Chapter 2
The Use of Plant Cell Biotechnology for the Production of Phytochemicals

Ara Kirakosyan, Leland J. Cseke, and Peter B. Kaufman

Abstract In this chapter, we bring together up-to-date information concerning plant cell biotechnology and its applications. Because plants contain many valuable secondary metabolites that are useful as drug sources (pharmaceuticals), natural fungicides and insecticides (agrochemicals), natural food flavorings and coloring agents (nutrition), and natural fragrances and oils (cosmetics), the production of these phytochemicals through plant cell factories is an alternative and concurrent approach to chemical synthesis. It also provides an alternative to extraction of these metabolites from overcollected plant species. While plant cell cultures provide a viable system for the production of these compounds in laboratories, its application in industry is still limited due to frequently low yields of the metabolites of interest or the feasibility of the bioprocess. A number of factors may contribute to the efficiency of plant cells to produce desired compounds. Genetic stability of cell lines, optimization of culture condition, tissue-diverse vs. tissue-specific site-specific localization and biosynthesis of metabolites, organelle targeting, and inducible vs. constitutive expression of specific genes should all be taken into consideration when designing a plant-based production system. The major aims for engineering secondary metabolism in plant cells are to increase the content of desired secondary compounds, to lower the levels of undesirable compounds, and to introduce novel compound production into specific plants. Recent achievements have also been made in altering various metabolic pathways by use of specific genes encoding biosynthetic enzymes or genes that encode regulatory proteins. Gene and metabolic engineering approaches are now being used to successfully achieve highest possible levels of value-added natural products in plant cell cultures. Applications through functional genomics and systems biology make plant cell biotechnology much more straightforward and more attractive than through previous, more traditional approaches.

A. Kirakosyan (✉)
University of Michigan, Ann Arbor, MI 48109-0646, USA
e-mail: akirakos@umich.edu

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2.1 Plant Cell Factories as a Source of High-Value Metabolites

The presence of valuable metabolites in plants has stimulated interest on the part of industry in the fields of pharmaceuticals (as drug sources), agrochemicals (for the supply of natural fungicides and insecticides), nutrition (for the acquisition of natural substances used for flavoring and coloring foods), and cosmetics (natural fragrances and oils). The bulk of the market products, such as secondary metabolites from higher plants, are collected from plants growing in the wild or from field-cultivated sources. In using a plant strategy, major issues are that these plants need a seasonal period of growth before harvesting is possible. Other issues here include a relatively short growing seasons in temperate regions, disease and insect predation, and high costs for labor and machinery. On the other hand, total chemical synthesis of several compounds is possible, but economically not feasible. Therefore, an alternative, economically viable, and environmentally sustainable production source for desired secondary metabolites is of great interest. In this regard, plant cell cultures can be an attractive alternative as a production system, as well as a model system, to study the regulation of natural product biosynthesis in plants so as to ultimately increase yields.

The commercial-scale use of plant cell cultures is now progressing rapidly despite many drawbacks and limitations that scientists have acknowledged. Earlier, Verpoorte et al. (1994) had shown that biotechnological application of plant cell cultures on a large scale may become economically feasible. The limitation here, however, concerns the high price of the final product. This is mainly attributed to the slow growth of plant cell cultures, making the depreciation costs of the bioreactor a major cost-determining factor in future attempts (Verpoorte et al., 1994).

The detailed monitoring of functional status of cells is now routinely performed for plant cell cultures in order to permit accurate assessment of growth and metabolite production rates. The availability of plant cells for quantitative measurement parameters makes possible the accurate assessment of a culture's status and places the analysis of cell cultures on a par with the detailed monitoring that has been successfully applied for commercial microbial fermentations. The collected information may enable identification and clearer understanding of the biological and chemical constraints within the process, as well as optimization of cell culture production, planning, costs, and scheduling activities. All of these factors are now considered in relation to scale, geometry, and configuration of the bioreactor. In addition, in vitro plant cell cultures are currently carried out for a diverse range of bioreactor designs, ranging from batch, airlift, and stirred tank to perfusion and continuous flow systems. For a small scale of operation, both the conventional and the novel bioreactor designs are relatively easy to operate. In contrast, for a larger scale of operation, problems of maintaining bioreactor sterility and providing an adequate oxygen supply to the cells have yet to be resolved (Vogel and Tadaro, 1997).

While industrial applications of plant cell cultures are still in progress, recently, some promising advances have already been achieved for the production of several high-value secondary metabolites through cell cultivation in bioreactors. For example the valuable progress has been achieved for paclitaxel (Taxol), where yields have

improved more than 100-fold using multifactorial screening strategy (Roberts and Shuler, 1997). Such progress, however, is not universal and many trials with different cell cultures initially failed to produce high levels of the desired products. The failure to produce high levels of desired metabolites by cell factories is still due to our insufficient knowledge as to how plants regulate metabolite biosynthesis.

Earlier, Zenk and coworkers (1997) suggested a strategy to improve the production of secondary metabolites in cell cultures that is now being used by many researchers. This strategy includes the following general steps: (1) plant screening for secondary metabolite accumulation; (2) use of high producer plants for initiation of callus cultures; (3) biochemical analysis of derived cultures for their variability and productivity; (4) establishment of cell suspension cultures; (5) analysis of metabolite levels in cell suspension cultures; (6) selection of cell lines based on single cells; (7) analysis of culture stability; and (8) further improvement of product yields.

