Expression, purification and properties of lycopene cyclase from *Erwinia uredovora**

Georg SCHNURR†, Norihiko MISAWA‡ and Gerhard SANDMANN§||

†Lehrstuhl für Physiologie und Biochemie der Pflanzen, Universität Konstanz, D-78434 Konstanz, Federal Republic of Germany,

‡Central Laboratories for Key Technology, Kirin Brewery, Kanazawa-ku, Yokohama-shi, Japan, and §Biosynthesis Group, J. W. Goethe Universität, P.O. Box 111932, D-60054 Frankfurt, Federal Republic of Germany

Lycopene cyclase, an enzyme responsible for the formation of cyclic carotenoids from acyclic precursors has been purified to homogeneity in an active state. The *Erwinia uredovora* lycopene cyclase gene (*crtY*) was over-expressed in *Escherichia coli*. From this recombinant strain the enzyme was purified by immuno-affinity chromatography and its cyclization activity characterized as a two-step reaction in which both sides of the lycopene molecule are cyclized to β -ionone rings with the monocyclic γ -

carotene as an intermediate. Furthermore, neurosporene as well as 1-hydroxylycopene were cyclized to β -zeacarotene and hydroxy- γ -carotene respectively. In contrast, neither 1,1'-dihydroxylycopene nor the tetra-*cis*-prolycopene were accepted as substrates. The cofactors involved in the reaction were either NADH or NADPH. K_m values were determined for lycopene and NADPH to be 1.8 μ M and 2.5 mM respectively.

INTRODUCTION

Carotenoids are lipophilic pigments with a yellow or red colour. They are essential in photoautotrophic organisms, where they are located in the thylakoid membrane participating in the light harvesting process [1] as well as for protection against photo-oxidative damage [2]. Carotenoids are also present in some bacteria and fungi [3]. In general, the synthesis of these tetra-terpenes proceeds via the general terpenoid pathway, leading then to the formation of phytoene, the first carotene in the reaction sequence, which is formed by condensation of two geranylgeranyl diphosphate molecules. In the subsequent reaction steps four additional double bonds are introduced to yield lycopene [4]. Most of the end products that accumulate in different species and different plant tissues are cyclic carotenoids carrying either two β -ionone rings or one β -ionone ring and one ϵ -ionone ring. They all originate from the cyclization of lycopene by two different enzymes under the control of two different genes [5]. However, the sequential formation of the two β -rings in the reaction to β -carotene is catalysed by a single gene product [6].

Genes coding for a β -ring-forming lycopene cyclase have been cloned and sequenced from *Erwinia uredovora* [7], *E. herbicola* [8] and *Synechococcus* [9]. Enzymic studies with lycopene cyclase were previously performed with crude plastid preparations only, leading to very inconsistent results with respect to cofactor requirement and other characteristics [10]. So far, purification of this membrane-bound enzyme has resulted in loss of activity [11]. In contrast, expression in *E. coli* of two membrane-bound phytoene desaturases, which are also involved in the carotenoid biosynthetic pathway, resulted in the purification of homogeneous proteins suitable for enzyme studies [12,13].

A similar heterologous expression approach in *E. coli* has been followed in the present study. The gene encoding lycopene cyclase from the enterobacterium *E. uredovora* was employed and the resulting enzyme purified for the first time in an active state to homogeneity. This enzyme preparation facilitated the characterization of lycopene cyclization with respect to cofactor requirement and substrate specificity.

MATERIALS AND METHODS

Culture conditions of *E. coli*

E. coli strain JM101, the host of all plasmids used, was grown in Luria Bertani broth containing ampicillin (100 μ g/ml) or chloramphenicol (30 μ g/ml) or both as described by Maniatis et al. [14].

