

Plant cell biotechnology for the production of secondary metabolites

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Abstract: Plant cells, which in general are shear-stress tolerant, can be cultured on a large scale in stirred bioreactors. The costs of a natural product, at a production of 3g/l, would be about 430 US \$/kg. To increase yields metabolic engineering seems to be a promising approach, but requires the understanding of the regulation of secondary metabolism at all its levels: genes, enzymes, products, transport and compartmentation. From *Catharanthus roseus* already several genes have been cloned and successfully expressed in among others tobacco and *C. roseus*.

Introduction

The past two decades plant cell biotechnology has evolved as a promising new area within the field of biotechnology, focusing on the production of plant secondary metabolites. For most compounds of interest, e.g. morphine, quinine, vinblastine, atropine, scopolamine and digoxin, one has so far not been able to come to a commercially feasible process. In contrast with the production of antibiotics by micro-organisms, the plant is an already existing, although not always a reliable, source of these compounds. This has limited the amount of research put into developing alternative biotechnological production for each of the products mentioned. Fifty years of extensive research on the production of penicilline has led to yields of 50 g/l and more. Although in plant cell biotechnology the research efforts have been diluted over numerous plants, still for some products one has been able to considerably increase the production levels, e.g. shikonin (3.5 g/l) (1) and berberine (7 g/l) (2). Presently research particularly focuses on the possibilities to apply metabolic engineering to improve yields to commercially interesting levels.

Here we will briefly discuss the technological and economical feasibility of plant cell cultures for the production of secondary metabolites. Moreover, the approach of metabolic engineering for increasing product levels will be illustrated with some recent results obtained in the field of the terpenoid-indole alkaloid production.

Technological and economical feasibility

In literature plant cells are described as extremely sensitive for shear forces, necessitating the use of special low-shear bioreactors, e.g. air-lift bioreactors. However, in industry such bioreactors are not common, most processes are runned in stirred-tanks. As a consequence, such a bioreactor is preferable for plant cell cultures, it is the lowest cost process-unit.

More recent studies on the shear sensitivity of plant cells, among others in our own laboratories, have shown that in fact plant cells in general are quite shear-stress tolerant (3,4,5,6). This is supported by the fact that a series of large scale processes have been reported with plant cell cultures, e.g. shikonin production (1). Plant cells have even been

cultured in a 60 m³ stirred tank (7).

The technology being feasible, how about the economy? A number of papers has appeared on this (8,9,10,11,12). Assuming a yearly production of 3000 kg/year of a compound produced by a cell culture at a level of 0.3 g/l, resulted in a calculated price of 1500 US \$/kg (11,12). An increase of productivity with a factor 10 (i.e. 3 g/l) results in a price of 430 \$/kg. In both cases a fed-batch type of process was applied. These prices are high, but a number of natural products have even much higher prices (e.g. taxol, vinblastine and vincristine). However, most of the high-value specialty chemicals are produced at too low levels in the plant cell cultures. Their production must thus be increased to make an industrial process possible.

Improving production

Several strategies are being followed to improve yields of secondary metabolites in plant cell cultures. First of all the screening and selection of high producing cell lines and the optimization of growth and production media can be mentioned as common approaches. In case of shikonin and berberine with success (1,2), in many others (e.g. see the examples mentioned above) with limited success.

In the past years new approaches have been developed: the culturing of differentiated cells (e.g. shoots, roots and hairy roots), induction by elicitors and metabolic engineering.

With the culture of differentiated cells one has in most cases been able to get production of the desired compounds in levels comparable to that of the plant, however the culture of such differentiated tissues on a large-scale in bioreactors is a major constraint. For studies of the biosynthesis, such systems are very useful.

The second approach mentioned, the use of elicitors has been successful in several cases. However, it remains limited to a certain type of compound for each plant, compounds which most likely act as phytoalexins in these plants.

Therefore attention is more and more focused on metabolic engineering. How could metabolic engineering be used to increase yields? Several possibilities can be envisaged:

- increase activity of enzymes which are limiting in a pathway;
- induce expression of regulatory genes;
- block competitive pathways;
- block catabolism.

