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Dissertação

Avaliação de silenciamento gênico pós-transcricional (PTGS) de tropinona redutases em plantas de *Hyoscyamus muticus* L.

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Pelotas, Fevereiro de 2011

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Resumo

DALMAZO, GABRIEL OLLÉ. **Avaliação de silenciamento gênico pós-transcricional (PTGS) de tropinona redutases em plantas de Hyoscyamus muticus L. Dissertação (Mestrado)** – Programa de Pós Graduação em Ciência e Tecnologia Agroindustrial. Universidade Federal de Pelotas.

Um ponto de bifurcação na rota metabólica de tropano alcalóides (TA) é controlado pelas enzimas tropinona redutase 1 e 2 (TR1 e TR2). Tropinona, o substrato comum para estas enzimas, é reduzido por TR1 para formar tropina, hiosciamina e escopolamina ou por TR2 para formar pseudotropine e calisteginas. Hiosciamina e escopolamina são largamente utilizadas em medicina como anticolinérgicos, antieméticos, parassimpatolíticos e anestésicos. Calisteginas têm estrutura molecular semelhante a açúcares e são potentes inibidores específicos de glucosidases. A função dos alcalóides hiosciamina, escopolamina e calisteginas em plantas não é completamente entendida. Estudos recentes sugerem o envolvimento destes alcalóides na defesa da planta contra patógenos. A regulação da biossíntese de TA na planta tem atraído interesse não apenas quanto à aplicação na indústria farmacêutica, mas também com respeito à nutrição humana e fisiologia de plantas. No presente estudo avaliou-se uma estratégia transgênica, baseada em vírus, para induzir o silenciamento gênico pós-transcricional de *tr*1 e *tr*2 em plantas de *Hyoscyamus muticus* L. Observou-se que reduções significativas no acúmulo de transcritos para *tr*2 causaram um tremendo aumento no acúmulo de transcritos para *tr*1.

Palavras chave: metabolismo secundário, tropano alcalóides, calisteginas, PVX

Abstract

DALMAZO, GABRIEL OLLÉ. **Evaluation of post-transcriptional gene silencing (PTGS) of tropinone reductases in *Hyoscyamus muticus* L. plants. Dissertation (Master Degree in Agroindustrial Science and Technology).** Universidade Federal de Pelotas.

The tropane alkaloids (TA) pathway has a branch-point controlled by the enzymes tropinone reductase 1 and 2 (TR1 and TR2). Tropinone is the common substrate for these enzymes and is reduced either by TR1 to form tropine, hyoscyamine and scopolamine or by TR2 to form pseudotropine and calystegines. Hyoscyamine and scopolamine are largely used in medicine as anticholinergic, antiemetic, parasympatholytic and anaesthetic. Calystegines mimic different sugars and are potent and specific inhibitors of glucosidases. The function of hyoscyamine, scopolamine and calystegines in the plants is not fully understood. Recent studies suggest that they are involved in the plant defense against pathogens. Regulation of TA biosynthesis *in planta* has attracted interest not only in view of its applications in the pharmaceutical industry, but also in respect to human nutrition and plant physiology. In the present study a virus-based transgenic approach devised to induce PTGS of *tr*1 and *tr*2 in whole transformed *Hyoscyamus muticus* plants is evaluated. It was observed that a significant reduction in transcript accumulation for *tr*2 caused a tremendous increase in transcript accumulation for *tr*1.

Key-words: Secondary metabolism, tropane alkaloids, calystegines, PVX

Apresentação

Esta dissertação de mestrado consiste em uma introdução geral sobre tropano alcalóides e de um artigo científico onde são apresentados os principais resultados de pesquisa experimental realizada em laboratórios e casas de vegetação. Em conformidade com as normas da UFPel e do Programa de Pós-graduação em Ciência e Tecnologia Agroindustrial para elaboração de Dissertações e Teses esta dissertação de mestrado foi redigida em língua inglesa.

General Introduction

Alkaloids represent a diverse group of low-molecular-weight compounds that are related only by the occurrence of a nitrogen atom in the heterocyclic ring. Plants are estimated to produce approximately 12,000 different alkaloids that can be found in about 20% of plant species (Facchini 2001; Ziegler and Facchini 2008). Alkaloids found in plants represent one of the largest groups of natural products, and most pharmacological products are provided by this group of secondary metabolites (Hashimoto and Yamada 1994).

The tropane alkaloids atropine, scopolamine and its precursor hyoscyamine, which are found mainly in *Duboisia*, *Datura*, *Hyoscyamus*, *Atropa* and *Scopolia*, together with their semi-synthetic derivatives are used as parasympatholytics that competitively antagonize acetylcholine. Anticholinergics are generally used as mydriatics and for controlling saliva secretion , gastric acidity, as well as to slow gut motility and prevent vomiting. Besides their medicinal use they possess hallucinogenic and poisonous properties. In applied medicine, they are used to relieve the symptoms of Parkinson's disease, to dilate the pupils, increase the heart rate and to counteract organophosphate poisoning that causes smooth muscle relaxation (Sevon et al. 2001; Palazon et al. 2008). Another group of tropane alkaloids are the recently identified calystegines, consisting of a nortropane skeleton containing several hydroxy groups in different positions. These compounds function as selective inhibitors of glucosidases, galactosidases, xylosidases, and lysosomal enzymes which act on oligosaccharide metabolism; they are more widely spread than hyoscyamine and scopolamine in the plant kingdom, occurring mainly in the Solanaceae and Convolvulaceae.

The genera *Atropa, Datura, Duboisia, Hyoscyamus,* and *Scopolia*, which belong to the Solanaceae, are especially rich sources of calystegines (Drager et al. 1994; Asano et al. 2000). The possibility of toxicity caused by ingestion of plants containing

calystegines was considered by Asano et al (2000). They proposed that calystegines together with swainsonine were responsible for the intoxication caused in cattle ingesting Solanaceae e Convolvulaceae plants. Although calystegines are known as potent inhibitors of glycosidase, its role in cattle intoxication is not yet elucidated and in most cases, swainsonine is pointed as the responsible for the disturbs. In other hand, glycosidase inhibition can be used to treat diabetic patients, by stopping the fast increase of glucose just after carbohydrates ingestion. Therefore, calystegines may be revealed as an important compound to diabetic treatment, once they can regulate the activity of enzymes involved in carbohydrates digestion, thus slowing its absorption (Keiner and Drager 2000; Asano 2003; Drager 2004). Recently studies have demonstrated the potential of using calystegines as anti-cancer drugs. Calystegine B_3 specific inhibits a cytoplasmic α-mannosidase (Man2C1) implicated in non-lysosomal catabolism of free oligosaccharides derived from N-linked glycans accumulated in the cytosol. Suppression of Man2C1 expression reportedly induces apoptosis in carcinoma cells (Tian et al. 2008).

Bioactive compounds with privileged structures are highly sought paradigms in drug development. Functionally, a privileged structure is a molecular scaffold that can accommodate various pharmacophores arranged to promote interaction with biological targets (Costantino and Barlocco 2006). Though many molecules have been synthetically designed, nature remains the largest source of highly sophisticated biologically active privileged compounds because presumably they play a key role in increasing the survival fitness of organisms (Williams et al. 1989; Koehn and Carter 2005), with over 12,000 structurally characterized members, plant alkaloids are important privileged compounds with many pharmacological activities (Costantino and Barlocco 2006)

The availability of bioactive alkaloids is frequently impaired because they are usually complex molecules that cannot be synthesized chemically and occur at very low abundance and with restricted tissue distribution thereby hindering their isolation

and evaluation (Dixon 2001). A good example is the scarcity of vincristine, the cancer chemotherapeutic compound in *Catharanthus roseus*, which exists at concentrations that only reach 0.0003% by dry weight. The organic synthesis has advanced and created several methodologies for constructing bisindole alkaloids (Kuboyama et al. 2004), however total or semi-synthesis of many other alkaloids remains challenging and far from being practical at the industrial level.