How does this strategy work and does it raise the bars of current modern plant biotechnology? Here, we will trace in detail the main points of such a strategy in order to show how these steps may work and what limitations may still occur when they are employed in modern plant biotechnology. As a part of such strategy, the primary effort has been devoted to the development of cultures from elite germplasm so as to take advantage of the wide range of biosynthetic capacities within cultures. This has been achieved either by selection or by screening germplasm for highly productive cell lines, as for example, in production of Taxol from *Taxus* cell cultures (Kim et al., 2005). On the other hand, several limiting factors can play crucial role for successful use of plant cell cultures in biotechnology. These limiting factors can include light intensity and quality; temperature; length of culture period, including kinetics of production; concentration and source of major limiting nutrients such as phosphate, carbon, and nitrogen; and concentration and source of micronutrients, vitamins, and plant growth regulators.

The other point concerns optimization of cell culture conditions. This has been carried out for a variety of media formulations and environmental conditions. The *Plackett and Burman* technique was particularly useful in these cases. It allows for testing of multiple variables within a single experiment (Plackett and Burman, 1946). This method relies on the following characteristics: (1) each variable is tested at a high level in half of the test cultures, or at a low level or not at all in the other half; (2) any two variables are tested in 25% of the test cultures; (3) both will be excluded in 25%; and (4) only one variable is tested in the remaining 50% of the test cultures. Since the production of secondary metabolites can be followed by HPLC or GC, a medium can be selected that supports good cell growth and production of secondary metabolites. The role of the cell cycle in plant secondary metabolite production must also be considered.

Screening of cell cultures for metabolite high productivity is carried out on several levels. In some cases, high-producing cell clones are obtained from single cells, and subsequently, these are used for screening high-producing strains. For rapid selection of high-producing cells, some simple techniques are applicable. A good example is flow cytometry, which may be useful. This technique is based on the fact

that cells contain fluorescent products (e.g., thiophenes), and therefore, it is possible to separate these (marked) cells from others. However, some problems may occur with cell line stability, especially as a result of cell differentiation or morphogenesis. Therefore, such stability problems of cell lines have probably made researchers reluctant to develop extensive screening programs, leaving this as the last step prior to an industrial application (Verpoorte, 1996). The fluorescent proteins from a wide variety of marine organisms have initiated a revolution in the study of cell behavior by providing convenient markers for gene expression and protein targeting in living cells and organisms. The most widely used of these fluorescent proteins, the *green fluorescent protein* (*GFP*), first isolated from the jellyfish *Aequorea victoria*, can be attached to virtually any protein of interest and still fold into a fluorescent molecule. Fluorescent proteins are increasingly being employed as noninvasive probes in living cells due to their ability to be genetically fused to proteins of interest for investigations of localization, transport, and dynamics. Martin Chalfie, Osamu Shimomura, and Roger Y. Tsien share the 2008 Nobel Prize in Chemistry for their discovery and development of molecular probe uses of the green fluorescent protein. To date, many plant cells, along with other organisms, have been selected using GFP as a marker for gene expression.

Alternatively, selection of high-producing cell lines by culturing cells on media containing certain additives, such as biosynthetic precursors or toxic analogues, also may be applied (Verpoorte, 1996). In this case, some instability of many precursors or toxic effects of some constituents on the cells is, however, possible. Therefore, it is not possible to use a universal screening platform for plant cell cultures. Instead, a specific screening for a particular plant cell culture must be employed in order to produce specifically desired metabolites.

Whether with plant cell cultures or with intact plants, the key to success in discovering naturally occurring phytochemicals rests on bioassay-guided fractionation and purification procedures. Generally, screening of both natural products and synthetic organic compounds has led to impressive advances in the identification of active agents. High-throughput screens and sensitive instrumentation for structural elucidation have greatly reduced the amount of time and the sample quantity that are required for analysis.

Still, the main criterion for future biotechnological success is connected to the biosynthetic capacity of cell factories. It is well known that the biosynthesis of plant secondary metabolites could be up- or downregulated by biotic and abiotic factors. In order to unravel the regulation of plant metabolism by such environmental stimuli, it is important to elucidate the factors that control the accumulation of secondary metabolites in plants. Therefore, nowadays, scientists are carrying out intensive research efforts to identify and apply limiting factors that can ultimately increase plant cells' biosynthetic capacities. With such research, attention has also been given to the accumulation and storage of desired secondary metabolites in plant cells. Secondary metabolites in plants, and perhaps in tissue cultures, are usually stored intracellularly, as for example, in vacuoles or multicellular cavities. Thus, transporters probably play an important role in the sequestration of secondary metabolites (Kunze et al., 2002).

Biotic factors are among the environmental factors that affect to a large extent the production of phytochemicals. Therefore, it is highly probable that there is a relationship with defensive responses that is manifested either in phytoalexin production or in the production of compounds produced along one of the signal transduction pathways. An approach to characterize the biotic parameters that may elicit the plant's defensive mechanisms may be revealed by an analysis of the expression of certain genes involved in the process and by correlation of gene induction with particular metabolite levels.

In addition to the strategy described above, new approaches based on genetic and metabolic engineering have been successfully introduced (Verpoorte and Alfermann, 2000). Consequently, the development of an information base on genetic, cellular, and molecular levels is now a prerequisite for the use of plants or plant cell cultures for biotechnological applications for the following reasons. First, a better understanding of the basic metabolic processes involved could provide key information needed to produce high-value metabolites. Second, many biosynthetic pathways in plants are extensive and complicated, requiring multiple enzymatic steps to produce the desired end-product. So, when engineering secondary metabolism in plant cells, the primary aim should be to increase the content of desired secondary compounds, to lower the levels of undesirable compounds, and finally to introduce novel compound production into specific plants. This kind of research must, therefore, focus on metabolic regulation by first establishing the pathways at the level of intermediates and enzymes that catalyze secondary metabolite formation (metabolic pathways profiling). The subsequent step is the selection of targets for further studies at the level of genes, enzymes, and compartments. Such studies on regulation of metabolite biosynthesis might eventually lead to the derivation of transgenic plants or plant cell cultures with an improved productivity of the desired compounds. Aside from practical applications with such organisms, the knowledge gained will be of interest in connection with studies on the adaptive/functional roles of secondary metabolism in plants. These are covered in the next section that deals with functional genomics.

