EPOXYGENASE EXPRESSION IN SOYBEAN AND BIOLOGICAL EFFECTS OF EPOXY FATTY ACIDS

Purnima Kamlakar Wagh
University of Kentucky, waghpk@email.uc.edu

Right click to open a feedback form in a new tab to let us know how this document benefits you.

Recommended Citation
https://uknowledge.uky.edu/gradschool_theses/384

This Thesis is brought to you for free and open access by the Graduate School at UKnowledge. It has been accepted for inclusion in University of Kentucky Master's Theses by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.
ABSTRACT OF THESIS

EPOXYGENASE EXPRESSION IN SOYBEAN AND BIOLOGICAL EFFECTS OF EPOXY FATTY ACIDS

Epoxy fatty acids (EXA) are valuable to industry as they are used in synthesizing plasticizers such as of poly vinyl chloride, resins, adhesives, coating materials such as paint, lubricant, lubricant additives, insecticides, insect repellants, crop oil concentrates and formulations of carriers for slow release pesticides and herbicides. There is interest in developing commercial oilseeds accumulating epoxy fatty acids to at least 50% of the seed oil. Soybeans are the most widely cultivated oilseed and its oil has high levels of linoleic acid which can be a substrate for epoxygenase enzymes. Cahoon et al., expressed a cytochrome P450 enzyme (CYP726A1) from *Euphorbia lagascae* in soybean somatic embryos and found that the epoxy fatty acid, vernolic acid, reached ~8% of the total fatty acids in transgenic somatic embryos. Rabbit Livers possess a cytochrome P450, CYP2C2, which catalyzes the same epoxidation reaction as the *E. lagascae* enzyme but might be less likely to be influenced by regulatory machinery in plant cells. This CYP2C2 gene was placed in a plant expression vector under a seed-specific promoter and used to transform soybean, *Glycine max*, somatic embryos. The ten putative transgenic clones observed after 4-5 weeks were separated and proliferated under selection. β-glucuronidase (GUS) assays and PCR analyses performed on selected clones were positive. However vernolic acid in total lipids and specific lipid classes was not detected as analyzed by GC. *In vitro* enzyme assay performed on microsomes isolated from mature somatic embryos at three weeks of maturation using [14C] 18:2 PC as substrate showed presence of [14C] methyl vernoleate. Preliminary analyses on toxicity of epoxy fatty acids and corresponding diols in bacteria, yeast and caco-2 cells showed that leukotoxin diol (LD) most toxic.

KEYWORDS: Epoxy fatty acid (EXA), rabbit epoxygenase (CYP2C2), Soybean (*Glycine max*), β-glucuronidase (GUS), radiolabeled linoleoyl phosphatidyl choline [14C] 18:2 PC

Purnima Kamlakar Wagh
August 1st, 2006

Copyright © Purnima Kamlakar Wagh 2006
EPOXYGENASE EXPRESSION IN SOYBEAN AND BIOLOGICAL EFFECTS OF EPOXY FATTY ACIDS

By

Purnima Kamlakar Wagh

David Hildebrand, Ph.D.
Director of thesis

Steven Post, Ph.D.
Director of Graduate Studies

August 1st, 2006
RULES FOR THE USE OF THERSES

Unpublished theses submitted for the Master’s degree and deposited in the University of Kentucky Library are as a rule open for inspection, but are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but quotations or summaries of parts may be published only with the permission of the author, and with the usual scholarly acknowledgements.

Extensive copying or publication of the thesis in whole or in part also requires the consent of the Dean of the Graduate School of the University of Kentucky.

A library that borrows this thesis for use by its patrons is expected to secure the signature of each user.
THESIS

Purnima Kamlakar Wagh

The Graduate School
University of Kentucky
2006
EPOXYGENASE EXPRESSION IN SOYBEAN AND BIOLOGICAL EFFECTS OF EPOXY FATTY ACIDS

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Nutritional Sciences Department, The Graduate School at the University of Kentucky

By

Purnima Kamlakar Wagh

Lexington, Kentucky

Director: Dr. David Hildebrand, Professor of Agronomy

Lexington, Kentucky

2006

Copyright © Purnima Kamlakar Wagh 2006
Acknowledgements

I sincerely thank my advisor Dr. David Hildebrand for giving me an opportunity to work in his lab. His constant support and guidance helped me tremendously to shape my work. He strongly motivated me to pursue the task after repetitive failures.

I would also like to thank all my lab members who made this thesis possible. In particular I would like to thank Dr. Keshun Yu who helped me exhaustively for the enzyme assays.

I wish to thank my committee members, Dr. Brett Spear and Dr. Michael Toborek for the useful discussions and co-operation. I would like to thank Dr. Brett Spear for providing the caco-2 cells for toxicity experiments and Dr. Toborek for giving me an opportunity to work in his lab and conduct the toxicity experiments.

Last but not the least I would like to thank my family and friends for their support and motivation.
# TABLE OF CONTENTS

Acknowledgements........................................................................................................ iii

List of Tables.................................................................................................................. v

List of Figures................................................................................................................ vi

List of Files.................................................................................................................... vii

Introduction.................................................................................................................... 1

Literature review............................................................................................................. 2

Chapter 1: Expression and characterization of rabbit epoxygenase in soybean somatic embryos

A. Introduction................................................................................................................. 12

B. Materials and Methods............................................................................................. 13

C. Results......................................................................................................................... 39

D. Discussion.................................................................................................................... 62

E. Conclusions................................................................................................................ 66

F. Future Prospects........................................................................................................ 67

Chapter 2: Toxicity testing of epoxy and diol compounds

A. Introduction................................................................................................................. 68

B. Materials and Methods............................................................................................. 70

C. Results......................................................................................................................... 75

D. Discussion.................................................................................................................... 82

E. Conclusions................................................................................................................ 84

Appendix......................................................................................................................... 85

References..................................................................................................................... 86

Vita................................................................................................................................. 91
List of Tables

Table 1: The distinction of P450 type epoxygenases from desaturase ............... 4
Table 2: Sequencing primers for CYP2C2 ................................................................. 14
Table 3: Primers for NCoI site mutagenesis ......................................................... 18
Table 4: Primers for adding NCoI and Hpa I sites at 5’ and 3’ end respectively .... 20
Table 5: Primers for PstI site mutagenesis ............................................................... 22
Table 6: DNA extraction buffer recipe ................................................................. 26
Table 7: RT-PCR reaction components and quantities ........................................... 30
Table 8: Quantitation of phosphotidylcholine from the microsomes .................. 32
Table 9: Reaction components and quantities for in-situ labeling ....................... 35
Table 10: In vitro enzyme activity calculated by amount of product ................. 59
Table 11: Toxicity measurement of methyl vernoleate in E. coli ....................... 76
Table 12: Toxicity measurement of vernonia oil in E. coli ................................. 77
Table 13: Toxicity measurement of isoleukotoxin diol in E. coli ....................... 78
Table 14: OD values for controls ......................................................................... 79
List of Figures

Figure 1: Chemical structure of vernolic acid………………………………………….. 3
Figure 2: Intermediate cloning vector pPHI4752………………………………………… 15
Figure 3: Plant expression vector pCAMBIA 1201………………………………………. 16
Figure 4: CYP2C2 ligation in pPHI4752…………………………………………………. 40
Figure 5: CYP2C2 ligation in pCAMBIA1201………………………………………….. 41
Figure 6: PCR analysis of CYP2C2 expressing transgenic lines………………………… 44
Figure 7: GUS reporter gene assay on transgenic soybean somatic embryos……………… 46
Figure 8: GUS reporter gene assay on matured somatic embryos……………………….. 48
Figure 9: Comparison of total fatty acid levels of different transgenic lines……………… 50
Figure 10: Polar fraction fatty acid profile of transgenic lines…………………………… 51
Figure 11: Neutral fraction fatty acid profile of transgenic lines………………………… 52
Figure 12: RT-PCR of the matured somatic embryos at mid maturation stage……………… 54
Figure 13: Synthesis of $^{[14}C]$ 18:2 CoA …………………………………………… 56
Figure 14: In situ labeling of transgenic soybean microsomes…………………………….. 57
Figure 15: Phosphorimager of TLC plate after performing the enzyme assay……………… 60
Figure 16: Enzyme assay on transgenic soybean microsomes at three weeks…………… 61
Figure 17: Synthesis of isoleuktoxin diol………………………………………………… 72
Figure 18: The effect of MV, VA, LD, ESO, ELO, VO and SO on viability…………… 81
# List of Files

<table>
<thead>
<tr>
<th>File Name</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purnima_MSthesis.pdf</td>
<td>3.7 MB</td>
</tr>
</tbody>
</table>
Introduction

Vernolic acid is an epoxy fatty acid and has useful industrial and medical applications. Currently the need for epoxy fatty acid is supplied by petroleum derived sources and by chemically epoxidizing vegetable oil. The petroleum sources are being depleted day by day and the high cost of chemically modifying vegetable oil demands a useful and renewable source of epoxy fatty acids. Species like *Vernonia galamensis*, *Euphorbia lagasceae* and *Stokesia laevis* accumulate up to 80%, 75% and 70% of vernolic acid respectively in their seed oils. However growing these plants for obtaining large amounts of vernolic acid useful for commercial application is a difficult task because of their lack of good agronomic traits. Therefore it is of great interest to use molecular genetic approaches to clone putative cDNA’s encoding the genes responsible for vernolic acid synthesis and expressing them in oilseed crops. Over the last decade there has been much research in this area to obtain a commercially valuable crop accumulating high levels of vernolic acid in the seed oil. It has already been shown that transgenic *Arabidopsis thaliana* seeds accumulate up to 10% epoxy fatty acid when Δ-12 epoxygenase and Δ-12 desaturase were co-expressed. Similarly soybean somatic embryos were able to accumulate 8% epoxy fatty acid when P450 epoxygenase from *Euphorbia lagasceae* was expressed under a seed-specific promoter.

Epoxy fatty acids are also produced in our body by action of cytochrome P450 enzymes on linoleic acid. They are termed as leukotoxins; high concentrations of which are found to be toxic. Leukotoxin diol, which is formed by the hydrolysis of leukotoxin is found to be more toxic than the parent leukotoxin. The handling of transgenic plants accumulating large amounts of the epoxy fatty acid in seed oil will depend on its toxicity profile. Use of soybean meal as a food to the animal industry will depend on the toxicity of epoxy fatty acid. The main objective of this research is to study the expression and characterization of a mammalian epoxygenase gene in soybean somatic embryos. The minor objective is to test the toxicity of several epoxy compounds in *yeast*, *E. coli* and mammalian cells.
Literature Review

Vernolic acid Sources and Applications

Unusual fatty acids like epoxy fatty acids are valuable to industry. Vernolic acid (\textit{cis}-12, 13-epoxyoctadeca-\textit{cis}-9, 10-enoic) acid is an 18 carbon fatty acid molecule with the epoxy functional group present between the 12 and 13 position. Natural accumulators of the vernolic acid are \textit{Vernonia galamensis}, \textit{Stokesia laevis} and \textit{Euphorbia lagascae} (Gunstone, 1954; Badami and Patil, 1981; Kleiman et al., 1965) which accumulate up to 80\%, 70\%, 75\% vernolic acid of the seed oil. Two types of characterized epoxygenases have been discovered, a cytochrome P450 type epoxygenase and a desaturase type non-heme di-iron epoxygenase (Table 1). It has been found that epoxygenases from \textit{Stokesia} and \textit{Vernonia} are the desaturase type while that from \textit{Euphorbia} is cytochrome P450 type epoxygenases.

