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Molecular tailoring and boosting of bioactive secondary metabolites in medicinal plants

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1. Introduction

Although the production of most of the current medicines is based on chemical synthesis, more than 25% of the current prescribed drugs contains at least one active ingredient of plant origin (Kaufman et al 1999). Examples of important plant-derived pharmaceuticals include the anti-tumoral taxol and vinblastine, the antimalarial drug quinine and artemisinin, the analgesical morphine and codeine. In addition, it has been estimated that more than 80% of the world's population in developing countries depends primarily on herbal medicine for basic healthcare needs (Vines 2004). There is also a revival of traditional medicine in developed countries and an increase in the use of herbal remedies. The world market of herbal medicines, including herbal and raw material, has been estimated to have an annual growth rate between 5-15%. Total global herbal drug market is estimated as US $ 62 billion and it is expected to grow to US $ 5 trillion by the year 2050 (Joshi et al. 2004). At same time, there is a growing concern on loss of genetic diversity since about 75% of the 50,000 different medicinal plant species in use are collected from the wild (Edwards 2004). Moreover, to rely solely on wild spontaneous plants as a

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production system can be extremely dangerous, as shown recently by severe shortage problems of the antimalarial artemisinin (Scheindlin 2005). Additionally, bioactive plant compounds are produced generally at very low amount and, often, it is not economically convenient to extract them from natural sources.

Altogether these considerations have opened the way to a new renaissance in the field of genetic and metabolic engineering studies to modify biosynthetic pathways in medicinal plants and to enhance the production of bioactive phytopharmaceuticals.

Far to be exhaustive, the present review is aimed at presenting an updated view of the most recent advances in engineering tailored secondary metabolites in medicinal plants. Analysis and discussion will be restricted to the successes, the pitfalls, the bottlenecks and the evolution in the strategies that have been used or might be used to boost the synthesis of plant compounds that exert specific pharmacological actions and that may be used for specific health problems over short- or long-term intervals. Engineering of plant compounds, serving nutritional or health benefits arising from long-term use as food (nutraceuticals), will be not covered in the present review.

2. Evolution of diversity of secondary metabolites

Most of the plant compounds exerting pharmacological activity belongs to the class of small molecules (< 1000 Da), known collectively as secondary metabolites. To date, more than 100,000 different plant metabolites have been described, that represent less than 10% of the actual total present in nature (Wink 1999). The astonishing chemical and structural heterogeneity of plant secondary metabolites is the result of the increased evolutionary plant adaptability to an unstable and challenging environment. Besides their role in the plant's defence, secondary metabolites have also potential pharmacological effects in humans. In general terms, to promote plant survival under biotic and abiotic stress, structures of secondary metabolites have evolved to interact with molecular targets affecting cellular and physiological functions in competing microorganisms, plants and animals. In this respect, some plant secondary metabolites may exert their actions by resembling endogenous metabolites, ligands, hormones, signal transduction molecules, or neurotransmitters, thus having beneficial medicinal effects on humans due to the similarities in their potential target sites (*e.g.* central nervous system, endocrine system etc) (Wink and Schimmer 1999). For example, plant secondary products that are cytotoxic

towards plant pathogens could be useful also as antimicrobial medicines in humans, if not too toxic. Similarly, secondary metabolites active against plant herbivores, due to their neurotoxical activity, could prove to be beneficial in relieving disturbs related to the central nervous system in humans (i.e. as antidepressants, sedatives, muscle relaxants, or anaesthetics).

Much of the chemical and structural diversity of plant secondary metabolites derives from a limited number of backbone structures. As an example, the more than 40,000 different molecules ascribed to the class of isoprenoids, are all synthesized through the condensation of isopentyl diphosphate (IPP) and its allylic isomer, methylallyl diphosphate (DMAPP) (Croteau et al. 2000) and most alkaloids are derived through decarboxylation of amino-acid precursors (i.e. ornithine, lysine, tryptophan and histidine) (Facchini 2001). It is only the subsequent decoration of these common precursors, by addition of functional groups and/or modification operated by quite conserved enzymatic activities (e.g. hydroxylation, methylation, acetylation, glycosilation etc), that generates the amazing diversity of plant secondary metabolites and that imparts a genus- or species-specific chemical signature. Each biosynthetic route of secondary metabolism derives from primary metabolism and involves complex and often specific enzymatic reactions that lead to specific biosynthetic pathways to yield different classes of natural products. In many cases, the first reaction leading to the synthesis of a new secondary metabolite is pivotal to the formation of a new secondary biosynthetic pathway. If the resultant new encoded enzyme can form a secondary product that is advantageous for plant survival, the trait is inherited under selection pressure. In fact, there is increasing evidence that duplication of essential genes of primary metabolism is an important evolutionary mechanism for gene recruitment in secondary metabolism. During evolution, these duplicated genes acquired new functions and were optimized and diversified for their role in new pathways (Pickersky and Gang 2000; Noel et al. 2005; Pickersky et al. 2006). As it will discuss later, this feature implies that different strategies of genetic and metabolic engineering might be adopted according to which part of the pathway is rate-limiting for the synthesis of a specific bioactive secondary metabolites, i.e. enzymes involved in the synthesis of precursors or in the following decoration.

3. General concepts and molecular tools for metabolic engineering

Recent advances in plant biotechnology, molecular biology and genomics have created promising new opportunities in using plants as efficient, environmentally friendly and renewable chemical factories. The possibility of exploiting the biosynthetic capacity of plants to meet future demands for pharmaceuticals is dependent on a detailed understanding of the biochemical enzymatic reactions involved in secondary metabolites pathways, and on a thorough knowledge on the relative genes and their regulation.

In general terms, development of metabolic engineering strategies to boost the production of bioactive secondary metabolites in *in vivo* plants or in plant tissue cultures requires: i) a systematic expansions of the available molecular toolbox (i.e. cloned genes for enzymes involved in a specific pathway and regulatory genes), ii) detailed and accurate knowledge on the biochemistry of the metabolic pathways under study, including rate-limiting enzymatic reactions, on the cellular compartmentation of the specific compounds and their catabolism and iii) appropriate and efficient systems for genetic transformation of the plant of interest.

Presently, a major constraint in engineering plant secondary metabolite production is that only few genes of these pathways are known, especially for medicinal plants. Impressive progress has been made for model plants, especially *Arabidopsis thaliana*, for which emerging technologies, genomics, functional genomics, transcriptomics, metabolomics are providing efficient tools to identify biosynthetic and regulatory genes involved in the individual secondary metabolite pathway. Though an increasing number of genes involved in secondary metabolic pathways have been mined from the genome sequence of model plant *A. thaliana* and *Oryza sativa* or other crop plants, this knowledge is of limited values for secondary metabolism of medicinal plants, that is per definition species-specific. For medicinal plants, an attractive alternative to hunt genes involved in the biosynthesis of specific secondary metabolites is offered by random EST sequencing, as reported for *Stevia rebaudiana* (Brandle et al. 2002) and *Ocymum basilicum* (Gang et al. 2001). Sequencing of EST libraries highly enriched in the metabolic genes of interest, such as those from highly specialized cell types, (e.g. glandular trichomes or other specialized tissues) (Gang et al. 2001) or from elicited plant cells or tissues, may serve also as a short-cut to gene discovery in medicinal plants. This last approach has rescued several genes involved in the synthesis of the anticancer drug taxol in a cDNA library from *Taxus*

cuspidata cells induced for taxoid biosynthetic with methyl jasmonate (Jennewein et al. 2004). An ample repertoire of known and novel genes in the tobacco secondary metabolism has been obtained by transcriptional profiling by cDNA-AFLP of elicited plant cells, as reported for jasmonate-elicited tobacco BY-2 cells (Goossens et al. 2003) or *Catharanthus roseus* cells (Rischer et al. 2006).

