Biotechnological Aspects of Plant Lipids

(International Workshop) Carmona, Spain. 26 - 29 May 1993

Contributions and Abstracts of Plenary Lectures

The following are contributions to an international workshop on the Biotechnological Aspects of Plant Lipids held in Carmona in May 26-29, 1993, in Carmona, Spain. The meeting was organized by Juan Sánchez (Instituto de la Grasa, Sevilla, Spain) and John L. Harwood (University of Wales, Cardiff, U.K.) and was sponsored by the Fundación Ramón Areces, Madrid, Spain, with the financial support of International Olive Oil Council, Consejo Superior de Investigaciones Científicas (Spain), Cooperative Research for Plant Biology (U.S.A.), British Council (U.K.), Junta de Andalucía (Spain), Ayuntamiento de Carmona (Spain), Instituto de la Grasa (Spain) and Alexander von Humboldt Foundation (Germany). The plenary lectures, whose abstracts are also included here,will be published within the series Progress in Lipid Research in January 1994.

CONTRIBUTIONS

Characterizing some European olive oil varieties by volatiles using statistical tools

M.T. Morales and **R. Aparicio**
Instituto de la Grasa y sus Derivados (CSIC)
Avda. Padre García Tejero, 4. 41012-Sevilla

SUMMARY

Characterizing some European olive oil varieties by volatiles using statistical tools.

The volatile aroma composition of five virgin olive oil varieties corresponding to three different Mediterranean countries were studied. Chemical data of 63 compounds quantified by a dynamic headspace gas-chromatographic method were treated by Analysis of Variance (ANOVA), Stepwise Linear Discriminant Analysis (SLDA) and Factor Analysis (FA) in order to achieve a possible characterization. Statistical methodology was established to reduce the initial number of compounds to the 6 most important in discriminating between these varieties.

KEY-WORDS: Virgin olive oil - Varieties - Volatiles - Statistics.

1. INTRODUCTION

Virgin olive oil is a highly appreciated kind of oil due to its well-balanced and complex flavour. Volatile compounds are chiefly responsible for this flavour. In previous works (Gutiérrez, 1975) (Solinas, 1988) quantitative differences were found in a few volatile components when different olive varieties were studied.

Thus, a study of olive oils by means of their content in volatile compounds needs two procedures: an optimal method for the quantification of the volatile compounds and a mathematical procedure that allows the most significant peaks to be selected.

In the study of flavours, headspace methods are especially attractive as they measure the volatile substances in the ratios in which they are typically presented to the human senses. Hence the resulting chromatogram is regarded as a true representation of the composition of the starting material (Werkhoff, 1987). A great number of methods have been developed (Morales, 1992) and among headspace methods the dynamic headspace has the advantage that it includes a concentration step, thereby improving detection limits.

This paper analyses the characterization of virgin olive oils produced from five olive varieties harvested in Italy, Greece and Spain. The volatile components were quantified by a Dynamic Headspace Gas Chromatographic Method using Tenax TA, thermal desorption and cold trap injection.

A volatile chromatogram has a profile constituted by so many peaks that it is difficult to work with all of them. The ideal procedure would be to reduce the total number of peaks using only those peaks that give information about "varieties discrimination". Statistical methodology has been established to realize this objective using some uni- and *multi-variate statistical techniques: Analysis of Variance*, Factor Analysis and Linear Discriminant Analysis.

2. MATERIALS AND METHODS

2.1. Samples

Twenty eight virgin olive oil samples corresponding to two consecutive crops and harvested in three different Mediterranean countries were analysed in duplicate. The varieties studied were five: 8 samples of Coronaiki from Greece, 4 samples of Moraiolo and Racioppa from Italy, 6 samples of Arbequina and Picual from Spain.

2.2. Analytical procedure

Pre-concentration was carried out by a dynamic headspace (DHS) method (Morales, 1993). 0.5 g of virgin olive oil were heated at 40°C and swept with N_2 (200 ml/min) for 15 minutes. Tenax TA was used as the trap. Volatiles were thermally desorbed at 220°C onto a fused silica trap cooled at -110°C for 5 minutes. When cold this trap was flushed by heating at 170°C for 5 min. The volatiles that had condensed within the trap were transferred onto a fused silica Supelcowax 10 capillary column (60m, 0.32mm I.D., 0.5µm FT). The oven temperature was held at 40°C for four minutes and programmed to rise at 4°C/min to a final temperature of 240°C at which temperature it was held for 10 minutes. A FID detector was employed. Isobutyl acetate (3000 ppb) was added as internal standard to each sample of virgin olive oil.

2.3. Statistical analyses

Once the samples had been analysed the chromatograms were stored in a relational database and multivaria-

te data treatment was performed using SPSS (1986) and BMDP (Dixon, 1983) statistical packages.

ANOVA was performed to study the repeatability of the replicates. The step-wise procedure was used in the discriminant analysis (SLDA), where variables (peaks) were selected according to their F-to-enter value (Tabachnick, 1983) in order to avoid a good discrimination being achieved by chance. The final probability is a predictive one obtained after applying the jackknife algorithm.

The factor extraction method used in Factor Analysis was Principal Components Analysis (PCA), eigenvalues greater than 1.0 were employed as extraction criterion and varimax as rotation.

3. RESULTS AND DISCUSSION

The flow diagram of the work is displayed in figure 1. A first set of 63 peak areas was employed for the initial statistical analyses. The peak areas of each chromatogram were divided by the standard area, so avoiding the possibility that the results were affected by random variables in the quantification process.

Initially the significant differences between replicates were tested by an analysis of variance (ANOVA). Thus, a peak was not selected for the subsequent statistical analysis if its F significance was lower than 0.05. On the other hand, it is well-known that the chemical composition of olive oil can change over the years (Aparicio, 1988) so

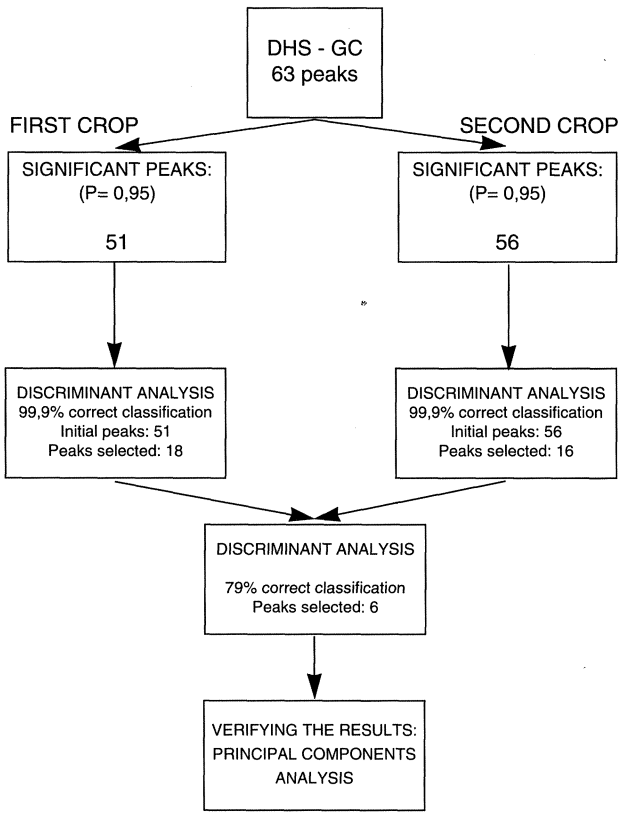

Fig. 1
Flow diagram

the repeatability studies were carried out independently for each year. In the first year the initial set of 63 peaks was reduced to 51 whilst 56 peaks were selected in the second. 48 of the peaks were identical in both years.

Subsequently, SLDA was independently applied to the datasets of both years. 99.9% of correct classifications in prediction were obtained in both cases. In the first year 18 peaks and in the second 16 peaks were used to classify the samples.

At this point, SLDA was again applied but this time using the samples from both years together and using the sum of the previously selected peaks; 18 plus 16. 79% of total correct classifications: 83.3% Picual, 83.3% Arbequina, 87.5% Coroneiki, 100% Moraiolo and 25% Racciopa, were obtained with only six peaks. Peaks 12, 13, 18, 36, 45 and 55 were selected in the discriminant equation. Figure 2 shows a computerized chromatogram where the varieties can be neatly distinguished by the profile of six peaks. Peaks 12, 13, 36 and 45 classify Greek and Spanish varieties and peaks 18 and 55 help to fit the equation and classify Italian varieties.

These volatile compounds were present in every sample but in different range of concentration. Table I shows the results of six selected volatile compounds. Mean values and 95% confidence intervals are reported.

Table II displays the chemical compound corresponding to each selected peak and the significance of F classifying the five varieties in both years. As can be seen different kind of chemical compounds participate in the discrimination. The F-values were lower of 0.05 in all cases showing there were differences between varieties.

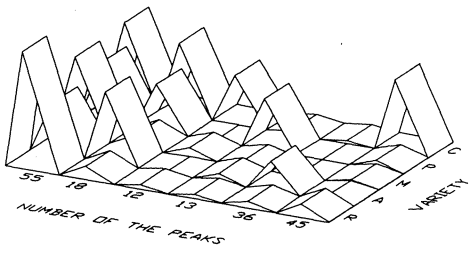

Fig. 2
Quantitative profile of the six selected peaks

To verify that the results obtained from previous studies were correct PCA were carried out with this set of six peaks. Using this method 80% of total variance was explained with three factors. The first eigenvector explained 33.2%, the second 26.5%, the third 19.8% and the remainder 20.5%. Figure 3 shows the 3D plot obtained. It is possible to distinguish each variety. At the top is situated the Picual variety, in the middle appears the Greek Coronaiki variety, at the left the Italian varieties and at the right the Arbequina variety. As can be seen only one Racciopa sample appears close to the Arbequina variety and only one Coronaiki sample close to the Italian varieties.

It can be concluded that it is possible to discriminate between varieties using volatile compounds and that the whole

Table I

Results of the six selected volatile compounds (mean values ± 95% confidence interval)[a]

Number of peak	Variety				
	Picual	Arbequina	Coronaiki	Moraiolo	Racioppa
12	trace	trace	0.24±0.06	0.04±0.03	0.02±0.01
13	0.57±0.18	0.09±0.02	0.04±0.01	0.06±0.02	0.05±0.02
18	0.50±0.14	0.71±0.30	0.89±0.15	0.15±0.08	0.15±0.03
36	trace	0.06±0.02	trace	trace	0.02±0.01
45	0.09±0.02	0.04±0.02	0.91±0.16	0.06±0.04	0.15±0.04
55	1.24±0.40	1.02±0.27	2.04±0.29	1.80±0.43	2.67±0.97

[a]Ratio of the compound peak area to the internal standard peak area.
trace: <0.01.

Table II

Chemical compounds corresponding to the six selected peaks. Significance of F classifying varieties in two crops.

Number of peak	Chemical Compound	Signif. of F classifying varieties	
		1991	1992
12	1,3 Hexadien 5-ine	<0.001	0.006
13	Alcohol (*)	0.003	0.012
18	1-penten-3-one	0.013	0.009
36	Unidentified	0.003	0.020
45	Furan 3-(4 methyl 3 pentenyl)	0.002	0.001
55	3-hexen-1-ol	0.049	0.042

(*) Identification in progress.

set of initial peaks is not necessary to achieve the characterization since it is possible to obtain a good discrimination between the five varieties studied from both harvests with a reduced set of only six compounds.

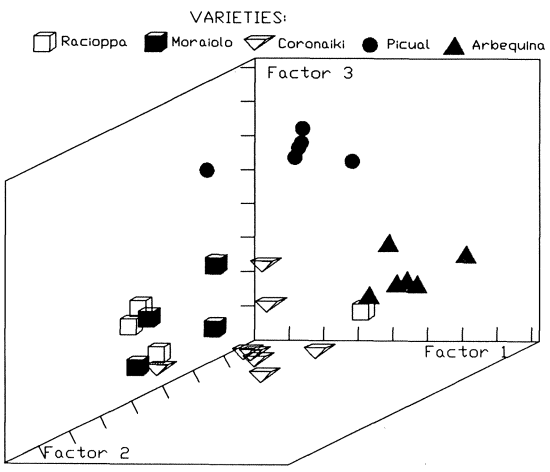

Fig. 3
3D plot of five species of virgin olive oil by three principal components

ACKNOWLEDGEMENTS

The authors would like to acknowledge their indebtedness to Mr. J.J. Rios for the mass spectrometry identification of the volatile compounds. This work has been supported by CICYT-SPAIN ALI-91-0786.

REFERENCES

Aparicio, R. (1988).- "Characterization of foods by inexact rules: the SEXIA expert system".- J. Chemometrics **3**, 175-192.

Dixon, W.J. (1983).- "BMDP statistical software".- University of California Press, Los Angeles (CA), USA.

Gutiérrez, R., Olías, J.M., Gutiérrez, F., Cabrera, J. and del Barrio, A. (1975).- "Los métodos organolépticos y cromatográficos en la valoración de las características aromáticas del aceite de oliva virgen".- Grasas y Aceites **26**, 21-31.

Morales, M.T., Aparicio, R. and Gutiérrez, F. (1992).- "Técnicas de aislamiento y concentración de volátiles de aceites vegetales".- Grasas y Aceites **43**, 164-173.

Morales, M.T. and Aparicio, R. (1993).- "The optimization by mathematical procedures of two dynamic headspace techniques for quantifying virgin olive oil volatiles".- Anal. Chim. Acta (in press).

Solinas, M., Angerosa, F. and Marsilio, V. (1988).- "Indagine su alcuni componenti dell'aroma degli oli vergini di oliva in relazione alla varietá delle olive".- Riv. Ital. Sostanze Grasse **65**, 361-368.

SPSS (1986).- "SPSS Users Guide".- 2nd Ed.- McGraw Hill Book Co., New York.

Tabachnick, B.G. and Fidell, L.S. (1983).- "Using Multivariate Statistics".- Harper & Row, New York.

Werkhoff, P. and Bretschneider, W. (1987).- "Dynamic headspace gas chromatography: concentration of volatile components after thermal desorption by intermediate cryofocusing in a cold trap.I. Principle and applications".- J. Chromatogr. **405**, 87-98.

The cloning and overexpression of *E. coli* acyl carrier protein

A. Lesley Jones [1], **Peter Kille** [1], **Jane E. Dancer** [2], **John L. Harwood** [*1]
1. Department of Biochemistry, University of Wales College of Cardiff, P.O. Box 903, Cardiff CF1 1ST, U.K.
2. Schering Agrochemicals Ltd., Chesterford Park, Saffron Walden, Essex CB10 1XL, U.K.

SUMMARY

The cloning and overexpression of *E. coli* acyl carrier protein.

Acyl carrier protein (ACP) from *E. coli* was cloned using degenerate PCR. The resulting clone was sequenced and translated to give a product which had the known amino acid sequence of *E. coli* ACP. The insert obtained was cloned into various expression systems, but no overexpression was obtained and sequencing of the insert revealed the presence of mutations.

KEY-WORDS: Acyl carrier protein (ACP) - E. coli - Fatty acid synthesis.

1. INTRODUCTION

Acyl carrier protein (ACP) is essential for fatty acid synthesis. Plants and bacteria contain Type II dissociable fatty acid synthases where each enzyme of the synthetic reactions is a separate polypeptide capable of catalysing individual steps independently [1]. The acyl chains undergoing the cyclical condensation, reduction, dehydration and further reduction steps of fatty acid synthesis are esterified to ACP through the prosthetic group, 4'-phosphopantetheine, during synthesis. By analogy with animal systems, ACP may also be involved in the mechanism of condensation.

ACP itself is a small (approximately 9kDa) acidic protein which is well conserved through evolution [2]. There is considerable homology between plant and bacterial ACPs, and the animal ACPs such as that from rabbit [2, 3], also show some homology even though they are associated with Type I multifunctional protein fatty acid synthases. Homology is particularly high around the serine residue through which the prosthetic group is attached [2] (Fig 1, residue 37).

E. coli ACP is used in many plant fatty acid synthase assays as a substitute for the appropriate plant ACP. There are two main reasons for this:

1. Large quantities of plant material are required to isolate relatively small amounts of protein. *E. coli* cell paste is a much more concentrated starting material.

2. *E. coli* has only one ACP isoform whereas different isoforms exist in different tissues of plants and isoforms active in one system may be inactive in another [2, 4]. *E. coli* ACP is usually active in all plant systems and often gives higher activity in fatty acid synthase assays than the native plant ACP [5].

Approximately 30mg of ACP can be isolated from 200g of *E. coli* wet cell paste in 3 days by standard protein purification techniques. However, assaying plant fatty acid synthesis requires relatively large amounts of ACP and it would be useful to have a more readily available supply. For this reason we have attempted to overexpress *E. coli* ACP in *E. coli* using various expression systems.

```
                                                                  10                  20                  30                  40
CCCGAATTCATGAGCACCATCGAAGAACGTGTGAAAAAAATTATT
                  MetSerThrIleGluGluArgValLysLysIleIle

      50          60          70          80          90
GGCGAACAGCTGGGCGTTAAGCAGGAAGAAGTTACCAACAATGCT
GlyGluGlnLeuGlyValLysGlnGluGluValThrAsnAsnAla

     100         110         120         130
TCTTTCGTTGAAGACCTGGGCGCGGATTCTCTTGACACCGTTGAG
SerPheValGluAspLeuGlyAlaAspSerLeuAspThrValGlu

     140         150         160         170         180
CTGGTAATGGCTCTGGAAGAAGAGTTTGATACTGAGATTCCGGAC
LeuValMetAlaLeuGluGluGluPheAspThrGluIleProAsp

     190         200         210         220
GAAGAAGCTGAGAAAATCACCACCGTTCAGGCTGCCATTGTCTAT
GluGluAlaGluLysIleThrThrValGlnAlaAlaIleAspTyr

     230         240         250     255
ATTAACGGAAACCAGGCGTAAAAGCTTGGG
IleAsnGlyHisGlyAla***
```

The primer regions are heavily underlined and the restriction enzyme sites built onto the end of the primers are single underlined. Rawlings and Cronan [7] confirmed our original assumption that the initial Met residue would be lost after translation but that no other amino acid residues would be processed from the N-terminus. The Ser residue through which the 4' phosphopantetheine group is attached is underlined.

Fig. 1
Sequence and translation of a DNA fragment coding for *E. coli* ACP

2. RESULTS AND DISCUSSION

The complete protein sequence of *E. coli* was first determined as long ago as 1968 [3], and a more recent sequence found only two amino acid differences from the original work [6]. Initially no nucleotide sequence data for this gene was available from the literature or the databases, so the protein sequence was used to design degenerate oligonucleotide primers encoding the N- and C-termini of the protein. 5' Extensions carried appropriate cloning sites (see Fig. 1). Genomic *E. coli* DNA was used as a template for a PCR reaction which yielded a single band on an agarose gel, of exactly the size predicted for the amplified ACP gene.

Subsequent cloning and sequencing of the above PCR product demonstrated that it encoded an ACP-like protein, but all clones had a frame-shift mutation with an insertion of one base in the region of the N-terminal primer (an extra A in region 34-40 for the sequence in Fig. 1). This extra base was removed by overlap extension mutagenesis and the product of this reaction cloned into the expression vector pKK 223-3. No overexpression of ACP was detected after cells transformed with the vector were induced. *In vitro* translation of the plasmid yielded no protein product of the appropriate size although a DNA fragment of the correct size was detected after digestion of plasmid DNA with the appro-

priate restriction enzymes. Thus, the cloning had worked but no protein was being produced.

The product of the overlap exension mutagenesis was sequenced in M13 to confirm its identity, and two families of clones were identified - one family with the full sequence (Fig 1) and one family with a deletion at nucleotide 41. It is possible that the failure to overexpress the gene was caused by the cloning of the mutated gene into the expression vector. Subsequently a similar strategy was used to introduce the PCR product into the vector pJLA503. This vector has a tightly controlled heat-inducible promoter and, thus, should avoid any problems caused by a toxic product being formed by a leaky promoter before induction. The gene was shuttled into pUC18 before being cloned into pJLA503, and while pUC18 was demonstrated to have a DNA insert of the correct size, pJLA503 did not. Possible reasons for the high mutation rate, and the seeming difficulty of overexpression of this gene are discussed below.

The DNA sequence for *E. coli* ACP was published recently [7]. This sequence agrees exactly with the sequence we report here apart from our predetermined primer regions. Translation of both of the open reading frames produces an identical polypeptide which disagrees with both of the amino acid sequences previously published [3, 6]. Vanaman et al. [3] reported Asp at position 24 and Jackowski and Rock [6] reported Asn at this position; Rawlings and Cronan [7] confirmed this residue to be Asn, as we did (Fig 1). Similarly at residue 43, Vanaman *et al* [3] reported Val and Jackowski and Rock [6] reported Ile; we found Val at this position, as did Rawlings and Cronan [7].

Rawlings and Cronan [7] observed that the natural *E. coli* ACP gene when cloned into *E. coli* has a high frequency of spontaneous deletions. This is consistent with the high mutation frequency observed in the fragment we isolated containing the ACP gene. They suggested that the DNA segment encoding ACP is somehow toxic to *E.coli* and thus selection pressure would be high as mutated organisms which did not express the insert would be at a great advantage, so *E. coli* with a mutated gene would rapidly take over the cultures. Rock and Cronan [8] also reported that a synthetic construct of this gene proved lethal to the organism and observed that all the ACP present was acylated, mainly with short chain fatty acids, suggesting that high levels of ACP in the cell caused scavenging of acyl chains which could be part of a toxic effect [8]. The question of why an *E. coli* gene should be toxic to its host organism when inserted into a plasmid is interesting *per se*, especially as it is possible to overexpress ACP genes from other organisms, such as spinach [9] without, apparently, affecting the growth or viability of the culture.

ACKNOWLEDGEMENTS

This is a LINK project with the SERC, AFRC and Schering Agrochemicals Ltd.