The economic production of these plants for obtaining high EXA levels is difficult due to a lack of good agronomic characteristics including the seeds maturing at different times, unsynchronized flowering, shattering, lodging and low seed yield. Also, depleting petroleum reserves and the high cost of chemically epoxidizing vegetable oils demands the need for renewable resources. There are ongoing efforts to make transgenic plants accumulating large amount of epoxy fatty acids.

Currently, epoxy fatty acids are obtained from petroleum sources and by chemical modification of vegetable oil such as soybean and linseed oil. The process for epoxy fatty acid synthesis is expensive and requires the use of a catalyst (Perdue et al., 1986). The oxirane moiety of the epoxy molecule is highly reactive, which is desirable for industrial applications. The oxirane content of vernonia oil is 4.1\%, epoxidized soybean oil is 7\% and epoxidized linseed oil is 8.9\% (Carlson and Chang, 1985). Chemical epoxidation of vernonia oil gives an oxirane content of 8.2\%. The higher the oxirane content the better it is.
Figure 1: Chemical structure of vernolic acid

cis-12,13-epoxy-octadec-cis-9-enoic acid
Table 1: The distinction of P450 type epoxygenases from desaturase type epoxygenases as indicated by CO and CN inhibitory reactions.

<table>
<thead>
<tr>
<th>CYT P450</th>
<th>Rxn is inhibited by CO</th>
<th>And CYT P450 reductase Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desaturase type epoxygenase</td>
<td>Rxn is inhibited by CN</td>
<td>--------------</td>
</tr>
</tbody>
</table>
One of the synthetic processes of epoxy fatty acid synthesis is by peroxygenase enzyme in the presence of a suitable oxygen donor. The peroxygenase isolated from a biological source and immobilized on a filter membrane was very active and the membrane containing the active enzyme was reusable. Thus, the use of enzyme as catalyst was found to be more benign and environmentally friendly than the traditional acid catalyst reactions (Piazza et al., 2000).

Epoxy fatty acids are used in the synthesis of epoxy resins, lubricant, lubricant additives, insecticides, insect repellents, crop oil concentrates, adhesives, coatings, slow release of pesticides and herbicides, metal coatings and plasticized phenolic resins (Perdue, 1989; Carlson and Chang 1985). Some non-shrinking dental composite material can also be made by molecules formed by ring opening of epoxides (Schweikl et al., 2002).

The various possible vernolic acid synthesis mechanisms in soybeans are:

- Peroxygenase which is non-P450, heme-containing protein that catalyzes the epoxidation reaction does not require NADPH, cytochrome P450 reductase, or cytochrome b5 (Blee and Schuber 1990), although it requires hydroperoxides as an oxygen donor (Laethem et al., 1992)
- P450 mono-oxygenase catalyzed the reaction which requires CoASH, ATP and NADPH and unlike peroxygenase, does not discriminate between cis and trans double bonds.
- Desaturase homologue like the di-iron clusters (van de Loo et al., 1993).
- Free peroxyl radicals formed by reaction of hydroperoxides with free transition metals or heme complexes.

(Blee and Schuber 1990) demonstrated an in vitro epoxidation of unsaturated fatty acids by partially purified peroxygenase from soybean microsomes. This enzyme catalyzed only the cis-double bond. Oxidation of linoleic acid was highly regio-selective and showed preference for 9, 10 position (> 72% coronaric acid) than 12, 13 (vernolic acid).
The soybean peroxynase also catalyzed the epoxidation of oleic acid. For epoxidation the hydroperoxides can also serve as substrates in co-oxidation reactions. The enzymatic co-oxidation reactions are catalyzed by lipoxygenase (Chan, 1971; Belvedere et al., 1983) and the non-enzymatic by transition metal or heme complexes (Gardner, 1989).

**Cloning of epoxygenases and their expression studies**

In order to understand the precise biochemical pathway of epoxy fatty acid synthesis and its incorporation into triacylglycerol, some of the epoxygenase genes from native plants were cloned and expressed in systems like yeast, *Arabidopsis thaliana*, *Nicotiana tabaccum*, soybean somatic embryos, etc.

When a Δ-12 epoxygenase gene from *Crepis palaestina Cpal2* was expressed in *Arabidopsis thaliana* under a napin seed-specific promoter, 6.2% epoxy fatty acid accumulated in seeds. There was a marked increase in oleic acid (18:1) and a decrease in linoleic (18:2) and _-linolenic acid in these plants. But when a Δ-12 desaturase from *C. palaestina* was co-expressed in *Arabidopsis thaliana* they found that the fatty acid profile was normalized and the epoxy fatty acid obtained increased up to two fold (Singh, Thomaeus et al., 2001).

When CYP726A1 from *E. lagascae* was expressed in yeast, tobacco callus and soybean somatic embryos, the amount of epoxy fatty acid accumulated in yeast was 1-5% (w/w) of total fatty acids, Δ \(^{12}\) epoxy fatty acid detected in tobacco callus was 15% (w/w), out of which vernolic acid was around 13% (w/w) and in soybean somatic embryos the amount of epoxy fatty was nearly 8% (w/w) of the total fatty acids. There was also a slight increase in 18:1 and a small decrease in 18:2 content as compared to untransformed embryos. The increase in 18:1 content was more in tobacco callus as compared to soybean somatic embryos probably because it is possible that soybean somatic embryos are more efficient in removing epoxy fatty acids from PC thereby facilitating its sequestration into triacylglycerol (TAG). It has been suggested that these unusual fatty
acids products inhibit the activity of native FAD2 (enzyme that converts oleic to linoleic) which might be a potential reason for increased oleic acid in tobacco callus (Cahoon, Ripp et al., 2002).

(Blee, Stahl et al., 1993) reported vernolic acid synthesis from linoleic acid by cytochrome P450 epoxygenases and peroxygenases in microsomes prepared from Euphorbia lagascae developing seeds. This cytochrome P450 catalyzed reaction produced exclusively cis-12(S), 13(R)-epoxy-9-(Z)-octadecenoic acid with high regio and enantioselectivity. Also coronaric acid was not found to accumulate in Euphorbia lagascae developing seeds. This is unlike the 12(R), 13(S)-isomer which occurs in the seed oils of some Malvaceae. The 9, 10-Epoxy-12-octadecenoic and 12, 13-epoxy-9-octadecenoic acids are also produced in humans by oxidation of unsaturated fatty acids, e.g. linoleic acid. These cytotoxic molecules are called leukotoxins and are produced by leukocytes (Blee and Schuber 1990).

Enzyme activity assays from microsomes isolated from developing endosperm of Euphorbia lagascae were reported by (Bafor, Smith et al., 1993). Microsomes were labeled in situ with [1-14C] linoleate and it was found that [1-14C] vernolate was formed on PC in the presence of NADPH as a co-factor. For short incubation times the vernolate was mostly confined to the PC fraction but as the incubation time increased the free vernolic acid dominated. Based on the kinetic studies following biosynthesis pathway for vernoloyl-triacylglycerol synthesis in endosperm of E. lagascae was suggested.

- Oleoyl-PC is deaturated to linoleoyl-PC
- Linoleoyl-PC is epoxidized to vernoleoyl-PC
- Vernolic acid at the sn-1 and sn-2 positions of PC is converted to free vernolic acid and lyso-PC
- The free vernolic acid is esterified to its CoA ester by acyl-CoA synthetase followed by its incorporation into triacylglycerol via the glycerol 3-phosphate pathway.
On the other hand, the vernolyl CoA reacts with diacylglycerol (DAG) catalyzed by diacylglycerol acyltransferase (DAGAT) to triacylglycerol (TAG) (Frazer et al., 2000). The acyl-CoA independent formation of triacylglycerol in Crepis palaestina, Ricinus communis, and yeast microsomes was reported by (Dahlqvist, Stahl et al., 2000). The enzyme which catalyzes this acyl-CoA independent TAG synthesis is phospholipid:diacylglycerol acyltransferase (PDAT). It has also been shown that in both microsomes from developing plant seeds and from yeast utilize phospholipids:diacylglycerol acyltransferase (PDAT) enzyme for TAG synthesis, which is CoA independent. In microsomes from developing Euphorbia seeds, evidence is presented that phospholipases remove vernolic acid from PC, but a slight PDAT activity was also detected. On the other hand, in microsomes from developing Crepis palaestina removal of vernolic acid from PC appears to mainly be a PDAT enzyme (Banas et al., 2000).

(Seither et al., 1996) characterized the epoxygenase activity in Vernonia galamensis. By cyanide and carbon monoxide inhibitor studies they found the epoxygenase from Vernonia is a desaturase type epoxygenase but not a cytochrome P450 type one. (Hatanaka et al., 2004); cloned an epoxygenase gene from Stokesia laevis and expressed it in yeast and Arabidopsis thaliana. In yeast, vernolic acid accumulated to 0.64% and in transgenic plants, vernolic acid accumulation was about 2.4%.

Epoxy molecules are naturally involved in the synthesis of the cutin polymer which covers most of the aerial parts of plants. Two different biochemical pathways for epoxy fatty acids have been reported. The P450 catalyzed reactions form epoxy fatty acids in spinach, pea, Helianthus tuberosus and Vicia sativa. Also the peroxygenase catalyzed reactions are described in soybean seedlings and Vicia species.

**Generation of transgenic plants and possible pitfalls**

Plants can be considered “green factories” for production of molecules. Plants are carbon-neutral i.e. not contributing to CO₂ emissions. Also plant growth requires only
sunlight, carbon dioxide, water and minerals. Transgenic plants that confer input trait help the farmers whereas those which confer output traits help the consumer. The development of these second generation transgenic plants with output traits can require significant amount of time (Napier, Haslam et al., 2006).

Soybean transformation


A marker free transgenic soybean was generated by simultaneous introduction of two T-DNA elements, one had the marker gene and the other T-DNA element had the borage Δ⁶ desaturase gene under a seed specific _-conglycinin promoter. Simultaneous delivery of these two cassettes led to production of 3.4-28.7% of _-linolenic acid (GLA) and 0.6-4.2% of stearidonic acid (STA) in soybeans. Four lines were free of selectable marker and a total of twenty nine transgenic soybean lines contained both the T-DNA elements (Sato, Xing et al., 2004).

Agrobacterium tumefaciens mediated cotyledonary-node transformation was reported by (Olhoft, Flagel et al., 2003). Use of hygromycin B resulted in rapid selection of transgenic shoots. The addition of thiol compounds like _-cysteine, dithiothreitol and sodium thiosulphate during co-cultivation significantly increased the A. tumefaciens infection and T-DNA transfer into cotyledonary node cells. Thus the overall transformation efficiency was about 16.4%.
Screening of 15 soybean cultivars having different genetic background for soybean transformation under hygromycin selection was reported by (Ko, Nelson et al., 2004). Among those 15 cultivars which were transformed by Agrobacterium three of them i.e. the Cisne, Council and the Kunitz were highly embryogenic. Induction of somatic embryos along the margins of the abaxial side of cultured cotyledons for these high embryogenic cultivars originated mainly from the actively dedifferentiating and necrotic tissue.

The biolistic process of plant transformation via particle bombardment allows the DNA coated gold particles to accelerate into the target tissue. DNA dissociates from the gold particles by the action of cellular fluids and can subsequently integrate into the genome of the organism. A fertile transgenic plant can be formed from the transformed cell. The selection of transformed apical meristematic cells of transgenic soybean can be accomplished with imazapyr, a herbicide molecule of the imidazolinone class (AragÃo, Sarokin et al., 2000).

Soybean transformation via particle bombardment of embryonic suspension culture tissue was reported by Finer and McMullen (1991). Histological studies to determine proliferating somatic embryo origin revealed that the embryos originated from the surface and the adjacent sub-surface tissue. The transformation efficiency determined by particle gun bombardment of a pUCGUS fragment in soybean somatic embryos and found that the conversion efficiency from transient to stable transformation is around 0.4%. For producing stable transformants they used a pHG1 fragment that contained both the hygromycin as well as the GUS gene. An average of three transgenic clones per bombardment was obtained.

