Application of Recombinant DNA Technology to Studies on Plant Secondary Metabolism

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Abstract

This review which is concerned with the application of recombinant DNA technology to studies on plant secondary metabolism, presents the more common plant transformation strategies and shows how these genetic approaches are being used in attempts to manipulate and increase the yield of secondary metabolites, both in cultures and in transformed plants. The different plant transformation strategies reviewed here are: infection with intact Agrobacteria; particle bombardment, vacuum infiltration and floral dip; viral vectors and finally protoplast fusion. The review continues with examples of the application of several of these transformation strategies in the manipulation of secondary metabolism. These are outlined under four subheadings which include developmentally regulated genes, addition of novel genes, down-regulation of specific genes and insertion of regulatory genes. Finally, under concluding remarks, reference is made to the advances achieved in the manipulation of plant secondary metabolism and how these approaches may impact on this new

1.- Introduction

Plants produce an array of natural products, the so-called secondary metabolites, which play a variety of roles such as pollinator attractants (e.g. pigments and scents), and defence molecules against attacks by animals and microorganisms. These substances are also important to man as a source of pharmaceuticals, fragrances, agrochemicals and food additives. However, despite great efforts by the chemical industry to mimic and synthesise specific plant secondary metabolites, little success has been achieved and plants still remain the major source of many vital medicinal compounds (Wink, 1990). As most of these compounds originate from plants, any factor (e.g. climatic, political, etc.) which affects the continued supply of these molecules will endanger world supply. In the late 1970s plant cell culture was seen as an alternative, or additional way of producing these compounds, since it was known that plant cells could be readily cultured and produce useful secondary metabolites (see Alfermann & Peterson, 1995). Nevertheless, the low yields obtained with cultured cells, often inferior to the amounts present in intact plants, provided a major drawback to their commercial exploitation. However, a few projects were successful, for instance the production of shikonin and berberine by cell cultures of Lithospermum erythrorhizon and Coptis japonica respectively (Fujita 1988).

Many strategies have been tried in attempts to increase product yield, including for instance the induction of differentiated cell cultures which are known to have a higher biochemical potential (Yeoman & Yeoman, 1996). Indeed, in some cases higher yields of

metabolic intermediates or end-products were attained using this approach. Indeed, established hairy root cultures transformed following infection with Agrobacterium rhizogenes displayed enhanced production of those secondary metabolites which occur naturally in untransformed roots, resulting in amounts of secondary compounds comparable or even higher than those present in intact roots (e.g. Sharp & Doran, 1990; Zárate, 1999). Over the last 10-15 years, the successful genetic transformation of plants has been reported in about 200 species including agricultural crops, trees, ornamentals, fruits and vegetables. Such genetic modification has improved specific crop traits, such as resistance to pathogens, to herbicides and to various environmental factors including drought and floods (Bajaj, 1999). Following on from this success genetic transformation of medicinal plants has been attempted, primarily to enhance the production of various pharmaceuticals, but also flavours, and pigments. Transgenic cultures and plants, have been reported for about 70 of these species (Bajaj, 1999), and interesting results are already appearing. Some of this research was directed towards the control of metabolism. In 1991 Bailey suggested that metabolic engineering of biosynthetic networks might be achieved by application of recombinant DNA methods, but also suggested how complex cellular responses to genetic perturbation could complicate the predictive metabolic design.

In this short review attention is focussed on the different plant transformation methods which have been used successfully and is followed by a series of examples of how these approaches have worked and how they may develop in the future.

2.- Plant transformation strategies

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. In this section the most common plant transformation strategies are presented, these include *Agrobacterium* mediated transformation and direct gene transfer. In addition, the applications, advantages and drawbacks of the various methods are also considered.

2a.- Infection with intact Agrobacteria

The gram-negative soil bacteria *Agrobacterium tumefaciens* and the related species *A. rhizogenes* are causal agents of the plant diseases crown gall tumour and hairy root respectively. These species, which belong to the Rhizobiaceae, are natural metabolic engineers able to transform or modify, mainly dicotyledonous plants (Tepfter, 1990), although there are reports on the infection of monocotyledonous plants (Hiei et al., 1994; Ishida et al. 1996), and *A. tumefaciens* has been reported to transform other bacteria and yeast (Bundock et al. 1995).

Agrobacterium rhizogenes has been used regularly for gene transfer in many dicotyledonous plants (Tepfer, 1990). Plant infection with this bacterium induces the formation of proliferative multibranched adventitious roots at the site of infection, the so-called "hairy roots" (Chilton et al., 1982). This infection is followed by the transfer of a portion of DNA i.e. T-DNA, known as the root inducing plasmid (Ri-plasmid), to the plant cell chromosomal DNA. In contrast the tumour inducing plasmid (Ti-plasmid) is present in *A. tumefaciens*.

