CHAPTER 21

FUNCTIONAL GENOMIC APPROACHES TO STUDY AND ENGINEER SECONDARY METABOLISM IN PLANT CELL CULTURES

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Abstract. Plants produce a wide range of secondary compounds, also referred to as natural products, which may have important functions in the plants adaptation to specific ecological niches or its responses to biotic and abiotic stresses. Some of these secondary metabolites turn out to be beneficial for humans as pharmaceuticals. Because of their unique and often complex chemical structures, synthesis of these natural compounds is frequently unfeasible or not economically justified. Therefore, many secondary metabolites are still extracted from whole plants. However, they are often produced only in certain tissues, at specific developmental stages or they are present in low concentrations. The possibility of growing medicinal plants, either as a whole, or as a specific tissue or even as plant cells in so-called tissue culture is intensively being investigated. Nevertheless, only few examples of commercial exploitation of plant cell cultures to produce a natural product exist, mainly due to the low yields and the instability of production rates commonly encountered in cell culture systems. Emerging tools such as metabolic engineering have added little to the production problem, since insight into the molecular mechanisms driving plant secondary metabolism at present is fairly limited.

Knowledge of the genetics of biosynthetic pathways and their regulation is thus of crucial importance to bypass the low yield of various secondary metabolites in plant cells. To facilitate gene discovery in plant secondary metabolism, in our department a comprehensive profiling approach has been developed that is based on functional genomics. This approach integrates cDNA-AFLP-based transcript profiling and targeted metabolic profiling. As this method requires no prior genetic knowledge or sequence databanks, it is applicable to any plant species to unravel the biosynthesis of any metabolite of interest. This knowledge will then allow for metabolic engineering, as well as pave the way for so-called 'combinatorial biochemistry', with which novel metabolites could be produced in plants.

Keywords: biosynthesis; secondary metabolites, genes; biotechnology

INTRODUCTION

Plants represent an immense biodiversity, with more than 250,000 known species existing within the plant kingdom. In addition they are very rich in metabolites of a

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huge chemical diversity, of which only a small fraction has actually been characterized. It is estimated that about 100,000 plant secondary metabolites or natural products have been identified. For the vast majority of natural products their function for the plant as well as their potential role in human health care is still to be elucidated. This study field, referred to as pharmacognosy, has over the years evolved from a mere descriptive botanical science to a discipline integrating biochemistry and molecular genetics (Phillipson 2003). This has led to the identification and biochemical characterization of several natural products, which in turn have led to the development of important novel drugs. As a proof of this, over one quarter of new drugs that have been approved in the last 30 years are based on a lead from a molecule from plant origin. Moreover, 9 of the top 20 selling drugs are derived from knowledge of plant secondary metabolites (Harvey 2000; Tulp and Bohlin 2002).

Although over the last decades the pharmacological industry had slightly turned away from natural products and had been shifting more to the 'combinatorial chemistry' approach (Craker and Gardner in press), it has now renewed its interest in plants, as well as in other lower organisms, to look for active natural products with a putative pharmaceutical importance (Müller-Kuhrt 2003). This is partly due to the limited success of the combinatorial-chemistry approach to deliver novel drugs. Indeed, it is clear that the search for active products will be far more successful using the enormous biodiverse library of molecules from living organisms compared to a random chemical library. Since plants and their natural enemies, such as insects, bacteria, nematodes and viruses, have co-evolved, plants produce a wide range of natural products that are involved in defence (Wink and Schimmer 1999). As plants have a sedentary life, and had to adapt to different environments, they developed systems of defence to various stresses. Other plant secondary metabolites serve as attractants for pollinators, or as protectors for UV irradiation. In principle the molecules involved in these actions can be seen as highly specific structures designed through evolution to interact with certain protein folds, but not with the majority of them. Thus, they form very good candidate molecules with pharmaceutical potential (Heimann and Bauer 1999).

For molecular biology and biotechnological applications, however, medicinal plants and their products still hold many hurdles: compound production is often species- or even genotype-specific, and their accumulation is limited to certain tissues or cell types and regulated by environmental or developmental factors. In addition, the function of most of the metabolites is not known, nor is it clear which of the secondary metabolites from the plant's metabolome are responsible for its (proclaimed) medicinal activity. Our current view on the plant metabolism is still rather linear, whereas it is likely that in reality metabolism will behave like a web, in which pathways and molecules will interact in a flexible and dynamic way. For molecular biology it has also been a problem that these 'exotic' medicinal plants have not yet been used extensively in research. Thus, there is little knowledge of, for example, their genomes and no experience with their tissue culture and genetic engineering. Yet it is clear that the new era of plant biology, activated by the tools of modern molecular biology, holds a lot of promise for medicinal plant research and will without any doubt help scientists unravel some of the secrets that medicinal

plants and their metabolites still keep (see also Verpoorte in press). Genetic approaches using the model plant *Arabidopsis thaliana* can serve as an example on how to move forward in this endeavour (Memelink 2005).

