PLANTS AS HOSTS FOR HETEROLOGOUS CYTOCHROME P450 EXPRESSION

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SUMMARY

Higher plant species, particularly tobacco, have been used as hosts for expression of both eucaryotic and procaryotic cytochromes P450. Strategies for subcellular localization and for providing access to a source of reducing equivalents have varied. While expression of the P450 gene can be confirmed by the appearance of mRNA or antigen, recovery of activity for *in vitro* studies is very difficult. Some plant-expressed heterologous cytochromes P450 dramatically alter the phenotype of the plant, so *in vivo* activity such as premature senescence or P450-specific alterations in herbicide sensitivity confirm that expression of a functional protein has occurred.

INTRODUCTION

With very few exceptions, eucaryotic cytochromes P450 are intrinsic membrane proteins. Their function in monooxygenase reactions is dependent on the proximity of another membrane-bound reductase protein that transfers reducing equivalents from NADPH for the reduction of one atom of O_2 during the catalytic cycle. Any system for heterologous expression of these proteins needs to take into account both localization and reductant availability if a functional enzyme system is the desired outcome. The two general solutions that have been used for laboratory studies of eucaryotic P450s is to express the protein in another eucaryote, or to engineer the protein to be suitable for expression in *E. coli* /1/. The former approach has been successfully carried out with plant P450s using yeast expression systems /2/. The advantage of these systems is that yeast is a eucaryote, so that appropriate membrane targeting of the nascent P450

polypeptide can occur. Isolation of the protein in the microsomal fraction is still possible, and because yeast has its own cyt P450 reductase, the P450 can be enzymatically active.

While not as multi-purpose as yeast expression for eucaryotic P450s, or *E. coli* expression for soluble enzymes, several recent advances have made plant heterologous expression of cytochromes P450 a technical reality. The motivation for carrying out this work is largely for the practical possibilities of modifying plant metabolic pathways. The goals are not to achieve overexpression of the P450, or to place it in an organism where it might be more amenable to study, but to alter the phenotype of the plant in some way. In this sense, the goals are similar to those in transgenic animals. While the goals are similar, the obstacles are as well. Like all higher eucaryotes, appropriate subcellular and tissue targeting are essential. The protein must end up in the endoplasmic reticulum of one type of cell in a particular tissue for the expression to be considered successful. In some cases expression of the protein in all tissues might be desirable, or at least a harmless side product.

One object of heterologous P450 expression in plants is to engineer relatively simple one-step metabolic pathways with a predictable outcome. Herbicide resistance, for example, can be conferred by a one-step modification of a herbicide catalyzed by an appropriate P450. Modification of more complex pathways, as might be desirable in a secondary metabolic pathway leading to a drug or drug precursor, is perhaps less technically feasible at present, but not beyond consideration. Related efforts are those which attempt to achieve some useful function by modifying a property, or the overall composition, of the plants secondary metabolite pool. Pest resistance or attractiveness, flavor, color and aroma of plant products are traits that might be modified by introduction of a P450. Nonetheless, the initial efforts at these may do more to illustrate the complexities of regulation in these pathways than to provide the desired modification. "Phyto-remediation", the use of plants as agents of bioremediation, is another bioengineered possibility especially attractive because it has the advantage of being a cheap and easily introduced remediation agent. Introduction of particular P450s, or increasing the expression levels of an endogenous P450 might be used as part of a plant bioremediation scheme.

The purpose of this paper is to compare three general strategies that have been used for plant expression of cytochromes P450. These

include simple expression with normal targeting to the endoplasmic reticulum membrane, normal targeting of a fused cyt P450 and P450 reductase protein, and organellar targeting of procaryotic P450. In each case there is a recurring theme of adequate reductant delivery, either as a means of accounting for low activity, or as a key factor in obtaining *in vivo* activity.