2.2 Applications Through the Use of Functional Genomics

Interdisciplinary approaches that are based on molecular biology and biochemistry led to rapid advances in the identification of biosynthetic genes, the elucidation of specific biosynthetic enzymes, and the identification of end-products. The complete genetic makeup of an organism has been generated in the plant sciences as well. Because of the success of large-scale quantitative biology projects such as genome sequencing (*genomics*), the suffix "omics" has been extended to other directions. *Proteomics* is now well-established as a term that refers to a study of the proteome. More recently, *metabolomics* has been introduced, which is now leading to an incredible amount of research on all kinds of primary and secondary metabolites (Cseke et al., 2006). Thus, quantitative and qualitative measurements of all kinds of cellular metabolites, or metabolomics, yield a global view of the biochemical

phenotype or phytochemical database for a plant organism. This can be used to differentiate phenotypes and genotypes at a metabolite level that may or may not produce visible phenotypes. Due to the diversity of plant secondary metabolites, it is generally accepted that there is no single analytical method employed that can provide sufficient visualization of the entire metabolome. Multiple technologies are therefore needed to measure the entire metabolome of a given plant sample. Most metabolomic approaches seek to profile metabolites in a nontargeted way, i.e., to reliably separate and detect as many metabolites as possible in a single analysis. This is technically challenging due to the diverse chemical properties and large differences in the abundance of the metabolites. In contrast, selective profiling of a certain group of compounds, which is also called *targeted metabolic profiling*, is relatively easy to perform.

One of the major applications of genome sequencing of plants is functional genomics. In simple words, an understanding of the function of genes and other parts of the genome is known as functional genomics. It is a field of molecular biology that attempts to make use of the vast amount of data produced by genomic projects (such as genome sequencing projects) to describe gene (and protein) functions and their interactions. Unlike genomics and proteomics, functional genomics focuses on the dynamic aspects, such as gene transcription, translation, and protein–protein interactions, as opposed to the static aspects of the genomic information such as DNA sequences or structures (Cseke et al., 2006). It aims to determine the biological function of every gene within a given genome. Functional genomics, then, refers to the development and application of global (genome-wide or system-wide) experimental approaches to assess gene function by making use of the information and reagents provided by structural genomics. Functional genomics includes function-related aspects of the genome itself, such as mutation and polymorphism analysis, as well as measurement of molecular activities. Together, all measurement modalities quantify the various biological processes and powers in order to enhance our understanding of gene, protein, and metabolite functions and their interactions (Fig. 2.1).

Functional genomics uses mostly modern techniques to characterize the abundance of gene products such as mRNAs and proteins. It is characterized by high-throughput or large-scale experimental methodologies combined with statistical or computational analysis of the results. Some typical technology platforms are *DNA microarrays* and *SAGE* (serial analysis of gene expression) for mRNA analysis, two-dimensional gel electrophoresis and mass spectrometry (MS) for protein analysis, and targeting and nontargeting mass spectrometry and nuclear magnetic resonance (NMR) analysis in metabolomics. Because of the large quantity of data produced by these techniques and the desire to find biologically meaningful patterns, *bioinformatics* is used here for this type of analysis of complex systems. Bioinformatics refers to the extraction of biological information from genomic sequence and the reconciliation of multiple data sets based on DNA and RNA microarrays. In connection with the above, a *DNA microarray* (also called a DNA chip or gene chip) is a piece of glass or plastic on which pieces of DNA have been affixed in a microscopic array to screen a biological sample for the presence of many genetic

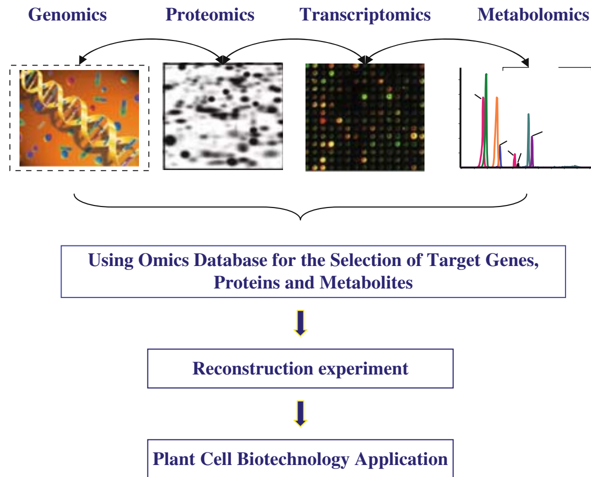

Fig. 2.1 Application of functional genomics tools in plant cell biotechnology

sequences simultaneously. The affixed DNA segments are known as *probes*. Thousands of identical probe molecules are affixed at each point in the array in order to make the chips effective detectors. Many microarrays use PCR products, genomic DNAs, BACs (bacterial chromosomes), plasmids, or longer oligos (35–70 bases) instead of short oligonucleotide probes of 25 bases or less. RNA microarrays are used to detect the presence of mRNAs that may have been transcribed from different genes and that encode different proteins. The RNA is converted to cDNA or cRNA. The copies may be amplified by RT-PCR (reverse transcriptase-polymerase chain reaction). Fluorescent tags are enzymatically incorporated into the newly synthesized strands or can be chemically attached to the new strands of DNA or RNA. A cDNA or cRNA molecule that contains a sequence complementary to one of the single-stranded probe sequences will hybridize, or stick, via base pairing (more so for DNA) to the spot at which the complementary probes are affixed. The spot will then fluoresce, or glow, when examined using a microarray scanner. The major components, then, of a functional genomics approach include *bioinformatics* (the global assessment of how the expression of all genes in the genome varies under changing conditions), *proteomics* (the study of the total protein complement expressed by a particular cell under particular conditions), and *reverse genetics* (deducing the function of novel genes by mutating them and studying mutant phenotypes).

