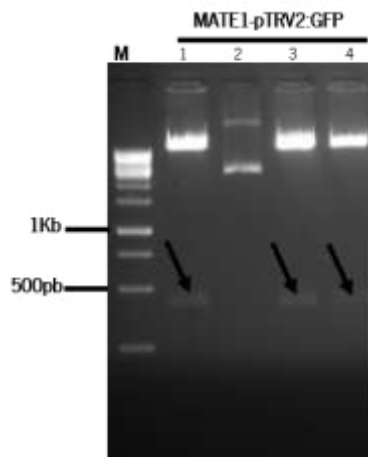


Figure 27 - Agarose gel electrophoresis of the double digestion of MATE1-pTRV2 with *EcoRI* and *XhoI*. M, Molecular weight marker, GeneRuler™ 1kb DNA Ladder (Thermo Scientific). Positive clones (arrows): 1, 3, 4.

The double restriction analysis performed on MATE1-pTRV2:GFP clones revealed three positive clones, exhibiting two different bands: one with the same size of the empty pTRV2:GFP, around 10kb, and a smaller band with a size lower than 500 pb compatible with the 413 bp of the *MATE1* fragment (Figure 27). Two of the positive clones were sequenced with the same primers designed to amplify the fragment of *MATE1* (Table 11). The resulting sequences were aligned with the fragment selected from the full length sequence of *MATE1*, and a perfect alignment was obtained, as shown in Figure 28.

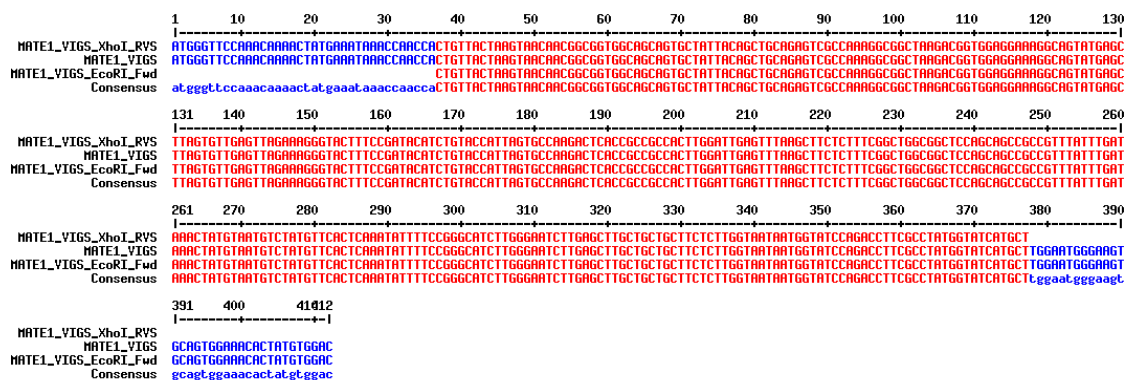


Figure 28 - Alignment between the nucleotide sequence of the *MATE1* fragment cloned in pTRV2:GFP with the original *MATE1* sequence.

3.2.2 *Agrobacterium* prick inoculation of *C. roseus* plants

The *Agrobacterium* used to inoculate *C. roseus* plants was previously transformed with each construct: MATE1-pTRV2:GFP, pTRV2-*ChlH*, pTRV2-MCS and pTRV1 vector. Plants inoculated

with pTRV2-*ChlH* were used as positive control, whereas, plants inoculated with the empty vector, pTRV2-MCS, were used as negative control. Plants were also inoculated with inoculation solution (Mocks).

Plants with 9 weeks were used for prick inoculation with *Agrobacterium*. The inoculation with the positive control allows to follow the effectiveness of VIGS experiments, since it produces a visible phenotype. Figure 29 shows plants 8 days after inoculation (left) and plants 20 days after inoculation (right) with pTRV2-*ChlH*, and it is possible to observe a photo-bleaching phenotype in the plants (right) characteristic of the down-regulation of the chelatase gene.

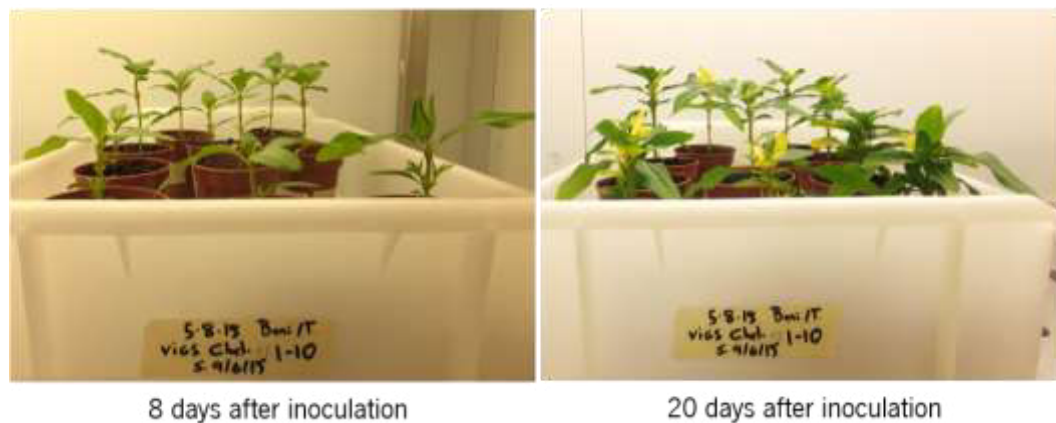


Figure 29 - *C.roseus* plants inoculated with the positive control pTRV2-*ChlH*. Plants 8 days after inoculation (left) do not present photo-bleaching, whereas plants 20 days after inoculation (right) display a clear photo-bleaching phenotype.