Vector construction and protein overexpression

Plasmid pUCCRT-Y was created for the overexpression of the lycopene cyclase. It was constructed by digestion of pUC19 with *Sph*I and *Kpn*I, then treated with Klenow enzyme and subsequently ligated in the presence of a *Sph*I linker, pGGCATGCC. In the resulting vector (pUC19SP) the ATG in the *Sph*I site is in frame with *lacZ'*. From pCAR16 [7], a 1.29 kb *Eco*RV–*Bam*HI fragment carrying the *E. uredovora crtY* gene was isolated, and then inserted into the *Hinc*II–*Bam*HI site of pUC19, yielding the plasmid pCART-Y. The hybrid plasmid pUCCRT-Y was then constructed by isolating a 1.21 kb *Nru*I–*Eco*RI fragment carrying the truncated *crtY* gene and ligation with a synthetic fragment from the cohesive end for *Sph*I containing the original sequence of *crtY* [7] from the initiation codon up to the blunt end of the *Nru*I site, and with a *Sph*I/*Eco*RI-digested pUC19SP treated with alkaline phosphatase. The recombinant protein was overexpressed in *E. coli* carrying pUCCRT-Y by inducing the culture at an attenuation (D_{600}) of 0.5 with 0.25 mM isopropyl β -D-thiogalactopyranoside (IPTG) at 28 °C overnight. Plasmid pACCAR-EBI was constructed by using the 3.66 kb *Kpn*I–*Dra*II fragment from pCAR-ADE [7] carrying the genes *crtE*, *crtB* and *crtI* from *E. uredovora*. After treatment with Klenow enzyme it was inserted into the *Eco*RV site of pACYC184.

Preparation of the affinity column

Cytoplasmic protein from the lycopene cyclase-negative *E. coli* strain JM101/pUC19 was prepared and used for the production of an antiserum as previously described [13]. The purified IgG

Abbreviation used: IPTG, isopropyl β -D-thiogalactopyranoside.

* This paper is dedicated to Professor H. Senger on the occasion of his 65th birthday.

|| To whom correspondence should be addressed.

fraction [15] was used and coupled to CNBr-Sepharose from Pharmacia in accordance with the supplier's protocol.

Isolation and purification of the recombinant *E. uredoovora* lycopene cyclase

IPTG-induced cells were harvested by centrifugation at 6000 *g* for 15 min at 4 °C. Pelleted cells were resuspended in 50 mM Tris/maleate buffer, pH 6.5, and broken by passage through a French press cell at 30 MPa. The viscosity of the resulting suspension was reduced by incubation with 10 µg/ml DNase for 30 min. After centrifugation at 105000 *g* for 1 h at 4 °C the resulting supernatant fraction was used for further protein purification. The lycopene cyclase in the supernatant was enriched by precipitating with 40% (w/v) ammonium sulphate. The protein was pelleted by centrifugation at 6000 *g* for 10 min at 4 °C and resuspended in 50 mM Tris/maleate buffer, pH 6.5. After incubation on ice for 15 min the insoluble fraction was collected by centrifugation at 6000 *g* for 5 min at 4 °C. The pellet was then resuspended in the same buffer containing in addition 1 mg/ml soybean lipids. After desalting by gel filtration (PD10 columns from Pharmacia) with the same buffer, the lipid extract was applied to an immunoabsorption column that bound the remaining *E. coli* proteins. This step was repeated after regeneration of the column with 5 volumes of 25 mM Tris/HCl buffer, pH 8, containing 0.5 M NaCl and 1 mg/ml lipids followed by 5 volumes of 0.1 M acetate, pH 4, containing 0.5 M NaCl in accordance with the Pharmacia protocol mentioned above. The pass-through eluate contained the purified lycopene cyclase. SDS/PAGE was performed by the method of Laemmli [16].

Production of the substrates

For the production of the substrate carotenes in *E. coli*, strain JM101 was transformed with different plasmids. pACCAR-EBI carrying the *E. uredoovora* genes *crtE*, *crtB* and *crtI* was used for lycopene production. pUCCRT-C carrying *crtC* from *Rhodospirillum rubrum* [17] in combination with pACCAR-EBI was used for the production of the hydroxylated lycopene derivatives, and pACCAR-EBI_{RC} [18] in combination with pUCCRT-Y was responsible for the production of β -zeacarotene. *E. coli* cells with these plasmids were incubated in acetone for 20 min at 50 °C and filtered. After addition of a saturated-NaCl solution to the acetone extracts, the carotenoids were partitioned into diethyl ether/petrol (b.p. 35–80 °C) (1:9, v/v). The diethyl ether/petrol phase was evaporated to dryness and the substrate carotenoids were purified as previously described [19]. γ -Carotene was isolated from the fungus *Allomyces arbusculus* accordingly.