To circumvent this problem other strategies are available for large scale production of secondary metabolites, plant cell and organ cultures have demonstrated good results in production of some metabolites (e.g., berberine, shikonin, ginsenosides and paclitaxel), but numbers of compounds that are produced commercially by cell culture technology are still very low (Rao and Ravishankar 2002). The production of secondary metabolites in plant cell and organ culture is partially impaired because undifferentiated plant tissue cultures are frequently unable to produce secondary metabolites as the intact plant. This is also the case of scopolamine production in undifferentiated *in vitro* cultures of Solanaceae, probably due to the specific location of some of the key enzymes involved in this biosynthetic pathway. The predicted localization and translocation of tropane alkaloids intermediate compounds in the roots of producing plants is presented in Fig. 1.. Suzuki et al. (1999) have demonstrated that the expression of the *pmt* gene was pericycle-specific, and it has also been shown that H6H is localized in the root pericycle (Hashimoto et al. 1991; Kanegae et al. 1994). In addition, Nakajima and Hashimoto (1999), have observed that TR proteins accumulate in the lateral roots of *Hyoscyamus niger*. Another possible reason for the low production of scopolamine in undifferentiated *in vitro* cultures could be that the auxin added to the callus and cell culture media for normal growth inhibits the activity of some of the key enzymes involved in scopolamine biosynthesis, such as PMT (Rothe et al. 2003).

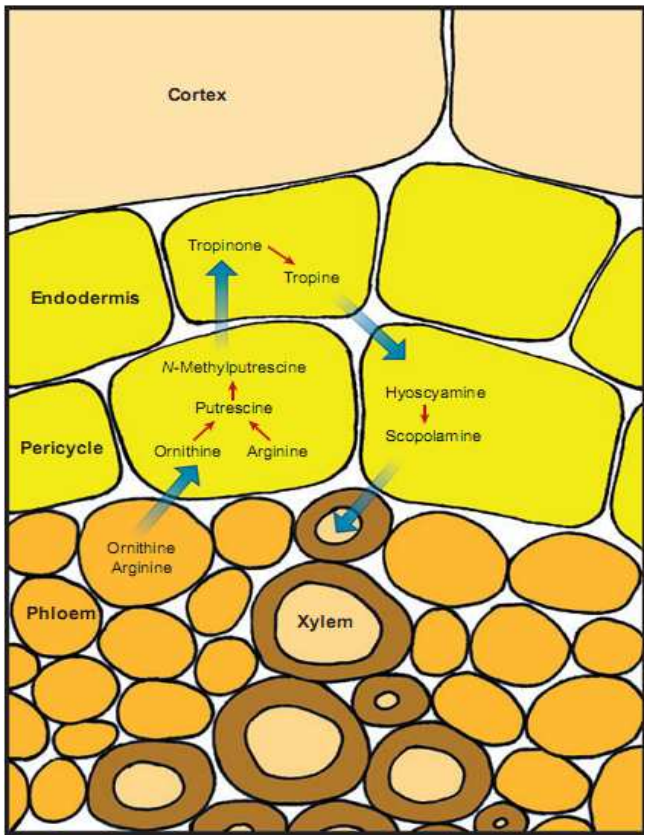

Figure 1. Putative spatial distribution and metabolic trafficking of alkaloids in the roots of producing plants. Red arrows (➔) indicates specific enzyme-catalyzed reactions; Blue arrows: Purported intercellular translocation of unspecified compounds. Source: Ziegler and Facchini (2008).

Regarding this limitation the genetic engineering of secondary metabolic pathways shows as a promising strategy for production of large scale natural products. This approach aims to either increase or decrease the quantity of a certain compound or group of compounds (Dixon 2001; Facchini 2001; Verpoorte, Heijden, and Memelink 2000). An enzymatic step in the pathway can be knocked out, for example, by reducing the level of the corresponding mRNA or by over expressing an antibody against the enzyme. Other approaches include the diversion of the flux into a competitive pathway. This technique permits endogenous biochemical pathways to be manipulated, resulting in the generation of transgenic crops in which the range, scope, or nature of a plant's existing natural products are modified to provide beneficial commercial, agronomic

and/or post-harvest processing characteristics (Kinney 1998). Metabolic engineering can provide various strategies to improve productivity, such as: a) increasing the number of production cells; b) Increasing the carbon flux through a biosynthetic pathway by overexpression of genes codifying for rate-limiting enzymes or blocking the mechanism of feedback inhibition and competitive pathways and c) decreasing catabolism (Palazon et al. 2008).

In this context, the TA pathway shows as a good model for testing transgenic strategies to modify the metabolic flux and to gain valuable insights into regulatory mechanisms that control pathway activity. The TA pathway is branched (Fig. 2), this characteristic is of special interest for testing the feasibility of transgenic strategies to re-direct the metabolic flux exclusively through the branch that leads to the formation of hyoscyamine and scopolamine (*tr*1 activity) or the branch that leads to the formation of calystegines (*tr*2 activity).

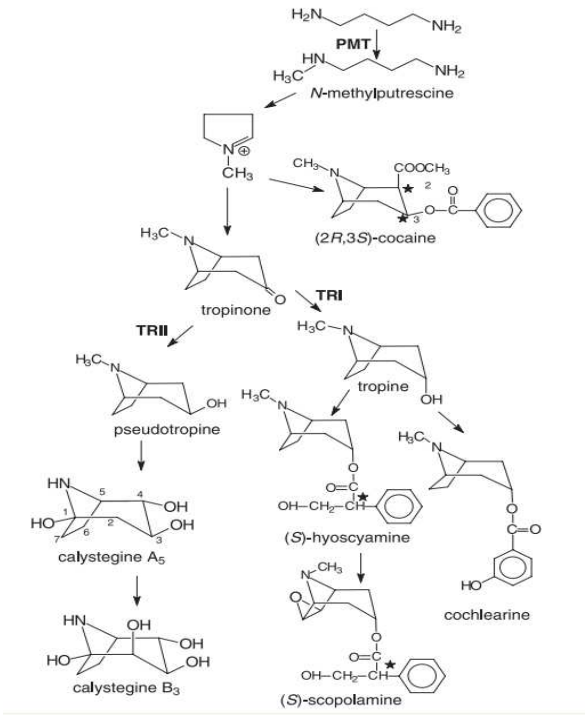

Figure 2. Tropane alkaloid pathway. **TRI**, tropinone reductase I; **TRII**, tropinone reductase II; **PMT**, putrescine *N*-methyltransferase. Source: Brock, Brandt, and Drager (2008).

The first manifestation of this approach was expression of the full-length complementary (or antisense) RNA transcripts. The strategy at the time was simply to invert the coding sequence of the target gene in a transgenic cassette. Later, the use of complementary double-stranded RNA (dsRNA) generated by inverted-repeat (IR) transgenes greatly increased the efficiency of gene silencing and became widely adopted in plant biotechnology. Meanwhile, the molecular fundamentals of RNA mediated gene silencing, known as RNA silencing, began to take shape. It appears that RNA silencing is not only a broadly used mechanism for gene regulation in plants, but also evolutionarily conserved in other branches of life. Both RNA interference (RNAi) in animals and quelling in fungi seem to operate under the same principles as RNA silencing (Frizzi and Huang 2010). The Post-Transcriptional Gene Silencing (PTGS) pathway is elicited by aberrant RNAs, RNA molecules lacking a polyA tail or 5' capping. They are thought to be derived from highly abundant transgenic sense or antisense RNA, viral RNA or truncated transcripts (Fig. 3) from complicated gene insertions or duplications (Frizzi and Huang 2010). These aberrant RNAs are converted into dsRNA by the RNA-dependent RNA polymerase, RDR6 (Dalmay et al. 2000), with the involvement of SGS3, SDE3 and WEX (Mourrain et al. 2000; Dalmay et al. 2001; Glazov et al. 2003). Originally identified in Drosophila, Dicers are RNaseIII enzymes that cleave (or 'dice') dsRNA into siRNAs. In *Arabidopsis*, a Dicer-like protein, DCL4, digests dsRNA into 21-nt siRNA duplexes (Dunoyer, Himber, and Voinnet 2005) that are then methylated at the 3' terminal nucleotide by a RNA methyltransferase, HEN1 (Boutet et al. 2003), used in all known small RNA pathways. One strand of the siRNA duplex is subsequently incorporated into the so called RNA-induced silencing complex (RISC). Although many of the components of the plant RISC have yet to be identified, the *Argonaute* protein (mainly AGO1) (Fagard et al. 2000; Morel et al. 2002), is known to be an integral part of the complex. Guided by the complementary 21nt siRNA, Argonaute cleaves the mRNA target.