The photo-bleaching allows to infer that the technique was applied correctly and it induced the silencing of the chelatase gene. In Figure 30 it is possible to see not only a plant inoculated with pTRV2-*ChlH* but also plants inoculated with two others constructs and with Mock treatment. As expected, no other plant besides the inoculated with pTRV2-*ChlH* presents a phenotype. The plants inoculated with Mock treatment demonstrate that the method of inoculation did not affect the survival of plants.

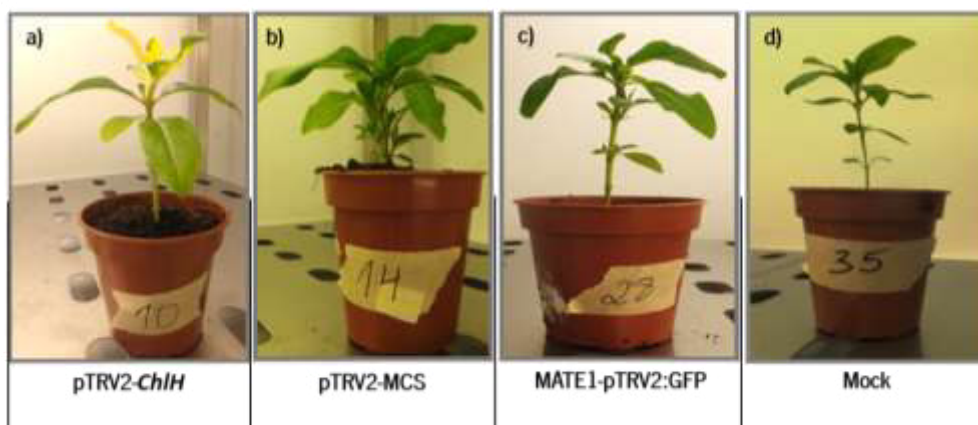


Figure 30 - *C. roseus* plants 20 days after inoculation with: a) positive control; b) negative control; c) MATE1-pTRV2:GFP; d) Mock treatment.

3.2.3 Analysis of the alkaloid profile and levels of the silenced plants

In order to quantify and evaluate the alkaloid profile of plants subjected to *MATE1* gene silencing, the alkaloids were extracted from control pTRV2-MCS plants and MATE1-pTRV2:GFP plants, 3 weeks after the inoculation, and were analysed by HPLC-DAD.

The analysis by HPLC-DAD allowed to detect the internal standard papaverine and the two main alkaloids present in the leaves of *C. roseus*, vindoline and catharanthine (Figure 31).

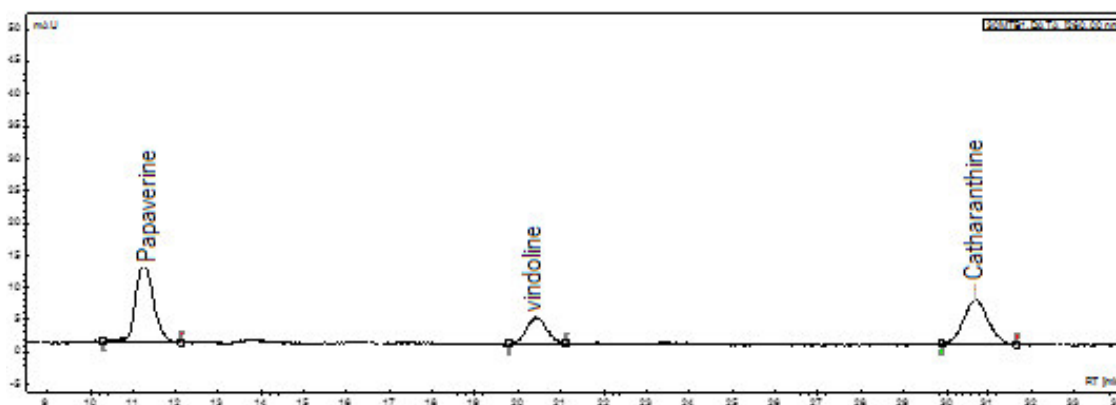


Figure 31 - Representative chromatogram of a leaf alkaloid extract registered at 260 nm. The three main peaks detected correspond to the papaverine, vindoline and catharanthine.

The alkaloids were identified based on the retention time and on comparison of UV spectra with those of authentic standards.

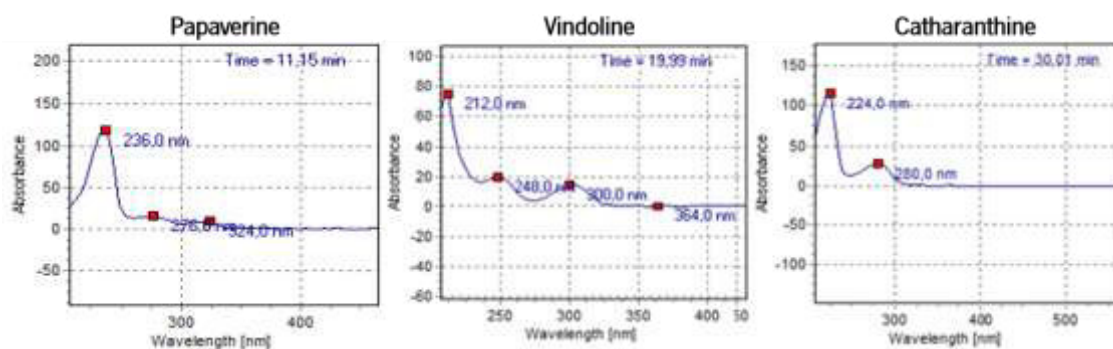


Figure 32 - Absorbance spectra of the peaks detected during HPLC-DAD analysis of leaf alkaloid extracts allowing the identification of papaverine, vindoline and catharanthine.

