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Heterologous expression and strategies for encapsulation of membrane-localized plant P450s

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Abstract Heterologous expression of plant P450 proteins is critical for functional definitions of their enzymatic activities as well as for producing natural products whose biosyntheses involve P450s. Over the past decade and a half, several expression systems, using bacterial, yeast and insect cells, have been utilized successfully for expression of P450s from different plant species. Extensive optimizations in each system have focused on the improvement of expression levels, and the enhancement of the redox environment for catalytic activity. In this review, we discuss the strengths and limitations of each system, as well as recent developments and applications of each system. We also discuss the principles behind Nanodisc technology, which utilizes an amphipathic "membrane scaffold protein" (MSP) to stabilize the soluble membrane protein-containing nanometer diameter phospholipid bilayers, and its potential applications in plant P450 research.

Keywords Baculovirus · cDNA expression · *E. coli* · Membrane scaffold protein · Yeast

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Introduction

Cytochrome P450 monooxygenases (P450s) are nearly ubiquitous in that they exist in all eukaryotes and many bacterial species (Feyereisen 1999; Nelson 1999; Werck-Reichhart et al. 2002; Kelly et al. 2003; Guengerich 2005). There is an ever-increasing interest in using these proteins to synthesize many types of natural products for human health and other industrial uses since many of these chemistries are beyond the reach of simple synthetic procedures and current extraction methods. Of particular interest are the plant P450s responsible for the in vivo synthesis of complex secondary metabolites historically isolated from tropical species that are becoming increasingly rarer. Examples of the array of these plant-derived compounds include taxoids that have antimitotic activity (paclitaxel), terpenoid indole alkaloids that have antineoplastic activities (vincristine and vinblastine), antihypertensive activities (reserpine and ajmalicine) and anti-arrhytmic activities (ajmaline), as well as phenolic compounds that serve as antioxidants (caffeic acid and its derivatives) and antimicrobial, antiproliferative and antidepressive compounds (Morant et al. 2003).

Except for the most abundant plant P450s, it is unfeasible to biochemically purify enough of these proteins for functional characterization due to their low abundance and cell-specific

expression patterns. And, for plants nearing extinction, purification is totally impossible (Schuler 1996; Dixon 1999; Morant et al. 2003). The interest in producing plant-derived compounds in large quantities has focused research efforts on expressing plant P450s in a variety of heterologous expression systems. In addition to their uses in metabolic engineering of natural plant products, heterologous cDNA-expression systems have become increasingly essential to the process of identifying physiological substrates for proteins in the P450 superfamily in most species. But, due to their requirements for redox partners, such as NADPH P450 reductase and cytochrome b_5/cytochrome b_5 reductase for the Class II ER-localized P450s and ferredoxin/ferredoxin reductase (or putaredoxin/putaredoxin reductase) for the Class I soluble P450s, heterologous expression of P450s by themselves normally results in no activity or very low activity if endogenous redox partners interface poorly with the heterologously expressed P450. As a consequence, individual heterologously expressed P450 proteins are either purified and reconstituted with their respective electron transfer partners or, they are co-expressed with appropriate redox partners. While co-expression obviates the need for extensive reconstitution, the yield of individual P450 proteins may be compromised by the limited translational potential of most heterologous expression systems. With clear knowledge about the expression conditions attenuating production of all components needed for activity determinations, optimal P450 catalytic activities are readily attainable.

Heterologous expression of the first functional mammalian P450 was reported more than two decades ago in Oeda et al. (1985) analyzing the activity of the rat CYP1A1 mediating aryl hydrocarbon hydroxylation of benzopyrene. Since that time, several commonly used expression systems have been developed for P450 expression in bacteria, yeast, insect cells and mammalian cells (Gonzalez and Korzekwa 1995). While mammalian cell systems are particularly useful to express mammalian P450s for drug and carcinogenesis research, the other three systems have all been widely used for expression of plant P450s with the yeast expression system being the most frequently used. Several factors that need to be considered when choosing among these systems for plant P450 expression include their expense, ease of use, codon preferences, post-translational modifications and membrane insertion mechanisms. Choices for individual P450s may vary depending on their compatibility with host cellular functions, protein stability and coupling efficiency with endogenous and/or overexpressed redox partners. Comparisons of the different heterologous expression systems for plant P450s, as well as a complete summary of all heterologously expressed plant P450s have been reviewed recently (Schuler and Werck-Reichhart 2003). All heterologously expressed *Arabidopsis* P450s are listed in Table 1. Other plant P450s expressed since this 2003 review are listed in Table 2.

While plant P450s expressed in bacterial systems normally need to be purified and reconstituted for activity assays, the microsomal P450–P450 reductase complexes expressed in eukaryotic systems can be readily assembled in micron-sized liposomes derived from subfractionated natural cellular membranes obtained from systems co-expressing both components. These liposomes allow heterologous P450s to effectively couple with endogenous or co-expressed P450 reductases but, due to their constrained micrometer-scale nature, some portion of the membrane proteins embedded within them remain sequestered in the solvent-inaccessible interior (Angrand et al. 1997). A recently developed membrane scaffold system that incorporates membrane proteins into stable soluble nanometer-scale structures (designated Nanodiscs) eliminates this limitation by inserting integral membrane proteins into lipid bilayers that are solvent-accessible on both surfaces (Bayburt et al. 1998; Bayburt and Sligar 2003; Civjan et al. 2003; Duan et al. 2004). Integral membrane proteins incorporated in this system are amenable to standard fractionation and protein display technologies that are typically applied to soluble proteins. Plant P450s assembled in this system are suitable for analysis of substrate binding in high-throughput formats and at lower protein concentrations than currently used in standard spectrophotometric substrate binding assays. When assembled with appropriate redox partners,