The Ri-plasmid shares large functional homologies with the Ti-plasmid and appears to have evolved from a common ancestor (Sinkar et al. 1987). The Ri-plasmid consists of a T-DNA, a border sequence and a virulence area. The T-DNA contains the *rol* (root loci) genes

A, B, C, D, which confer the capacity to differentiate into roots on transformed cells. Besides, different opine synthase genes are also present which activate the synthesis of different classes of opines, as well as their catabolism. Opines are unique natural substances, pseudoaminoacids such as octopine, nopaline and agrocinopine, which serve as a nutrient source of carbon and nitrogen, and as specific growth substances for the pathogenic bacteria. For further details the readers are referred to two elegant papers dealing with the different opines and their biochemistry (Petit et al., 1983; Dessaux et al., 1993). The virulence area containing different silent *vir* genes does not enter the plant genome but are required for T-DNA transfer. These genes are activated by wound tissue metabolites, such as lignin precursors and acetosyringone (Melchers et al., 1989) which may explain why tissue wounding appears to be a prerequisite for efficient infection. A large body of research has been devoted to the application of plant transformation and genetic modification using *A. rhizogenes*, in order to boost production of those secondary metabolites which are naturally synthesised in the roots of the mother plant. Transformed hairy roots mimic the biochemical machinery present and active in the normal roots, and in many instances transformed hairy roots display higher product yields.

In a similar fashion, *A. tumefaciens* is also able to infect plant material. A transfer T-DNA is inserted and its Ti-plasmid induces genetic and metabolic changes in the plant cell which result in crown gall disease. These alterations to the biosynthesis of plant growth regulators, which are involved in the regulation of cell division, provoke uncontrolled cell growth and tumour formation. As mentioned for *A. rhizogenes*, the Ti-plasmid of *A. tumefaciens* also harbours genes encoding enzymes responsible for the synthesis and catabolism of the novel aminoacid-like compounds *i.e.* opines which provide a nutrient source. The type of opine formed, such as octopine, nopaline, and agrocinopine, is dependent on the class of *Agrobacterium* strain used and often represents an identifying character to distinguish the strain of *Agrobacterium* employed.

Likewise, as described for the case of *A. rhizogenes*, the process of *A. tumefaciens* T-DNA transfer to the plant genome is triggered by the release of wound phenolic compounds, necessary for the activation of the various *vir* genes which facilitate infection. This complex process is elegantly described by Hooykass (2000). How the T-DNA integrates into the plant genome is not fully understood but it is considered to resemble illegitimate recombination. It appears as a random process with multiple copies often being inserted; nevertheless, once integrated, the T-DNA is maintained stably. Contrary to other gene insertion methods, the plant transgenic lines produced via infection with *Agrobacteria* often contain one copy or a low copy number of the T-DNA, although cell lines with multiple T-DNA copies at one of more loci in the genome can also be encountered. Besides, at much lower frequencies partial, truncated T-DNA copies may be present, or T-DNAs which are accompanied by fragments of the original binary vector. Following this strategy, integration of the T-DNA occurs at random positions in the genome, although there appears to be a preference to insert into transcriptionally active regions.

The importance and advantage of using the *Agrobacteria* system is that by genetic engineering and DNA recombinant technology it is feasible to remove most of the genes in the T-DNAs of both *A. tumefaciens* and *A. rhizogenes*. Subsequent cloning of the desired foreign gene(s) follows, which can then be co-transformed and integrated into the host genome after infection where it can encode specific enzymes dealing with the formation of wanted metabolites or other goals.

In conclusion *A. tumefaciens* has been used directly to genetically modify many plants, and in most cases this requires the tedious and time consuming process of *in vitro* culture and plant regeneration. In addition, two other major problems are encountered when using *Agrobacteria*, these are susceptibility and hypersensitivity of some plant cells or tissues. The

latter leads to tissue necrosis and cell death, although the use of antioxidants such as polyvinylpolypyrrolidone and dithiothreitol can restore plant viability and inhibit necrosis (Perl et al. 1996).

2b.- Particle bombardment

This is another technique of direct DNA delivery, successfully applied to achieve plant transformation; in particular it is effective with recalcitrant species such as most monocotyledons and some dicotyledons. Moreover, it has also been employed to transform animals, fungi and bacteria (Smith et al. 1992; Toffaletti et al. 1993; Johnston et al. 1990). Particle bombardment, also known as Biolistic (biological and ballistic), was developed and first described by Sanford et al. (1987) and Sanford (1988). The technique uses high velocity particles or microprojectiles coated with DNA to deliver exogenous genetic material into the target cell or tissue, which is then cultured *in vitro* and regenerated to produce mature transformed plants.