Functional genomics, used as a means of assessing phenotypes, differs from more classical approaches, primarily with respect to the scale and automation of biological investigations. A classical investigation of gene expression might examine how the expression of a single gene varies with the development of an organism *in vivo*. Modern functional genomics approaches, however, can examine how 1,000–10,000 genes are expressed as a function of development.

The massive expansion of available genomic information in plants allows researchers to push the limits as to what can be produced by a chosen organism. Such technology continues to hold great promise for the future of plant

biotechnology. We now may simultaneously analyze the expression or silencing of thousands of genes in plants or in plant cell lines, screen for high- and low-producer lines of the desired phytochemical(s), or determine the full spectra of metabolites. With advances in proteomics, we should also be able to simultaneously quantify the levels of many individual proteins or to follow posttranslational alterations that occur. What are now needed are analogous analytical methods for cataloging the global effects of metabolic engineering on metabolites, enzyme activities, and metabolite fluxes.

So far as we are aware, many limitations or drawbacks occur when investigators try to engineer plant cells. The question here concerns: what cannot be genetically engineered? Our imagination creates thousands of possible applications for plant genetic engineering. It is easy to imagine, for example, that we will be able to derive coffee beans with less caffeine and with hazelnut aroma. Theoretically, that is possible. However, nothing can be successfully accomplished here without unraveling relevant gene expression phenomena, proteins with multifunctional tasks, or metabolic networks in particular plant organisms. Let us consider the fact that there are many identical genes in plants, animals, microorganisms, and even in humans. However, they all have so many differences in terms of their functions. For this reason, complex traits involving multiple functions are still impossible to genetically engineer without the use of a systems biology approach. The systems biology approach has four known steps in general. The first step consists of gathering various high-throughput data sets in addition to legacy data sets. All of these data are then used in the second step to reconstruct the biochemical reaction networks that underlie the cellular function of interest. When such data are put into the format of a biochemically, genetically, and genomically structured database, they have a mathematical format consistent with the underlying physicochemical processes. This mathematically structured database can then be mathematically interrogated (step 3). Constraint-based methods can be used to perform such interrogation at the genome- and network-scale levels. The mathematical computations are then used to perform new experiments. In plant cell biotechnology, extensive metabolic profiling must be more systematic and involve considerable analysis in this case. Due to the productivity issue we have mentioned previously, gene or metabolic engineering must be based on a systems biology approach involving integrated metabolomics, proteomics, and transcriptomics approaches (Carrari et al., 2003; Dixon, 2005). Likewise, metabolic engineering (see below) is a potentially very powerful tool in plant cell biotechnology for the regulation of secondary metabolism in transgenic plants or plant cell cultures, with potential to have wide applications in the phytochemical industry or in agriculture (Verpoorte and Alfermann, 2000).

2.3 Metabolic Engineering

Plant metabolic engineering treats the cell as a factory and adds or optimizes kinds and amounts of metabolites within the cell for some specific design purpose. In other words, metabolic engineering refers to a targeted metabolic pathway being

elucidated in plant or bacterial organisms with the purpose of unraveling and utilizing this pathway for future modification of a plant's end-products. It is generally defined as the redirection of enzymatic reactions so as to improve the production of high-value constituents, to produce new compounds in an organism, to mediate the degradation of environmentally toxic compounds, or to create plants that become resistant to environmental stress factors. In addition, metabolic engineering may include not only the manipulation of endogenous metabolic pathways but also the transfer of metabolic pathways into new host organisms.

The main goals of metabolic engineering in industry or agriculture are the stimulation of the production of secondary metabolite end-products, biosynthetic precursors, polymers that have plant origin, and the derivation of new plant organisms with high salt or drought resistance in agriculture. It is not surprising that metabolic engineering applications in plant biotechnology in recent years have had incredible achievements in agriculture, industry, and medicine.

This multidisciplinary field draws concepts and methodologies from molecular biology, biochemistry, and genetics, as well as biochemical engineering. In addition, the extension of metabolic engineering to produce desired compounds in plant organisms may answer many fundamental questions applied to plant development, physiology, and biochemistry. For example, plant metabolic flux analysis in the primary carbon-based metabolic pathways presents fundamental information on the application of plant metabolic engineering that is based on a thorough knowledge of plant biochemistry and plant physiology. Plant metabolism itself concerns thousands of interacting pathways and processes that are regulated by environmental and genetic stimuli. Therefore, engineering even known metabolic pathways will not always provide the expected results. Despite major advances in metabolic engineering, still only a few secondary metabolic pathways have been enzymatically characterized and the corresponding genes cloned. In this context, the biosynthetic pathways for alkaloids, flavonoids, and terpenoids are presently the best characterized ones at the enzyme and gene levels. More successful cases of gene discovery have also been considered for the lipid biosynthetic pathway, where most genes in plants encoding enzymes for fatty acid biosynthesis have been cloned. This information was applied for eventual manipulation through modification of many fatty acids in transgenic plants by means of metabolic engineering. As for targeting metabolite manipulation, DellaPenna advocated the conversion or chemical modification of an existing compound(s), rather than attempting to increase flux through a metabolic pathway. Another example, he cites, claims that modifications made in the end-products or secondary metabolic pathways have been generally more successful than in cases where manipulation of primary and/or intermediary metabolic pathways is attempted (DellaPenna, 2001). Recent achievements have been made in the altering of various pathways by use of specific genes encoding biosynthetic enzymes or genes encoding regulatory proteins (Verpoorte and Memelink, 2002; Maliga and Graham, 2004). Most current metabolic engineering studies have focused on manipulations of enzyme levels and the effect of amplification, addition, or blockage of a particular pathway. A new area is the manipulation of cofactors, which play a major role in plant biochemistry and physiology and in the fermentation process

of several end-products. Additionally cofactors are essential for many enzymatic reactions.