**Improvement of soybean transformation protocol**

Improvement of soybean transformation protocols is important. Since soybean somatic embryogenesis is very useful in evaluating the seed-specific traits of the expressed transgene it is necessary to have optimal maturation.
Auxin and sucrose interaction in soybean somatic embryogenesis was evaluated by Lazzeri et al., (1988). A significant interaction between the two compounds has been found and that a precise balance between the two was necessary for optimal embryo production and normality. Also the germination ability of somatic embryos was more influenced by sucrose content than auxin content. Proliferation, maturation, desiccation methods in soybean somatic embryogenesis has vast effect on germination and subsequent conversion to whole plants. Liquid media lacking vitamin B5 and 2, 4-D hormone gave faster and better quality matured somatic embryos. Also the air'-drying treatment for desiccation gave best regeneration rate (Moon et al., 2003).

(Schmidt, Tucker et al., 2005) studied the effects of carbon, amino acid supplements, abscisic acid and basal salts on maturation. The first maturation medium reported is MSM6 wherein the embryos were placed on the solid medium having MS basal salts with 6% maltose and 0.5% activated charcoal. Another medium reported is FNLS3 which has FNL (Finer and Nagasawa) salts and 3% sucrose. The disadvantage with FNLS3 was that very few embryos would convert to plants. The limitation of FNLS3 media was solved by addition of 3% sorbitol. The sorbitol serves as a humectant and the embryos obtained are very large and can germinate into plants. The addition of 3% sucrose instead of maltose produced 61% more plants per milligram of initial tissue. The glutamine supplementation gave larger embryos and increased the oil and storage protein reserves and 1 mM methionine could be served as growth stimulant. Abscisic acid did not have any significant effect in counteracting the delay in maturation time. The glutamine supplementation promoted the accumulation of storage reserves and attainment of physiological maturity and thereby desiccation tolerance in somatic embryos.
Chapter 1

Expression and characterization of rabbit epoxygenase CYP2C2 in soybean somatic embryos

A. Introduction

In an effort to accumulate additional epoxy fatty acids in soybeans at levels higher than previous epoxygenase transgenics, we expressed a rabbit epoxygenase CYP2C2 in soybean somatic embryos. The CYP2C2 clone pH2 encodes a protein of 490 amino acids (Imai et al., 1988). The first twenty amino acids comprise the trans-membrane domain with a hydrophobic N terminal signal anchor sequence. The next eight amino acids form a relatively hydrophilic glycine rich region followed by a proline rich region which is essential for formation of a functional protein. The C terminal domain has the active site for catalysis (Doray, Chen et al., 2001). Eukaryotic P450 enzymes form 1:1 functional complexes with a reductase enzyme and require NADPH for catalytic activity. When P450 2C2 was expressed in yeast, COS cells, and insect cells, specific proline residues in proline rich regions were found important for proper folding and formation of a functional P450 (Kusano, Sakaguchi et al., 2001).

Transient transfection of COS-1 cells with P450 2C2 in the presence or absence of 1,2-epoxy-3,3,3-tri-chloropropane and linoleic acid gave both the epoxide metabolites 12, 13 and 9, 10 indicating the role of P450 2C2 in catalyzing epoxidation reaction. Proper folding of microsomal P450 monoxygenase and its redox partners is essential for the formation of an active enzyme. Also, microsomal P450 is speculated to interact with multiple components of ER (Laethem, Balazy et al., 1996).

It has long been known that the N terminal signal anchor sequences of P450 enzymes are necessary for membrane integration and act as ER retention signal. Recently (Szczesna-Skorupa and Kemper 2006) showed that BAP31, a ubiquitously expressed ER membrane protein forms a complex with P450 2C2 or the signal anchor sequence of 2C2
and these interactions affected both the localization and the expression level of P450 2C2, thus facilitating P450 2C2 retention in ER.

Soybeans are the second most widely cultivated crop in the US. Soybeans have 40% protein and 20% oil. Soybean oil has high amounts of linoleic acid, a substrate for epoxygenase enzymes. Researchers are interested in genetically engineering soybeans for medical and industrial applications. The transgenic plants generated from a single somatic cell or a group of somatic cells is called somatic embryogenesis. Soybean somatic embryogenesis has been used to generate transgenic plants and to evaluate seed-specific traits of the expressed transgene.

The main hypothesis of this study is that rabbit CYP2C2 is capable of producing vernolic acid (12(S), 13(R)-epoxy-9(\(z\)) octadecenoic acid) from linoleic acid in soybean somatic embryos. To test this hypothesis rabbit CYP2C2 was expressed in soybean somatic embryos.

**B. Materials and Methods**

**Sequencing CYP2C2 construct (pESC-trp vector containing CYP2C2)**

CYP2C2, a Rabbit cytochrome P450 epoxygenase (laurate omega-1 hydroxylase) was sequenced using the following primers in a Beckman Coulter sequencer.
Table 2: Sequencing primers for CYP2C2

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C2 seq R1</td>
<td>5' AGT GAA CAC GGG CCC ATA GAC TTT 3'</td>
</tr>
<tr>
<td>2</td>
<td>C2 seq R2</td>
<td>5' TTC ACT GCT TCA TAC CCA TGC ACC A 3'</td>
</tr>
<tr>
<td>3</td>
<td>C2 seq R3</td>
<td>5' CAA TTG CAG AAC TGT ACC CAG G 3'</td>
</tr>
<tr>
<td>4</td>
<td>C2 seq R4</td>
<td>5' ATG TAT CTC TGG ATC TCG TGC ACC GT 3'</td>
</tr>
<tr>
<td>5</td>
<td>C2 seq F1</td>
<td>5' CAT GGA GCT GTT TCT GTT CCT GAC T 3'</td>
</tr>
<tr>
<td>6</td>
<td>C2 seq F2</td>
<td>5' CCC AAG GGC ACA GAT GTA CTA ACA 3'</td>
</tr>
</tbody>
</table>
Constructing a seed-specific transformation vector with CYP2C2 gene

Figure 2: Intermediate cloning vector pPHI4752 having the seed-specific phaseolin promoter and terminator. CYP2C2 was cloned in this vector with Ncol and HpaI at 5’ end and 3’ end respectively.
Figure 3: Plant expression vector pCambia 1201. CYP2C2 with the seed-specific promoter and terminator was cloned in the multiple cloning site of this vector with Kpn I and Pst I at 5’ and 3’ ends respectively.
Cloning of CYP2C2 in pPHI4752

The CYP2C2 gene was cut from the pESC-Trp vector and ligated into a phaseolin cassette of pPHI4752 using *Nco*I and *Hpa*I. For a successful ligation, it was necessary to delete the *Nco*I site present 70 base pairs after the start codon in CYP2C2. To achieve this, the nucleotide thymine 72 was changed to cytosine 72 by PCR mutagenesis. This mutation does not change the amino acid Histidine 24. PCR mutagenesis was done employing the following primers.
Table 3: Primers for NCoI site mutagenesis

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C2 Mut F</td>
<td>5’ GGA AAC AGA GCC ACG GGG GAG GGA AGC TTC 3’</td>
</tr>
<tr>
<td>2</td>
<td>C2 Mut R</td>
<td>5’ GAA GCT TCC CTC CCC CGT GGC TCT GTT TCC 3’</td>
</tr>
</tbody>
</table>
For cloning of the CYP2C2 gene into pPHI4752 NcoI and HpaI sites were created at the 5’ and 3’ end of the CYP2C2 gene by PCR. The primers used for adding the restriction sites were as follows.
Table 4: Primers for adding *Nco*I and *Hpa*I sites at 5’ and 3’ end respectively

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5’ C2</td>
<td>5’ GCG GCC CAT GGA TCT GGT GGT AGT GCT G 3’</td>
</tr>
<tr>
<td>2</td>
<td>3’ C2</td>
<td>5’ GCC GGC GTT AAC CTC AGA CAG GAA TGA AGC TGA C 3’</td>
</tr>
</tbody>
</table>
The PCR mixture was composed of 10 X PCR buffers, 10mM dNTP mixture, 50mM MgSO4, 5’ and 3’ C2 primers 10 _M each, 2 _L template DNA (which was CYP2C2 in pESC-trp), Platinum Taq high fidelity 5 units/_L and H2O to make final volume of 50 _L. The program for PCR was (a) 94.C-2 min (b) 35 cycles of 94.C-30 sec, 55.C-30 sec, 68.C-90 sec (c) and final extension at 68.C for 5 min.

After performing PCR for adding the NCol and Hpal sites, the PCR product was ligated in a pGEM-T Easy-vector. Ligation was performed and DH5_ cells were transformed. Cells were plated on LB Amp plates (100 _g/mL). The gene was cut from the pGEM-T-Easy vector with NCol and Hpal and was ligated to pPHI4752 that was cut with the same enzymes (NCol is present at the end of the promoter and Hpal at the beginning of the terminator in the phaseolin cassette of pPHI4752).

Cloning of pPHI4752-CYP2C2 in pCAMBIA 1201

To ligate the phaseolin cassette with the CYP2C2 gene into pCAMBIA 1201 using Kpn I and Pst I sites it was decided to delete the Pst I site 125 bp from the start codon of CYP2C2. To achieve this, the nucleotide G129 was changed to C129. This mutation did not change the amino acid Leucine 44. The primers employed for PCR mutagenesis were as follows.
Table 5: Primers for *PstI* site mutagenesis

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C2PstIMutF</td>
<td>5’ CCA ATT CTT GGA AAC GTC CTC CAG TTA GAT TTT AAG GAC 3’</td>
</tr>
<tr>
<td>2</td>
<td>C2PstIMutR</td>
<td>5’ GTC CTT AAA ATC TAA CTG GAG GAC GTT TCC AAG AAT TGG 3’</td>
</tr>
</tbody>
</table>
*Kpn* I and *Pst* I were used for double digestion and 0.7 _L_ of calf alkaline phosphatase (1U/ _l_) and 1 _L_ of alkaline phosphatase buffer were used for removal of 5’ phosphate group. After gel extraction, ligation was performed at 15 °C for 3 h. *BamHI* was used as clean-up enzyme to facilitate the insert ligation in pCAMBIA 1201. After addition of *BamHI* the ligation mix was kept at 37 °C for 20 mins followed by *E. coli* transformation. The ligation mixture was plated on LB chloramphenicol plates with chloramphenicol from stock concentration as 10 mg/ml.

**DNA preparation for shooting**

Fifteen milligrams of gold (particle size 0.6 µM) was used for 9 shots. The gold particles were weighed and put in a 1.5 mL eppendorf tube. Then the particles were washed with 1 mL of 100% ethanol. Then they were washed boarded and kept in ice for 30 seconds. The tube was centrifuged for 5 min at 5000 rpm and the liquid was removed. One hundred eleven microliters of 100% ethanol was added and the tube was vortexed again. Then the liquid was divided into 35 _L_ aliquots, the gold was continuously mixed when making the aliquots ensuring homogeneity between the three aliquots. The three tubes were again centrifuged for 10 seconds at 5000 rpm. One milliliter of water was added to each tube and then centrifuged for 5 min at 2000 rpm. The water and ethanol was removed. Twelve microliters of DNA, 220 _L_ of water, 250 _L_ of CaCl₂ (2.5 M) and 100 _L_ of spermidine (0.1M) were added and the tubes were vortexed continuously during each addition. The tubes were placed on ice for 2 min and vortexed for 10 min followed by centrifugation for 5 min at 1000 rpm. The liquid was removed and 600 _L_ of 100% ethanol was added. The pellet was vortexed for a min and then centrifuged for 5 min at 1000 rpm and the liquid was discarded. Thirty six microliters of 100% ethanol was added and the tube was wash boarded for 5-7 sec and then kept on ice for 1 hour.