Production of the lipids

Soybean lipids with a 20% content of L- α -phosphatidylcholine were used for making a suspension together with the hydrophobic carotenoids. The lipids were first dissolved in chloroform and then the solvent was removed with a stream of nitrogen. Finally 50 mM Tris/maleate buffer, pH 6.5, was added and a suspension produced by sonicating for 30 min at 0 °C in the dark. To this suspension the substrate carotenes were applied.

Enzyme assay

The assay mixture (final volume 0.5 ml) contained 150 µl of lipid suspension (2.5 mg/ml) including the appropriate substrate, 5 mM NADPH and the purified enzyme (approx. 25 µg of protein) in 50 mM Tris/maleate buffer, pH 6.5. Incubations were in the dark at 30 °C for 4 h. This incubation period was still in the linear range with time. They were terminated by adding

methanol (1.5 ml) containing 6% (w/v) KOH and heated at 60 °C for 20 min. The remaining substrate and the products formed were extracted from the aqueous incubation mixture with diethyl ether/petrol (b.p. 35–80 °C) (1:9, v/v). Analysis of the reaction products was performed by HPLC on a Spherisorb ODS-1 5 µm column eluted isocratically with acetonitrile/methanol/2-propanol (85:10:5, by vol.) as eluent [12]. The separated carotenoids were detected with a Waters 994 diode array detector and spectra were directly recorded on-line.

Protein determination

Protein concentration was determined by the method of Bradford [20]. Protein concentrations were also estimated from SDS/PAGE by comparison with the staining intensity of a BSA protein standard (2, 5 and 10 µg), as reported by Sasiak and Rilling [21]. The relative amount of the expressed lycopene cyclase band on stained SDS/polyacrylamide gels was determined by using a flatbed scanner with densitometric software.

RESULTS

Expression and purification of lycopene cyclase

For optimal overexpression of lycopene cyclase protein in *E. coli*, a modified pUC19 vector was used into which the coding region of the *crtY* gene from *E. uredoovora* was cloned in frame with the ATG of *lacZ'*. Theoretically, a protein is formed containing the full-length lycopene cyclase with an extension of eight amino acids at the N-terminus originating from the pUC19 polycloning sites:

↓-beginning of crtY

1	2	3	4	5	6	7	8	9	10	11	12	13
Thr	Met	Ile	Thr	Pro	Ser	Leu	Gly	Met	Gln	Pro	His	Tyr

ATG ACC ATG ATT ACG CCA AGC TTG GGC ATG CAA CCG CAT TAT

↑----->

Analysis by SDS/PAGE indicated the presence of a prominent novel protein with a molecular mass of 43 kDa appearing after induction of *E. coli* cells with IPTG (Figure 1). This protein represented approx. 1–2% of the total *E. coli* protein. After breaking the cells in a French press followed by centrifugation, enzymic activity was detected not only in the pellet but also in the supernatant (Table 1). The amount of soluble lycopene cyclase was about 4-fold higher when *E. coli* was grown at 28 °C instead of 37 °C. Purification was performed on this soluble enzyme fraction, which was subjected to ammonium sulphate precipitation as the first step. This resulted in a considerable concentration of lycopene cyclase in the 40% precipitated fraction (Figure 1, lane 4). The ammonium sulphate pellet was suspended in a small volume of buffer and then centrifuged. The supernatant contained a range of contaminating proteins, whereas most of the lycopene cyclase stayed in the pellet (results not shown). However, after a second extraction by dissolving in a lipid-containing buffer and centrifugation (i.e. lipid extraction), almost half of the lycopene cyclase protein could be dissolved. The purification effect of this differential extraction can be seen in Figure 1, lane 5. In a final step the rest of the contaminating proteins were absorbed by passing the lipid-extractable proteins through an affinity column to which an antiserum against total *E. coli* proteins had been coupled. This procedure resulted in a homogeneous lycopene cyclase (Figure 1, lane 6). A typical

Figure 1 SDS/PAGE of fractions obtained during purification of lycopene cyclase

Lane M, molecular mass markers (molecular masses shown at left, in kDa); lane 1, whole cells without IPTG (amount of total protein 35 μ g); lane 2, whole cells with IPTG (35 μ g of protein); lane 3, French press supernatant (26 μ g of protein); lane 4, ammonium sulphate precipitate (9 μ g of protein); lane 5, fraction after lipid extraction (4 μ g of protein); lane 6, fraction after immunoadsorption (3 μ g of protein).