Metabolic engineering is becoming a powerful technology for the successful implementation of plant cell biotechnology in the future. This may be possible with the advances we already have mentioned above and some other important issues and key criteria that are cited as follows: (1) Metabolic flux analysis must be applied to well-documented and elucidated metabolic pathways; (2) extension of metabolic cross-talk between the desired metabolite pathway and other pathways for a possible direct impact on plant development and nutritional value must be considered; (3) identification of further elements in the complex regulatory network (such as transcription factors and their binding partners) needs to be examined; and (4) rigorous criteria must be developed for the assessment of the risk and benefit performance of engineered plants. Comprehensive studies in several directions may help to bring metabolic engineering out of the trial-and-error era and transform it into industrial applications.

Metabolic engineering approaches can be defined according to several different directions (Fig. 2.2). The first appropriate approach involves increases in the total carbon flux toward the desired secondary metabolite. In addition, decreasing the flux through competitive pathways is an alternative way to increase the biosynthesis of desired metabolite. Other possible directions involve the introduction of an *antisense gene* of the competing enzyme at the branch point, as well as overcoming *rate-limiting steps*, or blocking *competitive pathways*.

2.3.1 Increasing Total Carbon Flux Through Metabolic Pathways

Metabolic flux analysis determines the rate of carbon flow for each metabolic reaction in a biochemical pathway. A method to quantify flux through metabolite measurements is necessary for the analysis of original and modified pathways. Flux of carbon into a given metabolite pathway, diversion of metabolic flux at intermediate branches, and lack of final conversion at the end of a specific branch all may affect secondary metabolite production in plants. Therefore, it is important to identify points of possible flux limitation to be able to pursue pathway steps for genetic modification.

The biosynthesis of secondary metabolites in plants can be regulated by increasing the metabolic flux within cells through reconstruction experiments. In vivo, resource allocation is often accomplished by controlling the flux of branch point intermediates in metabolic networks. For example, Kleeb with coworkers used this approach to optimize an in vivo selection system for the conversion of prephenate to phenylpyruvate, a key step in the production of the essential aromatic amino acid phenylalanine (Kleeb et al., 2007). Careful control of prephenate concentration in a bacterial host lacking prephenate dehydratase, achieved through the provision of a regulable enzyme that diverts it down a parallel biosynthetic pathway, provides the means to systematically increase selection pressure on replacements of the missing catalyst. Successful differentiation of dehydratases, whose activities vary over

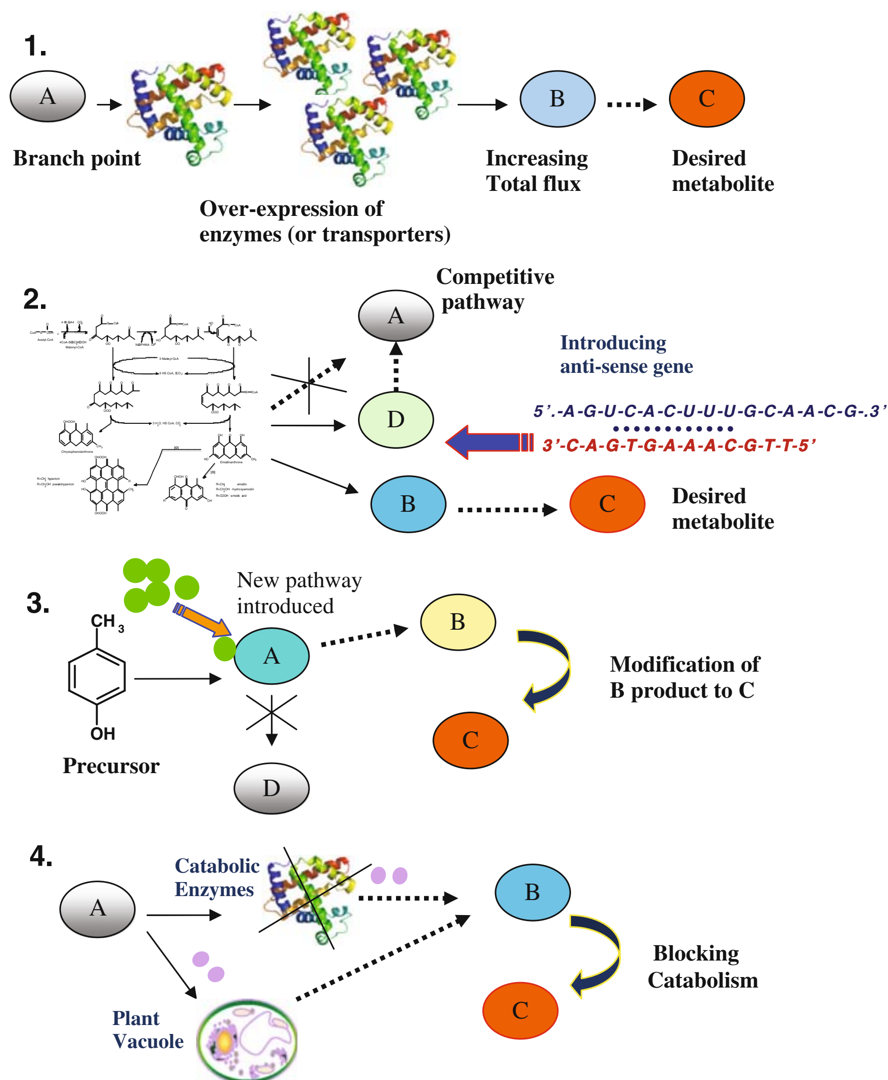

Fig. 2.2 Approaches in metabolic engineering: (1) increase in the total carbon flux at the branch point; (2) decrease in the flux through competitive pathways or introduction of an antisense gene of the competing enzyme; (3) regulation of desired metabolite yield either by competitive pathway determination and targeting of rate-limiting steps or by introduction of a new pathway; and (4) blocking catabolism either by increasing the transport of metabolites into the vacuole or by downregulation of catabolic enzymes

a >50,000-fold range, and the isolation of mechanistically informative prephenate dehydratase variants from large protein libraries illustrate the potential of the engineered selection strain for characterizing and evolving enzymes (Kleeb et al., 2007). This approach complements other common methods for adjusting selection pressure

and may be generally applicable to plant systems that are based on the conversion of an endogenous metabolite. There are several examples that have been reported for well-characterized rate-limiting enzymes of plants and their controversial role in the regulation of pathway flux (for review, see DellaPenna, 2001).

