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Genetic Transformation for Metabolic Engineering of Tropane Alkaloids

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1. Introduction

1.1 Tropane alkaloids: history and relevance

Historically, plants have been the major source of active compounds for the pharmaceutical industry. Today, plant extracts and its active principles represent 25% of the annual commercialized drugs in the United States (Kinghorn and Seo, 1996, Buttler 2004, Prakash Rout et al., 2009, Qurishi et al., 2010). Moreover, the World Health Organization has estimated that more than the 80% of the population in developing countries relies for their health care on traditional medicines mostly from plant origin (Canter et al., 2005). The plant active principles are, in general, a product of the plant secondary metabolism; they are low-molecular-weight compounds that in general participate in defence mechanisms against diseases (phytoalexins) or as attractants for pollinator insects (pigments and fragrances) (Canter et al., 2005). The production of each group of secondary metabolites, as counterpart of primary metabolites, is in general restricted to a limited number of species, and they usually are organ-specific and furthermore, tissue-specific.

According to their chemical properties, secondary metabolites could be classified in three main groups: a) terpenes, b) phenols and c) alkaloids. Terpenes and terpenoids are the primary constituents of the essential oils, widely used as natural flavour additives for food and fragrances. Phenols are a class of chemical compounds consisting of a hydroxyl group directly bonded to an aromatic hydrocarbon group that seem to be universally distributed in plants. They are essential for the growth and reproduction of plants, and are produced as a response for defending injured plants against pathogens. Finally, alkaloids are characterized for being alkaline nitrogenated organic compounds, derived from aminoacids, generally insoluble in water and soluble in alcohol, ether, chloroform, etc. Among the alkaloids, those derived from tropane have received particular attention for their properties.

1.2 Tropane alkaloids

Tropane alkaloids are distributed among the plant families *Orchidaceae*, *Dioscoreaceae*, *Proteaceae*, *Rhizophoraceae*, *Cruciferae*, *Eritroxilaceae*, *Euphorbiaceae*, *Convolvulaceae*, *Brassicaceae*, *Olacaceae* and *Solanaceae*. Their principal characteristic is the presence of a pyrrolic ring derived from the ornitine and arginine aminoacids by a chemical reaction catalyzed by ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) respectively (Figure 1). Ornithine and arginine also are the precursors of the polyamines putrescine, spermidine and spermine, which play a critical role in plant development by the regulation of the cell

division in plants (Theiss et al., 2002). On the other hand, putrescine, by the action of the enzyme putrescine N-methyltransferase (PMT; EC 2.1.1.53), is sequestered to the secondary metabolism being the first step of the tropane alkaloid biosynthetic pathway.

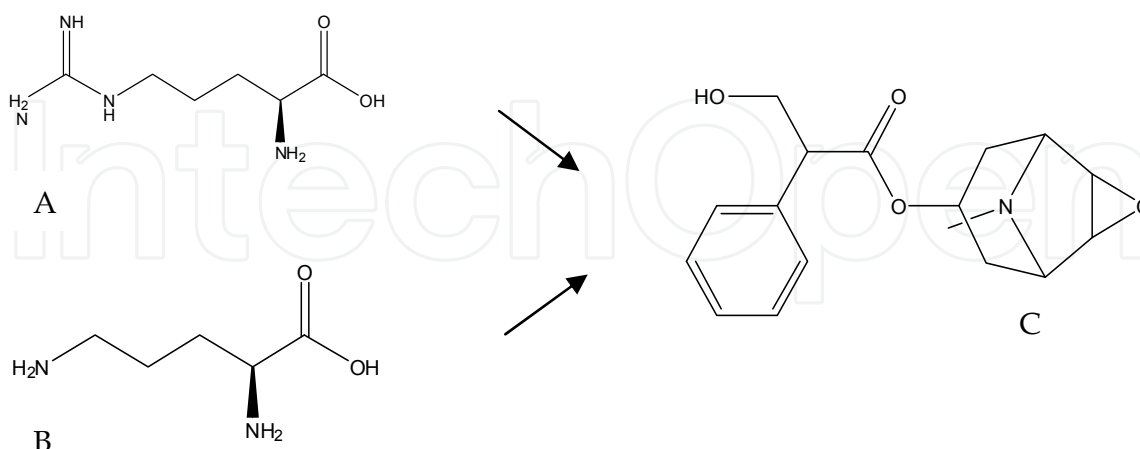


Fig. 1. Primary metabolism precursors: arginine (A) and ornithine (B) and the tropane alkaloid final product scopolamine (C).

