Chapter 16
Metabolic Engineering of Plant Cellular Metabolism: Methodologies, Advances, and Future Directions

Rafael Zárate, Nabil el Jaber-Vazdekis and Robert Verpoorte

16.1 Introduction

Plants produce a myriad of secondary metabolites with an immense number of applications for man (fragrances, phytochemicals, dyes, drugs, insecticides, toxins, etc.). For the producing plants these compounds are important for their interaction with the environment and their survival (symbiosis, pollinator attractants, defense against herbivores, and phytopathogens, etc.). Structures of an estimated 200,000 natural products have been elucidated (Dixon and Steele 2002) and each year approximately 4,000–5,000 novel compounds are characterized. At the same time, the determination, characterization, and sequence of genes involved in the biosynthesis of secondary metabolites are progressing at an increasing pace (Oksman-Caldentey and Barz 2002; Verpoorte and Alfermann 2000).

Metabolic engineering constitutes an important tool to improve the plant cell factory directed for the production of desired phytochemicals (Verpoorte and Alfermann 2000; Zárate and Yeoman 2001; Barampuram and Zhang 2011). In theory, any biosynthetic pathway is amenable to metabolic engineering, although in practice different positive outcomes have been reported although unsuccessful experiments are difficult to get published and thus might not be accounted for.

R. Zárate (✉) · N. el Jaber-Vazdekis
Instituto Canario de Investigación del Cáncer, Hospital Universitario La Candelaria,
Carr. EI Rosario 45, 38010, Santa Cruz de Tenerife, Spain
e-mail: rzarate@icic.es

N. el Jaber-Vazdekis
e-mail: njvazdekis@icic.es

R. Verpoorte
Natural Products Laboratory, Institute of Biology Leiden, University of Leiden,
P.O. Box 9502, 2300 RA, Leiden, The Netherlands
e-mail: verpoort@chem.leidenuniv.nl

S. Chandra et al. (eds.), *Biotechnology for Medicinal Plants*,
DOI: 10.1007/978-3-642-29974-2_16,

Metabolic engineering is possible. But what are the targets, why and what should one like to alter in the metabolism of plants? The following goals can be considered:

(a) Improved quality for producer (farmer)

1. Improved yield
2. Improved resistance against pests and diseases
3. Improved cold, salt, and drought resistance
4. Nitrogen fixation

(b) Improved quality for processing (industry)

1. Storage, increased shelf life
2. Water content
3. Unwanted by-products (e.g. amylopectine in starch production, lignin for cellulose production)
4. Higher level of speciality chemicals; e.g. medicines, biocides, flavors, fragrances, cosmetics, dyes
5. Novel compounds for drug development

(c) Improved quality for consumer

1. Taste food
2. Color food, flowers
3. Increased level of health improving compounds
4. Lower level of undesired compounds

Looking at this list of possibilities, one can clearly distinguish that the applications concern in part changes in primary metabolism and in part changes in secondary metabolism. Secondary metabolism is per definition species-specific, it serves the producing organism to survive within its ecosystem. In plants, that means that among others it is involved in defence against pests and diseases, and in attracting pollinators. The defence compounds are of different character, some are constitutively expressed (phytoanticipins), others are only biosynthesized after wounding or following infection (phytoalexins). That means that the regulation of secondary metabolism is, in part, developmentally regulated, and in part dependent on external (stress) signals.

The length and intricacy of secondary metabolite pathways differs considerably. For instance, the biosynthesis of a compound like the phytoalexin resveratrol consists of only a single step, starting from primary metabolism, catalyzed by one single enzyme, encoded by one single gene (Hain et al. 1990). Whereas the biosynthesis of a terpenoid indole alkaloid like vinblastine, includes at least 30 different steps, at least three different cell types and in each type of cell three different cellular compartments, and thus is also regulated by transport (Van der Heijden et al. 2004; Roytrakul and Verpoorte 2007). Because of the fact that secondary metabolism is plant species-specific, the knowledge about most pathways is limited and few pathways have been fully elucidated to all levels of intermediates, enzymes and genes.

These aspects have to be kept in mind in considering metabolic engineering of plant secondary metabolism. Another consideration is whether one wants to increase or decrease the flux through a pathway. The latter could, for example, be of interest in case of undesired (toxic) compounds, or to switch off certain pathways that compete with the pathway of interest. Also, catabolic pathways might be of interest to cut, in order to increase the level of a desired compound. The aim in all these cases is to reduce the level of the protein of interest by an antisense or RNAi approach, or by overexpressing an antibody of the protein in the plant.

In case that one wants to increase the level of a compound, it has to be kept in mind that in most cases not all genes of the biosynthetic pathway are known. Engineering long pathways thus first needs quite some work to elucidate the pathway. In case of inducible pathways, one might also consider the engineering of the signal transduction pathway leading to the induction of the pathway of interest, e.g. overexpression of a regulatory gene such as a transcription factor.

To produce new compounds for a plant, one can add extra steps to an already existing route, or introduce a new enzyme for the plant that catalyzes an early step in a route, e.g. a terpene synthase, resulting in the formation of a novel molecular skeleton for that plant, which might be further processed by enzymes naturally occurring in that plant to yield a novel product for the plant, or maybe even a totally novel compound. Such a recombinatorial biochemistry approach aiming at production of novel compounds would be of interest for developing novel leads for drug development. It might also be of interest for increasing the resistance of the plant against pests and diseases; however, from the point of view of safety, it might need quite some work to prove that the new compound is not toxic for the consumer. Producing a known compound in another plant might be of interest for several reasons. The most obvious is the introduction of health promoting compounds in food plants, e.g. the vitamin A in rice (Ye et al. 2000). Another reason could be that the target plant has better properties for producing the compound than others. The *Atropa belladonna* producing scopolamine is such an example (Yun et al. 1992).

Anyway in principle there are infinite possibilities, but in practice they are limited by: public acceptance, safety issues, lack of knowledge of biosynthetic pathways, or viability of plants with altered metabolism.

The major area for application on the short term is thus the production of medicinal compounds, either known or new, in plants or plant cell or tissue cultures. At present, the only commercial application is in altered flower colors.

In this chapter, the more common plant transformation techniques are reviewed; furthermore, the different genetic approaches and advances made on the genetic engineering of biosynthetic pathways are also presented, as well as various new points that should be addressed for a more predictable outcome of the genetic engineering of plant species. Finally, under future directions, references are made to the expected impacts of new or more comprehensive approaches, such as, functional genomics, or systems biology, to be likely applied in future genetic engineering programs of target plant species.

16.2 Methodology

Several methods for stable or transient genetic transformation of plants or plant cells have been developed (Potrykus 1991; Hansen and Wright 1999; Lorence and Verpoorte 2004). These include: *Agrobacterium*-mediated transformation, particle bombardment, floral dip transformation, agrodrench, viral vectors, protoplast transformation, and ultrasound. These are the main techniques for the genetic transformation of plants, and many of them have also been applied for the transformation of secondary metabolite pathways in an attempt to alter the metabolic pathways of target compounds (Zárate 2010).

16.2.1 Agrobacterium-Mediated Transformation

Two different *Agrobacterium* species i.e. *A. tumefaciens* and *A. rhizogenes* are routinely used for genetic transformation of plants. These belong to the *Rhizobiaceae* family and are the causal agents of the plant diseases crown gall and hairy root, respectively. Diseases caused by the presence of bacterial DNA, the transferred DNA (T-DNA) within the plant cells, which are involved in the production of plant growth regulators, auxin and cytokinin type, which participate in the induction of tumor or hairy roots (Hooykaas 2000). These bacteria have been considered natural metabolic engineers for their capacity to transfer genes into the target plant cells, thus showing their ability to genetically cross kingdoms (Chilton et al. 1977; Tepfter 1990). Moreover, these bacteria have been reported to transform mainly dicotyledonous plants, although there are records on their use to also transform monocotyledonous plants (Ishida et al. 1996), and in some instances even yeast and animals (Bundock et al. 1995; Michielse et al. 2005; Kunik et al. 2001).

Agrobacterium-mediated transformation occurs after bacterial infection of the plant cells or tissues. Upon infection, the T-DNA, which can be engineered to harbor the genes of interest, gets inserted into the plant nuclear DNA. Similarly, other components of the bacterial plasmids, the Ti-plasmid from *A. tumefaciens* (tumour inducer) or the Ri-plasmid from *A. rhizogenes* (root inducer) are also involved in this process. Both plasmids show large functional homologies and appear to have evolved from a common ancestor (Sinkar et al. 1987). These plasmids also possess a virulence region, with various silent *vir* genes that do not enter the plant genome but are essential for the T-DNA transfer. These genes are activated by lignin precursors and acetosyringone, wound tissue metabolites, which may explain why tissue wounding is necessary for efficient infection (Melchers et al. 1989). On the other hand, the *onc*-genes and *rol*-genes encode enzymes involved in the production of plant growth regulators, as well as other opine synthase genes that activate the synthesis of different classes of opines, as well as their catabolism. These are unique natural metabolites, pseudoaminoacids, which serve as a nutrient source of carbon and nitrogen for the pathogenic bacteria (Dessaux et al. 1993).

Agrobacterium transformation requires an acidic pH (5–6), the presence of phenolic inducers produced by wounded tissue, and more recently it has been reported that light also enhances *Agrobacterium* transformation (Zambre et al. 2003). Nevertheless, how the T-DNA integrates into the plant nuclear genome is not totally understood, but it seems to resemble illegitimate recombination, and it is conducted in a similar way in dicot and monocot plants (Hooykaas 2000). Integration of the T-DNA occurs at random positions in the genome, although it shows preferences for transcriptionally active regions. Once integrated, the T-DNA is maintained stably. Contrary to other gene insertion techniques, the plant transgenic lines produced via *Agrobacterium*, often contain one copy or a low copy number of the T-DNA, although cell lines with multiple T-DNA copies can also be found.

Recently, a novel transient assay has been developed and efficiently established, based on the cocultivation of young *Arabidopsis thaliana* seedlings with *A. tumefaciens* in the presence of a surfactant. This new method does not require any dedicated equipment and can be carried out within 1 week from sowing seeds to protein analysis. This Fast Agro-mediated Seedling Transformation (FAST) was used successfully to express a wide variety of constructs driven by different promoters in *Arabidopsis* seedling cotyledons in diverse genetic backgrounds (Li et al. 2009). The FAST assay offers particular advantages: (i) only routine techniques and reagents are used which breaks the constraint of specialized devices, such as a particle gun reducing the overall cost of the experiments; (ii) unlike particle bombardment and protoplast transfection where high quality plasmid DNA has to be prepared each time, *A. tumefaciens* cells used in this assay can be stored indefinitely and can be repeatedly generated from the glycerol stock before use; (iii) this transient assay could achieve higher cotransformation efficiency for two constructs when they are simultaneously carried in the same agrobacterium cell; (iv) small-scale assay produces sufficient proteins for downstream analysis (e.g. Western blot), and the protein production can be easily scaled up for other applications; (v) the use of 4-day-old *Arabidopsis* seedlings instead of mature plants allows for rapid screening with minimal manipulations, which could even be adapted for a 96-well plate format.

Analogously, important crops, such as cereals have been modified using *Agrobacterium*-mediated transformation employing cocultivation of highly embryogenic callus tissue or healthy immature embryos with *A. tumefaciens* being considered one of the critical factors in successful genetic transformation of crop plants (Shrawat and Good 2011).

16.2.2 Particle Bombardment

This is a direct DNA delivery technique developed by Sandford et al. (Klein et al. 1987; Sandford et al. 1987; Sandford 1988), also referred to as biolistics (biology + ballistics). This technique employs high velocity particles or microprojectiles

coated with DNA to deliver exogenous genetic material into the target cell or tissue, which is then cultured in vitro and regenerated to produce mature transformed plants.

The particles, of gold or tungsten, are of small size (0.5–5 μm) but large enough to have the necessary mass to be accelerated and able to penetrate the target cell or tissue carrying the coated DNA on their surface. The microprojectiles are DNA coated following for instance the $CaCl_2$ protocol, with the addition of spermidine to protect the DNA (Klein et al. 1987). Nevertheless, a recent report describes the use of *Agrobacterium* as coating material which has been used successfully (Cordero-Mesa et al. 2000). The microprojectiles are propelled under partial vacuum, using helium or CO_2 pressure to produce the necessary blast to propel the coated particles. Particle penetration may be controlled by altering different parameters, and instrument setup. These include the particle size, the distance between sample holder and target, pressure applied to thrust the particles, the presence of a retaining screen employed to disperse the particle before hitting the target, as well as the biological stage of the cells or tissues to be transformed.