The particles, either tungsten or gold, are of small size (0.5-5 µm) but big enough to have the necessary mass to be accelerated and able to penetrate the cell wall carrying the coated DNA on its surface which once integrated into the cell nucleus can be expressed. Gold particles are chemically inert, although rather costly, and present more uniformity. Tungsten particles, although with some phytotoxicity (Russell et al. 1992), and of more variable size, are adequate for most studies. Furthermore, the chosen microprojectile should also have good DNA affinity but, at the same time, be able to release it once it has hit the target. DNA coating of surface sterilized particles can be accomplished by binding the DNA, using for instance the calcium chloride method, (Klein et al. 1988) with the addition of spermidine to protect the DNA. However, recently a report describes the novel use of *Agrobacterium* as coating material for the microprojectiles which are then shot into the target tissue, this procedure has resulted in the stable transformation of strawberry plants (Cordero-Mesa et al. 2000). Once coated the particles are ready for shooting and in some cases macrocarriers are employed to support and accelerate the particles. The macrocarrier is retained by a screen or stopping plate and the particles continue travelling and collide with the target. Most frequently, microparticles are accelerated under partial vacuum, and helium pressure may be employed to produce the necessary blast to propel the coated microprojectiles (Finer et al. 1999). Often a spreading screen or metal mesh of specific pore size is located between the point of blast and the target to ensure spreading of the coated particles to achieve a more even collision with the target.

Particle penetration can be controlled by altering several parameters, these include the size of the particles, the distance between the sample holder and the target, the pressure applied to blast the particles, or the presence of a spreading screen used to disperse the particles before hitting the target. In many instances, these parameters are varied to achieve the best conditions to transform a specific material.

The DNA, delivered utilising this direct gene strategy, can be expressed after reaching the nucleus. Presumably most of the coated particles are either degraded or inactivated and only a few reach the nucleus, where some would become stably integrated and expressed and in other cases be expressed in an unexpected manner. Furthermore, frequently fragments of the plasmid used to harbour the gene(s) of interest are also inserted together with intact plasmids resulting in some cases to single insertions or more often multiple insertions. However, a high copy number of the introduced gene(s), does not result in or imply a higher gene expression.

The expression of the gene(s) is dependent on the nature of the DNA as well as the final physical rearrangement of the foreign DNA in the plant genome, the so-called position

effect. When landing in a transcriptionally active region it may be expressed at a high rate, whereas if it integrates within a non-active area, gene expression may be reduced. This appears to indicate that direct DNA delivery by means of particle bombardment does not show a preference for insertion sites, contrary to *Agrobacterium* mediated transformation where DNA tends to be inserted in transcriptionally active regions (Ingelbrecht et al. 1991).

2c.- *Vacuum infiltration and floral dip*

The vacuum infiltration DNA delivery technique utilises *Agrobacterium tumefaciens* as the DNA vector carrier, together with plants of *Arabidopsis thaliana* at an early flowering stage. The plants are uprooted, and *A. tumefaciens* applied to the intact plants by vacuum infiltration which is achieved by immersion in an appropriate liquid growth medium containing the *Agrobacterium*. Subsequent to replanting, growth and collection of seeds assessment for transformants in the progeny is made by selection using antibiotics or herbicides (Bechtold et al. 1993). Using this procedure, it has been shown, in some detail, that the ovules of *A. thaliana* appear to be the target for *A. tumefaciens* in transformed plants (Ye et al. 1999).

A clear advantage of this approach is that it avoids the necessity for tissue culture and plant regeneration, although it has to be remembered that this strategy has only been successful with a few species, mainly *A. thaliana* but recently with *Medicago truncata*. Clearly species which develop a large number of flowers together with a higher number of set seeds following infiltration are best. In the case of *A. thaliana*, approximately 1-5% would be transformants, equal to 394 transformed seeds per infiltrated plant (Ye et al. 1999).

A novel modification of the vacuum infiltration procedure and a simple method to transform *A. thaliana* is the floral dip (Clough & Bent, 1998). This technique of floral dipping eliminates the labour-intensive vacuum infiltration process. Plants are collected at an early flowering stage, when most of the flowers are still immature. The attached flower buds are immersed in a solution containing a mixture of *A. tumefaciens* cells, sucrose and a surfactant. The appropriate amounts and ratio of these two latter ingredients appears to be crucial for a successful transformation. Furthermore, repeated dipping in *A. tumefaciens* as well as covering the infected plant for one day after bacterial infection, was shown to increase transformation about twofold (Clough & Bent, 1998). After reports of the success of this technique several research groups have applied *A. tumefaciens* by spraying yielding satisfactory results.