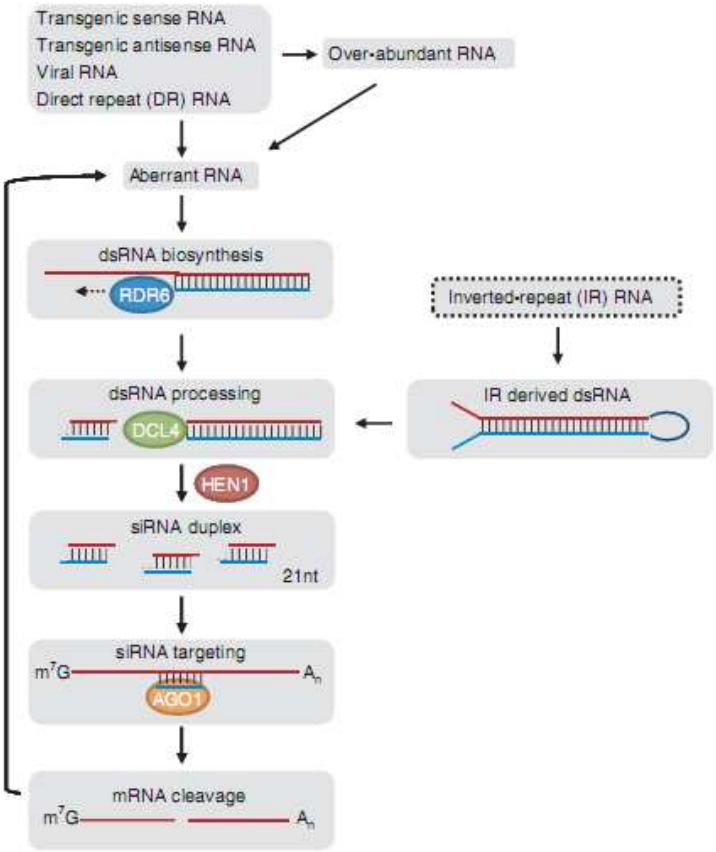

Figure 3. PTGS pathway. RNA-dependent RNA polymerases (**RDR**); Dicer-like RNaseIII type proteins (**DCL**); RNA methyltransferase (**HEN1**) and Argonaute proteins (**AGO**). Source: Frizzi and Huang (2010).

Based in the problematic demonstrated and the available tools to circumvent secondary metabolic scarcity, in a previous study *Hyoscyamus muticus* plants were stable transformed through *Agrobacterium* harbouring the transformation binary vector pBIN19 (Bevan 1984), with the PVX-amplicon expression cassettes PVX-*gus-astr*1-*npt*II and PVX-*gus-astr*2-*npt*II, designed to silence the endogenous genes *tropinone reductase* 1 (*tr*1) and 2 (*tr*2), respectively; and also T1 *H. muticus* plants, stable transformed with the dominant selectable marker gene *neomycin phosphotransferase* II (*npt*II), that confers high levels of resistance to kanamycin, were used as control. This approach aims to re-direct the metabolic flow towards one or another branch of the

metabolic pathway as shown in Fig. 2. This strategy applies to *Hyoscyamus muticus* plants with both active enzymes. The objectives of the present study were to evaluate the feasibility of transgenic approaches aiming the control of metabolic pathways. The hypothesis of cross-silencing, given the similarity between enzymes coding sequences was also evaluated. In this context, the main results of our study is presented in the following pages as a scientific article to be published.

ARTIGO

“Controlling the expression of tropinone reductases genes in Hyoscyamus muticus L. plants”

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Controlling the expression of tropinone reductases genes in *Hyoscyamus muticus* L. plants

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ABSTRACT

The tropane alkaloids (TA) pathway has a branch-point controlled by the enzymes tropinone reductase 1 and 2 (TR1 and TR2). Tropinone is the common substrate for these enzymes and is reduced either by TR1 to form tropine, hyoscyamine and scopolamine or by TR2 to form pseudotropine and calystegines. Hyoscyamine and scopolamine are largely used in medicine as anticholinergic, antiemetic, parasympatholytic and anaesthetic. Calystegines mimic different sugars and are potent and specific inhibitors of glucosidases. The function of hyoscyamine, scopolamine and calystegines in the plants is not fully understood. Recent studies suggest that they are involved in the plant defense against pathogens. Regulation of TA biosynthesis *in planta* has attracted interest not only in view of its applications in the pharmaceutical industry, but also in respect to human nutrition and plant physiology. In the present study a virus-based transgenic approach devised to induce PTGS of *tr*1 and *tr*2 in whole transformed *Hyoscyamus muticus* plants is evaluated. It was observed that a significant reduction in transcript accumulation for *tr*2 caused a tremendous increase in transcript accumulation for *tr*1.

Key-words: secondary metabolism; PXV; RT-qPCR; tropane alkaloids

1. INTRODUCTION

The plant genera *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus*, and *Scopolia*, which belong to the Solanaceae, accumulate the tropane alkaloids (TA) hyoscyamine and/or scopolamine, known to have anticholinergic, antiemetic, parasympatholytic (competitively antagonize acetylcholine), anaesthetic, and many other actions (Griffin and Lin 2000; Aehle and Drager 2010). They also accumulate calystegines, a unique subgroup of the TA class, termed nortropane alkaloid, characterized by the high degree of hydroxylation and absence of an N-methyl substituent, which is present in hyoscyamine and scopolamine (Molyneux et al. 1993). Calystegines mimic different sugars and are potent and specific inhibitors of glucosidases. They are widely distributed in plants of Solanaceae, Convolvulaceae, Brassicacea and Moraceae, including edible plants that do not accumulate hyoscyamine and scopolamine, such as potato, sweet potato, tomato, eggplant, chilli peppers, physalis fruits, mulberries, and scurvy-grass. Whether calystegines are toxic, beneficial or innocuous to humans is unknown (Asano 2003; Asano et al. 1997; Brock et al. 2006). The function of hyoscyamine, scopolamine and calystegines in the plants is not fully understood. Recent studies suggest that they are involved in the plant defense against pathogens (Chaudhuri et al. 2009; Abdel-Motaal et al. 2010).

TA are not chemically synthesized because the structural complexity of the molecules and plants remain as the main source, despite the low productivity that makes their extraction both difficult and expensive. As alternative systems, cell culture produces TA at trace levels only and root cultures, despite capable to produce as much as the plants, are not used in industrial production because large scale root culture are difficult to maintain (reviewed by Leonard et al. 2009).

Regulation of TA biosynthesis (Fig. 1) *in planta* has attracted interest not only in view of its applications in the pharmaceutical industry, but also in respect to human nutrition and plant physiology. Characterization of alkaloid biosynthetic genes (*pmt*, *tr*1, *tr*2, *h*6*h*) allows engineering of alkaloid pathways to generate plants with tailored

alkaloid profiles for basic research and for commercial production (reviewed by Kutchan et al. 2008). In this context, silencing of either *tr*1 or *tr*2 genes appears as an interesting strategy to re-direct the metabolic flux exclusively through the branch that leads to the formation of the bioactive alkaloids hyoscyamine and scopolamine. Alternatively, TA metabolic flux could be redirected exclusively towards the formation of calystegines, which are known as strong inhibitors of glucosidase activity.

The amino acids L-arginine and L-phenylalanine are the building blocks leading to TA (Fig. 1). Because putrescine is metabolized to other types of polyamines such as spermidine and spermine, the N-methylation of putrescine catalyzed by putrescine N-methyltransferase (PMT) is the first committed step in the biosynthesis of TA (Robins et al. 1997). Tropinone is reduced either by tropinone reductase 1 (TR1) to tropine, the biosynthetic precursor of hyoscyamine (Hashimoto et al. 1992; Koelen and Gross 1982) or by tropinone reductase 2 (TR2) to pseudotropine, the biosynthetic precursor of calystegines (Robins et al. 1994). Hyoscyamine 6b-hydroxylase (H6H) is bifunctional, catalyzing both the monooxygenation of hyoscyamine to 6b-hydroxyhyoscyamine and the subsequent epoxidation to scopolamine (Hashimoto and Yamada 1986).

Several *pmt* genes have been isolated from *Nicotiana tabacum*, *Atropa belladonna*, *Hyoscyamus niger*, *N. sylvestris*, and *N. attenuate* (Hibi et al. 1994; Shoji et al. 2000; Suzuki et al. 1999a; Winz and Baldwin 2001).The genes *tr*1 and *tr*2 have been characterized from *Datura stramonium* and *Hyoscyamus niger* (Nakajima et al. 1993b; Nakajima et al. 1999) and the gene *tr2* also from *Solanum tuberosum* (Keiner et al. 2002). The gene *h*6*h* has been characterized from *H. niger* and *A. belladonna* (Matsuda et al. 1991; Suzuki et al. 1999b).