Table 1 Functionally expressed *Arabidopsis* P450s

P450	Expression system	Vector/Host cells	References
CYP71A19	Insect	pFastbac1/Sf9	Annamalai and Schuler (unpublished)
CYP71A20	Insect	pFastbac1/Sf9	Annamalai and Schuler (unpublished)
CYP73A5	Insect	pVL1392/Sf21	Mitzutani et al. (1997)
	Insect	pFastbac1/Sf9	Duan et al. (2004)
CYP74A1	*E. coli*	pQE30/M15	Laudert et al. (1996)
CYP74B2	*E. coli*	pGEX/unknow	Bate and Rothstein (1998)
CYP75B1	Yeast	pYES2/INVSc1	Schoenohm et al. (2000)
CYP79A2	*E. coli*	pSp19g10L/JM109	Wittstock and Halkier (2000)
CYP79B2	*E. coli*	pCWori+/	Hull et al. (2000)
	E. coli	pSP1910L/C43	Mikkelsen et al. (2000)
CYP79B3	*E. coli*	pCWori+/DH5α	Hull et al. (2000)
CYP79F1	*E. coli*	pSp19g10L/JM109	Hansen et al. (2001)
	E. coli	pSp19g10L/C43	Hansen et al. (2001)
CYP79F2	Yeast	pYeDP60/WR	Chen et al. (2003)
	Yeast	pYeDP60/WAT11	Chen et al. (2003)
CYP83A1	Yeast	pYeDP60/WAT11	Bak and Feyereisen (2001)
CYP83B1	Yeast	pYeDP60/WAT11	Bak et al. (2001), Bak and Feyereisen (2001)
CYP84A1	Yeast	pYeDP60/WAT11	Humphreys et al. (1999)
CYP85A1	Yeast	pYeDP60/WAT11	Shimada et al. (2001)
CYP85A2	Yeast	pYeDP60/WAT11	Kim et al. (2005), Nomura et al. (2005), Shimada et al. (2003)
CYP86A1	Yeast	pYeDP60/WR	Benveniste et al. (1998)
	Insect	pFastbac1/Sf9	Duan and Schuler (2005)
CYP86A2	Insect	pFastbac1/Sf9	Duan and Schuler (2005)
	Yeast	pYeDP60/WAT11	Benveniste et al. (2006)
CYP86A4	Insect	pFastbac1/Sf9	Duan and Schuler (2005)
CYP86A7	Insect	pFastbac1/Sf9	Duan and Schuler (2005)
CYP86A8	Yeast	pYeDP60/WAT11	Wellesen et al. (2001)
	Insect	pFastbac1/Sf9	Duan and Schuler (2005)
CYP88A3	Yeast	pYeDP60/WAT1	Helliwell et al. (2001)
	Yeast	pYeDP60/WAT21	Helliwell et al. (2001)
CYP88A4	Yeast	pYeDP60/WAT11	Helliwell et al. (2001)
	Yeast	pYeDP60/WAT21	Helliwell et al. (2001)
CYP89A5	Insect	pFastbac1/Sf9	Ali and Schuler (unpublished)
CYP90B1	*E. coli*	pCWori+/JM109	Asami et al. (2001, Fujita et al. (2006)
CYP94B1	*yeast*	pYeDP60/WAT11	Benveniste et al. (2006)
	Insect	pFastbac1/Sf9	Civjan et al. (unpublished)
CYP94B2	Yeast	pYeDP60/WAT11	Benveniste et al. (2006)
CYP94B3	Yeast	pYeDP60/WAT11	Benveniste et al. (2006)
CYP94C1	Yeast	pYeDP60/WAT11	Benveniste et al. (2006)
	Insect	pFastbac1/Sf9	Civjan et al. (unpublished)
CYP96A1	Yeast	pYeDP60/WAT11	Benveniste et al. (2006)
CYP96A2	Yeast	pYeDP60/WAT11	Benveniste et al. (2006)
CYP96A3	Yeast	pYeDP60/WAT11	Benveniste et al. (2006)
CYP96A4	Yeast	pYeDP60/WAT11	Benveniste et al. (2006)
CYP96A9	Insect	pFastbac1/Sf9	Palacio et al. (unpublished)
CYP96A10	Insect	pFastbac1/Sf9	Palacio et al. (unpublished)
CYP97B3	Yeast	pYeDP60/WAT11	Benveniste et al. (2006)
CYP98A3	Yeast	pYeDP60/WAT11	Schoch et al. (2001)
	E. coli	pCWori/DH5α	Rupasinghe and Schuler (unpublished)
CYP701A3	Yeast	pYe22/G1315	Helliwell et al. (1999)
CYP704A2	Yeast	pYeDP60/WAT11	Benveniste et al. (2006)
CYP707A1–A4	Yeast	pYeDP60/WAT11	Kushiro et al. (2004)
	Insect	pFastbac1/Sf9	Saito et al. (2004)
CYP709C1	Yeast	pYeDP60/WAT11	Kandel et al. (2005)
CYP710A1	Insect	pFastbac1/Sf9	Morikawa et al. (2006)

Table 1 continued

P450	Expression system	Vector/Host cells	References
CYP710A2	Insect	pFastbac1/Sf9	Morikawa et al. (2006)
CYP711A1	Yeast	pYeDP60/WAT11	Benveniste et al. (2006)
CYP734A1	Yeast	pYeDP60/WAT11	Turk et al. (2003)
CYP735A1	Yeast	pYES2/YPH499	Takei et al. (2004)
CYP735A2	Yeast	pYES2/YPH499	Takei et al. (2004)

this system is also suitable for analysis of substrate metabolism and NADPH consumption rates (Duan et al. 2004).

In this review, we highlight some of the differences and similarities between the available heterologous expression systems and summarize the recent applications of these systems to functional characterizations of plant P450 activities. We also review background relevant to this Nanodisc technology and discuss its potential application in plant P450 characterizations.