Hyoscyamine and scopolamine are the end products of the tropane alkaloid biosynthetic pathway having both pharmacological applications for its action in the parasympathetic nervous system. Scopolamine, a 6,7- β -epoxide of hyoscyamine, is derived from hyoscyamine by the intermediate form 6 β -hydroxyhyoscyamine (Fig. 2). The pharmaceutical industry employs hyoscyamine, and its racemic form atropine, to obtain hyoscine N-butyl bromide that is used for its antispasmodic action since 1951. This semisynthetic derivative has pharmacological proprieties similar to those from scopolamine but with less activity and more adverse effects (Tytgat, 2007). Figure 2 compares the industrial process for producing the atropine racemic form of hyoscyamine, and the scopolamine biosynthetic pathway in plants.

1.2.1 Scopolamine

Scopolamine is one of the earlier alkaloids purified from plant sources, described by Albert Ladenburg in 1880. It is the most valuable of the tropane alkaloids being its worldwide demand 10-fold the demand of the sum of hyoscyamine (its precursor) and atropine (the semisynthetic drug) (Moyano et al., 2003). As atropine, scopolamine is an anticholinergic drug, besides it also has anti-muscarinic activity. Anticholinergic drugs are, in general, competitive and reversible inhibitors from acetylcholine with effects at parasympathetic level. Because of that pharmacological activity scopolamine has a number of uses in medicine. Its primary use is for the therapy of nausea (transdermal patches), motion sickness and intestinal cramping. Also, it is used for ophtalmic purposes (to induce mydriasis and cycloplegia) and as a general depressant in combination with narcotic painkillers. It also has secondary uses are a preanesthetic agent, a drying agent for sinuses, lungs and related areas and to reduce motility and secretions in the gastrointestinal tract (tinctures). Uncommonly, it is also used for some forms of Parkinsonism, combined with opioids (e.g.: with morphine). The production and purification of scopolamine is extremely expensive which have made that several groups have focused in finding scopolamine analogs or synthetic forms.