**Shooting protocol**

Shooting was performed with 10 ng of DNA per particle preparation. The shooting was performed once at 1100 psi. Twenty five to thirty embryo clumps were
selected and placed on a D20 plate 5 days before shooting. For particle bombardment the DNA coated gold particles were placed on macro-carriers. When the pressure of 1100 psi was reached the rupture disc broke and the gold particles traveled with a high velocity and hit the embryo clumps placed on the D20 plate. The stopping screen prevents the macro-carriers from being carried away with the gold particles.

**Transformant selection and recovery protocol**

The embryos were kept on D20 (without selection) plates for 1 week after shooting. Then they were transferred to FNL proliferation media with hygromycin at 20 mg/L. The transformed embryos were sub-cultured in liquid media every 2 weeks with hygromycin (20 mg/L) selection. After 7-10 weeks in FNL-H20, each clump identified as independent transgenic line was transferred to an individual flask and was proliferated for about 1.5-2 months. After enough tissue of each transgenic line was obtained, it was used for analysis. Two or three embryo clumps were transferred to maturation media for further analysis on matured somatic embryos.

**GUS Reporter gene assay**

50mM Na- Phospahate, pH 7.0       4.095g Na2HPO4
0.5mM K-Ferricyanide              2.538g NaH2P04
0.5mM K- Ferrocyanide            164.6 mg
10 mM EDTA                        20mL of 0.5M stock
0.05% Triton X-100               500 µL

The following ingredients were dissolved into 150 ml volume. Buffer was dispensed into 15mL aliquots and stored at -20°C. The aliquot was subsequently diluted to 100 mL and was stored at 4°C. 35mg of X-gluc dissolved in 150 µL of dimethyl formamide (DMF) was added to the diluted buffer just before use. The sample was incubated in 600 µl of X-gluc solution for 24hrs at 37°C (Jefferson 1987)
DNA isolation from somatic embryos:

The embryos were kept frozen in sterile Eppendorf tube until ready for DNA extraction. Following is the DNA extraction buffer recipe.
Table 6: DNA extraction buffer recipe

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity/Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM Tris</td>
<td>5mL (2M stock)</td>
</tr>
<tr>
<td>20 mM EDTA</td>
<td>4mL EDTA (0.5mM)</td>
</tr>
<tr>
<td>0.5M NaCl</td>
<td>10 mL NaCl (5M stock)</td>
</tr>
<tr>
<td>0.5% SDS</td>
<td>2.5 mL SDS (20%)</td>
</tr>
<tr>
<td>0.5% -mercaptoethanol (BME)</td>
<td>0.5% volume BME was added immediately before using the buffer</td>
</tr>
</tbody>
</table>
Two to three medium sized embryo clumps were ground in liquid nitrogen with a pre-cooled mortar and pestle. Fifty microliters of DNA extraction buffer was added and the tissue was crushed thoroughly. Around 150 \text{ _L} of buffer was added again and the samples were crushed for one min. Seventy five microliters of chloroform was added and the mixture was vortexed for 10 sec. The samples were kept at room temperature for 10 min and centrifuged at 10,000 rpm for 10 min. The clear upper layer was transferred to a separate tube and 100 \text{ _L} of phenol and 100 \text{ _L} of chloroform were added. The samples were mixed by gentle inversion and spun at 10,000 rpm for 5 min. To the upper layer 120 \text{ _L} of isopropanol was added and mixed by gentle inversion. The samples were incubated at room temperature for 10 min and then spun at 12,500 rpm for 10 min. The pellet obtained was washed with 80\% ethanol (200 \text{ _L}) and samples were centrifuged at 12,500 rpm for 5 min. The centrifuge tubes were inverted on paper towels to remove excess liquid and the DNA pellet was re-suspended in 50 \text{ _L} of DEPC treated water.

**Total Lipid Extraction from matured transgenic somatic embryos**

Matured transgenic somatic embryos were weighed and transferred to a glass test tube. Five hundred microliters of sodium methoxide was added and the samples were crushed using a glass rod. The samples were incubated on a shaker for 15 min and 500 \text{ _L} of hexane was added to each tube. The samples were vortexed and the upper hexane layer was transferred into a new test tube. Subsequently 400 \text{ _L} of 0.9\% KCl was added to each tube. Again the samples were vortexed and the upper layer of hexane was removed into new tubes. The hexane was dried under a stream of nitrogen gas and the samples were re-suspended in 200 \text{ _L} of hexane.

**Separation of total lipids into polar and neutral fractions**

Approximately 0.5 g of matured somatic embryos was weighed and lipids were extracted according to (Bligh and Dyer 1959). In brief, 500 \text{ _L} of chloroform:methanol (2:1) and 500 \text{ _L} of 0.9\% KCl was added and the tube was vortexed well. After centrifugation the lower chloroform phase was removed and the aqueous phase was re-
extracted with 500 _L of chloroform. The chloroform layers were combined and were blown to dryness by nitrogen gas. Then 2 mL of chloroform was added to re-suspend the lipids. For separation of polar and neutral lipids, the total lipid re-suspended in chloroform was passed through a silica column. The neutral lipids were eluted by 2 mL of chloroform and then the polar lipids were eluted using 3 mL of chloroform: methanol: water (1:3:1 v/v/v). Both the solvent fractions were evaporated and re-suspended in hexane. The neutral lipids were analyzed by TLC using a solvent system of hexane: MTBE: acetic acid (75:25:1 v/v/v) and for the polar lipids were separated using a solvent system of chloroform: methanol: water (65:25:4 v/v/v). 

_Vernonia_ oil and free vernolic acid were used as standards for the neutral lipid fractions and soy lecithin was used as a standard for phosphatidyl choline. The bands corresponding to PC were scrapped off and methylated using sodium methoxide. No bands corresponding to free vernolic acid or vernolic acid TAG was observed by TLC analysis so the neutral lipids were also methylated with sodium methoxide. The methyl esters were analyzed by GC.

**Transcript analysis from transgenic matured somatic embryos**

One hundred fifty milligrams of tissue was ground in liquid nitrogen with pre-cooled mortar and pestle. One milliliter of Trizol reagent (Invitrogen) was added. The homogenized tissue was transferred to an RNase free centrifuge tube and centrifuged at 12,000 _g_ at 4 ºC for 15 min. The supernatant contained RNA and the pellet contained protein, extracellular membrane and polysaccharides. Supernatants were transferred to fresh centrifuge tubes and 300 _L_ of chloroform (per 1 mL of Trizol reagent) was added. The centrifuge tubes were shaken vigorously by hand for 15 seconds and were incubated at room temperature for 2-3 min. The mixture was centrifuged at 12,000 _g_ for 15 min at 4 ºC. The mixture was separated into a lower phenol-chloroform phase, a middle interface and a colorless aqueous phase. RNA, which remained exclusively in aqueous phase was transferred to a new centrifuge tube. 750 _L_ of isopropanol (per 1 mL of Trizol reagent) was then added. The samples were mixed by hand inversion and they were incubated at room temperature for 20 min. The mixture was centrifuged at 11,000 rpm for 10 min at 4 ºC. The RNA pellet obtained was washed with 75% ethanol (1 mL ethanol
per 1 mL Trizol) at 11,000 rpm for 5 min. After centrifugation the ethanol was removed and the pellet was air dried. The RNA pellet was re-suspended in 100 _L of DEPC treated water and the RNA was stored at -80 °C. No RT control was performed to ensure no genomic DNA contamination in RNA samples.

**RT-PCR**

The total RNA extracted from the CYP2C2 expressing matured somatic embryos was used for c-DNA synthesis by M-MLV reverse transcriptase. The following components were added:
Table 7: RT-PCR reaction components and quantities

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo (dT) primers 1_M</td>
<td>1 _L</td>
</tr>
<tr>
<td>1 ng to 5 _g of total RNA</td>
<td>3 _L</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1 _L</td>
</tr>
<tr>
<td><strong>H₂O</strong></td>
<td><strong>7 _L</strong></td>
</tr>
</tbody>
</table>
The mixture was heated to 65 °C for 5 min and quickly chilled on ice. The tube contents were collected by centrifugation. Then to the mixture 4 _L of 5X First-Strand Buffer, 2 _L of 0.1 M DTT, 1 _L of RNaseOUT Recombinant Ribonuclease Inhibitor were added. The contents were mixed gently and incubated at 37 °C for 2 min. Then 1 _L of 200 units of M-MLV RT was added and the samples were incubated at 37 °C for 50 min. The total reaction volume was 20 _L. The reaction was inactivated by heating at 70 °C for 15 min. The cDNA synthesized was used for PCR reactions. The RNA complementary to the cDNA was removed by adding 1 _L (2 units) of E. coli RNase H followed by incubation at 37 °C for 20 min.

For PCR, 5 _L of 10X PCR Buffer, 1.5 _L of 50mM MgCl2, 1 _L of 10 mM dNTP mix, 1 _L of 5’C2 - 5’ GCGGCCCATGGATCTGGTAGTGCTG 3’, 1 _L of 3’C2- 5’ GCGGCG GTAAACCTCAGACAGGAATGAAGCTGAC 3’, 0.4 _L of Taq DNA polymerase (5 U/ _L), 2 _L of c-DNA, 38.1 _L of H2O. The program for PCR was (a) 94 °C-2 min (b) 35 cycles of 94 °C-30 sec, 55 °C- 30 sec, 68 °C- 90 sec (c) and final extension at 68 °C for 5 min.

Microsomal assays

(Bafor, Smith et al., 1993) Around 2-3 g of fresh tissue (matured somatic embryos- 24 days) were collected and gently ground in a mortar and pestle with 10 parts (v/w) of 0.1 M potassium phosphate buffer, pH 7.2 containing 0.1% bovine serum albumin, 0.33 M sucrose and 1000 units catalase/mL on ice. The homogenate was filtered through a double layer of Miracloth and centrifuged at 20,000 _g for 15 min at 4 °C. After centrifugation the lipid pad was removed and the supernatant was filtered through a single layer of Miracloth. The supernatant was then centrifuged at 105,000 _g for 90 min at 4 °C in an ultracentrifuge. The resulting microsomal pellet was re-suspended in 1 mL of 0.1 M potassium phosphate buffer (pH 7.2).
Table 8: Quantitation of phosphatidylcholine from the microsomes isolated from matured somatic embryos

<table>
<thead>
<tr>
<th>Sample</th>
<th>g protein corresponds to 1 nmol PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>8131P1</td>
<td>3.2 g protein corresponds to 1 nmol PC</td>
</tr>
<tr>
<td>8132P5</td>
<td>5.0 g protein corresponds to 1 nmol PC</td>
</tr>
<tr>
<td>8132P2</td>
<td>1.48 g protein corresponds to 1 nmol PC</td>
</tr>
<tr>
<td>8136</td>
<td>3.58 g protein corresponds to 1 nmol PC</td>
</tr>
<tr>
<td>Control</td>
<td>0.21 g protein corresponds to 1 nmol PC</td>
</tr>
</tbody>
</table>
**Isolation and quantitation of microsomal PC**

Note: 14 _g of protein corresponds to approximately 1 nmol PC. There were two replications for each sample.