Figure 2 Conversion of different carotene substrates by purified lycopene cyclase

Substrates were (A) lycopene, (B) γ -carotene, (C) 1-hydroxylycopene and (D) 1,1'-dihydroxylycopene. The applied substrate in each experiment is indicated by a broken line. Peak 1, lycopene; peak 2, γ -carotene; peak 3, β -carotene; peak 4, 1-hydroxylycopene; peak 5, hydroxy- γ -carotene; peak 6, 1,1'-dihydroxylycopene. Substrate concentrations in the assays were 4 μ M.

Table 1 Purification of the recombinant lycopene cyclase from *E. uredoovora*

Step	Volume (ml)	Activity (nmol/h)	Lycopene cyclase (% total protein)	Specific activity (nmol/h per mg)	Purification (fold)
Homogenate	100	2.1	2	0.6	1
Supernatant*	100	0.54	1	0.3	0.5
(NH ₄) ₂ SO ₄	3	0.42	53	14.7	25.5
Lipid extract	1	0.18	82	22.8	41
Immunoadsorption	1	0.05	100	26.7	48

* The specific activity of the corresponding membrane pellet was 0.24 nmol/h per mg.

purification starting from 1 litre of *E. coli* cells is documented in Table 1. The specific activity increased over the whole purification procedure, reaching a final value of 26.7 nmol/h per mg of protein. This represents a 48-fold purification. The optimum pH for the cyclization reaction was determined as 6.5. It was determined with a buffer containing Mes (50 mM), Tris (100 mM) and acetate (50 mM) in the pH range 5.5–8.5 at intervals of 0.5 pH units.

Substrate specificity of lycopene cyclase

Different carotenes were assayed as potential substrates for the *E. uredoovora* lycopene cyclase. HPLC traces separating the substrate (broken lines) from the product(s) after termination of the reaction are shown in Figure 2. The spectra of the reaction

Figure 3 Absorbance spectra of the different reaction products of lycopene cyclase

The spectra were recorded on-line from the products of the enzymic reaction with purified lycopene cyclase after separation by HPLC as outlined in Figure 1. They correspond to (A) γ -carotene, (B) β -carotene, (C) β -zeacarotene and (D) 1'-hydroxy- γ -carotene.

Table 2 Cofactor dependency of the lycopene cyclase reaction

Enzymic reactions were performed with the purified lycopene cyclase. Applied concentrations were: for the substrate lycopene, 4 μM ; for Mn^{2+} , 6 mM; for Mg^{2+} 4 mM; and for all the cofactors mentioned, 2 mM.

Addition	Lycopene cyclase activity (% conversion to β -carotene)
Control	2.8
FAD	0.7
NAD^+	1.8
NADH	39.5
NADP^+	3.1
NADPH	41.3
AMP	2.6
ADP	2.5
ATP	2.5

Figure 4 Dependence of lycopene cyclization on the concentration of the substrate lycopene, and determination of the K_m

The concentration of NADPH was 5 mM.

products were recorded on-line from the elution peaks (Figure 3).

The purified lycopene cyclase is able to convert lycopene (peak 1) as substrate efficiently via the intermediate γ -carotene (peak 2) to all-*trans* β -carotene and a *cis* isomer (peak 3 and adjacent peak) (Figure 2A). These carotene products were identified by co-chromatography with authentic standards and by their absorption spectra (Figures 3A and 3B respectively). The intermediate γ -carotene was also accepted as a substrate (Figure 2B). Furthermore, we introduced 1-hydroxylycopene (peak 4) and 1,1'-dihydroxylycopene (peak 6) into the assay mixture (Figures 2C and 2D). From the spectrum of the reaction product (peak 5) with absorbance maxima at 435, 460 and 492 nm (Figures 3C), we can conclude that 1'-hydroxy- γ -carotene is formed from 1-hydroxylycopene. In contrast, 1,1'-dihydroxylycopene was not accepted as a substrate (Figure 2D). ζ -Carotene and a precursor of lycopene and prolycopene with a 7,7',9,9'-tetra-*cis* configuration were also not converted. How-