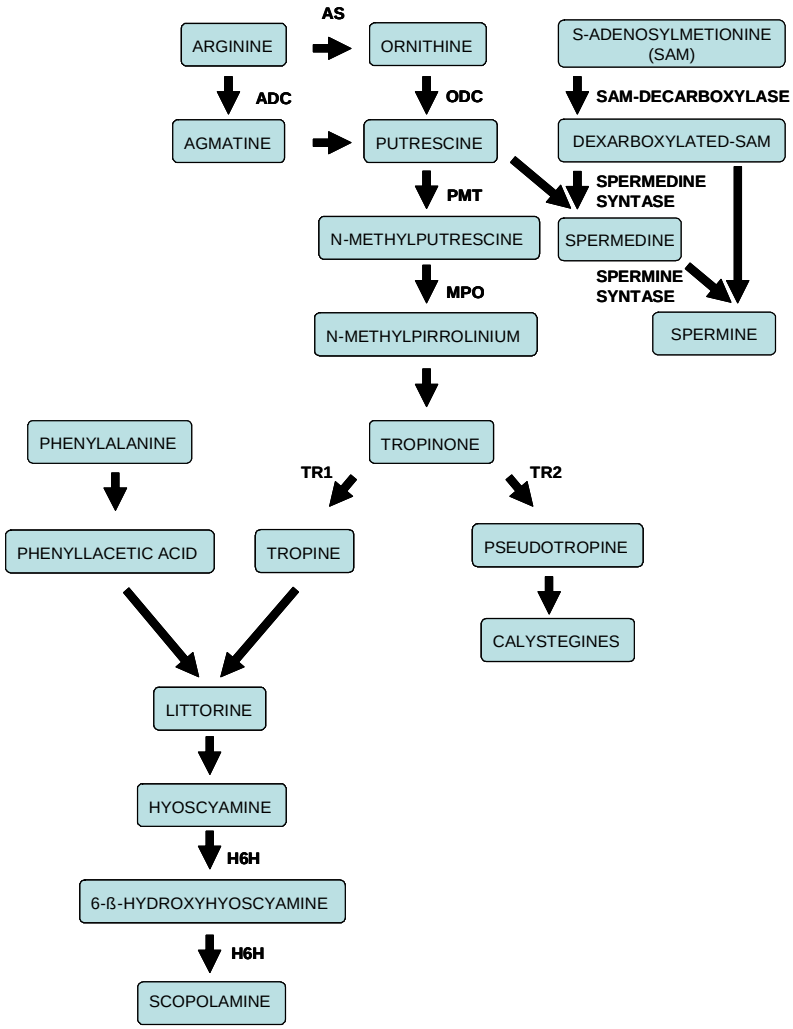

Figure 1 Metabolic pathway of tropane alkaloids (**TA**) synthesis. **AS** arginase (EC 3.5.3.1); **ODC** ornithine decarboxylase (EC 4.1.1.17); **ADC** arginine decarboxylase (EC 4.1.1.19); **PMT** putrescine N-methyltransferase (EC 2.1.1.53); **MPO** N-methylputrescine oxidase (EC1.4.3.21); **TR1** tropinone reductase 1(EC 1.1.1.206); **TR2** tropinone reductase 2 (EC 1.1.1.236); **H6H** hyoscyamine-6β-hydroxylase (EC 1.14.11.11). Arrows only represent chemical origins.

In post-transcriptional gene silencing (PTGS), the transgene is transcribed but the transcripts fail to accumulate as a result of sequence-specific targeting and destruction. PTGS which is based on a transgene comprising a cDNA of replicating

potato virus X (PVX) RNA, termed an 'amplicon', induces PTGS in every plant expressing a replicating amplicon. Besides, amplicon-mediated PTGS is stably inherited through subsequent generations (Angell and Baulcombe 1997). Amplicons carrying part of an endogenous gene sequence can consistently silence the corresponding gene and can phenocopy a previously characterised mutation (Angell and Baulcombe 1999).

Here we present the outcomes of a transgenic approach devised to induce PTGS of *tr*1 and *tr*2 in whole transformed *Hyoscyamus muticus* plants with cDNA of replicating potato virus X (PVX) RNA carrying the endogenous genes *tr*1 or *tr*2, in antisense orientation, from *Hyoscyamus niger.* Due to the high similarity of these genes and considering the RNA silencing mechanism elucidated so far, it was expected that by silencing *tr*1 gene, *tr*2 might be also silenced and vice-versa. To test this hypothesis we developed an efficient protocol to obtain plants producing enough roots to study transcripts accumulation of the above mentioned genes.

2. MATERIAL AND METHODS

2.1. Plant material

T_1 *Hyoscyamus muticus* plants, originally produced at John Innes Centre (Nora 2005), stable transformed through Agrobacterium harbouring the transformation binary vector pBIN19 (Bevan 1984), with the PVX-amplicon expression cassettes PVX-*gus*-*astr*1-*npt*II (plant line E03) and PVX-*gus*-*astr*2-*npt*II (plant lines U01, U02, U03 and U07), designed to silence the endogenous genes *tropinone reductase* 1 (*tr*1) and 2 (*tr*2), respectively; and also T_1 *H. muticus* plants, stable transformed with pBIN19, with the dominant selectable marker gene *neomycin phosphotransferase* II (*npt*II) that confers resistance to kanamycin, were used as control (plant line C03).

[G.D1] Comentário:

2.2. Plant culture

Twenty seeds from each plant line were transferred to a 10 mL syringe. The syringe was loaded with 10 mL absolute ethanol and agitated by hand for 2 min. The

[G.D2] Comentário:

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ethanol was replaced with 10 mL 3% sodium hypochlorite. The syringe was attached to a shaker and agitated at 150 RPM, for 25 min. The sodium hypochlorite solution was unloaded and the seeds were washed three times in 10 mL sterile water in the laminar flow cabinet to prevent recontamination. After washing, the seeds were placed in Petri dishes and left to dry inside a laminar flow cabinet. After that seeds were placed on ½ MS (Murashige and Skoog 1962) complemented with 3 $g.L^{-1}$ gelrite and 5 $g.L^{-1}$ sucrose, at pH 5.8, in Petri dishes (Fig. 2a), and incubated in an automatic growth cabinet set to seven cycles: 18 h in the light at 25 ℃ followed by 6 h in the dark at 6 ℃.

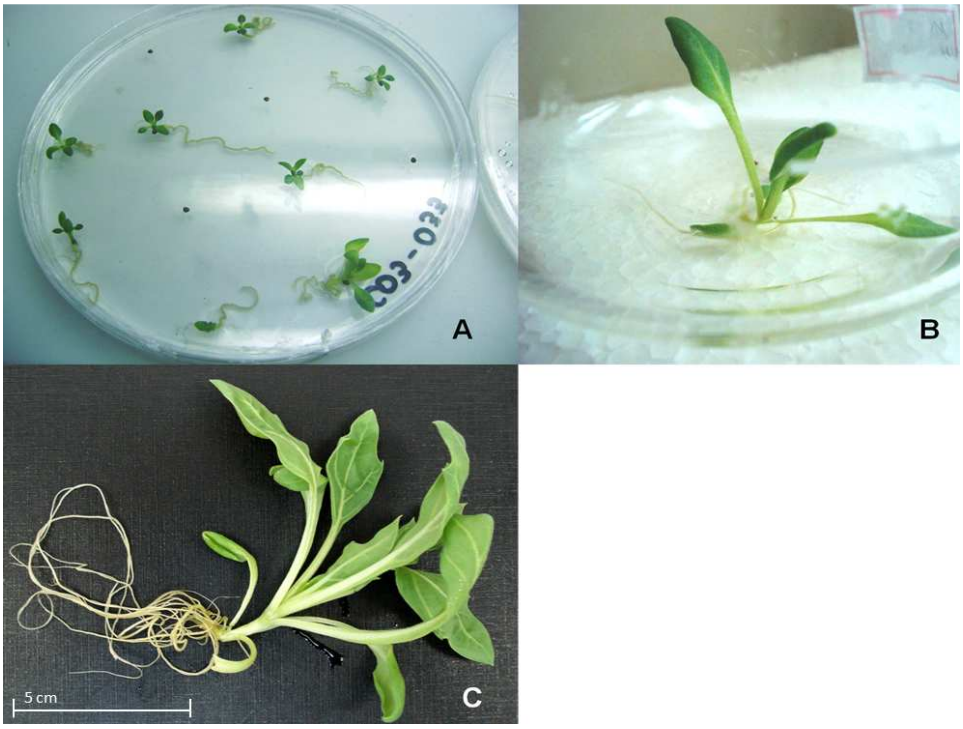

Figure 2 Production of *Hyoscyamus muticus* L. plants for analysis: A) Seedlings on solid medium in a Petri dish, ready for culturing on liquid medium; B) Plantlet on liquid medium in Erlenmeyer flask; C) Plantlet ready for RNA isolation from the roots.

