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Research review paper

Improvement of hairy root cultures and plants by changing biosynthetic pathways leading to pharmaceutical metabolites: Strategies and applications

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ABSTRACT

A plethora of bioactive plant metabolites has been explored for pharmaceutical, food chemistry and agricultural applications. The chemical synthesis of these structures is often difficult, so plants are favorably used as producers. While whole plants can serve as a source for secondary metabolites and can be also improved by metabolic engineering, more often cell or organ cultures of relevant plant species are of interest. It should be noted that only in few cases the production for commercial application in such cultures has been achieved. Their genetic manipulation is sometimes faster and the production of a specific metabolite is more reliable, because of less environmental influences. In addition, upscaling in bioreactors is nowadays possible for many of these cultures, so some are already used in industry. There are approaches to alter the profile of metabolites not only by using plant genes, but also by using bacterial genes encoding modifying enzymes. Also, strategies to cope with unwanted or even toxic compounds are available. The need for metabolic engineering of plant secondary metabolite pathways is increasing with the rising demand for (novel) compounds with new bioactive properties. Here, we give some examples of recent developments for the metabolic engineering of plants and organ cultures, which can be used in the production of metabolites with interesting properties.

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Introduction

Over the last decades natural products, which are produced by plants and microorganisms, became more important for pharmaceuticals, crop-protection agents (Naumann, 2000) and agrochemicals (Jeschke, 2010). The production of secondary metabolites with beneficial properties in food and cosmetics industry, as well as medicinal applications, has therefore received tremendous attention. While the identification of active substances is one major point, the demand for large amounts of individual compounds or plant extracts or pulverized plant materials as drugs is the second issue that needs to be addressed. In many cases it is an issue that the source plant cannot be easily cultivated or that the plant material is protected. For example, hairy roots of Devil's claw (*Harpagophytum procumbens*) are alternative cultivation systems of a plant from Namibia, which so far cannot be

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cultivated. It was shown that these organ cultures, like many others already known before (reviewed in Georgiev et al., 2007; 2010), can be cultivated in bioreactors and that they produce the bioactive compounds, harpagide and harpagoside (reviewed in Georgiev et al., 2013), which have been made responsible for the anti-inflammatory effect of the plant's extract.

In addition, the variation in secondary metabolites extracted from naturally grown plants is very high due to unfavorable environmental conditions, such as biotic and abiotic stresses and seasonal changes. For these reasons it is obvious that alternative methods for the production of bioactive plant metabolites are needed. This led to the development of cell and hairy root culture systems with enhanced production of secondary metabolites. Furthermore, these cultures can be upscaled for growth in bioreactors leading to more and reproducible biomass production. Examples of high-value molecules produced by hairy roots are the anticancer drug camptothecin, the antimalarial compound artemisinin, the anti-inflammatory verbascoside and the anticholinergic scopolamine (reviewed in Georgiev et al., 2012). Another interesting feature of hairy roots is that they may accumulate metabolites that are not detectable in mother plants (Gyurkovska et al., 2011; Pollier et al., 2011). Many approaches have been established so far to increase secondary metabolite production in cultures of a given plant species. Among these are: 1) generation of cell or organ cultures with subsequent selection for high metabolite production; 2) elicitation of cultures with stressors or chemicals to enhance production; 3) genetic engineering of cell or hairy root cultures with heterologous genes (see below); and 4) transformation of whole plants (e.g., *Arabidopsis*) or plant explants. The literature is full of reports on such culture systems for production of a single metabolite or a family of related compounds. Also, there is a wealth of reviews compiling these data (e.g., Chandra and Chandra, 2011; Ono and Tian, 2011; Georgiev et al., 2010, 2012). In this review only some aspects of genetic engineering of hairy roots and plants to achieve tailored metabolite production will be discussed.

The question arises which transgenic strategies will lead to the desired metabolic changes in plants or cultures. In general, it should be possible to transform the plant material in question with one of the classical strategies, for example *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, or via protoplast or chloroplast transformation by particle bombardment. The latter can be also used for other organelles. While transformation and regeneration of the desired plant species is sometimes difficult and usually time consuming, the alternative to create cell or organ cultures can often circumvent this problem.