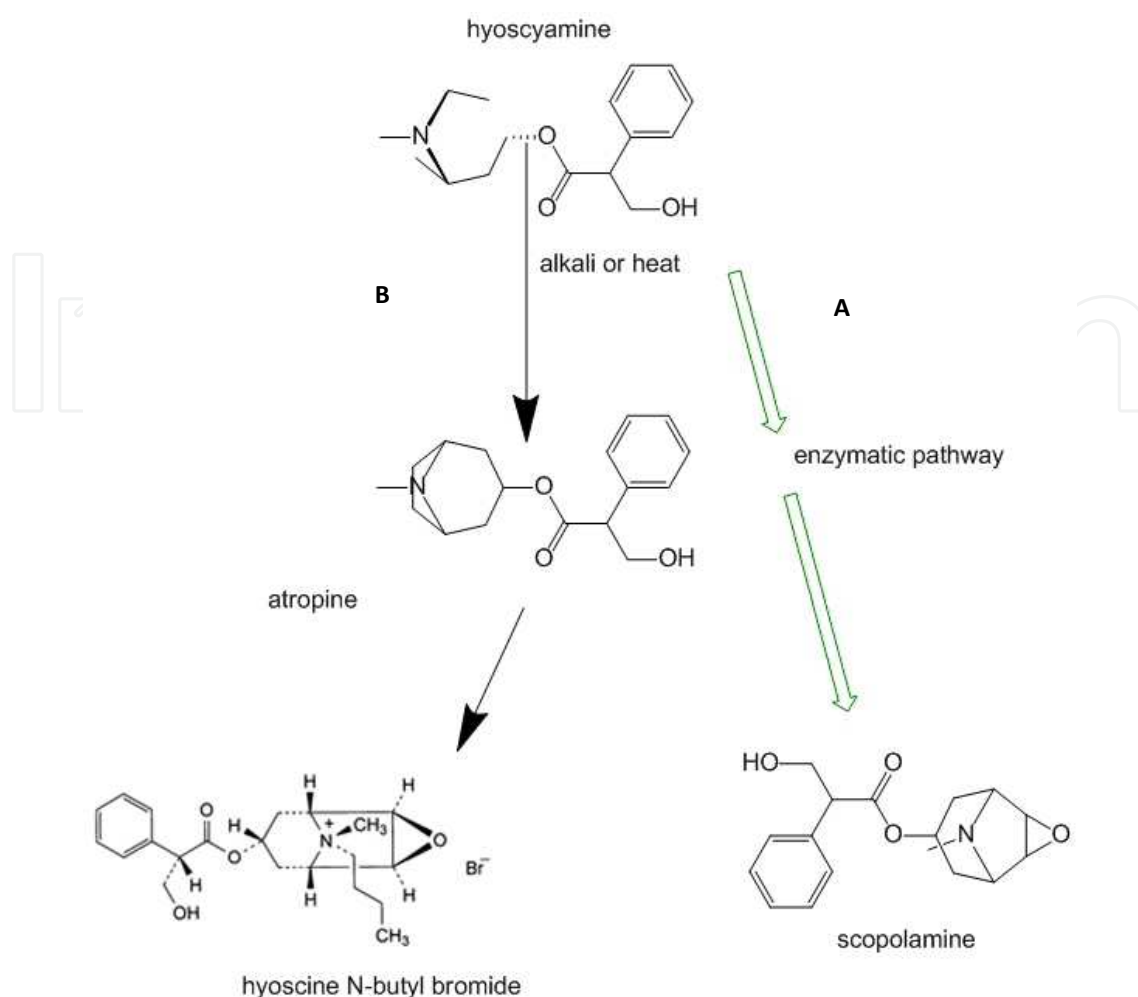


Fig. 2. The racemic hyoscyine N-butyl bromide is obtained from natural sources as hyoscyamine (A) or by semi-synthesis from atropine (B). Scopolamine is the final product in the tropane alkaloid pathway being hyoscyamine its precursor.

1.2.3 Biosynthetic pathway, key points: PMT and H6H

As was mentioned above, scopolamine and hyoscyamine are the most relevant tropane alkaloids widely in use. Their metabolic pathway starts in putrescine, a polyamine that is shared by several metabolic pathways (e.g.: pyridinic alkaloids). The role of putrescine methyl transferase (PMT; EC 2.1.1.53) is to sequester putrescine from the polyamine pool, which is the cross-point between the primary and secondary metabolism towards the tropane alkaloid pathway. The enzyme PMT catalyses the reaction from putrescine to N-methyl putrescine with S-adenosyl methionine as methyl donor (Zhang et al., 2004, Stenzel et al., 2006). It was described by Mizusaki et al. (1973) in tobacco for the first time, afterwards it has also been described in *Hyoscyamus* sp. (Hashimoto et al., 1989, Suzuki et al., 1999). The last part of the tropane alkaloid biosynthetic pathway is the enzymatic reaction catalyzed by hyoscyamine-6 β -hydroxylase (H6H), a 2-oxoglutarate dependent dioxygenase (EC 1.14.11.11, MW: 41.000 \pm 1.0, optimal pH 7.8) that catalyses the hydroxylation of hyoscyamine to scopolamine in two steps. The first step is the hydroxylation of hyoscyamine to 6 β -hydroxyhyoscyamine, a reaction that requires 2-oxoglutarate, Fe²⁺, molecular oxygen, and ascorbate. The second step is the epoxidation of the intermediate

6 β -hydroxyhyoscyamine producing the 6,7- β -hyoscyamine epoxide (scopolamine) (Hashimoto and Yamada, 1986 and 1987).

2. Strategies for scopolamine production

The chemical complexity of tropane alkaloids turns almost impossible its substitution by other compounds or the *de novo* synthesis. To date, alkaloids are extracted from the macerated leaves of wild plants growing in greenhouses. The whole process, performed under Good Manufacture Practices (GMP), makes the final product very expensive thus the search of an alternative production strategy is a priority. The alternative methods comprise biotechnology approaches as the genetic transformation for metabolic engineering to overexpress the key proteins in the biosynthetic pathway and the establishment of *in vitro* organ, tissue and plant cultures from scopolamine producer species.