SIMPLE EXPRESSION, NORMAL TARGETING

The most straightforward approach to plant expression was taken by Saito *et al.* /3/. These authors prepared tobacco transformed with the rabbit CYP2C14, under control of the phloem-specific mannopine synthase TR2' promoter. The CYP2C14 protein appears to be involved in steroid hydroxylation, specifically the 16-α-hydroxylation of testosterone. There were no modifications made to this protein, and it was assumed that the subcellular localization was endoplasmic reticulum. There was expression of this protein in the plant as measured by mRNA levels and Western blots, and the expression was localized in the internal phloem tissue, as expected from the TR2' promoter. These plants had a marked phenotype, however, characterized by a tendency to rapidly senesce and flower. The alkaloid 2-propenylpyrrolidine also accumulated in these plants, but the investigators concluded that this was a secondary effect of the rapid senescence and not due to metabolic activity of the P450. *In vitro* enzymatic activity of the plant-produced enzyme was not measured, presumably partly because of the difficulty in obtaining microsomes from the phloem tissue. Clearly expression of this protein led to an altered phenotype in these plants; however, it is unclear whether this phenotype is solely a consequence of CYP2C14 monooxygenase activity or an unexpected result of the foreign gene expression.

Another example of this type of expression has been carried out with the CYP72 gene from Madagascar periwinkle (*Catharanthus roseus*). This protein was expressed in both tobacco and *Arabidopsis thaliana*, and tissue from the transgenic plants was tested for nine different enzymatic activities to ascertain whether one could be a physiological role for CYP72 /4/. The lack of any activity with these compounds may be because none is a good substrate for CYP72, but the authors point out that an alternative explanation is poor coupling between the P450 and reductase in the transgenic plant, and there is no simple way to distinguish between these alternatives.

FUSED CYTOCHROME P450 AND P450 REDUCTASE

Another strategy that has been used takes more into account the need for adequate electron transfer from reductant to the P450. The rat CYP1A1 gene was fused with a yeast NADPH:P450 oxidoreductase gene /5/. This combined gene had previously been shown to produce a microsomal enzymatically active fused enzyme in a yeast expression system /6/. In addition to the expected CYP1A1 dealkylation of ethoxycoumarin and hydroxylation of benzo[*a*]pyrene, the yeast-produced hybrid enzyme was capable of three separate reactions on the herbicide chlortoluron. When this enzyme was expressed in tobacco, under the control of the cauliflower mosaic virus 35S promoter, the resulting plants had no obvious change in phenotype. Microsomal fractions from these plants exhibited enzymatic activity characteristic of CYP1A1, demonstrating that it is possible to recover the heterologous P450 activity from a transformed plant. More importantly, the plants exhibited resistance to the herbicide chlortoluron, demonstrating that it is possible to predictably alter the phenotype of the plant with a heterologously expressed mono-oxygenase enzyme.

ORGANELLAR TARGETING

Targeting of P450s to organelles is an alternative approach to the two already described, and is particularly useful for procaryotic P450s, which are all soluble proteins. Cytochromes P450 are found in mitochondria of animals, particularly in steroidogenic tissue such as the adrenal cortex. To date, no P450s have been found in any plant organelles although the N-terminal sequence of the CYP74 gene suggests it is plastid-localized /7/. Several P450s from the actinomycetes have appeared amenable to chloroplast targeting because they are able to use the plant chloroplast ferredoxin as a reductant. *In vitro* reconstitution experiments show that many of the *Streptomyces* P450s are functional using a chloroplast ferredoxin as an immediate reductant /8/, and in some cases capable of >50% monooxygenase efficiency using this heterologous electron donor /9/. This is in contrast to the well characterized bacterial $P450_{CAM}$, for which spinach ferredoxin does not support significant catalytic activity despite the favorable electrochemical relationship between its Fe-S cluster and the P450 heme. Within the chloroplast, soluble ferredoxin

is localized in the stromal space, as are many other soluble proteins, including ribulose-bis-phosphate carboxylase (rubisco) and the other enzymes of the Calvin-Benson cycle. Ferredoxin functions in photosynthesis as an electron transfer agent between the low potential final donor of photosystem I and the ferredoxin:NADP oxidoreductase, the enzyme ultimately responsible for the production of NADPH in photosynthesis. Ferredoxin is a branch point in the utilization of photochemically produced reducing equivalents, also serving as a reductant for the essential reactions of sulfite reductase, nitrite reductase and fatty acid desaturase, to name a few. Appropriate redox partner pairing with a P450 that is correctly compartmentalized should provide a functional monoxygenase system.