Most of the coated particles are either degraded or inactivated, and many do not reach the nucleus. The DNA can be expressed after reaching the nucleus and getting stably integrated in the genome. Gene insertion using biolistic technique does not show a preference for insertion sites and when landing in a transcriptionally active region, it may be expressed at a high rate; whereas, if it integrates in a nonactive region, gene expression may be reduced or absent. In contrast to *Agrobacterium*-mediated transformation where DNA tends to be inserted in transcriptionally active sites (Ingelbrecht et al. 1991), biolistic technique generates higher copy numbers of the inserted DNA which often results in gene silencing at higher frequencies.

Biolistics offer unique advantages over conventional techniques such as *Agrobacterium* (Leech et al. 2000). For instance, (1) the ability to transfer foreign DNA directly into regenerable cells, tissues or organs; (2) the instrument allows fine tuning and control, permitting precise targeting of DNA coated particles to specific cells or tissue areas; (3) it does not show host specificity, hence it offers the potential to transform any plant species, and it has been successfully applied for the transformation of recalcitrant species such as many monocots and some dicots; (4) it permits a simultaneous bombardment of different gene constructs, finding accounts whereby even 12 different gene constructs were bombarded and expressed in plant cells (Hadi et al. 1996); (5) the input DNA size is not a limitation and it is possible to bombard almost any plasmid, although larger plasmids tend to disintegrate after bombardment resulting in a poor transformation efficiency; (6) this technique has been successfully applied for the transformation of not only plant cells but even animal, bacteria, yeast and fungi (Smith et al. 1992; Wang et al. 2001; O′Brien and Lummis 2004; Olmedo-Monfil et al. 2004). This technique has been successfully used to easily deliver plasmid DNA into the epidermis of *Arabidopsis* leaves comparing with *Agrobacterium* infiltration, a method that does not work efficiently in *Arabidopsis* leaf tissue. This approach was characterized by its high efficiency, reproducibility, and suitability for the robust

transient expression of a variety of functional *Arabidopsis* proteins with diverse biological activities and subcellular localization specificities. Furthermore, this procedure also allows for the coexpression of numerous proteins, tagged and/or untagged, by bombardment with multigene expression constructs (Ueki et al. 2009).

16.2.3 Vacuum Infiltration and Floral Dip

Both methods can be considered to be simple techniques for the genetic transformation of *Arabidopsis thaliana*, although other plant species have also been successfully transformed following these methods (Curtis and Nam 2001; Curtis 2005). Species with large number of flowers together with a higher number of set seeds following infiltration are the best candidates. The major advantage of these methods is that they make it possible to transform these species without necessitating the labor-intensive plant tissue culture or regeneration steps required with other techniques such as *Agrobacterium*-mediated transformation or particle bombardment.

Vacuum infiltration was first developed (Bechtold and Pelletier 1998) and involves the growth of *Arabidopsis thaliana* to an early flowering stage (prior to anthesis). The plants are uprooted, and submerged in cloned *A. tumefaciens* liquid cultures, which after vacuum infiltration acquires the bacterium thus carrying the transgene(s) of interest. Plants are replanted and further grown, and seeds collected for assessment of transformants employing appropriate selection agents. Following this procedure, it has been demonstrated that the ovules of *A. thaliana* are the target for *A. tumefaciens* in transformed plants (Ye et al. 1999).

Similarly, floral dip is a variation of the vacuum infiltration method, developed as a simplified method for *Agrobacterium*-mediated transformation of *A. thaliana* (Clough and Bent 1998). It also employs *A. tumefaciens* as the DNA vector carrier, here floral dipping into the liquid bacterial culture is performed avoiding the vacuum infiltration process. Again, plants are susceptible to transformation when flowers are still immature; the attached flower buds are immersed in a solution containing a mixture of *A. tumefaciens* cells, sucrose and a surfactant. The appropriate amounts and ratio of the latter ingredients are important for a successful transformation. This method allows repeated dipping in the transformation mixture after a few days; besides, covering the plants with a plastic dome after infection to maintain humidity was also reported to increase transformation by 2-fold (Clough and Bent 1998). Transformants could be obtained at a high rate, 0.5–3% of all progeny seed, and for multiple *Arabidopsis* ecotypes. The success of the floral dip method implies that the germ-line tissues to be transformed should be exposed and accessible to the *Agrobacterium*. It was reported that female tissues such as developing ovules within the gynoecium of young flowers are the primary target of *Agrobacterium*-mediated floral-dip transformation of *Arabidopsis* (Desfeux et al. 2000). Besides, it has also been reported that *A. thaliana* transgenic

plants obtained following floral dip transformation presents genetic stability (Labra et al. 2004).

A further modification of the above methods has been the application of *A. tumefaciens* by spraying. The results indicate that the floral spray method of *Agrobacterium* can achieve rates of *in planta* transformation comparable to the vacuum-infiltration and floral dip methods. The floral spray method opens up the possibility of *in planta* transformation of plant species which are too large for dipping or vacuum infiltration (Chung et al. 2000).

Plant transformation using these techniques produces genetically uniform progeny and more importantly obviates the somaclonal variation often encountered with tissue culture and regeneration. In addition, practicing these methodologies does not require costly or precise equipment being approachable by many laboratories. Nevertheless, the major drawback is that few species appear susceptible to transformation following these methods, although further research may provide new attractive possibilities, for example, transformation of banana meristematic tissue after vacuum infiltration (Acereto-Escoffié et al. 2005), and other potential plant species such as the medicinal plants *Catharanthus roseus* or *C. pusillus* which produce large number of flowers and many seeds per follicles (Zárate et al. 2001).

16.2.4 Agrodrench

This is a newly devised technique developed as an agroinoculation method for virus-induced gene silencing (VIGS) (Ryu et al. 2004). It is known that virus-induced gene silencing is an RNA-mediated posttranscriptional gene-silencing mechanism that protects plants against foreign gene invasion (Baulcombe 1999), and it represents a powerful tool, for instance, for plant functional genomics which allows knocking out gene expression of target plant genes in some plants, following an RNA silencing mechanism via small interference RNA. One advantage of VIGS is that it does not alter the gene itself, but transiently suppresses the expression of the gene via degradation of mRNA transcripts.

It has been developed employing tobacco rattle virus (TRV)-derived vectors, in conjunction with *A. tumefaciens* which harbors the designed TRV-VIGS constructs. For agrodrenching, the cloned bacterium is applied as a solution to the soil adjacent to the rhizosphere of previously grown 3–4-week-old seedlings, after infection gene silencing was demonstrated. Furthermore, it was established that an avirulent *A. tumefaciens,* and *Agrobacterium*-mediated transformation is required for agrodrench method of agroinoculation, and that agrodrench was more effective than the leaf infiltration method of agroinoculation (Ryu et al. 2004).

Furthermore, the agrodrench technique was employed to carry out a virus-induced gene silencing using Tobacco rattle virus (TRV)-based VIGS vector. This method was used to demonstrate that the duration of gene silencing by VIGS can occur for more than 2 years and that TRV is necessary for longer duration VIGS.

Also, inoculation of TRV-VIGS constructs by both agrodrench and leaf infiltration greatly increased the effectiveness and duration of VIGS (Senthil-Kumar and Mysore 2011). These results demonstrated the transmittance of VIGS to progeny seedlings via seeds. Moreover, the transmittance of VIGS to the progeny will be useful in studying the effect of gene silencing in young seedlings.

This method proved also to be a better choice for high-throughput gene silencing and for plants which appear difficult to infiltrate. On the other hand, this technique shuts down transcript levels to a greater degree in roots than leaf infiltration does, indicating advantages when roots are to be engineered.

Although this technique has been recently described for virus-induced gene silencing in primarily Solanaceous species, and for functional genomic analyzes, its employment might soon be broadened to other plant species and likely for the genetic modification of metabolic pathways by gene silencing.

16.2.5 Viral Vectors

Another approach to genetically transform plants has been the employment of viral vectors, thus different plant viruses have been used for the insertion of genes into a variety of plant species. The majority of plant viruses have an RNA genome, and just a few are of single or double stranded DNA. The main drawback is that viruses can propagate easily and spread to other plant parts and even to other neighboring plants. Nevertheless, their use offers interesting advantages such as being highly multiplicative, production of large number of copies of the transgene, as well as its expression (Porta and Lomonosaff 2002).

One of the early plant viruses used was the cauliflower mosaic virus (CaMV), a double stranded DNA virus that replicates through an RNA intermediate (Gronenborn et al. 1981). Using this virus extra DNA insertions are difficult to achieve, and often molecular recombinations rapidly eliminate the inserted transgene. In addition, replication of this virus requires an RNA step which can introduce errors because the inverse transcriptase does not have a proof-reading activity.

Similarly, regarding RNA viruses, two viruses, i.e. the tobacco mosaic virus (TMV) and the cowpea mosaic virus (CMV) have been successfully employed (Wilson and Davies 1992).

Two different strategies have been followed to clone and insert foreign gene(s) using plant viruses; either to replace the coat protein of the virus by a transgene or to insert the gene(s) next to the initiation codon of the coat protein that will then express the product of the inserted gene and the coat protein. Nonetheless, it has been observed that the inserted gene(s) can be removed by the virus, thus returning to its original form.

Several drawbacks have been reported when using plant viruses as vectors. These include: their instability, manifested by the loss of infectivity when compared to intact viruses and elimination of the transgene when this is larger than

1 kb. It appears that the ease of insert elimination might be an evolutionary strategy of the virus to avoid the accumulation of unwanted genomic material which does not offer any advantage to the host.

16.2.6 Protoplast Transformation

Protoplasts are osmotically fragile plant cells deprived of their cell wall, also referred to as naked cells. Upon removal of the cell wall, protoplast can behave like animal cells which by nature have no cell wall barrier. Protoplasts have the potential ability to redifferentiate into whole plants due to their totipotency, and this has been exploited for genetic modification of plants; however, there exist many recalcitrant plant species which have not been able to be transformed by this means, either because of failure to isolate viable protoplast or impossibility of regeneration.

Generally, removal of the cell wall is achieved by treatment of plant material (leaves, tissue cultures, suspended cells, etc.) with a designed cocktail of enzymes, such as cellulases, pectinases, and/or hemicellulases, in an incubation medium of the right osmolarity to avoid cell burst. In addition, the physiological status of the source tissue also influences the release of viable protoplasts, and several other factors influence protoplast release, including the extent of thickening of cell walls, temperature, duration of enzyme incubation, pH of the enzyme solution, gentle agitation, and nature of the osmoticum (Sinha et al. 2003).

Insertion of transgenes into protoplast can be achieved either by fusion of different protoplasts from different species, or DNA insertion into the protoplast through the plasma membrane. Several approaches have been devised for this gene insertion; these include: (a) *chemical techniques*, such as the use of PEG (polyethylene glycol), Ca^{++}-DNA coprecipitation or use of liposomes; (b) *electrical techniques*, where electrical pulses are applied to the protoplasts resulting in an increase in the protoplast permeability which facilitates DNA transfer; (c) *microinjection*, whereby DNA is physically injected into the protoplast nucleus or cytoplasm, although the presence of vacuole (comprising 90% of total cell volume) can produce cell death if they become damaged releasing hydrolases and toxic metabolites.

Although other genetic transformation techniques have shown increasing success, the use of protoplasts has also demonstrated to be effective in genetically transforming both monocot and dicot species. Furthermore, it has also proved suitable for other applications such as to investigate plant cell responses to growth regulators, genetic basis of developmental regulation and specificity, regulation of osmotic water transport across cell membranes, intracellular responses to drought and salinity stress, transient gene expression, and proteomics. A comprehensive review of the current status of plant protoplasts in genetic engineering has been recently published (Davey et al. 2005).

This technique has also been used for the transformation of fungal species, i.e. *Ozonium* sp. a producer of taxol and taxol-related compounds (Zhou et al. 2008). The authors were able to obtain protoplasts using a cocktail of commercially available enzymes, which were then successfully transformed using PEG and regenerated into mature fungus, thus establishing a model to efficiently transform via protoplast transformation different fungal species.

16.2.7 Ultrasound (Sonoporation)

Ultrasound is a sound whose frequency is higher than that perceptible to the human ear, although many animal species rely on ultrasound for their survival. It shows multiple industrial, medical, chemical, and biotechnological applications. Nevertheless, ultrasound can destroy microbial and other cells and this well-known effect has perhaps discouraged research on beneficial effects of ultrasound on many biological applications. Potentially, finely tuned ultrasound can enhance diffusive transport both within and outside a cell to influence rates of reactions and yields of metabolites (Christi 2003), and has been frequently used as a means for genetic transformation in animal cells and tissues. Nonetheless, there are few reports about the use of ultrasound in plant cells or tissues transformation. It seems that the cell wall of plant cells is the big obstacle for gene transfer using this technology (Liu et al. 2005).