REFERENCES

1. Harwood, J.L. (1988). -"Fatty acid metabolism".- *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **39**, 101-138.
2. Slabas, A.R. & Fawcett, T. (1992). -"The biochemistry and molecular biology of plant lipid biosynthesis".- *Plant Molecular Biology* **19**, 169-191.
3. Vanaman, T.C., Wakil, S.J. & Hill, R.L. (1968).-"The complete amino acid sequence of the acyl carrier protein of *Escherichia coli*".- *J. Biol. Chem.* **243**, 6420-6436.
4. Ohlrogge, J.B., Browse, J. and Somerville, C.R. (1991).-"The genetics of plant lipids".- *Biochim. Biophys.* Acta **1082**, 1-26.
5. Simoni, R.D., Criddle, R.S. & Stumpf, P.K. (1967) -"Purification and properties of plant and bacterial acyl carrier proteins".- *J. Biol. Chem.* **242**, 573-581.
6. Jackowski, S. & Rock, C.O. (1987). -"Altered molecular form of acyl carrier protein associated with beta-ketoacyl carrier protein synthase II (fabf) mutants".- *J. Bacteriol.* **169**, 1469-1473.
7. Rawlings, M. & Cronan, J.E. (1992). -"The gene encoding *Escherichia coli* ACP lies within a cluster of fatty acid biosynthetic genes".- *J. Biol. Chem.* **267**, 5751-5754.
8. Van den Boom, T. & Cronan, J.E. (1989). -"Genetics and regulation of bacterial lipid metabolism".- *Ann. Rev. Microbiol.* **43**, 317-343
9. Beremand, P.D., Hannapel, D.J., Guerra, D.J., Kuhn, D.N. & Ohlrogge, J.B. (1987). -"Synthesis cloning and expression in *E. coli* of a spinach ACP-1 gene".- *Arch. Biochem. Biophys.* **256**, 90-100 .

Cloning of the acetyl-coenzyme A carboxylase gene from the cyanobacterium, *Anacystis* R2

Nora W. Lem
Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, CANADA. V6T 1Z3.

1. INTRODUCTION

Cyanobacteria occupy a unique evolutionary position between prokaryotes and photosynthetic eukaryotes. An organism similar to extant single cell cyanobacteria may have been the endosymbiont precursor of higher plant chloroplasts. This evolutionary relationship is supported by ultrastructural, physiological and biochemical lines of evidence - including lipid and fatty acid metabolism (Lem and Stumpf, 1984). Therefore, cyanobacteria may be ideal organisms with which to use molecular biology techniques to investigate the control of fatty acid synthesis and desa-

turation and to provide insight into metabolism in higher plant cells.

Three major questions dominate the field of higher plant lipid metabolism.

1. What are the genetic and biochemical controls which govern fatty acid synthesis and the fatty acid composition of higher plant tissues?
2. What is the mechanism of fatty acid desaturation?
3. How can the fatty acid and lipid composition of higher plant tissues be modified?

In order to contribute to the available data in answer to these questions, the gene for acetyl-CoA carboxylase, the first step in fatty acid synthesis, has been cloned from the cyanobacterium, *Anacystis* R2, by complementation of *Escherichia coli* L8, a temperature sensitive mutant.

2. MATERIAL AND METHODS

Strains and culture conditions. *E. coli* L8 (LA2-22) was a gift from Dr. David Silbert (Washington University School of Medicine, St. Louis, Missouri) and was maintained on Luria-Bertani (LB) agar or grown on LB broth at 30 C. This strain has a temperature sensitive phenotype which results from synthesis of a defective protein of acetyl-CoA carboxylase (see later). Growth of L8 is not observed at non-permissive temperatures (> 37-40 C) in media which are not supplemented with fatty acids (Silbert *et al.,* 1976).

Anacystis R2 (*Synechococcus leopoliensis*) was obtained from the University of Toronto Culture Collection and grown on BG11 medium (30 C; 16 h light and 8 h dark cycle, 24 μEinsteins M^{-2} sec^{-1}).

Cloning of *Anacystis* R2 acetyl-CoA carboxylase. *Anacystis* R2 chromosomal DNA was extracted and purified by CsCl density gradient centrifugation (Sambrook et al., 1989). The chromosomal DNA was digested to completion with *Bam HI* and ligated onto *Bam HI* digested dephosphorylated pUC13. Competent L8 cells were made and electroporated with chimeric plasmid prior to plating onto LB agar plates containing ampicillin (200 μg ml^{-1}; 30 C). Ampicillin resistant colonies were picked and plated onto M9 minimal medium containing agar plates (Sambrook et al., 1989). After two days of incubation at 40 C, the colonies were inoculated into LB broth and grown for analysis of plasmids and proteins.

Enzyme assay. Acetyl-CoA carboxylase was assayed essentially according to Eastwell and Stumpf (1983).

3. RESULTS AND DISCUSSION

Twelve clones of *E. coli* L8 complemented in the mutation (as indicated by their ability to grow at 40 C on minimal medium) were obtained. Restriction analysis of the plasmids obtained from these clones indicated that the size of the inserts ranged from ca. 600 bases to 2200 bases in length.

Assays for enhanced levels of acetyl-CoA carboxylase and temperature stable acetyl-CoA carboxylase activity are currently in progress.

The *fab*E gene associated with the temperature sensitive mutation in *E. coli* L8 has been cloned and sequenced (Muramatsu and Mizuno, 1989) and shown to encode the structural gene for biotin carboxyl carrier protein (BCCP; Kondo et al. 1991). In the temperature sensitive L8 mutant, there is a G to A transversion in this gene which results in a Gly^{100} to Ser change in the amino acid sequence of the protein. Biotinylation of the protein is altered. The gene encoding the biotin carboxylase enzyme is located immediately downstream from this BCCP structural gene and these two genes appear to function as an operon. These genes are located at min. 72 of the *E. coli* genome.

In contrast, the genes for the alpha- and beta-subunits of carboxyl transferase are located at min. 4.3 and min. 50, respectively, of the *E. coli* genome although they are cotranscribed (Li and Cronan, 1992).

The organization of the genes for acetyl-CoA carboxylase in *E. coli* poses interesting questions with respect to control of transcription and the evolution of the positioning of these genes. The non-associated nature of the protein subunits of the *E. coli* acetyl-CoA carboxylase differs from the "associated" acetyl-CoA carboxylase complex in higher plants. It will be important to examine the acetyl-CoA carboxylase genes, to characterize the protein complex and enzyme activity in *Anacystis* R2 and to compare them with both *E. coli* and higher plants.

REFERENCES

Eastwell, K.C. and Stumpf, P.K. (1983).–"Regulation of plant acetyl-CoA carboxylase by adenylate nucleotides"–. Plant Physiol. **72**, 50 - 55.

Kondo, H., Shiratsuchi, K., Yoshimoto, T., Masuda, T., Kitazono, A., Tsuru, D., Anai, M., Sekiguchi, M. and Tanabe, T. (1991). –"Acetyl-CoA carboxylase from *Escherichia coli:* Gene organization and nucleotide sequence of the biotin carboxylase subunit".– Proc. Natl. Acad. Sci. **88**, 9730 - 9733.

Lem, N.W. and Stumpf, P.K. (1984). –"In vitro fatty acid synthesis and complex lipid metabolism in the cyanobacterium, *Anabaena variabilis.* I. Some characteristics of fatty acid synthesis".– Plant Physiol. **74**,134 - 138.

Li, S.-J. and Cronan, J. E., Jr. (1992). –"The genes encoding the two carboxyltransferase subunits of *Escherichia coli* acetyl-CoA carboxylase".– J. Biol. Chem. **267**, 16841-16847.

Muramatsu, S. and Mizuno, T. (1989). –"Nucleotide sequence of the fabE gene and flanking regions containing a bent DNA sequence of *Escherichia coli*".– Nucl. Acids Res. **17**, 3982.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989).– "Molecular Cloning: A Laboratory Manual".– (2nd edition).– Cold Spring Harbor Laboratory Press.

Silbert, D.F., Pohlman, T. and Chapman, A. (1976). –"Partial characterization of a temperature-sensitive mutation affecting acetyl coenzyme A carboxylase in *Escherichia coli* K-12".– J. Bacteriol. **126**,1351-1354.

cDNA cloning and overexpression of 3-oxoacyl-ACP reductase from *Brassica napus* seed

José M. Martínez-Rivas, Neil C. Thomas, Diane Chase, Tony Fawcett, Antoni R. Slabas*
Lipid Molecular Biology Group, Department of Biological Sciences, University of Durham, South Road, Durham DH1 3LE, U.K.

SUMMARY

cDNA cloning and overexpression of 3-oxoacyl-ACP reductase from *Brassica napus* seed.

cDNA clones encoding NADPH-linked 3 oxoacyl-ACP reductase (EC 1.1.1.100) were isolated from a *Brassica napus* (rape) developing seed library using a probe derived from a full length cDNA clone from *Arabidopsis thaliana* leaf.

There is strong sequence homology, both at the nucleotide and amino acid level, for the cDNAs coding for this enzyme from *Brassica napus* seed and *Arabidopsis thaliana* leaf.

Northern blots analysis was performed using mRNA isolated from rape leaves and seeds, and demonstrated that the expression of this gene in seeds is aproximately 20 times higher than in leaves.

Expression of this gene in an appropriate *E. coli* expression vector resulted in the accumulation of the 3-oxoacyl-ACP reductase protein, which was accompanied by an increase in the enzymatic activity. The overexpressed protein was thus soluble and biologically active.

Antibodies against avocado β-keto reductase enzyme have been previously reported to be non-cross reactive with the rape protein. On western blot of crude rape seed, no positive signal is observed using the rabbit anti avocado antibody; however the same antibody strongly recognized the overproduced rape β-keto reductase in *E. coli.* probably as a result of a higher concentration of the rape protein.

KEY-WORDS: 3-oxoacyl-ACP reductase - Fatty acid synthesis - Brassica napus.

1. INTRODUCTION

The biosynthesis of fatty acids in plants is catalyzed by a type II dissociable fatty acid synthetase (for a review see Slabas and Fawcett, 1992). Many of the components have been purified but not in sufficient quantities to allow for detailed structural studies. 3-Oxoacyl-ACP reductase (β-keto reductase) catalyses the first reduction step in fatty acid biosynthesis. Two isoforms of the enzyme, one NADPH-linked and the other NADH-linked, have been reported to be localised in plastids. Recently, the isolation of a full length clone of this enzyme from *Arabidopsis thaliana* has been reported (Slabas *et al.* 1992). The present paper describes the cDNA cloning and overexpression of this enzyme from *Brassica napus* seed in order to make future structural studies.

2. MATERIALS AND METHODS

Standard molecular biology techniques were followed. Enzyme assays were performed according Sheldon *et al.* (1992).

3. RESULTS AND DISCUSSION

A 0.2 kb fragment corresponding to the 5' part of the mature protein of the *Arabidopsis thaliana* β-keto reductase cDNA clone was used as a probe to screen a λ-ZAP II rape seed cDNA library. Several clones were isolated and the largest sequenced. The degree of identity between the encoded proteins of the *Brassica napus* seed and *Arabidopsis thaliana* leaf cDNA clones was extremely high at the nucleotide and amino acid level.

Northern blot analysis was performed using mRNA isolated from rape leaves and seeds showing that the expression of this gene in seeds is aproximately 20 times higher than in leaves (Fig. 1).

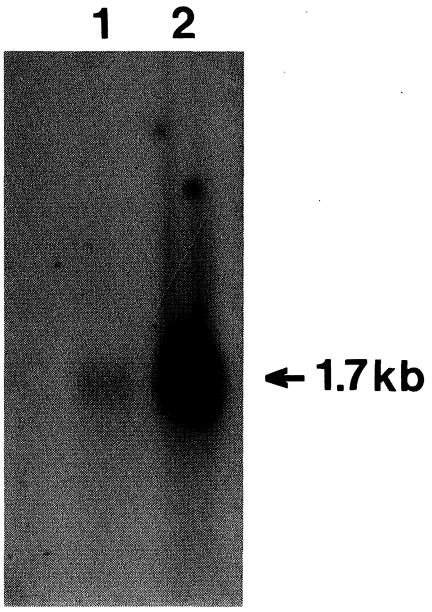

Fig. 1
Equal amouns (1μg) of poly(A)+RNA isolated from rape leaves (lane 1) and rape seed (lane 2) were probed with the rape seed β-keto reductase cDNA clone using the following conditions. Hybridisation: 50% formamide, 5xSSPE, 0.1% SDS, 42°C. Washing: 0.1xSSC, 0.1% SDS, 65°C.

The rape seed β-keto reductase cDNA clone were overexpressed in a *E. Coli* expression vector for the production of large quantities of protein for enzymatic studies. A PCR fragment corresponding to the mature protein of this clone was inserted into *Bam* HI / *Nco* I digested pET-11d vector and transformed into *E. Coli* BL21 (DE3) for expression of

β-keto reductase. The quantification of the level of expression by SDS-PAGE showed that 5% total soluble protein correspond to β-keto reductase (Fig. 2a).

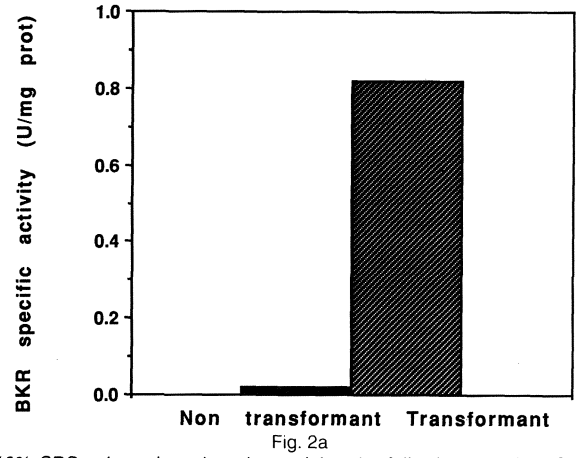

Fig. 2a
10% SDS-polyacrylamyde gel containing the following samples: Crude extracts from *E. Coli* BL21 (DE3) induced with IPTG. Lane 1: protein from a non-transformed control culture. Lanes 2,3: from transformed cells. Lanes 4,5,6,7: 1000, 500, 200 and 100 ng of bovin serum albumin as standard, respectively.

On the other hand, antibodies raised against avocado β-keto reductase (Sheldon et al. 1990) are non-cross reactive with the rape protein but strongly recognise the overproduced rape enzyme in *E. Coli,* probably as a result of a higher concentration of the rape protein.

To check if the overexpressed enzyme is biologically active, crude extracts from *E. Coli* BL21 (DE3) induced with IPTG transformants were obtained and assayed for, β-keto reductase. High levels of activity compared with non transformants were observed (Fig. 2b). The overexpressed enzyme will be purified and used to raise an antibody and also for future structural studies.

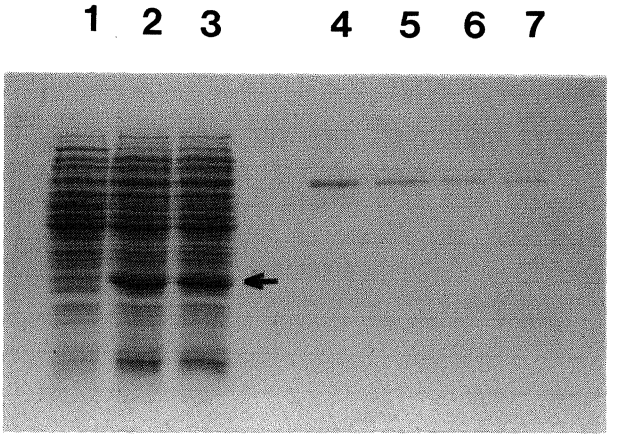

Fig. 2b
Levels of β-keto reductase specific activity detected in crude extracts from *E. Coli* BL21 (DE3) transformants and non-transformants induced with IPTG.

ACKNOWLEDGEMENTS

We wish to thank the EC for a postdoctoral fellowship to J.M.M.-R. and AFRC for a research studentship to N.C.T.

BIBLIOGRAPHY

Sheldon, P.S., Kekwick, R.G.O., Sidebottom, C., Smith, C.G., Slabas A.R. (1990).- "3-oxoacyl-(acyl carrier protein) reductase from avocado (*Persea americana*) fruit mesocarp".- Biochem. J. **271**, 713-720.

Sheldon, P.S., Kekwick, R.G.O., Smith, C.G., Sidebottom, C., Slabas A.R. (1992).- "3-0xoacyl-(ACP) reductase from oilseed rape (*Brassica napus*)".- Biochim. Biophys. Acta **1130**, 151-159.

Slabas, A.R., Chase, D., Nishida, 1., Murata, N., Sidebottom, C., Safford, R., Sheldon P.S., Kekwick, R.G.O., Hardie, D.G., Mackintosh, R.W. (1992).– "Molecular cloning of higher-plant 3-oxoacyl-(acyl carrier protein) reductase".- Biochem. J. **283**, 321-326.

Slabas, A.R. and Fawcett T. (1992).- "The biochemistry and molecular biology of plant lipid biosynthesis".- Plant Mol. Biol. **19**,169-191.

Solubilization and partial purification of the acyl-CoA elongase from developing rapeseeds (*Brassica napus* L.)

Anne Creach and **René Lessire***
IBC–CNRS. 1 rue Camille Saint-Saëns. 33077 Bordeaux-Cedex. FRANCE.

SUMMARY

Solubilization and partial purification of the acyl-CoA elongase from developing rapeseeds (*Brassica napus* L.).

The $C_{18:1}$-CoA elongase has been studied using a 15000 g particulate fraction from developing rapeseeds. The synthesis of very long chain monounsaturated fatty acids (VLCMFA) was stimulated by Triton X-100 whereas N-octyl-β-D-glucopyranoside had no effect and deoxycholate strongly inhibited the formation of VLCMFA. A Triton X-100 : Protein ratio (w:w) of 2.5 was efficient to solubilize the acyl-CoA elongase. Using affinity, anion exchange and filtration chromatographies, the solubilized $C_{18:1}$-CoA elongase has been partially purified. Based on specific activity, the rapeseed acyl-CoA elongase has been purified at least 5 fold.

KEY-WORDS: Brassica napus - Acyl-CoA elongase - Erucic acid - Enzyme purification.

1. INTRODUCTION

Erucic acid is an important renewable material in oleochemical industries (Princen and Rothfus, 1984) and the increase of erucic acid level in rapeseed oil is an important commercial goal. Acyl-CoA elongase(s) and acyl transferase are key enzymes for the obtention of high erucic acid rapeseed oil. Previous studies of cruciferous seeds strongly suggested that the very long chain monounsaturated fatty acids (VLCMFA) could be formed from $C_{18:1}$-CoA by successive additions of malonyl-CoA (Downey and Craig, 1964) carried out by a membrane-bound elongation system (Agrawal and Stumpf, 1985, Murphy and Mukherjee, 1988, Taylor et al.,

1991) as previously demonstrated in the leaves for the saturated very long chain fatty acid synthesis (Cassagne and Lessire, 1978, Agrawal et al., 1984). It has been also shown that four intermediate reactions are involved in the elongation process and the metabolic intermediates have been characterized (Fehling and Mukherjee, 1991).

Despite of the partial purification of the $C_{18:0}$-CoA elongase from epidermal leek cells (Bessoule et al., 1989) and of the $C_{18:1}$-CoA elongase from *L. annua* (Fehling et al., 1992), the structure and the mechanisms of the different enzymic reactions involved in the elongation process remain unknown. This paper reports the solubilization and the partially purification of the acyl-CoA elongase from developing rapeseeds.

2. EXPERIMENTAL PART

- **Material:** High erucic acid *Brassica napus L.* were grown at INRA-Rennes and seeds were harvested 5 to 8 weeks after flowering. [2-14C] malonyl-CoA came from NEN.
- **Methods:** The preparation of the 15000g particulate fraction and the $C_{18:1}$-CoA elongation measurement have been described previously (Creach and Lessire, 1992).
- **Solubilization of proteins:** The 15000g pellet, about 0.5 mg of proteins, was incubated with different Triton X-100: protein ratios (w:w) or using the optimal ratio of 2.5. The treated fraction was centrifuged at 150000g for 15 min and the supernatant was used as the solubilized fraction.
- **Enzyme purification:** The solubilized proteins were loaded onto a C_{16}-CoA Agarose column previously equilibrated by a 0.08M Hepes pH 7.2 buffer containing 0.02% Triton X-100 and 2% ethylene glycol. Then, the column was washed and the elongation activity was eluted by a 0.5M NaCl concentration. The fractions containing the $C_{18:1}$-CoA elongation activity were pooled and submitted to Ultrogel 34 AcA and DEAE chromatographies as reported earlier (Bessoule et al., 1989).

3. RESULTS

Effects of detergents on the $C_{18:1}$-CoA elongation

The effects of detergents, on the elongation of $C_{18:1}$-CoA were studied using a 15000g particulate fraction as enzyme source at concentrations below their cmc. The elongation activity was stimulated by increasing concentrations of Triton X-100 from 0.06 mM to 0.3 mM, and was multiplied by 2 times at a detergent concentration of 0.3mM (figure 1) as compared to the activity of untreated membranes. Under these conditions the synthesis of $C_{22:1}$ was greatly increased from 1.65 to 4.28 nmoles/mg/h. The VLCMFA synthesis was practically not modified in the presence of octyl-β-D glucopyranoside and the detergent did not significantly modify the label distribution among the fatty acids (Figure 1).

The deoxycholate even at low concentrations inhibited the elongation activity.

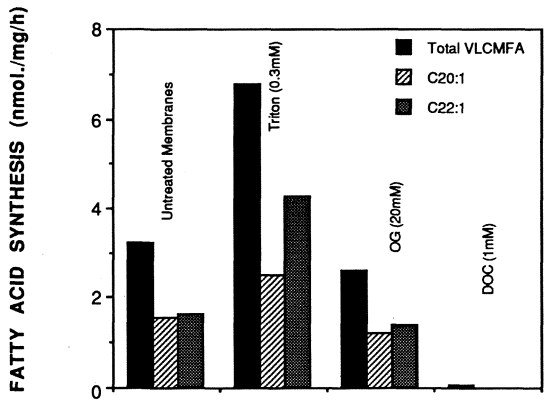

Figure 1: Effects of detergents upon $C_{18:1}$-CoA elongation.
The $C_{18:1}$-CoA elongation has been measured using:
19 µM [2^{14}C] malonyl-CoA, 0.5mM NADH, 0.5mM NADPH, 2mM DTT, 1mM $MgCl_2$, 9.2 µM oleoyl-CoA, 0.08 Hepes buffer, pH 7.2 containing 100 µg proteins in the presence or not of detergent in a final volume of 0.1 mL. The detergent concentrations used were Triton X-100 0.3mM, octyl-β-D glucopyranoside (OG), 20mM and deoxycholate (DOC) 1mM. The incubation medium was incubated for 1 hour at 30°C and the VLCMFA were extracted and analyzed by radio-GLC.