With only few exceptions, widely used medicinal plants have not received the extensive studies devoted to food crops or model plants, and therefore, for this group of plants there is a poor and scattered characterization of plant secondary metabolites pathway at the level of biosynthetic intermediates and enzymes (Briskin 2000).

Finally, although an efficient transfer delivery system, using either *Agrobacterium rhizogenes* or *A. tumefaciens,* is already available for several important medicinal plants, including *Atropa belladonna*, *Catharanthus roseus, Papaverum somniferum, Artemisia* spp, members of the family *Solanaceae*, *Taraxacum platycarpum*, *Taxus* spp. *Echinacea*, *Scrophularia*, *Digitalis*, *Thalictrum*, *Salvia* spp and others (Bajaj 1999), many medicinal plants are still not prone to genetic transformation, often because of the lack of intensive experimental work. However, in many cases the bottleneck is not genetic transformation *per se* but rather the unfeasibility of *in vitro* organogenesis. This limitation can be overcome by establishing stable transformed cell lines or hairy roots for large-scale production in bio-fermentors.

4. Boosting the metabolic flux towards a target compound

Provided that all the biochemical and genetic information of a metabolic pathway leading to an end-product of interest are available, along with a suitable transformation system for a specific medicinal plant, the choice of the most convenient strategy of metabolic engineering to enhance biosynthesis of one or more secondary metabolites depends on the specific metabolic pathway, but, basically may be achieved through: i) over-expression of a gene encoding an enzyme known to be rate-limiting; ii) down-regulation of a gene encoding the first enzyme of a lateral competitive chain or involved in catabolic reactions; iii) increase of intracellular compartmentation of the interested compound, to avoid feedback control and/or cellular toxicity, and iv) over-expression of multiple genes or coordinate over-expression by regulatory genes (Fig. 16.1).

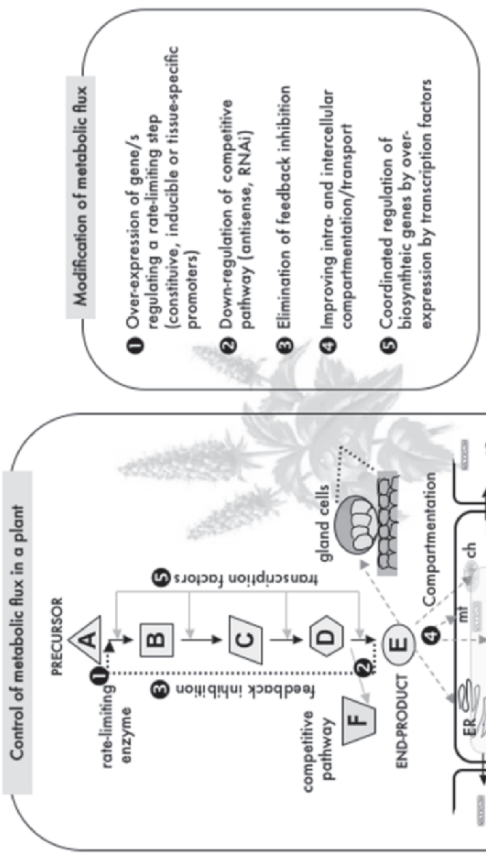

Fig. 16.1. Control of metabolic flux in a plant and possible strategies of metabolic engineering. **n**, nucleus; **mt**, mitochondria; **ch,** chloroplast; **V**, vacuole; **ER**, endoplasmic reticulum.

Pioneer work in engineering metabolically medicinal plants was based on over-expressing one or a few genes thereby overcoming specific rate-limiting steps in the pathway, or to shut down competitive pathways and to decrease catabolism of the product of interest. However, identifying rate-limiting reactions in one specific pathway is often difficult and, besides, the level of the end-product accumulation can be controlled by more than one enzymatic activity. Secondly, attempts have been made to change the expression of regulatory genes that control multiple biosynthetic genes. Recent large-scale studies of gene expression have fuelled the present knowledge on mechanisms that govern transcriptional regulation of complex metabolic pathway. All these strategies will be discussed in the following paragraphs, reporting the most recent results obtained for medicinal plants (Table 16.1).

4.1 One is better than nothing: over-expression of single genes for rate limiting reactions

A typical metabolic engineering approach generally focuses on a particular metabolic intermediate or product. A critical evaluation of the current knowledge on that specific biosynthetic pathway may identify candidates for rate-limiting enzymatic reactions and open the way to a possible strategy of genetic engineering of the identified limiting target gene. As mentioned before, the main constraint for applying this principle in plants, and especially for medicinal ones, is that detailed and unequivocal information on precursors, metabolic intermediates and the relative conversion enzymes are still lacking and, therefore, in many cases, it is virtually unfeasible to establish unambiguously which step is rate-limiting in a specific metabolic pathway. Current up-graded analytical technologies, such as genome sequencing, microarray analysis and sophisticated metabolic profiling, have ameliorated the predictability of metabolic engineering, but still the final effect of manipulating a single gene is under a strict control of the cellular homeostasis. Despite the trial-and error approach adopted principally at the beginning of the metabolic engineering era, the relatively simple way to introduce single genes for rate limiting enzymes, also from

Table 16.1. List of medical plants engineered for the production of secondary metabolites.

Plant species	Target gene	Source	Target compounds	Strategy	Results	References
Atropa belladonna	Hyosciamine 6β-hydroxylase (H6H)	*H. niger*	Tropane alkaloids	Constitutive over-expression in plant	Accumulation of scopolamine	Yun et al. 1992
	Putrescine N-methyl transferase (PMT)	*N. tabacum*	Tropane alkaloids	Constitutive over-expression in plant	No major effects in alkaloid production	Rothe et al. 2003
	Triptone reductase I + II	*D. stramonium*	Tropane alkaloids	Constitutive over-expression in hairy roots	Accumulation of tropine, pseudo- tropine, scopolamine and calystegine	Richter et al. 2005
Cinchona officinalis	Tryptophan decarboxylase (TDC) + Strictosidine synthase (STR)	*C. roseus*	Terpenoid indole alkaloids	Constitutive over-expression in hairy roots	Accumulation of high levels of tryptamine, strictosidine and TIA derivatives quinine and quinidine	Geerlings et al. 1999
Catharanthus roseus	Tryptophan decarboxylase (TDC) + Strictosidine synthase (STR)	Endogenous	Terpenoid indole alkaloids	Constitutive over-expression in cells	Increased accumulation of tryptamine, strictosidine and derivatives in STR transgenic cells; no effects of TDC overexpression	Canel et al. 1998

Plant species	Target gene	Source	Target compounds	Strategy	Results	References
	Tryptophan decarboxylase (TDC)	Endogenous	Terpenoid indole alkaloids	Constitutive over-expression /antisense in call	Increased tryptamine conitent in overexpressing calli; no effects in TIA accumulation	Goddijn et al. 1995 Hughes et al. 2004b
	3-hydroxy-3-methylglutaryl-CoA reductase (HMGR)	Hamster	Isoprenoids/ Terpenoids	Constitutive over-expression in hairy roots	Accumulation of high levels of campesterol and serpentine	Ayora-Talavera et al. 2002
	Anthranilate synthase α subunit (ASα)	*A. thaliana*	Terpenoid indole alkaloids	Inducible over-expression in hairy roots	High extent of tryptophan and triptamineaccumulation; no effect on TIA derivatives	Hughes et al. 2004b
	Anthranilate synthase α subunit (ASα) + Tryptophan decarboxylase (TDC)	*A. thaliana* Endogenous	Terpenoid indole alkaloids	Inducible over-expression in hairy roots	High accumulation of tryptamine; no TIA derivatives accumulation	Hughes et al. 2004a
	Anthranilate synthase β subunit (ASβ) + Anthranilate synthase α subunit (ASα)	*A. thaliana* *A. thaliana*	Terpenoid indole alkaloids	Inducible over-expression in hairy roots	Content of tryptophane and triptamine increased dramatically upon induction; no effect on TIA derivatives	Hong et al. 2006
	Anthranilate synthase β subunit (ASβ) + Anthranilate synthase α subunit (ASα) + Tryptophan decarboxylase (TDC)	*A. thaliana* *A. thaliana* Endogenous	Terpenoid indole alkaloids	Constitutive / inducible over-expression in hairy roots	High increase of tryptophane and triptamine. Changes in TIA accumulation profiles	Hong et al. 2006
	ORCA 3	Endogenous	Terpenoid indole alkaloids	Activation tagging/ constitutive over-expression in cells	Changes in metabolic flux to indole precursors; tryptamine, tryptophan and TIA accumulation	van der Fits and Memelink 2000

Table 16.1. (Continued).