Plant transformation following these two methods produces genetically uniform progeny and obviates the somaclonal variation associated with tissue culture and regeneration. Furthermore, these methodologies are not `high tech´ and do not require costly equipment. Unfortunately, very few plant species apart from *A. thaliana* seem to respond to these methods of transformation (Bent, 2000)

2d.- *Viral vectors*

Several plant viruses have been used as vectors for the insertion of foreign gene(s) into a variety of plant species. However, the quest for a plant virus to enable an easy conversion has not yet been achieved. The large majority of plant viruses have an RNA genome, and just a few are of single or double stranded DNA. Viruses offer large advantages on transient gene expression, they are highly multiplicative, originate a large number of copies of the transgene as well as its expression; moreover, the viruses can propagate easily and spread rapidly to other plant parts and to other plants.

One of the first plant viruses studied was the cauliflower mosaic virus (CaMV) a double stranded DNA virus that replicates through an RNA intermediate (Gronenborn et al. 1981). Despite being considered an ideal candidate because of its small size (8kb) and being

double stranded (DNA), extra DNA insertions are difficult to achieve and often molecular recombinations rapidly eliminate the inserted transgene. Besides, replication of the virus is complex and requires an RNA step which can introduce errors because the inverse transcriptase does not possess a known proof-reading activity, also the CaMV host range is rather limited. Nevertheless, two RNA viruses have been successfully used as vectors. These are the tobacco mosaic virus (TMV) and cowpea mosaic virus (CPMV) (Wilson & Davies, 1992). Two different approaches have been used to clone and insert foreign gene(s) using plant viruses. One is to replace the coat protein of the virus by a transgene or insert the gene(s) next to the initiation codon of the coat protein which will then express the product of the inserted gene and the coat protein. However, in some instances, it has been observed that the inserted gene(s) was removed by the virus thus returning to its original form. Furthermore, the propagation of the virus and the inserted transgene in the plant depend on the presence of a coat protein (movement protein MP) which is needed to spread the transgene within the plant.

Several drawbacks have been reported by those using plant viruses as vectors. These include: their instability, evidenced by the lost of infectivity when compared with intact viruses, and elimination of the transgene when this is larger than 1kb. Unfortunately, the mechanism(s) of viral elimination is not fully understood and therefore not controllable. Indeed, it seems that the ease of insert elimination might be an evolutionary strategy present in the virus to avoid the accumulation of unwanted or unnecessary genomic material which does not provide any advantage.

2e.- Protoplast Fusion

Protoplasts are plant cells in which the cell wall has been removed. Therefore protoplasts can behave like animal cells which have no cell wall barrier. Plant regeneration from single protoplasts is possible due to the totipotency of plant cells, hence plant regeneration from a single cell.

Removal of the cell wall is achieved by treating the plant material (leaves, tissue cultures, suspended cells, etc.) with a cocktail of enzymes including pectinases, cellulases, and/or hemicellulases (Warren, 1991) in an appropriate incubation medium of the right osmolality. After removal of the cell wall, the protoplasts must be kept immersed in a solution of the appropriate concentration to prevent bursting of protoplasts. Also the protoplasts must be incubated in a culture medium of the correct osmolality until wall formation occurs.

Different approaches exist for the insertion of transgenes into protoplasts, either by fusion of different protoplasts or DNA insertion into the protoplast through the plasmamembrane (Lindsey, 1992). These include: (a) chemical techniques, (b) electrical techniques or (c) microinjection. (a) *Chemical Techniques*: several methods have been described, polyethylene glycol (PEG), Ca^{++}-DNA co-precipitation and liposomes. PEG is the most widely used, employing solutions of 10-15% PEG, with high calcium content and high pH. After mixing isolated DNA and protoplasts, followed by different washes, DNA protoplast fusion takes place. Here PEG alters the plasmamembrane properties causing reversible permeabilization enabling exogenous macromolecules to enter the cell cytoplasm. Ca^{++}-DNA co-precipitation: formation of a co-precipitate of plasmid DNA and calcium phosphate is required. On contact with protoplasts, the co-precipitate trespasses the cell membrane in the presence of high calcium concentration and high pH. Liposomes, these are negatively-charged spheres of lipids, are also employed for DNA transfer. DNA is first encapsulated into the liposomes and these are fused with protoplasts employing PEG as a fusogen. (b) *Electrical Techniques*: electrical pulses are applied to the DNA protoplast

mixture provoking an increase in the protoplast permeability to DNA. It is much simpler than the chemical method giving attractive results. However, the electrical pulses must be carefully controlled as cell death can occur above a certain field threshold. These pulses create the transient formation of micropores in the lipid bilayer which last for a few minutes, allowing time for DNA uptake. (c) Microinjection: is an old technique originally designed to transform animal cells, later it gained importance and interest in transforming plant cells. However, in plant cells the existence of a tough cell wall, a natural rigid barrier, as well as the presence of vacuoles which can produce cell death after breakage due to the release of hydrolases and toxic metabolites, and in some instances where vacuoles surround the nucleus make microinjection impossible. Therefore, protoplast rather than intact plant cells are more suitable for microinjection, and thus subsequent genetic modification (Songstad et al. 1995). Clearly, this method is rather labour intensive and requires specialised microequipment for the manipulation of host protoplasts and DNA. However, some success in transforming both monocotyledonous and dicotyledonous species has been achieved employing this technique.