Solubilization of the $C_{18:1}$-CoA elongase

The solubilization of the elongation activity from the 15000g pellet prepared from developing rapeseeds was carried out using different Triton X-100: protein ratios from 1 to 6. After two hours of solubilization, the treated membranes were centrifuged at 150000g for 15 min using a micro ultracentrifuge and the $C_{18:1}$-CoA elongase activity was measured in the pellets and the corresponding supernatants. The best results were obtained using a detergent: protein ratio (w/w) of 2.5. Under these conditions, 60% of the proteins were recovered in the solubilized fraction (Figure 2)

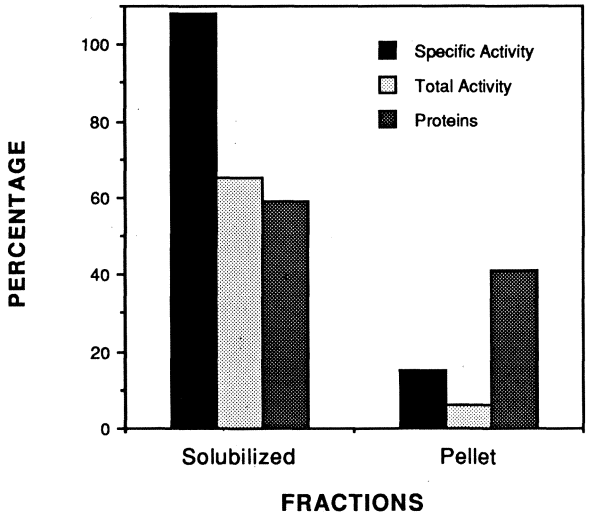

Figure 2: Solubilization of the $C_{18:1}$-CoA elongase activity.
The 15000g particulate (about 0.5mg of proteins) fraction were incubated with Triton X-100 (detergent/protein ratio : 2.5). The treated fraction was centrifuged 15 min at 150000g and the elongation activity was measured in the subsequent supernatant (solubilized) and pellet fractions as indicated in figure 1. The value 100 has been given to the elongation activity measured using untreated membranes as enzyme source.

and the total and specific activities remained about the same as those observed for untreated membranes. Moreover, little elongase activity was recovered in the corresponding pellet (Figure 2) indicating that the acyl-CoA elongase was efficiently solubilized.

Partial purification of the C18:1-CoA elongase

The C_{16}-CoA agarose chromatography profile shows that the major elongation activity was retained on the column and was eluted as a single peak. The specific activity was about 2 times that of the specific activity of the solubilized enzyme and the fractions containing the highest activity represented 3.6% of the membrane proteins. The $C_{18:1}$-CoA elongation activity peak was eluted close to the void volume during the filtration chromatography and that the elongation complex presented an apparent molecular mass of about 350 KDa. The specific activity of the $C_{18:1}$-CoA elongase in the Triton X-100 micelles was about 60 times higher than compared to the activity in the native membranes. The partially purified acyl-CoA elongase recovered after ultrogel filtration and anion exchange chromatography had still a specific activity of 2.45 nmoles/mg/h corresponding to an increase of more than 5 fold and represented only 0.05% of the membrane proteins. This relative high value was obtained although the partially purified fraction was highly delipidated as shown by the phospholipid analysis indicated that the partially purified fraction was highly delipidated and the analysis. The enzyme was still able to synthesize acyl-CoAs. Moreover, the SDS-electophoresis analysis of the partially purified fraction indicate that the elongation complex was constituted of different proteins having molecular weights ranging from 50 to 70 KDa.

4. DISCUSSION

The results reported here show that the $C_{18:1}$-CoA elongase from developing rapeseeds has been solubilized and partially purified. The Triton X-100 stimulated the VLCMFA synthesis and appeared to be the best detergent for the solubilization of the elongating complexes. This detergent has been also successfully used for the solubilization of the leek epidermal cell elongase (Lessire et al., 1985) and of the *L. annua* $C_{18:1}$-CoA elongase (Fehling et al., 1992).

The $C_{18:1}$-CoA elongase from developing rapeseeds presents an apparent molecular mass of 350 KDa which is identical as reported for the other plant elongases (Bessoule et al., 1989, Fehling et al., 1992).The fact that the partially purified enzyme was dissociated upon SDS treatment and showed the presence of 50-70 kDa proteins suggest, that the $C_{18:1}$-CoA elongase is an enzymatic complex constituted of different proteins as the other purified elongating systems (Bessoule et al., 1989, Fehling et al., 1992).

ACKNOWLEDGEMENTS

The financial support of ONIDOL and CETIOM is gratefully acknowledged.

BIBLIOGRAPHY

Agrawal V.P., Lessire R. and Stumpf P.K. (1984). –"Biosynthesis of very long chain fatty acids microsomes from epidermal cells of *Allium porrum L".*– Arch. Biochem. Biophys. **230**, 580-589.

Agrawal V.P. and Stumpf P.K. (1985). -"Elongation systems involved in the biosynthesis of erucic acid from oleic acid in developing *Brassica juncea* seeds".– Lipids **20**, 361-366.

Bessoule J.J., Lessire R. and Cassagne C. (1989). –"Partial purification of the acyl-CoA elongase of *Allium porrum* leaves".- Arch. Biochem. Biophys. **268**, 475-484.

Cassagne C. and Lessire R. (1978). –"Biosynthesis of saturated very long chain fatty acids by purified membrane fractions from leek epidermal cells".– Arch. Biochem. Biophys. **191**, 146-152.

Creach A., Lessire R. and Cassagne C. (1992). –"Biosynthesis of very long chain fatty acids by subcellular fractions of developing rapeseeds in Metabolism, structure and utilization of plant lipids".- p 144 - A. Cherif, D.B. Miled-Daoud, P. Mazliak, A. Maoui, M. Zarrouk, (Eds), CNP, Tunis.

Downey R.K. and Craig B.M. (1991). –"Genetic control of fatty acid biosynthesis in rapeseed (*Brassica napus*)".– J. Am. Oil. Chemists'Soc. **41**, 475-478.

Fehling D. and Mukherjee K.D. (1991). –"Acyl-CoA elongase from higher plant (*Lunaria annua*). Metabolic intermediates of very long chain acyl-CoA products and substrates specificity.– Biochim. Biophys. Acta **1082**, 239-247.

Fehling E., Lessire R., Cassagne C. and Mukherjee K.D. (1992).– "Solubilization and partial purification of constituents of acyl-CoA elongase from *Lunaria annua".*–Biochim. Biophys. Acta **1126**, 88-94.

Lessire R., Bessoule J.J. and Cassagne C. (1985). –"Solubilization of C_{18}-CoA and C_{20}-CoA elongases from *Allium porrum* L. epidermal cell microsomes".– FEBS Lett. **187**, 314-320.

Murphy D. and Mukherjee K.D. (1988). –"Biosynthesis of very long chain monounsaturated fatty acids by subcellular fractions of developing seeds".– FEBS Lett. **230**, 101-104.

Taylor D.C., Weber N., Barton D.L., Underhill E.W., Hogge L.R., Weselake R.J. and Pomeroy M.K. (1991). –"Triacylglycerol bioassembly in microspore-derived embryos of *Brassica napus* L. cv Reston".– Plant Physiol. **97**, 65-79.

Princen L.H. and Rothfus J.A. (1984). –"Development of new crops for industrial raw materials".– J. Am. Oil. Chemists'Soc. **61**, 281-289.

Cloning and expression of a cyanobacterial Δ6-desaturase

Avutu S. Reddy [1], **Michael L. Nuccio** [1], **Lisa M. Gross** [1], **Martine Freyssinet** [2], **Terry L. Thomas*** [1]
1. Department of Biology, Texas A&M University, College Station, TX 77843, USA.
2. Rhône-Poulenc Agrochimie, Lyon, France.

SUMMARY

Cloning and expression of a cyanobacterial Δ6-desaturase.

A cyanobacterial gene encoding Δ6-desaturase was cloned by expression of a *Synechocystis* genomic cosmid library in *Anabaena*, a cyanobacterium that lacks Δ6-desaturase but does have linoleic acid (18:2), the substrate for this enzyme. Expression of the *Synechocystis* Δ6-desaturase gene in *Anabaena* resulted in the accumulation of γ-linolenic acid (GLA) and octadecatetraenoic acid (18:4). The predicted 359 amino acid sequence of the *Synechocystis* Δ6-desaturase shares limited sequence similarity with other reported cyanobacterial and plant desaturases. Analysis of three overlapping cosmids revealed a Δ12-desaturase gene linked to the Δ6-desaturase gene. Expression of *Synechocystis* Δ6- and Δ12-desaturases in *Synechococcus,* a cyanobacterium deficient in both desaturases, resulted in the production of linoleic acid and γ-linolenic acid.

KEY-WORDS: C18 fatty acid desaturation - γ-linolenic acid - Synechococcus sp. strain PCC 7942 - Octadecatetraenoic acid.

1. INTRODUCTION

Long-chain fatty acids (≥C18) are significant components or precursors of the constituents of plant seed oils. Linoleic (18:2$\Delta^{9,12}$) and α-linolenic (18:3$\Delta^{9,12,15}$) acids are usually obtained from oil seed crops. However, most seed oils are deficient in γ-linolenic acid (GLA; 18:3$\Delta^{6,9,12}$), a primary precursor of a large family of C20 compounds. GLA is the result of desaturation of linoleic acid catalyzed by the enzyme Δ6-desaturase. We investigated the feasibility of obtaining a Δ6-desaturase gene from a heterologus source which could, in turn, be used to transform plants to obtain seed oils containing GLA. The unicellular cyanobacterium *Synechocystis* PCC 6803 was chosen as a source for the Δ6-desaturase because: (i)*Synechocystis accumulates GLA to a level greater than 20% of the total fatty acid mass* (Wada et al., 1990); (ii)*Cyanobacteria have aerobic desaturases similiar to plants* (Wada et al., 1990); and (iii)*production of polyunsaturated fatty acids in higher plants and cyanobacteria occurs after fatty acids are esterified to glycerolipids* (Jaworski, 1987).

Most of the fatty acid desaturases (except plant Δ9-stearoyl acyl carrier protein desaturase) are intergral membrane proteins, a property that makes them difficult to purify and subsequently clone (Arondel et al., 1992). Therefore, we developed a molecular genetic strategy to isolate a Δ6-desaturase gene from *Synechocystis.* A *Synechocystis* cosmid library was constructed and conjugated into wild-type *Anabaena* PCC 7120, a cyanobacterium deficient in Δ6-desaturase, to identify gain-of-function *Anabaena* transconjugants that produce GLA and therefore contain a functional *Synechocystis* Δ6-desaturase gene. With this approach, we cloned a Δ6-desaturase gene from *Synechocystis* and verified its expression in another cyanobacterium, *Synechococcus* PCC 7942.

2. MATERIALS AND METHODS

Cyanobacterial strains, *Synechocystis* PCC 6803, *Anabaena* PCC 7120 and *Synechococcus* PCC 7942 were grown photoautotrophically at 30°C in BG-11 medium (Rippka et al., 1979) under illumination of incandescent lamps (60 μEinsteins.m^{-2}.s^{-1}). Standard molecular biology techniques were performed as described (Ausubel et al., 1989). The total genomic DNA from *Synechocystis* PCC 6803 was partially digested with *Sau3 AI,* ligated into the cosmid vector, pDUCA7 (Buikema and Haselkorn, 1991) and packaged *in vitro.* The packaged phage were propagated in *E.coli* DH5α(mcr$^-$) containing the helper plasmid, pRL528 encoding *Ava I* and *E. coli* 47II methylases (Elhai and Wolk, 1988).Conjugation of *Synechocystis* cosmid library into *Anabaena* was performed as described (Elhai and Wolk, 1988). Fatty acid methyl esters were prepared from transconjugants (Dahmer et al., 1989) and were analyzed by Gas Liquid Chromatography.

3. RESULTS AND DISCUSSION

Conjugation of a *Synechocystis* cosmid library into the filamentous cyanobacterium *Anabaena,* which lacks GLA but does contain linoleic acid, the precursor to GLA, resulted in the gain-of-function expression of GLA and octadecatetraenoic acid (18:4). Two independent transconjugants, AS13 and AS75, were identified that expressed significant levels of GLA and which contain cosmids, cSy13 and cSy75, respectively. These cosmids overlap in a region approximately 7.5 kb in length. A 3.5 kb Nhe I fragment of cSy75 (Fig. 1A) was recloned into the vector pDUCA7 to create cSy75-3.5 and transferred to *Anabaena,* resulting in the production of GLA.

Two Nhe I/Hind III subfragments (1.8 kb and 1.7 kb) of the 3.5 kb Nhe I fragment of pSy75-3.5 were subcloned into pBluescript (Fig. 1A) and then mobilized into a conjugal expression vector, pAM542, in both forward and reverse orientations with respect to a cyanobacterial *rbcLS* promoter (Fig.1B) and were introduced into *Anabaena* by conjugation. Transconjugants containing the 1.8 kb fragment in the forward orientation (pAM542-1.8F) produced significant quantities of GLA and octadecatetraenoic acid. The nucleotide sequence of the 1.8 kb fragment was determined and an open reading frame encoding a polypeptide of 359 amino acids was identified (data not shown).

We isolated a cosmid, cSy7 containing a Δ12-desaturase gene by screening the*Synechocystis* library with an oligonucleotide synthesized from the published Δ12-desaturase gene sequence (Wada et al., 1990). We subcloned a 1.7 kb

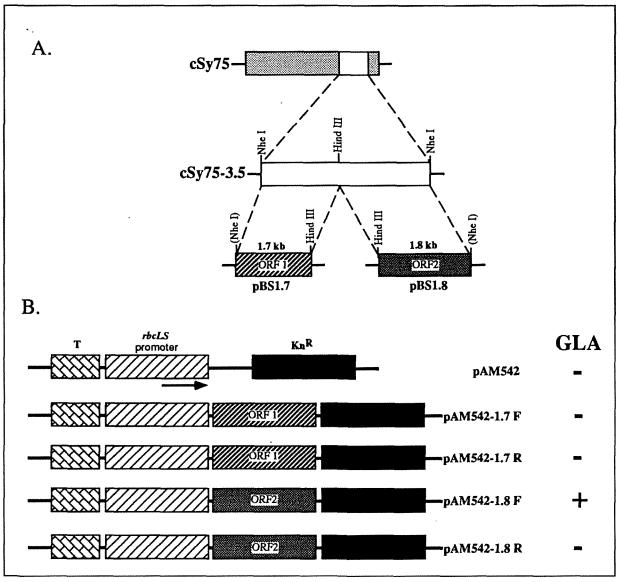

Fig. 1

Identification of Δ^6-desaturase gene. **A**. Map of cosmid cSy75. The origin of subclones, cSy75-3.5, pBS1.7 (ORF 1) and pBS1.8 (ORF 2) are indicated by the dotted diagonal lines. Two open reading frames are indentified within the 3.5 kb fragment of cSy75-3.5. Restriction sites that have been inactivated are in parenthesis. **B**. Determination of correct open reading frame encoding Δ^{6}-desaturase. The two subclones (pBS1.7 and pBS1.8) carrying open reading frames (ORF 1 and ORF 2 respectively) were introduced into pAM542, a conjugal expression vector (9.4 kb) containing a cyanobacterial *rbcLS* promoter, in both forward and reverse orientations. ORF, open reading frame; F, forward orientation; R, reverse orientation; GLA, γ–linolenic acid; '–' and '+' indicate presence and absence of GLA, respectively, in transgenic *Anabaena.*

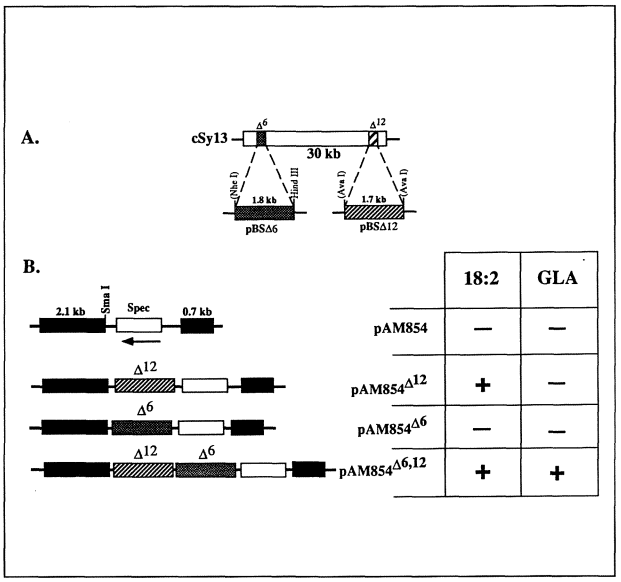

Fig. 2

Modification of fatty acid composition of *Synechococcus* PCC 7942. **A**. Map of cSy13 showing the origin of two subclones carrying the Δ^{6}- and Δ^{12}-desaturase genes. Restriction sites that have been inactivated are in parenthesis. **B**. Expression cassetes containing the Δ^{6}- and Δ^{12}-desaturase genes used to transform *Synechococcus.* Two black boxes represent recombination sites in the chromosome of *Synechococcus.* 18:2, linoleic acid; GLA, γ-linolenic acid '–' and '+' indicate presence and absence of fatty acid respectively, in transgenic *Synechococcus.*

Ava I fragment from this cosmid containing the Δ^{12}-desaturase gene into pBluescript to create pBSΔ12. Using this as a probe, we found that the cSy13 not only contains a Δ^{6}-desaturase gene but also a Δ^{12}-desaturase gene (Fig.2A).

We cloned Δ^{12}- and Δ^{6}-desaturase genes individually and together into pAM854 (Bustos and Golden, 1991), a shuttle vector that contains sequences necessary for the integration of foreign DNA into the genome of *Synechococcus* (Fig.2B). Transformants with pAM854-Δ6 &Δ12 produced both linoleate and GLA, whereas transformants with pAM854-Δ12 expressed linoleic acid. This confirms that the 1.8 kb insert of the clone, pBΔ6 contains both coding and promoter regions of the *Synechocystis* Δ^{6}-desaturase gene. Furthermore, two functional genes (Δ^{6}-and Δ^{12}-desaturases) involved in the C18 fatty acid desaturation pathway are linked in the *Synechocystis* genome.

In conclusion, the success of the gain-of-function approach described here makes possible the identification of other cyanobacterial fatty acid desaturases for which there is no selectable phenotype. The triad of *Synechocystis, Anabaena* and *Synechococcus* provide an opportunity to isolate most genes involved in fatty acid metabolism in cyanobacteria.

Production of transgenic plants with the *Synechocystis* Δ^{6}-desaturase is in progress.

ACKNOWLEDGEMENTS

The work was supported by grants from Rhône-Poulenc Agrochimie and BioAvenir.

REFERENCES

1. Arondel,V., Lemieux, B., Hwang, I., Gibson, S., Goodman, H.M. and Somerville, C.R. (1992).–"Map-based cloning of a gene controlling omega-3 fatty acid desaturation in *Arabidopsis*".– Science **258**, 1353-1355.
2. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (1989) Current Protocols in Molecular Biology. Greene Publishers/Wiley-Interscience, New York.
3. Buikema, J.W., Haselkorn, R. (1991). –"Isolation and complementation of nitrogen fixation mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120".– J. Bact. **173**, 1879-1885.
4. Bustos, S.A., Golden, S.S. (1991). –"Expression of the *pSbDII* Gene in *Synechococcus* sp. strain PCC 7942 requires sequences downstream of the transcription start site".– J. Bact. **174**, 7525-7533.
5. Dahmer, M.L., Fleming, P.D., Collins, G.D., Hildebrand, D.F. (1989). –"A rapid screening technique for determining the lipid composition of soybean seed".– J. Am. Oil Chemists'Soc. **66**, 543-548.
6. Elhai, J., Wolk, C.P. (1988). –"Conjugal transfer of DNA to cyanobacteria".– Meth. Enzymol. **167**, 747-754.
7. Jaworkski, J.G. (1987).– Biosynthesis of monoenoic and polyenoic fatty acids. In: The Biochemistry of Plants Vol 9, pp 159-174, Stumpf, P.K., (ed.) Academic Press, Orlando, FL.
8. Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., Stanier, R.Y. (1979). –"Generic assignments, strain histories and properties of pure cultures of cyanobacteria".– J. Gen. Microbiol. **111**, 1-61.
9. Wada, H., Gombos, Z., Murata, N. (1990). –"Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation".-Nature **347**, 200-203.

Wild and cultivated *Borago officinalis* L.: Sources of gamma-linolenic acid

M. del Río (a), **J. Fernández-Martínez** (b), **A. de Haro*** (b)
(a) C.I.D.A., Apdo. 4240, 14080 Córdoba (Spain).
(b) Instituto de Agricultura Sostenible (CSIC), Apdo. 4084, 14080 Córdoba (Spain).

SUMMARY

Wild and cultivated *Borago officinalis* L.: Sources of gamma-linolenic acid.

One hundred and eighty five borage (*Borago officinalis* L.) accessions of cultivated and wild germplasm collections, from different Spanish origins, were evaluated for gamma-linolenic (GLA) and other fatty acids and seed characters. White flowered cultivated genotypes had higher contents of GLA than blue flowered wild material. No correlation was found between oil content and GLA. Genotypes with high GLA content, low levels of erucic acid and high oil content were selected.

KEY-WORDS: Gamma-linolenic acid - Fatty acid composition - Borago officinalis - Germplasm collections.

1. INTRODUCTION

Gamma linolenic acid (GLA) (C:18, n-6, 9, 12, all *cis*) is a essential fatty acid in increasing demand for its clinical and pharmaceutical applications.

The most common commercial source of GLA for pharmaceutical uses has been the oil of evening primrose seeds (*Oenothera biennis*).

Borage seed is also an alternative source which shows some interesting characteristics: higher oil content in the seed (24-34% as compared to 14-25% in *Oenothera*), as well as higher GLA proportion in the seed oil 23% in borage and 10.5% in *Oenothera.*

White flowered borage is currently used as a vegetable in the North of Spain. However, no attention has been paid till now in Spain to study borage as a source of GLA. Moreover, the only germplasm evaluated for other traits has been the white flowered materials used as a vegetable, neglecting the wild blue flowered material which could offer potential advantages of adaptability to semi-arid conditions.

Therefore, the objective of this work was to evaluate the existing variability for oil, GLA content and other seed characteristics of different sources of borage germplasm: white flowered borage cultivated in Spain and blue flowered material collected from roadside populations.

2. EXPERIMENTAL

2.1. Material

The material evaluated in this study consisted of borage seed from 185 accessions originating from two different spanish sources.