Quantification of vindoline and catharanthine, per dry weight (DW), was performed by using calibrations curves obtained with external standards, and the values were normalized using papaverine as internal standard. The levels of vindoline in leaves of plants inoculated with MATE1-pTRV2:GFP was $1.57 \mu\text{g mg}^{-1}$ DW, almost 2 fold less than in plants inoculated with pTRV2-MCS, the empty vector, which was $3.21 \mu\text{g mg}^{-1}$ DW (Figure 33). To confirm if the values are significantly different it was applied the Student's t-test, since we are comparing the means of two groups. The values of vindoline production in plants inoculated with MATE1-pTRV2:GFP and with pTRV2-MCS were significantly different ($P < 0.001$).

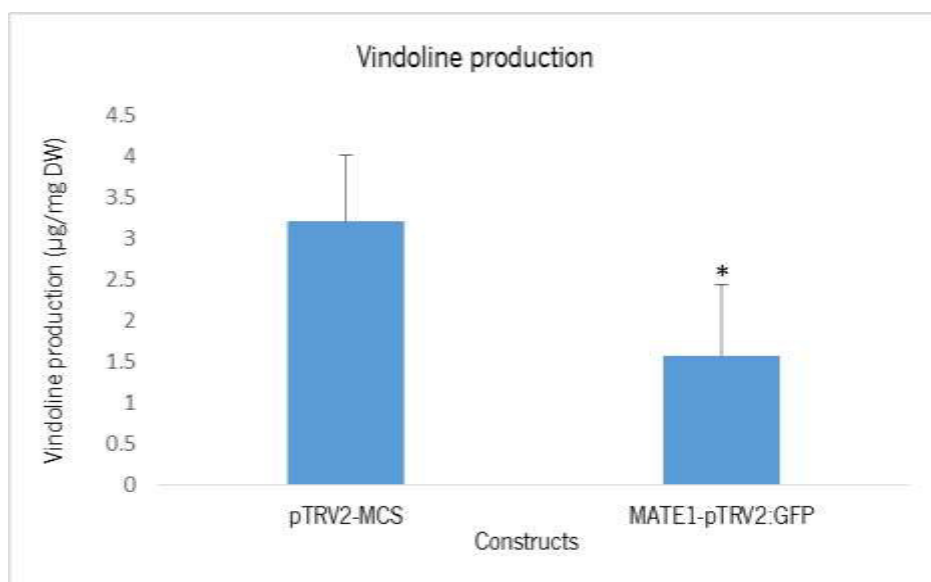


Figure 33 - Quantification of vindoline levels in the leaves of plants inoculated with the empty vector (pTRV2-MCS) and MATE1-pTRV2:GFP. Values are means of 10 plants. Asterisk indicate significant differences between vindoline production in plants inoculated with pTRV2-MCS and with MATE1-pTRV2:GFP, using Student's t-test ($P < 0.05$)

The levels of catharanthine in the leaves of plants inoculated with MATE1-pTRV2:GFP was 0.45 $\mu\text{g mg}^{-1}$ DW, almost 5 fold less than the $\mu\text{g mg}^{-1}$ DW observed in leaves of plants inoculated with pTRV2-MCS, the empty vector (Figure 34). The levels of catharanthine were significantly different between plants inoculated with pTRV2-MCS and MATE1-pTRV2:GFP ($P < 0.001$).

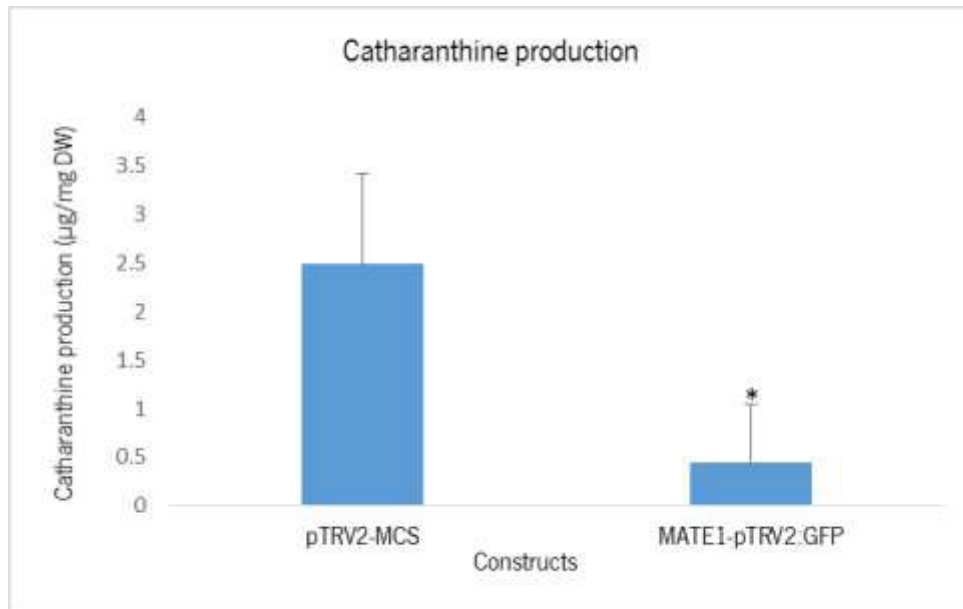


Figure 34 - Quantification of catharanthine levels in the leaves of plants inoculated with the empty vector (pTRV2-MCS) and with MATE1-pTRV2:GFP. Values are the mean of 10 plants. Asterisk indicate significant differences between values of catharanthine production in plants inoculated with pTRV2-MCS and with MATE1-pTRV2:GFP using Student's t-test ($P < 0.05$)

4. Discussion

In the present work, the genes *MATE1* and *CYP2*, though to be involved respectively in alkaloid vacuolar accumulation and the final, bottleneck biosynthetic steps of the anticancer alkaloids from *C. roseus*, were cloned into the binary vector pCAMBIA2301m and used to transform *C. roseus* cells. Hypocotyls from *C. roseus* were transformed with each gene, individually, via *Agrobacterium* mediated transformation, and transgenic *callus* lines were successfully established. Despite several attempts, it was not possible to induce organogenesis and regenerate transgenic plants. *Callus* transformation was proven by using the GUS assay.