3.- Approaches, systems and metabolites

In the previous section a series of plant transformation strategies were outlined. Here, an attempt will be made to show how these approaches have been used or can be used to manipulate secondary metabolism. It is generally accepted that one of the chief problems encountered when cultured plant cells or tissues are used to accumulate secondary metabolites is low yield of product. Lately, and with the rapid advances made in recombinant DNA technology, it is now possible to tailor secondary metabolism to obtain higher yields of the designated product and to shed some light into the control of these complex metabolic processes. Indeed, to this end many enzymes have been purified, characterised and cloned, and many biosynthetic steps recognised and subsequently manipulated.

3a.- Developmentally regulated genes
It is well known that many secondary metabolites are restricted to a specific organ, tissue or cell type, which may be the site of synthesis, or accumulation or both. Therefore, it would appear that some genes are only expressed in specific tissues, for instance, it is known that some metabolic pathways are confined to roots. For example tropane alkaloid biosynthesis is known to be developmentally regulated and occurs in a specific cell type (Nakajima & Hashimoto, 1999; Suzuki et al., 1999). Thus, *Agrobacterium rhizogenes* transformed hairy root cultures have been used to try to increase the yields of these metabolites, which are naturally synthesised and accumulated in intact plant roots. Indeed, for this reason hairy roots have been employed to study production of root metabolites in a range of species. Hairy roots of *Rubia tinctorum* (van der Heijden et al. 1994) produce comparable amounts of anthraquinones to intact roots with nordamnacanthal, which displays antifungal activity, as the major accumulated compound. Similarly, hairy root cultures of the endangered species *Atropa baetica* (Zárate, 1999) display high accumulation of the major tropane alkaloids, atropine (±hyoscyamine) and scopolamine, with atropine levels similar to intact non-transformed roots. Surprisingly, scopolamine levels were 4-fold higher than intact roots, suggesting a much higher H6H activity (hyoscyamine 6ß-hydroxylase) (Hashimoto et al., 1991) the enzyme responsible of the conversion of hyoscyamine into scopolamine (Fig. 1). Furthermore, and in contrast to other reports on hairy roots of *A. belladonna* producing

tropane alkaloids (Falk & Doran, 1996, Sharp & Doran, 1990), hairy roots of *A. baetica* released considerable amounts of these metabolites into the surrounding liquid medium, and this product accumulation did not seem to be growth associated. Currently, research is underway investigating the effect of overexpressing the *h6h* (hyoscyamine 6ß-hydroxylase) gene under the control of a constitutive CaM 35S promoter in a transgenic hairy root culture of *A. baetica* (Zárate unpublished).
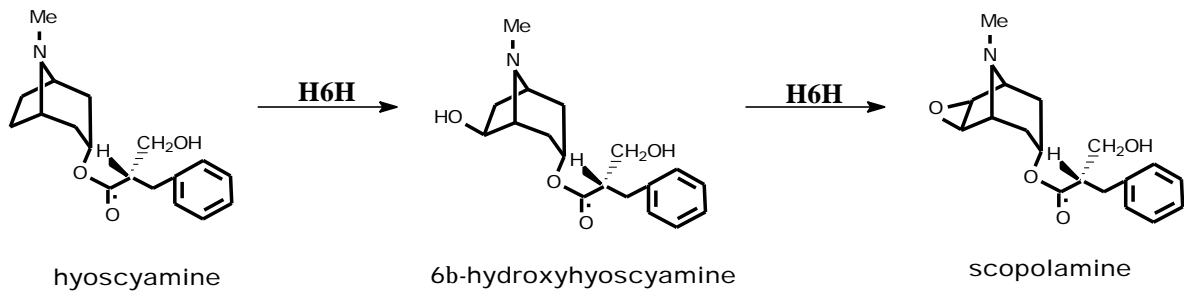
Figure 1: Reactions catalysed by hyoscyamine 6β–hydroxylase, the last steps in the metabolic pathway leading to the synthesis of tropane alkaloids in various Solanaceous species. The enzyme is bifunctional, displaying hydroxylase as well as epoxidase activity.