The first was formed by 130 cultivars and populations of white flowered material cultivated as a vegetable, and provided by the Center of Agricultural Research of Rioja for this study. This material was evaluated and selected for morphological and agronomical traits at the Center of Agricultural Research of Rioja (Domínguez *et al.* 1991). Based on this evaluation entries were classified in six groups (Table I).

Table I
Characteristics of white flowered entries used in this work

Group	Plant habit	Maturity	Seed production	Nº of entries
I	erect	late	low	23
II	erect	very late	low	26
III	postrate	average	average	28
IV	semierect	early	average	22
V	erect	very late	high	17
VI	semierect	late	high	14

The second group of Spain consisted of 55 wild entries of spontaneus blue-flowered roadside populations collected in different localities of Andalusia (Southern Spain).

2.2. Methods

Seed weight was determined by weighing 100 seeds. Oil content was determined by nuclear magnetic resonance (NMR) using a Newport Analyser MK III A wide line (NMR) instrument. Fatty acid composition of solvent-extracted oil from a bulk of 10 seeds/entry was determined by gas liquid chromatography using esterification as described by Fernández Martínez and Knowles (1982). The gas chromatographic analyses were performed using a Shimadzu gas chromatograph model GC-9A fitted with a flame ionisation detector (FID). A semi capillary column (25 m x 0.53 mm ID) packed with acidified polietilenglicol silica was used for the separation of methylated esters.

3. RESULTS AND DISCUSSION

Table II gives the oil and fatty acid content and seed weight of the different populations of borage evaluated in this study. Considering all the entries, there was an important range of variation for most of the characters studied.

Table II
Mean and range of 1000 seed weight, oil content and fatty acid composition (%) of white and blue flowered borage populations.

Borage populations	n	Seed weight (g)	Oil content (%)	Fatty acid content (%)	
				γ-Linolenic	Erucic
WF I	23	15.6	34.3	22.2	1.7
		12.9-20.1	32.1-36.3	14.1-26.6	1.3-2.3
WF II	26	15.2	34.1	22.1	1.6
		12.7-19.2	31.7-35.8	8.7-27.1	1.0-2.0
WF III	28	15.1	34.5	22.3	1.7
		10.8-20.0	32.4-37.8	19.6-25.7	1.4-2.1
WF IV	22	14.7	34.2	23.6	1.6
		12.4-17.8	32.1-37.2	15.9-27.1	1.2-1.9
WF V	17	15.1	34.4	25.1	2.5
		13.8-17.7	31.2-38.0	21.9-28.6	1.3-3.3
WF VI	14	15.3	34.4	23.9	1.8
		12.8-19.0	32.4-37.2	21.3-26.8	1.2-2.7
BF	55	14.0	32.5	18.9	1.8
		9.3-18.1	26.7-35.4	12.6-26.5	0.6-2.9

n= number of entries, WF = white flowered, BF = blue flowered.

Seed weight showed an overall range of variation from 9.3 to 20.1g. Wild, blue flowered (BF), material showed lower values for this characters than the cultivated white flowered (WF) population. Oil content varied from 26.7% to 38 %.

The group V of WF material showed the higher content of GLA with a mean value of 25.17% and a maximum value reaching 28.6%. An additional advantage of this group is its high seed production and higher level of oil content, with maximun levels of 38%. These ranges are wider than those published in other studies, for example Muuse et al. (1980) and Galwey and Shirlin, (1990).

Oil content did not show any significant correlation with the content of any fatty acid although it was positively correlated, 0.70, with seed weight.

This absence of significant correlation between oil content and fatty acids is favorable because it indicates that it is possible to select material with high oil, high GLA and low erucic acid content.

In conclusion, the results presented in this work show a great deal of variability for the most relevant quality traits in borage; oil and GLA content. WF population, especially group V, showed consistently higher values of GLA than BF populations.

Entries with about 27% of GLA, oil content higher than 34% and erucic acid values lower than 2.5% have been selected.

These selections are being multiplied for further use in agronomic studies and breeding programs.

BIBLIOGRAPHY

Domínguez, V., Arce, P. and Santamaria, J. (1991).– "Diferenciación de diez fenotipos de borraja (*Borago officinalis* L. flor blanca) en una población de dicha especie cultivada".– Actas de Horticultura VIII Jornadas de Selección y Mejora de Plantas Hortícolas.– Pontevedra. Spain, pp 313-317.

Fernández Martínez, J. and Knowles, P.F. (1982).– "Maternal and embryo effects on the oleic and linoleic acid contents of sunflower oil".– Proc 10th Int Sunf Conf Surfers Paradise. Australia, 241-243.

Galwey, N.W. and Shirlin, A.J. (1990).– "Selection of borage (*Borago officinalis*) as a seed crop for pharmaceutical uses".– Heredity **65**, 249-257.

Muuse, B.G., Essers, M.L. and Van Soest, L.J.M. (1988).– "*Oenothera* species and *Borago officinalis*: sources of gamma-linolenic acid".– Netherlands J. Agric. Sci. **36**, 357-363.

Medium-chain acyl-ACP thioesterase is not the exclusive enzyme responsible for early chain-lenght termination in medium-chain fatty acid synthesis

Ricardo Schuch, Monika Brummel, Friedrich Spener
Institut für Biochemie, Universität Münster and Institut für Chemo- und Biosensorik, Wilhelm-Klemm-Str. 2, D-48149 Münster, Germany.

SUMMARY

Medium-chain acyl-ACP thioesterase is not the exclusive enzyme responsible for early chain-lenght termination in medium-chain fatty acid synthesis.

With the aim to elucidate the regulating mechanisms involved in the biosynthesis of medium-chain fatty acids we investigated the substrate specificity of the ß-ketoacyl-ACP synthases (KAS) in extracts obtained from developing seeds of *Cuphea lanceolata*, a crop producing up to 90% decanoic acid in seed triacylglycerols. Reactions of ß-ketoacyl-ACP synthases were carried out in absence and presence of cerulenin (100 µM) and started by addition of a primer, either acetyl-CoA or acyl-ACPs of chain-lengths varying from C_2 to C_{16}. The elongation was monitored by the criterion of incorporation of radioactively labelled malonate from [2-^{14}C]malonyl-CoA into acyl-ACPs. The reaction products were separated by 2.5 M urea-PAGE, electroblotted onto a PVDF-membrane and visualised by autoradiography. The elongation of each primer was quantitatively evaluated by densitometrically scanning of the autoradiograms. The results show that KAS III of *C. lanceolata* has a high preference for acetyl-CoA, but can, though in small amounts, catalyse elongation reactions of acyl-ACPs up to C_6. Experiments in absence of cerulenin show that in *C. lanceolata* seed extracts ß-ketoacyl-ACP synthases as a whole hardly elongate C_{10}-ACP, a special feature that can be attributed to a low specificity of KAS I for this substrate.

KEY-WORDS: Condensing enzymes - Cuphea - Cuphea lanceolata - ß-ketoacyl-ACP synthases.

1. INTRODUCTION

Recently a specific acyl-ACP thioesterase was described in the seeds of California bay (Pollard et al., 1991) that correlates with the high contents of lauric acid in the triglycerides of these seeds. Such a medium-chain acyl-ACP specific thioesterase is also present in *Cuphea* seeds, containing high levels of medium-chain fatty acids (Dörmann et al., 1993). Nevertheless, other mechanisms have been suggested for the early termination of chain elongation in the

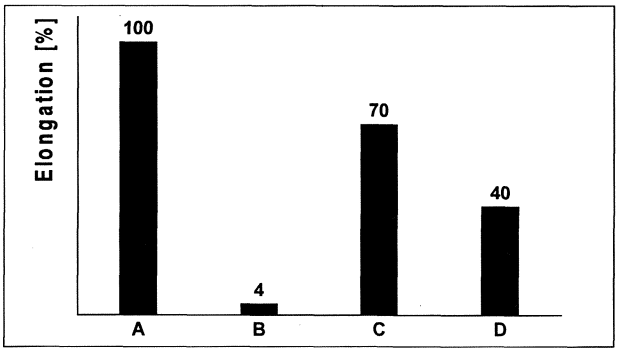

Fig. 1
Effect of octanoyl (8:0)-ACP and free ACP on the inhibition of condensing enzymes during pre-incubation of *Cuphea lanceolata* seed extract with cerulenin. A, elongation of 8:0-ACP in absence of cerulenin (= 100 %); B, elongation of 8:0-ACP using a seed extract pre-incubated with 100 μM cerulenin; C, elongation of 8:0-ACP using a seed extract simultaneously pre-incubated with 100 μM cerulenin and 8:0-ACP; D, elongation of 8:0-ACP using a seed extract simultaneously pre-incubated with 100 μM cerulenin, 8:0-ACP and free ACP. In all cases the reaction was started with the addition of [2-^{14}C]malonyl-CoA, from which [2-^{14}C]malonyl-ACP is produced *in situ*.

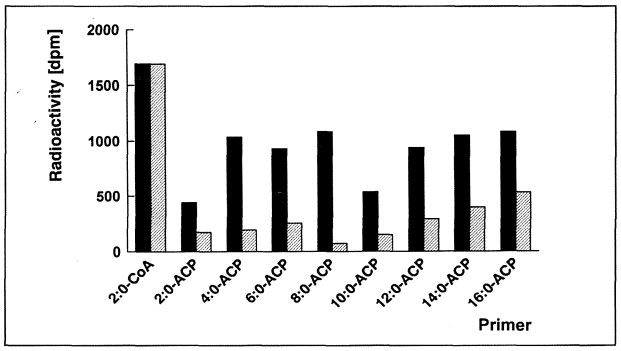

Fig. 2
Specificity of overall condensing enzymes for acyl-ACPs in *Cuphea lanceolata* seed extract. The products of reactions in absence (■) and presence (▒) of 100 μM cerulenin were separated by 2.5 M urea-PAGE, electroblotted onto PVDF-membrane and visualised by autoradiography. Autoradiograms were scanned densitometrically and the radioactivity of elongation products was estimated from the relative area of corresponding spots. Acyl groups are represented by number of carbon atoms:number of double bonds.

synthesis of fatty acids in plants. One of these mechanisms is related to the action of ß-ketoacyl-ACP synthases (KAS), the condensing enzymes. Three condensing enzymes - KAS I, KAS II and KAS III - showing different substrate specificities and sensitivities towards the antibiotic cerulenin have been already described (Shimakata and Stumpf, 1982; Clough et al., 1993). With the aim to elucidate the role of the condensing enzymes in the regulation of medium-chain fatty acid synthesis, we investigated their presence and substrate specificity in seed extracts of *Cuphea lanceolata,* a crop producing up to 90% decanoic acid in seed triacylglycerols.

2. EXPERIMENTAL PART

One elongation step of the fatty acid synthase (FAS) reaction was followed by a two-step assay of condensing enzymes. Reactions were carried out with a *C. lanceolata* seed extract containing all FAS enzymes, in absence and presence of 100 μM cerulenin, and started by addition of a primer, either acetyl-CoA or acyl-ACPs of chains-lengths from C_2 to C_{16}. Primer acyl-ACPs were synthesised from *E. coli* ACP. The elongation was monitored by the criterion of incorporation of radioactively labelled malonate from [2–^{14}C] malonyl-CoA, from which [2-^{14}C]malonyl-ACP was synthesised *in situ,* into acyl-ACPs. In the first step of the assay the reduction factors NADPH and NADH were omitted. After incubation of the assay mixture for 5 min at 30 °C the reaction was stopped by addition of 10 % trichloroacetic acid (TCA) to precipitate the ß-ketoacyl-ACP, the direct product of the condensing reaction. In the second step TCA-precipitated ß-ketoacyl-ACP was resuspended and thus reduced to acyl-ACP in the presence of NADPH and NADH catalysed by a seed extract pre-treated with N-ethyl maleimide (NEM). This NEM-treated extract contained all FAS reducing enzymes but no condensing activity. After precipitation with 10 % TCA the elongated radioactively labelled acyl-ACP was separated by 2.5 M urea-PAGE, electroblotted onto a PVDF-membrane and visualised by autoradiography. Autoradiograms were finally scanned densitometrically for quantitative evaluation of the elongation steps.

3. RESULTS AND DISCUSSION

The substrate specificity of condensing enzymes of *C. lanceolata* was investigated directly in seeds extracts, without prior purification of the enzymes. In order to discriminate the elongation activity of the individual condensing enzymes, the reactions were carried out in absence and presence of 100 μM cerulenin. In these concentrations cerulenin completely inhibited KAS I and only partially KAS II, whereas KAS III was insensitive. To assure an efficient inhibition a pre-incubation of the seed extract with the antibiotic was included. As shown in Fig. 1, the presence of acyl-ACPs during pre-incubation must be avoided as they preferentially bind to the active site of KAS I, thus having a protective effect against cerulenin action. This protective effect can be reduced by adding free ACP. The ACP attachment to KAS I apparently modifies the active site of the enzyme in a way to promote cerulenin binding and, consequently, reduce the preference for acyl-ACP binding. Thus in all experiments *C. lanceolata* seed extracts were pre-incubated solely with cerulenin prior to the addition of the other substrates.

For the analysis of the reaction products acyl-ACPs were separated by 2.5 M urea-PAGE, electroblotted onto PVDF-membrane, visualised by autoradiography, and the autoradiograms were scanned densitometrically. As shown in Fig. 2, acetyl-CoA (2:0-CoA) was strongly elongated to 4:0-ACP in absence as well as in presence of cerulenin, whereas acetyl-ACP (2:0-ACP) was a less efficient substrate for this reaction. The antibiotic could not inhibit the incorporation of radioactively labelled malonate into product butyryl-ACP (4:0-ACP) when acetyl-CoA was the starter substrate. This demonstrates that *C. lanceolata* seeds con-

tain a cerulenin insensitive condensing enzyme, KAS III, which uses directly acetyl-CoA as substrate for the production of butyryl-ACP. As observed from the reactions in presence of cerulenin, KAS III elongates 4:0- and 6:0-ACPs as well, but at smaller extents. Interestingly, under action of the antibiotic 8:0-ACP was practically not elongated by the condensing enzymes in *C. lanceolata* seed extracts (see also Fig. 1). Moreover, under inhibition acyl-ACPs from C_{10} to C_{16} were elongated in increasing extents. These elongations must be attributed to KAS II action, since KAS I is completely inactive and KAS III cannot elongate acyl-ACPs higher than C_6. As KAS I is completely inhibited by 100 μM cerulenin, we conclude that 8:0-ACP is the exclusive substrate for this enzyme. In absence of cerulenin acyl-ACPs from C_4 to C_{16} were elongated at almost similar extents, with the significant exception of 10:0-ACP, apparently a poor substrate for the condensing enzymes of *C. lanceolata.*

Considering these results we postulate that the high contents of decanoic acid found in *C. lanceolata* seeds are attained because the KAS I in these seeds has a low affinity for 10:0-ACP and thus supplies sufficient substrate for the medium-chain acyl-ACP thioesterase described earlier (Dörmann et al., 1993).

REFERENCES

Dörmann, P., Spener, F. and Ohlrogge, J.B. (1993).—«Characterization of two acyl-acyl carrier protein thioesterases from developing Cuphea seeds specific for medium-chain- and oleoyl-acyl carrier protein»- Planta **189,** 425-432.

Clough, R.C., Matthis, A.L., Barnum, S.R. and Jaworski, J.G. (1993).—«Purification and characterization of 3-ketoacyl-acyl carrier protein synthase III from spinach».—J. Biol. Chem. **267,** 20992-20998.

Pollard, M.R., Anderson, L., Fan, C., Hawkins, D.J. and Davies, H.M. (1991).— «A specific acyl-ACP thioesterase implicated in medium-chain fatty acid production in immature cotyledons of Umbellularia californica»—Arch. Biochem. Biophys. **248,** 306-312.

Shimakata, T. and Stumpf, P.K. (1982).—«Isolation and function of spinach leaf β-ketoacyl-[acyl-carrier-protein] synthases»- Proc. Natl. Acad. Sci. USA **79,** 5808-5812.

Oil seed phospholipases are involved in the metabolism of unusual fatty acids

Antoni Banas [1], Ulf Ståhl[2], Elisabeth Tillberg [2], Sten Stymne[2]*

1. Department of Plant Physiology, Agricultural and Teachers University, ul. Prusa 12, 08-110 Siedlce, Poland.
2. Department of Plant Physiology, Swedish University of Agricultural Sciences, P.O. Box 7047, S-750 07 Uppsala, Sweden.

SUMMARY

Oil seed phospholipases are involved in the metabolism of unusual fatty acids.

Microsomal membranes from developing oil seeds catalysed the acyl hydrolysis of phospholipids containing different types of radioactive acyl groups. The specificity for a particular acyl groups differed considerably between phospholipases fron the different plant species. Phospholipid acyl-hydrolase activities that selectively removed medium chain, hydroxylated and epoxidated acyl groups from phosphatidylcholine were demonstrated in incubations with microsomal membranes prepared from tissues accumulating such unusual fatty acids. The results strongly suggest that phospholipases are involved in removing unusual fatty acids from membrane lipids in developing oil seeds.

KEY-WORDS: Phospholipase - Triacylglycerol - Oil seed - Biosynthesis - Microsomes.

1. INTRODUCTION

A number of plant species have seeds triacylglycerols containing unusual fatty acids. These acids are often major acyl constituents of the triacylglycerols but are absent, or occur at very low levels, in the phospholipids of the seeds. Since diacylglycerol is a common precursor for both membrane lipids and triacylglycerols in the seeds, the question arises how the exclusion of the unusual fatty acids from the phospholipids is accomplished.

The oxidated fatty acids ricinoleic (12-hydroxy-octadeca-9-enoic acid) and vernolic acid (12-epoxy-octadeca-9-enoic acid) are the dominating acyl groups in the triacylglycerols of *Ricinus communis* (castor bean) and *Euphorbia lagascae* seeds, respectively, but represents only a few percentage of the acyl groups in the phospholipids of the seeds. Recent studies have shown that ricinoleic and vernolic acids are synthesised by hydroxylation and epoxidation of precursor acyl groups esterified in phospholipids, notably phosphatidylcholine, in microsomal preparations from developing seeds of *R. communis* and *E. lagascae* (Bafor *et al.* 1991, 1993). Although the phospholipids were substrates for the synthesis of the oxidated acyl groups, the newly synthesized hydroxy and epoxy acyl chains were removed from the phospholipids by microsomal phospholipid acyl hydrolase(s) and recovered mostly as free fatty acids. It was further demonstrated that *sn*-2-oleoyl-phosphatidylcholine, which is the precursor for ricinoleate synthesis, was not hydrolyzed in microsomal preparations from developing *R. communis* seeds (Bafor *et al.*,1991). Microsomal preparations from plant tissues which did not accumulate oxidated fatty acids were also capable to quite specifically hydrolyze *sn*-2-ricinoleoyl-phosphatidylcholine as well as phosphatidylcholine esterfied with hydroperoxidated linoleate at *sn*-2 position (Banas *et al.,* 1992).

To more fully characterize the properties of microsomal phospholipases from different plant species we have presented a comprehensive range of phospholipid substrates to microsomal phospholipases prepared from developing embryos and endosperms of a number of different plant species. The tissues selected for membrane preparations accumulate triacylglycerols with different types of fatty acids.

2. MATERIALS AND METHODS

[1-^{14}C]Ricinoleic acid and [1-^{14}C]vernolic acid were synthesised biochemically from precursor [1-^{14}C]fatty acids by the microsomal Δ^{12}hydroxylase from *R. communis* endosperm and the microsomal Δ^{12}epoxidase from *E. lagascae* endosperm, respectively. [1-^{14}C]Acyl-phosphatidylcholine was synthesized biochemically [in case of radioactive ricinoleic and vernolic acyl groups (Banas *et al.*, 1992)], or chemically (Kanda and Wells, 1981), from radioactive fatty acids and palmitoyl-lysophosphatidylcholine. [^{14}C]Acyl-phosphatidic acid was synthesised by treatment of corresponding [^{14}C]acyl-phosphatidylcholine with phospholipase D (from *Streptomyces chromofuscus*). Microsomal preparations, phospholipase assays and analytical procedures were essentially as described before (Banas *et al.*, 1992).

3. RESULTS AND DISCUSSION

Microsomal phospholipases were assayed in microsomal fractions prepared from developing embryos or endosperms of five different plants: *Brassica napus* (oilrape), *Ulmus glabra* (elm), *Cuphea procumbens, Ricinus communis* (castor bean) and *Euphorbia lagascae.* The fatty acid composition of the triacylglycerols of the five tissue types used for miscrosomal preparations are shown in Table I. Whereas *B. napus* embryos contain only fatty acids that are common for both membrane lipids and triacylglycerols, the tissues from the other species plant species contain unusual 'triacylglycerol specific' fatty acids. *U. glabra* and *C. procumbens* embryos are extremely rich in capric acid (10:0) whereas *E. lagascae* and *R. communis* endosperms have high amounts of vernolic acid and ricinoleic acid, respectively.