Seedlings on stage of four leaves (Fig. 2A) were transferred onto 10 mL liquid medium (MS, 2.5 $g.L^{-1}$ sucrose, pH: 5.8) in 500 mL Erlenmeyers, and incubated in a

growth room at 22 ℃ ± 2 ℃, 70 % ± 5 % of relative humidity (RH), light intensity of 22 µmol.m^{-2}.s^{-1} ± 2 and 16 h photoperiod, until the plants produced reasonable amounts of roots (Fig. 2B and 2C). Secondary roots were harvested, frozen in liquid nitrogen and stored at - 80 ℃ until analysis.

2.3. Measurement of mRNAs accumulation of target genes by RT-qPCR

Total RNA was isolated from 100 mg of frozen roots, ground to a fine powder under liquid nitrogen, from each *H. muticus* plant, using *Concert Plant RNA Reagent* (Invitrogen™, catalogue number 12322012) according to manufacturer instructions. Total RNA was treated with *DNAse* I™ (New England Biolabs Inc.™, Part Number: M0303L) and reverse-transcribed into cDNA using *High Capacity cDNA Reverse Transcription Kit* (Applied Biosystems™, Part Number 4375575 Rev. E) which includes random hexamer primers suitable to reverse transcribe 18S rRNA transcripts that lacks PolyA tail. The quantity and quality of the RNA and cDNA was assessed spectroscopically (NanoVue Plus Spectrophotometer, GE Healthcare™) and by electrophoresis on agarose gel. Gene sequences from *tr*1 and *pmt* genes were obtained at Gene Bank (Benson et al. 2005), accession numbers AB026544 and AB018572 respectively. The nucleotide sequence from *tr*2 was cloned through RACE protocol in a previous study (Nora, 2005). Gene-specific primers were designed using *Perl Primer*™ (Marshall 2004). Due to the similarity of *tr*1 and *tr*2 gene sequences, they were aligned using AlignX (Vector NTI®10), and primers binding high similarity regions shared by both sequences were avoided to ensure primer specificity. The same procedure was adopted with *pmt* sequence, which is very similar to *spds*1 *and spds*2 (spermidine syntase 1 and 2), accession number AB006690 and AB006691 respectively. The criteria adopted for primer design were as follows: a) primer size between 20 bp and 26 bp, b) melting temperature between 60 ℃ and 65 ℃, c) percentage of guanine-cytosine (GC) content between 40 % and 60 %, d) average size of amplified fragments between 100 bp and 250 bp, e) low GC-content at the 3' end of

[LN4] Comentário: Include catalogue number

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the primer, f) avoidance of amplicon secondary structures (hairpin) predicted with *MFold*™ software (Zuker 2003). Primers were tested for specificity and expect PCR product prior to RT-qPCR. Melting curves were evaluated and only primers giving single peaks were used (Table 1.). RT-qPCR was performed with Stratagene MX3005P cycler (Stratagene, Agilent Technologies) using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen®., Cat. No. 11733-046) according to manufacturer instructions. The amplification reaction was carried out in a total volume of 25 µL, containing 2 µM of each primer, 6.25 µL of PCR Master Mix SYBR®Green, 1 µL of cDNA (approximately 100 ng) and water to make up the final volume. Samples were loaded in 96 well optic plates (Stratagene® Catalogue Number 410088) and covered with optic adhesives (Stratagene® Catalogue Number 401425). Thermal cycle conditions were as follows: denaturing at 50 ℃ for 2min and 95 ℃ for 2 min, followed by 40 three-step cycles (95 ℃ for 15 s, 60 ℃ for 30 s and 72 ℃ for 1 min) and final extension at 72 ℃ for 5min. Relative quantificatio n of each single gene expression was performed using the comparative quantification cycle method, as described by Livak and Schmittgen (Livak and Schmittgen 2001). For each cDNA, 18S *rRNA* was used as a reference gene to quantify cDNA abundance (at the same dilution as mentioned above). Results for accumulation of transcript were expressed as a variation of the normalized quantification cycle Cq (ΔCq), commercially named threshold cycle (Cq), from treatment plants (E and U) compared to control (C) originating a ΔΔCq value. Results were graphically expressed as fold induction ($fold\ induction = 2^{-\Delta\Delta Cq}$). Control plants served as baseline for determining relative quantification. Statistic analysis (Fisher's Test for ANOVA and Duncan's Test for comparing means of treatment) was performed on the variate ΔCq, considered equivalent to the variate fold induction for statistical purpose, using the software Sanest (Zonta and Machado 1991).

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Table 1. Oligonucleotides sequences used in the quantification of tropinone reductase 1, 2 and putrescine-*N*- methyltransferase transcripts.

primer name	primer sequence	target gene	amplicon size	Tm (°C)
tr1-F-234	CGACATCGTATTGGGCAC	*tr1*	234	60,85
tr1-R-234	TGGAGCAACTGAATTGACC			60,21
tr2-F-139	GAGGCTGCTTATCACTTATCTA	*tr2*	139	58,17
tr2-R-139	ATTGCTCCTTTGGTTGCTC			60,9
pmt-F-230	TAAGGGTTCTGTCAATTATGCG	*pmt*	230	60,77
pmt-R-230	AACTCAACCAAATCCCTAGC			59,98

3. RESULTS

3.1. Cultured plants

Total RNA was isolated from the whole root system of individual plants (Fig. 3).

Figure 3 Representative *Hyoscyamus muticus* L. plants of each of the six independent plant lines at the stage the RNA was isolated from the roots. Transformed with PVX-*astr*2: A) U03 (n=4), B) U01 (n=5), C) U07 (n=7), E) U02 (n=5); PVX-*astr*1: D) E03 (n=4); and empty pBIN19: F) C03 (n=9), where 'n' represents the number of biological replicates.

3.2. Accumulation of transcripts from *tr*1, *tr*2 and *pmt* genes

The overall expression patterns of both constructs in individual plants are presented on Fig. 4. The increase in *tr*1 activity is present in all plant lines with the PVX-as*tr*2 construction (U), these lines also present a pattern related to the other two genes assayed, showing in almost all biological repetitions an decrease of RNA expression of both *tr*2 and *pmt* genes. The plant line with the PVX-as*tr*1 construct (E) presented a less predictable pattern. The syngenic plants E03-4 and E03-14 segregated the PXV-as*tr1* transgene and present low accumulation of *tr*1 transcript. This indicates that this line (E03) naturally produces less amounts of *tr*1 transcripts compared to control (C03). Conversely the transgenic E plants present an increase in *tr*1 activity and a decrease in *tr*2 activity. The expression pattern of *pmt* gene in U plants, except in U03-6, U03-12 and U02-10 plants, is reduced in comparison to control plants.