PRODUCTION OF PLANT SECONDARY METABOLITES

The problem with interesting secondary metabolites identified in medicinal plants has mainly been on the level of the production. In general there are three options:

- to pursue the chemical synthesis of the compound if the structure is known
- to use extraction from the plant, either from the wild harvest, through cultivation of the plant, or using tissue cultures
- to produce the metabolite in a heterologous (plant or non-plant) system.

All these approaches, however, have their own specific problems and challenges. Chemical synthesis, for example, is often not possible or not economically feasible, since most natural products are quite complex structures. Sometimes, however, it is feasible, based on the knowledge of the action of the natural product, to produce a synthetic substitute with the same action. Recently such an example emerged from research on artemisinin, where the active site from the molecule, a pharmacophoric peroxide bond in a unique 1,2,4-trioxane heterocycle, formed the basis to develop a synthetic peroxide antimalarial drug (Vennerstrom et al. 2004).

Intact plant-based production systems have the problem that in most cases the natural product is present at low levels, or accumulates only in a specific tissue and at a specific vegetative growth stage or upon certain growth or environmental conditions. Furthermore, collecting material from the wild, not always in a sustainable way, can lead to over-collection of endangered species as well as to habitat destruction. Nevertheless, about two thirds of all medicinal plants are still collected from the wild (Edwards 2004). The problem with whole-plant extraction is also that the pharmaceutical industry prefers homogeneous samples with more or less constant levels of the active ingredient, which cannot be ensured from random wild-sampling. The domestication of these plants would be a valuable alternative, giving rise to a more controlled environment, and thus more stable production. But this has been shown not always to be possible, as also discussed in some other chapters in this book.

Plant tissue culture was believed to be the answer to these production problems, but unfortunately this has not delivered up to the expectations. The first problem encountered is that in cultures the natural product often does not accumulate at all, or at very low concentration. One obvious reason here can be that the natural product itself has a high intrinsic cellular toxicity. Different kinds of culture systems have been exploited, with varying success (for a review see for example Vanisree et al. 2004). Hairy roots have traditionally often been used, because they give a lot of material that is easy to maintain, but again not all natural products will accumulate in these hairy-root cultures (Kim et al. 2002). Tissue cultures also pose the problem that they sometimes have a slow growth rate, and that they are vulnerable to epigenetic changes, so-called somaclonal variation, and thus are not stable in their production level. The limited knowledge on the biosynthesis of natural products and its regulation, forms a bottleneck for using tissue culture in combination with

advanced tools such as metabolic-pathway engineering. This will be needed in order to pursue a biotechnological approach whereby plant cell tissue cultures could be genetically engineered to produce profitably a natural product of choice (Oksman-Caldentey and Inzé 2004). However, it should be added that tissue culture is already bringing enormous advantages as well, apart from being used as a production system, such as for the delivery of disease-free material and the conservation and multiplication of rare species through efficient regeneration procedures (Rout et al. 2000).

Heterologous production of metabolites of interest can only be done if the enzymes involved in the biosynthesis of the compound are known and the pathway is not to complex, so that it can be transferred to another system. This approach has been used successfully in micro-organisms such as bacteria, fungi and yeast, to produce, e.g., antibiotics, vitamins and amino acids. It is clear that this field will benefit enormously from recent progress in genomics (Hermann 2004). One such example is the production of amorphadiene, the precursor of artemisinin, an anti-malarial compound, in *Escherichia coli* (Martin et al. 2003). However this strategy may not always be suitable for production of more complex chemical molecules. Also the method requires growth fermentors, which demand a rather high upfront investment as compared to growing plants. Lower plants such as seaweeds might also hold promises for heterologous production of drugs (Qin et al. 2005).

BIOTECHNOLOGY AND SECONDARY METABOLITES

Biotechnology in its broadest sense includes plant tissue culture, as discussed above; the use of molecular markers both for breeding and fingerprinting purposes; the use of molecular tools to study gene expression; as well as the use of all this information for genetic engineering of plants.

Genetic fingerprinting could be a powerful tool in the field of medicinal plants, to be used for example for correct germplasm identification. In addition, when linked to emerging tools such as metabolomics and proteomics, which could be seen as a fingerprinting technique on the plant's metabolites or protein composition, it cannot only give data on phenotypic variation, caused by growth conditions or environmental factors, but also yield data on the genes involved in the biosynthesis, as will be discussed further (Fridman and Pichersky 2005). The use of molecular markers in breeding is a widespread technique for commodity crops such as the cereals, but for medicinal plants there are only a few reports. In cannabis for example, markers have been identified linked to high THC or CBD content (Mandolino and Carboni 2004). It is clear that we can expect more of this kind of work in the future, as the tools of genomics are becoming more and more popular and available.