2.1 Metabolic engineering

The in-depth understanding of biosynthetic pathways, along with the increasing number of cloned genes involved in biosynthesis, enable the exploration of metabolic engineering as a potential effective approach to increase the yield of specific metabolites. It could be achieved by enhancing rate-limiting steps or by blocking competitive pathways. Metabolic engineering is a multidisciplinary powerful tool that uses from bioinformatics, genetics, biochemistry, systems biology, molecular biology, biochemical engineering, etc, for re-direct the metabolic flux generally in order to increase secondary metabolite production yields (e.g.: alkaloids). It also could be useful for a rational and directed modification of metabolic pathways to understand their design and regulation and to study the impact of intermediates and end products in a specific organism.

When the improvement of a productive process is the main goal, the *a priori* knowledge of the metabolic pathway is fundamental. The next step is to know the kinetic of growth of the biomass that produces the product of interest, the parameters that could reflect that growth, and the election of the parameters that will led to the optimization of the process. Metabolic engineering uses different strategies such as improving the precursors production, re-directing one or more enzymatic reactions in a metabolic pathway, widening the metabolic capacities by stress or increasing the enzymatic activities, etc. Also a reliable strategy is to direct the carbon flux towards the biosynthetic pathway by the overexpression of the genes codifying the limiting enzymes, blocking the feedback inhibition mechanism or the competitive pathways, diminishing catabolism, etc..

2.1.1 Hairy root culture initiation and maintenance

Hairy roots are obtained by infection of the plant with different *Agrobacterium rhizogenes* strains (e.g.: LBA9402 and LBA15834) that harbor the Ri vector (In: *Agrobacterium-mediated genetic transformation: history and progress*, present book). They are characterized by their indefinite and fast growth and its higher chromosomal stability than *in vitro* clonal multiplication. The first hairy root cultures were established at the end of the 1980's (Oksman-Caldentey and Strauss, 1986, Hamill et al. 1987, Oksman-Caldentey et al., 1987). Since then, it has been established hairy roots from numerous species, among them scopolamine-producing species (*Hyoscyamus*, *Brugmansia*, *Duboisia*, *Atropa*, and *Scopolia*, etc). In general, hairy roots are initiated from axenic plants by infection with *A. rhizogenes* the hairy root tips usually appear in the infected areas approximately after one week of

infection, being a chimera between the infected and non-infected cells. Once initiated, they are maintained by transfer to selective media without the addition of plant growth regulators. The hairy root cultures are subcultured in the same medium approximately every 20 days. When the process is performed in liquid medium it is carried on under agitation in an orbital shaker (100 rpm) with 16 h- photoperiod using cool white fluorescent lamps.

As we have said before, the synthesis of tropane alkaloids is produced in the pericycle of roots (Hashimoto et al., 1991) being the final products translocated to the aerial part of the plant. It is not surprising then that the establishment of hairy roots was considered as an alternative strategy for scopolamine production (Oksman-Caldentey et al., 1991, Oksman-Caldentey and Arroo, 2000, Palazón et al., 2003 and 2008). It was reported that *Datura stramonium* hairy roots de-differentiated and loose their ability to synthesize scopolamine when treated with auxines (Palazón et al., 1995). On the other hand, undifferentiated cultures (calli) do not produce scopolamine proving the need of some degree of tissue specialization (Moyano et al., 1999; Jouhikainen et al., 1999). Evidently, the tropane alkaloid synthesis requires root organization when scopolamine is the end product.

Even though hairy roots are scopolamine producers the yields achieved are low, which has fostered the quest of an alternative production strategy. In the 1990's the first studies about the influence of plant growth regulators on scopolamine yields were performed. The effect of the addition of GA₇ on kinetics of growth and alkaloid accumulation in two different *Brugmansia candida* hairy root clones have demonstrated that GA₇ (10⁻⁴ to 10⁻¹ mg.l⁻¹) increased the scopolamine/hyoscyamine ratios in the early phases of growth, the sum of scopolamine plus hyoscyamine per flask decreased during the same growth period. Indeed the addition of GA₃ produced higher growth rates but lower alkaloid concentration (Pitta Alvarez & Giulietti, 1997).

2.1.2 Elicitation

The term elicitor was first introduced to describe the action of biomolecules able to induce phytoalexin production. In general, elicitors induce defence systems and increase the resistance to pathogens in plants. Also, pathogen biomolecules derived from the pathogen cell wall (exogenous elicitors), and compounds released from plants by the action of the pathogen, triggers that defence response (Angelova et al., 2006).