Plant species	Target gene	Source	Target compounds	Strategy	Results	References
Mentha piperita L	Deoxyxylulose phoshate Reducto isomerase (DXR) Menthofuran synthase (MFS)	Endogenous	Monoterpenes	Constitutive over-expression /Co-suppression in plants	Accumulation of essential oils or selective reduction of undesiderable monoterpenes obtained by DXR over-expression and MFS reduction respectively	Mahmoud and Croteau 2001
	Menthofuran synthase (MFS)	Endogenous	Monoterpenes	Constitutive over- / antisense expression in calli	Menthofuran suppression increased accumulation of menthone and menthol. Over-expression led to opposite results	Mahmoud and Croteau 2003
	Limonene 3-hydroxylase	Endogenous	Monoterpenes	Constitutive over-expression/co-suppression in plants	Accumulation of limonene by co-suppression; no effects by over-expression	Mahmoud and Croteau 2004
	4-S limonene synthase	*M. spicata*	Monoterpenes	Constitutive over-expression in plant	No effect in essential oil accumulation	Mahmoud and Croteau 2004
	4-S limonene synthase	*M. spicata*	Monoterpenes	Constitutive over-expression in plant	Accumulation of total monoterpenes and of intermediate compound pulegone. Same results in *M. arvensis*	Diemer et al. 2001
	4-S limonene synthase	*M. spicata*	Monoterpenes	Constitutive over-expression in plant and protoplasts	Changes in essential oil profile (high menthone and methofuran, low menthol)	Kransnyansky et al. 1999
Papaver somniferum L.	Codeinone reductase (COR)	Endogenous	Alkaloids	Down regulation by RNAi in plant	Accumulation of non-narcotic alkaloid reticuline	Allen et al. 2004
	Berberine Bridge Enzyme (BBE)	Endogenous	Benzylsoquinoline alkaloids	Constitutive antisense expression in plant	Changes in intermediate alkaloid pathways. Major change in alkaloid profiles occurred in latex	Frick et al. 2004

Plant species	Target gene	Source	Target compounds	Strategy	Results	References
Eschscholzia californica Cham	Berberine Bridge Enzyme (BBE)	*P. somniferum*	Benzophenanthridine alkaloids	Constitutive antisense expression in cells	Changes in of benzophenatridine alkaloids content and major effects on cell growth and aminoacid synthesis	Park et al. 2002, 2003
	N-methyl-coclaurine 3'-hydroxylase (CYP80B1)	*P. somniferum*	Benzophenanthridine alkaloids	Constitutive over-/ antisense expression in cells	Reduced accumulation of benzophenatridine alkaloids and effects on cell growth and aminoacid synthesis	Park et al. 2002
Hyosciamus niger	Putrescine N Methyltransferase (PMT) + 6β hydroxylase (6H6)	*N. tabacum* Endogenous	Tropane alkaloids	Constitutive over-expression in hairy roots	Accumulation of high levels of scopolamine. Best results in trasformants with both PMT and 6H6 genes	Zhang et al. 2004
Artemisia annua	Farnesyl disphosphate synthase (FDS)	*G. arboreum*	Isoprenoid	Constitutive over-expression in hairy roots	Increased artemisinin content	Chen et al. 1999
				Constitutive over-expression in plant	Accumulation of artemisinin, higher in plant tissues than in roots	Chen et al. 2000
Duboisia myoporoides-D. leichhardti	Putrescine N Methyltransferase (PMT)	*N. tabacum*	Alkaloids	Constitutive over-expression in plant	Accumulation of N-methyl putrescine accumulation. No effect in tropane alkaloids content	Moyano et al. 2002

heterologous organisms, has yielded remarkable successes even for medicinal plants, especially for alkaloids and isoprenoids.

4.1.1 Engineering plant alkaloids

Alkaloids ascribed about 12,000 different nitrogenous compounds, found in about 20% of plant species, many of which are largely used as pharmaceuticals, such as the antitumoral vincristine and vinblastine, the narcotic morphine, the anti-cough depressant codeine, the oral antibacterial sanguinarine (Facchini 2001).

Several attempts have been reported on modifying expression of genes involved in the nicotine content in *Nicotiana tabacum*, which can be considered a model plant and a medicinal plant at the same time. Over-expression of tropinone reductase and hyoscyamine-6β-hydroxylase (*h6h*) has enhanced the synthesis of nicotine and related pyridin alkaloids (Rocha et al. 2002). As far as medicinal plants, the constitutive expression of tobacco *pmt* gene, encoding the putrescine N-methyltransferase, in *A. belladonna* plants and hairy roots did not significantly alter the alkaloid level (Sato et al. 2001), suggesting that the conversion of the putrescine and N-methylputrescine, the first two intermediate compounds in the synthesis of scopolamine from ornithine, are not rate-limiting for the synthesis of this compound. However, a 3-4-fold increase of scopolamine, a tropane alkaloid used as anticholinerginic sedative agent that acts on the parasympathetic nervous system, was achieved by over-expressing the *h6h* gene in *A. belladonna* (Yun et al. 1992). The H6H enzyme has a dual enzymatic activity since it catalyzes the hydroxylation of hyoscyamine to 6-β-hydroxyhyoscyamine and its further epoxidation to scopolamine (Facchini 2001).

Other alkaloids thoroughly studied for their pharmacological impact are those produced in *C. roseus*, the common Madagascar periwinkle. This plant species produces the monomeric alkaloids serpentine and ajmalicine, used as a tranquilizer and to reduce hypertension, respectively. Dimeric alkaloids from periwinkle, vincristine and vinblastine, and their semi-synthetic derivates, including vinorelbine and vinflunine, are used extensively in the treatment of many cancers. Dimeric alkaloids are synthesized at very low levels in the periwinkle and are restricted to specific leaf cell type (St Pierre et al. 1999). Synthesis of these bioactive secondary metabolites is quite complex since it involves two separate pathways, the terpenoid and the indole pathway, necessary for the synthesis of a converging intermediate, the strictosidine, and a total of over 20 different enzymes. Enzymatic studies coupled with expression analysis have demonstrated that activity of the tryptophan decarboxylase (*tdc*),

converting the tryptophan in tryptamine, coincides with alkaloid accumulation, while strictosidine synthase (STR) activity is relative stable (Meijer et al. 1993). Either *tdc* or *str* genes have been overexpressed in *C. roseus* cells or hairy roots in an attempt to enhance the level of terpenoid indole alkaloids (TIAs). Constitutive expression of *str* in cell culture proved to be useful to increase the alkaloid levels (Canel et al. 1998), while *tdc* overexpression triggered accumulation of the intermediate tryptamine, with no significant increase in TIAs (Goddijn et al. 1995). Alkaloid profiling has been also modified by over-expression of the *tdc* gene in other medicinal plants such as *Cinchona officinalis*, for quinine production (Geerlings et al. 1999) and *Peganum harmala,* producing psychotropic alkaloids (Berlin et al. 1993).