PC 17:0 was added to the glass test tubes and the chloroform was blown off. Microsomes were added to the test tubes and the tubes were vortexed. To the microsomes 500 _L of chloroform:methanol (2:1, v/v) and 500 _L of 0.9% KCl were added and the test tubes were vortexed well. The test tube was centrifuged and the lower chloroform phase was removed into a new test tube. The aqueous contents of the tube were re-extracted with 500 _L of chloroform, and both chloroform layers were combined. The chloroform layers were blown down to 50 _L with nitrogen and then loaded on a silica gel plate with soy lecithin as a standard (50 _g). The TLC was run in a solvent system of chloroform:methanol:acetic acid:water (85:17:10:3.5, by vol). The plate was dried and sprayed with primulin and the band corresponding to PC with soy lecithin was scrapped off and placed into a test tube. The scrapings were methylated with 500 _L of sodium methoxide for 45 min and then 1 mL hexane was added and the tubes were vortexed. The upper hexane layer was removed into a new tube and the procedure was repeated with another 1 mL hexane. The hexane layers were combined. The hexane was washed with 1 mL of 0.9% KCl and the hexane was blown off to 100 _L and was transferred to a GC vial and the samples were run on GC with fatty acid methyl ester standards (FAME standards). For quantitation, the amount of area corresponding to 16:0, 18:0, 18:1, 18:2 and 18:3 fatty acids for each of the lines was added. The total area was divided by the PC 17:0 areas. The number obtained was multiplied by the volume of PC 17:0 added in the reaction.

**Synthesis of radiolabeled linoleoyl CoA from linoleic acid and CoASH using Acyl CoA synthetase**

(Gang, Lavid et al., 2002) Fivety microCuries (corresponding to 1.1 _ 10^8 dpms) of [1-14C] linoleic acid was placed into a test tube and blown dry under stream of nitrogen
gas. The following compounds in 100 mM MOPS-NAOH (pH 7.5) were then added to the test tube: 100 \_L 1% w/v Triton X-100, 100 \_L 50 mM CoASH, 100 \_L 100 mM ATP, 100 \_L 10 mM DTT and 100 \_L 100 mM MgCl\_2. The mixture was vortexed and sonicated for 10 min in an ultrasonic bath. Then acyl-CoA synthetase (0.25 units) in solid form directly taken from vial) was added to the test tube and the reaction volume was brought to 1 mL by adding 100 mM MOPS-NaOH pH 7.5. The test tube was flushed with argon and teflon was placed around the mouth of the test tube and it was sealed tightly to prevent oxidation. The test tube was placed in a shaker incubator for 5 h at 35 \textdegree C at 200 rpm. The reaction progress was monitored by TLC using hexane:MTBE:acetic acid (60:40:1 v/v/v) and also by measuring the radiation by Geiger counter. [1-\textsuperscript{14}C]Linoleoyl-CoA was purified by a reverse phase column chromatography. In detail, the column was washed with 5 volumes of methanol, distilled water and 2\% ammonium acetate. The reaction mixture was then loaded on the column and was washed with 4 mL of 2\% ammonium acetate, and 6 mL of distilled water. The remaining water was removed by blowing air in the column and then [1-\textsuperscript{14}C]18:2-CoA was eluted with 10 mL of methanol followed by 2 mL of butanol. The wash and elution were scintillated using scintillation counter. A small sample of [1-\textsuperscript{14}C]18:2-CoA was then run on reverse phase TLC (butanol:acetic acid:water, 63:10:27, v/v/v) and normal phase TLC (hexane:MTBE, 50:50, v/v) to check the purity of the labeled 18:2-CoA.

Note: amt of radiation recovered was about 110 million dpms dissolved in 12.5 mL solvent (11,000dpm/ \_L).
Table 9: Reaction components and quantities for \textit{in-situ} labeling of microsomal $[^{14}\text{C}]18:2$-PC

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector control microsomes</td>
<td>70 nmol</td>
<td>163 mL</td>
</tr>
<tr>
<td>$[1^{14}\text{C}]18:2$ CoA</td>
<td>90 nmol</td>
<td>1.25 mL</td>
</tr>
<tr>
<td>BSA</td>
<td></td>
<td>6 mg</td>
</tr>
<tr>
<td>CoASH</td>
<td>0.8 mol</td>
<td>8 mL</td>
</tr>
<tr>
<td>phosphate buffer</td>
<td>100 mM</td>
<td>29 mL</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>200 mL</td>
</tr>
</tbody>
</table>
In-situ labeling of microsomal $^{14}$C18:2-PC

**Notes:** 50 _Ci of $^{14}$C18:2-CoA was in 12.5 mL of chloroform. Only 10\% of the volume (i.e. 5 _Ci corresponding to 1.25 mL) was used for synthesis of $^{14}$C18:2-PC. Five million dpm of radioactivity was recovered from 11 million of starting material

$55\ _{Ci} = 1\ _{Mole}$ ----i.e. $5\ _{Ci} = 0.0909\ _{mol} = 91\ \text{nmol}.$

(Bafor, Smith et al., 1993) Vector control microsomes were incubated with $^{14}$C labeled 18:2-CoA, BSA and CoASH in total volume of 200 _L at 25 °C for 20 min. Then 500 _L of chloroform:methanol (2:1, v/v) and 500 _L of 0.9\% KCl was added and the tube was vortexed well. After centrifugation the lower chloroform phase was removed and the aqueous phase was re-extracted with 500 _L of chloroform. The chloroform layers were blown to a final volume of 50 _L with nitrogen. This was then loaded on a silica gel plate and then run on TLC with soy lecithin as a standard (50 _g). The solvent system was chloroform:methanol:acetic acid:water (85:17:10:3.5, by vol). The plate was allowed to dry and was wrapped in a plastic sheet and kept in a phosphorimager screen overnight. After visualizing the plate with a phosphorimager and Image-Quant software, the plate was sprayed with primulin and viewed under UV light. The band corresponding to PC was scrapped and placed in a silica column. The $[1-^{14}$C]18:2-PC was eluted with chloroform:methanol:water (1:1:1, v/v/v). The solvent was evaporated under a stream of nitrogen and re-suspended in 2 mL of toluene:ethanol (1:1, v/v).

**Enzyme assay**

(Bafor, Smith et al., 1993) Microsomes equivalent to 15 nmol of PC, 0.5 _Ci corresponding to 9 nmol of substrate i.e. $[^{14}$C]18:2-PC and 20 _L of 50 mM NADPH to a final volume of 300 _L in phosphate buffer was used for the reaction. Initially the radiolabeled substrate was dried in a glass tube and resuspended in phosphate buffer (pH 7.2). The samples were sonicated for 15 min and 20 _L NADPH and 15 nmol of microsomes were immediately added to the reaction. The reaction mixtures were incubated at 25 °C with constant shaking at 180 rpm for 2 h. The positive control for the
reaction was *Vernonia* microsomes and the negative control was vector control microsomes. After 2 h the lipids were extracted into chloroform according to (Bligh and Dyer 1959) as described previously. The chloroform extracts were blown to dryness by nitrogen gas. Subsequently, 200 _L_ of diazomethane was added to the samples and the test tubes were vortexed thoroughly. The solvent was immediately evaporated and 500 _L_ of sodium methoxide was added to the test tubes for methylation. The test tubes were vortexed well. Ideally the diazomethane methylates all the free fatty acids and sodium methoxide methylates the polar lipids and triacylglycerol. After 45 min of constant shaking, the fatty acid methyl esters were extracted using hexane. Briefly, 1 mL of hexane was added to the tube and vortexed. The upper hexane layer was removed into a new tube and the procedure was repeated with another 1 mL of hexane. The hexane layers were combined. The hexane was washed with 1 mL of 0.9% KCl. The hexane was evaporated with nitrogen and the samples were re-suspended in 7-8 drops of hexane. The samples were loaded on the TLC plates and the TLC was run with hexane:MTBE:acetic acid as solvent system (85:15:1, v/v/v). Non-radiolabeled 18:2 FAME and vernolic acid FAME standards were used. The TLC plate was wrapped in a plastic sheet and placed overnight in phosphorimager screen. The plate was scanned in phosphor imager and the histogram was viewed using Image-QuaNt software. The plate was sprayed with primulin and the non-radiolabeled standards were viewed under UV light. The bands corresponding to vernolic acid standard were observed. For further confirmation those bands were scrapped from the TLC plate and were eluted through silica column by using 2 mL of MTBE. To obtain radiolabeled vernolic acid FAME standard, $[1^{14}C]$vernoloyl-CoA was methylated using sodium methoxide and was extracted using hexane. The samples in MTBE were evaporated and re-suspended in hexane and was run on TLC with radiolabeled vernolic acid FAME standard using hexane:MTBE:acetic acid (85:15:1, v/v/v). After TLC the plate was wrapped in plastic and was kept overnight in phosphorimager screen. The plate was scanned under phosphorimager and viewed by Image QuaNt software. In separate experiments, microsomes were incubated with $^{14}C$ labeled 18:2-CoA, BSA, NADPH and CoASH instead of $^{14}C$18:2-PC and proceeded with the lipid analysis as described above. The enzyme activity was calculated by the
Image QuaNt software. The density of the bands of product and substrate was analyzed by IQ software. Enzyme activity was calculated by following formula:

\[
\text{Density of band of product/ density of band of substrate} \times 100
\]
C. Results

Sequencing of rabbit epoxygenase CYP2C2

An amino acid identity of 99.8% was found in 490 amino acid overlap when compared to rabbit cytochrome P450 (laurate omega-1 hydroxylase) mRNA, complete cds, clone pH2. (M19137 –NCBI sequence). [Conflict P17_L17 and P471_L471; Leighton et. al., 1984]. The 435\textsuperscript{th} cysteinyi residue was thought to serve as fifth ligand to the heme iron.

Plasmid construction result

In the multiple cloning site of the plant expression vector pCAMBIA 1201, CYP2C2 gene driven by a seed-specific phaseolin promoter was constructed. The gene and the promoter-terminator cassette insertion was confirmed by restriction analysis using \textit{KpnI} and \textit{PstI} enzymes and PCR analysis by 5' C2 and 3' C2 primers (Figure 3 and 4).
Figure 4: CYP2C2 ligation in pHI4752 confirmed by digestion with NCo I and Hpa I. Lane 1: marker and Lane 2: digestion product.
Figure 5: CYP2C2 ligation in pCAMBIA1201 confirmed by *Kpn* I and *Pst* I digestion. Lane 1: marker and Lane 3: digestion product.
Generation of transgenics

There are a few native species like the *Vernonia*, *Euphorbia* and *Stokesia* which synthesize a large amount of epoxy fatty. Due to the problems of their poor good agronomic characteristics there is a need of transgenic plants producing epoxy fatty acids. Transgenic plants accumulating epoxy fatty acids will be an excellent renewable resource. They will reduce the pressure on petroleum imports and on the other hand will be eco-friendly.

Soybeans are an excellent commercial crop. They have abundant linoleic acid which is a substrate for epoxygenase enzymes. Soybean somatic embryogenesis has been extensively studied over past decade. The efficiency of soybean transformation has been about one percent, which is very low and due to this the analysis and generalization of expression patterns becomes very difficult. Since then, there has been much effort to modify the protocol for generating large numbers of transgenic events with focus on better quality embryos and better regeneration capacity. Some of the parameters, like the gold particles used for shooting and pressure of particle bombardment have been changed from 1 micron and 1350 psi to 0.6 micron and 1100 psi respectively (Parrott, 2006). One of the reasons for reducing the gold size for particle bombardment would be better delivery into the target tissue.