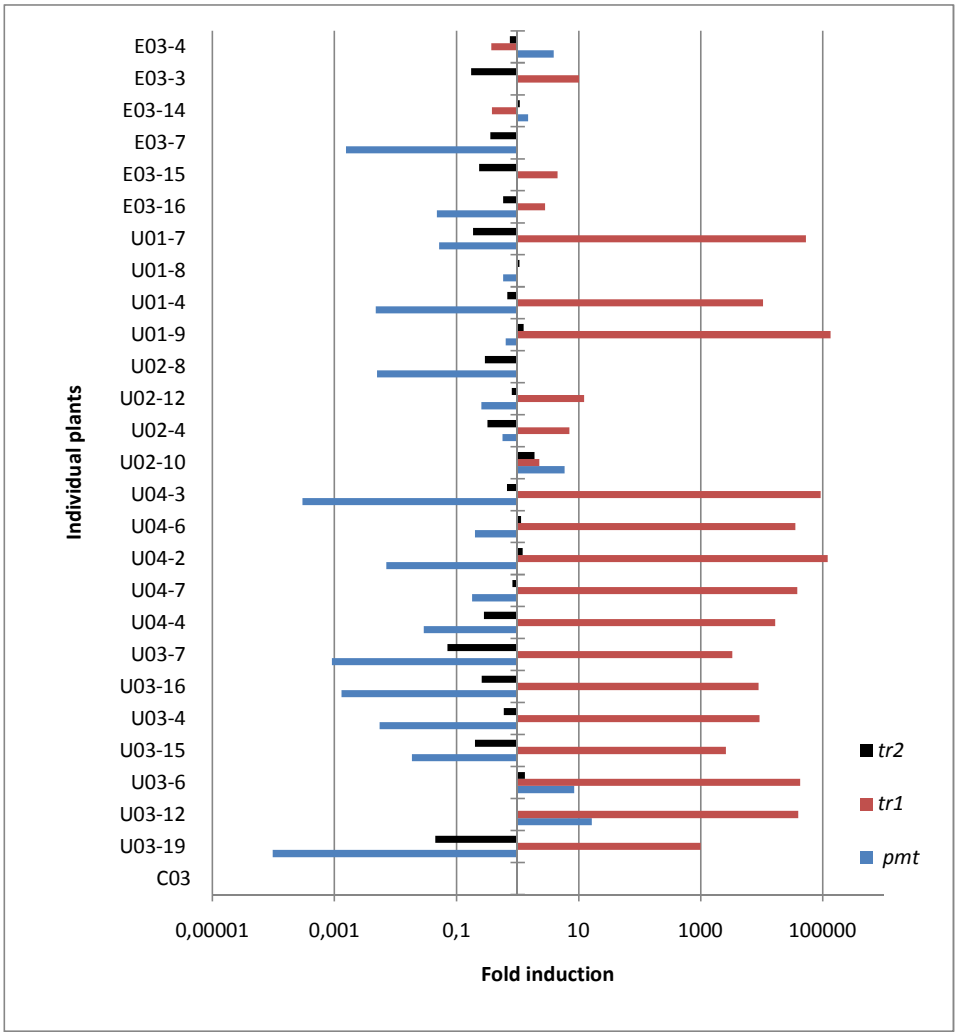

Figure 4. Accumulation of transcripts (fold induction) from the genes tropinone reductase 1 (*tr*1), tropinone reductase 2 (*tr*2), and putrescine methyl transferase (*pmt*) in *Hyoscyamus muticus* L. individual plants, stable transformed with either PVX-as*tr*1 (plant line E03) or PVX-as*tr*2 (plant lines U01, U02, U03 and U07), to induce post-transcriptional gene silencing of *tr*1 or *tr*2, respectively. The average ΔCq of control plants (C03) was used as base line.

In the plants transformed with PVX-as*tr*2 (U) the accumulation of transcripts from the *tr*2 gene was slightly reduced compared to control (C), while the accumulation of transcripts from the *tr*1 gene was tremendously increased (Fig. 5). Both group of

plants, PVX-as*tr*1 (E) and PVX-as*tr*2 (U), did not differ from control (C) in terms of accumulation of transcripts from *pmt* gene. Surprisingly, the accumulation of transcripts of *tr*1 gene in PVX-as*tr*1 (E) plants was the same as in the control plants while the accumulation of transcripts of *tr*2 gene was lower than in the control plants.

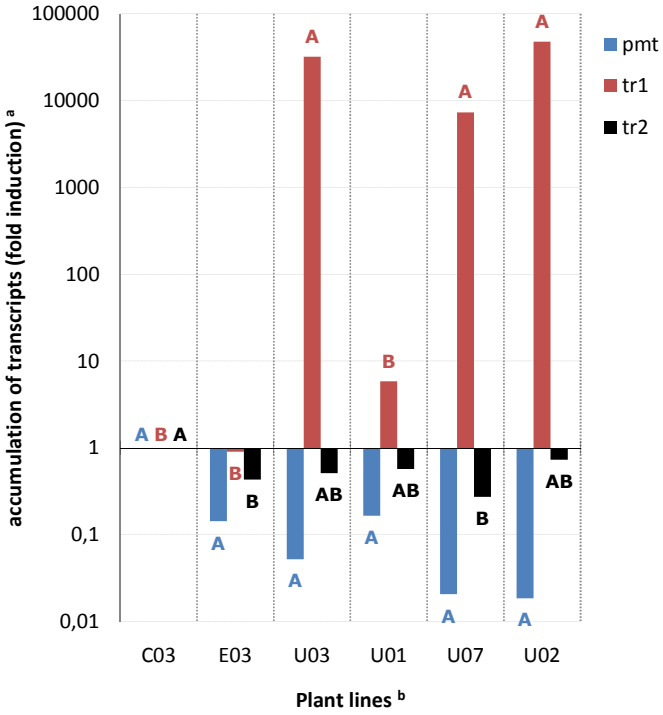

Figure 5 Accumulation of transcripts from the genes tropinone reductase 1 (*tr*1), tropinone reductase 2 (*tr*2), and putrescine methyl transferase in T_1 *Hyoscyamus muticus* L. plants, stable transformed with viral constructs designed to induce post-transcriptional gene silencing of *tr*1 and *tr*2 genes.

a) Accumulation of transcript expressed as fold induction was obtained by RT-qPCR according to the equation $\text{fold induction} = 2^{-\Delta\Delta Cq}$ where Cq denotes cycles of quantification. b) The plants were transformed with cDNA of replicating potato virus X (PVX) RNA carrying the endogenous genes tr1 [E03 (n=4)] or tr2 [U01 (n=5), U02 (n=5), U03 (n=4), U07 (n=7)] from Hyoscyamus niger, in antisense orientation. Control plants (C03=9) were transformed only with the marker gene nptII that confers resistance to kanamycin. “n” represents the number of biological replicates for each plant line. d) Means of treatment with the same letter do not differ from each other ($P > 0.05$) by Duncan’s test. The variate ΔCq instead of the variate fold induction was used to compare means of treatment.

4. DISCUSSION

Our results provide valuable insights and raise interesting questions regarding to metabolic homeostasis of TA biosynthesis in *Hyoscyamus muticus* transgenic plants. The most remarkable observation was the exponential increase in *tr*1 transcripts accumulation in plants transformed to silence *tr*2 gene. Nakajima (1993a) screening *H. niger* cDNA libraries isolated four *tr*2 clones but none *tr*1 clone, indicating a low concentration of *tr*1 transcript in this genus. In a subsequent study, Nakajima (1999) determined the activities of both TR enzymes in *Hyoscyamus niger* plants. The results indicated that TR2 enzyme has an activity 2-3 fold higher than TR1 enzyme, being TR2 the main responsible for tropinone conversion in the TA pathway. Based on that is plausible to assume that a small reduction of *tr*2 transcription, and consequent decrease in TR2 enzyme activity, could increase the accumulation of tropinone. The hypothesis of tropinone recycling leading to the production of upstream metabolites was considered, but reports don't indicate the conversion of tropinone into other compounds rather than tropine and pseudotropine *in vivo*. Thus, the increase in *tr*1 seems to be a response of the pathway facing an abnormal accumulation of tropinone. Previous studies have shown that the TA pathway is regulated at transcriptional level. Moyano (2003) demonstrated that just the increase of *pmt* transcripts, rather than the amount of PMT enzyme, up regulated the entire TA pathway leading to a higher tropane alkaloid production. Regulatory mechanisms in secondary metabolite pathways have been discussed in many scientific publications. Allen (2004) via RNAi silencing successfully shut down the codeinone reductase (COR) gene in *Opium poppy*, in response, specific upstream alterations were observed, with some rate-limiting enzymatic steps being down regulated rather than others. The main hypothesis point transcriptional regulation triggered by specific substrate accumulation. Another raised point of view is the spatial distribution of enzymatic complexes in secondary metabolic pathways, involving specific transport channels that may also being down regulated. In *Hyoscyamus muticus* plants PMT and H6H, which catalyze the last steps in the

biosynthesis of the tropane alkaloid scopolamine, are localized in the pericycle of the roots (Suzuki et al. 1999b; Suzuki et al. 1999a; Hashimoto et al. 1991). In contrast, TRI resides in the endodermis and nearby cortical cells (Nakajima and Hashimoto 1999); thus, tropane alkaloids intermediates must also traffic between different cell types. Another interesting fact led us to consider a feedback control hypothesis. Reductions in *pmt* transcripts were detected in almost all PVX-as*tr*2 transformed plants (U), suggesting a response to a supposed accumulation of the downstream compound tropinone and leading to negative feedback of upstream enzymes (e.g. putrescine-*N*-methyltransferase).