At the end of the 1990's several groups started the study of the effect of elicitors on secondary metabolism. Hence, it was evident that biotic elicitors (produced by pathogenic microorganisms or released from their cell walls by plant enzymes) were able to induce changes in secondary metabolites patterns. Also, the release of certain compounds, such as cellulose or pectinase and molecules active in the signal transduction pathway (salicylic acid, jasmonic acid), could also promote the plant defence response (Benhamou, 1996; Guo et al., 1997; Lawrence et al., 2000). That response includes the production of phytoalexins (pathogenesis related proteins), protease inhibitors and a variety of other defence compounds (among them alkaloids). *In vitro* plant cell cultures have been used for studying the influence of elicitors on secondary metabolism (Zabetakis et al., 1998, Jung et al., 2003, Marconi et al., 2008), and also their influence on tropane alkaloid metabolism particularly in hairy roots (Pitta Alvarez et al., 2000, 2001, Ajungla et al., 2009). Non-conventional elicitors (e.g.: acetate buffer, pectinase, *Hormonema* sp. homogenate), increased intracellular hyoscyamine content (200-300%), and the release of scopolamine (up to 1500%) and hyoscyamine (up to 1100%) in *B. candida* hairy roots (Pitta-Alvarez et al., 2003). Thus, elicitors

represent a promising and inexpensive alternative to increase production of hyoscyamine and scopolamine by *in vitro* culture.

3. Metabolic and genetic engineering

Molecular biology, as a tool for DNA manipulation, could be used for engineering metabolic pathways. Figure 3 shows the usual strategies employed for overproducing a specific metabolite (C).

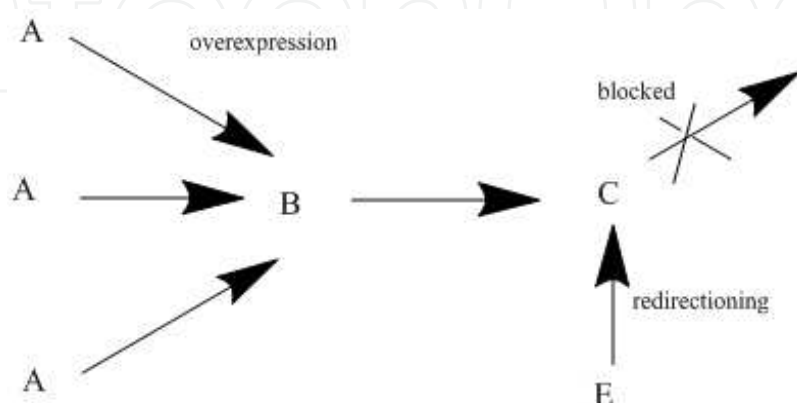


Fig. 3. Strategies for increasing secondary metabolite production by engineering metabolism.

The strategies metabolic engineering uses to increase secondary metabolite production are a) the overexpression of secondary metabolite precursors (A); b) the overexpression of genes whose products are rate-limiting (B), c) the creation of new branches in the biosynthetic pathway (E to C), d) the blocking of reactions (C to D) redirecting them to the C pathway, among others. Also, there are other strategies for overproducing C such as a) manipulation of regulatory genes, b) a positive or negative regulation of gene expression, c) selection of mutants with increased expression of metabolites, d) use of specific promoters for tissue/organ specific expression, e) iRNA technology and gene silencing. Each of the strategies above mentioned has unpredictable consequences in the cell homeostasis.

3.1 Overexpression of key enzymes

Overexpression of a key enzyme in the biosynthetic pathway is one of the strategies used to increase secondary metabolite production. In general, the overexpressed enzyme catalyzes a bottleneck step. In the tropane alkaloid pathway the key enzyme is H6H. As was mentioned above, H6H catalyzes two reactions, the hydroxylation at position 6, rendering an intermediate 6 β -hydroxyhyoscyamine; and the epoxidation that leads to the end product scopolamine. Therefore, overexpression of H6H is an attractive strategy for poor scopolamine producing species with hyoscyamin-rich accumulation. *In vitro* shoot and callus cultures shown very low concentration of H6H and, hence, scopolamine production. On the contrary, hairy root cultures from scopolamine-producing species have high concentration of H6H. The difference in the expression of this enzyme could be attributed to the specialization of root pericycle for producing H6H. Several attempts were made in order to isolate and overexpress the H6H cDNA in hairy roots for scopolamine production. The *h6h* cDNA was isolated from *B. candida*, *H. muticus*, *A. belladonna*, *Duboisia* hybrid with high producing scopolamine capacity (Matsuda et al., 1991, Jouhikainen et al., 1999, Cardillo et