The period of bombardment to obtaining transgenic plants takes about six months. Several precautions must be taken to prevent cross contamination between the flasks. After six months from shooting as per the protocol described in the materials and methods, the transgenic soybean somatic embroys were ready for analysis. Ten individual transgenic lines were obtained from two shooting sessions. Enough sister clones of each line were developed.
Genomic DNA extraction and PCR analysis of transgenic somatic embryos

Genomic DNA was extracted from the 10 independent transgenic lines as described under materials and methods. The PCR amplification of genomic DNA by CYP2C2 gene specific primers 5' C2 and 3' C2 showed the presence of 1.5 Kb CYP2C2 gene (Figure 6).
Figure 6: PCR analysis of CYP2C2 expressing transgenic lines with 5’ and 3’ primers (sequence is given in materials and methods). Lanes 1-10: genomic DNA from different transgenic lines. C- untransformed embryos with CYP2C2 specific primers. M- marker lane.
**GUS reporter gene assay**

Transgenic somatic embryos from each of the 10 independent transgenic lines were tested for GUS gene by the β-glucuronidase GUS reporter gene assay. All the lines were found to be GUS positive (Figure 5).
Figure 7: GUS reporter gene assay on transgenic soybean somatic embryos expressing CYP2C2. The plant expression vector pCAMBIA 1201 used for transformation had a GUS gene. The GUS gene was expressed in the transgenic soybean embryos as depicted by the blue staining. Picture represents some of the transgenic lines expressing the GUS gene.
Histodifferentiation and maturation

All 10 transgenic lines which were identified to be positive by PCR were then matured in histodifferentiation and maturation media. From two to three globular stage embryos, fifty to sixty matured somatic embryos of each line were recovered. However because of contamination issues two lines were lost and one of the lines being pretty weak was not able to mature well (Figure7).
Figure 8: GUS reporter gene assay on matured somatic embryos at mid maturation. These are some representative transgenic soybean somatic embryos.
Lipid analysis of matured somatic embryos

Total lipids extracted from matured somatic embryos at three weeks and after desiccation did not give any epoxy fatty acid. Also 18:2 fatty acid levels did not change much as compared to vector control. The total lipids extracted were separated into polar and neutral lipid fractions. When a very concentrated sample was injected for GC analysis a minute peak was seen at the retention time of vernolic acid standard in the samples, which appeared quite like the background peaks. After extensive GC-MS analysis and determination of the mass spectra for the compound it was found that the peak was 11-eicosanoic acid but not vernolic acid. Also the polar and the neutral lipid fractions isolated from total lipids for some transgenic lines did not show any traces of vernolic acid (Figure 9, 10, 11).
Analysis of total lipids from matured somatic embryos

Figure 9: The comparison of total fatty acid levels of different transgenic lines. Data is the mean of two repetitions. C: control which is total lipids from untransformed embryos. The numbers denote total lipids from different transgenic lines.
Analysis of polar lipids from the matured somatic embryos

Figure 10: The polar fraction fatty acid profile of transgenic lines. Data is the mean of two repetitions. C: control which is polar fraction from untransformed embryos and the numbers are polar lipid fraction from transgenic matured somatic embryos.
Analysis of neutral lipids from the matured somatic embryos

Neutral Fatty Acid Profile

Figure 11: The neutral fraction fatty acid profile of transgenic lines. C: control which is neutral fraction from untransformed embryos and the numbers are neutral lipid fraction from transgenic matured somatic embryos.
RT-PCR analysis of matured somatic embryos

The RNA extracted from the matured embryos at mid maturation showed the presence of CYP2C2 mRNA. The transcript level of CYP2C2 was compared to endogenous 18S rRNA used as an internal control. The highest expressers of the transgene will be compared with the enzyme activity assay (Figure 8).
<table>
<thead>
<tr>
<th>Positive control</th>
<th>Negative control</th>
<th>8136</th>
<th>8131P1</th>
<th>8164P1</th>
<th>8132P2</th>
</tr>
</thead>
</table>

Figure 12: RT-PCR of the matured somatic embryos at mid maturation stage. Positive control is plasmid DNA with CYP2C2 specific primers. Negative control is untransformed embryos with CYP2C2 and 18S rRNA primers. All the numbers represent different transgenic lines expressing CYP2C2 as well as 18S rRNA.
Microsome labeling:

The microsomes were incubated with [1-\(^{14}\)C]18:2-CoA and CoASH for 20 min. After this short incubation majority of the [1-\(^{14}\)C]18:2 was found in the membrane. The acyl exchange system is presumably responsible for driving this into membrane PC pool.
Figure 13: The above figures represent a phosphorimage of TLC depicting synthesis of \(^{14}\text{C} 18:2 \text{ CoA}\). On the left hand side is the reverse phase TLC showing the migration of \([1-^{14}\text{C}] 18:2 \text{ CoA}\). On the right hand side is a normal phase TLC where the \([1-^{14}\text{C}] 18:2 \text{ CoA}\) is stationary.
Figure 14: Phosphorimage of TLC plate following $[1^{14}\text{C}]$ 18:2 incorporation into PC after *in situ* labeling of soybean microsomes with $[1^{14}\text{C}]$ CoA.
Enzyme activity assay

(Blee, Stahl et al., 1993) reported that 12(S), 13 (R) and 12(R), 13(S) isomer of vernolic acid was formed in developing seeds of E. lagascae by action of cytochrome P450 epoxygenase and peroxigenase activity, respectively. The 12(S), 13(R) isomer is known to accumulate in triglycerides of developing seeds on the other hand the 12 (R), (S) enantiomer is a metabolite involved in plant defense (Kato et al., 1993) and is metabolized by soluble epoxide hydrolase.

(Bafor, Smith et al., 1993) reported epoxygenase activity in microsomes from endosperm of developing E. lagascae. Microsomes were labeled in-situ with [1-14C]oleate or [1-14C]linoleate which were mainly recovered in phosphotidylcholine (PC) fraction. In a time course study they found [1-14C] vernolate was mostly confined to PC at short time intervals, but with longer incubation times they found free vernolic acid dominated.

[1-14C] 18:2 CoA was synthesized using [1-14C] 18:2 as a substrate (Figure 12). Almost all the [1-14C] 18:2 was converted into [1-14C] 18:2 CoA. The microsomes were isolated from the transgenic matured somatic embryos at three weeks of maturation. Then the protein concentration was determined by modified Lowry assay as described earlier. The amount of PC in the microsomes of the transgenic lines was determined (Table 3). The microsomes were labeled with [1-14C] 18:2 using [1-14C] 18:2 CoA (Figure 13). Within 20 min most of the [1-14C] 18:2 was found in the membrane.

[1-14C] Linoleoyl-PC substrate was incubated with microsomes isolated from various transgenic lines in presence of suitable co-factors. Vernonia microsomes were used as a positive control and vector control microsomes as a negative control. After 2 h incubation fatty acids were extracted and were subsequently methylated and were run on TLC. A band corresponding to [1-14C] methyl vernoleate was seen.
Table 10: The *in vitro* enzyme activity calculated by amount of product formed as compared to the unreacted substrate in various transgenic lines.

<table>
<thead>
<tr>
<th>Line</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>8131 P1</td>
<td>0.865</td>
</tr>
<tr>
<td>8132 P2</td>
<td>1.83</td>
</tr>
<tr>
<td>8136</td>
<td>1.005</td>
</tr>
<tr>
<td>Vector control</td>
<td>0.315</td>
</tr>
<tr>
<td>Vernonia microsomes</td>
<td>0.78</td>
</tr>
</tbody>
</table>
Figure 15: Phosphorimager of TLC plate after performing the enzyme assay on three weeks old transgenic soybean microsomes following in situ labeling of microsomal PC. Lanes 1-2 represent 8131P1 transgenic line. Lanes 3-4 are 8132P2 transgenic line. Lane 5 is cold 18:2. Lane 6 is cold methyl vernoleate. Lanes 7-8 are 8136 transgenic line. Lanes 9-10 are vector control. Lanes 11 and 12 represent Vernonia microsomes.
Figure 16: Phosphorimager of TLC plate after performing the enzyme assay on three weeks old transgenic soybean microsomes following in situ labeling of microsomal PC. Left hand side is the sample and on the right hand side is the standard. 

$[^{14}\text{C}]$ methyl vernoleate
D. Discussion

In an *in vitro* reaction microsomes isolated from matured somatic embryos at three weeks gave vernolic acid when [1-\(^{14}\text{C}\)] 18:2 PC was used as a substrate in presence of suitable co-factors. We suspect that the vernolic acid formed in this reaction is due to CYP2C2 activity.

Lipids isolated from matured somatic embryos at three weeks did not show any vernolic acid as detected by GC. One of the speculations for not detecting vernolic acid could be that the epoxy fatty acid formed maybe degraded *in vivo*. The substrate is a membrane fatty acid so the epoxy fatty acid synthesis would also be in the membrane. The epoxy molecules are quiet unstable and also its accumulation in the membranes can be toxic to plants (Spiekermman et al., 2003).

There is a concrete biological evidence for epoxidation of C18 cutin monomers *in vivo* by plant peroxygenases (Lequeu, Fauconnier et al., 2003).

Millar et al., (2000) reported that most of the native species accumulate unusual fatty acids like the epoxy and the hydroxy fatty acids in the form of triacylglycerol. In the case of castor (*Ricinus communis*) 18:1-OH fatty acid accounts for 90% of total fatty acids out of which 85% is found in TAG and 5% is in the membranes despite of the fact that the membrane phosphotidylcholine is the substrate for 18:1-OH synthesis. One of the reasons for this might be that the polar oxygenated group of the hydroxyl fatty acid may be incompatible in the membrane hydrophobic environment. Thus membrane editing of these unusual fatty acids and its selective channeling into the storage lipids might be a highly specific and efficient phenomenon. Likewise in the case of epoxy fatty acids in native plant species like *Vernonia, Crepis* and *Euphorbia*; most of epoxy fatty acid is seen in the oil bodies and 2% is in the membrane FA pool. Thus it can be well hypothesized that the accumulation of these unusual FA in the membrane might disrupt the membrane integrity and impair seed development and germination. This might not be a potential problem in transgenic plants accumulating moderate amounts of these fatty
acids, but for very high accumulators it might be necessary to introduce additional enzymes such as acyl transferases and phospholipases which will help direct these FA into oil bodies.

Cytochrome P450 2C2 is a resident ER protein. It contains ER retention signals that keep it from getting recycled. Localization of ER resident proteins can be achieved by direct retention in the ER or by retrieval of protein to ER after transport to the pre-Golgi section. The retrieval is a receptor mediated process. However the mechanism of direct retention of CYP2C2 in the ER is not yet established. Studies with chimera of full length CYP2C2 (490 amino acid) and GFP revealed that both the N-terminal signal anchor sequence and the C-terminal cytoplasmic domain is necessary for ER retention (Szczesna-Skorupa, Chen et al., 2000).

Recently it has been shown that the diacylglycerol acyl transferase DGAT1 and DGAT2 resident ER protein from tung Aleurites, are in different compartments of ER. Thus we can speculate that CYP2C2 expressed in transgenic soybean tissue can be present in a specific domain of the ER where the substrate availability can be varying, since the ER membrane may not be homogeneous.

The biochemical evidence of futile cycling of unusual fatty acids like vernolic acid and ricinoleic acid by \(-\)oxidation was shown by (Moire, Rezzonico et al., 2004) The fatty acid degradation was determined by analysis of polyhydroxyalkanoate PHA synthesized in the peroxisomes. In plants, the \(-\)-oxidation occurs in the peroxisomes or the glyoxysomes which are specialized peroxisomes in germinating seed. The free fatty acids are triggered by acyl CoA synthetase to acyl-CoA. Followed by a series of steps involving trans-2-enoyl-CoA, L-3-hydroxyacyl-CoA, 3-ketoacyl-CoA intermediates finally give acetyl-CoA and acyl-CoA reduced by 2 carbons. The reaction generates ATP molecules which are used as an energy source by the plants. The acetyl-CoA generated joins the citric acid cycle. The end products like oxaloacetate formed in this cycle are precursors for gluconeogenesis.
(Eccleston and Ohlrogge 1998) measured the _- oxidation degradation products by $^{14}$C-Acetate labeling when lauroyl-acyl carrier protein thioesterase (MCTE) was expressed in oilseed rape plants.

