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PRODUCTION AND CHARACTERISATION OF MONOCLONAL ANTIBODIES TO PHYTOENE SYNTHASE OF *LYCOPERSICON ESCULENTUM*

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Key Word Index—*Lycopersicon esculentum*; carotenoids; monoclonal antibodies; phytoene synthase.

Abstract—Monoclonal antibodies have been prepared against the tomato (*Lycopersicon esculentum* Mill.) fruit ripening-enhanced phytoene synthase (PSY1). The antigen was prepared as a β-galactosidase fusion protein by cloning a 1.13 kb fragment of *Psy1* cDNA into pUR291, followed by transformation of *E. coli*. The fusion protein, induced by IPTG, was purified by preparative SDS-PAGE and used to elicit an immune response. The cell lines were screened for cross-reactivity against β-galactosidase- phytoene synthase fusion protein in *E. coli* extracts using western blotting and ELISA detection procedures. Positive clones were further screened for their ability to cross-react with the mature phytoene synthase protein on western blots as well as their ability to inhibit enzyme activity. Eleven monoclonal lines were obtained. Nine of these, all of the IgM isotype, exhibited strong responses to phytoene synthase of ripe tomato fruit on western blots, but did not inhibit enzyme activity effectively. The other two lines (IgG/1a 2 isotypes) inhibited phytoene synthase activity in ripe tomato stroma, but produced a poor response to the protein on western blots. The monoclonals identified a ripe fruit phytoene synthase of 38 kDa, exclusively located in the chromoplast. In contrast, antibodies were unable to detect microbial phytoene synthases, nor phytoene synthase of maize leaf, tomato chloroplast or mango fruit extracts, either on western blots or from inhibition of phytoene synthase activity. However, they did cross-react with a 44 kDa protein from carrot leaf stroma and with three different proteins (44, 41, and 37 kDa) in carrot root. Cross-reactivity was also found with a 37 kDa protein from pumpkin fruit stroma.

INTRODUCTION

Phytoene synthase catalyses the first committed step in carotenoid biosynthesis, whereby geranylgeranyl pyrophosphate (GGPP) is converted into phytoene via the intermediate prephytoene pyrophosphate. Phytoene is colourless, but subsequent desaturations of the molecule increase the number of conjugated double bonds to yield coloured, acyclic carotenes such as lycopene, the pigment responsible for the characteristic red colour of ripe tomato fruit [1]. Many carotenoid genes have been cloned [2–6], but the regulatory mechanisms operating in carotenoid formation are still poorly understood.

The ripening of tomato fruit is an excellent system in which to investigate the regulation of carotenoid biosynthesis, as quantitative and qualitative changes in several isoprenoid classes, including carotenoids, occur during tissue-specific plastid differentiation. Phytoene synthesis appears to be a key step in carotenoid formation in ripening fruit [7] and its manipulation influential on other isoprenoids [8].

The tomato fruit ripening-enhanced cDNA cloned pTOM5 [9] encodes phytoene synthase and has been designated *Psy1* [10]. The inability of transgenic tom-

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Abbreviations: FPP = farnesyl pyrophosphate; GGPP = geranylgeranyl pyrophosphate; IPP = isopentenyl pyrophosphate; IPTG = isopropyl β-D-thiogalactopyranoside; mab = monoclonal antibody; PMSF = phenylmethylsulphonyl fluoride; PVDF = polyvinylidene difluoride; TBST = Tris-buffered saline/Tween; TBS = Tris-buffered saline.

ato plants possessing antisense RNA to pTOM5 [11] fruit and of a yellow flesh, *r* mutant variety [12] to produce phytoene in ripe fruit, despite both having the normal array of carotenoids in green tissues, has led to speculation that two or more phytoene synthases exist in tomato [13]. Further support for this hypothesis has come from the discovery that a clone with strong homology to pTOM5, clone F [14], has been shown to be expressed predominantly in green tissues and designated *Psy*2 [15].

The practical difficulties associated with the isolation of low-abundance enzymes of carotenoid biosynthesis have made it virtually impossible (with the exception of *Capsicum* fruit [16]) to isolate sufficient homogeneous proteins to elicit specific immune responses. The prolific cloning of carotenoid genes over recent years, however, has enabled substantial amounts of pure proteins to be acquired following heterologous gene expression in *Escherichia coli*. Despite this, the number of antisera to higher plant carotenogenic enzymes remains small, or the response is non-specific, and so immunological detection of these enzymes is not used routinely.