Yeast expression

Yeast (*Saccharomyces cereviseae*) represents the first system reported to successfully express mammalian (Oeda et al. 1985; Sakaki et al. 1985) and plant (Urban et al. 1994) P450 proteins. Due to several subsequent studies optimizing parameters for P450 expression, it has now become the system most frequently used for functional expression of plant P450s with almost two-thirds of all heterologously expressed *Arabidopsis* P450s expressed in yeast (Table 1); some have also been expressed in *E. coli* and insect systems (Table 1). Clear advantages of the yeast system for P450 expression include the lower costs of culture media, rapid growth, reasonable expression levels of correctly configured heme-containing P450s (as defined by carbon monoxide (CO) difference spectra (Omura and Sato 1964)) and the availability of strains over-expressing the appropriate P450 reductases needed for catalytic activity assays. Most importantly, many studies have now described the parameters needed to optimize this system for plant P450 expression and catalytic activity.

In one of the first reports describing this system, an engineered pYeDP60 yeast expression vector that uses a galactose-inducible GAL10-CYC1 hybrid promoter for expression of plant P450 cDNAs was coupled with endogenous or overexpressed yeast P450 reductase (Urban et al. 1994). In later studies, a WAT11 strain, which overexpresses *Arabidopsis* NADPH-dependent P450 reductase ATR1, and a WAT21 strain, which overexpresses *Arabidopsis* ATR2, were constructed to overcome coupling deficiencies with the endogenous *S. cerevisiae* P450 reductase. In each of these strains, the chromosomally integrated *Arabidopsis* P450 reductase provides the redox environment that is optimal for plant P450 activities. Transformations of P450-pYeDP expression plasmids (Pompon et al. 1996) into these strains has allowed for the assembly of compatible P450–P450 reductase complexes and subsequent activity determinations.

Subsequent studies focused on optimizing the yeast expression system by altering codons (often GC-rich sequences) that frequently impair gene expression in recombinant yeast (Batard et al. 2000; Hehn et al. 2002). The *modus operandi* of the translation machinery and higher frequency of codons designated as rare in yeast codon frequency charts near the 5′ of plant cDNAs has made it possible to limit codon reengineering of P450 and P450 reductase cDNAs for improved protein expression to the first third of each ORF (Batard et al. 2000) and does not entail complete reengineering of the full-length sequences. Examples where this strategy has successfully improved protein expression in yeast include several monocot P450s (e.g., maize CYP73A17, wheat CYP86A5) and P450 reductase (e.g., wheat TAR1) that have strong biases in GC-rich codons and some dicot P450s that have clusters of low usage codons close to their translation start sites (e.g., *Helianthus tuberosus* (Jerusalem artichoke) CYP73A1). Another example includes a monocot

Table 2 Heterologously expressed plant P450s[a]

Species	P450	Expression system	References
Ammi majus L	CYP73A41	Yeast	Hubner et al. (2003)
Artemisia annua L.	CYP71Av1	Yeast	Teoh et al. (2006)
Coptis japonica	CYP719	Yeast	Ikezawa et al. (2003)
	CYP80B2	Yeast	Ikezawa et al. (2003)
Glycine max (soybean)	CYP71D8	Yeast	Shibuya et al. (2006)
	CYP82A2	Yeast	Shibuya et al. (2006)
	CYP82A3	Yeast	Shibuya et al. (2006)
	CYP82A4	Yeast	Shibuya et al. (2006)
	CYP93E1	Yeast	Shibuya et al. (2006)
Medicago truncatula	CYP81E7	Yeast	Liu et al. (2003)
	CYP81E9	Yeast	Liu et al. (2003)
Pinus taeda (loblolly pine)	CYP720B1	Yeast	Ro et al. (2005)
	CYP720B2	Yeast	Ro et al. (2005)
	CYP750A1	Yeast	Ro et al. (2005)
	CYP704C1	Yeast	Ro et al. (2005)
Parsley	CYP73A10	Yeast	Koopmann et al. (1999)
Pea	CYP88A6	Yeast	Davidson et al. (2003)
	CYP88A7	Yeast	Davidson et al. (2003)
	CYP701A10	Yeast	Davidson et al. (2003)
Petunia	CYP76A4	Yeast	Tamaki et al. (2005)
	CYP92B1	Yeast	Petkova-Andonova et al. (2002)
Potato	CYP51G1-Sc	Yeast	O'Brien et al. (2005)
Rice	CYP714D1	Yeast	Zhu et al. (2006)
	CYP74A (OsAOS)	Yeast	Ha et al. (2002)
	CYP74C	*E. coli*	Kuroda et al. (2005)
Picea sitchensis (Sitka spruce)	CYP716B1	Yeast	Ro et al. (2005)
	CYP716B2	Yeast	Ro et al. (2005)
Sinapis alba	CYP79B1	*E. coli*	Naur et al. (2003)
Taxus	Taxoid 2α-hydroxylase	Yeast	Chau et al. (2004)
	CYP725A-like taxoid 7β-hydroxylase	Yeast	Chau and Croteau (2004)
	CYP725A3	Yeast	Jennewein et al. (2003)
	CYP725A-like Taxadiene 5-alpha hydroxylase	Yeast/Insect	Jennewein et al. (2004)
Tomato	CYP85A1	Yeast	Bishop et al. (1999)
	CYP85A3	Yeast	Nomura et al. (2005)
	CTP710A11	Insect	Morikawa et al. (2006)
Wheat	CYP71C6v1	Yeast	Xiang et al. (2005, 2006)
	CYP709C1	Yeast	Kandel et al. (2005)

[a] Functionally expressed plant P450s previously summarized in Schuler and Werck-Reichhart (2003) are not listed here

P450 (e.g., wheat CYP86A5) where expression was further improved by altering clusters of low usage codons in its more central coding sequences (Hehn et al. 2002). Later studies focused on improving P450 expression in yeast for structural studies and created a water-soluble form of the *H. tuberosus* CYP73A1 (*t*-cinnamic acid hydroxylase) by replacing its N-terminal sequence with a 25-amino acid amphipathic peptitergent sequence (PD1) (Schoch et al. 2003) initially designed for the solubilization of intrinsic membrane proteins (Schafmeister et al. 1993). This PD1–CYP73A1 fusion protein expressed at high levels in yeast and was subsequently used for NMR-based analysis of substrate positioning in its active site (Schoch et al. 2003).