Another interesting data is related to the behaviour of the PVX construct. Angell and Baulcombe (1999) demonstrated that PVX mediated PTGS does not result in total abolition of target mRNA, rather a gradient of silencing intensity is observed among transformed plants. In some plants (e.g. U02-10) the expression patterns was slightly affected while in other it was strongly affected (e.g. U02-1). This behaviour can be partially explained by the characteristics of PVX mediated PTGS.

The results obtained, specially related to *tr*1 transcription, raise interesting questions regarding to metabolic regulation of TA pathway in *Hyoscyamus muticus* plants, making further studies worthy .and well funded. Proteomic and metabolomic analyses are essential for the complete understanding of the observed events and represent the next step in our studies.

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REFERENCES

Abdel-Motaal FF, El-Zayat SA, Kosaka Y, El-Sayed MA, Kashima R, Maeda Y, Nassar MSM, Ito S (2010) Antifungal activities of hyoscyamine and scopolamine against two major rice pathogens: Magnaporthe oryzae and Rhizoctonia solani. Journal of General Plant Pathology 76 (2):102-111. doi:10.1007/s10327-010-0225-6

Aehle E, Drager B (2010) Tropane alkaloid analysis by chromatographic and electrophoretic techniques: An update. J Chromatogr B 878 (17-18):1391-1406. doi:10.1016/j.jchromb.2010.03.007

Allen RS, Millgate AG, Chitty JA, Thisleton J, Miller JAC, Fist AJ, Gerlach WL, Larkin PJ (2004) RNAi-mediated replacement of morphine with the nonnarcotic alkaloid reticuline in opium poppy. Nature Biotechnology 22 (12):1559-1566. doi:10.1038/nbt1033

Angell SM, Baulcombe DC (1997) Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. Embo J 16 (12):3675-3684

Angell SM, Baulcombe DC (1999) Potato virus X amplicon-mediated silencing of nuclear genes. Plant J 20 (3):357-362

Asano N (2003) Naturally occurring iminosugars and related compounds: Structure, distribution, and biological activity. Curr Top Med Chem 3 (5):471-484

Asano N, Kato A, Matsui K, Watson AA, Nash RJ, Molyneux RJ, Hackett L, Topping J, Winchester B (1997) The effects of calystegines isolated from edible fruits and vegetables on mammalian liver glycosidases. Glycobiology 7 (8):1085-1088

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL (2005) GenBank. Nucl Acids Res 33 (suppl_1):D34-D38. doi:10.1093/nar/gki063

Bevan M (1984) Binary Agrobacterium Vectors for Plant Transformation. Nucleic Acids Res 12 (22):8711-8721

Brock A, Herzfeld T, Paschke R, Koch M, Drager B (2006) Brassicaceae contain nortropane alkaloids. Phytochemistry 67 (18):2050-2057

Chaudhuri K, Das S, Bandyopadhyay M, Zalar A, Kollmann A, Jha S, Tepfer D (2009) Transgenic mimicry of pathogen attack stimulates growth and secondary metabolite accumulation. Transgenic Res 18 (1):121-134. doi:10.1007/s11248-008-9201-8

Griffin WJ, Lin GD (2000) Chemotaxonomy and geographical distribution of tropane alkaloids. Phytochemistry 53 (6):623-637

Hashimoto T, Hayashi A, Amano Y, Kohno J, Iwanari H, Usuda S, Yamada Y (1991) Hyoscyamine 6*B*-hydroxylase, an enzyme involved in tropane alkaloid biosynthesis, is localized at the pericycle of the root. Journal of Biological Chemistry 266:4648-4653

Hashimoto T, Nakajima K, Ongena G, Yamada Y (1992) Two tropinone reductases with distinct stereospecificities from cultured roots of *Hyoscyamus niger*. Plant Physiol 100 (2):836-845

Hashimoto T, Yamada Y (1986) Hyoscyamine 6-Beta-Hydroxylase, a 2-Oxoglutarate-Dependent Dioxygenase, in Alkaloid-Producing Root Cultures. Plant Physiol 81 (2):619-625

Hibi N, Higashiguchi S, Hashimoto T, Yamada Y (1994) Gene-Expression in Tobacco Low-Nicotine Mutants. Plant Cell 6:723-735

Keiner R, Kaiser H, Nakajima K, Hashimoto T, Drager B (2002) Molecular cloning, expression and characterization of tropinone reductase II, an enzyme of the SDR family in Solanum tuberosum (L.). Plant Mol Biol 48 (3):299-308

Koelen KJ, Gross GG (1982) Partial-Purification and Properties of Tropine Dehydrogenase from Root Cultures of Datura-Stramonium. Planta Med 44 (4):227-230

Kutchan TM, Frick S, Weid M (2008) Engineering Plant Alkaloid Biosynthetic Pathways: Progress and Prospects. In: Hans J. Bohnert HN, Norman GL (eds) Advances in Plant Biochemistry and Molecular Biology, vol Volume 1. Pergamon, pp 283-310

Leonard E, Runguphan W, O'Connor S, Prather KJ (2009) Opportunities in metabolic engineering to facilitate scalable alkaloid production. Nat Chem Biol 5 (5):292-300

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25 (4):402-408. doi:10.1006/meth.2001.1262

Marshall OJ (2004) PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. Bioinformatics 20 (15):2471-2472. doi:10.1093/bioinformatics/bth254

Matsuda J, Okabe S, Hashimoto T, Yamada Y (1991) Molecular-Cloning of Hyoscyamine 6-Beta-Hydroxylase, a 2- Oxoglutarate-Dependent Dioxygenase, From Cultured Roots of Hyoscyamus-Niger. Journal of Biological Chemistry 266:9460-9464

Molyneux RJ, Pan YT, Goldmann A, Tepfer DA, Elbein DA (1993) Calystegines: a novel class of alkaloid glycosidase inhibitors. Archives of Biochemistry and Biophysics 304:81-88

Moyano E, Jouhikainen K, Tammela P, Palazon J, Cusido RM, Pinol MT, Teeri TH, Oksman-Caldentey KM (2003) Effect of pmt gene overexpression on tropane alkaloid production in transformed root cultures of Datura metel and Hyoscyamus muticus. J Exp Bot 54 (381):203-211

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473-497

Nakajima K, Hashimoto T (1999) Two tropinone reductases, that catalyze opposite stereospecific reductions in tropane alkaloid biosynthesis, are localized in plant root with different cell-specific patterns. Plant Cell Physiol 40 (11):1099-1107

Nakajima K, Hashimoto T, Yamada Y (1993a) cDNA encoding tropinone reductase-II from *Hyoscyamus niger*. Plant Physiol 103:1465-1466

Nakajima K, Hashimoto T, Yamada Y (1993b) Two tropinone reductases with different stereospecificities are short-chain dehydrogenases evolved from a common ancestor. Proc Natl Acad Sci U S A 90 (20):9591-9595

Nakajima K, Oshita Y, Kaya M, Yamada Y, Hashimoto T (1999) Structures and expression patterns of two tropinone reductase genes from *Hyoscyamus niger*. Biosci Biotechnol Biochem 63 (10):1756-1764

Nora L (2005) A virus-based transgenic approach to engineer the production of tropane alkaloids and calystegines in *Hyoscyamus muticus* plants. PhD, University of East Anglia (Available from the British Library), Norwich, UK

Robins RJ, Abraham TW, Parr AJ, Eagles J, Walton NJ (1997) The biosynthesis of tropane alkaloids in *Datura stramonium*: The identity of the intermediates between *N*-methyl-delta1-pyrrolinium salt and tropinone. J Am Chem Soc 119:10929-10934

Robins RJ, Bachmann P, Peerless ACJ, Rabot S (1994) Esterification reactions in the biosynthesis of tropane alkaloids in transformed root cultures. Plant Cell Tissue and Organ Culture 38 (2-3):241-247

Shoji T, Yamada Y, Hashimoto T (2000) Jasmonate induction of putrescine N-methyltransferase genes in the root of Nicotiana sylvestris. Plant Cell Physiol 41 (7):831-839

Suzuki K, Yamada Y, Hashimoto T (1999a) Expression of Atropa belladonna putrescine N-methyltransferase gene in root pericycle. Plant Cell Physiol 40 (3):289-297

Suzuki K, Yun DJ, Chen XY, Yamada Y, Hashimoto T (1999b) An Atropa belladonna hyoscyamine 6 beta-hydroxylase gene is differentially expressed in the root pericycle and anthers. Plant Mol Biol 40 (1):141-152

Winz RA, Baldwin IT (2001) Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. IV. Insect-induced ethylene reduces jasmonate-induced nicotine accumulation by regulating putrescine N-methyltransferase transcripts. Plant Physiol 125 (4):2189-2202

Zonta EP, Machado AA (1991) Manual do SANEST: Sistema de análise estatística para microcomputadores. DMEC/IFM/UFPEL, Pelotas, RS, Brasil

Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31 (13):3406-3415. doi:10.1093/nar/gkg595

General references:

Asano, N. 2003. Naturally occurring iminosugars and related compounds: Structure, distribution, and biological activity. *Current Topics In Medicinal Chemistry* 3 (5):471-484.