al., 2005; Canter et al., 2005, Oksman-Caldentey et al., 2007, Palazón et al., 2008). All the isolated sequences revealed high homology (>80%). A strategy used is to introduce in low-producing scopolamine species numerous copies of their own H6H gene. All the protocols have several common steps. First, the *h6h* cDNA is cloned into the binary vector and amplified in *Escherichia coli*. Then, *A. rhizogenes* is transformed with the binary vector obtained and used for re-introducing, by agrotransformation, that *h6h* cDNA into the genome of the same species from which it was isolated. However, the results were not successful because the H6H overexpressing clones obtained did not have a significant increase on alkaloid production. Those results could be attributed to an upstream regulation of alkaloid biosynthesis, to a rate limiting speed of H6H or to a deficiency of precursors.

Another strategy focus on the lack of substrates in the tropane alkaloid pathway. Thus, the carbon flux is redirect from the primary to the secondary metabolism, being PMT the key and pivot enzyme between both metabolisms. There is a strong expression of the *pmt* gene in the pericycle of *Atropa belladonna* roots that is suppressed by the addition of exogenous auxins (Suzuki et al., 1999, Hibi et al., 1994, Palazón et al., 1995). There are several protocols describing the establishment of PMT overexpressing hairy roots. Nevertheless the results obtained are as disappointing as those obtained overexpressing H6H. The first report of PMT overexpression in hairy roots was with *Hyoscyamus albus* (Hashimoto et al., 1989). Tracer-feeding studies with radioactive aminoacids demonstrated that putrescine is the precursor of tropane alkaloids. Sato et al. (2000) have reported the overexpression of PMT in *Atropa belladonna* hairy roots revealing an increase in polyamines pools. However, the alkaloid profile remained unchangeable. On the other hand, the overexpression of PMT from *Nicotiana tabacum* in *Duboisia hybrid* hairy roots (yielding scopolamine) produced an increase of *pmt* gene expression that is not reflected in the alkaloid production (Moyano et al. 2000). Also, there are reports of heterologous tests with non-tropane alkaloid producing species such as *Solanum tuberosum* (Stenzel et al., 2006) and *Nicotiana sylvestris* (Sato et al., 2001). The overexpression of PMT in *N. sylvestris* increased the nicotine concentration, probably for a higher metabolic flux towards the tropane and pyridinic alkaloid pathways. However, when the *pmt* gene was overexpressed in the tropane alkaloid producer *A. belladonna* (Sato et al., 2001) and the *Duboisia hybrid* (Moyano et al., 2002), no significant increase in scopolamine was observed suggesting that methylputrescine is not the rate limiting substrate in the tropane alkaloid pathway.

The overexpression of only one enzyme in a complex metabolic net could not be sufficient to increase some secondary metabolite expression. Particularly, there are several works about the overexpression of more than one of the enzymes involved in the alkaloid tropane pathway (Zhang et al., 2004, Liu et al., 2010, Chunxian et al., 2011). However, not significant scopolamine yields were attained. The unsuccessful results could be attributed to the transgenic transformation processes itself. The mechanism of integration of transgenes into plant DNA is poorly understood, the integration of many genes at one or a few loci could not happen by chance. Also, multiple copies of one or more transgenes can result in postranscriptional gene silencing and turn unstable and reduce gene expression (Matzke & Matzke; 1998; Palazón et al., 1998; Bulgakov et al., 2004; Kutty et al., 2011). Evidently, *h6h* played a more important role in stimulating scopolamine accumulation than *pmt*. Nevertheless, when *pmt* redirects the carbon flux to the tropane pathway and *h6h* is overexpressed there is an accumulation of scopolamine (Zhang et al. 2004).