Gene transfer by ultrasonication employs the same simple procedure irrespective of the nature of the plant material to be transformed. The protoplasts, suspension cells or small pieces of tissue are suspended in a few millilitres of sonication medium in a microcentrifuge tube. Plasmid DNA (and possibly carrier DNA) is then added, and after rapid mixing the samples are ready for sonication. The pulses of ultrasound of selected intensity and duration are delivered by ordinary machines used for homogenization of various tissues. Finally, the cells are transferred to fresh growth medium (Liu et al. 2005). Success on the insertion of plasmid DNA has been reported for sugar beet and tobacco protoplasts after a brief exposure to 20 kHz ultrasound (Joersbo and Brunstedt 1990).

Currently most studies are focused on sonication-assisted *Agrobacterium*-mediated transformation (SAAT) in plant cells or tissues (Weber et al. 2003). SAAT is a new technology which involves subjecting the plant tissue to brief periods of ultrasound in the presence of cloned *A. tumefaciens*. It has been demonstrated that for all tissues tested, the SAAT treatment greatly increased the levels of transient expression. In addition, it was established that SAAT treatment tremendously improved the efficiency of *Agrobacterium* infection by introducing large numbers of micro-wounds into the target plant cells or tissues which facilitated gene insertion (Trick and Finner 1997; Tang 2003).

16.3 Advances on Metabolic Engineering of Secondary Metabolite Pathways

Metabolic engineering is the redirection or modification of one or more biosynthetic steps (enzymes) for either the production of new compounds, enhancement of existing metabolites or halting the accumulation of a specific product, either by knocking out specific genes or by degradation of that metabolite.

On the other hand, the scientific advances made by a combination of biochemistry, genetics, and molecular biology (i.e. recombinant DNA technology) on biosynthetic pathway studies of target secondary metabolites have allowed the identification, function determination, cloning, and expression of many genes, which has shed some light on the complexity of secondary metabolism networks and their regulation. This knowledge has permitted to engineer metabolic pathways to yield tailored compounds, but has also resulted in successes, as well as in unexpected disappointments (Zárate and Yeoman 2001; DellaPenna 2001). Nonetheless, the future to elucidate and manipulate metabolic pathways is very challenging and further success is clearly foreseen.

Under the following subheadings, an attempt has been made to review the major strategies conducted to tailor secondary metabolism, highlighting some of the most current and interesting results, avoiding repeating the biotechnology of those products referred in this book. Furthermore, references to several important aspects which are often overlooked but should be addressed for successful metabolic engineering are also presented.

16.3.1 Gene Silencing

Although the first undesired results of gene silencing were considered disappointing, later it was realized its great potential to specifically block the expression of endogenous genes, and it is now considered a powerful tool to knockout genes in a given pathway (Waterhouse et al. 2001a, b; Lessard et al. 2002).

Downregulation or blockage of specific genes forms another approach to manipulate secondary metabolic pathways to either avoid formation of competitive intermediates or to completely turn off metabolic pathway branches or undesired metabolite synthesis. An enzymatic step in a pathway can be knocked out or downregulated by reducing the level of the corresponding mRNA either via antisense, cosuppression or employing RNAi (RNA interference), or by activating the synthesis of an antibody against the enzyme.

The antisense gene approach, whereby a gene sequence (cDNA), is fused in the inverted orientation to a plasmid, containing an upstream promoter and a downstream terminator sequence was the initial approach in the 1980s. The resulting mRNA becomes double stranded, and depending on the size of the inserted DNA, the double stranded mRNA could be partial or along all its length. Thus, the

ribosome finds it difficult to process mRNA and none or very little protein is produced. Consequently, a pioneering work on the genetic manipulation of the flavonoid flower pigments in *Petunia hybrida* was reported by inserting an antisense chalcone synthase gene (a key enzyme for anthocyanin and flavonoid biosynthesis), which resulted in pigmentation changes of the transgenic plant flowers (Van der Krol et al. 1988). Further on this strategy but altering two genes, antisense insertion of chalcone synthase and dihydroflavonol-4-reductase genes in torenia plants resulted also in an altered flower pigmentation (Aida et al. 2000a, b). Following a similar approach, engineering the 7-xanthosine methyl transferase gene in antisense orientation via *A. tumefaciens* transformation for the production of caffeine free coffee plants has also been attempted (Moisyadi et al. 1998). More recently, antisense-mediated downregulation of putrescine *N*–methyltransferase (PMT) in transgenic *Nicotiana tabacum* plants and hairy root cultures resulted in elevated levels of anatabine, and a clear decrease in nicotine (Chintapakorn and Hamill 2003). In *Catharanthus roseus* antisense has also been used in an attempt to elucidate the participation of the enzyme isochorismate synthase (ICS) in the formation of 2,3-dihydroxybenzoic acid, indicating that in *C. roseus* the biosynthesis of this metabolite probably involves a multienzyme pathway (Rodríguez-Talou et al. 2001; Zárate and Verpoorte 2007).

RNA interference (RNAi) is a newer strategy for gene silencing, displaying a posttranscriptional gene silencing mechanism. However, it represents the oldest and most ubiquitous antiviral system in plants (Sharp 2001). RNAi refers to the introduction of homologous double stranded RNA (dsRNA) to specifically silence a gene. The presence of dsRNA is responsible for producing the interfering activity, the dsRNA is cleaved into 21–23 bp short interfering RNA (siRNA) by an enzyme called Dicer, thus producing multiple molecules from the original dsRNA. These form a RISC complex (RNA-induced silencing complex) that after becoming activated targets the homologous transcript by base pairing interactions and cleaves the mRNA. The readers are referred to comprehensive reviews on RNA silencing in plants for further information on this topic (Waterhouse et al. 2001a, b; Baulcombe 2004; McGinnis 2010).

RNAi offers important advantages compared to the antisense approach for being highly specific, remarkably potent since only a few dsRNA molecules per cell are required for effective interference and dsRNA can cause interference in cells and tissues away from the site of introduction and its effects are maintained through the progeny.

The chalcone synthase gene has also been silenced following the RNAi technique, thus the flower colour of the garden plant *Torenia hybrida* was successfully modulated. These results demonstrate that RNAi is a powerful technique for flower colour modulations of commercially important garden plants (Fukusaki et al. 2004).

An elegant piece of research worth quoting has been the genetic engineering of the opium poppy whereby morphine was replaced by the non-narcotic alkaloid reticuline by RNAi (Allen et al. 2004, Page 2005). Silencing of the codeinone reductase (COR) in *Papaver somniferum* plants was achieved employing a chimeric hairpin RNA construct designed to silence all members of the multigene

COR family. The precursor alkaloid (*S*)-reticuline, 7 enzymatic steps upstream of codeinone, accumulated at the expense of morphine, codeine, oripavine and thebaine. (*S*)-reticuline is a potential substrate for the synthesis of various bioactive compounds (antimalarial or anticancer) but its availability is limited, though not in these silenced poppy plants which show (*S*)-reticuline as the major metabolite. The eight enzyme branch leading to morphine can be downregulated in response to the loss of the penultimate enzyme, COR. This represents the most dramatic example of gene silencing-induced feedback in secondary metabolism ever reported (Allen et al. 2004). These authors suggest three possible processes responsible for the results observed, even though the full chain of events is still unelucidated, i.e. (a) the build up of COR substrates–codeinone and neopinone–might switch negative feedback on earlier enzyme or transport step(s); (b) or might inhibit transcription of those genes; and (c) loss of COR enzyme from a larger enzyme complex might disable other enzyme reactions associated with this complex. The initial expectation of this research was the accumulation of thebaine and oripavine following silencing of COR. The unexpected accumulation of (*S*)-reticuline demonstrates that the morphinan-alkaloid pathway can be coordinately regulated independently of the benzylisoquinoline pathway.

Similarly, RNAi has also been employed to further elucidate the metabolic pathway of caffeine synthesis, which has been proposed to involve three distinct *N*-methyltransferases, xanthosine methyltransferase (XMT), 7-N-methylxanthine methyltransferase (MXMT; theobromine synthase), and 3,7-dimethylxanthine methyltransferase (DXMT; caffeine synthase). Silencing of CaMXMT1 expression in *Coffea arabica* and *C. canephora* plants by the double stranded RNAi method showed that not only transcripts for CaMXMT1 but also for CaXMT1 and CaDXMT1 were reduced, indicating that indeed caffeine was synthesized in vitro by the combination of their three gene products although the major pathway of caffeine synthesis is mediated by the theobromine synthase, CaMXMT1. The resulting transgenic tissues and plantlets exhibited a marked reduction of theobromine and caffeine contents, indicating that RNAi can be attractively used for low or free caffeine plants (Ogita et al. 2004). Furthermore, in comparison with the above-mentioned antisense approach for caffeine-free plants, the construction of transgenic coffee plants in which the expression of the gene encoding theobromine synthase (CAMXMT1) was repressed by RNAi has been attempted (Ogita et al. 2003). This resulted in a major caffeine reduction of up to 70%, indicating that it should be feasible to produce coffee beans that are intrinsically deficient in caffeine following this strategy. Similarly, in *Camellia sinensis* an *Agrobacterium*-mediated silencing of caffeine synthesis through root transformation was carried out to suppress the expression of the caffeine synthase gene, by means of a RNAi construct, resulting in a marked reduction in both caffeine and theobromine contents in young shoots of tea seedlings after root transformation through *Agrobacterium* infiltration (Mohanpuria et al. 2010).

In the same fashion, Carotenoid Cleavage Dioxygenase 4 (CCD4) activity was studied in potato (*Solanum tuberosum*) in order to check whether the carotenoid content would be affected after manipulation. The expression level of the potato

CCD4 gene was downregulated using an RNA interference (RNAi), resulting in a 2- to 5-fold increased carotenoid content (Campbell et al. 2010).

More recently, a novel gene-silencing system using the chimeric transcriptional repressor, which is a designated chimeric repressor gene-silencing technology (CRES-T) has been developed. This system can induce a phenotype that was not expressed by antisense or gene knockout lines by suppressing expression of target enzyme genes in the pathway through modification of a metabolic regulator (Hiratsu et al. 2003). The CRES-T system can convert a transcriptional activator into a dominant repressor simply by fusion of the EARrepression domain with the activator, referring as chimeric repressor (Shikata and Ohme-Takagi 2008 and references therein). An outstanding advantage of the CRES-T system to other gene-silencing technologies, namely antisense, RNAi, and T-DNA insertion, is that the repressive activity of the chimeric repressor can overcome those of the endogenous and functionally redundant transcription factors, resulting in the induction of a dominant negative phenotype.

16.3.2 Homologous and Heterologous Overexpression of Biosynthetic Encoding Genes

Progress on enzyme purification, characterisation, and encoding genes determination of a myriad of secondary metabolism pathways has allowed the genetic engineering of biosynthetic networks. In an attempt to tailor and boost secondary metabolite yields, the overexpression of a biosynthetic enzyme encoding gene was the first approach conducted, which was further developed by the overexpression of multiple, even heterologous genes.

Thus, the manipulation of flavonoid and anthocyanin biosynthesis was the first example of genetic engineering, since the pathway was well characterized and the results could easily be observed by flower colour changes. Several genes have been overexpressed aiming at obtaining different flower colour; for instance, the dihydroflavonol reductase gene from maize or *Gerbera* into *Petunia*, as well as the introduction of alfalfa chalcone reductase gene into *Petunia* resulted in clear flower colour changes (Dixon and Strack 2003 and references therein). Moreover, based on the antioxidant activity of these metabolites, attempts have also been made to increase their yields in tomato. Thus, the overexpression of *Petunia* chalcone isomerase into tomato led to a 78-fold increase of flavonol levels in tomato peel, and 21-fold increase in tomato paste; demonstrating that it is feasible to enhance the yield of health beneficial metabolites by overexpression of genes (Muir et al. 2001). Tomatoes have also been metabolically engineered to enhance the aroma and flavor compound *S*-linalool by heterologous overexpression of *Clarkia breweri S*-linalool synthase (LIS) gene, under the control of the tomato late-ripening-specific E8 promoter. The accumulation of *S*-linalool and 8-hydroxylinalool was increased in ripening fruits. Apart from the difference in

volatiles, no other phenotypic alterations were noted, including the levels of other terpenoids, such as γ- and α-tocopherols, lycopene, β-carotene, and lutein (Lewinsohn et al. 2001).