Another example of metabolic engineering of plant alkaloids is the modification of the synthesis of several important medicinal compounds belonging to the benzylisoquinoline alkaloid class, such as the antimicrobial berberine and sanguinarine, which has been attempted in *Coptis japonica* and the California poppy (*Eschscholzia californica* Cham). Constitutive expression of the scoulerine 9-*O*-methyltransferase (*smt*) gene in *C. japonica* cell cultures resulted in 20% higher SMT activity and a small increase in the accumulation of protoberberine alkaloids (Sato et al. 2001). Analogously, expression of *smt* gene in cultured California poppy cells led to a diversion of the metabolic flux towards the protoberberine alkaloid columbamine and away from benzophenenthridine (Sato et al. 2001).

Taken together these studies, have demonstrated that, more than increase the synthesis of a specific alkaloid, genetic modification of a single gene can divert this pathway, but much remains to be learnt before this class of bioactive compounds might be rationally engineered. As it will be discussed later in § 4.2.1, one possibility is to identify and over-express regulatory genes able to activate in a coordinate fashion the multiple genes involved in this complex pathway.

4.1.2 Engineering plant isoprenoids

Isoprenoids are the largest and most diverse family of natural products, with very diversified structures and chemical size. All the terpenoids have been long believed to be synthesized solely in the cytosol by condensation of units of isopentenyl diphosphate (IPP) and its allyl isomer dimethylallyl diphosphate (DMAPP), both originated from acetyl-CoA through the classical mevalonate (MVA) pathway, until the recent discover of an alternative plastidial pathway to IPP synthesis, starting from the 1-deoxy-D-xylulose phosphate (DXP). The cytosolic pathway supplies IPP for the

synthesis and subsequent precursor for the biosynthesis of sesquiterpenes and triterpenes, while monoterpenes, diterpenes and tetraterpenes are produced through the plastidial pathway (Mahmoud and Croteau 2002). This large family of compounds includes essential molecules, such as carotenoids, giberellins, abscisic acid, brassinosteroids, sterols and the phytol chains of chlorophyll, tocopherols and quinones. However, the majority are secondary metabolites of pharmacological interest, such as the volatile components of essential oils produced by the *Lamiaceae* species, and complex molecules like the anticancer drug taxol.

Monoterpenes comprise the major components of the essential oils of the *Lamiaceae* family, to which belong several medicinal and aromatic plants (sage, peppermint, basil to mention few of them). Biosynthesis of essential oils has been thoroughly studied in peppermint, which can be considered a model system for monoterpenes of the whole family. Synthesis of monoterpenes in mint is localized in glandular trichomes and originates in the leucoplasts of the secretory cells of these highly specialized structures. The flux through the monoterpene pathway has been modified in mint by over-expression of a gene encoding the deoxyxylulose phopshate reductoisomerase (DXR), which control the first committed step in the plastidial DXPS pathway (Mahmoud and Croteau 2001). Most transgenic plants accumulated more essential oils than control plants, with an increase up to 50%, without remarkable changes in the monoterpene composition compared to wild type plants. Furthermore, because the monoterpene synthase steps might be rate limiting (Wise and Croteau 1999), modified expression of these genes could increase yield and change composition of the monoterpenoid essential oil, as it has been demonstrated for ectopic expression of a 4S-limonene synthase in peppermint (*Mentha x piperita* L) and cornmint plants (*Mentha arvensis*) (Diemer et al. 2001; Krasnyanski et al. 1999).

A successful example of the metabolic engineering of cytosolic isoprenoids biosynthesis is the genetic modification of the artemisinin biosynthesis pathway. Artemisinin, extracted from the traditional Chinese herb *Artemisia annua* L, which has been used in China to treat fevers since A.D. 150, came into vogue as a modern malaria treatment after studies in Vietnam showed it reduced deaths from the illness by 97 percent. Most of the current pharmacological treatments against malaria are inefficient due to the selection of resistant forms of the parasite. The World Health Organization now recognizes that drugs combining antibiotics with artemisinin provide the most rapid defence against malaria and are the only ones to which the parasites has not developed resistance (WHO and UNICEF: Malaria World Report, 2005: http://rbm.who.int/wmr2005/-html/1-2.htm). Artemisinin is currently extracted from natural plants with a

very low content. Attempts to produce it by cell or organ tissue have been unsuccessful and chemical synthesis is not economically convenient (Abdin et al. 2003). Chemically, artemisinin is an endoperoxide sesquiterpenoid lactone (Klayman 1985), produced in the MVP pathway by two consecutive condensations of IPP with DMAPP by a farnesyl diphosphate synthase (FPPS), forming FPP, which is subsequently converted in artemisinin by a sesquiterpene cyclase (Bouwmeester 1999). Increased accumulation in artemisinin has been achieved by over-expressing the gene *fpps* in *A. annua* hairy roots and plants. In transgenic plants, the concentration of artemisinin was approximately 8-10 mg/g DW, about two- to three–fold higher than that in control plants, while in hairy roots the content of this sesquiterpene was 2-3 mg/g DW, which is three- to four-fold higher than in control roots (Chen et al. 1999; 2000). Optimization of enhanced production of this interesting plant secondary metabolites is, however, far to be reached, due to the involvement of a total of twelve genes, nine of which have already been cloned (Martin et al. 2003). The recent funding of research groups in USA by the Bill & Melinda Gates Foundation in 2004 may speed up the development of a genetically engineered form of artemisinin (Purcell 2006).

To the class of isoprenoids belongs also the tricyclic diterpene taxol (paclitaxel), initially isolated from the bark of *Taxus brevifolia*. Taxol is a potent antimitotic agent, acting by stabilizing microtubules and promoting microtubule assembly, rather than by depolymerizing microtubules, as found for vincristine and vinblastine. Since taxol is only available as a natural product, sources other than the bark of *T. brevifolia* have been investigated, including production of taxol from plant tissue culture. For a long period taxol was produced by chemical semi-synthesis from its precursor, 10-deacetylbaccatin III, extracted from leaves and twigs of the European yew, *T. baccata*, but presently Bristol-Meyer produced it solely from cell cultures of *T. chinensis*. Taxol is now used to treat ovarian, breast, and lung cancer and it has been prescribed to more than 1 million of patients. Despite the strong interest in this valuable natural anti-tumoral plant compound, successes in engineering its biosynthetic pathway are limited by the large number of enzymes involved (nineteen enzymatic steps from the universal diterpenoid progenitor GGPP) and lack of known regulatory genes. However, increased demands for taxol and its high cost have recently accelerated research towards the elucidation of its biosynthetic pathway, the responsible enzymes and underlying genes (Schoendorf et al. 2001; Jennewein et al. 2004), that may provide many promising targets for metabolic engineering of the production of taxol and its precursors.

4.2 Down-regulation of competitive or catabolic reactions

This approach deserves the same considerations that have been reported for over-expressing single rate-limiting genes. Though a down-regulation approach has been successfully applied in model and crop plants to remove undesirable plant compounds, in medicinal plants the main bottleneck for this technology is again the lack of thorough information on branching of complex biosynthetic pathways as well as on the endogenous plant catabolic degradation system of these compounds.