Organellar targeting is accomplished in plants by a so-called transit peptide or targeting presequence /10/. This is a sequence of amino acids that allows for recognition and uptake of the polypeptide into the organelle. The transit peptide is often cleaved resulting in a mature intraorganellar protein. Nuclear encoded, chloroplast targeted plant proteins include the small subunit of rubisco, and the chlorophyll *a/b* antenna protein. The targeting sequence from these proteins can be attached to the amino-terminus of a bacterial P450 and successful uptake into the chloroplast can be accomplished. This has been achieved with cytochrome $P450_{SU1}$ (CYP105A1) from *Streptomyces griseolus* /11/.

Expression of this protein in transgenic tobacco results in a phenotype that is unchanged from control plants unless a substrate for the P450 is applied /12/. $P450_{SU1}$ is capable of metabolizing a variety of sulfonylurea herbicides /9/, and when these compounds are applied to plants expressing this P450, the effect can be dramatic. One compound in particular, R7402 (2-methylethyl-2,3-dihydro-*N*-[(4,6-dimethoxypyrimidin-2-yl)aminocarbonyl]-1,2-benzoisothiazole-7-sulfonamide-1,1-dioxide), is activated from a non-phytotoxic to a phytotoxic compound by its *N*-dealkylation mediated by $P450_{SU1}$. Application of R7402 to transgenic plants which have chloroplast-localized $P450_{SU1}$ is lethal. The compound has minimal effect when applied to plants which have comparable levels of cytoplasmically localized $P450_{SU1}$. These findings demonstrate that chloroplast localization of $P450_{SU1}$ is essential to obtain R7402 sensitivity; the interpretation is that the P450 has a competent reductant (ferredoxin) in the chloroplast and not in the cytoplasm. Use of appropriate tissue-specific promoters allows this lethal effect to be sequestered to particular tissues; it can be used as a

chemical treatment to destroy specific tissues as in a chemical male sterilizing agent /12/.

In principal, plant mitochondria should also provide a subcellular compartment in which a bacterial P450 could be functional. There is adequate low potential reductant, although there is no obvious step involving a soluble ferredoxin which could be used as a reductant for the P450. In steroidogenic tissue in animals, such as the adrenal cortex, mitochondria contain a special reductase and ferredoxin, known as adrenodoxin reductase and adrenodoxin respectively, which provide this electron transfer function. No analogous system has been found in plants. Uptake into plant mitochondria by a targeting presequence has been shown to be possible with $P450_{SCC}$ and soybean cotyledon mitochondria /13/. Functional expression in plant mitochondria may require coordinated expression of more than one heterologous protein in order to engineer a system capable of monooxygenase activity. This may be more desirable than chloroplast targeting for some applications, such as activity in roots or other non-photosynthetic tissue.

CONCLUSIONS

The examples of plant expression of P450s presented here show that a range of approaches may be taken to achieve functional expression of a monooxygenase activity in a higher plant. While it is possible to monitor expression by some sensitive phenotypic measures such as herbicide sensitivity, it is not clear how efficient the expression of active proteins is in any of the systems described here. In the tobacco expression of $P450_{SU1}$, estimates are that this efficiency (active P450 out of totally expressed P450) is quite low /12/. Before it is possible to carry out large scale reengineering of plant metabolic pathways, one of the biggest challenges will be to provide more control over how well these recombinant proteins are expressed.

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