The VIGS technique was performed to silence the *MATE1* gene and the results demonstrate a significant reduction in the levels of the main leaf alkaloids of *C. roseus*, strongly suggesting the involvement of MATE1 in vacuolar alkaloid accumulation in *C. roseus* leaf cells. A discussion of the most important aspects and results of this work is provided below.

4.1 Biotechnological approach

The high medical value and extremely low yields of VLB and VCR in *C. roseus* are the main reasons to extensive studies in the missing steps of TIA biosynthesis and in the multiple steps of transmembrane transport of TIA intermediates (Pan et al., 2012). Previous studies in our group allowed to identify a number of very strong candidate genes to important functions within the late steps of TIA pathway (Carqueijeiro 2013). These candidates were found on a differential transcription profiling of leaf idioblast cells, since it is there that the late steps of VLB biosynthesis are thought to take place. CYP2 is a P450 differentially expressed in idioblasts and is therefore a candidate to the first step of the conversion of AVLB into VLB, and also to the first step of the conversion of VLB into VCR. On the other hand, MATE1 was the most abundant MATE transporter identified in the vacuole proteome (Carqueijeiro 2013) and is therefore a strong candidate to TIA transtonoplast transport. In fact, the alkaloid nicotine was shown to be transported into the vacuole of tobacco cells by a MATE transporter acting as a proton antiport, the exact mechanism shown to be responsible for TIA accumulation in the vacuoles of *C. roseus* leaf cells (Carqueijeiro et al., 2013).

The approach chosen here to understand the role of these genes was by its overexpression in *C. roseus* cells and plants. To date, genetic transformation of *C. roseus* has been mostly confined

to hairy roots and suspension cells and it has been successful only for the production of some of the TIA monomers (e.g. ajmalicine and serpentine). There is no report about significant production of the dimeric VLB and VCR in cell cultures or hairy roots. The reason can be the highly complex cell-type specific TIA compartmentation, with at least four different cell types and as many subcellular compartments involved and with inherent intercellular and intracellular metabolite translocations. Both cell and hairy root cultures do not cover all the leaf cell types and, therefore, to increase the production of VLB and VCR, gene transformation into whole plants is required (Pan et al., 2012). Accordingly, one of the main objectives of this thesis was to optimize an efficient protocol for the production of regenerated transgenic *C. roseus* plants. In spite of many efforts it was not possible to achieve this goal, although it was possible to generate stable transgenic *calli* lines.

4.2 *Agrobacterium* mediated transformation of *C. roseus* hypocotyls

The hypocotyls of *C. roseus* were the explant chosen to be transformed by the *Agrobacterium*-mediated transformation method, despite the reputation of *C. roseus* of being recalcitrant to transformation by *Agrobacterium* strains. This method was chosen since the transformation protocol is well-established, is easily performed and requires a minimum equipment. Moreover, transgenic plants obtained using *Agrobacterium* often contain simple copy insertions (Tzfira and Citovsky, 2006). Nevertheless, a transformation process is successful only combined with an effective selection method for transgenic tissues and a protocol to regenerate the transgenic plants at a reasonable frequency.

Recently, a successful protocol of transformation and regeneration was published by Wang et al., (2012), and this protocol was used as the starting point in this work to perform the transformation of explants with the *MATE1* and *CYP2* genes and to perform the regeneration of plants from transgenic hypocotyls. However, throughout the work several improvements were required that will now be discussed.

The first obstacle was the excessive proliferation of *Agrobacterium* that was not allowing the proliferation of *callus* (Figure 17). To overcome this, the antibiotic concentration of carbenicillin was increased to 500 mg L⁻¹ and another antibiotic was tested, 500 mg L⁻¹ cefotaxime. Both antibiotics were effective in removing *Agrobacterium* but, as observed in many species, also in *C. roseus* the high concentrations of antibiotics inhibited the regeneration of the plants (Figure 18).

Investigation in several species, such as *Antirrhinum majus* (Holford, 1992), *Arabidopsis* (Nishida et al., 1993), *Picea glauca* (Tsang et al., 1989) and *Populus* (Howe et al., 1994), showed that cefotaxime in concentrations from 250-500 mg L⁻¹ had an inhibitory effect. About the effect of carbenicillin, several reports also demonstrated that it has a negative influence at concentrations ranging from 250-1000 mg L⁻¹ in *Antirrhinum majus* (Holford, 1992), *Arabidopsis* (Nishida et al., 1993), *Delphinium* (Leifert et al., 1992), *Nicotiana tabacum* (Lin et al., 1995) and *Vitis* (Colby and Meredith, 1990). Since so many plants respond negatively to high concentrations of both antibiotics and the *C. roseus* explants showed no signs of any type of cell growth in the presence of the high concentrations of antibiotics, it was decided to reduce the concentration to the originally used and, as alternative, the concentration of the *Agrobacterium* culture was decreased from an OD of 0.8 to 0.2-0.3.

Regarding the sonication effect in improving the efficiency of the transformation process it did not seem to be acting in this case. Explants that were submitted to sonication, especially those submitted to 5 or 10 minutes presented a translucent and fragile aspect and showed extreme difficulty to generate *callus*. Only explants subjected to 1 minute of sonication presented some capacity to generate *callus*. One possible explanation is that the sonication power, as well as the periods of sonication were too high for the *C. roseus* hypocotyl explants.

4.3 *Callus* induction in *C. roseus* hypocotyls

The method selected to try the regeneration of whole plants from hypocotyls was through indirect organogenesis, which is controlled by the balance of plant hormones and other factors added to the culture medium. The main difficulty in the regeneration of plants from hypocotyls was to gather suitable conditions capable of inducing the differentiation of shoots.