For improving the metabolic pathways (Georgiev et al., 2010; Chandra and Chandra, 2011) it is essential to know as much as possible not only the pathway to be manipulated, but also the related pathways. There are three basic aims in engineering of biosynthetic pathways: to increase the content of a desired metabolite, to reduce unwanted by-products or to produce new structures that do not occur in the plant species, which is the origin of the culture. Pioneering work on engineering of hairy roots started in the 1970s for example with the experiments of Hamill et al. (1990), who were able to increase nicotine accumulation in tobacco by overexpressing a yeast gene. While much work on hairy roots from different plant species has followed, which is summarized in many reviews, we decided to focus on some recent developments on targeted changes in metabolism in the following paragraphs.

In this paper we use the term “hairy root” for hairy root lines that were induced with wild type *Agrobacteria* and thus are transformed only with the transfer DNA of the root inducing plasmid (pRi). Hairy root lines carrying additional transgenes are referred to as “engineered hairy roots”. This can be done by hairy root induction with wild type *Agrobacteria* on transformed plants or by co-transformation. For the second strategy wild type plants and *Agrobacteria* harboring both a pRi and a binary plasmid with the gene(s) desired for the engineering approach are used for hairy root induction (Simpson et al., 1986). Usually 20–30% of the emerging hairy roots carry the desired construct additionally to the root inducing genes.

This review will discuss some general concepts for bioengineering, e.g., introduce the necessity to think of endophytic microorganisms as possible producers of interesting substances and give examples for strategies to achieve altered metabolic patterns. The production of α-tocopherol and how that can be achieved in plants and plant cultures will be described as one example for increasing a desired metabolic pathway. In addition, the expression of bacterial genes in plants with respective consequences on metabolite patterns will be discussed. Also, some thoughts how to reduce either unwanted or toxic pathways will be presented.

Metabolic engineering — some approaches

Concepts for metabolic engineering

The potential of plants to produce pharmaceutically valuable bioactive products is high, but it needs to be exploited and optimized. For the modulation of biosynthetic pathways some general issues have to be taken into account (Georgiev et al., 2010; Chandra and Chandra, 2011). Problems for metabolic engineering of biosynthetic pathways come from 1) rate-limiting enzymatic steps, 2) competing pathways for the same substrate or intermediate, 3) compartimentation of pathways and thus the need for transport of the metabolite across the membranes, 4) co-factors for the reaction are limiting and 5) feedback-inhibition of individual enzymes by accumulating end-products. These bottlenecks in the natural biosynthesis suggest themselves as targets for bioengineering. Of course, the alteration of specific pathways will demand that other, eventually occurring problems will be taken into account.

The increase of a desired metabolite can be achieved if a single metabolic step can be identified, which is rate limiting. Subsequently, the gene encoding for this enzyme can be overexpressed. Ideally, such transgene will lead to the overproduction of a desired metabolite. When the expression of a specific gene in a pathway should be increased, using the gene from the same plant is sometimes disadvantageous, because it might lead to the phenomenon known as co-suppression (Matzke and Matzke, 1995). A solution could be to use genes from a different organism, but with the same function. In some cases it can be beneficial to downregulate a competitor pathway to increase the metabolic flow into the desired direction (e.g., Davies et al., 2003).

Changing metabolite biosynthetic pathways can be achieved by transformation with, for example, microbial genes. Thus, it has been shown that bacterial halogenases can be expressed in plant hairy roots, leading to the production of novel halogenated alkaloids (Runguphan et al., 2010; see also Altering the pattern of metabolites by heterologous gene expression). Transformation of foreign or homologous genes into the nuclear DNA limits the number of genes that can be co-expressed, but plastid transformation could be used to express polycistronic messages (Lu et al., 2013).

Generally, the biosynthesis of secondary metabolites has to be regulated in a timely and spatial manner, because of the large energy costs associated. The plant cannot provide the complete range of secondary metabolites needed for survival of stress situations at all times. Therefore, good knowledge on the regulation of the pathway in question is required. Genes for transcription factors controlling several steps in a pathway are therefore good candidates for transformation and increasing yields of a given metabolite (reviewed in Yang et al., 2012), when this pathway is complex and therefore difficult to engineer. This has been shown to work in the metabolic engineering of anthocyanins in cauliflower, where the expression of one MYB-family transcription factor led to the formation of pink colored heads (Chiu et al., 2010).

Synthesis of secondary metabolites by plants or endophytes?

Endophytes are coming more and more into the focus of researchers due to their capacity to produce diverse classes of plant-associated secondary metabolites (Aly et al., 2013) such as the antimicrobial agent

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