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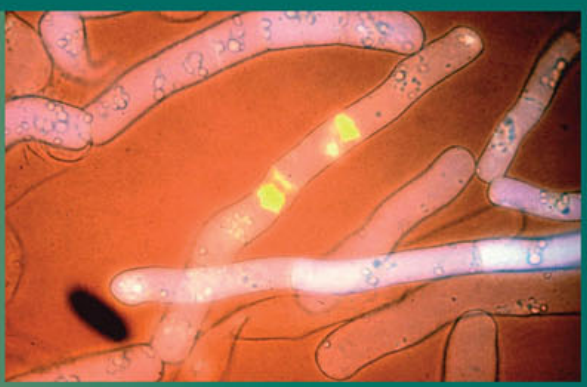
Editors

Applications of Plant Metabolic Engineering



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Applications of Plant Metabolic Engineering

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CONTENTS

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INTRODUCTION

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In the past years the interest in plant secondary has increased rapidly. Three major reasons can be mentioned for this. First because plants are a major source for the production of medicines and the development of novel medicines; second because plants contain health promoting secondary metabolites, third because of the interest in the resistance of plants against pests and diseases in which the secondary metabolism plays a crucial role. Seven years ago we edited a book (Verpoorte and Alfermann 2000) on the engineering of plant secondary metabolism. A general overview was given of plant secondary metabolism, and the strategies one could envisage for engineering plant secondary metabolite pathways. Furthermore, a number of examples were presented describing the state-of-the-art of engineering plant secondary metabolism. Now we have again compiled a series of papers on the engineering of plant metabolism.

Obviously in the past period quite a few applications have been reported. Some of them were successful, others were less successful and the unsuccessful ones we will probably never hear of. Reasons for failure are often basic biological problems: the regeneration of transgenic plants from transformed cells, and the stability of transformed cell lines or transformed plants. The toolkit for transformation and overexpressing genes has improved and consequently the number of successful transformations increased. However, the major difficulties concern the fact that the biosynthetic pathways involved proved to be much more complicated than originally thought. Engineering a single step may result in an increase of the immediate product but not necessarily in an increase of the final product of the pathway. As we discussed in the previous book, problems of pathway architecture, interaction between various pathways in the total metabolic network, enzyme complexes, compartmentation, feedback inhibition, and regulation all play an important role. It means that unraveling pathways on all levels should have the highest priority. Eventually this might enable us to design efficient approaches to pathway engineering.

PATHWAY ELUCIDATION

The key to genetic engineering is the detailed knowledge of the pathways of interest. The step-by-step approach for elucidation of pathways remains an important, though elaborate, tool in biosynthetic studies. Retrobiosynthetic studies and labeling experiments have shown to be excellent tools to confirm pathways on the level of intermediates (e.g. Eisenreich et al., 2004). Once the intermediates are known, one has to identify the enzymes involved. However, the isolation of enzymes catalyzing the individual steps of a pathway is hampered by, among others, low levels of the enzyme, instability of the enzyme, and problems in obtaining the substrate for measuring activity. Consequently many of the secondary metabolite pathways still have quite a few black boxes, for which paper chemistry has proposed intermediates, but for which no actual experimental evidence exists.

To elucidate pathways various molecular biological approaches have been advocated. Many are based on making "mutants" by knocking out genes (transposon tagging, RNAi, etc.). However, the problem is the identification of the steps which have been blocked in a mutant. Plants in which an essential biosynthetic gene for the flower pigments is affected are immediately observed by eye. In a split second one can screen hundreds of plants for the flower color. However, in case of a colorless metabolite in roots or leaves elaborate analytical methods are needed to identify a mutant. This explains why the flavonoids/anthocyanin biosynthesis is one of best known biosynthetic pathways (Springob et al., 2003).

FUNCTIONAL GENOMICS

Because of the problems in pathway mapping, functional genomics was thought to be a way to elucidate secondary metabolite pathways on all levels from genes to products. Functional genomics aims at determining the function of genes. Transcriptomic data, proteomic data, metabolomic data and physiological functions are all matched through biostatistical methods and bioinformatics. In case of organisms with a known genome sequence such an approach may be successful. But lack of sequence data is a major constraint in studying secondary metabolism in non-model plants.

Proteomics is not the panaceae to solve these problems, as only a small percentage of all proteins will be observed. Particularly low abundance proteins will not be observed (Jacobs et al., 2000, 2005; Chen and Harmon 2006). Secondary metabolism often only represents a small part of the total metabolism, e.g. the energy needed for the biosynthesis of alkaloids was found to be less than 1% of the total metabolism in the development of *Cinchona* seedlings (Aerts et al., 1990, 1991). The enzymes involved may be below the level of detection. For example in proteomics of *Catharanthus roseus* cell cultures some 100 proteins were found to be induced when alkaloid biosynthesis was turned on. Only two of these are known indole alkaloid biosynthesis enzymes (Jacobs et al., 2000, 2005). About 60 had homology with peptide sequences from primary metabolite genes from other plants, whereas

the peptides of about 40 proteins did not match with any known sequence. To identify the genes encoding these proteins and determine their function would be quite difficult and elaborate.