One speculation for vernolic acid not being accumulated in transgenic soybean expressing CYP2C2 might be that the vernolic acid formed is getting degraded via _- oxidation. A technique like $^{14}$C labeling as described above can help identify the degradation products of vernolic acid by _-oxidation.

Another speculation can be that there are no specific phospholipases that can cleave vernolic acid from the phosphatidylcholine. Or there can be no active DGAT that can convert vernoloyl-CoA to diacylglycerol.

It might be better to try to find enzyme activity _in vivo_ by feeding [1-$^{14}$C]18:2-CoA as a substrate to the matured embryos. If we observe good activity of CYP2C2 as compared to the controls, this might mean the endogenous substrate for the epoxidation reaction might be limiting. In this case, it would be better to co-express a desaturase gene that can normalize the substrate levels. The fact that we did not see any reduction in 18:2-PC levels, rules out the possibility that the substrate might be limiting.

If the enzyme does not show any activity _in vivo_, it might indicate that either the soybean P450 reductase is not active or there is no proper interaction between the P450 enzyme and the reductase _in vivo_ resulting in an incomplete electron transfer reaction, which is necessary for coupling and oxygen incorporation into the substrate. Our _in vitro_ assays indicate that the P450 reductase might not be a limiting factor for the epoxyxygenation reaction.

One reason for not getting vernolic acid in transgenic soybean embryos can be somaclonal variation, wherein some sort of genetic variability like a base change, etc. is induced by plant tissue culture techniques. A hormone 2, 4-D which is used in proliferation media is well known to induce somaclonal variation. Since such sort of
variation does not induce morphological changes, it would be better to apply some plant screening procedures. The very fact that we got CYP2C2 activity in vitro rules out the possibility of somaclonal variation of CYP2C2 in vivo.

It would be useful to compare the transcript level of CYP2C2 from our transgenic soybeans to Vernonia epoxygenase which is native plant species. If the expression of CYP2C2 is more than Vernonia epoxygenase it means that gene expression is not a problem. CYP2C2 expression is less than Vernonia means screening of some additional clones is required. Because of time constraint additional screening of clones was not possible. Also CYP2C2 expression in heterochromatin region might be another reason which can affect its transcript abundance in transgenic soybean embryos. But fact that we observe more CYP2C2 activity in transgenic embryos in an in vitro assay as compared to Vernonia microsomes suggests that the transgene expression is not an issue.
E. Conclusions

CYP2C2 is a cytochrome P450 enzyme which requires P450 reductase for its action. When CYP2C2 under a seed-specific phaseolin promoter was expressed in soybean somatic embryos, transgenic somatic embryos expressing CYP2C2 transcript were obtained. The lipid analysis revealed no significant decrease in 18:2 fatty acid which is substrate for the CYP2C2 enzyme. The levels of 18:2 were same as controls. Also when total lipids were separated into polar and neutral fractions, no vernolic acid was seen in any of these lipids classes as determined by GC. Microsomes isolated from matured somatic embryos incubated in presence of [1-14C] 18:2-PC and NADPH showed epoxyxygenase activity with formation of vernolic acid from 18:2. Since no activity was detected in vivo it may be that vernolic acid is formed in situ and degraded rapidly by _-oxidation. Another possibility is that CYP2C2 in our transgenic embryos is present in a particular domain of the ER where substrate availability is variable due to non-homogeneity of the ER membrane.
F. Future Prospects

With a view to make soybeans accumulating large amounts of vernolic acid, we expressed a rabbit epoxygenase in soybean somatic embryos. On analysis we did not see any vernolic acid in the lipid fractions of the lipids isolated from matured somatic embryos. Upon isolating the mirosmes and doing an in vitro enzyme activity assay, we saw the presence of vernolic acid with no vernolic acid in the negative control. This result is very promising as it signifies the activity of CYP2C2 in vitro. It would now be important to characterize the biochemical pathway of vernolic acid accumulation in vivo. By feeding [1-14C] 18:2 to the somatic embryos, it would be interesting to know if vernolic acid is formed in vivo which would tell us about the enzyme activity in vivo. It is possible that vernolic acid formed in vivo is rapidly degraded by biochemical pathway like - oxidation. Then, as described by (Eccleston and Ohlrogge 1998) we can identify the degradation product of -oxidation. On the other hand, if vernolic acid is getting accumulated but it is at low levels, then we need to expresses additional genes that can facilitate vernolic acid built-up in triacylglycerol. If we are able to make transgenic soybeans accumulating large amounts of vernolic acid it would be an economic and renewable commercial source of this unusual fatty acid. Additionally it would help us understand the biochemical machinery required for accumulating other unusual fatty acids in transgenic plants.
Chapter 2

Biological Effects of Epoxy fatty acids and diol

A. Introduction

Leukotoxin (9,10-epoxy-12-octadecanoate) and isoleukotoxin (12,13-epoxy-9-octadecanoate) also known as vernolic acid are mono-epoxides of linoleic acid. They are produced from linoleic acid by cytochrome P450 enzymes and by reactive oxygen species in human body. There are various native plant species that accumulate up to 80% vernolic acid in their seed oils. However to date we do not know why and how these plants accumulate such high levels of this unusual fatty acid. In animals, most of the CYP enzymes are present in the liver. They are used in metabolizing drugs and synthesizing steroids and cholesterol.

Intravenous injection of leukotoxin at a very high concentration (50 mg/kg) in test dogs caused severe hemodynamic changes causing death in a very short time (Fukushima et al., 1988). This shows the profound cardiovascular effects of leukotoxin at high concentrations. Pulmonary edema was caused in lungs of Wistar rats when intravenous injection of leukotoxin at a dose of 100 mmol/kg was administered. Linoleate treated control rats did not show any effect (Hu et al., 1988). In the skin of burn patients the plasma leukotoxin levels are very high, thus indicating the role of leukotoxin in tissue destruction (Kosaka, Suzuki et al., 1994).

Epoxide hydrolase enzymes are members of _/-_hydrolases. These enzymes are present in abundance in liver and kidney. Four epoxide hydrolases are known: leukotriene epoxide hydrolase, cholesterol epoxide hydrolase, microsomal epoxide hydrolase and soluble epoxide hydrolase (Grant, Storms et al., 1993). Among these four epoxide hydrolases, the microsomal and the soluble epoxide hydrolases are present in animal tissue at high levels. The soluble epoxide hydrolase activity is very high as compared to microsomal epoxide hydrolase.
Murine and human soluble epoxide hydrolases were cloned and expressed in Sf-21 cells that are low in endogenous epoxide hydrolase activity (Moghaddam, Grant et al., 1997). This was the first report that the diol was more toxic than the precursor epoxy fatty acid. An effect of methyl leukotoxin/isoleukotoxin and corresponding diol on the viability of Sf-21 cells was measured. Methyl leukotoxin/isoleukotoxin were toxic to cells expressing human or mouse sEH, were midly toxic to hmEH expressing Sf-21 cells and were not toxic to lacZ expressing cells. On the other hand, diol was toxic to all cell types (Moghaddam, Grant et al., 1997).

Various symptoms associated with leukotoxin toxicity, such as pulmonary edema, cardiac arrest, vasodilation, mitochondrial dysfunction, capillary damage, and nitric oxide elevation would not be because of leukotoxin but due to the diol. Leukotoxin diol affects the mitochondrial membrane integrity. The diol shows cytotoxicity by its action on mitochondria. It has been suggested that diol particularly affects mitochondrial inner membrane permeability and causes cell death. Cyclosporin A, which is an inhibitor of mitochondrial permeability transition, inhibits diol induced mitochondrial membrane disruption (Sisemore et al., 2001). On the contrary the diols metabolized from arachidonic acid epoxide are not toxic. Swiss Webster mice administered leukotoxin diol showed massive alveolar edema and hemorrhage thus the mice died with acute respiratory distress syndrome (ARDS) like distress syndrome (Zheng, Plopper et al., 2001).

CYP2C9 is a key enzyme for linoleic acid epoxidation in human liver microsomes. Leukotoxins are known to cause inflammation. Thus CYP2C9 can be a potential therapeutic target for reducing inflammation caused by leukotoxins and their respective diols (Draper and Hammock 2000). CYP2C9 is involved in linoleic acid epoxidation. Dietary fatty acids like the linoleic acid caused a marked increase in expression of CYP2C9 which in-turn caused endothelial cell activation by increasing the reactive oxygen species. When endothelial cells from porcine pulmonary artery were treated with 60 _M and 90 _M of leukotoxin and leukotoxin diol, a severe effect as a
A measure of cellular oxidative stress of the leukotoxin and diol was found only at high concentration i.e. 90 \text{ _M} (Viswanathan, Hammock et al., 2003).

To our knowledge there have been no reports on the toxicity of various epoxy and hydroxy fatty acids such as epoxidized linseed oil, epoxidized soybean oil, castor oil, vernonia oil, etc. Toxicity measurements of such epoxy and hydroxy molecules in yeast, \textit{Escherichia coli} and mammalian cells will be useful in elucidating its biological impact in living systems. It would be useful to know how these compounds affect cell viability and membrane function.

Caco-2 cells which are intestinal epithelial cell monolayers are a model system for testing various drugs. We therefore used these cells to test the toxicity of our compounds. These cells are derived from colon carcinoma. The differentiated Caco-2 cells have characteristics similar to mature enterocyte brush border microvilli. The cells exhibit paracellular (for polar, water soluble compounds) and transcellular mobility (relatively hydrophilic compounds).

**B. Materials and Methods**

The following compounds were studied for their toxicity in \textit{E. coli}.

- ELO: Epoxidised linseed oil
- ESO: Epoxidised soybean oil
- LD: Leukotoxin diol
- RM: Racemic Mixture of various epoxides

**Method:** The above mentioned compounds were studied at 5\% (w/v) concentration for measuring toxicity. Luria bertini LB liquid media was used for the study in \textit{E. coli}. Compounds were dissolved in ethanol and dispersed in the media. Tween 20 at a final concentration of 0.1\% (w/v) was also used to bring the test compounds into the solution. The final concentration of ethanol was 1 \% (v/v).
The following compounds were tested for their toxicity in yeast

ESO: Epoxidised soybean oil
ELO: Epoxidised linseed oil
HCO: Hydroxylated castor oil
MV: Methyl vernolate
CO: Castor oil
RM: Racemic mixture of various epoxides
LD: Leukotoxin diol
SO: Soybean oil
VA: Vernolic Acid
VO: Vernonia oil

Method: The above mentioned compounds were studied at 5% (w/v) concentration for measuring toxicity. Yeast (YPAD) liquid media was used for the study in yeast. The compounds were dissolved in ethanol and dispersed in the media. Tween 20 at final concentration of 0.1% (w/v) was also used to bring the test compounds into the solution. The final concentration of ethanol was 1% (v/v).

Synthesis of isoleukotoxin diol:

130 mg of vernolic acid was placed in a test tube. 4 mL of tetrahydrofuran: H2O (3:1) was added to it. 1 mL of 5% of perchloric acid was also added. The reaction was carried out for 3 hours at room temperature with constant shaking. The diol formation was checked by TLC using a diol standard and using methyl tertiary butyl ether (MTBE): Hexane (30:70) as a solvent system.
Figure 17: The TLC of the isoleukotoxin diol. The left lane is vernolic acid standard. The middle lane is sample and on the right lane is the diol standard.
Toxicity determination:

At the concentration of 2% all the compounds except LD were tested in *E. coli* and no toxicity was found (Dr. Keshun Yu, unpublished data). When the compounds at 5% (w/v) concentration were used for the study, it was difficult to get the test compounds into the solution. Often a lipid layer floating above the media was apparent. Since the compounds did not get into the solution, the results were inconclusive. So the experiment was re-designed for some of the compounds using a different approach.