Figure 5 Lineweaver-Burke plot to determine the K_m for NADPH

The concentration of the substrate lycopene was 5 μM .

ever, neurosporene, which from its structure resembles half the lycopene molecule, was cyclized at one side to give β -zeacarotene as reaction product. The optical absorbance spectrum is shown in Figure 3(D) with typical maxima at 406, 428 and 460 nm.

Cofactor requirements

Using the purified enzyme we investigated presumptive cofactor requirements. Three different adenosine phosphates as well as oxidized and reduced nicotine nucleotides, including FAD, were assayed. The basic reaction without any addition was very low (Table 2) and also much lower than that determined in the crude homogenate. On addition of either NADH or NADPH, more than a 10-fold stimulation in activity was observed. The other compounds exerted no effect, as indicated in Table 2. A possible involvement of oxygen was tested by performing the assay under anaerobic conditions, but the reaction did not seem to be affected.

Enzyme kinetics

Enzyme kinetic studies were performed with the purified lycopene cyclase to determine the K_m values for the principal substrate lycopene and also the K_m for the cofactor NADPH. With increasing concentrations of lycopene the activity increased and a 90 % saturation was reached at 3.5 μM (Figure 4). The double-reciprocal plot with seven data points from the linear range resulted in a good linear regression with a correlation coefficient of 0.98, and enabled the determination of a reproducible K_m value for lycopene of 1.8 μM . The $V_{\max}$ related to the amount of lycopene cyclase in the assay was 32.3 nmol/h per mg. The K_m was also determined for the substrate neurosporene in the same way, resulting in a value of 6.3 μM . Similar enzyme kinetics studies were carried out with various concentrations of NADPH (Figure 5). Saturation was reached with 4 mM NADPH. From the Lineweaver-Burke plot the K_m for the cofactor NADPH was determined as 2.5 mM and $V_{\max}$ as 30.5 nmol/h per mg of protein. Very similar results were obtained with NADH instead of NADPH (results not shown).

Scheme 1 (A) Substrates and products of the lycopene cyclase reaction; (B) carotenoids that are not converted.

DISCUSSION

It is extremely difficult to extract and purify membrane-bound carotenogenic enzymes from plants and micro-organisms. The amounts present are very low and solubilization of the enzymes with detergent is often extremely detrimental to their activity [4]. Only in the case of the membrane-bound phytoene desaturase and capsanthin synthase have active enzymes previously been isolated from *Capsicum* fruit chromoplasts, which are specialized organelles accumulating high levels of carotenoids [22,23]. An alternative strategy for isolating carotenogenic enzymes from any other carotenogenic organism from which the gene has been isolated is to start with heterologous overexpressed enzymes. However, these enzymes may be inactive when sequestered in inclusion bodies and an additional reactivation step must be performed. This has been achieved for two different phytoene desaturases [12,13]. A lycopene cyclase was previously overexpressed by using the gene from *E. herbicola*, but activity of the enzyme was not reproducible. Purification of an active protein was therefore not possible [11].

It was shown for phytoene desaturase from *E. uredovora* that a small portion of this enzyme can be found in a supernatant fraction after cell disruption [12]. Therefore the strategy followed in the present work was to overexpress lycopene cyclase in a way that after breaking the cells by French press treatment an active

enzyme could be isolated from the supernatant after ultra-centrifugation. Its amount and also its activity was increased by growing *E. coli* at lower temperature, as also occurred for several other recombinant proteins [24]. Under these conditions the specific activity of the crude supernatant (Table 1) was six times higher than a crude lycopene cyclase preparation from *Capsicum* chromoplasts [25].