However, silencing of genes encoding key enzymes of relevant secondary metabolite pathways has been proved successful in some medicinal plants, either by antisense, co-suppression or RNAi technology. A significant decrease in the level of the undesirable menthofuran has been achieved in peppermint (*Mentha* x *piperita* L) through antisense suppression of the *mfs* gene, coding for the cytochrome P450 (+) menthofuran synthase (Mahmoud and Croteau 2001). Menthofuran reaches levels in environmentally stressed peppermint plants considered unacceptable by the pharmaceutical and industrial companies, because confer a bitter flavour to mint essential oils and promote off-color on storage. Accumulation of limonene in peppermint plants was achieved by co-suppression of the limonene-3-hydroxylase gene (Mahmoud and Croteau 2004). Down-regulation of the putrescine N-methyltransferase (PMT), the first committed step in both pyridine and tropane alkaloids, has been reported to reduce nicotine level in tobacco plants, with a concomitant unexpected increase in anatabine, whose synthesis does not require PMT (Chintapakorn and Hamill 2003). Antisense RNA-mediated suppression has been also reported for two genes involved in the biosynthesis of benzophenanthridine alkaloids in California poppy (Park et al. 2002), respectively the berberine bridge enzyme (BBE) and the N-methylcoclaurine 3'-hydroxylase (CYP80B1). These results have provided insight into the complex regulation of the benzylisoquinoline alkaloid biosynthesis, to which belong important pharmacological compounds, e.g. morphine, codeine, sanguinarine, rather than effectively increased specific alkaloids. BBE has been also knocked out by an antisense approach in opium poppy, resulting in an altered ratio of alkaloids in latex but not in roots (Frick et al. 2004).

RNAi technology has been also used recently in medicinal plants. A recent paper from Allen and coworkers (2004) reported the metabolic engineering of morphine biosynthesis in opium poppy (*Papaver somniferum* L.) by using RNAi to block the gene coding for the codeinone reductase (COR), the enzyme that catalyzes the reduction of codeinone to codeine and morphinone to morphine. Several COR genes are present in

the opium poppy, but the authors designed a hybrid RNAi construct to knock out all known members of this multigene family. Chemical analysis of the silenced transgenic lines showed that the amount of morphine was substantially lower and that, instead, higher level of (*S*)-reticuline, an intermediate compound located seven steps upstream the COR conversion in the morphine pathway. The authors speculated that this unexpected result might be due to feedback inhibition of the preceding enzymes or transporters, due to the resulting accumulation of codeinone and morphinone due to suppression of the COR or caused by other unpredictable transcriptional down-regulation of the up-stream enzymes. Alternatively, based on recent findings on the presence in plants of metabolon, multienzyme complex capable of metabolic channelling (Winkel 2004), a more fascinating explanation for this unexpected upstream control might be related to disruption of the morphinan metabolon caused by the silencing of COR, thus preventing the formation of its intermediates and resulting in the accumulation of (*S*)-reticuline. The relevance of metabolon formation in metabolic engineering of plant secondary metabolites will be discussed again when facing and solving possible pleiotropic effects associated to metabolic engineering (see § 6). Though not a medicinal plant *in sensu strictu*, in *Coffea canephora* RNAi has been used successfully to reduce the content of the alkaloid caffeine (Ogita et al. 2003).

As far as the feasibility of improving the synthesis of the end-product of interest through inhibiting eventual catabolic reactions, at our knowledge there are no examples in medicinal plants. Catabolism and degradation often occur simultaneously with synthesis, but, unfortunately, only the synthetic capacity has received sufficient attention in engineering secondary metabolites. Obviously, any strategy that is able to inhibit or avoid these degradative or catabolic processes may enhance total yield of a desired metabolite. However, catabolism of a secondary metabolite of interest may be avoided indirectly by promoting proper compartmentation through engineering genes involved in transport towards specialized cellular storage organelle (such as vincristine and vinblastine that are accumulated in the vacuole) or by a judicial choice of cell- tissue specific plant promoters, as it will be discussed in § 5.

4.3 Orchestrated regulation of sets of genes involved in the biosynthesis of plant secondary metabolites

Since it has been realized that the manipulation of single genes is of limited value in metabolic engineering, attention has shifted then towards

more complex and sophisticated strategies in which several steps in a given pathway are modified simultaneously to achieve optimal flux. Multipoint metabolic engineering is now beginning to replace single-point engineering as the best way to manipulate metabolic flux. This can be achieved either by over-expressing and/or suppressing several enzymes simultaneously or through the use of transcriptional regulators to control several endogenous genes.

4.3.1 Two or few are better than one: introducing multiple genes

Unlike bacteria, plants cannot normally co-express genes from polycistronic messengers. Introducing multiple genes into a target plant is quite challenging but not technically impossible, as elegantly proved for the increase in provitamin A in rice plants (Ye et al. 2000) or lignin pathway in forest tree (Li et al. 2003). Co-expression of multiple transgenes in a single tobacco plants was achieved by crossing independent transformed plants that expressed three different monoterpene synthases from lemon (Lucker et al. 2004), with an increase in endogenous terpenes as well as changes in the terpenoid profiles compared to untransformed wild-type plants. This is also the first report of transgenic plants expressing multiple foreign enzymes competing for the same substrate.

A nine-fold increase in the sedative compound scopolamine has been recently reported by simultaneously over-expressing the *pmt* and *h6h* genes, respectively encoding an up-stream and a down-stream rate-limiting enzyme in hairy roots of *Hyoscyamus niger* (Zhang et al. 2004). Compared to hairy roots over-expressing the single gene, the transgenic hairy roots expressing both *pmt* and *h6h* genes produced significantly higher levels of scopolamine, with the best line produced 411 mg/liter scopolamine, which was over nine times more than that the content of wild type roots (43 mg/liter). Transgenic hairy roots of *C. roseus* co-expressing a tryptophan decarboxylase (TDC) and a feed-back resistant anthranilate synthase subunit (ASα) have been produced (Hughes et al. 2004a). Interestingly, while the TDC line showed no significant increase in tryptamine level, hairy roots co-expressing the two genes synthesize much as six-fold amount of this crucial precursor in the synthesis of vincristine and vinblastine. This example offers another way of improved metabolic flux towards a desired end-product by mutagenesis of genes encoding enzymes subjected to a feed-back control. In fact, by over-expressing a mutated form of the AS α–subunit in periwinkle hairy roots, it has been proved that

this enzyme regulates the flux to tryptophan, a common precursor for alkaloid accumulation (Hughes et al. 2004b). Other possibilities of multiple gene co-expression have been attempted in plants other than medicinal ones, such as artificial gene-clusters introduced in plants using a vector system based on intron and intein-encoded endonuclease (Thomson et al. 2002) or the expression of polyproteins able to self-cleave, recently used to engineer ketocarotenoids in higher plants (Ralley et al. 2004). Finally, the success in plastidial transformation, although limited to a restricted number of plant species, opens also the possibility of introducing multiple genes assembled in polycistrons in the prokaryotic-like chloroplast genome, as it will be discussed in § 5.

4.3.2 Transcription factors

Although many successful results in increasing biosynthesis of plant secondary metabolites have been obtained by over-expression or down-regulation of single genes, in many cases, over-expression of an enzyme upstream in the pathway of the desired metabolite does not lead to increased production of that product, since secondary metabolites generally are not products of single genes, but are the results of multi-step, multi-enzymatic processes. Recent large-scale studies of gene expression coupled to metabolomic analyses have revealed that there is a coordinated metabolic regulation, mediated by regulatory genes that control the expression of the series of enzymes involved in a particular pathway. It has been demonstrated that the transcriptional control of the genes involved in metabolic pathways is the primary mechanism to regulate the final concentration of secondary metabolites in plants. Therefore, the identification of transcription factors (TFs) and relative genes able to simultaneously and coordinately controlling the transcription of multiple genes of a specific pathway is of great interest as biotechnological tool to enhance the production of plant bioactive molecules (for excellent reviews see Gantet and Memelink 2002; Broun 2004). The use of specific TFs would avoid the time-consuming steps of acquiring knowledge about all enzymatic steps of a poorly characterized biosynthetic pathway and, at the same time, allows the coordinated transcription of many genes belonging to a specific metabolic pathway.