Dietary carotenoids offer important health benefits (protection against prostate cancer, age-related macular degeneration and cardiovascular diseases), and attempts for the metabolic engineering of potato tubers (*Solanum tuberosum* and *S. phureja*) containing enhanced levels of β-carotene and lutein have been undertaken (Ducreux et al. 2005 and references therein]. A phytoene synthase gene (*crtB*) from the bacterium *Erwinia uredovora* cloned using a tuber-specific patatin promoter in conjunction with a plastid targeting sequence, has been overexpressed in potato tubers. This resulted in an enhanced accumulation of β-carotene and lutein with total carotenoid levels increasing 6.25-fold and 4-fold in *Solanum tuberosum* and *S. phureja,* respectively. Similarly, attempts to increase isoprenoids (carotenoids and phytosterols) have been successfully performed in tomato by metabolic engineering of the mevalonate (MVA) and nonmevalonate (MEP) isopentenyl diphosphate-forming pathways. Independent overexpression of the two genes 3-hydroxymethylglutaryl CoA (*hmgr-1*) from *A. thaliana*, and 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*) from *Escherichia coli* resulted in an increased carotenoid content with phytoene and β-carotene exhibiting the greatest increases (Enfissi et al. 2005). Further information can be found in a recent comprehensive review, which presents the current state of the molecular tools for the metabolic engineering of carotenoid in plants (Sauret-Güeto et al. 2003).

Another group of health promoting metabolites which has received immense attention for their metabolic engineering is one of the fatty acids. Particularly long-chain polyunsaturated fatty acids (PUFA) among which eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) are of major importance due to their multiple important roles in human health and nutrition. These provide protection from cardiovascular diseases, obesity, diabetes (type II) as well as in an important number of mental illnesses such as schizophrenia and depression; they also participate in retinal development, and nerve and brain development of growing fetuses and infants, antiinflammatory responses, and against some types of cancer (Browning 2003; Simonopoulus 1991; Horrobin 1999; Simonsen et al. 1998; Trautwein 2001).

Different oilseed crops have been genetic engineered in an attempt to alter the fatty acid content aiming mainly to enhance n-3 and/or n-6 PUFA yield. This has been achieved either by overexpressing or suppressing single genes (i.e. desaturases, elongases), and in most cases, seed-specific promoters have been used to restrict the changes to the storage oils of seeds (Thelen and Ohlorgge 2002). Linseed plants have been engineered by the heterologous expression of three genes encoding a Δ6-desaturase, a Δ6-elongase, and a Δ5-desaturase which resulted in enhanced levels of arachidonic and eicosapentaenoic acids (Abbadi et al. 2004). Likewise, canola plants have been engineered to alter their PUFA content. Transgenic canola lines that expressed in seeds the Δ6 and Δ12-desaturases isolated from the fungus *Mortierella alpina*, and the Δ15-desaturase from canola (*Brassica napus*) were generated. Seed oil accumulated stearidonic acid (SDA),

the highest yield was up to 23% of the lipids F1 seed. The total omega-3 content in the seed lipids (α-linolenic acid and SDA) exceeded 55% of the seed lipids, whereas the total omega-6 fatty acid content of the seed lipids (γ-linolenic and linoleic acids) was 22% (Ursin 2003).

Furthermore, the expression of the borage Δ6-desaturase in transgenic nonoleaginous plants i.e., tobacco and *Arabidopsis* resulted in the production of gamma linolenic and stearidonic acids (Sayanova and Napier 2004; Sayanova et al. 1997). *Arabidopsis* was further engineered for the production of PUFA (Qi et al. 2004). Here, sequential transformation of this plant with genes encoding a Δ9-specific elongating activity from *Isochrysis galbana*, a Δ8-desaturase from *Euglena gracilis,* and a Δ5-desaturase from *Mortierella alpina* resulted in the production of substantial quantities of arachidonic and eicosapentaenoic acids in this higher plant, suggesting the construction of an alternative sustainable source of fish oils. On the other hand, cotton has also been engineered by hpRNA-mediated silencing (hair pin RNA) of the *ghFAD2-1* Δ12-desaturase which raised the oleic acid content from 12 to 38%. Analogously, silencing of the *ghSAD-1* Δ9-desaturase gene, substantially increased stearidonic acid from the normal 2 to 40%. Intercrossing of these two plants resulted in a wide range of unique intermediate combinations of palmitic, stearic, oleic, and linoleic acids (Liu et al. 2002).

In another example, the endogenous *FAD2* gene was downregulated in *Brassica carinata* plants resulting in the desaturation of oleic acid to enhance substrate availability for the biosynthesis of erucic acid, via elongation with a significant increase in the relative proportions of erucic acid (12–27%) and other very long-chain fatty acids (Jadhav et al. 2005).

Similarly, using *Agrobacterium rhizogenes* mediated transformation, hairy root cultures of *Echium acanthocarpum* were established as the first example of a *Boraginaceae* species transformation for fatty acid studies and production. This system is capable to accumulate a large range of polyunsaturated fatty acids including stearidonic acid and gamma-linolenic acid, which are the precursors of important fatty acids, i.e. EPA and DHA (Cequier-Sánchez et al. 2011). This stable system, capable to produce economically important fatty acid, has been genetically manipulated overexpressing a Δ6-desaturase gene resulting in a clear increase of SDA amounts (unpublished).

The heterologous coexpression of the nematode *Caenorhabditis elegans* genes Δ6-elongase together with the Δ6- and Δ5-desaturases has been attempted using yeast. Following feeding with linoleic and alfa linolenic acid of the transgenic yeast, small but significant accumulation of arachidonic and eicosapentaenoic acids was recorded (Beauduin et al. 2000). Further information is available in recent comprehensive reviews dealing with the production of PUFA in transgenic plants, which demonstrate the feasibility of establishing transgenic plants for the supply of fish oils (Cahoon and Kinney 2005; Napier et al. 2004; Jaworski and Cahoon 2003).

16.3.3 Multiple Expressions of Transgenes

The possibility to overexpress homologous or heterologous genes in different plant systems has been highlighted and presented above. Progress has been further achieved whereby entire biosynthetic pathways have been engineered, resulting in the accumulation of the target metabolites, and attempts are being undertaken to further extend on the manipulation of entire pathways confirming that multigene transfer allows researchers to face challenges previously impossible (Naqvi et al. 2010).

One of the initial successes in genetic engineering an entire pathway was the production of β-carotene in rice endosperm (Ye et al. 2000), also known colloquially as the golden rice. The entire β-carotene biosynthetic pathway, vitamin A precursor, was introduced and expressed into rice endosperm in a single transformation effort with three vectors harboring four transgenes, i.e. *psy* plant phytoene synthase; *crt-1* bacterial phytoene desaturase; *lcy* lycopene β-cyclase and *tp* transient peptide. The transformed rice grains were yellow colored indicating the accumulation of carotenoids, and in some lines β-carotene was the only carotenoid detected.

Verberne et al. (2000) introduced isochorismate synthase (*entC* from *E. coli*) and isochorismate pyruvate lyase (*pmsB* from *Pseudomonas fluorescens*) with a chloroplast targeting signal into tobacco. This results in plants constitutively producing salicylate. The transformed plants were shown to be more resistant against viral, microbial, and fungal infections.

Hallard et al. (2000) reported the overexpression of the strictosidine synthase and tryptophan decarboxylase genes from *Catharanthus roseus* in hairy roots of *Weigela styriaca*. This plant makes secologanin, the precursor for the terpenoid indole alkaloids, and due to the presence of the two mentioned genes, the hairy roots made small amounts of ajmalicine and serpentine, two terpenoid indole alkaloids which are the major alkaloids of *C. roseus* roots, showing that it is feasible to introduce the production of alkaloids in nonalkaloid plants from a different family.

Transgenic *A. thaliana* plants expressing the entire biosynthetic pathway for the tyrosine-derived cyanogenic glucoside dhurrin has been achieved following the insertion of the cytochrome P450 enzymes CYP79A1, CYP71E1, and the glucosyltransferase UGT85B1 genes from *Sorghum bicolor* (Tattersall et al. 2001; Kristensen et al. 2005). Dhurrin is a plant cyanogenic glucoside that plays a role in defence against herbivore attack, and it is absent in *A. thaliana* plants but highly abundant in *S. bicolor*. Only in transgenic *A. thaliana* plants expressing the three transgenes, dhurrin was the single metabolite to accumulate in high abundance, together with minor amounts of p-hydroxybenzylglucosinolate. The presence of this metabolite also provided a clear deterrent effect against adults and larvae of flea beetles. In addition, the high accumulation of dhurrin did neither appear to provoke any physiological problem for the transgenic *A. thaliana* nor significant changes to the transcriptome and metabolome. Nonetheless, when incomplete pathways were introduced, metabolic crosstalk or detoxification reactions were

found to induce significant changes in plant morphology, the transcriptome and metabolome. These results demonstrate that insertion of a full pathway is feasible in *A. thaliana* resulting in the production of the cyanogenic glucoside dhurrin with marginal inadvertent effects on the transcriptome and metabolome.

16.3.4 Transcription Factors

Currently a different and novel approach to engineering the expression of biosynthetic pathway genes is the employment of regulatory genes, i.e. transcription factors, that control multiple genes in a pathway and are able to regulate or induce various genes concurrently, representing the most important strategy for studying secondary metabolic pathways mainly for their ability to activate multiple genes simultaneously (Grotewold 2008; Iwase et al. 2009; Hussain et al. 2011).

Transcription factors are regulatory proteins that modulate the expression of specific set of genes through sequence-specific DNA binding to motifs usually in gene promoters and protein–protein interactions, showing homologies with mammalian transcription factors (i.e. protein encoded by the proto-oncogene *c-MYB* and basic-Helix-Loop-Helix protein encoded by the proto-oncogene *c-MYC*) (Davies and Schwinn 2003; Broun 2004). They can function as repressors (less frequent) or activators (more abundant) of gene expression, provoking a decrease or increase in the mRNA synthesis by RNA polymerase II. These proteins regulate gene transcription depending on tissue type and/or in response to internal signals; moreover, external signals (UV light, microbial elicitors, etc.) may also induce production of internal signals (Vom Endt et al. 2002).

The first pioneering discovery in plants was the determination of the transcription factors C1 and R of the maize flavonoid-anthocyanin pathways which were shown to regulate this biosynthetic pathway (Dooner et al. 1991). The anthocyanin pathway has been extensively studied and other transcription factors have been determined in *Anthirrinum*, factor *DELILA* (Goodrich et al. 1992), and in *Petunia*, factors *ANTHOCYANIN2* (*AN2*) and *JAF13* (Quattrocchio et al. 1998; Quattrocchio et al. 1999). Similarly, in *Arabidopsis* two transcription factors, *PRODUCTION OF ANTHOCYANIN PIGMENT1* (*PAP1*) and *PAP2* have been identified (Borevitz et al. 2000). Recently, also in *A. thaliana* in the proanthocyanidin (condensed tannins) pathway the presence of the gene regulators *Transparent testa 2* (*TT2*) and *TT8* has been determined (Nesi et al. 2000; Nesi et al. 2001). Moreover, with the completion of the *Arabidopsis* genome sequence, and due to the well-conserved DNA-binding domains, the identities of the complete set of *Arabidopsis* transcription factors can be characterized in the near future. On the other hand, in a different metabolic pathway, i.e. the biosynthesis of the medicinally important terpenoid indole alkaloids (TIAs) vincristine and vinblastine in *Catharanthus roseus* the determination of various transcription factors such as *ORCA2* and *ORCA3* (octadecanoid responsive *Catharanthus roseus* AP2) have been reported (Memelink et al. 2001a, b).

