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Review

Pattern of gene and enzyme in secondary pathways of medicinal plants

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Medicinal plants have been the subjects of man's curiosity since time immemorial. Approximately 80% of the people in the world's developing countries rely on traditional medicine for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts. Bioactive compounds currently extracted from plants are used as pharmaceuticals, agrochemicals, flavor and fragrance ingredients, food additives, and pesticides. The secondary metabolites are known to play a major role in the adaptation of plants to their environment, but also represent an important source of pharmaceuticals. The utilization of plant cells for the production of natural or recombinant compounds of commercial interest has gained increasing attention over past decades. Tissue culture technology is a new molecular tools for plant metabolic engineering to increase the production of valuable compounds. New genomic approaches and efficient gene isolation methods applied to difficult secondary pathways in medicinal plant metabolism will undoubtedly expand the range and precision of manipulations via transgenesis, providing potentially superior material for the breeder. Understanding of secondary metabolism at the enzyme level is a prerequisite for metabolic engineering of medicinal plants, which potentially leads to yield improvement of pharmaceutically important secondary products. The production of secondary metabolites can be enhanced using bioreactors. Bioreactors offer a great hope for the large-scale synthesis of therapeutically active compounds in medicinal plants.

Key words: Enzyme, gene, medicinal plants, secondary pathways.

INTRODUCTION

A biosynthetic investigation may be stimulated or awakened as a result of interest in the pharmaceutical activity of a compound (Mehrafarin et al., 2010). Metabolic pathways include the principal chemical, mostlyenzyme-dependent, reactions that an organism needs to keep its homoeostasis (Koolman and Roehm, 2005). Preliminary progress has been made towards engineering alkaloid production in *Papaver somniferum* (Facchini et al., 2004). In 1991, Bailey defined metabolic engineering as "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technologies. Today, the availability of the complete genome sequence for several plants, together with the Development of powerful techniques for the transformation and stable or transient expression of genes in plants brings plant metabolic engineering as a strong alternative to classical chemical synthesis for the production of pharmaceuticals and other important industrial compounds. The utilization of plant cells for the production of natural or recombinant compounds of commercial interest has gained increasing attention over past decades (Canter et al., 2005). The choice of a production plant for metabolic engineering depends on the specific metabolite to be produced and whether the necessary precursors are already present (Wagner et al., 2004). The development of plant metabolomics which deals with all cellular metabolites has been recently recognized as an important sector of post-genome science. The general idea of 'metabolomics' or the 'metabolome' was first defined several years ago in the field of microbiology (Tweeddale et al., 1998) and its

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importance in plantscience was pointed out soon after Trethewey, 2004). Today, metabolomicsis also a powerful tool in drug discovery and development; for instance, in the identification of drug metabolites or biomarkers for organ-specific toxicities.

However, very little is known about how plants synthesize these substances, and almost nothing is known about how the synthesis is regulated at the genetic level. The questions on gene expression of plant secondary products are still widely open. Since information about enzymes involved in the biosynthesis of secondary metabolites is a prerequisite for metabolic engineering of medicinal plant (De-Eknamkul, 1999). Even in the absence of any visible change in a cell orindividual plant, metabolomics, which allows phenotyping by exhaustive metabolic profiling, can show how cells respond as a system. Plant metabolomics is of particular importance because of the huge chemical diversity of plants compared with microorganisms and animals. The number of metabolites from the plant kingdom has been estimated at 200,000 (Dixon and Strack, 2003) or even more (Trethewey, 2004). Even a single plant species such as *Arabidopsis thaliana* might produce 5000 metabolites (Facchini et al., 2004).

CHOICE OF PLANT SYSTEM FOR METABOLIC ENGINEERING

Whole plants compared to animals, cells in culture (plant or animal), or microbes, whole plants provide a cheap and simple platform for large-scale mass production. Transgenic plants can be grown, maintained, and harvested using equipment already available from classical agricultural practices. The choice of a production plant for metabolic engineering depends on the specific metabolite to be produced and whether the necessary precursors are already present. Specialized organs (e.g., leaf hairs, glands, or trichomes) can be used both to sequester compounds and to provide for an accessible source for metabolite extraction (Wagner et al., 2004). There is a growing concern in using crop species for the production of phytochemicals, particularly pharmaceuticals, in areas where those crops are grown for food consumption purposes. Thus, plant species that does not hybridize with major crops or with wild relatives are gaining importance for the purposes of genetic manipulation.