The genetic transformation with numerous in-tandem genes could be troublesome. For hairy root induction and *pmt* and *h6h* cDNA insertion, at least three tandem transformations

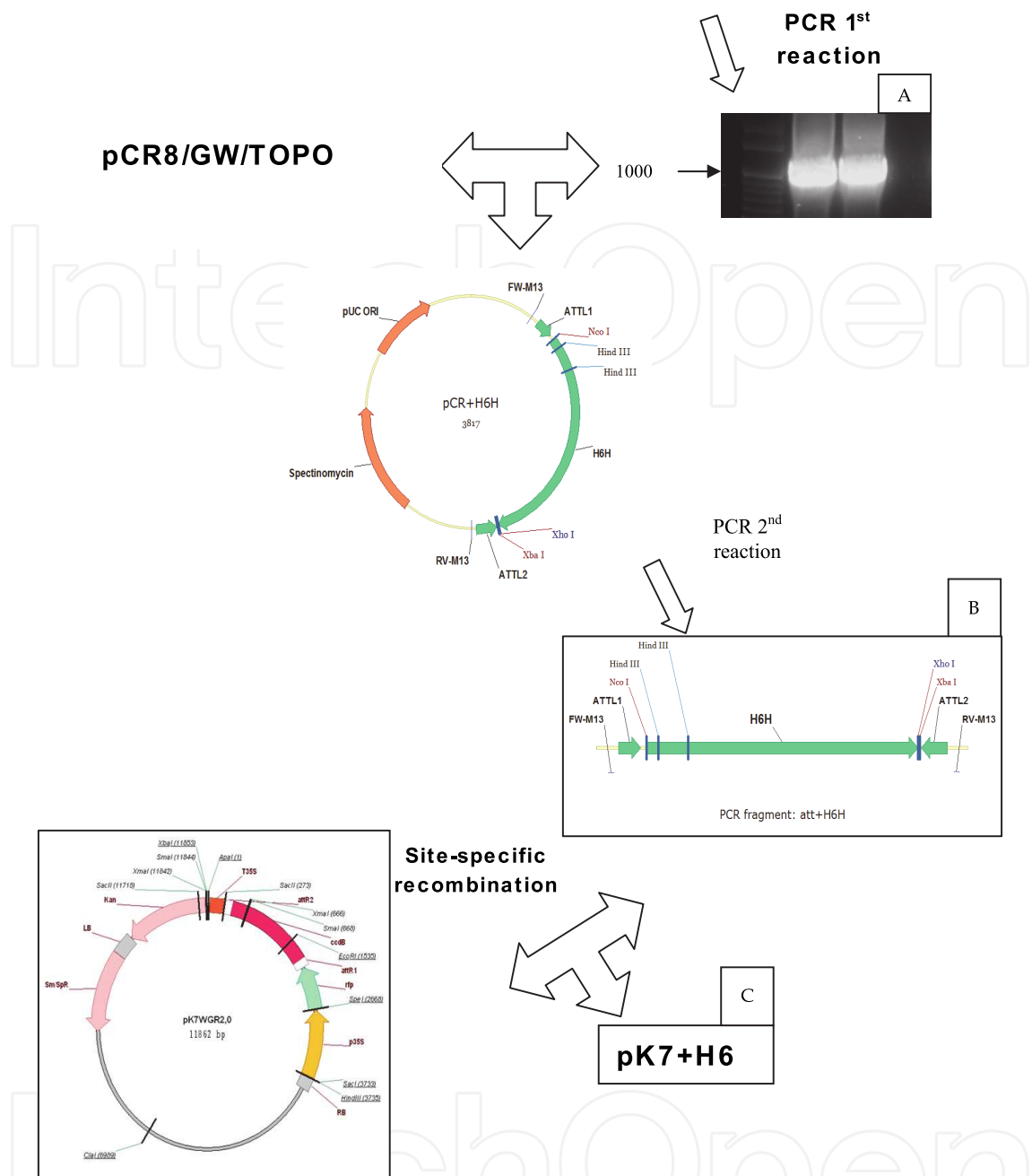


Fig. 4. Scheme of the site-specific recombination between the PCR product harboring *pmt* or *h6h* cDNA and the binary vector. During the first PCR round (PCR 1st Reaction), the H6H (or PMT) cDNA is isolated and purified from an agarose gel electrophoresis (A). The PCR fragment is inserted between the specific attachment sites sequences (att sites) into the Entry pCR8/FW/TOPO vector producing the plasmid pCR+H6H (B). The second round of PCR (PCR 2nd Reaction) is carried out with the M13 forward and reverse primers in order to amplify a cDNA fragment encoding the sequence of interest flanked by the att left and right recombination sequences. These sequences permit the site-specific recombination between the extended PCR fragments and a Destination binary plasmid (pK7WG2.00) following the Gateway protocol. LR Clonase II enzyme MIX is used to catalyze the site specific recombination reaction which is performed according to the manufacturer's protocol (Invitrogen). The final product is the binary destination vector pK7+H6H (C).