Table I

Fatty acid composition of triacylglycerols isolated from developing embryos of *B. napus, U. glabra* and *Cuphea procumbens* and developing endosperms of *E. lagascae* and *R. communis*

	Fatty acid distribution (mol %)								
Plant Species	8:0	10:0	16:0	18:1	18:2	18:3	Verno-leate	Ricino-leate	Others
Brassica napus	0	0	4	63	18	13	0	0	2
Ulmus glabra	11	68	4	5	4	0	0	0	8
Cuphea procumbens	1	89	2	3	1	0	0	0	4
Euphorbia lagascae	0	0	4	22	10	0	57	0	7
Ricinus communis	0	0	2	5	6	1	0	85	1

The release of [^{14}C]fatty acids from different *sn*-2-[^{14}C]acyl-phosphatidylcholine substrates incubated with microsomal preparations from the various plant species is presented in Fig. 1. The phospholipid acyl hydrolases in

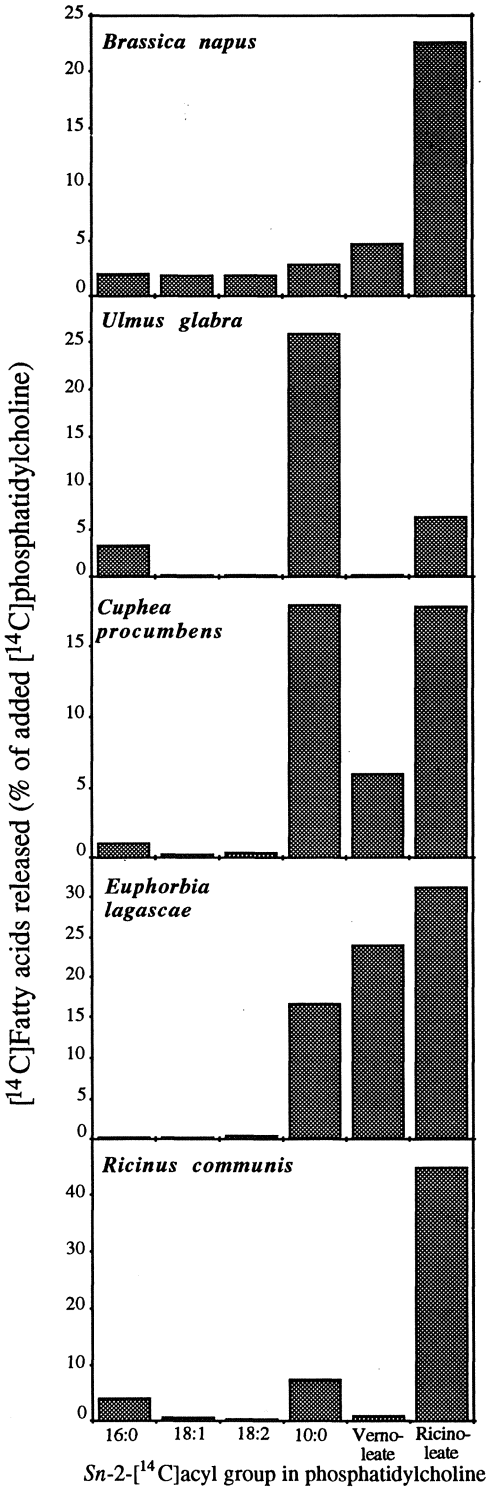

Fig. 1
The release of [^{14}C]fatty acids from different sn-2-[^{14}C]acyl-phosphatidylcholine substrates by phospholipases in microsomal preparations from developing embryos of *B. napus, U. glabra* and *C. procumbens* and developing endosperms of *E. lagascae* and *R. communis.* Each incubation contained microsomal membranes equivalent to 25 nmol of microsomal phosphatidylcholine and 4 nmol exogenous [^{14}C]acyl-phosphatidylcholine. Incubations were performed at 30°C for 120 min.

membranes from all plant species had negligable or low activities with phosphatidylcholine containing the 'common' (*i.e.* palmitoyl, oleoyl and linoleoyl) acyl groups. *Sn*-2[^{14}C]ricinoleoyl-phosphatidylcholine was a particularly good substrate for the *R. communis* and *E. lagascae* phospholipases, although enzymes from all plant species except *U. glabra* could efficiently hydrolyse this substrate. *Sn*-2[^{14}C]vernoleoyl-phosphatidylcholine was a very good substrate for the phospholipid hydrolase from *E. lagascae* but was very poorly utilized by the *R. communis* enzyme. *Sn*-2-[^{14}C]caproyl-phosphatidylcholine was a good substrate for *U. glabra* and *C. procumbens* enzymes, although *E. lagascae* enzyme(s) also hydrolyzed this compound at a high rate.

When different molecular species of *sn*-1-[^{14}C]acyl-phosphatidylcholine were tested as substrates for the phospholipases, similar activities were generally observed with these substrates as with corresponding *sn*-2-[^{14}C]acyl-phosphatidylcholine positional isomers (data not shown).

Sn-1-[^{14}C]acyl-lysophosphatidylcholine presented to the membrane fractions were metabolized to both ^{14}C-free fatty acid and [^{14}C]acyl-phosphatidylcholine, indicating the presence of both lysophospholipid hydrolase and transacylase activities (data not shown). The relative rate of hydrolysis of the different molecular species of lysophosphatidylcholine depended on the [^{14}C]acyl constituent in a similar manner as the rate of utilisation of [^{14}C]acyl-phosphatidylcholine substrates. However [^{14}C]oleoyl-lysophosphatidylcholine could, contrary to the [^{14}C]oleoyl-phosphatidylcholine, also serve as a substrate for the phospholipid hydrolases (data not shown).

Sn-2-[^{14}C]acyl-phosphatidic acid was a much less efficient substrates than *sn*-2[^{14}C]acyl-phosphatidylcholine with corresponding [^{14}C]acyl groups (data not shown).

The release of ^{14}C-fatty acids from *sn*-1- and *sn*-2-[^{14}C]acyl-phosphatidylcholine and from *sn*-1-[^{14}C]acyl-lysophosphatidylcholine was not affected by addition of EGTA or Ca^{2+} (data not shown), indicating no requirement for Ca^{2+} by the involved phospholipid hydrolases. This fact, together with the observed non-specificity for any particular *sn*-position of the acyl group, are in accordance with catalytic activities of phospholipases of B type. Phospholipase B associated with membrane fractions has previously been described from yeast (Ichimasa *et al.,* 1984). However, no reports have demonstrated such pronounced acyl specificity by phospholipases B, or any other type of phospholipases, as is here shown for the plant enzymes.

REFERENCES

Bafor, M., Smith, M.A., Jonsson, L., Stobart, K. & Stymne S. (1991).– "Ricinoleic acid biosynthesis and triacylglycerol assembly in microsomal preparations from developing castor-bean (*Ricinus communis*) endosperm".– Biochem. J. **280,** 507-514.

Bafor, M., Smith, M.A., Jonsson, L., Stobart, K. & Stymne S. (1993).– "Biosynthesis of vernoleate (cis-12-epoxyoctadeca-cis-9-enoate) in microsomal preparations from developing endosperm of *Euphorbia lagascae*".– Arch. Biochem. Biophys. **303,** 145-151.

Bafor, M. & Stymne S. (1992).– "Substrate specificities of glycerol acylating enzymes from developing embryos of two *Cuphea* species".– Phytochem. **31,** 2973-2976.

Banas, A., Johansson, I. & Stymne, S. (1992).– "Plant microsomal phospholipases exhibit preference for phosphatidylcholine with oxygenated acyl groups".– Plant Sci. **84,** 137-144.

Ichimasa, M., Morooka, T. & Niimura, T. (1984).– "Purification and properties of a membrane-bound phospholipase B from bakers yeast (*Saccharomyces cerevisiae*)".– J. Biochem. **95,** 137-145.

Kanda, P. & Wells, M.A. (1981).– "Facile acylation of glycerolphosphocholine catalyzed by trifluoroacteic anhydride".– J. Lipid Res. **22,** 877-879.

Use of olive cultures to evaluate triacylglycerol synthesis

Andrew J. Rutter [1], Juan Sánchez [2], John L. Harwood [1]*

1. Department of Biochemistry, University of Wales College of Cardiff, Cardiff, CF1 1ST, U.K.
2. Instituto de la Grasa, C.S.I.C., 41012 Seville, Spain.

SUMMARY

Use of olive cultures to evaluate triacylglycerol synthesis.

Olive cultures have been established which show good rates of triacylglycerol synthesis and accumulation. Microsomal fractions isolated from such cultures have proven to be useful model systems for examining the regulation of olive oil quality. Intact cultures or subcellular fractions prepared therefrom have been used to study the effect of a commonly used pesticide, namely dimethoate, on triacylglycerol synthesis and consequently any effects which its use may have on olive oil quality.

KEY-WORDS: Dimethoate - Lipid biosynthesis - Olive - Tissue culture - Triacylglycerol.

1. INTRODUCTION

Olive oil is a staple in the diet of Mediterranean people and is appreciated worldwide for its fragrant and delicate flavour. The major component of plant oils is triacylglycerol and it is the acyl composition of triacylglycerol that determines the quality of a vegetable oil. In olive oil, the major fatty acids are palmitic acid (10-15%) and oleic acid (70-75%). Olive oil is a valuable edible oil due to its very high proportion of oleic acid, which together with some minor components gives it its distinctive flavour characteristics and excellent nutritional qualities (Kiritsakis, 1990).

Triacylglycerol is formed mainly via the Kennedy pathway (Stymne, 1987) in which glycerol 3-phosphate is successively acylated at the *sn*-1 and *sn*-2 positions to yield phosphatidate. Phosphatidate is then hydrolysed to diacylglycerol and a third and final acylation yields triacylglycerol. The acylation reactions within the pathway are known to occur on the endoplasmic reticulum. In contrast to the extensive information on edible oil synthesis in seeds, little work has been done on oleaginous fruits such as olive, despite its high commercial value. The reason behind this experimental neglect could relate to the fact that fruits at the

correct stage of development, when the triacylglycerol biosynthetic enzymes are active, are not as readily available as ripening seeds. Moreover, there are often problems in preparing active subcellular fractions and enzyme extracts because of the high amounts of polyphenols and tannins in the mesocarp of these fruits.The advantages of tissue cultures are that they provide a year round supply of tissue for experimentation. Secondly, the absence of a cuticular layer, with its attendant phenolic and other toxic compounds, makes the preparation of active fractions easier. Thirdly, it is relatively easy no test individual parameters on tissue cultures with regard to possible effects on lipid synthesis. Active microsomal fractions have been prepared from olive cultures (Rutter 1992a,b), and these have been used to study the details of triacylglycerol synthesis and its regulation in olives.

The production of olives in Europe is threatened by an insect pest complex which causes losses of some 15%, eqivalent to about £450 million a year. European growers are annually spending £55million a year on pest control, half of which is related to pesticide use. The damage caused by these pests (and their control by chemicals) results in a reduction in yield and quality of oil and fruits. Olive tissue cultures and subcellular fractions prepared therefrom have been used to study the effect of dimethoate, a commonly used organophosphorous insecticide on lipid biosynthesis and consequently any effects it may have on olive oil quality.

2. EXPERIMENTAL

2.1. Tissue cultures

Olive cultures were established (Williams,1991) and maintained on Murashige and Skoog medium (Murashige 1962) supplemented with an auxin and cytokinin concentration of 12μM 2,4-dichlorophenoxyacetic acid and 0.56μM 6 benzylaminopurine riboside. The cultures were grown at 25°C with a 12 hour light/dark cycle. These conditions produced a good rate of growth with a doubling of weight of tissue approximately every 21 days.

2.2. Incubations using whole cultures

Intact cultures were incubated with dimethoate, or sterile water as a control, and [^{14}C]acetate (Amersham International) as described in the legends to the figures. Lipids were extracted by the method of Garbus (1963). Individual lipid classes were separated using thin-layer chromatography and chloroform:methanol:acetic acid:water (70:12.5:8:3 by volume) or petroleum ether:diethyl ether:acetic acid (80:20:2 by volume) as solvents. Identification was made by co-chromatography with authentic lipid standards. Fatty acid methyl esters were prepared using 2.5% H_2S0_4 in dry methanol and quantified by radio-gas liquid chromatography, using a 1.5x4mm column containing Supelco 10% SP-2330 on 100/120 Supelcoport coupled to a gas-flow proportional counter.

2.3. Microsomal incubations

Microsomal fractions were made according to the method of Sánchez *et al.* (1992) and incubated with [^{14}C]oleoyl-CoA in the presence of 300 μM glycerol 3-phospate, 75 μM palmitoyl-CoA and 100 μM oleoyl-CoA. Incubations were stopped with 0.15M acetic acid and lipids were extracted using a modified Bligh and Dyer extraction method (Griffiths *et al,* 1988). Individual lipid classes were separated using a one dimensional double development method of thin layer chromatography, and radioactivity was determined using scintillation counting.

3. RESULTS

Using olive cultures, the effects of dimethoate on lipid biosynthesis have been examined *'in vivo'* with intact callus cultures and *'in vitro'* with microsomal fractions. The effect of dimethoate in intact cultures is shown in Table I, where the pattern of fatty acid labelling with [^{14}C]acetate is altered. An important point to note is that the results are compiled from three separate experiments so it is necessary to consider each pesticide concentration in turn and examine the differences between control and treated cultures separately. At 50μM and 100 μM, dimethoate did not have any effect on the total incorporation of radiolabel into lipids, and at 50 μM the differences in fatty acid labelling are a decrease in the labelling of linoleate with an increase of radiolabel found in palmitate. At 100 μM dimethoate, there was a decrease in the labelling of oleate and linolenate with a concomitant increase in the labelling of palmitic acid. The difference in the labelling of linoleate was unexpected and further experiments will be undertaken in order to explain the effect. At 500 μM, the incorporation of acetate was dramatically reduced to about 10% of the control and there were large changes in the labelling pattern. In this case, there were large decreases in the labelling of stearic and oleic acid and significant radiolabelling of palmitoleic acid. Even though there were relative increases in the labelling of linoleate and linolenate, because the total labelling was reduced then consequently the overall labelling of these fatty acids was also lower. These results indicate that dimethoate may be influencing the step in fatty acid synthesis that yields stearate (and other C18 acids) from palmitate, which is regulated by the enzyme ß-ketoacyl-ACP synthetase II. Dimethoate may inhibit this enzyme causing a reduction in stearate and oleate and a build up in palmitate, and at 500 μM, it appears that palmitate accumulates enough to be desaturated to palmitoleate. In the experiment using microsomal fractions, dimethoate did not effect the incorporation of [^{14}C]oleoyl-CoA into lipids but it did alter the distribution of labelling among lipid classes. There was a decrease in the labelling of phospatidylcholine and triacylglycerol with an increase in the percentage of radiolabel found in non-esterified fatty acids (Table II).

Table I
The effect of dimethoate on the labelling of fatty acids from [^{14}C]acetate in olive callus cultures

	Fatty acid labelling (%)					
	16:0	16:1	18:0	18:1	18:2	18:3
50 μM dimethoate						
Control	35.6	0.0	11.6	30.3	13.1	9.3
Test	40.8 *	0.0	15.7	29.2	9.3	3.3 **
100 μM dimethoate						
Control	29.1	0.0	4.7	44.9	13.2	8.2
Test	34.4 *	0.0	4.1	35.8 **	20.2 **	5.5 *
500 μM dimethoate						
Control	46.0	0.0	15.7	29.9	5.7	2.6
Test	42.9	19.8 **	6.2 **	11.1 **	10.8 *	4.4 **

Olive cultures were incubated dimethoate for 72 hours. For 50 μM treatments, [^{14}C]acetate was added at the beginning of the experiment and for 100 μM and 500 μM treatments, [^{14}C]acetate for the final 24 hours of the incubation.
* Indicates a significant difference with $p<0.10$
** Indicates a significant difference with $p<0.05$

Table II
The effect of dimethoate on the incorporation of [^{14}C]oleoyl-CoA into glycerolipids by microsomal fractions from olive cultures

Dimethoate (μM)	Incorporation (nmol/mg protein/hr)	Distribution of label (%) PC	DAG	NEFA	TAG
0	74.0	17.67	19.77	54.66	2.36
10	87.6	11.02	17.19	63.73	1.35
50	85.2	4.12	17.57	75.46	0.58

Abbreviations: PC, phosphatidylcholine; DAG, diacylglycerol; NEFA, non-esterified fatty acids; TAG, triacylglycerol.

4. DISCUSSION

The results from these experiments with olive tissue cultures agree with similar experiments carried out with olive fruits (de la Vega *et al*, 1993). Thus, olive tissue cultures provide a good experimental system for studying lipid biosynthesis and its regulation in olives. These experiments also indicate that dimethoate can effect lipid biosynthesis in that it can inhibit the synthesis of oleic acid, the most abundant fatty acid in olive oil, and that it can reduce the deposition of triacylglycerol. These results show that the use of dimethoate could have serious implications not only on the yield, but also on the quality of olive oil.

REFERENCES

Griffiths, G., Stymne, S. and Stobart, K. (1988).– "The utilization of fatty-acid substrates in triacylglycerol biosynthesis by tissue-slices of developing safflower (*Carthamus tinctorius L.*) and sunflower (*Helianthus annus L.*) cotyledons".– Planta **173**, 309-316.

Kiritsakis., A.K. (Ed.) (1990).– Olive Oil.- American Oil Chemist's Society, Champaign, Illinois.

Murashige, T. and Skoog, F. (1962).— "Revised medium for rapid growth and bioassay with tobacco tissue cultures".– Physiol. Planta **15**, 473-497.

Rutter A.J., Sánchez, J. and Harwood, J.L. (1992a).– "Triacylglycerol synthesis by microsomal fractions from olive cultures".– Biochem. Soc. Trans. **20,** 181.

Rutter, A.J., Sánchez, J. and Harwood, J.L. (1992b).- "Biosynthesis of triacylglycerol by microsomal fractions from olive cultures".– in Metabolism, Suucture and Utilization of Plant Lipids, pp.51-54. A.Cherif, (Ed.) Centre National Pédagogique, Tunis.

Sánchez, J., Del Cuvillo, M.T. and Harwood, J.L. (1992).-"Glycerolipid biosynthesis by microsomal fractions from olive fruits".- Phytochemistry **31**, 129-134.

Stymne, S. and Stobart, A.K. (1987).—"Biosynthesis of Triacylglycerols".— in The Biochemistry of Plants, vol. 9 Stumpf, P.K. and Conn, E.E., Eds pp 175-214, Academic Press, New York.

de la Vega, M.G., Harwood, J.L. and Sánchez, J. (1992).– "Effect of dimethoate on lipid metabolism in olive fruits".— in Metabolism, Structure and Utilization of Plant Lipids, pp 368-371. A Cherif, (Ed.) Centre National Pédagogique, Tunis.

Influence of the "greenhouse effect" on the lipid composition of wheat (*Triticum aestivum* L.) seeds

Mark Williams [1], Peter R. Shewry [2], John L. Harwood [1*]
1. Department of Biochemistry, University of Wales College of Cardiff, Cardiff, CF1 1ST, U. K.
2. Long Ashton Research Station, Bristol, BS18 9AF, U. K.

SUMMARY

Influence of the "greenhouse effect" on the lipid composition of wheat (*Triticum aestivum* L.) seeds.

Few studies have been conducted in order to investigate comprehensively the "greenhouse effect" on wheat growth and even less on the alterations to the lipid content of mature wheat grains which are induced by such environmental changes. The objective of this study was to define any changes in wheat grain acyl lipids as a result of alterations in environmental growth conditions. In fact, qualitative and quantitative changes were observed. Most noticeable was the observation that elevated atmospheric carbon dioxide levels enhanced the accumulation of grain acyl lipids, whereas elevated temperature had the opposite effect. Marked alterations in the acyl moieties of the monoacyl phospholipids (starch-located) were also observed. Such changes will in turn affect the quality of the flour derived thereof.

KEY-WORDS: Wheat lipids - Greenhouse effect - Fatty acid composition - Temperature.

1. INTRODUCTION

Increases in the combustion of fossil fuels together with rapid deforestation have been responsible for the increasing atmospheric levels of carbon dioixide. Computer model systems are predicting that the present atmospheric carbon dioxide levels will double, rising to 700ppm by the middle of the twenty-first century, along with climatic

changes principal among which are increased atmospheric temperatures and concomittent changes in rainfall patterns (Bolin *et al.,* 1986). There is, therefore, a need to understand how vegetation will change and concern is mounting particularly as to the possible effects on plant growth and development. Wheat is an important crop commodity in temperate areas of the world and yet there have been few studies of the greenhouse effect on crop production in the field. Even less has been reported about the effect of such environmental changes on wheat acyl lipid composition (Williams *et al.,*1993). It is, however, well recognised that the effect on crop yield will indeed be complex (Warrick *et al.,*1986).

Wheat actually possesses a relatively low lipid content in its seeds (2.5 to 3.5% dry weight). However, lipid metabolism is important from three standpoints. First, acyl lipids, as components of membranes, influence the overall growth and development of the plant. Second, the acyl lipids of the seeds are important either as sources of essential fatty acids or because of their influence on the properties of the flour produced during milling (Karpati *et al.*, 1990). Thirdly, wheat germ oil is an economically viable product. Wheat seed lipids can be divided into non-starch (the principle component of which is the storage lipid triacylglycerol) and starch lipids. The latter are derived from the starchy endosperm of the grain and consist primarily of monoacyl phospholipids such as lysophosphatidylcholine and lysophosphatidylethanolamine which are known to affect profoundly the baking properties of flour.

2. MATERIALS AND METHODS

Triticum aestivum L. cv. Hereward was cultivated in field greenhouses at the Rothamstead Experimental Station and harvested in July, 1991. The various growth conditions included: normal and elevated carbon dioxide levels (350ppm and 700ppm, respectively); low nitrogen and high nitrogen fertilizer application; ambient temperature and 4°C above ambient temperature. Whole wheat grains were milled and then homogenised with acid-washed sand using a pestle and mortar and chloroform/methanol (2:1, v/v). Non-starch lipids were extracted using cold water-saturated butanol. Starch lipids were, thereafter, extracted from the homogenate using prolonged treatment with boiling watersaturated butanol (Hargin and Morrison, 1980). The lipid extracts were purified by thin-layer chromatography using pre-coated silica gel G plates and a polar solvent system consisting of chloroform/methanol/acetic acid/water (170:30:20:7, by vol.). Individual lipid classes were identified by co-chromatography with authentic lipid standards and their identity confilmed by specific spray reagents (Kates, 1986). Fatty acid methyl esters were produced from total lipid fractions and purified lipid classes by acid-catalysed transmethylation, as previously reported (Williams *et al.,* 1991). These were analysed with a Perkin-Elmer F33 gas chromatograph using a glass column (4.0mm x 2.0m) packed with EGSS-X (15% w/w) on chromosorb WAW (120 mesh) at 175°C. Quantification was achieved using heptadecanoic acid as an internal standard.

3. RESULTS AND DISCUSSION

Whole wheat grains which had been harvested from plants that had been exposed to the various growth conditions possessed considerable differences in the quantity of accumulated grain acyl lipids (Table I). Elevated atmospheric carbon dioxide levels enhanced the accumulation of non-starch lipids (1597±174 mg fatty acid g^{-2} fresh weight), the major components of which were triacylglycerol and, to a lesser extent, the glycosylacylglycerides digalactosyldiacylglycerol and monogalactosyldiaclglycerol (data not shown). At normal atmospheric carbon dioxide levels the amount of non-starch lipid was considerablely less (1096±109 mg fatty acid 100g fresh weight). It has been regularly observed that elevated carbon dioxide (a substrate for photosynthesis) levels increases photosynthesis by inhibiting photorespiration in C3 plants. Furthermore, elevated carbon dioxide levels have been shown previously to enhance wheat growth (Havelka *et al.,* 1984). Therefore, as shown in Table I, the increase in grain lipids was not unexpected.