The first two possibilities require the expression of genes yielding active enzymes, the latter two approaches blocking of genes by antisense genes. In all cases the respective biosynthetic pathway has to be known on the level of products, enzymes and genes, as well as the regulation on all these levels, including aspects as compartmentation and transport. In our studies of the production of terpenoid indole alkaloids we have cloned genes coding for tryptophan decarboxylase (TDC) (13), strictosidine synthase (SSS) (14) and NADPH:cytochrome P-450 reductase (15). The latter enzyme serves all cytochrome P-450 enzymes in the plant, among others geraniol-10-hydroxylase (G10H), a key enzyme in the terpenoid part of the alkaloid biosynthesis. The gene encoding for G10H has not yet been cloned, despite the fact we have been able to purify the enzyme to homogeneity (16). The cloning of the gene is hampered by the fact that the plant contains many, very similar, P-450 genes. By means of PCR, 18 closely related genes coding for P-450 enzymes were detected in *Catharanthus roseus* (17).

The *tdc*-gene has been expressed in tobacco plants, resulting in an active enzyme (18,19). These plants produce upto 1% of DW in tryptamine, i.e. similar levels as found for the tryptamine-derived indole alkaloids in plants producing such compounds. The activity of anthranilate synthase, the first committed enzyme of the tryptophan-pathway from chorismate,

was not increased in the tryptamine producing transgenic tobacco plants (19). Plants can apparently make about 1% of its DW in tryptophan derived compounds with its normal primary metabolic machinery. Introduction of the *tdc*-gene into *C. roseus* resulted in callus cultures showing up to 10-fold increased levels of TDC activity and a concomitant increase of tryptamine levels, but no significant increase of strictosidine or other alkaloids (20). This supports other observations that the terpenoid part of the pathway is also a limiting factor. The *tdc*- and *sss*-genes are both single copy genes. Both are repressed by auxins and induced by elicitation. Because of this similar regulation, they might be controlled by one trans-acting factor. Identification of this factor and the encoding gene is of great interest, as such a regulatory gene might also control further steps in the biosynthetic pathway. The cloning of such a regulatory gene would avoid the need to clone a large number of genes coding for the individual steps in the biosynthetic pathway.

In *C. roseus* upon elicitation a 25-50% increase is observed in the activity of AS, TDC and SSS (Moreno et al. in preparation), whereas the activities of PAL decreases and chalcone synthase is not affected. However, the major change is observed in the content of phenolics, 2,3-dihydroxybenzoic acid (DHBA) being the major compound formed (Moreno et al. submitted). At the same time a strong induction is seen of the enzyme isochorismate synthase, which convert chorismate into isochorismate. In bacteria this pathway is known to lead to DHBA. This compound has antimicrobial activity, particularly in combination with UV-light and has also antifeedant properties. It is thus likely to be a major defense compound. Phytoalexin-biosynthesis in *Cinchona* cell cultures (anthraquinones) also use chorismate as precursor as well as mevalonic acid (21). In *Tabernaemontana* species mevalonate is used for the triterpene biosynthesis after elicitation, channelling away this precursor from the alkaloid pathway (22). Blocking such pathways by means of antisense genes might be of interest to increase the availability of these precursors for the alkaloid biosynthesis.

Catabolism is another important factor in the accumulation of the alkaloids. Studies feeding ¹⁵N or ¹⁴C labeled alkaloids to *Tabernaemontana divaricata* and *C. roseus* cell cultures showed that at a certain point of the growth phase the rate of catabolism equals the de-novo biosynthesis (23,24). Identification of the enzymes involved and the cloning of the encoding genes is thus of interest to eventually use antisense gene technology to block catabolism and thus increase the production.

Conclusions

Plant cell cultures provide an excellent system for studying biosynthesis of secondary metabolites. For the large scale production of these compounds in most cases production is too low for a commercialization. To improve yields metabolic engineering offers promising perspectives, but requires the understanding of the regulation of the secondary metabolite pathways involved on the levels of products, enzymes and genes, including aspects as transport and compartmentation. Unraveling the regulation requires a joint effort of several disciplines, e.g. phytochemistry, plant physiology, cell biology and molecular biology.

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