The most well documented study of coordinated expression of biosynthetic genes in a medicinal plant driven by a TF is offered by the over-expression of ORCA3 in *C. roseus* cultured cells. The gene for ORCA3 (octadecanoid-responsive Catharanthus AP2-domain protein 2), a jasmonate-responsive gene, APETALA2 (AP2)-domain transcription factor from *C. roseus*, was isolated by T-DNA activation tagging (van der

Fit and Memelink 2000; 2001). *Orca3* over-expression in *C. roseus* cells resulted in enhanced expression of several metabolite biosynthetic genes and, consequently, in increased accumulation of terpenoid indole alkaloids (TIAs). Expression of TIA biosynthetic genes *tdc*, *str*, s*gd*, *cpr*, and *d4h* was increased in the *Orca3* over-expressing line, whereas *g10h* and *dat* genes were not induced, suggesting that they are not controlled by ORCA3. Genes encoding the α subunit of AS (AS α) and DXS, enzymes involved in primary metabolism leading to TIA precursor synthesis, were, instead, induced by *Orca3* over-expression. Transgenic cells that over-express ORCA3 accumulate significantly more tryptophan and tryptamine, and accumulate indole alkaloids when fed with the precursor secologanin (van der Fit and Memelink 2000). These data indicate that although ORCA3 is an important regulator of TIA biosynthesis, it is not sufficient to regulate the complete pathway.

Though not in medicinal plants, the feasibility of using regulatory genes for increasing the production of plant secondary metabolites other than alkaloids has been demonstrated in several other studies, as for phenylpropanoids by ectopic expression of plant *myb* and *bHLH* TF genes in *A thaliana*, *Petunia* or *N. tabacum* (Barkovich and Liao 2001; Schijlen et al. 2004). Studies in a number of systems suggest that many transcriptional regulators are capable of faithfully recognizing their homologous target genes in heterologous species, due to the conservation among the members of the same TF family. Therefore, it might be expected that over-expression in medicinal plants of TFs from other plants could be effective in modifying their metabolic profiles. To the MYB family belongs a novel TF, OsMYB4, isolated in rice, which has been recently described as able to increase cold and freezing tolerance when over-expressed in *Arabidopsis thaliana* plants (Vannini et al. 2004). Microarray analysis has revealed that *OsMYB4* over-expressing *Arabidopsis* plants express constitutively stress-responsive genes as well as genes involved in the metabolic pathway of secondary metabolites. Particularly, over-expression of *Osmyb4* gene in *Arabidospis* induces transcription of seven out of eight genes of the chorismate and aromatic amino acid biosynthesis, including also the chorismate mutase, the first enzyme responsible of the lateral chain leading to the synthesis of tyrosine and phenylalanine. Moreover *OsMYB4* controls the transcription of the genes for the phenylalanine ammonia-lyase (PAL), the cinnamate-4-hydroxylase (C4H) and the 4-coumarate:CoA ligase (4CL), involved in the phenylpropanoid biosynthesis (Immacolata Coraggio, personal communication). Over-expression of this transcription factor has increased the synthesis of chlorogenic acid in *Nicotiana tabacum* (Docimo et al. 2005) and of rosmarinic and salvianolic acid in *Salvia sclarea* (A. Leone

unpublished results), both compounds belonging to the class of phenylpropanoids.

Another large family of plant TFs is the WRKY proteins, which seems to exist exclusively in plants and low eukaryotes. The transcription of WRKY genes is strongly and rapidly upregulated in response to wounding, pathogen infection or abiotic stresses in numerous plant species (Eugelm et al. 2000). The WRKY family has 74 members in *Arabidopsis* (Ulker and Somssich 2004) and it would be interesting to exploit the role of the various members in triggering biosynthesis of bioactive secondary metabolites.

While the studies reported above have demonstrated that enhanced synthesis of plant secondary metabolites may be achieved through regulation of transcription of key biosynthetic genes or TFs, the formation of these plant compounds is a complex and dynamic process that involves multiple subcellular compartments, such as cytosol, endoplasmic reticulum, vacuoles and others, highlighting the central role of post-transcriptional regulation of secondary biosynthetic pathways. The relevance of these last aspects in successful metabolic engineering for plant molecules of therapeutic activity will be discussed in details in the next paragraph.

5. Improving transport and compartmentation

As discussed previously, in many species, the production of the astonishing number of secondary metabolites in plant cells relies on very well organized expression patterns of multiple genes. While gene expression is, in most cases, regulated at the transcriptional level and often influenced by the environment, the diversification of end-products, starting from few precursors and central intermediates, also depends on compartmentation of enzymes and reactions at the organ/tissue, cellular and subcellular level. Reasons for such tight developmentally related organization include the necessity to sequester toxic compounds in safe locales of the plant and the cell, the local availability of intermediates in some organs and tissues, the need to deliver end-products in plant parts where physiological and ecological interactions with other organisms, such as pollinators or pests, take place. Except for some notable cases, the lack of organization in undifferentiated cell cultures is often the cause of the failure of secondary metabolite production *in vitro* (De Luca and St Pierre 2000; Oksman-Caldentey and Inzé 2004). On the other hand, the comprehension, and possible manipulation, of cellular and molecular

mechanisms underlying secondary metabolite biosynthesis, transport and storage, is necessary for the adoption of successful metabolic engineering approaches and biotechnological applications.

By contrast with prokaryotic cells, plant cells present a number of compartments delimited by different kinds of membranes, that are responsible not only for marking the boundaries of production and/or storage sites, but also for intra- and intercellular transport.

It is now well known that isoprenoid production in plant cells is based on two parallel pathways leading to the same precursors but different end-products. Although some exchanges between the two pathways are possible, the acetate-mevalonate pathway, responsible for the production of isopenthenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) in the cytoplasm, leads to the synthesis of sesquiterpenes, triterpenes and other compounds, while, the GAP-pyruvate pathway determines the synthesis of IPP and DMAPP in plant plastids, where most hemi-, mono-, di- and tetraterpenes are produced and in various cases also accumulated (Croteau et al. 2000). Various highly specialized tissues and cells, such as glandular epidermis of flowers, resin blisters and ducts, glandular trichomes, secretory cavities and idioblasts, are involved in isoprenoid synthesis in higher plants, reflecting a complex pattern of organization at the tissue/cellular level at the basis of specialized functions and uses of end-products (McCaskill and Croteau 1997). The 3-hydroxy-3-methylglutaryl-coenzyme-A reductase (HMGR), a central enzyme in the acetate-mevalonate pathway, is present in different isoforms, encoded by small multigene families. Such isoforms show different tissue and developmental expression patterns and respond differently to environmental stimuli (McCaskill and Croteau 1998). Recent results in mint (Turner and Croteau 2004; Croteau et al. 2005) demonstrated that synthesis of menthol and other monoterpenes is not only compartimentalized at the tissue/cellular level, being confined to the glandular cap cells of secretory stage peltate glandular trichomes of the aerial part of the plant, but also at the subcellular level. In fact, different biosynthetic enzymes were immunolocalized in four cellular compartments, i.e. plastids (leucoplasts), endoplasmic reticulum, mitochondria, and cytoplasm (Fig. 16.2).