And, for the successful expression in yeast of P450s that are highly conserved across many kingdoms, (e.g., CYP51 family (Nelson et al. 2004)), endogenous yeast orthologues capable of interfering with expression and activity assays were eliminated by creating gene disruption strains. Use of these engineered strains, such as ERG11 disrupted in its endogenous CYP51 sterol

demethylase gene, has allowed for optimization of the wheat CYP51 obtusifoliol 14α-demethylase differentiation of its activity from its mammalian and yeast counterparts (Cabello-Hurtado et al. 1999). In this study, five-fold increases in expression resulted in wheat CYP51 levels that are as high as 1.5% of the total microsomal protein making it evident that the nature of the N-terminal anchor can significantly influence expression levels, folding and/or stability of heterologously expressed P450s in yeast. Interestingly, replacement of the N-terminal signal sequence on the wheat CYP51 with the N-terminal signal of the endogenous yeast CYP51, which should theoretically improve expression in yeast, significantly reduced the levels of functional wheat CYP51 in yeast microsomes and in contrast, replacement with the N-terminal signal of sorghum CYP51 allowed for the high level functional expression.

More recent attempts at optimizing the yeast P450 expression system have focused on improving its use in whole cell biocatalyst systems (Jiang and Morgan 2004). In these studies, which represent modifications on the protocols of Pompon et al. (1996), expression of *Arabidopsis* ferulate 5-hydroxylase (F5H, CYP84A1) activity was enhanced 2.6-fold by improving growth conditions using modified two-stage growth and induction protocols and another 30% by adding heme precursors such as δ-aminolevulinic acid (ALA) (Jiang and Morgan 2004).

Several other vectors and strains have been used for the expression of plant P450s in yeast. Example of these include *Arabidopsis* CYP701A3 (*ent*-kaurene oxidase) that was expressed in the G1315 yeast strain with the pYe22 expression vector originally designed for expression of *Rhizopus oryzae* glucose amylase in yeast (Helliwell et al. 1999). Although the efficiency of this vector/strain system for the expression of CYP701A3 was not discussed in detail in this study, the fact that subsequent work by this group switched to use of the previously described pYeDP60/WAT11/WAT21 vector/strain system suggests that the pYEDP60 vector is better for expression (Helliwell et al. 2001). Another example is *Arabidopsis* CYP75B1 (flavonoid 3′-hydroxylase, F3′H) that was expressed in the pYES2/INVSc1 vector/strain system (Schoenbohm et al. 2000). One distinct advantage of this vector is that it is commercially available and already suitable for the TOPO-Gateway™ technology (Invitrogen) that greatly facilitates subcloning of individual cDNAs. It is likely that the more commonly used pYeDP60 vector will also be engineered into Gateway destination vectors in the near future.

The ease of working with this *S. cerevisiae* system has recently been exploited to allow reconstitution of a significant part of the pathway for the diterpenoid taxol in yeast (DeJong et al. 2006). Expression of eight recombinant proteins representing four distinct enzyme targets (including P450s, diterpene cyclases, acyl transferases, prenyltransferases) was capable of generating taxadiene and small amounts of taxadien-5α-ol with taxadiene 5α-hydroxylase being the rate-limiting P450 that reduces levels of this final taxadien-5α-ol product. Pathway restrictions encountered in the expression of the first P450 (10β-hydroxylase) in this pathway were overcome by expression of the homologous Taxus P450 reductase and resulted in seven-fold increases in activity compared to expression in the presence of the endogenous yeast P450 reductase (Jennewein et al. 2005).

Besides *S. cerevisiae*, the yeast *Pichia pastoris* has also been used for functional expression of a number of plant P450s. *P. pastoris* is an especially interesting system for expression of P450s for structural analysis since it utilizes a defined minimal medium for growth and a strong methanol-inducible alcohol oxidase (*AOX1*) promoter for expression of heterologous proteins up to several grams per liter of culture (Rosenfeld 1999). Because previous attempts to express the cassava (*Manihot esculenta* Crantz) CYP79D1 and CYP79D2 in *E. coli* had been unsuccessful, these P450s were expressed in *P. pastoris* system where they accumulated at levels equivalent to those obtained in *S. cerevisiae* for other P450s (Andersen et al. 2000; Anderson and Moller 2002). Sequencing of the *P. pastoris*-expressed CYP79D1 protein obtained in these studies indicated that its asparagine residues had been glycosylated during expression process. It is not yet known whether these post-translational modifications occur on this P450 in its native cassava

but, importantly, they do not interfere with CYP79D1's capacity to generate oximes.

Insect cell expression

Another eukaryotic expression system, the baculovirus-infected insect cell culture system, has fewer problems with codon usage and more limited post-translational modifications that might detrimentally affect expression and functionality of P450s and P450 reductases. For P450s, this system also has the distinct advantage that substrate binding and activities can be measured in both cell lysates and microsomal preparations without the need for further purification. Although used less frequently than the yeast system for plant P450 expression, this system has been wildly used for expression of many mammalian and insect P450s, and, in several plant cases, such as *Taxus baccata* (yew) taxol hydroxylases (Jennewein et al. 2001, 2004), *Berberis stolonifera* (barberry) berbamunine synthase (CYP80A1) (Kraus and Kutchan 1995; Kutchan 1996), and more recently, *Arabidopsis* and tomato sterol C-22 desaturases (CYP710As) (Morikawa et al. 2006), it has represented the best alternative to yeast expression for P450s that are not stably expressed in lower eukaryotic systems.