The maize transcription factors *LC* and *C1* have been specifically expressed in the fruits of tomato plants to increase flavonoid levels. Expression of both genes was needed and sufficient to upregulate the flavonoid pathway in the fruit flesh, a tissue that normally does not produce any flavonoids. High levels of kaempferol and, to a lesser extent, naringenin were recorded, and all flavonoids detected were present as glycosides. Anthocyanins were present in *LC/C1* tomato leaves but absent in ripe *LC/C1* fruits. Furthermore, RNA analysis of ripening fruits revealed that, except the chalcone isomerase, all of the structural genes required for the production of kaempferol-type flavonols and pelargonidin-type anthocyanins were strongly induced by the LC/C1 transcription factors (Bovy et al. 2002). In another case, several transcription factors from monocot and dicot species, i.e. the maize *C1* and *B-PERU* genes (MYC family of transcription factors) and *DELILA* gene from *Antirrhinum* as well as *MYB-PH2* from *Petunia hybrida* (MYB family of transcription factors) were overexpressed in white clover (*Trifolium repens*). The transformed plants exhibited enhanced anthocyanin accumulation in a range of tissues. In particular, one plant transformed with the *B-PERU* gene, displayed a unique pattern of anthocyanin accumulation in the leaf. The accumulation of anthocyanin in this plant was closely associated with the crescent of leaves, which is normally white. The red pigmentation declined in intensity in the oldest leaf stage. The *B-PERU* message was detected in all leaf stages of this white clover plant. Furthermore, this anthocyanin pattern was shown to be heritable (de Maijnik et al. 2000).

Regarding the TIAs biosynthetic pathway in *C. roseus*, overexpression of *ORCA3* in suspension cultures was sufficient to induce several genes controlling the biosynthesis of indole precursors resulting in enhanced expression of several biosynthetic genes (*Tdc*, *Str*, *Cpr*, *Sgd*, *D4 h*) and, consequently, in increased accumulation of TIAs, after feeding loganin. Nevertheless, the biosynthetic genes *G10 h* and *Dat* were not induced, suggesting that these genes are not controlled by *ORCA3*. Besides, two genes involved in primary metabolism leading to TIA precursor synthesis (*As* α and *Dxs*) were also induced by *ORCA3*. These results show that *ORCA3* is indeed a regulator of primary as well as secondary metabolite biosynthetic genes involved in TIAs biosynthesis (Zárate and Verpoorte 2007; Memelink et al. 2001a; van der Fits and Memelink 2000).

These data show that expression of specific transcription factors can more efficiently redirect the metabolism of plant cells by acting simultaneously and coordinately on different metabolic events. The manipulation of transcription factors have also shown that they are involved not just in secondary metabolism, but in cell differentiation as well as growth and development (Montiel et al. 2003). Furthermore, one undesired effect of the overexpression of transcription factors is that it can negatively affect plant growth and development. This may be due to the sensitivity of certain cell types to the simultaneous overexpression of genes, which not only operate on the target metabolic step but may divert to unwanted points. This may be controllable by restricting transcription factor activity to limit the potential deleterious effects. This could be achieved by an appropriate choice of the promoter, which would then impede the occurrence of undesirable effects.

In another attempt, transcription factors have been widely used for engineering stress tolerance in plants (Hussain et al. 2011) and even different floral traits (Shikata and Ohme-Takagi 2008 and references therein). The manipulation of native plant regulatory networks therefore represents a new era for genetically modified crops.

16.3.5 Further Considerations in Metabolic Engineering

So far we discussed the methods needed for transforming plant cells, and the successful experiments in this field by silencing or overexpressing structural genes encoding proteins that catalyze certain reactions in a biosynthetic pathway or overexpressing regulatory genes. However, it is also clear from the results so far, that just overexpression of one or more structural genes, not always result in the desired effect for increasing productivity. This is due to the complexity of most biosynthetic pathways (Verpoorte et al. 2000). A number of reasons can be mentioned: Stability, Viability cells, Pathway architecture, Enzyme regulation, Metabolic channels, Substrate availability, Cofactor availability, Sub-cellular, and cellular compartmentation and Transport.

16.3.5.1 Stability

In plant cell cultures a continuous selection pressure exists for fast growing cells. Overexpressing an energy-consuming secondary metabolite pathway is a negative trait in such an environment. Thus, long-term stability of transgenic cell lines might be a problem. For example, *Catharanthus roseus* cell lines overexpressing some structural genes were found to maintain increased levels of the overexpressed proteins, but the initially increased levels of alkaloids, eventually went down to much lower levels, comparable with the wild-type cells (Whitmer et al. 2003 and unpublished results).

16.3.5.2 Viability

The production of high levels of certain compounds may lead to toxic effects. Many times changes in the production of secondary metabolites in plants lead to impaired growth, or plants which are more susceptible for pests and diseases. Overexpression of the isochorismate lyase gene, for example, in tobacco plants leads to impaired growth, probably due to lack of vitamin K production in the plant (Verberne et al. 2000 and unpublished results). Other examples have been reported about reduced growth rates after genetic engineering metabolic pathways that may compete with essential primary pathways.

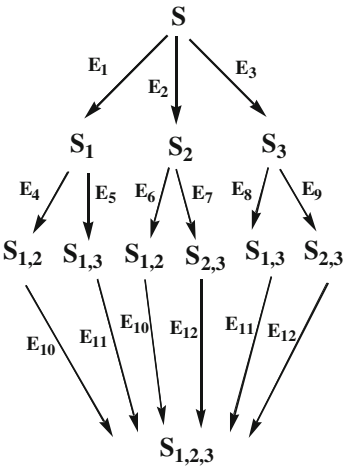

Fig. 16.1 Schematic biosynthetic network. S is a basic skeleton to which the functional groups 1, 2, and 3 are added. E_{1-12} are enzyme catalyzed steps. With high enzyme substrate specificity, 12 different enzymes are required to produce $S_{1,2,3}$, and with very low substrate specificity 3 enzyme would suffice (taken from Verpoorte and Alferman (2000) with permission from the authors and publisher)

16.3.5.3 Pathway Architecture

In a simple pathway consisting of a series of consecutive steps, it is easy to determine a possible rate limiting step and overexpress the gene concerned. However, in more complex networks, as it is most common in plants, an extensive knowledge about the specificity of every enzyme involved is needed to decide for the approach to be taken to improve the production of a certain desired compound (Verpoorte et al. 2000). Like in Fig. 16.1, the specificity of the enzymes is an important factor, is each step catalyzed by a separate enzyme, or are three enzymes with less specificity involved? For example, for making more of compound S2,3 in the latter case, only one single enzyme (E1) needs to be knockedout, whereas in case of a number of different enzymes being involved, several candidates needs to be considered (E1, E3 and E12). Moreover, competitive pathways may exist, or catabolism of the products of interest may be an important factor.

16.3.5.4 Enzyme Regulation

Regulation is not only on the level of the genes, but also on the level of the enzymes, e.g. activation of the enzyme via phosphorylation, feedback mechanisms, feed-forward mechanisms. The simple overexpression of a protein is thus not necessarily sufficient to come to an active enzyme. In case of feedback and feed-forward mechanisms, one might choose for genes encoding proteins that lack these properties, e.g. from other organisms or by protein engineering. For instance, to increase tryptophan levels in plants, overexpression of the first committed enzyme in the tryptophan biosynthesis, anthranilate synthase (ASA), requires a gene which is not sensitive for allosteric inhibition by tryptophan. However, still the effect might be limited as tryptophan induces the first enzyme in the

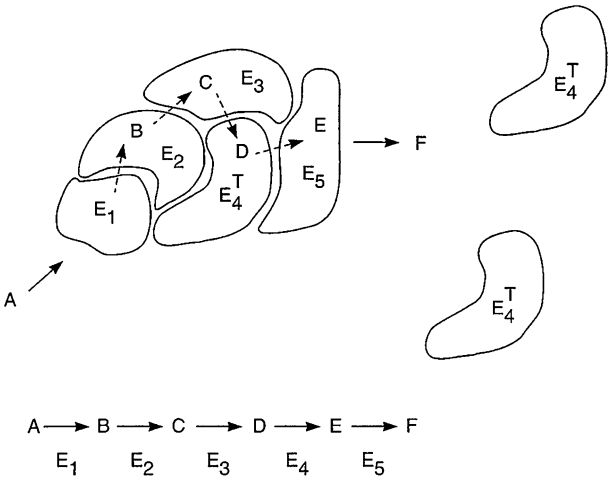

Fig. 16.2 Enzyme aggregates may play an important role in the regulation of pathways (taken from Verpoorte et al. 1999 with permission from the authors and publisher)

phenylalanine biosynthesis, also requiring chorismate, thus increasing the flux into that competitive pathway.

16.3.5.5 Metabolic Channels

Often enzymes occur in aggregates with other enzymes (metabolons, or metabolic channels). The cartoon of Fig. 16.2 illustrates this point. The aggregate problem is the same as concluding that there is more than one limiting step in a pathway, and consequently, by overcoming one, immediately the next one will be observed. Only by overexpressing all limiting enzymes involved, an increase can be achieved. Recently Choi et al. (2011) postulated that besides water and lipids a third phase is present in cells. This consists of Natural Deep Eutectic Solvents (NADES) that are made up from certain combinations of sugars, sugar alcohols, organic acids, amino acids, and choline derivatives. Such liquids have a clear structure like a liquid crystal and are excellent solvents for proteins as well as all kind of medium to low polar metabolites, up to several orders of magnitude better than in water. In fact, metabolons or metabolic channels could be such NADES in which both enzymes and substrates are freely moving, thus explaining the synthesis of complex water insoluble compounds. Such NADES could be in the ER and be attached to various membranes. The presence or absence of such a medium might also be a restricting factor in biosynthetic pathways. For studying biosynthesis the consequence is that *in-planta* enzymes might behave differently in such an environment than in water in which one is used to determine enzyme characteristics.

16.3.5.6 Substrate Availability

In the terpenoid indole alkaloid biosynthesis it has been found that the terpenoid part of the pathway is a major limiting factor. Overexpression of later steps in the pathway has only limited effect. By feeding the terpenoid precursor loganin, for example, an enormous production could be achieved in transgenic cell cultures of *C. roseus* overexpressing TDC and/or STR (Whitmer et al. 2003; Verpoorte et al. 1999; Whitmer et al. 1998; Whitmer et al. 2002a, b, c). But also substrates as nitrogen and methyl donors may be limiting in certain steps.

16.3.5.7 Cofactor Availability

Many enzymes require cofactors such as NAD(P)H, H_2O_2, ATP, also here the plant cell has the possibility to control the production of compounds on the level of the enzymes.

16.3.5.8 Subcellular and Cellular Compartmentation

All the factors mentioned above concern enzyme catalysed chemical reactions in the pathway itself, but also other regulatory mechanisms do play an important role. Intra and intercellular compartmentation are such regulatory mechanisms. The biosynthesis of terpenoid indole alkaloids in *C. roseus* is an excellent example of such compartmentation in which different steps of the pathway occur in different cellular compartments (plastids, chloroplasts, cytosol, vacuoles) and some of the later steps are even in different cells (Van der Heijden et al. 2004; St. Pierre et al. 1999).

16.3.5.9 Transport

Inter and intracellular compartmentation means that transport through cellular membranes and through membranes of organelles play a major role in controlling the flux through a pathway. This transport can be through physical phenomena (concentration or pH, driven diffusion), and/or the participation of active transporters that can translocate substrates and products of enzyme reactions to the right compartment (Yazaki 2005). Roytrakul et al. showed that in *C. roseus* vacuoles active ABC-transporters of the MDR-(efflux) and MRP-type (influx) are involved in selective bidirectional transport of various indole alkaloids through vacuolar membranes (Roytrakul and Verpoorte 2007). All alkaloids and iridoids tested showed a different accumulation in the vacuoles caused by different affinity of the various transport systems for the individual alkaloids. Accumulation in vacuoles is thus dependent on the activity of transporter proteins. This can be illustrated by the results of feeding tryptophan and secologanin to tobacco cells overexpressing two indole alkaloid biosynthetic genes (tryptophan decarboxylase and strictosidine

synthase) which resulted in the production of alkaloids, but instead of being stored in vacuoles, they were excreted from the cells into the medium, showing the importance of transport (Hallard et al. 1997). The overexpression of a specific berberine ABC-transporter gene from *Coptis japonica* (Shitan et al. 2003) in *Catharanthus roseus* (Pomahacova et al. 2009) surprisingly showed improved uptake by the transformed cells of ajmalicine, but no effect at all on berberine uptake, showing the complexity of the transport systems.

Considering all these possibilities the conclusion is that the overexpression of a single structural gene may not lead to the desired effect. To overcome all the above-mentioned problems regulatory genes have been overexpressed. Indeed, it was shown that this approach may lead to the upregulation of a series of structural genes, but not necessarily to an increased flux through the pathway.

The final conclusion is thus that to come to successful projects for the upregulation of the production of a certain desired compound, detailed information is required on all steps in the metabolic pathways concerned. Adding a single step to a pathway may result in the formation of the product of the enzyme, in fact that has so far been the most successful approach.