are needed. New and simple DNA transfer methods simplify the process as, for example, the Gateway system®, based on bacteriophage Lambda site-specific recombination system (Karimi et al., 2002; Attanasov et al., 2009; Dubin et al., 2008, Xu & Quinn, 2008). Figure 4 shows a protocol designed in our lab for the production of a PCR fragment for *h6h* cDNA ready to recombine into the destination vector independently of the antibiotic gene resistance.

4. New approaches

In this chapter, we have reviewed some of the most relevant strategies for improving tropane alkaloid biosynthesis such as the establishment of scopolamine overproducing organ cultures, the elicitation and the genetic transformation with homologous genes. Nevertheless, the knowledge generated and the strategies in use have demonstrated that the tropane alkaloid metabolism is immersed into a complex net of metabolic pathways with a delicate equilibrium quite difficult to be manipulated. Modern system biology tools, like elicitation and overexpression, allow the carbon flux redirection with some limitations. These margins cannot be overcome and decelerate the development of a competitive and sustainable production platform. Those troubles and limitations have fostered new strategies based on functional genomics (Goossens et al., 2002; Goossens & Rischer, 2007, Oksman-Caldentey & Inzé, 2004) such as biotransformation.

Biotransformation is one of those new strategies. The production of scopolamine and other alkaloids was studied in engineered *N. tabacum* hairy roots overexpressing the *h6h* cDNA after feeding the cultures with exogenous hyoscyamine (Hakkinen et al., 2005). The results obtained have shown an efficient uptake of hyoscyamine from the culture medium and a higher rate of bioconversion of hyoscyamine to scopolamine (up to 85% of the total scopolamine being released to the culture medium). Moreover, it was also evident an enhanced production of various nicotine alkaloids suggesting that the regulation of the alkaloid production is probably more complex than presently known.

Another approach was the bioconversion of hyoscyamine to scopolamine using recombinant *Saccharomyces cerevisiae* that expresses the *h6h* cDNA isolated from *B. candida* as a biocatalyzer. Transformed *S. cerevisiae* CEM PK2 expressing H6H as heterologous protein was able to grow into yeast base medium supplemented with hyoscyamine. However, the results have shown a low ability of hyoscyamine conversion to scopolamine (Cardillo et al., 2005 and 2008).

5. Conclusions

In the last two decades plant biotechnology has made considerable advances in the quest of a scopolamine and other tropane alkaloids productive process. Several groups have explored a wide spectrum of strategies that have led to the exhaustive knowledge of the tropane alkaloid pathway, its limiting steps and some of the regulation pathways. It is evident that genetic transformation is a promissory tool for engineering tropane alkaloid biosynthetic metabolism in order to produce high amounts of scopolamine. Combining genetic transformation and metabolic engineering would be a powerful strategy to re-direct the metabolic flux towards that biosynthetic pathway. It became clear, from the results already published, that the overexpression of *pmt* gene and/or *h6h* gene not only stimulated the conversion of hyoscyamine to scopolamine, but also the capacity of these hairy root lines

to synthesize both tropane alkaloids. However, the yields obtained up to now did not reach those of the current scopolamine productive process. Future research could be done considering the higher structural diversity of tropane alkaloids that could be functional to create new metabolic pathways and biological active products. A possible strategy would be cloning and engineering those new metabolic pathways in heterologous organism to produce a chemical structure diversity for generating new compounds with scopolamine-like activity (Harvey, 2000, Butler, 2004, Potterat & Hamburger, 2008).

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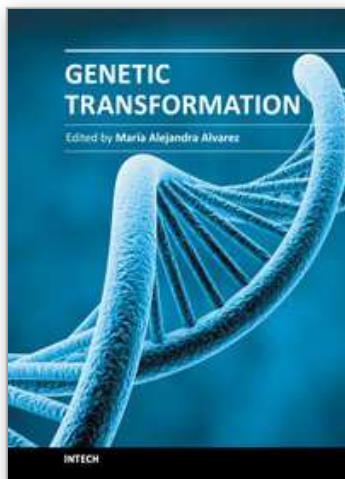
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