Disadvantages that are usually cited for this system include the facts that insect cell culture medium is relatively expensive, that construction of recombinant viruses can be relatively labor-intensive and time-consuming and that batch-to-batch variations in heterologous expression are sometimes significant. Another disadvantage occasionally cited is that the endogenous P450 reductase levels in baculovirus host cell lines, such as Sf9 and Tn5 cells, are normally insufficient for overexpression of P450 catalytic activities. This last disadvantage can be overcome by purifying and reconstituting heterologously expressed plant P450s with purified vertebrate and plant P450 reductases (Kraus and Kutchan 1995; Mizutani et al. 1997). Sometimes, as in the case of *Berberis* CYP80A1, the proportions of various products (e.g., the R,S and R,R stereoisomers of *N*-methylcoclaurine corresponding to berbamunine and guattegaumerine, respectively) have varied depending on the source of the P450 reductase with the homologous *Berberis* P450 reductase producing higher amounts of berbamunine than that obtained with heterologous insect *Spodoptera frugiperda* P450 reductase (Kutchan 1996). This disadvantage can also be circumvented and even turned to a researcher's advantage by co-expressing P450s with increasing amounts of P450 reductases and other electron transfer partners that couple well with the plant monooxygenases (e.g., *E. californica* P450 reductase, Pauli and Kutchan 1998; *Taxus* P450 reductase, Jennewein et al. 2001, 2005; Musca domestica P450 reductase, Duan et al. 2004) and produce the desired ratios of stereoisomers. For some P450 activities, the highest P450 activities have been obtained when P450s are expressed along with their homologous P450 reductases as exemplified by the efficient coupling of *Taxus* 10β-hydroxylase with *Taxus* P450 reductase (Jennewein et al. 2001, 2005).

With purification and reconstitution problems eliminated by use of the insect cell expression system, challenges still exist in carefully optimizing co-expression parameters. Infection of insect cell culture with too much recombinant P450 virus results in insufficient electron transfer to the P450 under analysis and, vice versa, infection with too much recombinant P450 reductase virus results in low P450 activities due to overexpression of P450 reductase and underexpression of the desired P450. To better describe the expression optimizations needed for functional analysis of P450 activities, Duan et al. (2004) tested a variety of co-expression conditions for *Arabidopsis* CYP73A5. With translation capacities limited in Sf9 cells (just as they are in other expression systems), variations in the multiplicity of infection (MOI) values ranging from 1 to 5 for the recombinant P450 virus produced approximately equal levels of P450 when expressed alone. But, at higher levels of recombinant virus, P450 expression decreased significantly due to limitations in the transcription and translation capacities of these cells. When co-expressed with insect P450 reductase at MOI ratios above 1:0.2 (P450:P450 reductase), the yield of P450 protein progressively decreased as the yield of P450 reductase increased with ratios of 1:1 and 1:2

producing the highest levels of *t*-cinnamic acid hydroxylase activity (Duan et al. 2004). Subsequent analysis on a number of *Arabidopsis* P450s (e.g., CYP71A19, CYP71A20, five CYP86A subfamily members, CYP89A5, CYP94B1, CYP94C1, CYP96A9 and CYP96A10) in this system has indicated that nearly all are expressed at significant levels at MOI values in the range of 0.5–5. The only exceptions are CYP71A19 and CYP71A20 that express well at MOI values of 6–10. As a consequence of these and other optimizations, most *Arabidopsis* P450s are now co-expressed with insect P450 reductase at a fixed MOI ratio of 1:0.5. Monitoring of the integrity of P450 catalytic sites in this insect system using CO difference spectra (Omura and Sato 1964) has indicated that at least two of the *Arabidopsis* P450s (e.g., CYP71A19 and CYP71A20) are not stably folded unless co-expressed with a compatible P450 reductase. In its absence, CO difference peaks for incorrectly configured proteins occur at 420 nm rather than the desired 450 nm.

The insect system has also been used to heterologously express *Arabidopsis* ATR1 and ATR2 (Mizutani and Ohta 1998) and NADH-dependent cytochrome b_5 reductase (Fukuchi-Mizutani et al. 1999) in quantities sufficient for purification and reconstitution. Combinations of these with NADH, NADPH and other proteins in their electron-transfer chains have demonstrated that these electron transfer proteins are highly selective in use of their electron donors: NADH-dependent cytochrome b_5 reductase utilizes NADH but not NADPH and NADPH-dependent ATR1 and ATR2 utilize NADPH but not NADH. Importantly and contrary to other eukaryotic electron transfer chains, both *Arabidopsis* P450 reductase and cytochrome b_5 reductase are capable of reducing cytochrome b_5 providing two avenues for transfer of reducing equivalents to terminal acceptors in microsomes (e.g., P450s, fatty acid desaturases). The extent to which NADH supplementation enhances P450 activities in Sf9 cells co-expressing P450s with all three of its electron transfer partners has not yet been analyzed. These reconstitution systems clearly represent valuable tools for the study of the P450-related microsomal electron transfer and the individual physiological roles of the diversified P450 electron transfer systems in higher plants.

Various P450s expressed in human cells have been reported to be modified by phosphorylation (CYP2B1, CYP2B4, CYP2E1), glycosylation (CYP19A1), nitration (CYP4A subfamily) and ubiquitination (CYP3A4, CYP2E1) (Aguiar et al. 2005). Little is known about the extent of post-translational modifications occurring on plant P450s. But it is known that the degree of post-translational modification varies substantially among these heterologous expression systems. Most relevant to our discussion of baculovirus-mediated expression is the fact that insect cells do not produce terminally sialylated complex glycans that are found on many proteins expressed in mammalian cells (Hooker et al. 1999). But, they do myristoylate, palmitoylate and phosphorylate heterologously expressed proteins such as endothelial nitric oxide synthase (Busconi and Michel 1995).