Goossens and co-workers (Goossens et al., 2003; Oksman-Caldentey et al., 2004; Rischer et al. 2006) developed a cDNA-amplified fragment-length polymorphism method that in combination with targeted metabolomics can be used to identify genes involved in certain pathways. Indeed it was shown that in this way a number of genes involved with the induction of alkaloid biosynthesis in *Catharanthus roseus* can be identified, though many of them are primary metabolism related genes, and not directly involved in the pathway. Genes with sequences not matching any known genes are candidates for structural genes of species specific pathways, but it requires extensive further studies to identify the precise role.

Metabolomics, the latest of the – omics family, aims at the qualitative and quantitative analysis of all metabolites in an organism (Fiehn, 2001; Rochfort, 2005; Ryan and Robards, 2006). Metabolomics can be considered as the chemical characterization of a phenotype, and is thus an important tool in functional genomics. It can be used to measure the levels of compounds under different conditions. By correlating these data with proteomic and transcriptomic data one may get information about genes involved in the regulation of pathways and the structural genes involved.

The integration of all the – omics data and physiological data, i.e. taking a holistic view at the organism at all levels without a starting hypothesis, is a novel approach to biological research now known as systems biology. Also for plants this approach is now recognized as a very promising way to study for example plant interaction with insects or microorganisms (Oksman-Caldentey et al., 2004; Sweetlove and Fernie, 2005; Verpoorte et al., 2005).

Even though the various tools of functional genomics can be helpful in identifying genes involved in secondary metabolite pathways, none of them is capable of identifying all intermediates, proteins or genes involved in a pathway. Besides problems of low concentrations, the major problem is that in a living system, the changes in levels of transcripts, activity of enzymes and level of metabolites have different dynamics. The final result of an induction at gene level is only observed many hours or days later, if one even at all can speak about a final result in a dynamic system.

COMPARTMENTATION

The compartmentation of secondary metabolite biosynthetic pathways has received much attention in the past years. Several reviews on this topic have been published (e.g. Kutchan, 2005; Yazaki, 2005). If we take *Catharanthus roseus* as an example it has been shown that both intra- and intercellular compartmentation do play an important role. The early terpenoid precursors from the MEP-terpenoid pathway and geraniol-10-hydroxylase are made in different cells (internal phloem parenchyma) than the other important precursor tryptamine (epidermis). The last step of the

biosynthesis of the terpenoid precursor secologanin occurs also in these epidermis cells. Strictosidine synthase present in the vacuoles of these cells catalyzes the condensation of tryptamine and secologanin to yield the intermediate strictosidine, which is the starting point for several different pathways leading to different types of terpenoid indole alkaloid skeletons (for a review see van der Heijden et al. 2004). The branch leading to vindoline is present in other specialized cells (ideoblasts and laticifers), thus requiring intercellular transport of strictosidine or a later product from the vindoline branch.

Concerning the intracellular compartmentation, it is known that plastids are the source of the terpenoid precursors and tryptophan. Decarboxylation of tryptophan occurs in the cytosol, whereas strictosidine is produced in the vacuole from the precursors secologanin and tryptamine in the vacuole. Further steps are again outside the vacuole. The required glucosidase, for example, is localized in the ER (Geerlings et al, 2000), whereas a crucial step in the vindoline biosynthesis occurs in green chloroplasts (for a review see van der Heijden et al., 2004; Kutchan, 2005). This has implications for engineering alkaloid production in the native host of the pathway. One needs to express the gene in the correct compartment and the correct type of cell, otherwise no or little effect is achieved. But even more important, it means that the flux through a pathway is not only controlled by structural genes catalyzing a chemical reaction, but also by transport from the site of production of a precursor to the site of the next enzyme.

TRANSPORT

Because of the different compartments involved in biosynthetic pathways, the intermediates need to be reallocated to the proper compartment. Reallocation is a complex phenomenon in plants and plant cells. Diffusion is always involved in the reallocation of compounds. Affinity for lipid membranes (lipophylic properties of a compound) and intra- and extracellular fluids (hydrophilic properties of a compound) are important factors for diffusion driven transport through membranes (Blom et al. 1991). On top of that active transport through membranes may occur through e.g. a proton antiport mechanism or ABC-type of transporters (such as proteins belonging to the PDR, MRP and MDR families). For example from measuring transport of alkaloids and iridoids into isolated *C. roseus* vacuoles, we concluded that bidirectional transport occurs through different type of transporters (MRD out and ABC and MRP proteins in) with quite different rates for the different *C. roseus* alkaloids and secologanin (Roytrakul, 2004; Roytrakul and Verpoorte, 2007). In other cell organelles and the cell membranes similar processes might occur. Furthermore, conjugation of compounds with e.g. glutathione under the influence of glutathione transferases and peroxidases may play a role in the vacuolar transport of certain compounds (Dean and Devarenne, 1997; Grotewold 2004; Yazaki 2005). Transport is thus extremely complex as besides diffusion driven transport, different types of active transport are involved, with different directions

and for each single compound a different selectivity. Biosynthetic rates might thus very well be controlled on the level of transport.