The first step in the protocol of regeneration of Wang et al., (2012), selected as a starting point was the induction of dedifferentiation and the consequent formation of a mass of proliferating cells, the *callus*. This was not achieved following the medium and conditions described in the publication, and significant modifications were introduced to be able to obtain the *callus*, beginning with the changing of the hormone ratios. It is known that specific auxin to cytokinin ratios give rise to an unorganized growing and dividing mass of *callus* cells, and that this ratio varies greatly according to the kind of plant being cultured, the culture conditions, and the form of the phytohormone used (Gaspar et al., 1996). Thus, different ratios were tested wherein the levels of

2.4D, an auxin, were increased from 1 mg L⁻¹ to 3 mg L⁻¹, keeping the NAA (auxin) concentration in 1 mg L⁻¹ and maintaining or increasing the zeatin (cytokinin) from 0.1 to 1 mg L⁻¹. The full description of the media is on table 8. None of the media with the different hormone contents tested showed a significant increase of *callus* generation, which may be justified by the amount of factors, above described, that influence the phytohormonal behaviour, as well as the difficulty to determine the specific effects of any particular phytohormone, since one hormone appears to be able to modify the synthesis of other classes of hormones (Gaspar et al., 1996) (Figure 19, media B to G).

Further attempts were made using the initially phytohormone ratios described by Wang et al., (2012) but introducing *myo*-inositol, citric acid and ascorbic acid. The addition of these compounds to the medium allowed to achieve an effective generation of *callus*. *Myo*-inositol was added for its role in the regulation of cellular metabolism and growth, although the mechanism by which stimulates cell growth is not fully understood (Loewus and Murthy, 2000; Nunes et al., 2006). Several protocols report its utilization in plant media used for inducing regeneration. Murashige (1974) reported that shoots usually produce enough *myo*-inositol, but unorganised tissues grow slowly without a small quantity of this compound added to the medium. Whereas citric acid and ascorbic acid were added to the medium in an attempt to resolve the explant browning that was notorious in all the explants, quickly after the transfer to *callus* induction media, and ultimately leading to the death of tissues. Antioxidants such as ascorbic acid or citric acid, reduce oxidative stress and prevent oxidation of phenolic compounds. For the same purpose, the explants were placed in the dark (Maxwell et al., 2013). The use of ascorbic acid was experimented, for example by Ndakidem et al (2014) that tested a range of ascorbic acid concentration to control the browning of *B. huillensis* and the best result was achieved supplying the medium with 200 - 250 mg L⁻¹. In currently work the result was positive with low concentration, so it was not necessary to use concentrations as high.

In addition to the introduction of the *myo*-inositol and the antioxidants, also was applied the nurse culture, which may have contributed to an increased generation of *callus*. Since reports support the active use of nurse culture in plant regeneration (Komai, et al., 2006)

It is not possible identify which of the changes was more prominent for the induction of *callus* but all together allowed achieve the goal.

4.4 Regeneration attempts of *C. roseus* transgenic plants

To try to regenerate whole *C. roseus* plants by indirect organogenesis, the next step after the proliferation of *callus* was the induction of redifferentiation, wherein from undifferentiated cells emerge the first organs. To start this process, the *calli* formed were transferred to a shoot induction medium. The first medium tested was also the one proposed by Wang et al., (2012), which did not produce the desired effects. Again, several variations were tested, the phytohormone balance was redefined and alternative phytohormones, TDZ and zeatin, were tested. The full description of the media tested is on table 10. TDZ, a phenyl urea-type plant growth regulator that has been investigated for cytokinin activity was one of the hormones introduced in the media. The efficient effect of TDZ on *in vitro* cultures has been reported in many species for shoot regeneration (Babaei et al., 2014). Yildirim and Turker, (2009) reported that TDZ was more successful than BA and kinetin for shoot regeneration in meadowsweet, and Lu, (1993) also reported that TDZ play a major and distinctive role on adventitious shoot regeneration compared with purine-type cytokinins, in carnation and rose. Uranbey, (2005) indicated that TDZ was more effective than BA on shoot regeneration in all hypocotyls, cotyledon, and stem explants of henbane. In our lab previous works by Cardoso (2009) achieved *C. roseus* regeneration through somatic embryogenesis using TDZ at 7.5 μM and 5 μM . Despite all successful reports, TDZ did not have the desired effect on *C. roseus calli*. TDZ was tried at various concentrations, in which the highest was 7.5 μM and the lowest was 0.1 μM , and it was also tested alone and associated with BA and NAA. The most obvious effect was an increased proliferation of the *callus* (Figure 22). In fact, several publications report this effect at concentration $\geq 0.1 \mu\text{M}$, so this may be a possible explanation for *callus* proliferation instead of shoot regeneration (Huetteman and Preece, 1993).

4.5 Establishment of transgenic *callus* cultures

To confirm that the proliferating *callus* lines obtained were composed of transformed cells, a histochemical analysis of GUS activity was carried out in putative kanamycin resistant transgenic *callus*. A non-destructive protocol adapted from Jefferson et al (1987) was used, which allows to maintain the culture of *callus* after the test. GUS positive blue coloration was visibly detected in *callus* stained with X-gluc reagent, confirming the transgenic nature of the cells. As can be seen in Figures 23, 24 and 25, the blue coloration covers much of the callus and in all transformations performed, and so, it is possible to infer that the transformations were successful. Therefore, the

optimized *callus* induction protocol enabled the establishment of proliferating transgenic *callus* that will be later evaluated for the impact in TIA levels and profile of the overexpression of MATE1 and CYP2, and can be used for further experiments.