Bacterial expression

Some of the advantages that bacterial expression systems share with yeast systems are their use of inexpensive culture media and rapid growth of host cells. However, since these are prokaryotic in nature, they have distinctly different codon preferences than most eukaryotes and more codon usage problems encountered in expressing plant P450s. In an increasing number of examples, high level expression of membrane-bound P450s has been achieved only after substantial modification of the N-terminal signal sequences. For expression of mammalian P450s, the two most frequently used strategies for optimizing expression are recoding, deletion of N-terminal hydrophobic sequences (Barnes 1996; Guengerich et al. 1997) and fusion with *omp*A targeting sequences that direct the expressed proteins to bacterial lipid bilayers (Pritchard et al. 1998). For expression of plant P450s in *E. coli*, only the first approach altering N-terminal sequences has been used. Examples of this include CYP74 proteins that have been expressed in *E. coli* at high levels after deletion of their N-terminal chloroplast transit sequences (Laudert et al. 1996; Bate and

Rothstein 1998). Others include ER-targeted P450s (e.g., *Arabidopsis* CYP79A2 (Wittstock and Halkier 2000), CYP79B2 (Hull et al. 2000; Mikkelsen et al. 2000), CYP79B3 (Hull et al. 2000) and CYP79F1 (Hansen et al. 2001)) that have been expressed in *E. coli* after modifications enriching the AT content of their 5′ coding sequences and eliminating RNA secondary structures as described in Barnes et al. (1991) for expression of the bovine CYP17α protein. Most of these plant P450 expressions have used either the pCWori+ or pSp19g10L plasmid (Barnes 1996) that have *lac* or *lac-derived* promoters. The latter vector also has a short leader sequence (g10L) of T7 bacteriophage gene *10* that has proved to be an excellent leader sequence for many types of proteins in *E. coli* (Olins et al. 1988). Even with enrichment of their AT-richness, addition of phage leader sequences and construction of chimeric coding sequences with eight N-terminal amino acids from CYP17α, some plant P450s such as CYP79F2, CYP79D1, CYP79D2 and CYP79B1 have still been problematic. While all of these are from the CYP79 family, other members in this family, such as CYP79A1, CYP79A2, CYP79B2, CYP79B3 and CYP79F1, have been expressed in *E. coli* successfully. For those that have remained problematic, most difficulties have been overcome by switching to yeast expression systems (Bak et al. 1998; Andersen et al. 2000; Chen et al. 2003).

Except for certain nonclassical P450s such as members in CYP74 family, which do not require electron transfer partners (Laudert et al. 1996; Bate and Rothstein 1998), bacterially expressed P450s need to be purified and reconstituted with appropriate redox partners to achieve any sort of catalytic activity. In some cases, P450–P450 reductase fusion proteins have been constructed as in the example of *Catharanthus roseus* C4H where the N-terminal P450 ORF is linked in-frame to *C. roseus* P450 reductase via a ST linker (Hotze et al. 1995). Although this strategy may not be the most optimal system for extensive kinetic analysis, this has made it possible to construct bacterial culture-based bioreactor systems. One of the most recent examples of the successes of this expression strategy has been the synthesis of plant flavonols from phenylpropanoid acid precursors in *E. coli* (Leonard et al. 2006). For this, a chimeric *C. roseus* flavonoid 3′,5′-hydroxylase-P450 reductase (F3′5′H-CPR) fusion protein was created and co-expressed in *E. coli* with 4-coumaroyl:CoA-ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3β-hydroxylase (FHT) and flavonol synthase (FLS) to produce plant flavonols including kaempferol, quercetin and myricetin.

One of the most important advantages of bacterial expression systems is that they can be used to produce large quantities of proteins for structural studies. For the mammalian membrane-anchored P450s, this has been possible only after reengineering of their coding sequences for optimal expression in *E. coli*. To date, the major modification has been to delete the N-terminal anchor sequence that converts the integral membrane protein into one that binds peripherally to membranes and causes in the subcellular location of the engineered protein to vary depending on the ionic strength of the extraction buffer (vonWachenfeldt et al. 1997; Wester et al. 2003a, b; Williams et al. 2000, 2003; Scott et al. 2001, 2003; Schoch et al. 2004). The one exception to this strategy has been CYP3A4 that had only residues 3–23 deleted from its N-terminal transmembrane domain (Yano et al. 2004). Additional modifications on most of these P450s have included the addition of His_4-tags to the C-terminus for nickel-affinity purification and, for mammalian CYP2C5 and CYP2C9, modification of seven amino acids in the F-G loop region to eliminate potential interactions with the lipid bilayer (vonWachenfeldt and Johnson 1995; Williams et al. 2003). Rupasinghe and Schuler (unpublished) have used similar approaches to express *Arabidopsis* plant P450s CYP98A3 in *E. coli*. The modifications added for successful expression have included deletion of the N-terminal hydrophobic sequences and addition of MA to the N-terminus. Expression of this construct in the pCWori in *E. coli* DH5α strain (vonWachenfeldt et al. 1997) yielded 25 mg P450 per liter of culture media as assayed by the CO difference analysis. As a comparison, expression of same protein and other plant P450s in yeast and insect cells could only yield few hundred µg protein per liter of culture media (Rupasinghe et al. unpublished).