Genetic transformation of medicinal plants has been exploited using two major tools: *Agrobacterium rhizogenes*, to produce hairy roots, either with a gene of interest or not (see e.g. Chen et al. 1999; Sevon and Oksman-Caldentey 2002), and secondly, *Agrobacterium tumefaciens* to obtain stable transformants. This latter could be used for two purposes. The first one is to solve cultivation problems that these plants might encounter when domesticated. This can include, e.g., herbicide

tolerance (Choi et al. 2003) or approaches to engineer pathogen resistance (Chen and Punja 2002). Secondly, genetics transformation is needed for metabolic engineering, as will be discussed more in detail in the next paragraph. Although numerous reports have been published about genetic engineering of medicinal plants (see Canter et al. 2005, and references therein), to our knowledge there still is not a stable genetically modified medicinal plant in cultivation, although field trials have been done with a transgenic poppy to evaluate pollen flow (Chitty et al. 2003).

FUNCTIONAL GENOMICS AS A TOOL TO STUDY SECONDARY METABOLISM

In principle each natural product is formed by chemical transformations of small and larger molecules through a number of enzymatic reactions. To understand how a natural product is synthesized, the enzymes involved in these reactions need to be identified and the complex network of regulations and interactions understood. This identification can be done on a gene and genome level, as discussed below. This might, however, have the disadvantage that it does not always give information on the nature of the encoded enzyme, that is, which reaction it is biochemically performing. Nevertheless, based on homology comparisons, often a function can be attributed to a newly discovered gene. Nowadays we are seeing a shift in gene studies, going from single-gene studies towards pathways and to whole-genome studies. New and powerful tools in functional genomics can thus be used in combination with metabolomics to elucidate biosynthetic pathways of natural products (Oksman-Caldentey and Inzé 2004).

The basic question in this research field is to identify all the players involved in the biosynthesis of a natural product, both on the enzyme level and on the regulation level, so that the road is paved for metabolic engineering. The general concept behind metabolic engineering is that certain pathways within a biosynthesis network could be stimulated, or favoured over others, by over-expressing a crucial, for example rate-limiting, enzyme. In simple cases this approach has led to good results. Lee et al. (2004), for example, have shown that upon over-expression of a squalene synthase gene they obtained a higher biosynthesis of triterpenes and phytosterols in *Panax ginseng*. Also in food crops metabolic engineering has been done; one recent example includes the increase in the flavonoids and carotene content in tomato, obtained through RNAi-mediated suppression of the DET1 gene (Davuluri et al. 2005). Also in tomato, over-expression of a transferase, involved in the synthesis of chlorogenic acid, has shown to give rise to accumulation of this antioxidant that protects against age-related degenerative diseases when supplied in an animal diet (Niggeweg et al. 2004). In addition, transcription factors regulating a whole pathway could also prove effective (Broun 2005).

Alternatively, silencing a gene giving rise to a specific enzyme in a side branch of a certain pathway, can also lead to accumulation of a certain metabolite. Allen et al. (2004) have shown that they could silence the pathway leading to morphine in *Papaver somniferum*, with the positive side effect that reticuline and its methylated forms accumulated. Reticuline is a potential substrate for the synthesis of anti-malarial and anti-cancer products. Another nice example is the engineering of the

monoterpene biosynthesis in mint (Mahmoud and Croteau 2001; Mahmoud et al. 2004).

Metabolic engineering can also be used to block the synthesis of unwanted metabolites. The manipulation of the caffeine content in coffee plants is such an example (Ogita et al. 2003). With this approach toxin production in medicinal plants could also be regulated. Extracts of *Ginkgo biloba*, for example, contain a toxic component, ginkgolic acid. For the final commercial product there usually is a norm for these toxic compounds, which are (partially) removed during the extraction, but if varieties could be engineered with low contents of these toxins, this would reduce production costs.

The availability of genes from various plants, either related or not, encoding different biosynthetic enzymes, also opens the possibility for so called 'combinatorial biochemistry'. It is envisioned that the introduction of a gene involved in the biosynthesis of one compound in one plant, could find other suitable substrates in another plant, so that new, non-natural homologues of known secondary metabolites can be formed that were previously not synthesized in either of these plants. Such an approach has been successful in lower organisms such as, e.g., *Streptomyces* (McDaniel et al. 1995). That the concept is likely to function also in plants can be concluded from hybridization experiments, such as happened in the case of potato, where a new glycoalkaloid was produced in a cross between *Solanum brevidens* and *Solanum tuberosum* that was not present in the parental varieties (Laurila et al. 1996).