ENGINEERING IN MEDICINAL PLANTS

Increasing the production of active phytochemical constituentsis a well-established target for genetic manipulation but presents some severe challenges. In particular, the metabolic pathways by which active compounds are biosynthesized are mostly poorly understood, and relatively few genes for key enzymatic or regulatory steps have been isolated (Peter et al., 2005). New genomic approaches and efficient gene isolation methods applied to difficult secondary pathways in medicinal plant metabolism will undoubtedly expand the range and precision of manipulations via transgenesis, providing potentially superior material for the breeder (Peter et al., 2005).

The progress in understanding the genetics, biochemistry and molecular biology of plant secondary metabolism pave the way to novel approaches, such as “metabolic engineering”and “molecular pharming”. Detailed knowledge of the biosynthesis of selected metabolites, however, enables the heterologous expression of multi-step pathways for the improvement of food plants or the production of specific compounds in both prokaryotic and eukaryotic microorganisms, which attributes a bottle-neck role to the characterization of relevant enzymes and genes (Ververidis et al., 2007). Genetic transformation, the recent advances and developments in plant genetics and recombinant DNA technology have helped to improve and boost research into secondary metabolite biosynthesis. A major line of research has been to identify enzymes of a metabolic pathway and then manipulate these enzymes to provide better control of that pathway.

Transformation is currently used for genetic manipulation of more than 120 species of at least 35 families, including the major economic crops, vegetables, ornamental, medicinal, fruit, tree and pasture plants, using Agrobacterium mediated or direct transformation methods (Birch, 1997). Plant metabolic engineering involves the manipulation of existing metabolic pathways by either increasing or diverting flux to desire or from undesired products, respectively, or the generation of chemical entities not normally found in the plant production system through the introduction of genes from other organisms. Characterization of metabolic pathways is a multi-disciplinary activity. It requires the identification of metabolic intermediates and the demonstration of a plausible reaction sequence, followed by the isolation and characterization of the individual enzymes responsible (Jain and Agrawal, 1994).

Nevertheless, there are examples of pathway engineering leading to improvements of potential value in the breeding of medicinal plant (Charlwood and Pletsch, 2002). A recent article illustrating the challenges and opportunities of this approach describes a nine fold enhancement in production of the sedative compounds copolamine in hairy root cultures of *Hyoscyamus niger* (*black henbane*), brought about by simultaneously over expressing two genes encoding the rate-limiting upstream and downstream biosynthetic enzymes (Zhang, 2004). Various metabolic pathways within a cell form the cell's metabolic network. In the metabolic pathway, a substrate enters depending on the needs of the cell, that

is, the specific combination of concentrations of the anabolical and catabolical end products. Yun (1992) increased the production of scopolamine in *Atropa belladona*, from the naturally occurring chemical precursor hyoscyamine, by transformation with the enzyme hyoscyamine b-hydroxylase from Hyoscyamus. Essential elements in the tool box of the metabolic engineer are mechanisms to eliminate or over express gene activity. Facchini et al. (2000), toward the metabolic engineering of benzylisoquinoline alkaloid biosynthesis in *Opium poppy* and related species. A threefold enhancement in production of the putative anti-malarial, anti-cancer agent artemisinin has been reported in transgenic Artemisia plants over expressing farnesyldiphosphate synthase, the enzyme immediately preceding the first committed biosynthetic step (Chen, 2000).