Besides transport also storage is an important aspect of secondary metabolite production. Vacuoles are storage organelles, but import of the products is required. For example overexpression of the terpenoid indole alkaloid pathway genes encoding tryptophan decarboxylase and strictosidine synthase in tobacco cells in combination with feeding of the precursor secologanin did not result in any storage of the products. Instead the products were excreted into the medium, which is opposite to the situation in *C. roseus* cells where the alkaloids are stored in the vacuole (Hallard et al., 1997).

In this introduction I will not try to give a complete overview of all aspects of compartmentation, transport and storage. I only want to conclude that the green factory in many aspects is very similar to an industrial factory, (e.g. a factory assembling cars). Both require energy for the production process, transport from the sites of the production of building blocks to the site where these are assembled to yield the final product and a storage site for the stock of the final product. It might thus be possible to apply technical engineering strategies to plan plant metabolic engineering.

TARGETS FOR METABOLIC ENGINEERING

Metabolic engineering is possible, but what are the targets? Why should one like to alter the metabolism of plants?

The following goals can be mentioned:

- Improved quality for producer (farmer)
  - Improved yield
  - Improved resistance against pests and diseases
  - Improved traits for cultivation and harvesting
- Improved quality for processing (industry)
  - Storage of food
  - Suppress level of unwanted products (e.g. toxic compounds) or improve quality of product (e.g. starch, lignins)
  - Higher level of specialty chemicals, e.g. for medicines
  - Fiber quality
  - Biofuel viscosity, stability
- Novel compounds for drug development (industry)
- Improved quality for consumer
  - Taste of food
  - Color of food or flowers
  - Increased level of health improving compounds
  - Lower level of undesired compounds

Looking at this list of possibilities it is clear that the applications concern changes in primary metabolism or in secondary metabolism. It also implies that choices have to be made, e.g. does one go for yields or quality (Morris and Sands 2006; Singh et al. 2006). Secondary metabolism is per definition species specific, it serves the producing organism to survive in its ecosystem. In plants it is, among others, involved in defense against pests and diseases, and in attracting pollinators. Furthermore taste, flavor and color of our food are related to secondary metabolism. Also various health effects of food are connected with secondary metabolites. The defense compounds are of different character, some are constitutively expressed (phytoanticipins), others are only biosynthesized after wounding or in infection (phytoalexins) (Zhao et al. 2005). That means that the regulation of secondary metabolism in part is developmentally regulated, in part is dependent of external (stress) signals.

Starting from ubiquitous primary metabolites as precursors the number of steps in secondary metabolite pathways differs considerably. The biosynthesis of the phytoalexin resveratrol from ubiquitous primary metabolites consists of only a single step, catalyzed by one single enzyme, encoded by one single gene (Hain and Grimmig, 2000). Whereas the biosynthesis of an indole alkaloid like vinblastine, includes at least 30 different steps, at least three different cells types and four different cellular compartments, and consequently also is regulated by transport systems (van der Heijden et al. 2004; Pasquali et al., 2006). Because secondary metabolism is species-specific, the knowledge about most pathways is limited, and very few pathways in plants have been fully elucidated to all levels of intermediates, enzymes and genes.

STRATEGIES

For developing a strategy for metabolic engineering of plant secondary metabolism, one has to keep all the above mentioned aspects in mind. There is a clear difference in approach for increasing or decreasing the flux through a pathway.

Decreasing a flux could for example be of interest in case of undesired (toxic) compounds, or to cut 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. To decrease a flux, the level of the protein of interest can be decreased by an antisense or RNAi approach or by overexpresssing an antibody of the selected enzyme of the target pathway. As long as not any vital pathway is knocked out, this approach should be easy with a good chance of success.

To increase the level of a compound, one needs to know the pathway into much detail to be able to select targets for engineering. This should result in the identification of possible sites for modification, e.g. overcoming limiting steps. As mentioned above, only a few genes of plant secondary metabolite pathways are known. Engineering long pathways thus requires extensive studies to elucidate the pathway. One may also consider the use of microbial genes to achieve certain reactions in plants for which the encoding plant genes are not known yet. The production of salicylate in plants by overexpression of microbial genes is such an example (Verberne et al. 2000).