4.6 Silencing of MATE1

VIGS is a technique widely used to investigate gene function in plants difficult to regenerate and, therefore, it should greatly facilitate the discovery and characterization of novel genes implicated in TIA metabolism (Liscombe and O'Connor, 2011). Based on this ability, the technique was applied to try to understand the role of *C. roseus* MATE1, which was a strong candidate to the vacuolar accumulation of the major TIAs present in the leaves of *C. roseus* (Carqueijeiro et al., 2013).

Recently, Liscombe and O'Connor (2011) developed for *C. roseus* a VIGS method using the pTRV vector system and demonstrated its utility in functional analyses of *C. roseus* genes by silencing three known steps in vindoline biosynthesis. This method proved to be successful and it has been repeatedly used with the purpose to uncover missing steps of the TIA pathway in *C. roseus*.

Despite the growing interest and use of this technique, it is necessary to be aware of its limitations, including, the not always uniform gene silencing and, therefore, a large number of replicate plants need to be analysed to obtain consistent results. Also, miss-target gene silencing can occur with some frequency. Hence it is primordial to choose a fragment of a gene that produces specific siRNAs targeting of the gene of interest (Senthil-kumar and Mysore, 2014). To deal with these limitations, the choice of the fragment to silence was made carefully, and ten replicas were performed for each construct.

The inoculation method may affect the survival of plants and consequently the VIGS outcome, so the selection of inoculation method is an important decision to the success of the technique. With this in mind, the method successfully tested by Liscombe and O'Connor (2011) was selected, wherein, instead of using a forceps dipped in the *Agrobacterium* to pinch just below the apical meristem and the youngest leaf pair, a syringe needle was used to prick and leave a drop of *Agrobacterium* culture in the same places (Figure 10) (Liscombe and O'Connor, 2011). This modification did not interfere with the survival of the plant since all the plants used in the experiment developed normally.

Recently, it was also shown that factors such as temperature and age of the plant influence the efficiency of VIGS (Sung et al., 2014). In *C. roseus*, high temperature (30°C) strongly inhibited VIGS, but low temperature (16°C) inhibited plant growth (Sung et al., 2014). So, the plants during the VIGS experiment were under at an intermediate temperature, at 25 °C. Concerning the age of plants, it seems that efficacy is not affected but is reduced in old leaves (Sung et al., 2014). Therefore, the plants were inoculated after the appearances of the first true leaves.

Here, the process of cloning, inoculation of plants and control chelatase silencing leading to photo-bleaching were successfully achieved, indicating that MATE1 silencing might also have been successful (Figure 29). Therefore, the extraction and quantification of alkaloids from putatively silenced *C. roseus* plants was performed. The method to detect and quantify the TIAs was HPLC analysis coupled to UV-VIS DAD, since it is particularly useful for a first analysis of the alkaloids profile and it is quite efficient for its quantification (Hisiger and Jolicoeur, 2007). The analysis of the absorbance spectra of the three peaks obtained allowed the identification, by comparison with authentic standards, of the internal standard papaverine and of the *C. roseus* TIAs vindoline and catharanthine (Figure 32), which are the most abundant TIAs in the plant leaves.

The data from HPLC analysis showed different levels of alkaloids in the plants inoculated with the empty vector and with the MATE1 gene. The results also showed a high standard deviation explained by the high variability of the alkaloid levels in each plant. Each plants is unique and so the alkaloid concentration in a plant, plant cells or tissues is also not equal, neither constant with time. The significantly lower levels of vindoline and catharanthine in plants inoculated with MATE1-pTRV2 comparing with the levels of plants inoculated with the empty vector may be a strong indication that MATE1 is involved in the transtonoplast transport of TIAs, possibly of vindoline and catharanthine. The statistical significance of the values was confirmed by application of the Student's t-test, and the alkaloid levels in plants inoculated with MATE1-pTRV2:GFP and with pTRV2-MCS were significantly different ($p < 0.05$).

Although it is still early to confirm the role of MATE1 in vacuolar accumulation of TIAs, this work brings us a step closer. However, further analysis will be essential.

5. Conclusions and Future Perspectives

In this work, the *C. roseus* genes *MATE1* and *CYP2*, putatively involved in the vacuolar accumulation of TIAs and the late, bottleneck, biosynthetic steps of VLB and VCR respectively, were sub-cloned into a binary vector and used to transform *C. roseus* hypocotyls through Agrobacterium-mediated transformation, leading to the establishment of transgenic *callus* lines. Moreover, silencing of *MATE1* using VIGS induced a significant decrease in the levels of the major TIAs accumulated in the leaves of *C. roseus*, strongly supporting the role of *MATE1* in the vacuolar accumulation of these TIAs in the leaves of the plant.

The regeneration of transgenic plants is still ongoing, since this showed to be a recalcitrant process. Until now, a successfully method to generate *callus* was achieved and the attempts to regenerate whole plants remain unsuccessful. The *callus* lines generated were tested by GUS assay and its transformation was confirmed. As future perspectives, besides testing other media composition to achieve regeneration, the transgenic *callus* lines should be analysed concerning the levels and profile of alkaloids present. The stable *callus* transformed lines can also be used to perform other assays such as elicitation assays, in an attempt to understand the effects and roles of *MATE1* and *CYP2*.

In order to investigate the functional role of *MATE1*, the downregulation of its gene was performed by VIGS. The silencing was carried out through inoculation of *C. roseus* plants with a viral vector modified to carry a fragment of the *MATE1* gene. The putatively *MATE1* silenced plants showed significantly lower levels of the major TAs accumulated in the leaves of *C. roseus*, vindoline and catharanthine. This result strongly supports a role of *MATE1* gene in the vacuolar transtonoplast transport of vindoline and catharanthine. Further analysis by qPCR must be done to confirm the silencing of *MATE1*. Moreover, an HPLC–Mass Spectrometry (HPLC-MS) analysis will be performed in order to assess if there is any increment or decrement of other alkaloids present in low amounts.