USING ENZYME IN METABOLIC PATHWAY

In biochemistry, a metabolic pathway is a series of chemical reactions occurring within cell, catalyzed by enzymes, resulting in either the formation of a metabolic product to be used or stored by the cell, or the initiation of another metabolic pathway (then called a flux generating step). Many pathways are elaborate, and involve a step by step modification of the initial substance to shape it into the product with the exact chemical structure desired (Koolman and Roehm, 2005). The usefulness of enzymological techniques in elucidating the biosynthetic enzymes of secondary metabolites is found in medicinal plants. Understanding of secondary metabolism at the enzyme level is a prerequisite for metabolic engineering of medicinal plants, which potentially leads to yield improvement of pharmaceutically important secondary products (Wanchai, 1999). Novel enzymes involved in the biosynthesis of plant secondary metabolites have been discovered from both differentiated plants and *in vitro* cultures of medicinal plants. The complexity and species diversity also represent an important genetic bank from which potential medicinal, agricultural and other commercial products could be derived. Gene shuffling technologies can produce a large number of enzyme variants, by shuffling fragments from an existing library (Jestin and Kaminski, 2004). Directed alteration of enzyme activity and substrate specificity can broaden the repertoire of metabolites produced in plants and other organisms (Peng et al., 2010).

However, differentiated plants and organs have also been used successfully for enzymological studies of biosynthetic pathways of secondary products. For example, the discovery of the enzymes geranylgeraniol-18-hydroxylase from *Croton sublyratus* leaves, dopamine-secologanin condensing enzymes from *Alangium lamarckii* leaves (De-Eknamkul et al., 1999) and 1,2-dehydro reticuline reductase from *P. somniferum* seedlings. *Anchusa officinalis* cell cultures producing high content of rosmarinic acid have been used for searching the entry-point enzymes of rosmarinic acid pathway. *Eschscholtzia californica* and *Thalictrum bulgaricum* cell cultures producing benzophenanthridine alkaloids have been used for elucidating the biosynthetic pathways of sanguinarine, chelirubine (De-Eknamkul et al., 1999) and macarpine. Lu¨cker et al. (2004) reported that, in the flowers of transgenic tobacco (*Nicotiana tabacum*) plants expressing three different monoterpene synthases, the levels of products corresponding to the three enzymes were high but did not affect the level of the endogenous linalool production. Enhancing the precursor supply for the biosynthesis of isoprenoids in the mevalonate pathway has been attempted mainly by altering expression levels of the gene encoding 3-hydroxy-3-methylglutaryl-Co A reductase (HMGR), which is considered to be a rate-limiting step in the pathway (Chappell et al., 1995). HDR is a key enzyme in methyl erythritol phosphate (MEP) pathway which catalyzes the last reaction of biosynthesis of Isopentenyl diphosphate (IPP). The cloning and characterization of AaHDR will be helpful to understand more about the function of HDR at the level of molecular genetics. It will also play an important role to unveil the biosynthetic mechanism of artemisinin precursors and provide a candidate gene for metabolic engineering of the artemisinin biosynthesis pathway in *Artemisia annua* (Peng et al., 2011).

Subcellular targeting of enzymes has demonstrated that terpenoid precursors in subcellular compartments are not as strictly separated as previously thought and that multistep pathway engineering is feasible, even across cell compartments. These engineered plants show that insect behavior is influenced by terpenoids. Terpenoids are derived from two Biosynthesis pathways. Both pathways lead to the formation of the C5 unit's isopentenyl diphosphate (IDP) and its allylic isomer dimethyl allyl diphosphate (DMADP), the basic terpenoid biosynthesis building blocks. In both compartments, IDP and DMADP are used by prenyl transferases in condensation reactions to produce larger diphosphates, such as the monoterpene precursor geranyl diphosphate (GDP), the sesquiterpene precursor farnesyl diphosphate (FDP) and the diterpene and C40 carotenoid precursor geranyl geranyl diphosphate (GGDP).

IMPROVEMENT IN GENETIC ENGINEERING WITH TISSUE CULTURE

Advances in tissue culture, combined with improvement in genetic engineering of pharmaceuticals, nutraceuticals and other beneficial substances (Hansen and Wright, 1999). Recent advances in the molecular biology, enzymology and fermentation technology of plant cell

cultures suggest that these systems will become a viable source of important secondary metabolites (Abdin, 2007). Genome manipulation is resulting in relatively large amounts of desired compounds produced by plants infected with an engineered virus, whereas transgenic plants can maintain constant levels of production of proteins without additional intervention (Abdin and Kamaluddin, 2006). Large-scale plant tissue culture is found to be an attractive alternative approach to traditional methods of plantation as it offers controlled supply of biochemical's independent of plant availability (Sajc et al., 2000). Current developments in tissue culture technology indicate that transcription factors are efficient new molecular tools for plant metabolic engineering to increase the production of valuable compounds (Gantet and Memelink, 2002).