The molecular mass of the newly expressed protein from the supernatant was 43 kDa (Figure 1), which represents the value calculated from the gene sequence [7]. Most of the same protein was found to be integrated into the membrane in about a 10-fold higher concentration (results not shown). The behaviour of the soluble lycopene cyclase during purification indicated that the enzyme might be complexed with membrane lipids. This would also explain the enzymic stability and high activity of our expressed protein because activity of phytoene desaturase from *Synechococcus* could be enhanced by addition of various lipids to the purified protein [13]. Furthermore the values in Table 1 can be used to calculate the activity per amount of enzyme protein during purification. They come close to a specific activity of 26.7 (Table 1) for the purified protein. This indicates that hardly any inactivation of our lycopene cyclase occurred during the purification to a homogeneous protein (Figure 1).

The mechanism of lycopene cyclization to β -carotene, which involves proton attack at C-2 and C-2' with a carbonium ion

intermediate and proton loss from C-5, was established from $^2\text{H}_2\text{O}$ -labelling experiment with intact cells [26,27]. The slightly acidic conditions that we found for optimum activity favour electrophilic proton addition as the initial step for the formation of the ionone ring. As both ends are cyclized by a single enzyme [6,7] and γ -carotene must consequently be an equal substrate, the recognition sites in the substrate molecule should be restricted to both terminal regions. Furthermore the double bonds at C-1 or C-1' should be essential for cyclization. With the purified enzyme and the assay *in vitro* that has been developed it was possible to elucidate, for the first time, which carotenes can serve as substrates for lycopene cyclase. Besides lycopene, conversion of γ -carotene to β -carotene (Figure 2) and also formation of β -zeacarotene from neurosporene (Figure 3) demonstrate that lycopene cyclase recognizes each half of the substrate molecule separately. 1-Hydroxylycopene and 1,1'-dihydroxylycopene originate from lycopene by addition of water to the double bonds at C-1 and C-1'. Depending on the availability of one of these double bonds, only 1-hydroxylycopene served as a substrate for cyclization (Figures 2C and 2D). The observation that the 7,7',9,9'-tetra-*cis* prolycopene is not suitable as a substrate for the *Erwinia* lycopene cyclase can be explained by the shape of this isomer, in which the steric arrangement prevents ionone ring formation. Scheme 1 illustrates the chemical structures of substrates that can be converted by lycopene cyclase and the corresponding end products (Scheme 1A). Mechanistically, cyclization is the same regardless of whether lycopene, 1-hydroxylycopene, γ -carotene or β -zeacarotene is the substrate. The requirement is that at least one end group has reached the lycopene level of desaturation. Consequently, 1,1'-dihydroxylycopene cannot be accepted as a substrate (Scheme 1B).

The affinity of lycopene cyclase for the principal substrate lycopene is quite high, as indicated by a K_m of $1.8\ \mu\text{M}$ (Figure 4) and $6.3\ \mu\text{M}$ for neurosporene. These are the only K_m values for any lycopene cyclase available until now. As different affinities were determined for either lycopene or neurosporene, we can assume that the degree of desaturation at the side of the carotene molecule that is not immediately cyclized exerts an effect on the binding of the carotene substrate to the active site. Although there are reports from work with crude systems *in vitro* that FAD or NADP stimulate lycopene cyclization [28], the reaction mechanism as outlined above is independent of any cofactor. With the purified lycopene cyclase, we found that NAD(P)H is needed for optimum activity (Table 2). Nevertheless the K_m values show that the affinity for NAD(P)H is less than one-thousandth of that for lycopene (Figures 4 and 5). As the reaction is independent of oxygen and conversion of prolycopene did not occur, the role of NAD(P)H must be totally different from that in *Narcissus* chromoplasts, where it is involved in an isomerization process [29]. The amino acid sequence of the *E. uredoovora* lycopene cyclase deduced from the gene sequence exhibits a $\beta\alpha\beta$ dinucleotide-binding region typically found in NAD(P)H-dependent enzymes [30]. This motif is also present in

the lycopene cyclases from *E. herbicola* [8] and *Synechococcus* [9]. On the basis of the information available on lycopene cyclase and deduced from this present study, it can be suggested that NAD(P)H is an enzyme-modifying cofactor. As we have now developed a procedure to express and obtain large quantities of lycopene cyclase in a homogeneous and active state, further experiments on the interaction of NAD(P)H with the enzyme can be done. This work on a more detailed characterization of the catalytic mechanism, including inhibitor studies, is in progress.