Overall, the work developed gives a significant contribution to the possibility of performing future biotechnological improvements of *C. roseus* for increased levels of the anticancer VLB and VCR.

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7. Appendixes

Appendix 1. Modified Hanahan's protocol for the preparation of *E. coli TOP10* chemically competent cells.

Procedure

- Inoculate a single colony from a LB plate in 25 mL of LB medium. Incubate overnight at 37°C with vigorous shaking (200 rpm).
- Dilute the incubated culture in 225 mL (~1:100) of LB medium supplemented with 10 mM of MgCl₂ and 10 mM of MgSO₄. Incubate at 37°C with vigorous shaking until the culture reaches an A_{600nm} ≈ 0.5.
- Incubate the cells for 10 min on ice. Centrifuge at 2300 g (5000 rpm) for 10 min at 4°C to pellet the cells.
- Pour off supernatant. Remove the remaining supernatant by pipetting without disturbing the pellet. Resuspend the pelleted cells in 80 mL of chilled CCMB80 buffer.
- Incubate the resuspended cells for 20 min on ice. Centrifuge at 2300 g (5000 rpm) for 10 min at 4°C.
- Discard supernatant as described before. Resuspend the pelleted cells in 10 mL of chilled CCMB80 buffer.
- Test OD of a mixture of 200 µl SOC and 50 µl of the resuspended cells. Add ice cold CCMB80 buffer to the whole suspension to yield a final OD of 1.0-1.5 in this test.
- Incubate on ice for 45 min to 1 h. Prepare 50 or 100 µL aliquots into prechilled 1.5 mL tubes. Flash freeze the tubes in liquid N₂ and store at -80°C.

Solutions

CCMB80 buffer

Composition per 1 L

10 mM potassium acetate 10 mL of a 1 M stock solution

80 mM CaCl₂ 2.2H₂O 11.8 g

20 mM MnCl₂ 2.4H₂O 4.0 g

10 mM MgCl₂ 2.6H₂O 2.0 g

10% (v/v) glycerol 100 mL

Adjust pH down to 6.4 with 0.1N HCl.

Filter-sterilize (0.2 µm).

Appendix 2. Protocol for the preparation of *A. tumefaciens* EHA 105 chemically competent cells

Procedure

- Inoculate a single colony from a YEB with agar (1.8% (w/v), Liofilchem) plate in 5 mL of YEB medium. Incubate overnight at 28°C with vigorous shaking (200 rpm).
- Dilute 2mL of the incubated culture in 50 mL (1:25) of YEB medium. Incubate at 28°C with vigorous shaking until the culture reaches an $A_{600nm} \approx 0.5-1.0$.
- Cool down the culture on ice. Centrifuge at 3000 g for 5 min at 4°C to pellet the cells.
- Pour off supernatant. Remove the remaining supernatant by pipetting without disturbing the pellet. Resuspend the pelleted cells in 1 mL of chilled $CaCl_2$ 20mM.
- Prepare 50 or 100 μ L aliquots into prechilled 1.5 mL eppendorf tubes. Flash freeze the tubes in liquid N_2 and store at -80°C.

YEB medium

Composition per 1 L

0.5% (w/v) beef extract 5 g

0.1% (w/v) yeast extract 1 g

0.5% (w/v) peptone 5 g

0.5% (w/v) sucrose 5 g

Adjust pH to 7.2. Sterilize by autoclaving

Appendix 3. Nucleotide sequence of *MATE1*

>cra_locus_1952_iso_7_len_1704_ver_3

ATGGGTTCCAAACAAAACCTATGAAATAAACCAACCACTGTTACTAAGTAACAACGGCGGTGGCAGCAGTG
CTATTACAGCTGCAGAGTCGCCAAAGGCGGCTAAGACGGTGGAGGAAAGGCAGTATGAGCTTAGTGTTGA
GTTAGAAAGGGTACTTTCCGATACATCTGTACCATTAGTGCCAAGACTCACCGCCGCCACTTGGATTGAG
TTTAAGCTTCTCTTTTCGGCTGGCGGCTCCAGCAGCCGCCGTTTATTTGATAAACTATGTAATGTCTATGT
TCACTCAAATATTTTCCGGGCATCTTGGGAATCTTGAGCTTGCTGCTGCTTCTCTTGGTAATAATGGTAT
CCAGACCTTCGCCTATGGTATCATGCTTGGAAATGGGAAGTGCAGTGGAAACACTATGTGGACAAGCATAT
GGAGCACAAAACTAGACATGCTTGGAAATATATCTCCAAAGATCAACAATTCTTCTAACCCTAACAGGCA
TTGTTATTATGTTTGTGTACATATTCTCAAAACCAATCCTCCTTTTACTTGGCCAATCAGAAGCCATAGC
TGCAGCAGCTGCCTTATTTACTTATGGTCTTATCCCACAAATCTTTGCCTACGCAGCCAATTTCCCAATC
CAAAAGTTCTTACAAGCTCAAAGTATAGTGGCACCTAGTACTTATATATCAGCAGGAGCTATTGTTTTCC
ATGTTTTGTTTAGTTGGTTAGCAATTTATAAGGTTGGACTTGGATTGTTTCGGGGCATCTTTAGTATTGAG
TTTGTCTTGGTGGGTGTTGTGTGGTGGTTCAGTTTATTTATATTCTATATAGTGATAGGACTAAGGACACT
TGGCGTGGATTTAGTGTTGAAGCTTTCATGGACTTTGGAGCTTTTTTAAAGTTGTCTGCTGCTTCTGCTG
TTATGCTTTGTTTGAAGCTTGGTATTTTCAGATTCTTGTTCCTTTTGGCTGGAATGCTTCCTGATCCTAA
AATCGCTTTGGATTCCCTCTCCATTTGCATTACAATCTTGGGTTGGGTATTCATGATAGCCGTTGGATTTC
AATGCTGCTGCCAGTGTGAGAGTAGGGAATGAACTAGGGGCAGGACATCCAAGGGCAGCTGCATTTTCAG
TAGTAATAGTGACAACAATGTCATTCATAATAGCAGTGATAATATCATTAGTGGTACTTGCTTTGCGCTA
CAAAATTAGCTATATCTTTACCGAAGGTGAAGTTGTAAGCAATGCTGTTGCCGATATGTGTCCCTTGCTC
GCCATCACTCTTGTCTTAATGGAATTCAACCTGTTTTATCCGGTGTTGCTGTTGGATGTGGATGGCAAG
CTTTTGTGTGCATATGTGAACGTTGGCTGTTATTACATTGTTGGTATCCCAACCGGTGCACCTTCTTGGGTT
CTACTTCAAACCTTGAGCCAAGGGTATTTGGTCCGGTATGATTGGTGGGACATTGATGCAAACATTATTAT
CTCATCTGGTTTTACTTATCGAACGGACTGGAAAAAAGAGGTGGATATAGCTCAAAGTAGGTTGGATACAT
GGGAGGACAAGCCAAAAATCTTTGTCCAATGAATAA