Analysis of a wide range of secondary metabolites has significant advantages as compared to a study of final product(s) accumulation. However, this approach may require fairly comprehensive study, because it is based on complex mathematical formulations for metabolite network analysis. The data are gathered from extracellular measurements of biomass composition, quantification of secreted metabolites, substrate utilization, and intracellular measurements of carbon partitioning. Such flux analysis may have some limitations due to the complexity of mathematical modeling.

A very interesting model that organizes the flux analysis by grouping metabolites of similar biosynthetic origin has been proposed by Morgan and Shanks (2002). They have quantified temporal profiles of metabolites from several branches of the *indole alkaloid pathway* in *Catharanthus roseus* (L.) G. Don (Madagascar pink) hairy root cultures and were able to examine the distribution of flux around key branch points. As a result, this analysis provides crucial information, such as an estimate of total flux for all the secondary metabolites produced in a multi-branched pathway. Another good example is the regulation of *metabolic flux* to *cellulose*, a major sink for carbon in plants, as reported by Delmer and Haigler (2002). As for many pathways, it is still unclear where carbon flux is rate-limited in the complex cellulose biosynthetic pathway. Cellulose is an important component of the cell walls of higher plants. As a major sink for carbon on the earth, possible means by which the quality or the quantity of cellulose deposited in various plant parts might be manipulated by metabolic engineering techniques is a worthwhile goal (Delmer and Haigler, 2002). Thus, putative mechanisms for regulation-altered flux through this pathway, as well as multiple genes for cellulose biosynthesis and their regulation, provide targets for metabolic manipulations. However, possible variation in flux control under environmental influences must also be considered.

2.3.2 Introduction of an Antisense Gene of the Competing Enzyme at the Branch Point

Metabolic engineering of a zeaxanthin-rich potato by antisense inactivation and cosuppression of carotenoid epoxidation is a classical example for this approach (Romer et al., 2002). In order to provide a better supply of zeaxanthin in a staple crop, two different potato (*Solanum tuberosum* L.) cultivars were genetically modified. Sense and antisense constructs encoding zeaxanthin epoxidase have been transformed into the potato plant. Subsequently, zeaxanthin conversion to violaxanthin was inhibited. In this study, both approaches (antisense and cosuppression) yielded potato tubers with higher levels of zeaxanthin, up to 40 $\mu g \cdot g^{-1}$

dry weight. As a consequence of this metabolic engineering manipulation, the amount of violaxanthin was diminished dramatically, and in some cases, the monoepoxy intermediate, antheraxanthin, accumulated (Romer et al., 2002). Most of the transformants with higher zeaxanthin levels showed simultaneous increases in total carotenoid content (up to 5.7-fold). The increase in total carotenoids suggests that the genetic modification affects the regulation of the whole carotenoid biosynthetic pathway in potato tubers, involving substantial higher phytoene synthase and a slight increase of the β-carotene hydroxylase transcripts levels in tubers.

Recent work has also led to the identification of a transcriptional regulator that is possibly involved in the control of carotenogenesis (Welsch et al., 2007). Another mechanism controlling carotenoid levels in plant tissues is their degradation (Ohmiya et al., 2006). The generality of such a mechanism remains to be tested, but it could provide an additional approach for biotechnological improvement of carotenoid synthesis. This is important because carotenoids are members of one of the most diverse classes of natural compounds. Plant carotenoids are composed of a C40 isoprenoid skeleton with or without epoxy, hydroxy, and keto groups. They are high-value compounds in human nutrition as antioxidants and vitamin A precursors. In previous years, several metabolic engineering efforts have been undertaken in edible plants, again with the aim to improve their nutritional value (for review, see Giuliano et al., 2008).

2.3.3 Overcoming Rate-Limiting Steps

The most important aspects in metabolic engineering are to identify enzymes that may be involved in intermediate biosynthesis and subsequently to determine if any of these may occur at regulatory steps, or as now named *rate-limiting steps*. Such determinations may play a key role in future manipulation of secondary metabolite biosynthesis, because rate-limiting steps can be considered as *docking targets*.

For known metabolic pathways, the single-gene approach is an excellent way to find out where a rate-limiting step occurs. However, if pathway architecture is quite complicated, it raises the bar from the linear to a complex network. The analysis should therefore start with a step-by-step identification of all enzymatic activities that are specifically involved in the pathway. As we have mentioned, blockage of one pathway may lead to diversion of the substrate to alternative pathways. In such a situation, the identification of the rate-limiting step for biosynthesis of a particular metabolite may be difficult and become a “fishing expedition.” Therefore, pathway architecture is one of the important factors that will allow one to determine the most suitable approach for engineering plant cells. It may also be that the pathway is subject to developmentally controlled flux at entry, as for example, through the activity of transcription factors. Several other factors, such as regulatory mechanisms or compartmentation, can also play a significant role. Thus, regulatory mechanisms such as *feedback regulation* may affect secondary metabolite