In vitro cell culture offers an intrinsic advantage for foreign protein synthesis in certain situations since they can be designed to produce therapeutic proteins, including monoclonal antibodies, antigenic proteins that act asimmunogenes, human serum albumin, interferon, immuno-contraceptive protein, ribosomeun activator trichosantin, antihy persensitive drug angiotensin, leu-enkephalin neuropeptide, and human hemoglobin (Doran, 2000). The appeal of using natural products for medicinal purposes is increasing, and metabolic engineering can alter the production of pharmaceuticals and help to design new therapies. At present, researchers aim to produce substances with antitumor, antiviral, hypoglycaemic, anti-inflammatory, antiparasitic, antimicrobial, tranquilizer and immunomodulating activities through tissue culture technology.

IMPROVEMENT IN GENETIC ENGINEERING USING OF MICRO ORGANISM

Plants provide a treasure of secondary metabolites with medicinal or neutral ceutical value, but these compounds often can not be exploited adequately because of quantitative and other limitations. Additionally, only a few of these bioactive compounds are produced commercially as pure compounds in various quantities (Ververidis et al., 2007). The use of cell cultures had been considered for production purposes, but failed mostly due to poor performance and cost-benefit relations. Therefore, industrial scale production in bioreactor systems was only achieved for some few examples due to several bottle necks like long and complicated pathways, complex regulation mechanisms and the overall economic feasibility of such techniques. Producing natural compounds in engineered microorganisms has several economic and qualitative benefits, as high yields of a specific product, easy and nearly unlimited technical up scaling, simple downstream processing, access also to high complex pathways and compounds. Bioreactors are the key step towards commercial production of secondary metabolites by plant biotechnology. The bioreactor system has been applied for embryogenic and organogenic cultures of several plant species (Levin et al., 1988). Several compounds with high additive value such as isoprenoids and polyketides, flavors and fragrances, terpenoids and flavonoids could be the end products of such bioconversions. Prominent examples are the recent production of morphin alkaloids or the preparative formation of various flavonoids in *E. coli*. Recent progress concerning the production of flavonoids in *E. coli* and yeast cells is presented with special emphasis on the regulation of themetabolic grid, and the perspectives of this approach are summarized. Jeong et al. (2002) have established the mass production of transformed Panax ginseng hairy roots in bioreactor.

Hahn et al. (2005) has observed the production of ginsenoside from adventitious root cultures of Panax ginseng through large-scale bioreactor system (1 to 10 ton). Producing natural compounds in engineered microorganisms has several economic and qualitative benefits, as high yields of a specific product, easy and nearly unlimited technical up scaling, simple downstream processing, access also to high complex pathways and compounds. Several compounds with high additive value such as isoprenoids and polyketides, flavors and fragrances, terpenoids and flavonoids could be the end products of such bioconversions. Prominent examples are the recent production of morphin alkaloids or the preparative formation of various flavonoids in *E. coli*. Recent progress concerning the production of flavonoids in *E. coli* and yeast cells is presented with special emphasis on the regulation of the metabolic grid, and the perspectives of this approach are summarized.

However, Agrobacterium-mediated transformation offers several advantages over direct gene transfer methodologies such as the possibility to transfer only one or few copies of DNA fragments carrying the genes of interest at higher efficiencies with lower cost and the transfer of very large DNA fragments with minimal rearrangement (Shibata and Liu, 2000). The fact that important natural products are not only present in higher plants but are also produced by plant associated microorganisms has also been made for cytostatic compounds such as paclitaxel, vincristine and camptothecin.

CONCLUSION

To improve yields, metabolic engineering offers promising perspectives, but requires the understanding of the regulation of thesecondary metabolite pathways involved on the levels of products, enzymes and genes, includingaspects as transport and compartmentation. Engineering could lead to the improvement of many input

and output traits in crops. The production of secondary metabolites can be enhanced using bioreactors. Bioreactors offer a great hope for the large-scale synthesis of therapeutically active compounds in medicinal plants.

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