Alternative *E. coli* strains, such as C41(DE3) and C43(DE3), have been developed to avoid the toxic effects of overexpressing membrane proteins using the T7 bacteriophage promoter in the bacterial BL21 strain (Miroux and Walker 1996). These strains were selected for greater tolerance to expression of high levels of membrane proteins using several rounds of growth for cells transformed with vectors capable of expressing OCGP (mitochondrial oxoglutarate–malate transport protein) and F-ATPase (H^+-transporting F_1F_0ATPase) in the presence of IPTG. Using the first single mutant C41 (DE3) strain isolated, mammalian CYP2B4 has been expressed in quantities as high as 100 mg (2,000 nmol) per liter (Saribas et al. 2001). Using the second double mutant C43 (DE3) strain that has an even greater tolerance for membrane protein expression, *Arabidopsis* CYP79F1 has been expressed at 110 nmol per liter in TB media, which represents a 200-fold increase in activity relative to enzyme produced in the more standard JM109 strain (Hansen et al. 2001). These strains provide the opportunity to dramatically increase the yield of functionally expressed P450s.

Nanodisc technologies

One of the biggest challenges in the field of membrane proteins in general and P450s in particular has been the characterization of their structure and function in soluble, dispersable formats that are suitable for crystallographic tests and high-throughput substrate binding analyses. Significant advances have recently been made in developing nanotechnologies for the assembly of P450s into soluble Nanodiscs either alone for substrate binding titrations or with P450 reductases for activity assays. The core of this technology is a bioengineered amphipathic multi-helical membrane scaffold protein (MSP1) whose hydrophobic faces circumscribe the edges of small lipid bilayer and whose polar faces interact with polar aqueous solvents (Bayburt and Sligar 2002, 2003). When mixed with detergent-solubilized phospholipids, and upon detergent removal, the MSP1 protein self-assembles and encircles lipids and membrane proteins in a flat discoidal structure (termed Nanodisc) that is nominally 10 nm in diameter (Fig. 1). It has now been demonstrated that a wide variety of integral membrane proteins, either prepurified or heterologously expressed proteins embedded within cellular membranes from Sf9 insect cells or yeast cells, can be incorporated into the discoidal bilayer while retaining their native activities (Bayburt and Sligar 2002, 2003; Civjan et al. 2003; Duan et al. 2004). To date, this system has been used for encapsulating integral membrane proteins such as bacteriorhodopsin (bR) containing seven transmembrane helices (Bayburt and Sligar 2003), insect CYP6B1 (Civjan et al. 2003), mammalian CYP2B4 (Bayburt and Sligar 2002), and mammalian CYP3A4 (Baas et al. 2004) each containing a single transmembrane helix, G-protein coupled receptors containing seven transmembrane helices (Leitz et al. 2003) and human mitochondrial NADH/NADPH transhydrogenase containing 24 transmembrane domains (S.G. Sligar and C.D. Stout unpublished). And, using a co-assembly strategy with microsomes obtained from independent pools of Sf9 cells expressing *Arabidopsis* CYP73A5 or housefly P450 reductase, both of these proteins have been co-incorporated into Nanodiscs (Duan et al. 2004). With this *Arabidopsis* P450 as the first plant representative inserted into Nanodiscs, this system has a demonstrated potential for high-throughput analyses of substrate binding by spectral analysis of nanodisc samples arrayed in microtiter plates. With the bimolecular P450–P450 reductase complexes inserted in Nanodiscs, this system has demonstrated potential for defining activities on membrane protein complexes moved from complex membrane environments into defined lipid bilayer systems without the need for strong denaturing detergents. Evidence that the mild solubilization conditions used to transfer proteins from the membranes of heterologous expression systems into Nanodiscs maintain the integrity of P450 target proteins has been demonstrated by the ability of CYP73A5-containing Nanodiscs to appropriately bind carbon monoxide in CO difference analyses (Fig. 2A), bind *t*-cinnamic acid (*t*-CA) in type I binding analyses and, when co-assembled with P450 reductase, to metabolize

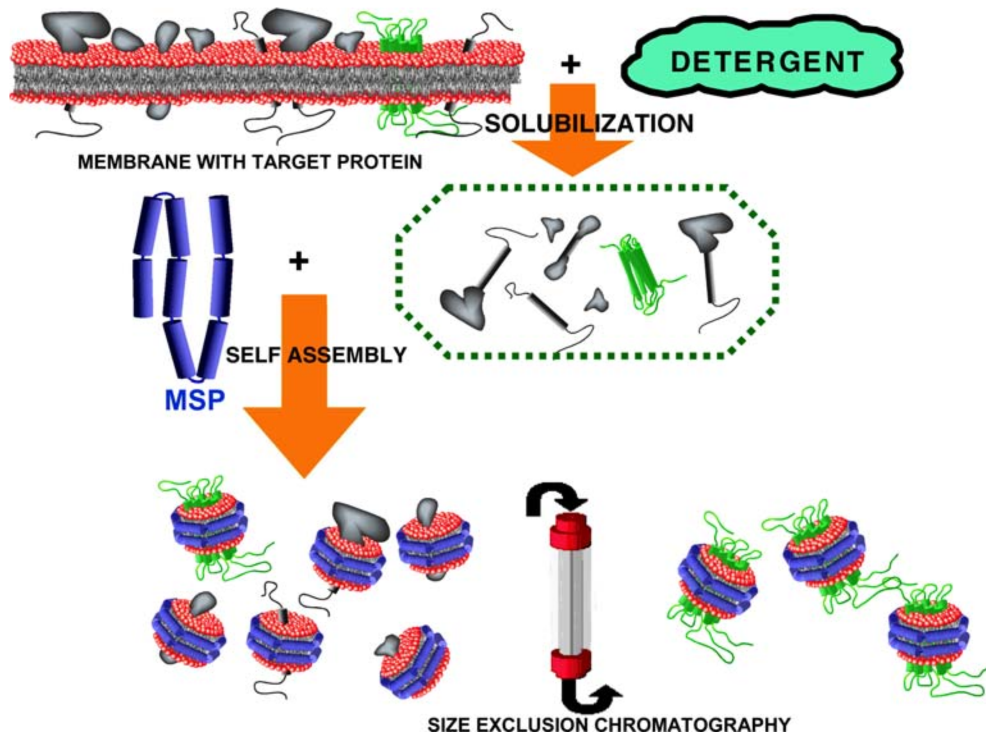

Fig. 1 Nanodisc assembly of membrane proteins into lipid bilayers. Heterologously expressed microsomal proteins are solubilized in a first step that uses mild detergents such as cholate for solubilization followed by assembly as detergents are removed with Biobeads. The assembled Nanodiscs are then separated from non-membrane proteins in a second step that binds His_6-tagged MSP1 Nanodiscs to nickel affinity matrices and these are size-fractionated by Sephadex size exclusion chromatography