16.3.6 Unraveling Biosynthetic Pathways

It is well known that the synthesis of plant secondary metabolites is a complex and highly regulated process that involves the participation of multiple factors such as subcellular compartments (vacuoles, endoplasmic reticulum, cytosol, plastids, nucleus, etc.); self regulation by endogenous feed-back and/or feed-forward mechanisms; transport of intermediates and end-products within cells, tissues and even organs; storage; catabolism, and chemical modifications such as acylation and glycosylation just to mention a few. Therefore, attempting to genetic engineering these complex networks requires a broad knowledge on each of the factors and control points participating in the biosynthesis of a target metabolite. In the previous sections, the general approaches conducted for the genetic engineering of secondary metabolite pathways have been outlined, as well as relevant published results; nevertheless, further considerations and attention to important parameters cannot be overlooked if a successful genetic manipulation is desired.

Indeed, it is not always possible to foresee the effect(s) of the knockout and/or overexpression of a biosynthetic enzyme(s) on an entire pathway, although large successes have been reported when overexpression and/or downregulation of single or multiple genes have been performed. Thus, it is evident that knowing the basic network of metabolite intermediates and enzymes involved in a pathway is crucial in unravelling secondary metabolism. However, further steps are required to keep advancing at an attractive pace. Particularly, it appears interesting to study the diverse regulatory mechanisms that control gene expression and transcriptional regulators (Memelink et al. 2001b) as well as identifying and characterizing the

diverse vital transport mechanisms and proteins governing many secondary metabolite routes which have been demonstrated to be of crucial importance (Yazaki 2005). It is also essential to look at the various postbiosynthetic events and mechanisms involved, occurring in plant secondary metabolites (Zhang et al. 2002) for a more controllable genetic engineering approach.

With the fast advancement of biological research and DNA technology, the entire genome of important plants species has been already sequenced as is the case of arabidopsis, rice, maize, poplar, soybean, tomato. Other genome sequencing programs are in progress, i.e., cotton, soya, sunflower, etc. Access to genomics information permits to characterize gene expression under different conditions, thus establishing with precision gene function, a research field referred to as functional genomics. Functional genomics aims at quantitatively determining the spatial and temporal accumulation of specific mRNA, proteins, and metabolites using high-throughput technologies (Oliver et al. 2002).

Thus, new approaches are being applied to unravel plant secondary metabolism on all levels from genes to metabolites, from genome via transcriptome and proteome to the metabolome. Linking data from the different levels should enable the identification of genes involved in the production of certain metabolites. Transcriptomics is nowadays a well-developed technology, the same applies for proteomics. However, using plants from which no sequences are known, studies of the proteome may end up with a large number of unknown peptide sequences, for which it will be difficult to determine the function of the encoding genes. For example, Jacobs et al. (2005) found about 100 proteins to be connected with the production of alkaloids in *C. roseus* cell cultures, but only 60 of these gave some peptide sequences that match known proteins, and of these, just 3 had direct connection with the terpenoid indole pathway. On the other hand, 40 unknown sequences were obtained. Many known proteins from the indole alkaloid biosynthesis were not detected, which might be due to the fact that they are membrane bound, or appear at too low levels to be detected in the presence of the primary metabolism proteins. Therefore, when working with nonsequenced plants other approaches need to be applied. These include for instance metabolite profiling (i.e. metabolomics) which intends to qualitatively and quantitatively determine all metabolites in an organism and in that way have a complete view of the metabolism as a network (Trethewey 2004). Metabolomics has emerged in the past years as a very useful technology platform in different fields, such as drug discovery, clinical diagnostics, plant biochemistry, and quality control of food and botanicals. It requires highly specific state of the art analytical equipment, such as LC–MS, GC–MS, MS–MS, and NMR to trace the metabolic changes following genetic manipulation of the target plant or after physiological or environmental alterations. By using biostatistical methods, metabolomic data can be correlated with transcriptomics and proteomics data, thus allowing a holistic view on all correlations between these data sets. Thus, for example allowing to find links between secondary metabolites biosynthesis and genes.

Such an integrated approach, which should be considered when developing strategies for metabolic engineering, is now known as systems biology. Systems

biology can be defined as the study of the mechanisms implicated in complex biological processes as integrated systems of many, varied, interacting components (DNA, RNA, proteins and cells). This involves (1) collection of large sets of experimental data; (2) proposal of mathematical models that might account for at least some significant aspects of this data set, (3) accurate computer solution of the mathematical equations to obtain numerical predictions, and (4) assessment of the quality of the model by comparing numerical simulations with the experimental data. However, full understanding of all collected data and design of the mathematical models are demanding tasks, but these can prove highly informative and attractive for a better design for a successful metabolic engineering of a target biosynthetic network (Kell 2004; Oksman-Cladentey et al. 2004). The major constraint in fact is what we call the transcriptome, proteome and metabolome of an organism, in fact is the sum of these "omes" of all individual cells that means cells with totally different functions, and consequently display a specific "ome" for that function. So to understand a cell and the organism we have to deconvolute the "omes" in the four dimensions (3 of space and 1 of time) which will be a major challenge for the coming years. Measuring fluxes rather than taking the snapshot approach of the present "omics" and single cell analysis will be key technologies for unravelling plant metabolism.

16.4 Future Directions

The early strategies used to boost secondary metabolite yields in the plant cell factory included cell line selection, nutrient regime, choice of culture system and conditions, level of plant growth regulators, precursor feeding, elicitation, removal of end-product. These were followed by the application of recombinant DNA technology and other biotechnological tools, resulting in spectacular advances.

During the past few years, great progresses in the metabolic and enzyme engineering for the production of pharmaceutical products using plant systems have been witnessed. Nonetheless, our knowledge on how secondary metabolites are synthesised and which genes govern and conduct these processes is far from complete, although more biosynthetic pathways are well understood and elucidated. Furthermore, in the near future new genes will become available, increasing the possibilities for further metabolic engineering of many new biosynthetic networks. However, plant metabolic engineering currently proceeds more by trial and error than by intelligent system design, a reality that need to be addressed with the application of the new tools and approaches such as those considered in subheading 16.3.6 of this chapter.

On the other hand, for positive metabolic engineering to be realised, natural product biosynthesis must be considered as a system of many interacting parts: promoters, transcription factors, enzymes, transporters, and intracellular structures, such as vesicles and membranes; ingredients which need particular attention when attempting to manipulate biosynthetic routes. In fact, it has been presented under

subheading 16.3.4 and can now be emphasized, the powerful use of transcription factors for the regulation of biosynthetic pathways. Consequently, the quest for new transcription factors in medicinal plant species appear as an attractive goal for the subsequent genetic manipulation.

Interdisciplinary cooperation between several research fields, such as biology, chemistry, instrumentation, computer science, physics, and mathematics are also required for a more foreseeable genetic engineering outcome, thus, avoiding many unwanted effects following the current strategies for the genetic modification of a target biosynthetic network.

It is known that the current knowledge of secondary metabolite pathways is limited, and this represents the major barrier for the successful genetic manipulation of pathways. Thus, improving this level of knowledge is a challenging goal and would provide precise and exquisite information to further understand the control mechanisms and processes involved in the functioning of a pathway. Nonetheless, despite these obvious obstacles, we believe that many stimulating progresses and tailor-made designs generating appealing results on the metabolic engineering of medicinal compounds biosynthetic routes are to be expected in the near future, and inevitable progress will continue. Furthermore, the enormous potential for the genetic engineering of plant secondary metabolism has been clearly demonstrated, and the possibility to obtain plants able to accumulate new products useful as specialty chemical is an attainable objective.

The metaphor of the cell factory is in fact quite interesting, if one compares the cell with a car factory, one may easily understand that one extra piece of equipment in the total production line will not lead to a higher output. Only when the complete infrastructure is functioning and all parts are on the right time on the right place and the energy supply is functioning, such a factory will have its maximum output. The same applies for the cell, all logistics must be functioning properly to enable the cell to perform optimally. And all cells must function in a coordinated way for an optimal performance of the organism. So engineering such a factory to perform better is a real challenge!

Acknowledgments The authors would like to thank Dr. Suman Chandra for the invitation to contribute to this book. RZ and NJV acknowledge Instituto Canario de Investigación del Cáncer and Agencia Canaria de Investigación Innovación y Sociedad de la Información, Spain for financial support.

References

Abbadi A, Domergue F, Bauer J, Napier JA, Welti R, Zähringer U, Cirpus P, Heinz E (2004) Biosynthesis of very-long-chain polyunsaturated fatty acids in transgenic oilseeds: constrains on their accumulation. Plant Cell 16:2734–2748

Acereto-Escoffié POM, Chi-Manzanero BH, Echeverría-Echeverría S, Grijalva R, James Kay A, González-Estrada T, Castaño E, Rodríguez-Zapata LC (2005) *Agrobacterium*-mediated transformation of *Musa acuminata* cv. "Grand Nain" scalps by vacuum infiltration. Sci Horticult 105:359–371

Aida R, Kishimoto S, Tanaka Y, Shibata M (2000a) Modification of flower color in torenia (*Torenia fournieri* Lind.) by genetic transformation. Plant Sci 153:33–42

Aida R, Yoshida K, Kondo T, Kishimoto S, Shibata M (2000b) Copigmentation gives bluer flowers on transgenic torenia plants with the antisense dihydroflavonol-4-reductase gene. Plant Sci 160:49–56

Allen RS, Millgate AG, Chitty JA, Thisleton J, Miller JAC, Fist AJ, Gerlach WL, Larkin PJ (2004) RNAi-mediated replacement of morphine with the nonnarcotic alkaloid reticuline in opium poppy. Nat Biotechnol 22:1559–1566

Barampura S, Zhang AJ (2011) Recent advances in plant transformation. Methods Mol Biol 701:1–35

Baulcombe D (2004) RNA silencing in plants. Nature 431:356–363

Baulcombe DC (1999) Viruses and gene silencing in plants. Arch Virol Suppl 15:189–201

Beaudoin F, Michaelson LV, Hey SJ, Lewis MJ, Shewry PR, Sayanova O, Napier JA (2000) Heterologous reconstitution in yeast of the polyunsaturated fatty acid biosynthetic pathway. Proc Natl Acad Sci USA 97:6421–6426

Bechtold N, Pelletier G (1998) In planta *Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. Meth Mol Biol 82:259–266

Borevitz JO, Xia Y, Blount J, Dixon RA, Lamb C (2000) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell 12:2383–2394

Bovy A, de Vos R, Kemper M, Schijlen E, Almenar-Pertejo M, Muir S, Colllins G, Robinson S, Verhoeyen M, Hughes S, Santos-Buelga C, van Tunen A (2002) High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes *LC* and *R1*. Plant Cell 14:2509–2526

Broun P (2004) Transcription factors as a tool for metabolic engineering in plants. Curr Op Plant Biol 7:202–209

Browning LM (2003) *n*-3 polyunsaturated fatty acids, inflammation and obesity-related disease. Proc Nutr Soc 62:447–453

Bundock P, den Dulk-Ras A, Beijersbergen A, Hooykaas PJJ (1995) Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. EMBO J 14:3206–3214

Cahoon EB, Kinney AJ (2005) The production of vegetable oils with novel properties: using genomic tools to probe and manipulate plant fatty acid metabolism. Eur J Lipid Sci Technol 107:239–243

Campbell R, Ducreux LJ, Morris WL, Morris JA, Suttle JC, Ramsay G, Bryan GJ, Hedley PE, Taylor MA (2010) The metabolic and developmental roles of carotenoid cleavage dioxygenase 4 from potato. Plant Phisiol 154(2):656–664

Cequier-Sánchez E, Rodríguez C, Dorta-Guerra R, Ravelo AG, Zárate R (2011) *Echium acanthocarpum* hairy root cultures, a suitable system for polyunsaturated fatty acid studies and production. BMC Biotechnol 11:42

Chilton MD, Drummond MH, Merlo DJ, Sciaky D, Montoya AL, Gordon MP, Nester EW (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorogenesis. Cell 11:263–271

Chintapakorn Y, Hamill JD (2003) Antisense-mediated down-regulation of putrescine *N*-methyltransferase activity in transgenic *Nicotiana tabacum* L. can lead to elevated levels of anatabine at the expense of nicotine. Plant Mol Biol 53:87–105

Choi YH, van Spronsen J, Dai Y, Verberne M, Hollmann F, Arends IWCE, Witkamp GJ, Verpoorte R (2011) Are natural deep eutectic solvents the missing link in understanding cellular metabolism and physiology? Plant Physiol 156:1701–1705