yield in plants. This is especially relevant with the single-gene approach. In contrast, with *heterologous gene overexpression*, a heterologous enzyme is shown to be operative and, because of this, may have no feedback inhibition by downstream products. Such an enzyme may be introduced from another source (Chartrain et al., 2000). Compartmentation also plays a major role in the regulation of secondary metabolite pathways because some important pathways occur in compartments (Verpoorte et al., 1999). for example, the biosynthesis of terpenoid-type indole alkaloids requires at least three compartments: the plastids for the terpenoid moiety and tryptophan, the cytosol for decarboxylation of tryptophan, and the vacuole for the coupling of tryptamine with secologanin (Verpoorte et al., 1999). Similar rules are shown for plant folate biosynthesis pathway, where it is split among cytosol, mitochondria, and chloroplasts. For example, in pea leaves, folate is distributed among mitochondria (highest concentration), chloroplasts, and a fraction consisting of the cytosol, nucleus, and vacuole (Gambonnet et al., 2001). Folates and their biosynthetic intermediates must therefore move in and out of organelles, thus requiring unraveling of its transport mechanisms. Since nothing is known about folate or its precursor carrier, identifying and cloning some transporters have been considered to be a priority for metabolic engineering of plant folate biosynthesis (Basset et al., 2005). It may be based either on modification of folate transport or on compartmentation. The engineering of folate transport, as reported by the same authors, is also a potential strategy to prevent and stockpile folate within an "inert" compartment like the vacuole. As the folate biosynthetic enzymes are not present in the vacuole, it may be possible to accumulate folate without feedback inhibition of its synthesis by directing folate import into this organelle (Basset et al., 2005).

Another example concerns plant polyketides and their biosynthesis. The plant polyketide synthases, like most enzymes, display broad substrate specificity. Using alternative substrates is the most straightforward and powerful approach to generate new polyketides in vitro. Initial efforts here also focus on how active site variation among enzymes making various molecules leads to product specificity. For example, modification of the octaketide-producing polyketide synthase from *Aloe arborescens* Mill. leads to a variety of octaketide products, which were produced by certain bacteria polyketide synthases (for review, see Yu and Jez, 2008). Similarly, three substitutions in chromone synthase, which make a pentaketide, triple the volume of the active site and result in synthesis of the nonaketide naphthopyrone from nine malonyl CoA molecules (Yu and Jez, 2008).

Deletion of a key biosynthetic enzyme can severely affect metabolite flux within a pathway. For example, the flow of precursors into the disrupted pathway often results in the accumulation of one or more intermediates upstream of the blocked step. This is because elevated concentrations of the substrate for the missing enzyme boost nonenzymatic background reactions and favor the appearance of enzyme variants with low substrate affinity. Such problems can be minimized, or even eliminated, through metabolic engineering, where, for example, excess substrate can be efficiently removed from cellular metabolism by providing a second enzyme to channel it away from the blocked step (Kleeb et al., 2007).

2.3.4 Blocking Catabolism or Competitive Pathways

Generally, metabolic pathways contribute to *catabolism* – the oxidative degradation of molecules – and *anabolism* – the reductive synthesis of molecules. In this regard, the catabolic or anabolic nature of the pathways must be revealed prior to any reconstruction experiment. Since little is known about catabolism in secondary metabolite pathways, there is an important question as to whether catabolism is an important factor in secondary metabolite pathways for limiting product accumulation. Interesting questions are also raised concerning the possible toxicity of some compounds to plant cells and the role of catabolism in detoxification mechanisms. In this context, naturally occurring storage compartments (e.g., vacuole(s) and plastid(s) in plant cells) may play a key role in preventing secondary metabolites from being catabolized. Catabolism thus may be an important factor in metabolic engineering. A remarkable observation was made in plant cell cultures of *C. roseus*by Dos Santos et al. (1994) concerning equality of the rate of catabolism with the rate of de novo compound biosynthesis. The phenomenon of catabolism in secondary metabolites has not been studied very extensively, and still few enzymes have been characterized in catabolism of most secondary metabolites (Verpoorte et al., 2000). Catabolism can be blocked by antisense genes or even by using some antibodies.

Blocking competitive pathways is also powerful tool to increase desired metabolite yield. Isoflavone levels in *Glycine max* (L.) Merr. (soybean) have been increased via metabolic engineering of the complex phenylpropanoid biosynthetic pathway (Yu et al., 2003). Phenylpropanoid pathway genes were activated by the expression of the maize C1 and R transcription factors in soybean seeds, which decreased genistein and increased the daidzein levels, with a small overall increase in total isoflavone levels. Cosuppression of flavanone 3-hydroxylase to block the anthocyanin branch of the pathway, in conjunction with C1/R expression, resulted in higher levels of isoflavones (Yu et al., 2003). The combination of transcription factor-driven gene activation and suppression of a competing pathway provided a successful means of enhancing accumulation of isoflavones in soybean seeds.

2.3.5 Inverse Metabolic Engineering

In contrast to classical metabolic engineering, where manipulation of known genes affects metabolic pathways with possible systematic changes, *inverse metabolic engineering (IME)* aims to identify and construct desired cell phenotypes of interest so as to incorporate them into appropriate host organisms. The concept of inverse metabolic engineering was first introduced by Bailey et al. (1996). The key element of this concept is identification of the molecular basis of a desired phenotype and its subsequent transfer to an appropriate host organism. Generally the following approaches may be involved: (1) the identification of desired phenotype, (2) determination of the influence of environmental or genetic factors on phenotype sustainability, and (3) alteration of the phenotype of the selected host by genetic manipulation.