This work was supported by a grant from the European Communities BIOTECH Programme, as part of the Project of Technological Priority 1993–1996.

REFERENCES

- 1 Siefertmann-Harms, D. (1987) *Physiol. Plant.* **69**, 561–568
- 2 Krinsky, N. I. (1979) *Pure Appl. Chem.* **51**, 649–660
- 3 Goodwin, T. W. (1980) *The Biochemistry of the Carotenoids*, vol. 1, Chapman & Hall, London
- 4 Sandmann, G. (1994) *Eur. J. Biochem.* **223**, 7–24
- 5 Kirk, J. T. O. (1967) In *The Plastids* (Kirk, J. T. O. and Tilney-Bassett, R. A. E., eds.), pp. 302–571, W. H. Freeman Co., London
- 6 Schnurr, G., Schmidt, A. and Sandmann, G. (1991) *FEMS Microbiol. Lett.* **78**, 157–162
- 7 Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K. and Harashima, K. (1990) *J. Bacteriol.* **172**, 6704–6712
- 8 To, K. Y., Lai, E. M., Lee, L. Y., Lin, T. P., Hung, C. H., Chen, C. L., Chang, Y. S. and Liu, S. T. (1994) *Microbiology* **140**, 331–333
- 9 Cunningham, F. X., Sun, Z., Chamovitz, D., Hirschberg, J. and Gantt, E. (1994) *Plant Cell* **6**, 1107–1121
- 10 Bramley, P. M. (1985) *Adv. Lipid Res.* **21**, 243–279
- 11 Hundle, B. S., O'Brien, D. A., Beyer, P., Kleinig, H. and Hearst, J. E. (1993) *FEBS Lett.* **315**, 329–334
- 12 Fraser, P. D., Misawa, N., Linden, H., Yamano, S., Kobayashi, K. and Sandmann, G. (1992) *J. Biol. Chem.* **267**, 19891–19895
- 13 Fraser, P. D., Linden, H. and Sandmann, G. (1993) *Biochem. J.* **291**, 687–692
- 14 Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 15 Clausen, J. (1969) in *Immunological Techniques for the Identification and Estimation of Macromolecules* (Work, T. S. and Work, E., eds.), vol. 1, pp. 397–572, North-Holland, Amsterdam
- 16 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 17 Armstrong, G. A., Leach, F., Alberti, M. and Hearst, J. E. (1989) *Mol. Gen. Genet.* **216**, 254–268
- 18 Linden, H., Misawa, N., Chamovitz, D., Pecker, I., Hirschberg, J. and Sandmann, G. (1991) *Z. Naturforsch.* **46c**, 1045–1051
- 19 Britton, G. (1985) *Methods Enzymol.* **111**, 113–149
- 20 Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- 21 Sasiak, K. and Rilling, H. C. (1988) *Arch. Biochem. Biophys.* **260**, 622–627
- 22 Hugueney, P., Römer, S., Kuntz, M. and Camara, B. (1992) *Eur. J. Biochem.* **209**, 399–407
- 23 Bouvier, F., Hugueney, P., d'Harlique, A., Kuntz, M. and Camara, B. (1994) *Plant J.* **6**, 45–54
- 24 Schein, C. H. (1989) *Bio/Technology* **7**, 1141–1147
- 25 Camara, B. and Dogbo, O. (1986) *Plant Physiol.* **80**, 172–174
- 26 Williams, R. J. H., Britton, G. and Goodwin, T. W. (1967) *Biochem. J.* **105**, 99–105
- 27 Britton, G. and Munday, A. P. (1980) *Dev. Plant Biol.* **6**, 345–350
- 28 Kushwaha, S. C., Kates, M. and Porter, J. W. (1976) *Can. J. Biochem.* **54**, 816–823
- 29 Beyer, P., Kröncke, U. and Nievelstein, V. (1991) *J. Biol. Chem.* **266**, 17072–17078
- 30 Scrutton, N. S., Berry, A. and Perham, R. N. (1990) *Nature (London)* **343**, 38–43