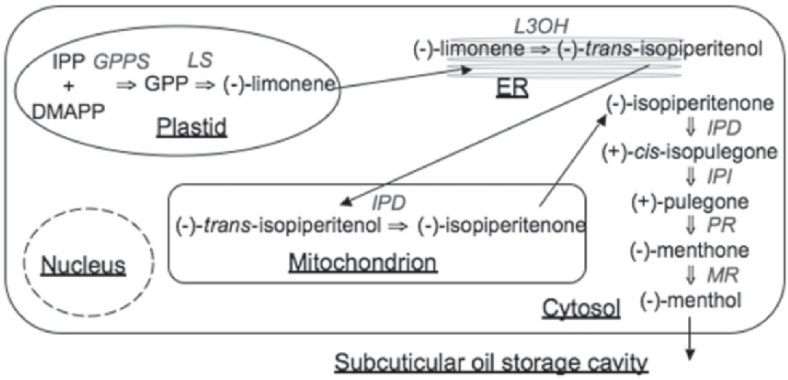

Fig. 16.2. Subcellular organization of (-)-menthol biosynthesis in the secretory gland cells of peppermint. Universal C_5 isoprenoid precursors IPP (isopenthenyl diphosphate) and DMAPP (dimethylallyl diphosphate) are produced in the plastid (leucoplast) through the GAP-pyruvate pathway. Subsequent enzymatic steps are localized in the plastid, the ER (endoplasmic reticulum), the mitochondrion, and the cytosol. Intermediates move between compartments and the end-product is secreted extracellularly in the subcuticular oil storage cavity. *GPPS* = geranyl diphosphate synthase; *LS* = (-)-limonene synthase; *L3OH* = (-)-limonene hydroxylase; *IPD* = (-)-*trans*-isopiperitenol dehydrogenase; *IPR* = (-)-isopiperitenone reductase; *IPI* = (+)-*cis*-isopulegone isomerase; *PR* = (+)-pulegone reductase; *MR* = (-)-menthone reductase. All enzymes are encoded by nuclear genes. Based on Croteau et al. (2005).

Results on enzyme localization were supported by those of transcripts and by the presence of specific signals in the correspondent gene sequences. Similarly, enzymes involved in subsequent steps of the biosynthesis of the diterpene taxol showed different subcellular localization (De Jong et al. 2006). A developmental regulated biosynthesis is also known for the tetraterpenes carotenoids, which is controlled by distinct regulatory mechanisms in chromoplasts of fruits and flowers and chloroplasts of green tissues (Hirschberg 2001).

Examples of various levels of organization patterns are also known for other secondary metabolite classes. The nicotine alkaloid in tobacco as well tropane alkaloids in other species are synthesized in roots and translocated to the aerial part for accumulation and storage (De Luca and St Pierre 2000; De Luca and Laflamme 2001; Yazaki 2006). Cells of different zones of the root, e.g. those of the pericycle and of the endodermis and outer cortex, harbour different enzymes and are specifically involved in different steps of tropane alkaloid biosynthesis in *A. belladonna* and other species (De Luca and St Pierre 2000; De Luca and Laflamme 2001). The first enzyme of the pathway leading to the synthesis of scopolamine (*S*-adenosyl-L-methionine-dependent putrescine-*N*-

methyltransferase) was localized in the perycicle (Suzuki et al.. 1999). Its presence in this tissue was linked to the unloading of the aminoacids ornithine and arginine, precursors of putrescine, from the vascular tissue into the pericycle (De Luca and St Pierre 2000).

The synthesis of the monoterpenoid indol-alkaloid vindoline (a precursor of the anticancer vinblastine) in *C. roseus* was stimulated by light and relied on the differential expression of several enzymes not only in various cell types, belonging to the internal phloem parenchyma of the young aerial organs, to the epidermis of developing leaves, and to leaf laticifers and idioblasts, but also in different subcellular compartments, such as the endoplasmic reticulum, the vacuole, the chloroplast, and the cytoplasm (McKnight et al. 1991; St Pierre and De Luca 1995; St Pierre et al. 1998; St Pierre et al. 1999; De Luca and St Pierre 2000; Irmler et al. 2000; Burlat et al. 2004; Kutchan 2005; Murata and De Luca 2005). Multiple cell types were also involved in the biosynthesis of various benzylisoquinoline-derived alkaloids in *P. somniferum* (opium poppy) (Weid et al. 2004; Kutchan 2005). For instance, some early enzymes for morphine biosynthesis were localized in phloem parenchyma cells of capsule and stem, whereas the late codeinone reductase in laticifers, where morphine accumulates. At the subcellular level, the biosynthesis of the lysine-derived quinolizidine alkaloids in lupins and other legumes occurs within the mesophyll chloroplasts of leaves, but further modifications, such as acylations, happen in the cytosol and the mitochondria, where the acyl donor is probably present. The final accumulation of end-products, however, is believed to occur in vacuoles of the epidermis cells of the leaf, where they can exert their defensive role (Suzuki et al. 1996; De Luca and St Pierre 2000). Indeed, the vacuole is the accumulation site of several toxic products, such as berberine in *C. japonica* and *Berberis wilsoniae,* nicotine in *Nicotiana spp.*, and others in various species (De Luca and St Pierre 2000; Hashimoto and Yamada 2003; Otani et al. 2005; Yazaki 2006).

An additional mechanism for "channeling" secondary metabolite production in plant cells was demonstrated with some phenylpropanoid biosynthetic enzymes (Kutchan 2005). Using advanced technologies, such as FRET (Fluorescence Resonance Energy Transfer), a non-invasive procedure for monitoring protein-protein interactions *in vivo*, and others, it was demonstrated the interaction and the (co)localization of two PAL isoforms with cytochrome P-450-dependent monoxigenase C4H, enzymes consecutively involved in the synthesis of coumaric acid starting from L-phenylalanine, in the endoplasmic reticulum and cytosol of tobacco cells (Achnine et al. 2004). Similarly, the interaction of chalcone synthase (CHS), chalcone isomerase (CHI) and dihydroflavonol 4-reductase (DFR)

was demonstrated not only *in vitro*, but also *in vivo,* particularly in specific subcellular compartments (*i.e.* the rough ER and vacuoles) of flavonoid synthesizing root cells (Burbulis and Winkel-Shirley 1999; Saslowsky and Winkel-Shirley 2001).

As discussed above, many secondary metabolite biosynthetic pathways show a cellular and/or a subcellular compartmentalization in different plant tissues and cell locales. This raises the question how intermediates and end-products move in a coordinated fashion from one tissue, cell, or subcellular compartment to another, and opens the way for additional engineering possibilities in transgenic cells.

An intercellular active transport mechanism based on specific ATP-binding cassettes (ABC) transporters was demonstrated in berberine producing *C. japonica* and *Thalictrum minus* cells (Yazaki et al. 2001; Sakai et al. 2002; Shitan et al. 2003; Terasaka et al. 2003a; Terasaka et al. 2003b). In *Berberis,* the terminal step of berberine biosynthesis occurs in vesicles that derive from ER and lately fuse with the central vacuole (Bock et al. 2002). Differences in transport mechanism with former species were attributed to the fact that while in *Berberis* berberine is produced and accumulates within the same cell, in *Coptis* and *Thalictrum* the production and accumulation sites are distinct (Yazaki 2005, 2006). In heterologous species, berberine can also be transported by a H^+-antiport mechanism (Yazaki 2005). An involvement of an ABC transporter has been identified in the leaves of *Nicotiana plumbaginifolia* in relation to the excretion of the diterpene sclareol onto the leaf surface (Jasinski et al. 2001), but it has been also hypothesized for nicotine in tobacco and for morphine intermediates in poppy (Yazaki 2005, 2006).