IME is a powerful framework for engineering cellular phenotypes (Bailey et al., 2002). Such cell phenotypes, for example, may be chosen based on the accumulation of a desired metabolite. In order to discover cells with the most desirable properties, the cells must be screened genetically to identify the genetic basis of the relevant phenotype. It allows determination of particular genetic modifications that could not be discovered with a more directed technique. Recent advances in functional genomics, described in Section 2.2, have dramatically improved our ability to relate changes in phenotype with associated changes in genotype. As a result, inverse metabolic engineering can be a method for discovering new genes to target with traditional metabolic engineering. Thus, the first step is to find the genes that underlie the relevant phenotypes. Genetic selection or screenings, together with conventional gene sequencing, may be used to identify such genes in mutations.

While IME was initially designed for prokaryotes, nowadays its application applies to plant or other eukaryotic organism's cells also. The best example described by Sauer and Schlattner (2004) concerns the ATP homeostasis exhibited by animal cells. A variable ATP turnover in these cells is achieved through temporal and spatial energy buffering, where phosphagen kinase systems (consisting of specific kinase and its cognate phosphagen) function as a large pool of high-energy phosphates that are used to replenish ATP during periods of high energetic demand. Thus, these authors suggest the use of recent advances and potentials of inverse metabolic engineering of cell types that do not normally contain such systems (bacteria, yeast, and plants) in conjunction with creatine or arginine kinase systems (Sauer and Schlattner, 2004). Beyond such applications in bioprocess engineering, engineering of phosphagen kinase systems is potentially important for medical and pharmaceutical applications. The advantage of inverse metabolic engineering may be more applicable if we can rationally modify a given phenotype to engineer cell behavior.

2.4 Development of Genetically Modified Plants That Express Resistance to Different Kinds of Abiotic and Biotic Stresses

Environmental stresses (e.g., high salt levels, low water availability that leads to drought, excess water that leads to flooding, or high- and low-temperature regimes) can adversely affect plant growth and productivity. The genetic or epigenetic responses of plants to these stresses are complex because they involve simultaneous expression of a number of genes or physiological reactions. Continuing efforts of scientists have resulted in engineering of plants resistant to high temperatures, low temperatures, and excess salinity. Some progress has also been achieved in generating plants resistant to water-deficit stress and to flooding. While such achievements are impressive, it is still a challenging task to understand complex functional genetic resistance responses to such stresses. Here, metabolic engineering can play an important role. The limiting factor in this aspect is the lack of information on what are the "useful genes", i.e., genes that would lead to better stress tolerance.

Metabolic engineering of rice leading to biosynthesis of *glycine betaine* and tolerance to salt and cold is one of the best examples in this field. Genetically engineered rice (*Oryza sativa* L.) with the ability to synthesize glycine betaine was established by introducing the *codA* gene for *choline oxidase* from the soil bacterium *Arthrobacter globiformis* (Sakamoto and Murata, 1998). This study indicates that the subcellular compartmentalization of the biosynthesis of glycine betaine was a critical element in the efficient enhancement of tolerance to salt and cold stresses in the engineered rice plants.

Metabolic engineering to accumulate osmoprotectants in plants may increase their drought and salinity tolerance. An effective mechanism to reduce damage from these stresses is brought about by the accumulation of high intracellular levels of *osmoprotectant compounds* including proline, ectoine, betaines, polyols, and trehalose. Engineering osmoprotectant biosynthesis pathways is a potential way to improve stress tolerance (Rontein et al., 2002). Several single genes for such osmoprotectant pathways have been successfully introduced into several plants, including rice (*O. sativa* L.), canola (*Brassica napus* L.), potato (*S. tuberosum* L.), tobacco (*Nicotiana tabacum* L.), and *Arabidopsis*, to improve their stress tolerance.

Another possible mechanism by which plants could survive salt stress is achieved by compartmentalization of sodium ions apart from the cytosol. Overexpression of a *vacuolar Na super*(+)/*H super*(+) *antiport* from *Arabidopsis thaliana* (L.) Heynh. in *Arabidopsis* plants promotes sustained growth and development in the soil/water environment. This salinity tolerance was correlated with higher-than-normal levels of *AtNHX1 transcripts*, protein, and vacuolar Na super(+)/H super(+) (sodium/proton) antiport activity, as reported by Apse et al. (1999). These results demonstrate the feasibility of metabolic engineering for salt tolerance in plants. Improving plant drought, salt, and freezing tolerance by gene transfer of a single *stress-inducible transcription* factor has also been reported to be successful (Kasuga et al., 1999). Overexpression of the cDNA encoding this transcriptional factor in transgenic plants activated the expression of many of these stress-tolerant genes under normal growing conditions and resulted in improved tolerance to drought, salt loading, and freezing.

Application of metabolic engineering to improve tolerance against UV radiation, intensive high light intensities, and high temperatures has been reported for *Arabidopsis* plants (Giuliano et al., 2008). Leaf carotenoids may have essential photoprotective roles, because they scavenge *reactive oxygen species (ROS)*, quench dangerous triplet states of chlorophyll, and participate in thermal dissipation of excess light energy. For example, zeaxanthin that is formed as a result of violaxanthin de-epoxidation under high irradiances enhances *nonphotochemical quenching (NPQ)* of chlorophyll fluorescence and protects membrane lipids from peroxidation. Thus, it is not surprising that genetically engineered *Arabidopsis* in which all leaf xanthophylls have been substituted with zeaxanthin shows more resistance to photooxidative stress and lipid peroxidation (Giuliano et al., 2008). However, due to genetic manipulations that may increase the levels of several β-xanthophylls simultaneously, each may have distinct photoprotective roles and therefore must be considered in future applications.

Improving resistance against pests or diseases also leads to improved yields. For resistance against pathogenic microorganisms, metabolic engineering can program for the expression of high levels of defense compounds, such as phytoalexins, or for pest resistance, improve the production of endogenous defense compounds (e.g., pathogenesis-related proteins), or introduce genes that produce new toxic compounds (e.g., the "B.T." gene from *Bacillus thuringiensis* that produces a toxic crystalline protein that interrupts digestion in many types of feeding insect pests) into plants to control insect predators.

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