Table I
The effect of varying growth parameters on the lipid composition of wheat grains

Environmental growth parameters			mg Fatty acid/100g fresh weight		
Temperature	Fertilizer	Carbon dioxide levels (ppm)	Non-starch lipids	Starch lipids	Total lipids
Amb T.+4°C	LN	350	1096±109	200±100	1302±23
Amb T.+4°C	LN	700	1597±174#	194±52	1778±174#
Amb T.	HN	700	1399±35	330±73	1729±41
Amb T.+4°C	HN	700	1193±181	118±5#	1301±100#
Amb T.	HN	350	1782±230	206±14	1989±234
Amb T.	LN	350	2144±196	384±14#	2528±202#

Abbreviations: LN= low nitrogen, HN= high nitrogen. Results show means±s.d. (n=3). Statistical significance (Student's t-test: #P<0.05)

Wheat grains cultivated at ambient temperature accumulated considerable amounts of both non-starch lipids and starch lipids, where as at the elevated temperature the wheat grains possessed smaller amounts of these lipids. This suggested that at the higher growth temperature less time was available for the accumulation of food reserves, as a result of the plant growing at a relatively faster rate than at ambient temperature.

The application of nitrogen fertilizer was also seen to markedly affect the lipid composition. Sionit *et al.* (1981) have reported that in wheat, morphological growth responses to the application of nitrogen fertilizer were influenced by the relative levels of atmospheric carbon dioxide. Table I shows that lipid composition was affected by the level of applied nitrogen fertilizer. In particular, plants grown at ambient temperature and normal atmospheric carbon dioxide level showed that at the lower nitrogen level the total lipid content was 2528±202 mg fatty acid 100g, whereas at the higher nitrogen level the total lipid content was 1989±234 mg fatty acid 100 g fresh weight. This data was most inte-

resting because this was one growth parameter that it is possible to control in field-grown crops.

Not only was the quantity of wheat grain lipids affected by the different growth parameters but also the quality. Non-starch lipids of mature wheat grains contain primarily linoleate as their major acyl moiety, although the starch monoacyl phospholipids also possess considerable amounts of palmitate (Morrison, 1988). And, in fact, it was these two acyl components that were found particularly to be influenced by changes in environmental growth conditions. Table II shows that carbon dioxide levels influenced the acyl moieties of starch lysophosphatidylcholine, whereby elevated atmospheric carbon dioxide was associated with a relative increase in palmitate with a corresponding decrease in linoleate (in the presence of a low nitrogen fertilizer).

Table II
The effect of varying growth parameters on the acyl composition of major starch lipid classes of wheat grains

Environmental growth parameters				Fatty acid distribution (% of total)				
Temperature	Fertilizer	Carbon dioxide levels (ppm)	Starch lipid class	16:0	18:0	18:1	18:2	18:3
Amb T.	LN	350	Lyso-PC	35±2	1±tr.	11±1	49±3	4±tr.
Amb T.	LN	700	Lyso-PC	45±3#	1±tr.	12±tr.	39±3#	1±1#
Amb T.+4°C	LN	700	Lyso-PC	37±0	1±tr.	9±tr.	51±tr.	3±tr.
Amb T.	LN	700	Lyso-PC	45±3#	1±tr.	12±tr.#	39±3#	1±1
Amb T.+4°C	HN	350	Lyso-PE	22±1	1±1	6±2	65±5	3±2
Amb T.	HN	350	Lyso-PE	26±1#	7±4	12±3#	49±9#	5±1
Amb T.	LN	700	Lyso-PC	45±3	1±tr.	12±tr.	39±3	1±1
Amb T.	HN	700	Lyso-PC	29±3#	1±tr.	7±tr.#	56±2#	5±tr.
Amb T.+4°C	LN	700	Lyso-PC	37±0	1±tr.	9±tr.	51±tr.	3±tr.
Amb T.+4°C	HN	700	Lyso-PC	33±2#	1±tr.	5±tr.#	57±1#	4±1

Abbreviations: See table 1. Lyso-PC=lysophosphatidylcholine, Lyso-PE= lysophosphatidylethanolamine, tr.=trace (<0.05%).
Results show means±s.d. (n=3).
Statistical significance (Student's t-test: #P<0.05).

Temperature affected the relative proportions of oleate and linoleate of both the non-starch neutral lipids and monogalactosyldiacylglycerol (data not shown), but the most marked effect was, once again, amongst the starch lipids, lysophosphatidylcholine and lysophosphatidylethanolamine. The relative proportions of linoleate increased generally at the expense of oleate and palmitate at the elevated temperature. The effect of nitrogen on acyl lipids was observed only with the starch lipids and even then the data was somewhat contradictory, depending on the temperature levels.

In general, the data reported here showed that there were significant alterations to the acyl lipid content of wheat grains as a result of exposure of the developing parental plant to the individual factors that contribute to the "greenhouse effect". These changes have important implications for properties of any flour derived from such seeds and, therefore, require further investigations to define the metabolic changes responsible for the compositional differences observed.

ACKNOWLEDGEMENTS

We are grateful to the A.F.R.C. for financial support and to Dr. David Lawlor (Rothamstead Experimental Station) for the supply of seeds.

REFERENCES

Bolin, B., Jager, J. and Doos, B.R. (1986).– "The Greenhouse Effect, Climatic Change and Ecosystems" in Scope Vol. **29**, pp. 1-32. Bolin, B., Doos, B.R., Jager, J. and Warrick, R.A. (Eds).– John Wiley, New York.

Hargin, K.D. and Morrison, W.R. (1980).– "The distribution of acyl lipids in the germ, aleurone, starch and non-starch endosperm of four wheat varieties".– J. Sci. Food Agric. **31**, 877-888.

Havelka, U.D., Wittenbach, V.A. and Boyle, M.G.(1984).– "$C0_2$.Enrichment effects on wheat yield and physiology".– Crop Science **24**, 1163-1168.

Karpati, E.M., Bekes, F., Lasztity, R., Orsi, F., Smied, I. and Mosonyi, A. (1990).– "Investigations of the relationship between wheat lipids and baking properties".– Acta Alimentia **19**, 237-260.

Kates, M. (1986).– "Techniques of Lipidology".– 2nd Ed.– Elsevier, Amsterdam.

Morrison, W.R. (1988).– "Lipids in cereal starches: A review".– J. Cereal Sci. **8**, 115.

Sionit, N., Mortensen, D.A., Strain, B.R. and Hellmers, H. (1981).– "Growth responses to CO_2 enrichment and different levels of mineral nutrition".– Agronomy Journal **73**, 1023-1027.

Warrick, R.A., Gifford, R.M. and Parry, M.L. (1986).– "The Greenhouse Effect, Climatic Change and Ecosystems" in Scope **29**, pp. 393-473. Bolin, B., Doos, B.R., Jager, J. and Warrick, R.A. (Eds.).– John Wiley, New York.

Williams, M., Francis, D., Hann, A.C. and Harwood, J.L. (1991).– "Changes in lipid composition during callus differentiation in cultures of oilseed rape (*Brassica napus L.*)"– J. Exptl. Botany **42**, 1551-1556.

Williams, M., Shewry, P.R. and Harwood, J.L. (1993).– "Influence of alterations in environmental $C0_2$ and temperature on wheat grain lipids".– Biochem. Soc. Trans. **21**, 182.

Changes in lipid metabolism, activity of superoxide dismutase and glutathione reductase during ageing and water deficit stress

E.M. Olsson* and **C. Liljenberg**
Department of Plant Physiology, University of Göteborg, S-413 19 Göteborg, Sweden.

1. INTRODUCTION

Water deficit stress is one of the main problems determining the yield of many crop plants. Water deficit stress has also been shown to influence lipid metabolism.

In recent years there has been increasing evidence that forms of activated oxygen play a central role in the senescence of leaves(Thompson 1987, Leshem,1988). In addition, the effects of water deficit stress, have been considered to be caused by the formation of oxygen radicals (Price et al.,1989). Although water deficit stress seems to accelerate many changes that resemble senescence, it has not clearly been shown that more or less identical processes are acting here as in senescence.

In this work, pea *(Pisum sativum)* plants were deprived of water for successive periods. The ageing process in

control plants were followed simultaneuosly. Leaves, from the stage of newly developed until the stage of visual senescence, were sampled.

The present work compares the lipid content and composition, lipid peroxidation, fluorescence reflecting photosynthetic activity and superoxide dismutase and glutathione reductase, enzymes active in the anti-oxidative defence, during ageing and during water deficit stress.

2. MATERIALS AND METHODS

Pea (*Pisum sativum*) were grown in vermiculite in pots, placed in nutrient solution. The second leaves, counted from the base of the plant, were used from plants of different ages. The leaves from the plants ageing without stress treatment were harvested at plant age 11, 20, 30, 35 and 40 days. Leaf abscission was shortly after day 40. The stress treated plants were harvested at plant age 20, 25 and 30. These plants were submitted to water deficit stress at day 11, 20 and 25. The harvest at day 20 and 25 was made before the stress treatment. Leaf abscission in the stress treated plants was shortly after day 30.

The sampled leaves were homogenised in a 100mM K_2HPO_4, pH 7.6. The extact was centrifuged by 15 000 x g for 5 minutes. The supernatant was used for further analysis:

Lipid peroxidation was determined as the concentration of thiobarbituric acid-reactive substances, equated with malonylaldehyd (MDA) (Heath and Parker, 1968) but quantified using 1,1,3,3 tetraethoxypropane as standard. Butylated hydroxytoluene (1%w/v) was included in the reaction mixture.

The activity of superoxide dismutase (SOD) was estimated using the xanthine-xanthine oxidase-nitro blue tetrazolium method (Halliwell,1975), quantified by the method of (Ginnopolitis and Ries, 1977).

The activity of glutathione reductase was determined by monitoring the oxidation of NADPH (Foster and Hess, 1982).

Lipid analysis: The leaves were homogenized in hot 2-propanol and the lipids were further extracted with chloroform: methanol 2:1. The total lipid extract was run on a silicic acid column. Neutral lipids were eluted with chloroform and polar lipids with acetone followed by methanol. The acyl lipids were converted to methyl esters by reacting with 2.5% HCl (g) in dry methanol (70°C, 1h.). Heptadecanoic acid was added as internal standard.The fatty acid methyl esters were separated and quantified by gas chromatography. Identifications were based on retention times relative to known standards.

Flourescence: Flourescence emission were recorded in vivo at -196°C using a SLM8000C spectrofluorometer. The exitation wavelength was 440nm.

3. RESULTS

As the leaves in both treatments began to senesce, there was an increase in the neutral lipid content and a decrease in the polar lipid content. In the drought stress treated plants, the activity of superoxide dismutase rapidly increased and then decreased with advancing senescence. The same occurred with glutathione reductase, but the activity peaked a few days later. A similar pattern could be seen in the plants without stress treatment; an increase in activity at the onset of senescence and a decrease with advancing senescence(Figure 1a, 1b).

Further, there was an increase in lipid peroxidation, in both treatments, starting at the onset of senescence and increasing with advancing senescence (Figure 2).

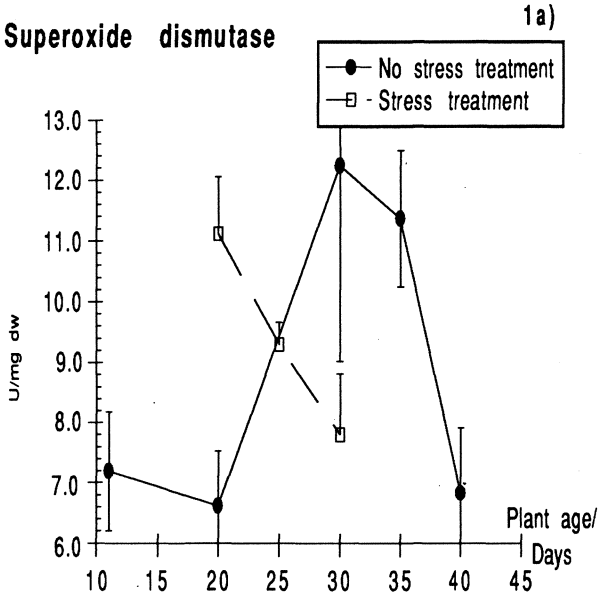

Fig. 1.a
Activity of superoxide dismutase

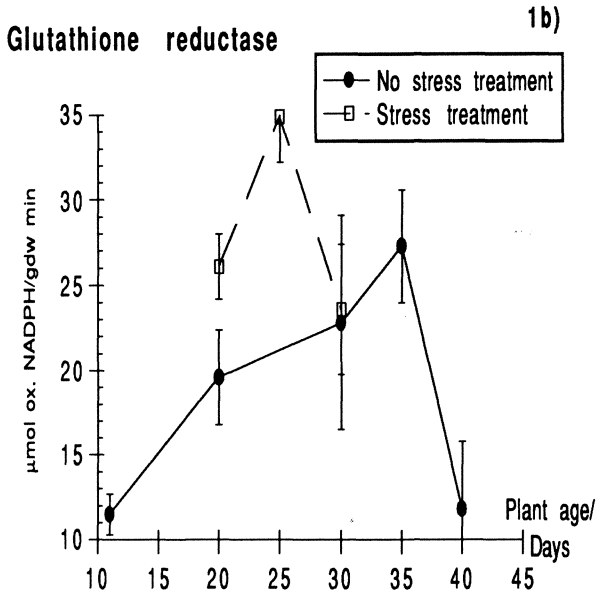

Fig. 1.b
Activity of glutathione reductase in pea leaves from plants of different ages

The ratio 685/735 from fluorescence emission spectra has been used as an indicator of stress effects on photosynthetic activity (Lichtenthaler, 1988). No change in fluorescence ratio could be detected at the onset of senescence in both treatments, while at advanced senescence in the stress treated plants an increased ratio could be seen (the ratio was not measured at advanced senescence in the plants without stress treatment).

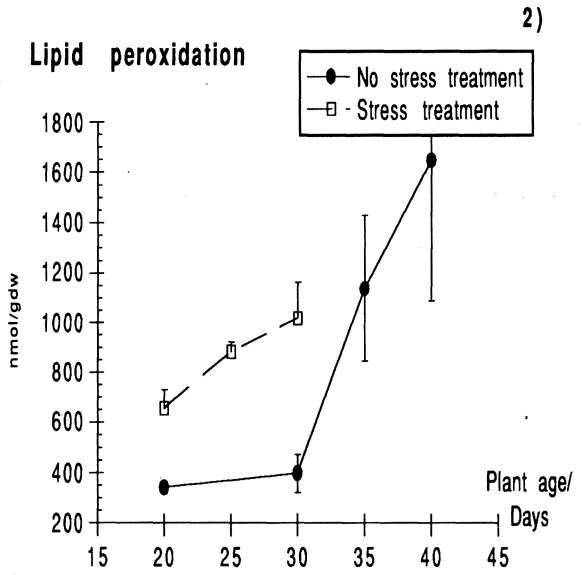

Fig. 2
Lipid peroxidation in pea leaves from plants of different ages

4. DISCUSSION

At senescence, as well as at water deficit stress, there are many reports on free radicals being detected in plants. Forms of highly reactive oxygen species, as well as other free radicals, are potentially highly destructive to plant cells. Superoxide radicals, singlet oxygen or hydroxyl radicals can indirectly or directly initiate lipid peroxidation leading to membrane deterioration. Fortunately, the plants have protecting mechanisms against these damaging oxygen species. Eg. superoxide radicals are dismuted to hydrogen peroxide by superoxide dismutase. Glutathione, a water-soluble anti-oxidant, is transformed from its oxidized form (GSSG) to its active, reduced form (GSH) by glutathione reductase. It has been suggested that the ability to limit the membrane deterioration processes by protection by enzymes or other anti-oxidants determines the tolerance of drought stress (Dhindsa and Matowe, 1981, Leprince *et al*, 1990).

The results in this work, a decrease in the polar lipid content and an increase in the neutral lipid content, as well as an increase in the level of lipid peroxidation in both treatments, indicates a disturbed lipid metabolism. The rapid increase in the level of lipid peroxidation and decrease in polar lipids are most probably a result of free radical mediated changes in the membranes. Also the increase in activity of glutathione reductase and superoxide dismutase at senescence indicates more free radicals being produced in the cells.

It has been suggested that superoxide is produced in the chloroplast as a result of leakage of electrons on to oxygen associated either with photosystem I acceptor or reduced ferredoxin (Halliwell, 1987). However, in this work, by looking at fluorescence emission ratio, reflecting changes in photosynthetic activity and used as a tool for stress detection, no alteration in the emission ratio could be seen simultaneously with the other changes occurring. Not until advanced senescence in the stress treated plants, the ratio increased.

The alterations in lipid content, the activity of superoxide dismutase and glutathione reductase, the lipid peroxidation as well as fluorescence emmission spectra all show a similar pattern during the senescence in the plants without stress treatment and in the stress treated plants, though of different magnitude and rate. This suggest that similar processes act as a result of water deficit stress as in senescence and that the stress initiates the senescence starting earlier and being more rapid.

REFERENCES

Dhindsa, R.S. and Matowe, W.(1981).– J.Exp. Bot. **32**, 79-91.
Foster J.G.and Hess J.L. (1982).– "Oxygen effects on maize leaf superoxide dismutase and glutathione reductase".– Phytochemistry **21**,1527-1532.
Ginnopolitis C.N. and Ries S.K. (1977).– "Superoxide dismutases. 1. Occurrence in higher plants".– Plant Physiology **59**, 309-314.
Halliwell B. (1975).– "The superoxide dismutase activity of iron complexes".– FEBS Letters **50**, 34-38.
Halliwell, B.(1987).– "Oxidative damage, lipid peroxidation and anti-oxidant protection in chloroplasts".– Chemistry and Physics of Lipids **44**, 327-340.
Heath R.L. and Parker L. (1968).– "Photoperoxidation in isolated chloroplasts. I.Kinetics and stoichiometry of fatty acid peroxidation".– Arch. Bioch. Biophys. **125**, 189-198.
Leprince, O., Deltour, R., Thorpe, P.C., Atherton, N.M. and Hendry, G.A.F. (1990).– New Phytol. **116**, 573-580.
Leshem Y.Y. (1988).– "Plant senescence processes and free radicals".– Free Rad. Biol. Med. **5**, 39-49.
Lichtenthaler, H.K.(1988).– "In vivo chlorophyll fluorescence as a tool for stress detection in plants, in Applications of Chlorophyll Fluorescence".– Lichtenthaler, H.K.,Ed., Kluwer Academic Publishers, Dordrecht, 129.
Price A.H., Atherton N.M., and Hendry G.A.F.(1989).– "Plant under droughtstress generate activated oxygen".– Free Rad.Res.Comms. **8**, No 1, 61-66.
Thompson J.E., Legge R.L. and Barber R.L. (1987).– "The role of free radicals in senescence and wounding".– New phytol. **105,** 317-344.

The determination of double bond positions in polyunsaturated fatty acids. Gas Chromatography / Mass Spectrometry of the diethylamide derivative

Ralf Nilsson, Peter Norberg, Conny Liljenberg
Department of Plant Physiology, University of Göterborg, S-413 19 Göteborg, Sweden.

Analysis of fatty acids by GC / MS requires some sort of derivatization due to assymetric peak shapes and double bond migration when analysing free fatty acids. Double bond migration also occurs when using the otherwise useful methyl esters. For determination of double bond positions a number of more or less useful methods have been presented derivatizing either at the carboxyl terminus or both at the carboxyl terminus and the double bonds. Derivatizing of the carboxyl terminus usually involves introduction of a nitrogen atom into the molecule, which stabilizes the positive charge and thereby reduces double bond migration. In the work presented on this poster, fatty acid diethylamides (FADEA) have been investigated as a possible derivative for determination of double bond positions in fatty acid mixtures.

Molecular cloning of a gene encoding acetyl-CoA carboxylase from *Brassica napus*

Wolfgang Schulte, Jeff Schell, Reinhard Töpfer
Max-Planck-Institut für Züchtungsforschung
Carl-von-Linné Weg 10, D-50829 Köln, Germany

Acetyl-CoA carboxylase (ACCase. EC 6. 4.1.2) is a biotin containing enzyme which catalyses the ATP dependent carboxylation of acetyl-CoA to form malonyl CoA. This reaction is the first comitted step in fatty acid biosynthesis and in animals, yeast, and plants is catalyzed by an ACCase of more than 200 kDa per subunit. In contrast, the E. *coli* enzyme is composed of three distinct polypeptides: biotin carboxylase (BC)-biotin carboxy carrier protein (BCCP)-carboxyltransferase (CT).

ACCase has been cloned from rat, chicken, yeast, and *E. coli* leading to the identification of conserved regions (e. 9. Kondo *et al.* 1991). Based on these conserved sequences degenerate oligonucleotides were prepared for Polymerase Chain Reaction (PCR). Using poly A^+ RNA from immature seeds of *Brassica napus* we amplified a 260 bp DNA fragment which was subsequently cloned and sequenced. It shows an open reading frame coding for 86 amino acids with 88.4% similarity to ACCase of rat or chicken. Screening a rapeseed genomic library we isolated a number of clones which hybridize strongly with the PCR-fragment. Restriction mapping showed at least three different classes of ACCase genes in rapeseed. One of the genomic clones was further subcloned and sequenced. Based on similarity to the corresponding rat protein the three different domains of ACCase are arranged in the same order: BC-BCCP-CT. The common motif in biotin binding proteins, -Met-Lys-Met-, which is the site of biotinylation, could be identified.

ACKNOWLEDGEMENTS

We gratefully acknowledge the excellent technical assistance of Jaqueline Bautor, Elke Filsak and Christa Höricke-Grandpierre. The research was funded by BMFT grant number 0319412D, including financiai support from Bayer AG, Deutsche Saatveredelung, Hoechst AG, Kleinwanzlebener Saatzucht AG and Norddeutsche Pflanzenzucht.

Changes in triacylglycerol storage and composition in cell cultures and somatic embryos of *Pimpinella anisum* L.

R.R. Theimer, U.F.M. Schöpf, H.R.J. Siegert, R. Radetzky
Bergische Universität/GH, FB9, Gaußstr. 20. D-5600 Wuppertal/Germany

Plant triacylglycerols (TAG) are synthesized in bulk only in embryonic storage tissues of maturing seeds or fruits which obviously poses experimental problems for controlled physiological and biochemical studies.

We therefore use submerged cell cultures of anise *(Pimpinella anisum* L.*),* an Apiaceae that stores in its endosperm cells TAG rich in petroselinic acid. Two lines of cell cultures were obtained from the hypocotyl of one anise seedling: Line 1 accumulates TAG in amounts comparable to those found in the seed storage tissues. In contrast, the cells of line 2 contain only very small amounts of TAG. The submerged cultures of these two lines also differ in both cell aggregation and growth characteristics. The fat storing line 1 produces small globular cell aggregates, is fast gro-

wing, and easily induced to form somatic embryos in high yields in liquid culture medium. The low fat storing line 2 of the anise cell cultures grows slower and forms aggregates with only a few cells.