CDNA-AFLP AS A FUNCTIONAL-GENOMICS TOOLS

In the Plant Systems Biology department (VIB/UGent, Belgium), cDNA–amplified fragment length polymorphism, has been the functional-genomics tool of choice to study gene expression profiles related to the biosynthesis of secondary metabolites. cDNA–AFLP has the advantage that it is an open tool, i.e., that no prior genomic data are needed (Breyne and Zabeau 2001; Breyne et al. 2003). Indeed, for most medicinal plants limited or even no information is available on genomic sequences, nor do cDNA libraries exist that could be used as a template for micro-arrays. The principle of cDNA–AFLP is as follows: mRNA is extracted from the tissue of choice; the mRNA is converted to cDNA and digested with two restriction enzymes. Adapters are ligated after which this pool of fragments is used for a selective round of amplification. As such, a high-resolution genome-wide profiling of transcripts can be visualized on acrylamide gels, which can then be analysed using software such as AFLP-Quantarpro (Keygene, Wageningen, The Netherlands).

Thus far, jasmonate-induced changes on the transcript and alkaloid profiles of tobacco BY-2 and *Catharanthus roseus* cell cultures have been monitored (Goossens et al. 2003; Rischer et al. in press). An inventory of hundreds of genes, potentially involved not only in alkaloid biosynthesis but also possibly in plant secondary metabolism in general, has been built. Thereafter, large-scale functional analysis of genes from this inventory, potentially involved in plant secondary metabolism, is performed. This includes the isolation of full-length open reading frames (FL-ORFs) and introduction and functional analysis of them in transgenic

plant cells. Tools to improve and speed-up functional analysis of candidate genes in transgenic plant cells, such as medium-throughput strategies for isolation of FL-ORFs, super-transformation of plant cells with reporter gene constructs, transient protoplast expression assays and micro-array facilities, have been designed and their use validated.

A FOCUS ON *ARTEMISIA*

A recent project in our department includes the study of the production of an anti-malarial compound, artemisinin in *Artemisia annua*. Artemisinin is currently still extracted from the plant itself, but due to the low amount of artemisinin in the plant there is a shortage in the production (Cyranoski 2004). In the case of *Artemisia*, it has been shown that the genetic variability linked to artemisinin content can be used to generate improved high-yielding varieties (Delabays et al. 2001); thus, it is likely that genetic factors will be identified that are involved in the low or high artemisinin trait. A biosynthetic pathway of artemisinin has been proposed, but most of the genes underlying the synthesis and the control of it, are not yet identified (Bertea et al. 2005; Bouwmeester et al. in press). In our department the biosynthesis of artemisinin will be studied using cDNA–AFLP gene profiling. The knowledge of genes activated upon artemisinin biosynthesis, combined with metabolome data from the same time points as used for the transcript profiling, will allow us to identify key genes, encoding enzymes and/or transcription factors involved in the biosysthesis of artemisinin and the regulation of this pathway. This information will be the basis for a more detailed study, which will ideally give the information needed to engineer a high artemisinin producing plant through metabolic engineering.

PUBLIC PERCEPTION

Since this chapter is finishing with the concept of metabolic engineering, which will require the genetic modification of medicinal plants, it might be worthwhile to say a few words on the subject of public perception in this area. In Europe the general perception of genetically modified food is rather negative, whereas for medical applications or medicines, produced by biotechnological means, consumers are more positive (Pardo et al. 2002). Medicinal plants, however, could form a separate class here. Often medicinal plants, or ‘herbs’ are seen as ‘natural’ medicines, and the typical consumer of medicinal plants might be reluctant to use a genetically modified herb. To our knowledge no studies are available, however, on the consumers of medicinal plants and their attitude towards genetic engineering. However, in cases where extracts from the plant are made and sold as drugs by the pharmacological industry, this would probably not affect consumer behaviour. As for environmental issues related to genetic modification in medicinal plants, it can be said that, if genetically modified medicinal plants will be grown, it will never be on the large scale as of some important food crops nowadays, such as maize and soybean. Appropriate measurements of growth can thus more easily be taken to avoid pollen flow to wild relatives of the herb. Intellectual-property issues, already

complex for medicinal plants, might also arise through the use of biotechnology, but this is covered in another part of this book (see chapter 9 by Van Overwalle).

CONCLUSION

Plant natural products have been a very productive source in drug development. The study of plant secondary metabolism is a fully expanding and challenging field in molecular biology and biotechnology, with many opportunities ahead. New tools of functional genomics combined with metabolomics and proteomics will revolutionize our knowledge on the pathways and enzymes involved in the synthesis of natural products, and thus allow a more focused approach for their production. With the increasing need for novel drugs for newly identified molecular targets, this field will likely become increasingly relevant. The appealing economic aspects of large-scale production of pharmaceuticals in plants could attract increasing investments and create new opportunities in this promising research field.

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