Progress has also been made in producing genetically modified plants to yield pharmaceutically important metabolites. For instance, transgenic *A. belladonna* plants have been obtained by introducing the *h6h* gene from *Hyoscyamus niger* under the constitutive control of the CaM 35S promoter following the *A. tumefaciens* method (Yun et al., 1992). The regenerated transgenic plants showed a dramatic change in alkaloid composition with scopolamine being the predominant alkaloid present in the aerial parts, and in the branched roots the highly efficient conversion of hyoscyamine into scopolamine was partially enhanced but only small increases were observed. In addition hairy roots of *Hyoscyamus muticus* have been genetically engineered to obtain enhanced amounts of scopolamine over hysocyamine (Jouhikainen et al., 1999). The 35S-*h6h* transgene encoding the H6H enzyme (Fig. 1) was inserted into *H. muticus* via *A. rhizogenes* infection. The authors reported a great variation in tropane alkaloid production among the different transformants with the best line producing over 100 times more scopolamine than the control. However, conversion of hyoscyamine into scopolamine was not total and hyoscyamine remained as the major alkaloid. Furthermore, the expression of H6H was found to be proportional to scopolamine production.

3b.- *Addition of novel genes*

This is another approach to induce organisms to replicate heterologous cDNA(s) and to express a foreign gene(s) in a host organism. Single step enzymes, as well as part of or all of a complex pathway can be transferred from one organism to another. Recently, an elegant report describes the insertion of a short peptide sequence originally isolated from frog skin, a magainin analog cDNA, to tobacco plants conferring on the plant higher resistance to blue mold disease (Li et al. 2001). A magainin analog peptide Myp30 was found to inhibit spore germination of the oomycete *Peronospora tabacina*, the causal agent of blue mold disease in

tobacco, *in vitro*, and growth of the bacterial pathogen *Erwinia carotovora* subsp *carotovora*, an organism lethal to young tobacco seedlings. Two plant expression constructs were made and introduced into tobacco plants. In one construct (AMY) the peptide was targeted to the cytoplasm, and in another construct (APM) the myp30 sequence was fused with the signal peptide sequence from a tobacco pathogenesis-related protein 1b which targets the Myp30 peptide to an extacellular location. It was found that leaves of tobacco plants expressing myp30 with the peptide directed to an extracellular location (APM) showed a significantly decreased susceptibility to *P. tabacina* infection when compared to control plants and plants with Myp30 peptide targeted to the cytoplasm (AMY). Contrarily, transgenic plants both, those expressing the peptide magainin analog extra or intracellularly, displayed significant resistance to the bacterial pathogen. This Indicates that expression of the gene encoding the magaining type peptide in transgenic plants can increase resistance to the pathogen.

Sekiguchi et al. (1999) have also shown that transformed hairy roots cultures of chilli pepper (*Capsicum frutescens* cv. cayenne) containing the CaM 35S promoter linked to the parsley PAL-2 cDNA displayed an increase in PAL activity at early and late stages of culture. These transformed roots also showed alterations to the metabolism of aromatic compounds such as ferulic acid and lignin-like substances increasing lignification of the induced hairy roots; similarly, the aminoacid content was also altered. Moreover, the increased PAL activity in this culture was associated with slow, abnormal growth of the transformed hairy roots which could be a consequence of increased lignification.

Successful attempts in which the insertion of more than one gene of a known pathway into a host organism have also been reported. For instance, following particle bombardment of tobacco leaves and plant regeneration the expression of two consecutive genes of the terpenoid indole alkaloid pathway of *Catharanthus roseus* ? a well known species able to accumulate the two potent anticancer drugs vincristine and vinblastine? encoding tryptophan decarboxylase (TDC) and strictosidine synthase (STR1) in tobacco plants has been reported (Leech et al. 1998). TDC and STR1 are two adjacent pathway enzymes that together form strictosidine which is an important intermediate of over three thousand indole alkaloids (Fig. 2), many of which possess important pharmaceutical properties. Both *tdc* and *str1* genes are absent in tobacco plants. Analysis of transgenic plants at the RNA and DNA levels demonstrated a range of integration events and steady-state transcript levels for both transgenes besides a 100% co-integration of both transgenes. A comparison of Southern and Northern data suggested that in 26% of the plants both *tdc* and *str1* were silent, 41% demonstrated a preferential silencing of either of the transgenes, with the remaining 33% of the plants expressing both transgenes. The authors did not observe a clear correlation between the number of integration events of a specific transgene and the levels of accumulated transcipt. Although the *tdc* and *str1* transgenes cointegrated into the tobacco genome differential expression of them was recorded. The resulting enzyme activities were indeed very high. TDC originated tryptamine levels of 5-119 µg per mg protein and represented a 5-fold increase as compared with previous published reports. In addition, STR1 activity was 1.1-6.1 µkatal per mg protein, which is 3-22 times higher than at present in *C. roseus*. Therefore, the introduction of these genes by particle bombardment generates diversity at the level of enzyme activity and for *tdc* at the level of product accumulation.