At the subcellular level, the vacuolar transport of berberine in *C. japonica* is dependent on a H^+-antiporter (Otani et al. 2005; Yazaki 2006). Similarly, it was suggested that a H^+-gradient-dependent transporter might have a role in vacuolar transport of anthocyanins in *A. thaliana* and tomato (Debeaujon et al. 2001; Mathews et al. 2003). On the other hand, an ABC transporter was found in maize tonoplast, being it required for anthocyanin accumulation in the vacuole (Goodman et al. 2004; Yazaki 2005). Interestingly, the same molecule, i.e. the barley flavonoid saponarin was imported in the vacuole via a H^+ antiporter in the homologous species, but through an ABC transporter in the heterologous species *Arabidopsis* (Frangne et al. 2002; Yazaki 2005). As previously mentioned, monoterpenoid production in mint proceeds from the plastids, to the endoplasmic reticulum, to the mitochondria, and finally to the cytoplasm, from where end-products are secreted to the subcuticolar cavity of the oil glands (Turner and Croteau 2004; Croteau et al. 2005). Simple diffusion of intermediates, somehow facilitated by their aqueous solubility and/or a

concentration gradient, seems the most likely mechanism in such intracellular transport, although some type of terpenoid carrier protein, a mitochondrial membrane pump, transient contacts between organelle membranes might help in some cases (Croteau et al. 2005). An ATP binding cassette transporter, however, has been hypothesized for active transport of end-products through the plasma membrane, because the final extracellular secretion is directional and selective for some monoterpene types.

Results discussed in this section not only indicate some interesting areas for future research, but also highlight some novel possibilities for metabolic engineering of secondary metabolite production in higher plants. The manipulation of genes encoding enzymes controlling critical biosynthetic steps is clearly important. However, based on recent results, it might be possible and probably necessary also to improve, through genetic engineering, the efficiency of long distance and cell to cell transport of metabolite intermediates and end-products, their extracellular secretion, and intracellular organelle to organelle movement (Hashimoto and Yamada 2003). The use of tissue specific promoters for developmentally regulated transgene expression, as well as the adoption of specific signals to address enzymes in the correct subcellular compartments are necessary in many cases.

Further, especially for the plastidial pathways, novel transformation procedures, such as the transformation of the plastome, might be used instead of the conventional nuclear transformation. In comparison with the latter, transgene expression in the plastidial genome shows some advantages, in this context mainly related to the expression level achievable and the possibility to express multiple genes in operons (Bock and Khan 2004). As far as the engineering of secondary metabolic pathways is concerned, plastidial transformation has been used so far in a few cases in tomato and tobacco: with bacterial, fungal and plant derived genes involved in carotenoid biosynthesis (Bock and Khan 2004), with the bacterial gene *ubiC* for p-hydroxybenzoic acid synthesis (Viitanen et al. 2004), and with a PAL (phenylalanine ammonia lyase) gene from *Arabidopsis* (C. Stettner, personal communication http://www.icongenetics.de). Other metabolic engineering applications were mainly related to the manipulation of the primary metabolism as well as to the production of recombinant proteins (Bock and Khan 2004).

6. Uncovering pleiotropic effects and avoiding failure in metabolic engineering of plant secondary metabolites

An increasing wealth of information is revealing that secondary metabolites are not solely involved in plant's defence mechanisms, but rather can also demarcate cellular and developmental differentiation. Therefore, to infer that secondary metabolites are not essential for the growth and development of a plant is an over-simplification that might underestimate eventual detrimental pleiotropic effects triggered by engineering secondary metabolites. Moreover, pleiotropic effects can rise because secondary metabolism forms a large interconnected network. Changes in the flux in one branch might lead to unexpected changes in other parts of the network.

Given the complexity and diversity of regulatory networks of secondary metabolism, it is impossible to make a generalization on how to introduce a novel high-flux pathway into a plant species without undesired effects. To avoid unexpected pleiotropic effects due to modifying one or few genes of a specific metabolic pathway, these interconnections have to be uncovered in advance, through accurate untargeted metabolic profiling beyond the strict boundaries of the pathway that is subjected to engineering (Trethewey 2004). Unexpected pleiotropic effects of modifying the expression of single or multiple genes in a plant are also revealed by microarray or proteomics analysis of the genetic modified plants. Integration of metabolomic and genomic tools have to be seen also as a tool to discover new key biosynthetic genes that can be engineered to enhance the production of bioactive natural compounds. Pleiotropic effects affecting plant growth and development may be less restrictive when engineering plant cells or hairy roots for scaling-up massive production of bioactive secondary metabolites by bio-fermentation.

Suggestions of how to avoid negative pleiotropic effects are offered by mechanisms by which plants naturally face this problem by transport and storage in a safe cell type (*e.g.* glandular cells of *Lamiacae* species) or other subcellular compartment (chloroplasts, vacuoles) or by secretion in the apoplast. For successful metabolic engineering of secondary metabolites in medicinal plants, tissue specificity and proper developmental regulation as well as proper subcellular localization of the desired product has to be taken into consideration. It follows that a judicial choice of the promoters, such cell or tissue-specific promoters, or contemporary over-expression of genes encoding intracellular transporters (as discussed in § 5) might be useful strategies.

Another way of optimising and increasing efficacy of metabolic engineering strategy comes from the recent discover that enzymes of complex metabolic pathways may be present in the cell in arrays of consecutive, physically associated enzymes assembled on membranes or other physical structures, to form multienzyme complexes, called metabolon (Winkel 2004; Jorgensen et al. 2005). Metabolon formation has to be seen as an evolutionary endogenous mechanism of plant cells to ensure efficient transformation of a common precursor into the end-product of a specific biosynthetic pathway by several different means. Firstly, catalytic efficiency is improved by bringing cooperating active enzymes into close proximity and, thereby, accelerating the time of synthesis of intermediates and reducing dilution of intermediates, which are both kinetic constraints of metabolite biosynthesis. At the same time, co-localization of multiple biosynthetic enzymes in a macromolecular complex secures a rapid conversion of potential labile/toxic intermediates into stable and not toxic molecules and, ultimately, prevents their secretion and probable degradation. Finally, metabolon formation might coordinate metabolic cross-talk by controlling either enzymes operating in different pathways or by a selection of intermediates shared between different metabolic pathways. It follows that gained knowledge on the molecular control of the metabolon formation and channeling of plant secondary metabolites will dramatically increase the potential of targeted metabolic engineering and enable the effective production of valuable phytopharmaceuticals.

7. Conclusions and perspectives

Despite the extensive work in the last few years, plant secondary metabolism remains still poorly characterized. Because metabolism is coordinated at many levels, the analysis of all regulatory levels is a prerequisite for comprehensive network analysis. Genetic maps of biosynthetic pathways are still far to be completed and very few regulatory genes of these pathways have been described and characterized, mainly in medicinal plants.

Technologies for gene discovery and plant transformation in medicinal plants are developing in advance to the understanding of the factors that control flux into specific routes of secondary metabolism. Knowledge of these factors is important to move from empirical to predictive metabolic engineering and is crucial to help bypass the low yield of various secondary metabolites in plants or cell cultures. However, it is becoming

increasingly clear that integrated analysis will be necessary in order to maximize understanding of metabolic networks. To date in plants this has only been attempted for few metabolic pathways or sub-networks. It is likely that the extension of such modular approaches will allow the identification of common regulatory motifs and enhance our understanding of metabolic regulation (Sweetlove and Fernie 2005).

As new genes for crucial enzymatic reaction are identified, proof-of concept may be obtained by preliminary testing the effect of their expression in *E. coli* (Willits et al. 2004) or other simple organisms. Another powerful tool towards predictive metabolic engineering is the rapid testing of pathway function of cloned genes, with unknown functions or for which kinetic parameters are unknown, by using functional chips, onto which mRNA-enzyme-fusion protein are immobilized (Jung and Stephanopoulos 2004).

Though integrative analysis in plants, based on correlations between genes, proteins and metabolites, is at the beginning, it remains likely that further advances in the development of network biology in the near future will give a tremendous impulse to successful application of metabolic engineering in medicinal and crop plants.

Acknowledgements

We apologize for omitting other interesting contributions to advancements in metabolic engineering of medicinal and crop plants, that have not be cited for lack of space or simply for our ignorance.

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