Study in *E. coli*:

DH5_ strain was streaked on a LB plate and the plate was incubated overnight at 37˚C in an incubator. A single colony was inoculated in 100 mL of LB liquid media and the culture was incubated in a 37 ˚C shaker incubator to a mid-exponential phase (OD of 0.6-0.8 at 600 nm). The OD values were optimal for response to toxic chemical testing (Kim, Park et al., 2003). The compounds were dissolved in ethanol (1% final concentration, v/v) and Tween 20 at final concentration of 0.05% (w/v) was used to bring the test compounds into the solution. Compounds were tested at 5 different concentrations (1 mg/mL, 100 _g/mL, 10 _g/mL, 100 ng/mL, 10 ng/mL). After addition of the compounds the OD at 600 nm was measured for a 24 h period at 0 h, 4 h, 10 h and 24 h time points.

Study in Caco-2 cells:

Caco-2 cells were a generous gift from Dr. Brett Spear. All the cell culture experiments were conducted in Dr. Toborek’s lab with extensive help from Dr. Ibolya. The basic cell culture medium consisted of Dulbecco’s modified Eagle’s medium DMEM:F12 (1:1) with 2.5 mM L-Glutamine and with 15mM HEPES buffer (HyClone laboratories) containing 10% (v/v) fetal bovine serum (FBS), 100 units per mL of penicillin and 100 _g per mL of streptomycin sulphate. The cells were grown at 37˚C in a humidified atmosphere with 5% CO2. For cytotoxicity assay, the cells were cultured in 24
well plates. The test compounds were dissolved in ethanol in such a way that the final concentration of ethanol is not more than 0.8%. The compounds were tested at final concentration of 1 mg/mL, 40 μg/mL, 10 μg/mL, 100 ng/mL. Following 24 hours of treatment the cells were washed with PBS to remove the test compound. MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at a final concentration of 1mg/mL was dissolved into the media and fed to the cells. The cells were incubated at 37°C for 1 hour. The media was gently aspirated from each well and the purple colored formazan was solubilized with 1mL of dimethyl sulfoxide (DMSO). The 24 well plates were incubated at room temperature on a shaker for 25 mins and the optical density was measured at 570 nm (Maher and McClean).
C. Results

Study in *E. coli*:

When the compounds (except leukotoxin diol) were tested at a concentration of 2% no toxicity was found (Dr. Keshun Yu, unpublished data). At 5% concentration the test compounds were found floating on the media and it was difficult for the test compounds to get into solution. The results obtained were inconclusive. So when the experiments were re-designed for MV, VO and LD using the concentrations of 1 mg/mL, 100 μg/mL, 10 μg/mL, 100 ng/mL, 10 ng/mL, no toxicity was found either.
Table 11: Toxicity measurement of methyl vernoleate in *E. coli*. Values represent optical density (OD) of bacterial test culture at time points shown in the table.

<table>
<thead>
<tr>
<th>MV</th>
<th>0 hrs</th>
<th>4 hrs</th>
<th>10 hrs</th>
<th>24 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mg/mL</td>
<td>0.899</td>
<td>1.33</td>
<td>1.566</td>
<td>1.86</td>
</tr>
<tr>
<td>100 μg/mL</td>
<td>0.887</td>
<td>1.168</td>
<td>1.405</td>
<td>1.698</td>
</tr>
<tr>
<td>10 μg/mL</td>
<td>0.743</td>
<td>1.177</td>
<td>1.387</td>
<td>1.736</td>
</tr>
<tr>
<td>1 μg/mL</td>
<td>0.749</td>
<td>1.172</td>
<td>1.431</td>
<td>1.729</td>
</tr>
<tr>
<td>100 ng/mL</td>
<td>0.784</td>
<td>1.214</td>
<td>1.432</td>
<td>1.782</td>
</tr>
</tbody>
</table>
Table 12: Toxicity measurement of vernonia oil in *E. coli*. Values represent optical density of bacterial test culture at time points shown in the table.

<table>
<thead>
<tr>
<th>VO</th>
<th>0 hrs</th>
<th>4 hrs</th>
<th>10 hrs</th>
<th>24 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mg/mL</td>
<td>0.901</td>
<td>1.548</td>
<td>1.701</td>
<td>1.927</td>
</tr>
<tr>
<td>100 µg/mL</td>
<td>0.816</td>
<td>1.394</td>
<td>1.629</td>
<td>1.836</td>
</tr>
<tr>
<td>10 µg/mL</td>
<td>0.821</td>
<td>1.165</td>
<td>1.421</td>
<td>1.756</td>
</tr>
<tr>
<td>1 µg/mL</td>
<td>0.806</td>
<td>1.181</td>
<td>1.392</td>
<td>1.745</td>
</tr>
<tr>
<td>100 ng/mL</td>
<td>0.826</td>
<td>1.257</td>
<td>1.43</td>
<td>1.736</td>
</tr>
</tbody>
</table>
Table 13: Toxicity measurement of isoleukotoxin diol (ILD) in *E. coli*. Values represent optical density of bacterial test culture at time points shown in the table.

<table>
<thead>
<tr>
<th>ILD</th>
<th>0 hrs</th>
<th>4 hrs</th>
<th>10 hrs</th>
<th>24 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mg/mL</td>
<td>0.977</td>
<td>1.306</td>
<td>1.408</td>
<td>1.73</td>
</tr>
<tr>
<td>100 µg/mL</td>
<td>0.86</td>
<td>1.167</td>
<td>1.436</td>
<td>1.677</td>
</tr>
<tr>
<td>10 µg/mL</td>
<td>0.879</td>
<td>1.217</td>
<td>1.457</td>
<td>1.725</td>
</tr>
<tr>
<td>1 µg/mL</td>
<td>0.85</td>
<td>1.243</td>
<td>1.421</td>
<td>1.698</td>
</tr>
<tr>
<td>100 ng/mL</td>
<td>0.866</td>
<td>1.225</td>
<td>1.43</td>
<td>1.705</td>
</tr>
</tbody>
</table>
Table 14: OD values of controls for above mentioned toxicity studies.

<table>
<thead>
<tr>
<th>Controls</th>
<th>0 hrs</th>
<th>4 hrs</th>
<th>10 hrs</th>
<th>24 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>TW+EtOH</td>
<td>0.874</td>
<td>1.436</td>
<td>1.705</td>
<td>1.869</td>
</tr>
<tr>
<td>TW</td>
<td>0.898</td>
<td>1.823</td>
<td>1.908</td>
<td>2.012</td>
</tr>
<tr>
<td>EtOH</td>
<td>0.838</td>
<td>1.49</td>
<td>1.749</td>
<td>1.945</td>
</tr>
<tr>
<td>Stock</td>
<td>0.844</td>
<td>1.524</td>
<td>1.867</td>
<td>2.23</td>
</tr>
</tbody>
</table>
Study in caco-2 cells:

The compounds were tested in caco-2 cells at 1 mg/mL, 40 \_g/mL, 10 \_g/mL, 100 ng/mL final concentration. Except leukotoxin diol none of the compounds showed toxicity at these concentrations. Leukotoxin diol showed severe toxicity at 1mg/mL final concentration.
Toxicity study in caco-2 cells

Figure 18: The effect of MV, VA, LD, ESO, ELO, VO and SO on viability of caco-2 cells. Results represent mean of three values. The asterisks denote statistically significant values (p<0.05).
D. Discussion

The epoxy compounds are known to have toxic and carcinogenic effects. The oxirane ring of the epoxy molecule is highly reactive. Epoxide hydrolases are enzymes that convert the parent epoxide into the diol. The epoxide hydrolases have several functions, including detoxification, catabolism and regulation of signaling molecules. Plant epoxide hydrolases play an important role in plant cuticle formation and are also involved in plant defense mechanism. Mammals have several epoxide hydrolases. The soluble epoxide hydrolase and microsomal epoxide hydrolase are concentrated in the liver (Morisseau and Hammock 2005).

The general scheme for leukotoxin and leukotoxin diol synthesis:

None of the epoxy compounds tested showed toxicity in yeast or E. coli at 2% concentration (Dr. Keshun Yu, unpublished data). Our data suggest that both yeast and E. coli may not have soluble epoxide hydrolase, an enzyme that converts epoxy fatty acids to their respective diols.

At similar concentrations the isoleukotoxin diol was toxic to mammalian cells. Isoleukotoxin diol might have affected the mitochondrial inner membrane permeability of the caco-2 cells thus releasing cytochrome-c and causing severe death of the mammalian cells. This conclusion is based on the toxicity of leukotoxin diol methyl ester in Sf-21
insect cells, wherein the diol affected the mitochondrial inner membrane permeability and thus caused release of cytochrome-c and subsequently cell death (Sisemore et al., 2001).
E. Conclusions

This data suggest that the diol may be more toxic than the precursor epoxide molecules. The preliminary studies in caco-2 cells suggests that the epoxide molecules ELO and ESO are toxic to cells at 40 \( \mu \text{g/mL} \) concentration. However at 1 mg/mL concentration no cytotoxicity of the epoxy compounds was observed, which was quiet unexpected. To further validate the data additional reps needs to be performed. Due to time constraint this confirmatory data could not be addressed in this thesis.
Appendix

Abbreviations:
CYP2C2: Rabbit cytochrome P450 epoxygenase
DTT: Dithiothreitol
ER: Endoplasmic reticulum
FA: Fatty acid
FAME: Fatty acid methyl esters
GC-MS: Gas chromatography – Mass spectrometry
GUS: β-Glucuronidase
MTBE: Methyl tert-Butyl ether
NADPH: Nicotinamide adenine dinucleotide phosphate (reduced form)
PC: Phosphatidyl choline
PCR: Polymerase chain reaction
RT: Reverse transcriptase
sEH: soluble epoxide hydrolase
TAG: Triacylglycerol
TLC: Thin layer chromatography
UV: Ultraviolet
References


Chan, H. W. S. (1971)." Singlet oxygen analogs in biological systems. Coupled oxygenation of 1,3-dienes by soybean lipoxidase." J. Am. Chem. Soc. 93(9); 2357-2358.


Vita

Name: Purnima Kamlakar Wagh

Date and Place of Birth: May 26\textsuperscript{th} 1982, Thane, India

Education:

Graduate Student: August 2004-December 2006
Department of Nutritional Sciences
University of Kentucky
Thesis Advisor: Dr. David Hildebrand

Undergraduate education: October 2000- May 2004
B. Tech. in Oils, Oleochemicals and Surfactants
UICT, Mumbai.

Professional and Research Experience:

• Extraction, processing and analysis of oils from non-traditional sources. Investigation of physicochemical and processing characteristics of the oil from non-traditional sources like \textit{Arachis sapota}, \textit{Garcinia indica}, \textit{Citrullus vulgaris} and \textit{Mangifera indica}, May 2002

• Fat splitting dynamics, Industrial project at GODREJ INDUSTRIES LTD. as an In-plant trainee, May 2003

• Synthesis of Sorbitan Esters (a Non-Ionic Surfactant) and analysis of its physicochemical properties, April 2004.

Awards/ Honors

• Mumbai Municipal Corporation scholarship awards during undergraduate education

• 2006 AOCS Biotechnology Division second place award for research entitled “Epoxy Fatty Acid Synthesis in Soybean Somatic Embryos Expressing a Mammalian Epoxycgenase Gene.”

Abstracts/ Posters

• Epoxy Fatty Acid Synthesis in Soybean Somatic Embryos Expressing a Mammalian Epoxycgenase Gene, AOCS conference, St. Louis, 2006.