Similarly, the following gene involved in the terpenoid indole alkaloid pathway of *C. roseus sgd* (strictosidine ß–D glucosidase) (Fig. 2) has been introduced via *A. tumefaciens* and expressed in suspended tobacco cells (R. Zárate et al., 2001). The recorded SGD activity in some of the cell lines appeared to be growth associated but in others was steady throughout culture growth, indicating continuous activation of the inserted transgene triggered by the CaM 35S constitutive promoter. Maximal SGD activity of ca. 170 pkat per mg protein was recorded following incubation of a crude protein extract of the transgenic culture.

Furthermore, the molecular data show, as seen in intact *C. roseus* cells, that SGD activity was associated with a protein conformation of 650kDa in size, and this was absent in control or transgenic lines
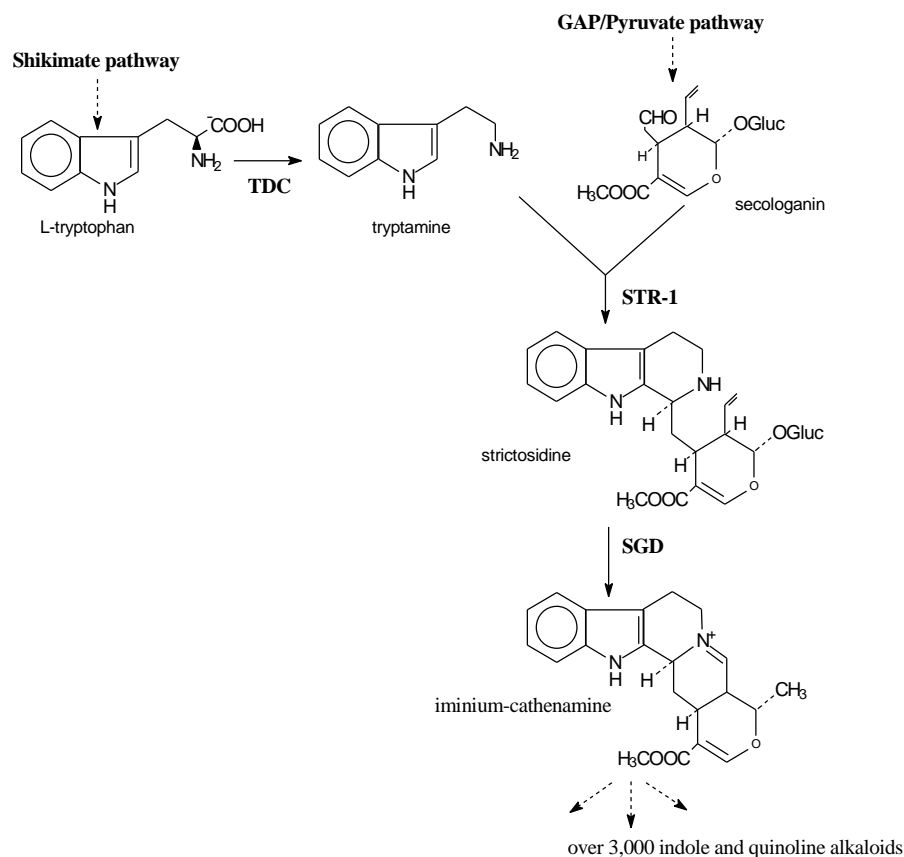
Figure 2: Partial illustration of the biosynthetic pathway of terpenoid indole alkaloids in *Catharanthus roseus* leading to the formation of the intermediate strictosidine, central precursor of over three thousand indole and quinoline alkaloids. (TDC tryptophan decarboxylase, STR-1 strictosidine synthase, SGD strictosidine glucosidase, GAP glyceraldehide-3-phosphate).

which failed to show any SGD activity. Furthermore, genetic transformation of the recalcitrant *C. roseus* plant via particle bombardment has resulted in insertion of the reporter genes (gus, gfp) (Zárate et al., 1999), suggesting that the insertion of the above mentioned terpenoid indole alkaloid genes could be usefully pursued.

There is also an account where a heterologous whole secondary metabolic pathway was expressed in a host plant (Ye et al. 2000) following *A. tumefaciens* mediated transformation. These authors introduced the entire ß-carotene biosynthetic pathway, vitamin A precursor, into rice endosperm in a single transformation effort with three vectors harbouring four transgenes; *psy* plant phytoene synthase, *crt-1* bacterial phytoene desaturase, *lcy* lycopene ß-cyclase, and *tp* transient peptide. In most cases the transformed endosperms were yellow indicating carotenoid formation, and in some lines ß-carotene was the only carotenoid detected. This elegant report illustrates how the nutritional value of a major staple food may be augmented by recombinant DNA technology.