t-CA into *p*-coumaric acid (Duan et al. 2004). In addition to making P450s and P450 reductases amenable to fractionations on affinity and sizing columns normally suited to soluble proteins, one clear advantage of this system is the stability of P450 proteins afforded by insertion into Nanodiscs. Separated from an array of undefined membrane components, CYP73A5-containing Nanodiscs incubated for 96 h at 25°C generate the same CO difference optima at 450 nm as at the beginning of the time course (Fig. 2A). But at longer times, (e.g., the 168 h time point in Fig. 2A), even this P450 shows some degree of destabilization with higher levels of P420 and lower levels of P450 suggesting some degree of unfolding in the catalytic site. Another clear advantage is that, without the high concentrations of microsomal proteins that are prone to scatter light in spectral assays for substrate binding, P450-containing Nanodiscs are capable of generating strong type I substrate binding profiles with lower amounts of P450 than standard microsomal assays. An example of this is shown in Fig. 2B where lauric acid binding spectra are compared for 30 pmol CYP86A8 assembled in Nanodiscs versus 100 pmol CYP86A8 in microsomes. Figures demonstrating the stability of P450-containing Nanodiscs to repeated fractionations on sizing columns and the purity of size-fractionated Nanodisc P450–P450 reductase complexes are included in Duan et al. (2004).

Currently, a number of ''extended'' and ''truncated'' MSP proteins which self-assemble into Nanodiscs larger than the typical MSP1 have been engineered (Denisov et al. 2004). These enable the incorporation of larger protein and protein–protein complexes into this type of nanobilayer and provides for the possibility of assembling larger macromolecular complexes containing P450s and other membrane integral proteins. Tagging methods for producing His-free MSP proteins have also been optimized (Baas

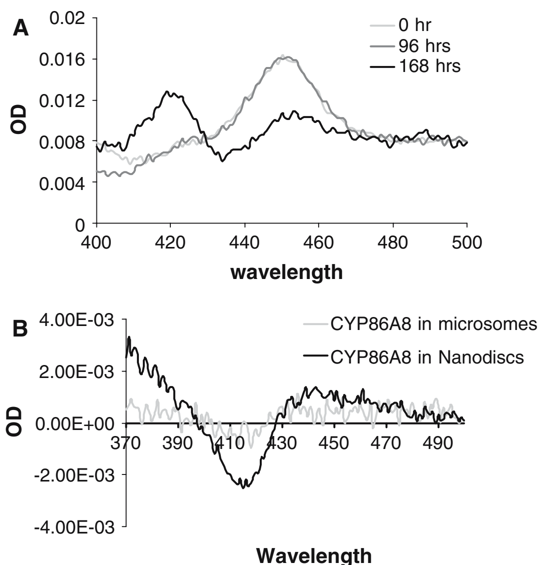

Fig. 2 Spectral analysis of P450-containing Nanodiscs. (**A**) Reduced CO-difference analysis of CYP73A5-containing Nanodiscs. Samples were stored at ambient temperature and CO-difference analyses were carried in standard cuvettes at 0 h (*light gray*), 96 h (*dark gray*) and 168 h (*black*). (**B**) Substrate binding analysis for CYP86A8 in Nanodiscs and microsomes. Type I-binding spectra were defined with either CYP86A8-containing Nanodiscs (30 pmol P450) or microsomes (100 pmol P450) incubated with 140 μM lauric acid

et al. 2004) and, when assembled with plant P450s tagged at their C-terminus with four histidines, these His-free MSP proteins provide promising opportunities for purification of homogenous P450-contaning Nanodiscs for future structure determinations.

Conclusions

The heterologous expression systems discussed above have been used to produce plant P450s in many different sorts of functional forms. Each system has its own advantages and disadvantages with the most frequently used and optimized yeast system expected to continue as the prime contender for functional characterizations. Even so, the baculovirus-insect cell system, which is being used for expression of a growing number of plant P450s, is coming to the forefront for its ease in manipulating electron transfer components and its less apparent codon constraints. With the opportunity to scale-up cultures in liquid suspension cultures, this system should prove valuable for future plant P450 research. Interestingly, in two recent independent studies aimed at characterizing the *Arabidopsis* P450 CYP707A subfamily (ABA 8′-hydroxylases), functional expressions were done in both yeast and insect cell systems (Kushiro et al. 2004; Saito et al. 2004) indicating that both systems are appropriate for functional analysis of these P450s. The bacterial system, although requiring substantial cDNA modification for functional P450 expression, will likely be the most ideal system for generating the large quantities of proteins needed for crystallization studies. Both the bacterial and yeast systems have the advantage of being readily scaled for fermentation production of natural products. In both reconstitution and co-expression assays, P450 reductases from several different kingdoms have been used as electron providers. Examples of this include use of yeast P450 reductase for CYP73A1 (Urban et al. 1994), rat P450 reductase for CYP79B2 (Hull et al. 2000) and housefly P450 reductase for CYP73A5 (Duan et al. 2004). The reasonable catalytic activities obtained in these three examples suggest that the P450 reductase functions are relatively conserved and not limiting to the catalytic activities of many plant P450s. Coupling these heterologous expression system with the new Nanodisc technologies dispersing overexpressed membrane proteins into monodispersed bilayers in their native form provides unique and exciting opportunities to produce "soluble" P450 proteins in defined complexes associated with lipid bilayers.

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