Asano, N., R. J. Nash, R. J. Molyneux, and G. W. J. Fleet. 2000. Sugar-mimic glycosidase inhibitors: natural occurrence, biological activity and prospects for therapeutic application. *Tetrahedron-Asymmetry* 11 (8):1645-1680.

Bevan, M. 1984. Binary Agrobacterium Vectors for Plant Transformation. *Nucleic Acids Research* 12 (22):8711-8721.

Boutet, S., F. Vazquez, J. Liu, C. Beclin, M. Fagard, A. Gratias, J. B. Morel, P. Crete, X. M. Chen, and H. Vaucheret. 2003. Arabidopsis HEN1: A genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. *Current Biology* 13 (10):843-848.

Brock, Andrea, W. Brandt, and B. Drager. 2008. The functional divergence of short-chain dehydrogenases involved in tropinone reduction. *Plant Journal* 54 (3):388-401.

Costantino, L., and D. Barlocco. 2006. Privileged structures as leads in medicinal chemistry. *Current Medicinal Chemistry* 13 (1):65-85.

Dalmay, T., A. Hamilton, E. Mueller, and D. C. Baulcombe. 2000. Potato virus X amplicons in arabidopsis mediate genetic and epigenetic gene silencing. *Plant Cell* 12 (3):369-379.

Dalmay, T., R. Horsefield, T. H. Braunstein, and D. C. Baulcombe. 2001. SDE3 encodes an RNA helicase required for posttranscriptional gene silencing in Arabidopsis. *Embo Journal* 20 (8):2069-2077.

Dixon, R. A. 2001. Natural products and plant disease resistance. *Nature* 411 (6839):843-847.

Drager, B. 2004. Chemistry and biology of calystegines. *Natural Product Reports* 21 (2):211-223.

Drager, B., C. Funck, A. Hohler, G. Mrachatz, A. Nahrstedt, A. Portsteffen, A. Schaal, and R. Schmidt. 1994. Calystegines as a New Group of Tropane Alkaloids in Solanaceae. *Plant Cell Tissue Organ Culture* 38 (2-3):235-240.

Dunoyer, P., C. Himber, and O. Voinnet. 2005. DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. *Nature Genetics* 37 (12):1356-1360.

Faccini, Peter J. 2001. Alkaloid biosynthesis in plants: Biochemistry, cell biology, molecular regulation, and metabolic engineering applications. *Annual Review of Plant Physiology and Plant Molecular Biology* 52:29-66.

Fagard, M., S. Boutet, J. B. Morel, C. Bellini, and H. Vaucheret. 2000. AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. *Proceedings of the National Academy of Sciences of the United States of America* 97 (21):11650-11654.

Frizzi, Alessandra, and Shihshieh Huang. 2010. Tapping RNA silencing pathways for plant biotechnology. *Plant Biotechnology Journal* 8 (6):655-677.

Glazov, E., K. Phillips, G. J. Budziszewski, F. Meins, and J. Z. Levin. 2003. A gene encoding an RNase D exonuclease-like protein is required for post-transcriptional silencing in Arabidopsis. *Plant Journal* 35 (3):342-349.

Hashimoto, Takashi, A. Hayashi, Y. Amano, J. Kohno, H. Iwanari, S. Usuda, and Yasuyuki Yamada. 1991. Hyoscyamine 6*B*-hydroxylase, an enzyme involved in tropane alkaloid biosynthesis, is localized at the pericycle of the root. *Journal of Biological Chemistry* 266:4648-4653.

Hashimoto, Takashi, and Yasuyuki Yamada. 1994. Alkaloid Biogenesis: Molecular Aspects. *Annual Review of Plant Physiology and Plant Molecular Biology* 45:257-285.

Kanegae, T., H. Kajiya, Y. Amano, Takashi Hashimoto, and Yasuyuki Yamada. 1994. Species-dependent expression of the hyoscyamine 6*B*-hydroxylase gene in the pericycle. *Plant Physiology* 105:483-490.

Keiner, R., and B. Drager. 2000. Calystegine distribution in potato (*Solanum tuberosum*) tubers and plants. *Plant Science* 150 (2):171-179.

Kinney, A. J. 1998. Manipulating flux through plant metabolic pathways. *Current Opinion In Plant Biology* 1 (2):173-178.

Koehn, F. E., and G. T. Carter. 2005. The evolving role of natural products in drug discovery. *Nature Reviews Drug Discovery* 4 (3):206-220.

Kuboyama, T., S. Yokoshima, H. Tokuyama, and T. Fukuyama. 2004. Stereocontrolled total synthesis of (+)-vincristine. *Proceedings of the National Academy of Sciences of the United States of America* 101 (33):11966-11970.

Morel, J. B., C. Godon, P. Mourrain, C. Beclin, S. Boutet, F. Feuerbach, F. Proux, and H. Vaucheret. 2002. Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell* 14 (3):629-639.

Mourrain, P., C. Beclin, T. Elmayan, F. Feuerbach, C. Godon, J. B. Morel, D. Jouette, A. M. Lacombe, S. Nikic, N. Picault, K. Remoue, M. Sanial, T. A. Vo, and H.

Vaucheret. 2000. Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* 101 (5):533-542.

Nakajima, K., and T. Hashimoto. 1999. Two tropinone reductases, that catalyze opposite stereospecific reductions in tropane alkaloid biosynthesis, are localized in plant root with different cell-specific patterns. *Plant and Cell Physiology* 40 (11):1099-1107.

Palazon, J., A. Navarro-Ocana, L. Hernandez-Vazquez, and M. H. Mirjalili. 2008. Application of metabolic engineering to the production of scopolamine. *Molecules* 13 (8):1722-1742.

Rao, S. R., and G. A. Ravishankar. 2002. Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnology Advances* 20 (2):101-153.

Rothe, G., A. Hachiya, Y. Yamada, T. Hashimoto, and B. Drager. 2003. Alkaloids in plants and root cultures of Atropa belladonna overexpressing putrescine N-methyltransferase. *Journal of Experimental Botany* 54 (390):2065-2070.

Sevon, N., S. Biondi, N. Bagni, and K. -M. Oksman-Caldentey. 2001. Transgenic *Hyoscyamus muticus* (Egyptian henbane). *Biotechnology in Agriculture and Forestry* 48:171-200.

Suzuki, K., Y. Yamada, and T. Hashimoto. 1999. Expression of Atropa belladonna putrescine N-methyltransferase gene in root pericycle. *Plant and Cell Physiology* 40 (3):289-297.

Tian, Y., J. Y. Ju, Y. Q. Zhou, Y. Liu, and L. P. Zhu. 2008. Inhibition of alpha-mannosidase Man2c1 gene expression suppresses growth of esophageal carcinoma cells through mitotic arrest and apoptosis. *Cancer Science* 99 (12):2428-2434.

Verpoorte, Robert, Robert van der Heijden, and Johan Memelink. 2000. Engineering the plant cell factory for secondary metabolite production. *Transgenic Research* 9 (4-5):323-343.

Williams, D. H., M. J. Stone, P. R. Hauck, and S. K. Rahman. 1989. Why Are Secondary Metabolites (Natural-Products) Biosynthesized. *Journal of Natural Products* 52 (6):1189-1208.

Ziegler, J., and P. J. Facchini. 2008. Alkaloid biosynthesis: Metabolism and trafficking. *Annual Review Of Plant Biology* 59:735-769.