By improved high resolution gas chromatography of the fatty acid methyl esters prepared from the storage TAG of line 1 anise cultures the presence of 22 different fatty acids (FA) could be shown ranging from C_{12} to C_{24} carbon skeletons. In particular, the method permits a clear separation of mono- and polyunsaturated FA in one mixture. Major FA in the TAG are linolic (57%), palmitic (22%) and oleic (10%) acid. Petroselinic acid, a major component (54%) of the oil in the genuine anise seeds used comprises less than 1% of the cell culture oil. But during somatic embryogenesis of the culture the portion of petroselinic acid in the storage TAG is strongly increased indicating changes in the enzyme equipment of the fat producing cells.

Feeding fatty acids (natural or unusual) to the cell cultures leads to corresponding changes in the composition of the lipid body TAGs. Incubation of line 2 anise cultures under identical conditions results in a strong increase in lipid bodies which, however, seem not to be utilized when the cells are starved in sucrose-free medium. Studies designed to elucidate the biochemical reasons for this difference are currently performed in this laboratory.

Studies on the enzyme-stability of lipase from rape (*Brassica napus* L.) seedlings

Andreas Hoppe and **Roland R. Theimer**
Bergische Universität/GH, FB9, Gaußstr. 20. D-5600 Wuppertal/Germany

During germination of oil seeds the mobilization of plant reserve oils that are stored in distinct cell structures (oil bodies, lipid bodies) appears to be a complex physiological process. In general, the enzyme lipase (triacylglycerol acyl hydrolase, EC 3.1.1.3.), is assumed to be responsible for the hydrolysis of lipid body triacylglycerols.

The basic aspects of the lipolysis in the cotyledons of *Brassica napus* L. are puzzling, especially since so far no convincing purification protocol for lipase has been reported. In the course of purification of the enzyme a rather uncommon dramatic decrease in the specific activity of lipase is generally observed which is hard to explain. Therefore, we studied the enzyme-stability of the rapeseed lipase under different conditions.

Specific activity of partially purified lipase is increased by treatment of the enzyme preparation with detergents such as Triton X-100. Furthermore, in the presence of detergents such enzyme preparations reveal good stability for at least 24 h between 4-21 °C retaining 50-80% of the initial lipase activity. But during zonal centrifugation in a sucrose density gradient (5-50% w/w, 4 °C, 116.000g, 14 h) identical enzyme preparations loose about 80% of the lipase activity applied. The remaining activity shows a peak at 1.148 kg/l which suggests that lipase is a high molecular lipolytic complex. In contrast, identical zonal centrifugation in the absence of detergents yields a recovery of 70-100% of the initial lipase activity.

Furthermore, when incubated for different periods of time at pH 6.5-11 lipase activity is very well preserved in the enzyme preparations, and at pH 11.4 (24 h, 4-21 °C) approximately 20% of the enzyme activity is lost. But upon zonal centrifugation at pH 11.4 up to 97% of the lipase activity disappear. Obviously at alkaline pH or after addition of detergents a lipolytically active complex dissociates into subunits that may be separated by centrifugation and possess no lipase activity. At pH 6.5 the complex appears to be stable as demonstrated by the recovery of up to 100% after centrifugation in the absence of detergents. Investigations into the nature of both the complex and its subunits are presently under way in this laboratory.

REFERENCES

(1) Hoppe A., Theimer, R.R.: Biol. Chem. Hoppe-Seyler 1989, **370**, 789.

(2) Hills M.J., O'Sullivan J.N.: Biochem.Soc.Trans. 1989, **17**, 480.

ABSTRACTS OF PLENARY LECTURES

A retrospective view of plant lipid research

Paul K. Stumpf
Department of Biochemistry and Biophysics
University of California, Davis, U.S.A.

In the late forties and early fifties, little was known in the area of lipid metabolism in animal and bacterial systems and even less in the area of plant systems. The techniques used to study lipid biochemistry were primitive, funding was essentially nonexisting, coenzyme A had not been discovered, and substrates now easily purchased were unknown. It was in this environment that research was begun in my laboratory at the University of California at Berkeley. As techniques were developed, and as knowledge was accumulated and funding became available, progress began to take shape. This progress, started in 1950, will be traced to about 1965.

As to the future, the knowledge base has simply exploded in the last five years as plant molecular biology began to influence the course of research in plant lipid research. In addition two additional developments occurred to set the stage, namely the concept of prokaryotic and eukaryotic lipid biosynthetic pathways in leaf tissue and the long sought resolution of the individual enzymes that make up the biosynthetic pathway of C16 and C18 fatty acids. With these in place, the future direction of plant lipid research will be a function of the imagination and innovative skills of the modern investigator. A number of trends will be discussed as well as some of the socio-economic implications that could affect agronomic industries in developing countries.

Silver ion and chiral chromatography in the analysis of triacylglycerols

William W. Christie
The Scottish Crop Research Institute, Ivergowrie,
Dundee, Scotland DD2 5DA

Silver ion chromatography has only recently regained some of its earlier importance for separation of molecular species, because of the availability of new stable columns for highperformance liquid chromatography (HPLC) for use in conjunction with evaporative light-scattering detection. Ready separation of natural triacylglycerols is possible with this technique, including those as highly unsaturated as linseed oil. When reversed-phase HPLC is used to complement silver ion chromatography, much more information is attainable. The HPLC technique has given a valuable new insight into the mechanism of silver ion chromatography. An ability to resolve diastereomeric chiral derivatives of diacyl-sn-glycerols by HPLC on silica gel has opened up a new method for stereospecific analysis of triacyl-sn-glycerols. Again, when this is used in conjunction with silver ion HPLC, much more information on the structure of oils is obtained. This is discussed in relation to olive oil.

High resolution ^{13}C-NMR. A technique for the study of lipid structure and composition

Frank D. Gunstone
Department of Chemistry, University of St Andrews,
St Andrews, Scotland

The application of high resolution ^{13}C nmr spectroscopy to the study of plant lipids will be discussed. A number of example will be considered including oils containing petroselenic acid, γ-linolenic acid, *n*-3 and *n*-6 acids, cyclic acids (malvalic, sterculic), and oxygenated acids (ricinoleic, vernolic. and other epoxy acids).

^{13}C nmr spectroscopy can also be used to distinguish triacylglycerols from phospholipids (PC and PE) and to analyse mixtures of glycerol esters (mono-, di-, and triacylglycerols, acelytated monoacylglycerols). The effect of changing the end group (acids, esters, nitrites, alcohols. acetates) will be reported.

Characterization of olive oils by an expert system (SEXIA)

Ramón Aparicio
Instituto de la Grasa y sus Derivados,
Avda. Padre García Tejero, 4. Sevilla 41012. Spain

The use of computers in the area of food technology has greatly increased in recent years. The most important reason for this expansion can be found in the increased possibility of computer-assisted decision making. In other words the computer is now more than just a depository for data: it may use the data to issue suggestions, advice and recommendations. One of the new fields of application is the characterization of foods, and the artificial intelligence and expert systems the subjects to get this objective.

SEXIA is an expert svstem that uses different methodological approaches to identify olive oils according to varieties, olive zones and denominations of origin. Its database stores more than 1.400 samples of four countries, Greece, Italy, Portugal and Spain, characterized up to fifty-three chemical parameters of different series: fatty acids, alcohols, triacylglycerols, hydrocarbons, sterols and methylsterols. The knowledge base keeps four different types of rules: inexact reasoning, relational and lineal equations and heuristic. The information is stored in nodes of a tree structure divided into levels. The top level is Country and the

bottom can be Zone. The searching strategy provides conclusions, associated with a confidence degree or a belief interval, at each level and the final goal. The computer can work with more than 200 rules during the identification process.

The characterization of European olive oils have been depicted on the geographical maps of the countries, where the figures —calculated by SEXIA— have been transformed in colours, and the similitudes and dissimilitudes among olive oils, gathered from different farms, can be easily distinguished.

Enzymes and genes of fatty acid biosynthesis

A.R. Slabas, T. Fawcett, B. Sinden, B. Simon, R. Swinhoe, K. Elborough, J.M. Martínez-Rivas
Plant Molecular Biology Group, Dept. Biological Sciences, University of Durham, U.K.

The biosynthesis of fatty acids is catalysed by two complex enzyme systems (1) Acetyl CoA carboxylase (ACC) (2) fatty acid synthetase (FAS). Both of these enzymes are located in plastids and the genes for them are nuclear encoded. Recently in collaboration with the group of Paul Whitfeld and Tony Ashton of CSIRO we have cloned a partial cDNA to wheat ACC. This shows strong homology to rat and chicken ACC. Several components of the rape FAS have been cloned including the β-ketoreductase and enoyl ACP reductase. Several enoyl reductase products are present in seed tissue and these show differential expression when investigated using 2D-Western blots.

The rape thioesterase has been cloned in three industrial laboratories and there is more than one type. Manipulation of the level of these thioesterases causes marked alterations in lipid content.

There have been rapid increases in our knowledge of the molecular control structure of lipid synthesis enzymes and genes in plants. The main challenge now in this area concerns membrane bound enzymes.

The initial reactions of fatty acid biosynthesis in plants

J. Jaworski[1], H. Tal[1], J. Ohlrogge[2], D. Post-Beittenmiller[3]
1. Chemistry Department, Miami University, OH 45056.
2. Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824.
3. Genetics Section, The S.R. Noble Foundation, P.O. Box 2180, Ardmore, OK 73402.

For the past several years, our understanding of how the fatty acid biosynthetic pathway functions in plants and bacteria has undergone a fundamental change as a result of the dicovery of an additional condensing enzyme, 3-ketoacyl-ACP synthase III (KAS III). Our characterization of this enzyme from *Spinacia oleracea* and its role in fatty acid synthesis will be reviewed.

The cerulenin insensitive KAS III has been purified to homogeneity from *Spinacia oleracea.* The size of the native protein was determined by gel filtration and had a Mr = 63,000. SDS-PAGE analysis of the purified KAS III produced a single band with a Mr = 41,000, suggesting that the native enzyme may be a homodimer. The substrate specificity of the purified enzyme suggested that KAS III catalyzes the initial condensation reaction of fatty acid biosynthesis. It was highly specific for the condensation of malonyl-ACP with acetyl-CoA. Acetyl-, butyryl-, and hexanoyl-ACP could not substitute for the malonyl-ACP.

In order to further examine the extent to which acetyl-ACP participated in fatty acid biosynthesis, the rate of labeling of those intermediate metabolic pools with ^{14}C-acetate by isoloated spinach chloroplasts was determined. In light-incubated isolated chloroplasts with the highest rates of fatty acid synthesis (greater than 800 nmol/h/mg chlorophyll), the rate of acetyl-ACP metabolism was at least 10-fold slower than the rate of butyrylACP metabolism. The relatively slow metabolism of acetyl-ACP provided in situ evidence that 1.) butyryl-ACP was formed principally from condensation of malonyl-ACP with acetyl-CoA.and 2.) acetyl-ACP was a minor participant in fatty acid biosynthesis. The changing level of acetyl-ACP observed in spinach leaves when shifted between the dark and light (Post-Beittenmiller et al., J. Biol. Chem. 266: 1858-1865 (1991)) can be attributed to changing rates of acetyl-ACP utilization by 3-ketoacyl-ACP synthase I. The rate of synthase I in turn changes in response to increased levels of malonyl-ACP in the light and the near absence of malonyl-ACP in the dark.

The KAS III has been from a spinach root cDNA library by probing with a 650 bp PCR fragment. The 1920 bp clone was sequenced and the deduced amino acid sequence showed identity of 48% and 45% to the putative KAS III of P. *umbilicalis* and KAS III of *E. coli* respectively. Northern analysis confirmed that this was a full length clone and Southern analysis indicated that it was a single copy gene in spinach.

Biosynthesis of very long chain fatty acids

Claude Cassagne, René Lessire, Jean-Jacques Bessoule, Patrick Moreau
IBC-CNRS. 1 Rue Camille Saint-Saëns, 33077 Bordeaux-France

1— Very long chain fatty acids (VICFA) are building blocks and precursors of the wax coating of virtually all the aerial parts of higher plants. They may also be abundant in the storage lipids of some seeds where they accumulate as triacylglycerols (most cases) or as "internal waxes" (rare). Roughly, the wax very long chain fatty acids are chiefly saturated, whereas those encountered in developing seeds are chiefly unsaturated, though in this case saturated VLCFA may also be observed.

2— VLCFA biosynthesis results from the elongation of (saturated or unsaturated) C18 fatty acids by strictly endomembrane-bound elongases.

3— There is an absolute requirement for malonyl-CoA; ACP is not involved,whereas long-chain acyl-CoAs (saturated or unsaturated) are the preferred substrates of the elongases. In some cases, the elongation of unidentified endogenous substrates has also been shown. The reductants are NADH and/or NADPH. Whether the various elongases (C18-elongase and C20-elongase), or the different reactions (reduction of 3-ketoacyl-CoA, or t2,3-enoyl-CoA) require distinct reductants is still being debated and awaits further examination.

4— The reaction sequence proposed to explain the elongation is largely extrapolated from what has been proposed (and not demonstrated...) in animals. Recent evidence obtained in leek and in honesty has suggested that the "classical" pathway (as far as an as yet unproven pathway may be called "classical") could be operative, and confirms the absence of transacylase for the binding of the substrate to the enzyme (s).

5— Partial reactions have been identified and characterized in very few cases. The demonstration of putative intermediates on the other hand, and the study of partial reactions on the other hand, give new insight into the overall elongation pathway.

6— Elongases have been (partly) purified in very few cases (leek, honesty, meadowfoam and rapeseed). In leek leaves, the acyl-CoA elongases account for 1% of the microsomal proteins. The 300 kDa purified elongase is resolved in four bands of 50-60 kDa upon SDS-PAGE. Antibodies have been raised against leek elongases: they immunoprecipitate 1% of the microsomal proteins and the total elongating activity.

7— In leek, C20-CoA is formed in the ER from C18-CoA and transferred to the GA where it is elongated to higher homologues by a C20-CoA elongase. The VLCFA are then transferred from the GA to the plasma membrane (PM) by the vesicular pathway and are excreted to the wax coating. A lowering of the temperature to 12°C blocks the VLCFA at the GA level, whereas the C18 fatty acids are still transferred to the PM.

Biosynthesis of polyunsaturated fatty acids

E. Heinz, P. Sperling, H. Schmidt
Institut für Allgemeine Botanik, Universität Hamburg, Ohnhorststr. 18, 2000 Hamburg 52

Various lines of evidence suggest that plant desaturases introduce second and third double bonds into C18-monoenoic acyl groups only after their incorporation as ester components into polar membrane lipids. This is in marked contrast to animal enzymes, which use acyl-CoA thioesters as substrates. Preferred substrates for plastidial desaturases are the acyl groups of monogalactosyl diacylglycerol (MGD), whereas microsomal enzymes are most active with phosphatidylcholine (PC) acyl residues. On the other hand, convincing evidence from in vitro experiments with exclusion of de/reacylation reactions was difficult to provide. To bring this long-lasting discussion to an end, we have synthesized 9-cis-octadecenyl ether analoguous phospho- and galactolipids which were used as substrates for *in vivo* and *in vitro* studies with microsomal and plastidial desaturation systems.

sn-1 and *sn*-2- (9-cis-octadecenyl) analogues of lysolecithin were acylated by microsomal membranes from sunflower achenes with [^{14}C]oleoyl-CoA. The *sn*-1- and *sn*-2-bound acyl group was desaturated with equal efficiency as was the alkenyl ether residue of a ^{3}H-labelled substrate. Photoautotrophic cell cultures from tomato incorporated isomeric *sn*-1- and *sn*-2-(9-cis-octadecenyl)-glycerol into PC and MGD. After 2 weeks of incubation the glycerol ether backbones were recovered from these lipids in desaturated form with two or three double bonds. Chemical derivatisation and mass spectroscopy showed that the dienyl ether residue carries the two double bonds in the 9,12-positions as found in linoleate.

To provide similar evidence for plastidial systems, the n-6 desaturase from envelopes of spinach chloroplasts was solubilized and incubated with labelled MGD having pro-(18:1/16:1) and eucaryotic (18:1/18:1) ester combinations as well as two esterless 9-cis-octadecenyl ether residues. HPLC of molecular species, showed that ester and ether substrates were desaturated to similar degrees resulting in products with a total of three and four double bonds.

The n-6 desaturase from spinach was purified and used for N-terminal amino acid sequencing. The use of various primers for PCR amplification enabled the isolation of a full length cDNA from mRNA. Apart from its leader peptide the derived amino acid sequence has homology with the desA-encoded desaturase from cyanobacteria. Efforts from different laboratories have contributed to a first complete collection of cDNAs (some by publications, some at present still by rumors), which encode all the enzymes required to desaturate oleate and linoleate in chloroplasts as well as in microsomes.

Biogenesis and function of storage lipid bodies in plants and their biotechnological exploitation

Denis J. Murphy
Department of Brassica & Oilseeds Research, John Innes Centre, Norwich, U.K.

The manipulation of storage product profiles in oilseeds is being carried out on both quantitative and qualitative levels. Quantitative manipulation by genetic and molecular approaches are aimed at altering oil: protein: starch ratios in seeds, particularly rapeseed. Qualitative manipulations of fatty profiles by molecular methods are aimed initially at the Δ_6 stearate desaturase, the Δ_{12} oleate hydroxylase and the acyltransferases of the triacylglycerol biosynthetic pathway.

The genetic regulation of quantitative variations in oil: protein: starch ratios in Brassica should yield information on

the overall regulation of storage product profiles in seeds. It is known that these are complex traits involving many different unlinked genes. It is likely therefore that the empirical genetic approach by itself will not yield a complete understanding of this problem. Another approach to quantitative manipulation is to isolate genes encoding key enzymes at branch points in the metabolic pathways involved in storage product formation. One approach we are taking here is to attempt to down-regulate the activity of acetyl-CoA carboxylase via the introduction of antisense copies of its gene under the control of a strong seed-specific promoter. The effects of such a down-regulation on the allocation of storage carbon to other pathways will then be observed.

Qualitative manipulations of fatty acid profiles are mainly aimed at engineering oilseed crops to produce novel, valuable industrial oils. To this end, we have cloned the gene encoding the Δ_6 stearate desaturase from coriander. This enzyme is responsible for the formation of the oleic acid isomer, petroselinic acid which has the potential to be a valuable industrial raw material, serving as a source of polymers and of detergents. At present, it is not possible to grow coriander as a high-yielding oil crop. A more attractive alternative is to transfer the gene for petroselinic acid into an already successful crop such as rapeseed. These experiments are now in progress in our laboratory. We are also using a number of differential screening methods to isolate the gene encoding the Δ_{12} oleate hydroxylase from castor bean. This enzyme is responsible for the formation of ricinoleic acid, which is a valuable industrial and pharmaceutical commodity with an established market.

In using genetic engineering to alter the fatty acid profile of seed oils, it is essential to ensure that there is no such alteration in the fatty acid profiles of the membrane lipids either of the seed or of other plant organs. In general, membrane lipids have a relatively narrow tolerance for changes in their fatty acid profiles. It is likely that the introduction of veryshort chain and very-long chain or saturated fatty acids into membrane lipids would seriously affect their function and could quite possibly be lethal to the plant. This can be avoided by using seed-specific promoters which direct the appropriate temporal and spatial gene expression in the transgenic plant. We have identified three classes of seed-specific gene exhibiting differential temporal expression during embryo development in rapeseed. Efforts are now underway to characterize those elements from seed-specific promoters which characterize a) their strength; b) their spatial regulation and c) their temporal regulation. In this way, it is hoped to produce a library of cassettes available for the specific manipulation of the different classes of seed storage product in transgenic plants.

It is hoped that this work will lead to the production of transgenic oilseed crops, synthesising a wide variety of useful industrial raw materials. In the short and medium term, this will provide an alternative to the practice of set-aside in developed countries. In the longer term, oilseeds will be the only remaining resource of hydrocarbons once our fossil hydrocarbon supplies are depleted over the next century.

Factors controlling medium-chain fatty acid synthesis in plastids from *Cuphea* embryos

Klaus-Peter Heise and **Jochen Fuhrmann**
Institut für Biochemie des Pflanze, University of Göttingen, Untere Karspüle 2, 3400 Göttingen, FRG.

Specific medium-chain fatty acids (about 30% capric and 50% lauric acid) in developing seeds of *Cuphea wrightii A. Gray* originate from non-green plastids. Their optimum biosynthesis *"in vitro"* requires intact plastids which are exogenously supplied with ATP as energy source and glucose 6-phosphate for maintaining the plastidial pyridine-nucleotide pool in reduced forms. In Cuphea plastids optimum formation of long-chain fatty acids occurs under alkaline conditions (pH>8), while medium-chain products accumulate in the neutral range (pH 7). In cell-free extracts neutral conditions favor not only medium-chain specific acyl-ACP thioesterases but also short-chain condensing enzyme (KAS) activities triggered by increasing ratios of acetyl-to malonyl-CoA at neutral pH. Treatment of Cuphea plastids with either cerulenin (inhibitor of 3-ketoacyl-ACP synthase I) or ADP (inhibitor of acetyl-CoA carboxylase) causes an accumulation of capric acid. In the absence of cerulenin unesterified lauric acid predominates. Transesterification on to CoA of neosynthesized fatty acids and recombination with Cuphea microsomes induce triacylglycerol incorporation of medium-chain fatty acids *"in vitro"*.

Review of wax biosynthesis in jojoba

Mike Pollard [1] and **Jim Metz** [2]
1. Agrigenetics Co., 5649 E. Buckeye Rd., Madison, WI 53716
2. Calgene Inc., 1920 5th St., Davis, CA 95616.

Jojoba seeds contain a unique wax ester as their principal storage lipid. The major components of the wax esters are *cis*-11-eicosenoic acid, *cis*-13-docosenoic acid, *cis*-11-eicosen-1-ol, *cis*-13-docosen-1-ol and *cis*-15-tetracosen-1-ol. Studies on the biosynthesis of this wax are reviewed. In particular, recent studies on the enzymology of the two steps required for the synthesis of wax esters, namely the acyl-CoA reductase and the acyl-CoA: fatty alcohol 0-acyltransferase will be covered. These reactions are unique to wax biosynthesis, are induced during embryo maturation, and are associated with particulate fractions. Both activities show minimal regulation by lipid end-products, although the acyl-CoA reductase activity is inhibited by its water-soluble end-products, NADP+ and CoASH. The acyl-CoA reductase activity is readily solubilised with the detergent CHAPS, and is sufficiently stable when solubilised to be purified to homogeneity. The reductase apparently catalyses conversion of the acyl-CoA to fatty alcohol without release of a fatty aldehyde intermediate. By comparison, the acyl-CoA: fatty alcohol 0-acyltransferase is not solubilised by the detergent CHAPS, though particula-

te activity can be recovered by a reconstitution protocol. Some comparisons are made between jojoba wax biosynthesis and the Kennedy pathway of triacylglycerol biosynthesis found in most oilseeds.