Christi Y (2003) Ultrasound the power of a silent gong. Biotechnol Adv 21:1

Chung M-H, Chen M-K, Pan S-M (2000) Floral spray transformation can efficiently generate *Arabidopsis* transgenic plants. Transgen Res 9:471–476

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Cordero-Mesa M, Jiménez-Bermúdez S, Pliego-Alfaro F, Quesada MA, Mercado JA (2000) *Agrobacterium* cells as microprojectile coating: a novel approach to enhance stable transformation rates in strawberry. Aust J Plant Physiol 27:1093–1100

Curtis IS (2005) Production of transgenic crops by the floral-dip method. Meth Mol Biol 286 (Transgenic Plants): 103–109

Curtis IS, Nam HG (2001) Transgenic radish (*Raphanus sativus* L. *longipinnatus* Bailey) by floral-dip method - plant development and surfactant are important in optimizing transformation efficiency. Transgen Res 10:363–371

Davey MR, Anthony P, Power BJ, Lowe KC (2005) Plant protoplasts: status and biotechnological perspectives. Biotechnol Adv 23:131–171

Davies KM, Schwinn KE (2003) Transcriptional regulation of secondary metabolism. Funct Plant Biol 30:913–925

de Majnik J, Weinman JJ, Djordjevic MA, Rolfe BG, Tanner GJ, Joseph RG, Larkin PJ (2000) Anthocyanin regulatory gene expression in transgenic white clover can result in an altered pattern of pigmentation. Aust J Plant Physiol 27:659–667

DellaPenna D (2001) Plant metabolic engineering. Plant Physiol 125:160–163

Desfeux C, Clough SJ, Bent AF (2000) Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the *Arabidopsis* floral-dip method. Plant Physiol 123:895–904

Dessaux Y, Petit A, Tempé J (1993) Chemistry and biochemistry of opines, chemical mediators of parasitism. Phytochemistry 34:31–38

Dixon RA, Steele CL (2002) Flavonoids and isoflavonoids—a gold mine for metabolic engineering. Trends Plant Sci 4:394–400

Dixon RA, Strack D (2003) Phytochemistry meets genome analysis, and beyond. Phytochemistry 62:815–816

Dooner HK, Robbins TP, Jorgensen RA (1991) Genetic and developmental control of anthocyanin biosynthesis. Annu Rev Genet 25:173–199

Ducreux LJM, Morris WL, Hedley PE, Shepherd T, Davies HV, Millam S, Taylor MS (2005) Metabolic engineering of high carotenoid potato tubers containing enhanced levels of β-carotene and lutein. J Exp Bot 56:81–89

Enfissi EMA, Fraser PD, Lois LM, Boronat A, Schuch W, Bramley PM (2005) Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. Plant Biotechnol J 3:17–27

Fukusaki E, Kawasaki K, Kajiyama S, An C-I, Suzuki K, Tanaka Y, Kobayashi A (2004) Flower color modulations of *Torenia hybrida* by downregulation of chalcone synthase genes with RNA interference. J Biotechnol 111:229–240

Goodrich J, Carpenter R, Coen ES (1992) A common gene regulates pigmentation pattern in diverse plant species. Cell 68:955–964

Gronenborn B, Gardner RC, Schaefer S, Shepherd RJ (1981) Propagation of foreign DNA in plants using cauliflower mosaic virus as vector. Nature 294:773–776

Grotewold E (2008) Transcription factors for predictive plant metabolic engineering: are we there yet? Curr Opin Biotechnol 10(2):138–144

Hadi MZ, McMullen MD, Finner JJ (1996) Transformation of 12 different plasmids into soybean via particle bombardment. Plant Cell Rep 15:500–505

Hain R, Bieseler B, Kindl H, Schroeder G, Stocker R (1990) Expression of a stilbene synthase gene in *Nicotiana tabacum* results in synthesis of the phytoalexin resveratrol. Plant Mol Biol 15:325–335

Hallard DAC, van der Heijden R, Verpoorte R, Lopez-Cardoso I, Pasquali G, Memelink J, Hoge JHC (1997) Suspension cultured transgenic cells of *Nicotiana tabacum* expressing tryptophan decarboxylase and strictosidine synthase cDNAs from *Catharanthus roseus* produce strictosidine upon feeding of secologanin. Plant Cell Rep 17:50–54

Hallard DAC, Palacios N, van der Heijden R, Memelink J, Verpoorte R (2000) Production of ajmalicine in transgenic *Weigela styriaca* tissue cultures expressing *tdc* and *str* cDNAs from

Catharanthus roseus. Chapter 6. Transgenic plant cells for the production of indole alkaloids, Hallard DAC, PhD thesis, Leiden, University, pp 69–85

Hansen G, Wright MS (1999) Recent advances in the transformation of plants. Trends Plant Sci 4:226–231

Hiratsu K, Matsui K, Koyama T, Ohme-Takagi M (2003) Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in *Arabidopsis*. Plant J 34:733–739

Hooykaas PPJ (2000) *Agrobacterium*, a natural metabolic engineer of plants. In: Verpoorte R, Alfermann AW (eds) Metabolic Engineering of Plant Secondary Metabolism. Kluwer Academic Publishers, The Netherlands

Horrobin DF (1999) Lipid metabolism, human evolution and schizophrenia. Prostaglandins Leukot Essent Fatty Acids 60:431–437

Hussain SS, Kayani MA, Amjad M (2011) Transcription factors as tools to engineer enhanced drought stress tolerance in plants. Biotechnology Prog 27(2):297–306

Ingelbrecht I, Breyne P, Vancompernetlle K, Jacobs A, van Montagu M, Depicker A (1991) Transcriptional interference in transgenic plants. Gene 109:239–242

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High frequency transformation of maize (*Zea mays*) mediated by *Agrobacterium tumefaciens*. Nat Biotechnol 14:745–750

Iwase A, Matsui K, Ohme-Takagi M (2009) Manipulation of plant metabolic pathways by transcription factors. Plant Biotechnol 26:29–38

Jacobs DI, Gaspari M, van der Greef J, van der Heijden R, Verpoorte R (2005) Proteome analysis of the medicinal plant *Catharanthus roseus*. Planta 221:690–704

Jadhav A, Katavic V, Marillia EF, Giblin EM, Barton DL, Kumar A, Sonntag C, Babic V, Keller WA, Taylor DC (2005) Increased levels of erucic acid in *Brassica carinata* by co-suppression and antisense repression of the endogenous *FAD2* gene. Metab Eng 7:215–220

Jaworski J, Cahoon EB (2003) Industrial oils from transgenic plants. Curr Opin Plant Biol 6: 178–184

Joersbo M, Brunstedt J (1990) Direct gene transfer to plant protoplasts by mild sonication. Plant Cell Rep 9:207–210

Kell DB (2004) Metabolomics and systems biology: making sense of the soup. Curr Opin Microbiol 7:296–307

Klein TM, Wolf ED, Wu R, Sanford JC (1987) High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327:70–73

Kristensen C, Morant M, Olsen CE, Ekstrom CT, Galbraith DW, Moller BL, Bak S (2005) Metabolic engineering of dhurrin in transgenic Arabidopsis plants with marginal inadvertent effects on the metabolome and transcriptome. Proc Natl Acad Sci USA 102:1779–1784

Kunik T, Tzfira T, Kapulnik Y, Gafni Y, Dingwall C, Citovsky V (2001) Genetic transformation of HeLa cells by *Agrobacterium*. Proc Natl Acad Sci USA 98:1871–1876

Labra M, Vannini C, Grassi F, Bracale M, Balsemin M, Basso B, Sala F (2004) Genomic stability in *Arabidopsis thaliana* transgenic plants obtained by floral dip. Theor Appl Genet 109:1512–1518

Leech MJ, Burtin D, Hallard D, Hillou F, Kemp B, Palacios N, Rocha P, O'Callaghan D, Verpoorte R, Christou P (2000) Particle gun methodology as a tool in metabolic engineering. In: Verpoorte R, Alfermann AW (eds) Metabolic Engineering of Plant Secondary Metabolism. Kluwer Academic Publishers, The Netherlands

Lessard PA, Kulaveerasingam H, York GM, Strong A, Sinskey AJ (2002) Manipulating gene expression for the metabolic engineering of plants. Metab Eng 4:67–79

Lewinsohn E, Schalechet F, Wilkinson J, Matsui K, Tadmor Y, Nam K-H, Amar O, Lastochkin E, Larkov O, Ravid U, Hiatt W, Gepstein S, Pichersky E (2001) Enhanced levels of the aroma and flavor compound *S*-linalool by metabolic engineering of the terpenoid pathway in tomato fruits. Plant Physiol 127:1256–1265

Li J-F, Park E, von Arnim AG, Nebenführ A (2009) The FAST technique: a simplified *Agrobacterium*-based transformation method for transient gene expression analysis in seedlings of *Arabidopsis* and other plant species. Plant Methods 5(6):1–15

Liu Q, Singh S, Green A (2002) High-oleic and high-stearic cottonseed oils: nutritionally improved cooking oils developed using gene silencing. J Am Coll Nutr 21:205S–211S

Liu Y, Yang H, Sakanishi A (2005) Ultrasound: mechanical gene transfer into plant cells by sonoporation. Biotechnol Adv 24(1):1–16

Lorence A, Verpoorte R (2004) Gene transfer and expression in plants. In: Balbás P, Lorence A (eds) Methods in Molecular Biology, Recombinant Gene Expression. Reviews and Protocols. Humana Press Inc, Totowa

McGinnis KM (2010) RNAi for functional genomics in plants. Brief Funct Genomics 9(2):111–117

Melchers LS, Regensburg-Tuink AJG, Schilperoort RA, Hooykaas PJJ (1989) Specificity of signal molecules on the activation of *Agrobacterium* virulence gene expression. Mol Microbiol 3:969–977

Memelink J, Kijne JW, van der Heijden R, Verpoorte R (2001a) Genetic modification of plant secondary metabolite pathways using transcriptional regulators. Adv Biochem Engineering/ Biotechnol 72:103–125

Memelink J, Verpoorte R, Kijne JW (2001b) ORCAnisation of jasmonate-responsive gene expression in alkaloid metabolism. Trends Plant Sci 6:212–219

Michielse CB, Hooykaas PJ, Van der Hondel CA, Ram AF (2005) *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. Curr Genetics 48:1–17

Mohanpuria P, Kumar V, Ahuja PS, Yadav SK (2010) *Agrobacterium*-Mediated Silencing of Caffeine Synthesis through Root Transformation in *Camellia sinensis* L. Mol Biotechnol doi:10.1007/s12033-010-9364-4

Moisyadi S, Neupane KR, Stiles JI (1998) Cloning and characterization of a cDNA encoding xanthosine-N7-methyltransferase from coffee (*Coffea arabica*). Acta Horticult 461:367–377

Montiel G, Breton C, Doireau P, Jay-Allemand C, Gantet P (2003) Transcription factors that regulate secondary metabolism biosynthesis pathways: key actors for plant eco-physiology and ontogeny. Rec Res Devel Plant Cell Physiol 1:83–98

Muir SR, Collins GJ, Robinson S, Hughes S, Bovy A, de Vos CHR, van Tunen AJ, Verhoeyen ME (2001) Overexpression of petunia chalcone isomerase in tomato results in fruits containing increased levels of flavonols. Nat Biotechnol 19:470–474

Napier JA, Sayanova O, Qi B, Lazarus CM (2004) Progress toward the production of long-chain polyunsaturated fatty acids in transgenic plants. Lipids 39:1067–1075

Naqvi S, Farré G, Sanahuja G, Capell T, Zhu C, Christou P (2010) When more is better: multigene engineering in plants. Trends Plant Sci 15(1):48–56

Nesi N, Debeaujon I, Jond C, Pelletier G, Caboche M, Lepiniec L (2000) The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of DFR and BAN genes in *Arabidopsis thaliana*. Plant Cell 12:1863–1878

Nesi N, Jond C, Debeaujon I, Caboche M, Lepiniec L (2001) The *Arabidopsis TT2* gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. Plant Cell 13:2099–2114

O'Brien J, Lummis SCR (2004) Biolistic and diolistic transfection: using the gene gun to deliver DNA and lipophilic dyes into mammalian cells. Methods 33:121–125

Ogita S, Uefuji H, Morimoto M, Sano H (2004) Application of RNAi to confirm theobromine as the major intermediate for caffeine biosynthesis in coffee plants with potential for construction of decaffeinated varieties. Plant Mol Biol 54:931–941

Ogita S, Uefuji H, Yamaguchi Y, Koizumi N, Sano H (2003) RNA interference: producing decaffeinated coffee plants. Nature 423:823