3c.- Down-regulation of specific genes

Many factors can affect a pathway leading to the synthesis and accumulation of a desired end product. These include control by rate-limiting enzymes ? often criticised since most of the enzymes in a pathway are coordinately regulated? , feedback inhibition, caused by the addition or endogenous excess of intermediates, and competition between pathways. Competitive metabolic pathways can be blocked selectively by means of transformation with antisense RNA, RNA transcribed in reverse orientation from ´sense´ or mRNA which binds the messenger sequences and prevents translation. This approach has been employed successfully for instance, caffeine-free coffee plants are being engineered by a research group in Hawaii led by Stiles (see brief report in Newscientist, Coghlan, 1998). These workers identified a master encoding gene (xanthosine-N7-methyl transferase), which governs caffeine production. Via *A. tumefaciens* transformation, the authors introduced an antisense gene construct of this encoding gene which virtually prevents caffeine production in regenerated tissues. Silencing of this gene in all the propagated material appears necessary as the coffee beans did not seem to possess much of the enzyme, although these are the main repositories of caffeine. Enzyme activity in untransformed coffee plants is present chiefly in the leaves, where caffeine synthesis seems to take place which is then transported to the beans. Therefore, the gene would need to be silent during the early developmental stages of the regenerated plants so that there would be no caffeine synthesis and therefore no transport to the beans.

Another interesting gene down-regulation using antisense RNA technology was that contained in an earlier report by van der Krol et al. (1988) who genetically manipulated the flavonoid flower pigments in *Petunia hybrida* by inserting an antisense *chs* (chalcone synthase) gene. This resulted in pigmentation changes in the transgenic plant flowers indicating the potential of this approach.

3d.- Insertion of regulatory genes

A completely different approach to the control of metabolism is by the insertion of regulatory genes or genes inducible by exogenous signals (i.e. elicitors, stress stimulus, etc.) which subsequently activate other gene(s) present in a given pathway, the signal transduction regulatory gene(s). It is widely accepted that biosynthesis of many secondary metabolites in plants can be induced by the stress inducing molecule jasmonate. In *C. roseus* the gene *Orca3*, a jasmonate-responsive APETALA2 (AP-2) domain transcription factor has been isolated (van der Fits & Memelink, 2000). Overexpression of *Orca3* in *C. roseus* cultures resulted in enhanced expression of several biosynthetic genes *tdc*, *str*, *sgd* (Fig. 2), *cpr* (cytochrome P450 reductase), and *dh4* (desacetoxy vindoline 4-hydroxylase) and resulted in enhanced accumulation of certain terpenoid indole alkaloids. However, other genes such as, *g10h* (geraniol 10-hydroxylase) and *dat* (acetyl CoA 4-O--deacetyl vindoline) were not induced, suggesting that these two latter genes were not controlled by *orca3*. These authors also reported that *Orca3* appears as a regulator of primary as well as secondary biosynthetic genes involved in the production of terpenoid indole alkaloids. These findings uncover a control mechanism(s) that may be operative in other cases of stress-responsive secondary metabolite biosynthesis, and opens up the possibility of applying this type of control to alter yields of secondary metabolites (for further reading on this topic see Memelink et al., 2000).

4.- *Concluding remarks*

In this review, we have attempted to present a range of the more common plant transformation strategies and to show how these various genetic approaches have been employed in attempts to manipulate and increase the yield of secondary metabolites both in cultures and in transformed plants.

Spectacular advances have been reported since an earlier publication (Yeoman & Yeoman, 1996) where the then current approaches for the manipulation of secondary metabolism were reviewed. In addition to the early strategies employed to increase secondary metabolite yield, such as cell line selection, choice of culture system, nutrient regime, level of plant growth regulators, elicitation, precursor feeding, removal of product, and culture conditions (light, pH, temperature, gaseous environment); the full application of recombinant DNA technology and other biotechnological tools are also being applied. This more focussed approach involves the identification of genes encoding metabolic enzymes which will allow full control of a given pathway, together with the manipulation of secondary metabolic pathways, by the insertion of heterologous gene(s) from one organism to another, and attempts to overexpress a single gene to alter the amounts of the end-product of interest.

Despite this surge of research activity the current achievements, although very encouraging, are still limited and many aspects of metabolic control are still unknown and need to be established (for example the involvement of signalling, compartmentalisation, pH, inhibition, activation, enzyme regulation, precursor feeding, feedback effects, etc.). This will take many years to provide a full understanding. Still, the new results are encouraging and stimulating further research which will no doubt provide a framework to give a better understanding of the control of the processes involved in the functioning of a pathway. Hopefully this will lead to a full appreciation of pathway control and eventually enable the manipulation of a chosen pathway to achieve a particular goal.

The application of these ideas and other new approaches, which will presumably emerge, will allow the further manipulation of pathways of different plant species of pharmaceutical or commercial interest and refine the biosynthetic potential of these organisms to make them more productive and profitable, or to alter them to obtain tailored metabolites. We are particularly hopeful that these obstacles will be soon overcome and progress will continue.

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