Lipid photosynthesis in olive fruits

Juan Sánchez
Instituto de la Grasa y sus Derivados, C.S.I.C.
Avda. Padre García Tejero, 4. 41012 Seville. Spain.

Developing olive *(Olea europaea)* fruits consist of two distint compartments: a fleshy pulp and a stony endocarp which encloses the seed. The pulp, in turn, consists of a thin outer layer of green cells (epicarp) and an inner layer of parenchimatous cells rich in oil (mesocarp). Both the seed and the pulp accumulate triacylglycerols, but the latter account for more than 95 % of the total oil in the fruit.

Due to the presence of active chloroplasts in their epicarp cells, olive fruits are capable of fixing atmospheric CO_2 and incorporating it into storage triacylglycerols, meaning that they are not as dependend as seeds on the supply of photosynthates from the leaves. Labelling experiments with ^{14}C-labelled acetate, bicarbonate and CO_2 have demonstrated that lipid synthesis in olives is light dependend, and that fruit photosynthesis can contribute significantly to the carbon economy of olives during the maturation period.

Diacylglycerol metabolism in chloroplast envelope membranes

Jacques Joyard, Eric Marechal, Agnès Malherbe, Maryse A. Block, Roland Douce
Laboratoire de Physiologie Cellulaire Végétale, URA CNRS nº 576, Départment de Biologie Moléculaire et Structurale, Centre d'Etudes Nucléaires de Grenoble et Université Joseph Fourier, 85 X, F-38041 Grenoble-cedex, France

Among their structural features, plastid polar lipids are characterized by the presence of various diacylglycerol backbones: some glycerolipid species contain almost exclusively C18 fatty acids whereas others contain C18 and C16 fatty acids respectively at the *sn*-1 and *sn*-2 position of the glycerol. To provide information on the mechanisms which could be involved in the formation of such specific structures. we have analyzed the functionning of two enzymes which manipulate diacylglycerol, namely the phosphatidate phosphatase and the 1,2-diacylglycerol galactosyltransferase (or MGDG synthase). In spinach chloroplasts, both enzymes are located on the inner envelope membrane.

The envelope phosphatidate phosphatase. which catalyzes the dephosphorylation of phosphatidic acid into diacylglycerol, has unique biochemical properties (optimum pH, sensitivity to cations...) and is strikingly different from its extraplastidial and animal counterparts. Among the possible mechanisms involved in the regulation of phosphatidate phosphatase activity, feedback inhibition probably plays a major role. Using isolated intact spinach chloroplasts, we have demonstrated that diacylglycerol is a powerful inhibitor of the enzyme (Ki 70 μM). All diacylglycerol molecular species analyzed presented the same effect on phosphatidate phosphatase activity. Obviously, inhibition of phosphatidate phosphatase by diacylglycerol would favor channeling of phosphatidic acid towards phosphatidylglycerol rather than towards galactolipids and sulfolipid.

We have also performed detailed kinetic studies of MGDG synthase partially purified from spinach chloroplast membranes. using mixed micelles containing diacylglycerol. CHAPS and phosphatidylglycerol. Two-substrate kinetics studies were performed with variable concentrations of UDP-galactose and dioleoylglycerol. The families of reciprocal plots obtained were shown to intersect at a single point of the 1/[substrate]-axis thus demonstrating that MGDG synthase is either a random or an ordered bireactant system. Because of the inhibition patterns obtained with UDP. MGDG synthase was shown to be more likely a random bireactant system. We have also demonstrated that the "surface dilution" kinetic model proposed by Deems *et al (J. Biol. Chem.,* 1975, **250**, 9013-9020) is valid for MGDG synthase assayed in mixed micelles as well as in envelope vesicles. In mixed micelles. diacylglycerol surface concentration (in mmol/nm^2) can also be expressed as mole fraction corresponding to the ratio [diacylglycerol] to ([phosphatidylglycerol] + [CHAPS] + [diacylglycerol]), which could be simplified as [diacylglycerol] to ([phosphatidylglycerol] + [CHAPS]). The experimental conditions we have set up lead to the description of defined equilibrium and kinetic parameters of the interaction of the envelope MGDG synthase with diacylglycerol and therefore allowed a comparison of the affinity of the enzyme for a wide range of diacylglycerol molecular species. The Km values obtained were ranging between 52 μM (1/112 in molar fraction), for dilinoleoylglycerol (18:2/18:2), to 416 μM (1/15 in molar fraction), for distearoylglycerol (18:0/18:0), but the differences observed were not really related to the unsaturation of the molecule since 18:2/18:2 was a much better substrate than 18:3/18:3. Dioleoylglycerol (18:1/18:1) and the diacylglycerol molecular species synthesized within chloroplasts, i.e. 18:1/16:0. were rather good substrate. The physiological significance of our observations will be discussed.

The role of lipids in the structure and function of photosynthetic membranes

W. Patrick Williams
School of Life Sciences, King's College London, Campden Hill, London W8 7AH, U.K.

A great deal of information exists in the literature relating to the possible roles of polar membrane lipids in the structure and function of photosynthetic membranes and their

stability under conditions of chilling and heat stress. Much of this information is in urgent need of reassessment.

With this in mind, the role of membrane lipids in the structure and function of photosynthetic membranes will be reviewed in the context of the physical properies of the lipids, and the different techniques available for their study in membrane systems. Emphasis will be placed on the extent to which the findings in such studies are able to provide answers to questions relevant to the thermal stability of photosynthetic membrane systems.

Special roles of inositol lipids in cell signaling and metabolic regulation

Guy A. Thompson, Jr.
Department of Botany, University of Texas,
Austin TX 78713, USA

Not long ago, the typical plant was considered to contain only one significant inositol lipid, phosphatidylinositol (PI). This phosphatide serves as a component of the membrane lipid bilayer where it participates along with the other lipid species in optimizing the physical properties of the membranes. Then, two PI derivatives, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate (PIP_2) were identified in animal cells and subsequently in plants as well. It is now known that PIP_2, although it comprises less than 1 mole % of the plant plasma membrane lipids and is essentially absent from other membranes, plays an extremely important role in regulating cellular metabolism.

PIP_2 is a key component in a major transmembrane signaling system which is probably present in all eukaryotic cells. Its hydrolysis by a receptor-initiated. G protein-activated, PIP_2-specific phospholipase C produces inositol trisphosphate (IP_3) and diacylglycerol (DAG), both of which have been proposed to act as second messengers in plants as they do in animal tissues.

My laboratory has studied various aspects of PIP_2-mediated signaling in the green alga *Dunaliella salina*. Hypoosmotic shock triggers a rapid 30% drop in PIP_2 and a concurrent rise in both IP_3 and DAG. After the initial peak of DAG, a second, more sustained rise was also measured. Analysis of the DAG molecular species at selected time intervals confirmed the source of the initial product to be PIP_2 and that of the more prolonged release to be phosphatidylcholine and *de novo* DAG synthesis.

The result of IP_3 and DAG formation in animal cells is the activation of Ca^{2+}-dependent protein kinases and protein kinase C, respectively. A Ca^{2+}-dependent protein kinase which has been well characterized in *D. salina,* and this kinase may be responsible for increased phosphorylation of a 29 kDa protein following hypoosmotic shock So far, no convincing evidence for protein kinase C action has been observed in *D. salina,* and its presence in higher plants is also doubtful. Alternative roles for the PIP_2-derived DAG are being sought.

Inositol phospholipids are also involved in an entirely different kind of membraneassociated structure. More than a hundred protein species are now known to be covalently linked to a modified glycosyl chain terminating in PI. The fatty acid chains of this PI moiety anchor the entire complex into the extracellular side of the plasma membrane.

Despite the widespread occurrence of PI-anchored proteins in animals, protozoa, and yeast, there are no published reports of their presence in algae or higher plants. However, in the last few months my laboratory has obtained evidence for the attachment of alkaline phosphatase to the surface of the aquatic duckweed *Spirodela oligorrhiza* by an inositol lipid-containing anchor. In this case, as reported earlier for *Dictyostelium discoideum,* analyses indicate that the protein is attached to the membrane by a ceramide phosphorylinositol-linked glycan chain rather than the more typical phosphatidylinositol moiety. Details of the structure will be presented.

Spirodela alkaline phosphatase activity increases 20-40 fold in leaves and roots after plants are placed in a medium deficient in inorganic phosphate. The newly synthesized enzyme is rapidly transported to the cell surface, where it becomes anchored in the plasma membrane with the protein moiety extending into the cell wall. In this location it is ideally placed to hydrolyze exctracellular organic phosphates which are often present in substantial amounts. Action by the alkaline phosphatase releases inorganic phosphate, which unlike most organic phosphates, can be readily absorbed by plant cells.

Accessible and exploitable diversity for oilseed breeding

Gerhard Röbbelen
Institut für Pflanzenbau und Pflanzenzüchtung,
Universität Göttingen, Germany

Breeders nowadays have gained access to any possible amount of genetic diversity. Natural barriers to recombine essential characters for new oilseed varieties are overcome with increasingly sophisticated biotechnology, e.g. by means of in vitro cell fusion or molecular gene transfer. Admittedly, several plant genotypes may still react recalcitrantly to established techniques well effective with other species. The major problem, however, which plant breeders have to solve is how to finally exploit such genetic materials for the development of new high yielding cultivars.

Quality of the produce is certainly a main requisit of an oilseed variety; compositions of seedoil and mealprotein are economically of almost equal importance. Likewise, such quality is required with the highest quantity possible; thus, all agronomic factors related to yield performance, including disease resistance, plant stress tolerance and others, codetermine the pace of breeding success.

Genetic progress achieved by selection within progenies depends on the structure of inherent diversity, i.e. the heri-

tability and number of genes involved, the complexity of intragenomic interactions or stage and tissue specificities of gene expression. Also, the breeder makes particular demands on analytical methods for his quantification of quality traits in question, recent progress in this field again being remarkable.

Altogether, genetic gain in selection cycles still remains to be a statistical function. All powerful new techniques, which led to an amazing acceleration of varietal improvement indeed, did not so far change the basic recognition that the development of successful oilseed varieties needs more than genetic diversity and lipid biotechnology. This will be exemplified with data from personal experience in oilseed breeding.

New sunflower mutants with altered fatty acid composition

Manuel Mancha
Instituto de la Grasa y sus Derivados. Sevilla. Spain

The fatty acid composition determines the physical properties and potential use of fats and oils. In the main oilseeds this composition is less than optimal to meet the requirements of the oils and fats industry. Consequently, there is increasing interest in producing oilseed crops with modified fatty acid composition. Although the expression in oilseed species of cloned genes for specific enzymes of lipid metabolism is promising, the fact that most enzymes have not been characterized and the mechanisms of regulation are little known makes the genetic engineering far from being a method of general use to modify the fatty acid composition. A different approach is the induction of genetic variability by mutagenesis followed by the identification and isolation of mutants with altered fatty acid composition. Some mutants with abnormal levels of palmitic, stearic, oleic, linoleic or linolenic acids have been obtained in soybean, rape, flax and sunflower.

Sunflower is an important oil crop in which only a high oleic acid mutant is available in addition to the normal lines. As a consequence of a mutagenesis program carried out by our group, a collection of mutant lines with different levels of specific fatty acids in the seed lipids have been obtained. This has been accomplished by treating sunflower seeds (M_1) with EMS, sodium azide or X-rays and obtaining the M_2 and M_3 generations. The analysis of more than 35,000 individual seeds using the halfseed technique and a new method for the one-step lipid extraction and methyl esters preparation from a small sample of cotyledon have made possible the identification of more than 20 mutants with altered fatty acid composition. The study of seven confirmed mutant lines with the most extreme phenotypes is under progress.

A review of the mutants that have been reported in different oilseeds crops and the first results of our mutagenesis program in sunflower will be presented. The possibilities of designing "tailor made" fats from sunflower seeds by crossing the different available lines including the new mutants will be discussed.

Biosynthesis of petroselinic acid and its production in transgenic plants

Edgar Cahoon, Peter Dörmann, John B. Ohlrogge
Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824. U.S.A.

Petroselinic acid, the *cis*-6-18:1, is an unusual fatty acid which is found at levels of up to 80% in the seeds of several Umbelliferae species. Petroselinic acid may have potential industrial value based on its physical properties or as a precursor of lauric and adipic acids. We have determined that the biosynthetic pathway of petroselinic acid formation in developing coriander endosperm involves desaturation of an acyl-ACP substrate. In order to determine if the desaturase positions the double bond placement relative to the carboxyl- or the methyl-terminal carbons, metabolism of (U-^{14}C)nonanoic acid (9:0) by coriander endosperm was examined. When supplied exogenously to endosperm slices the radiolabel was converted primarily to Δ 6-17:1. The presence of unsaturation at the Δ 6 rather than the, Δ 5 (or 12) position of 17:1 indicates that the acyl-ACP desaturase positions double bond insertion relative to the carboxyl end of its substrate. Using antibodies raised against the stearoyl-ACP desaturase of avocado, we have isolated a cDNA clone from coriander which encodes a 36 kDa acyl-ACP desaturase with 70% amino acid sequence identity to castor stearoyl-ACP desaturase. When this cDNA is expressed in transgenic tobacco under control of the 35S promoter, the transformed tissues produce petroselinic acid at a level of approximately 5% of total fatty acids.

Petroselinic acid production also offers a number of opportunities to examine the metabolism of unusual fatty acids in oilseeds. For example, we have asked whether other enzymes in Umbelliferae may have unique specificities. Since a major product of plastid fatty acid synthesis in coriander endosperm is apparently petroselinoyl-ACP, we have examined the specificity of the acyl-ACP hydrolase. Octadecenoyl-ACP with *cis*-double bonds at positions 6, 7, 8, 9, 10, 11, or 12 and elaidyl (*trans* Δ 9) were synthesized and used to characterize the substrate specificity of the hydrolase in various higher plants. In castor bean and spinach, the enzyme was found to be specific for the Δ 9 position and for *cis* configuration of the double bond. In coriander, a specific petroselinoyl-ACP hydrolase activity was found in endosperm in addition to the ubiquitous oleoyl-ACP hydrolase. This activity could be separated by anion exchange chromatography indicating that the two enzymes are represented by distinct polypeptides. Thus, coriander appears to have evolved (or adquired) a unique acyl-ACP hydrolase to accommodate the production of the high levels of petroselinoyl-ACP in its plastids.

Plant lipases and their applications in lipid transformations

Kumar D. Mukherjee

Federal Centre for Cereal, Potato and Lipid Research
Institute for Biochemistry and Technology of Lipids
Piusallee 68, D-48147 Münster, Germany

Plants are of considerable interest as source of lipases that could be used as biocatalysts for lipid biotransformations. Although the plant tissues are known to contain a variety of lipases, such as the non-specific lipid acyl hydrolases exhibiting combined action of phospholipases A_1 (EC 3.1.1.32), A_2 (EC 3.1.1.4), B (EC 3.1.1.5), glycolipase, sulfolipase and monoacylglycerol lipase, the lipases that appear to be most promising for lipid biotransformations are triacylglycerol lipases (EC 3.1.1.3) and phspholipase D (EC 3.1.4.4).

Highly active triacylglycerol lipases are conveniently isolated from germinating oilseeds, such as rapeseed and peanut, dormant oilseeds, such as castor bean, Vernonia anthelmintica and Vernonia galamanensis seeds, and cereal grains, such as oat, maize and rice bran. Partially purified triacylglycerol lipases have been used as such or after immobilization on suitable carriers as biocatalysts for reactions, such as hydrolysis and interesterification of plant oils and esterification of fatty acids. For example, in situ hydrolysis of the oil in castor bean by endogenous lipase has been utilized for the production of ricinoleic (12-hydroxy-9-cis-octadecenoic) acid under mild reaction conditions that do not lead to undesirable products, such as estolides and conjugated dienoic fatty acids. The ability of rapeseed lipase to discriminate against definite fatty acids as substrate has been utilized for the enrichment of gamma-linolenic (all-*cis*-6,9,12-actadecatrienoic) acid and all-*cis*-4,7,10,13,16,19-docosahexaenoic acid from the fatty acids of evening prinrose oil and cod liver oil, respectively, via selective esterification with *n*-butanol. Oat lipase has been used as catalist in the hydrolysis of plant oils for the preparation of polyunsaturated fatty acids. Hydrolysis of Vernonia galamanensis seed oil by endogenous seed lipase has been used to prepare *sn*-1,3-divernolin, from which vernolic (*cis*-12,13-epoxy-*cis*-9-octadecenoic) acid is obtained in high yield and purity.

Phospholipase D from cabbage has been used as catalyst for transphosphatidylation reactions of glycerolphospholipids in which the polar head groups, such as choline, ethanolamine, serine and glycerol are interchanged. Typically, tranphosphatidylation of phosphatidylcholines with glycerol yields phosphatidylglycerols that have potential use as artificial lung surfactant. Transphosphatidylation of phosphatidylcholines with serine provides phosphatidylserines that are used for the preparation of liposomes as drug delivery systems. Numerous other applications of phospholipase D transphosphatidylation reactions for the preparation of designed phospholipids have been reported.

Genetic resistance to disease and abiotic factors of genetically modified crops

Luis Carlos Alonso
Koipesol Semillas, S.A., Avda. San Francisco Javier, 24
Edificio Sevilla-1, 7º. 41018 Sevilla

In recent years, the transformation of higher plants using new techniques of manipulating DNA has opened up new possibilities for the development of varieties of the more important crops. The genetic characteristics most sought after have been those which confer a resistance to herbicides and pathogens. Interest has also been concentrated on those characteristics which alter the quality of foods, and others which could prove useful for the production of products for industrial uses.

Despite the great efforts made, the examples we have available show that there are several problems to be overcome before a transformed plant can be commercially useful. Apart from the stable expression of the genes introduced, the transformed varieties must show similar productivity to the conventional varieties.

Transformed plants must synthesise proteins and other metabolites which could alter their metabolism, in such a way that their productivity could be affected. Thus, to achieve transformed varieties with high productivity, it is probably necessary to undertake a complete programme of genetic improvement, until a balance is achieved which allows productivity and the expression of the new characteristic incorporated to be maximised.

The sunflower (*Helianthus annuus L.*) is a difficult plant to transform, given the difficulty of *in vitro* regeneration of the species. Even so, this oil-bearing plant is of enormous interest as a species susceptible to cultivation in different latitudes.

In our sunflower breeding program, we have had the opportunity to work with mutations which, though not introduced through genetic engineering, illustrate the difficulties involved when working with plants with special genetic characteristics. The first is genetic dwarfism, which furthermore has associated bad seed set, lack of resistance to drought, and susceptibility to different cryptogamic diseases. The second is the mutation wich confeers a high oleic acid content in the oil. This characteristic is associated to the susceptibility to necrosis of the capitulum through heat, and unusual suceptibility to chemical treatment of the plant and seeds. In both cases, competitive commercial varieties have been achieved by developing specific breeding subprogrammes, instead of simply introducing genes through back-crossing.

Oleogenic fungi

E. Cerdá-Olmedo and **J. Avalos**
Departamento de Genética, Universidad de Sevilla,
Sevilla, Spain

Considerable amounts of fat are found in many fungi belonging to various distant taxonomic groups. Fat consti-

tutes close to one half of the dry weight of some Mucorales, the common molds. These grow well on carbohydrate-rich industrial media and offer a biotechnological possibility to convert carbohydrates to lipids. In many cases the conversion rate (fat coefficient) exceeds 10% by weight.

The mycelia of the Mucoral *Phycomyces blakesleeanus* accumulate 41% fat (relative to the dry weight) when grown under certain conditions. This fat is made up very largely of fatty acids, free and sterified. The four most abundant are palmitic, oleic, linoleic, and γ-linolenic acid; their concentrations are very roughly similar, although the fatty acid composition depends on age, location and culture conditions; palmitoleic and stearic acids are found in smaller amounts. Little effort has been made to optimize fat production and to modify the fatty acid composition.

Sterols, free and sterified, constitute about 0.5% of the dry weight; ergosterol and episterol predominate, and cholesterol is absent.

Carotenoids are found in many fungi. *Phycomyces* contains ß-carotene, attractive as a widespread natural pigment, a provitamin A, and a protective agent against cancer, among other uses. No toxins have ever been found in this organism and there are reasons to believe that it is edible by mammals. *Phycomyces* is an appropriate organism to develop a carotene-rich oil. Fats are good vehicles for carotene and protect it against oxidation.

Procedures have been develop for the isolation of mutants, their analysis by complementation and recombination, and genetic transformation with both self-replicating and integrating DNA. The production of ß-carotene is exquisitely regulated, and genetic analysis has identified seven genes involved in this regulation. Most of the mutations are irrelevant for practical purposes. Recessive mutations at genes *carD, carF,* and *carS* result in vast increments in the carotene content. Double mutants *carS carF* increase the ß-carotene content more than 200-fold; further increases are produced by a genetic trick that allows the two sexes to coexist in the same mycelium. Nearly all the carotene in these strains is all *trans* ß-carotene.

Environmental factors which can alter lipid metabolism

John L. Harwood
Department of Biochemistry, University of Wales College of Cardiff. P.O. Box 903, Cardiff, CF1 1ST, U.K.

A wide variety of environmental factors have been shown to produce effects –sometimes lethal– on plant metabolism. These factors range from "natural" influences (such as light) over which Man has little control to xenobiotics which are used deliberately or which enter the environment accidently. Some factors such as temperature, sulphur dioxide, oxides of nitrogen and ozone can be classed as "natural" but their influence on plant growth is exacerbated by human activities. For each environmental factor two responses are important for the plant. First, there is the initial stress and how well a given species is able to survive that. Second, there is the ability of different plant types to acclimatise to particular conditions. Breeding or creation of transgenic plants which can survive a given environmental insult is of obvious importance to the agricultural industry.

In my talk I will concentrate on recent experiments which have furthered our understanding of the ways in which water deficiency, environmental temperature and pesticides can alter lipid metabolism.