Oksman-Caldentey K-M, Barz WH (2002) Plant Biotechnology and Transgenic Plants. In: Oksman-Caldentey K-M, Barz WH (ed) C.H.I.P.S. Texas, USA

Oksman-Caldentey K-M, Inzé D, Oresic M (2004) Connecting genes to metabolites by a systems biology approach. Proc Natl Acad Sci USA 101:9949–9950

Oliver DJ, Nikolau B, Syrkin-Wurtele E (2002) Functional genomics: high throughput mRNA, protein and metabolite analyses. Metab Eng 4:98–106

Olmedo-Monfil V, Cortes-Penagos C, Herrera-Estrella A (2004) Three decades of fungal transformation: key concepts and applications. Meth Mol Biol 267:297–313

Page JE (2005) Silencing nature′s narcotics: metabolic engineering of the opium poppy. Trends Biotechnol 23:331–333

Pomahacová B, Dusek J, Dusková J, Yazaki K, Roytrakul S, Verpoorte R (2009) Improved accumulation of ajmalicine and tetrahydroalstonine in *Catharanthus* cells expressing an ABC transporter. J Plant Physiol 166:1405–1412

Porta C, Lomonossoff GP (2002) Viruses as vectors for the expression of foreign sequences in plants. Biotechnol Gen Eng Rev 19:245–291

Potrykus I (1991) Gene transfer to plants: assessment of published approaches and results. Annu Rev Plant Physiol Plant Mol Biol 42:205–225

Qi B, Fraser T, Mugford S, Dobson G, Sayanova O, Butler J, Napier JA, Stobart AK, Lazarus CM (2004) The production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in transgenic plants. Nat Biotechnol 22:739–745

Quattrocchio F, Wing J, van der Woude K, Mol JN, Koes R (1998) Analysis of bHLH and MYB domain proteins: species-specific regulatory differences are caused by divergent evolution of target anthocyanin genes. Plant J 13:475–488

Quattrocchio F, Wing J, van der Woude K, Souer E, de Vetten N, Mol JN, Koes R (1999) Molecular analysis of the anthocyanin2 gene of petunia and its role in the evolution of flower color. Plant Cell 11:1433–1444

Rodríguez-Talou J, Verbene MC, Budi Muljono RA, van Tegelen LJP, Gonsalvez-Bernal B, Linthorst HJM, Wullems GJ, Bol JF, Verpoorte R (2001) Isochorismate synthase transgenic expression in *Catharanthus roseus* cell suspensions. Plant Physiol Biochem 39:595–602

Roytrakul S, Verpoorte R (2007) Role of vacuolar transporter proteins in plant secondary metabolism: Catharanthus roseus cell culture. Phytochemistry Rev 6:383–396

Ryu C-M, Anand A, Kang L, Mysore KS (2004) Agrodrench: a novel and effective agroinoculation method for virus-induced gene silencing in roots and diverse Solanaceous species. Plant J 40:322–331

Sanford JC (1988) The biolistic process. Trends Biotechnol 6:299–302

Sanford JC, Klein TM, Wolf ED, Allen N (1987) Delivery of substances into cells and tissue using a particle bombardment process. Trends Biotechnol 6:299–302

Sauret-Güeto S, Botella-Pavia P, Rodríguez-Concepción M (2003) Molecular tools for the metabolic engineering of carotenoids biosynthesis in plants. Recent Res Devel Plant Mol Biol 1:339–363

Sayanova OV, Napier JA (2004) Eicosapentaenoic acid: biosynthetic routes and the potential for synthesis in transgenic plants. Phytochemistry 65:147–158

Sayanova OV, Smith MA, Lapinskas P, Stobart AK, Dobson G, Christie WW, Shewry PR, Napier JA (1997) Expression of a borage cDNA containing an N-terminal cytochrome b_5 domain results in the accumulation of high levels of Δ6-desaturated fatty acids in transgenic tobacco. Proc Natl Acad Sci USA 94:4211–4216

Senthil-Kumar M, Mysore KS (2011) Virus-induced gene silencing can persist for more than 2 years and also be transmitted to progeny seedlings in *Nicotiana benthamiana* and tomato. Plant Biotechnol J 9:797–806

Sharp PA (2001) RNA interference. Genes Dev 15:485–490

Shikata M, Ohme-Takagi M (2008) The utility of transcription factors for manipulation of floral traits. Plant Biotechnol 25:31–36

Shitan N, Bazin I, Dan K, Obata K, Kigawa K, Ueda K, Sato F, Forestier C, Yazaki K (2003) Involvement of CjMDR1, a plant multidrug-resistance-type ATP-binding cassette protein, in alkaloid transport in *Coptis japonica*. Proc Natl Acad Sci USA 100:751–756

Shrawat AK, Good AG (2011) *Agrobacterium* tumefaciens-mediated genetic transformation of cereals using immature embryos. Methods Mol Biol 710:355–372

Simonopoulus AP (1991) Omega-3 fatty acids in health and disease and in growth and development. Am J Clin Nutr 54:438–463

Simonsen S, van't Veer P, Strain JJ (1998) Adipose tissue omega-3 and omega-6 fatty acid content and breast cancer in the EURAMIC study. Am J Epidemiol 147:342–352

Sinha A, Wetten AC, Caligari PDS (2003) Effect of biotic factors on the isolation of *Lupinus albus* protoplasts. Aust J Bot 51:103–109

Sinkar VP, White FF, Gordon MP (1987) Molecular biology of Ri-plasmid. A rev J Biosci 11:47–57

Smith FD, Harpending PR, Sanford JC (1992) Biolistic transformation of prokaryotes: factors that affect biolistic transformation of very small cells. J Gen Microbiol 138:239–248

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

Tang W (2003) Additional virulence genes and sonication enhance *Agrobacterium tumefaciens*-mediated loblolly pine transformation. Plant Cell Rep 21:555–562

Tattersall DB, Bak S, Jones PR, Olsen CE, Nielsen JK, Hansen ML, Hoj PB, Moller BL (2001) Resistance to an herbivore through engineered cyanogenic glucoside synthesis. Science 293:1826–1828

Tepfter D (1990) Genetic transformation using *Agrobacterium rhizogenes*. Physiol Plant 79: 140–146

Thelen JJ, Ohlorgge JB (2002) Metabolic engineering of fatty acid biosynthesis in plants. Metab Eng 4:12–21

Trautwein EA (2001) n-3 Fatty acids–physiological and technical aspects for their use in food. Eur J Lipid Sci Technol 103:45–55

Trethewey RN (2004) Metabolite profiling as an aid to metabolic engineering in plants. Curr Opin Plant Biol 7:196–201

Trick HR, Finner JJ (1997) SAAT: sonication-assisted *Agrobacterium*-mediated transformation. Transgen Res 6:329–336

Ueki S, Lacroix B, Krichevsky A, Lazarowitz SG, Citovsky V (2009) Functional transient genetic transformation of Arabidopsis leaves by biolistic bombardment. Nat Protoc 4(1):71–77

Ursin VA (2003) Modification of plant lipids for human health: development of functional land-based omega-3 fatty acids. Symposium: improving human nutrition through genomics, proteomics and biotechnologies. American Society Nutrition science, pp 4271–4276

Van der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289:295–297

Van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R (2004) The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Curr Med Chem 11:1241–1253

Van der Krol AR, Lenting PE, Veenstra J, Van der Meer IM, Koes RE, Gerats AGM, Mol JNM, Stuitje AR (1988) An anti-sense chalcone synthase gene in transgenic plants inhibits flower pigmentation. Nature 333:866–869

Verberne M, Verpoorte R, Bol J, Mercado-Blanco J, Linthorst HJM (2000) Overproduction of salicylic acid in plants by bacterial transgenes enhances pathogen resistance. Nat Biotechnol 18:779–783

Verpoorte R, Alfermann AW (2000) Metabolic engineering of plant secondary metabolism. In: Verpoorte R, Alfermann AW (ed) Kluwer Academic Publishers, Dordrecht, The Netherlands

Verpoorte R, van der Heijden R, Memelink J (2000) Engineering the plant cell factory for secondary metabolite production. Transgen Res 9:323–343

Verpoorte R, van der Heijden R, Ten Hoopen HJG, Memelink J (1999) Metabolic engineering of plant secondary metabolite pathways from the production of fine chemicals. Biotechnol Lett 21:467–479

Vom Endt D, Kijne JW, Memelink J (2002) Transcription factors controlling plant secondary metabolism: what regulates the regulators? Phytochemistry 61:107–114

Wang TT, Choi YJ, Lee BH (2001) Transformation systems of non-*Saccharomyces* yeasts. Crit Rev Biotechnol 21:177–218

Waterhouse PM, Wang M, Finnegan EJ (2001a) Role of short RNAs in gene silencing. Trends Plant Sci 6:297–301

Waterhouse PM, Wang M, Lough T (2001b) Gene silencing as an adaptive defence against viruses. Nature 411:834–842

Weber S, Friedt W, Landes N, Molinier J, Himber C, Rousselin P, Hahne G, Horn R (2003) Improved *Agrobacterium*-mediated transformation of sunflower (*Helianthus annuus* L.): assessment of macerating enzymes and sonication. Plant Cell Rep 21:475–482

Whitmer S, Canel C, Hallard D, Goncalves C, Verpoorte R (1998) Influence of precursor availability on alkaloid accumulation by transgenic cell lines of *Catharanthus roseus*. Plant Physiol 116:853–857

Whitmer S, Canel C, van der Heijden R, Verpoorte R (2003) Long-term instability of alkaloid production by stably transformed cell lines of *Catharanthus roseus*. Plant Cell Tiss Org Cult 74:73–80

Whitmer S, van der Heijden R, Verpoorte R (2002a) Effect of precursor feeding on alkaloid accumulation by a tryptophan decarboxylase overexpressing transgenic cell line T22 of *Catharanthus roseus*. J Biotechnol 96:193–203

Whitmer S, van der Heijden R, Verpoorte R (2002b) Effect of precursor feeding on alkaloid accumulation by a strictosidine synthase over-expressing transgenic cell line S1 of *Catharanthus roseus*. Plant Cell Tiss Org Cult 69:85–93

Whitmer S, van der Heijden R, Verpoorte R (2002c) Genetic engineering of the plant cell factory for secondary metabolite production. Indole Alkaloid production in *Catharanthus roseus* as a model. In: Oksman-Caldentey K-M, Barz WH (ed) Plant biotechnology and transgenic plants. Scitech publishing, New York

Wilson TMA, Davies JW (1992) Genetic engineering with plant viruses. CRC Press, Boca Raton, Florida

Yazaki K (2005) Transporters of secondary metabolites. Curr Opin Plant Biol 8:301–307

Ye GN, Stone D, Pang SZ, Creely W, Gonzalez K, Hinchee M (1999) *Arabidopsis* ovule is the target for *Agrobacterium in planta* vacuum infiltration transformation. Plant J 19:249–257

Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid free) rice endosperm. Science 287:303–305

Yun DJ, Hashimoto T, Yamada Y (1992) Metabolic engineering of medicinal plants: transgenic *Atropa belladonna* with an improved alkaloid composition. Proc Natl Acad Sci USA 89:11799–11803

Zambre M, Terryn N, de Clercq J, de Buck S, Dillen W, van Montagu M, van der Straeten D, Angenon G (2003) Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells. Planta 216:580–586

Zárate R (2010) Plant secondary metabolism engineering: methods, strategies, advances, and omics. In. Mander L, Lui H-W (ed) Comprehensive natural products II chemistry and biology, vol. 3. Elsevier, Oxford, pp 629–668

Zárate R, Dirks C, van der Heijden R, Verpoorte R (2001) Terpenoid indole alkaloid profile changes in *Catharanthus pusillus* during development. Plant Sci 160:971–977

Zárate R, Verpoorte R (2007) Strategies for the genetic modification of the medicinal plant *Catharanthus roseus* (L.) G. Phytochem Rev 6(2–3):475–491

Zárate R, Yeoman MM (2001) Application of recombinant DNA technology to studies on plant secondary metabolism. In: Bender L, Kumar A (eds) From Soil to Cell—A Broach Approach to Plant Life. Giessen Electronic Library, Germany

Zhang W, Curtin C, Franco C (2002) Towards manipulation of post-biosynthetic events in secondary metabolism of plant cell cultures. Enzyme Microbiol Technol 30:688–696

Zhou X, Wei Y, Zhu H, Wang Z, Lin J, Liu L, Tang K (2008) Protoplast formation, regeneration and transformation from the taxol-producing fungus *Ozonium* sp. African J Biotechnol 7(12):2017–2024