Appendix 4. Nucleotide sequence of *CYP2*

>cra_locus_4293_iso_2_len_2076_ver_3

ATGGATGTTGATATTCTTCTTTCTTCTTGTCTTGCGTTTTTTGGATGGGCAGCCATTTATTTCTCACTT
CAAGATTGAGCTTCGGGAAAAGAGCCAGACTTCCCCCAGGGCCTTATCCCTTTCCAGTCATCGGTAACAT
TTTTCAACTTGGCCAAAACCTAATCAGTCTCTACCAAACCTTGCCAAAACCTTATGGCCCTCTGATGTCC
CTTAAGCTTGGGAGCAACACAACCTGTGGTTGTTTCATCTCCAGCAGTCGCGAGAGAAGTACTACAGAAAA
ATGACCAGGTTTTTCTCAAGCCGAATAATCCCCCATGCAGCTGAAGCACATGCTCACCACAAGTACTCTAT
GGTCTGGTTGCAAGTTTCAGGCCTCTGGCGTAATCTCCGTAAGATAAGCAAAGAACACATGTTTGCAGCA
CAAAGGCTTGATGCTAGCGAAGGTCTTCGCCAAGAAAAGCTTCAAGAATTGCATGACTATTTGGTTTCGTT
GCAGCACTAGCAACAAAGCTGTCAATTTTGGTCAAACCTGCCTTTACGACATCTCTGAATTTTATATCGTC
CACTTTCTTTTCAGTTGATTTTGTCTGCTTATGGGTTCGGATTTCATCTCAAGAATTCAAGGACATTGTATGG
CGTATCCTGAAAAGTTATTCCACCCCCAATGTTGCAGACTATTTTCCGGTCCTCAAGTTCATGGATCCAC
AAGGCATTTTGCAGAAGAACACATTCTTATTTAGCAAAATGTTTGATATATTTGACAATATTATTAATGA
GCGACTGATGATGAGAGGTTTCATTGGACACTTCAAAGAAAAATGATTTACTAGAAGCTCTTCTCAATCAC
AGTGCCAATAATGAGTCTGAGTTCAGCCTCAATGAGCTCAAGCACATGCTTTTGGATTTATTTCTAGGAG
GGACTGAAACAACATCGACCACGCTGGAATGGGCTATGGCAGAGTTATTACGCAACCCTGAAAAATTAGA
AAGAGTTAGAGCTGAACTCCATCAAGTCATCGGGGAGAAGGAAATCATTCATGAATCAGATATCTCGAGG
CTTCCCTACTTGCAAGCAATAGTTAAGGAAACCTTTCGCCTTCACCCAATCGTTCCCTCTGTTAATTCCGC
ACAAAGCTGAGGCGGATGTAGAAATCAATGGCTACACTGTGCCCAAAAATTTCCAGATACTTATAAATGT
GTGGGCTTCTGGTAGAGATTCGGGGACATGGTTAGATCCTGAAACATTTTCCCCTGAACGATTCTTGCAT
AGTGAAATTGACATGAAAGGGCGGCATTTTGAGCTCATTCATTTGGGGCAGGTAGGAGAATATGTCCGG
GGTTACCTCTGGCTTATCGGACGCTTCATACAATGTTGGCAACTTGTATTCATAACTTTGATTGGAAGCT
TAAAGATGGAATGAAACCAGAAGACATTGACATGGAAGAAAAATATGGCCTGACTCTCCAGTTGGCGGTG
CCTTTAAATGTCATTTCCTGTAAACTCTGAT

Appendix 5. Fragment of MATE1 used in VIGS cloning

ATGGGTTCCAAACAAAACCTATGAAATAAACCAACCACTGTTACTAAGTAACAACGGCGGTGGCAGCAGTG
CTATTACAGCTGCAGAGTCGCCAAAGGCGGCTAAGACGGTGGAGGAAAGGCAGTATGAGCTTAGTGTTGA
GTTAGAAAGGGTACTTTCCGATACATCTGTACCATTAGTGCCAAGACTCACCGCCGCCACTTGGATTGAG
TTTAAGCTTCTCTTTCGGCTGGCGGCTCCAGCAGCCGCCGTTTATTTGATAAACTATGTAATGTCTATGT
TCACTCAAATATTTCCGGGCATCTTGGGAATCTTGAGCTTGCTGCTGCTTCTCTTGGTAATAATGGTAT
CCAGACCTTCGCCTATGGTATCATGCTTGAATGGGAAGTGCAGTGGAACACTATGTGGAC