In this article we describe the production of monoclonal antibodies to the *Psy1* gene product for immunological studies on carotenogenic tissues from tomato and other high-yielding carotenoid plants and microorganisms.

RESULTS AND DISCUSSION

*Expression of pUR*291-Psy1 *and purification of the phytoene synthase/β-galactosidase fusion protein*

The induced fusion protein was identified in two ways. First, by comparison of the proteins in extracts of *E. coli* JM101 possessing either pUR291, pUR291-*Psy*1 or no plasmid (Fig. 1). The pUR291-harbouring cells contained a 116 kDa protein corresponding to β-galactosidase (Fig. 1, lane 2). This protein was only a minor component in the extract of pUR291-*Psy*1 cells, but another protein, M_r 150,000, was present (Fig. 1, lane 4). Neither protein was present in extracts of untransformed JM101 cells (Fig. 1, lane 3). Secondly, the difference in M_r between the fusion protein (150 kDa) and that of β-galactosidase (116 kDa), i.e. 34 kDa, corresponds to that predicted for the phytoene synthase encoded by the truncated *Psy*1 cDNA (1.13 kb) ligated into pUR291.

Optimum expression of the fusion protein was achieved from a dense initial culture followed by 6–8 h growth in the presence of IPTG (0.1 mM). Typically, 1.0 g of cells yielded 0.7–1.0 mg of the fusion protein, which constituted about 0.2% of total cellular protein. The fusion protein was purified by preparative SDS-PAGE and used to elicit antibodies in mice as described in Experimental.

Cell extracts of the transformed *E. coli* did not exhibit *in vitro* phytoene synthase activity. Attempts to express *Psy*1 as a native enzyme, rather than as a fusion protein, were unsuccessful (data not shown), in contrast to other heterologously-expressed carotenoid genes such as phytoene desaturase [17] and lycopene cyclase [29].

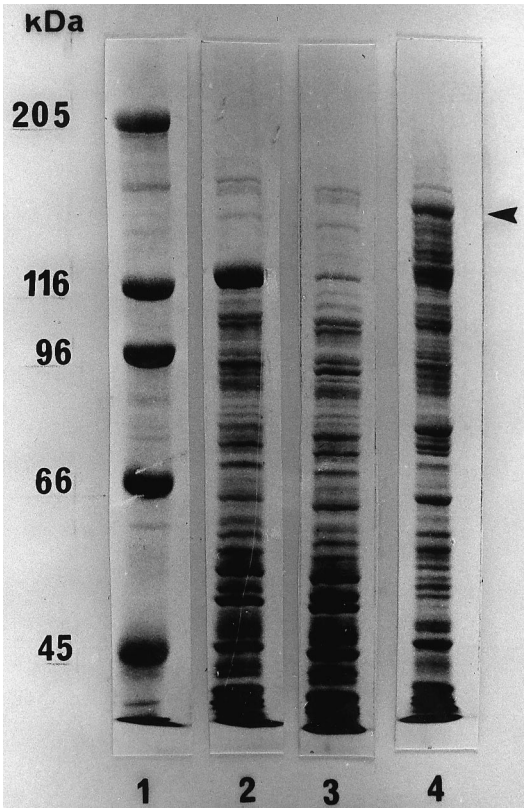

Fig. 1. Identification of the β-galactosidase/phytoene synthase fusion protein expressed by *E. coli* pUR291/*Psy*1 transformants. Protein extracts from lysed *E. coli* strain were electrophoresed on a 7.5% SDS-PAGE and stained for protein. Lane 1, molecular mass markers; lane 2, *E. coli* pUR291; lane 3, host *E. coli* JM101; lane 4, *E. coli* pUR291-*Psy*1. Arrow indicates the β-galactosidase/phytoene synthase fusion protein.

Selection and characterisation of hybridoma recognising phytoene synthase

Antisera from three mice immunised with the fusion protein were tested for cross-reactivity against the β-galactosidase/phytoene synthase protein and β-galactosidase itself by western blotting. The mice with the highest titre to the fusion protein were used in subsequent fusions for the production of hybridoma lines. In the first stage of screening, some 2000 lines showing a strong reaction to the fusion protein but a weak response to β-galactosidase, were selected for subculturing. These lines were further tested for their ability to cross-react with the mature phytoene synthase protein obtained from stromal extracts of ripe tomato fruit. A protein of apparent molecular mass

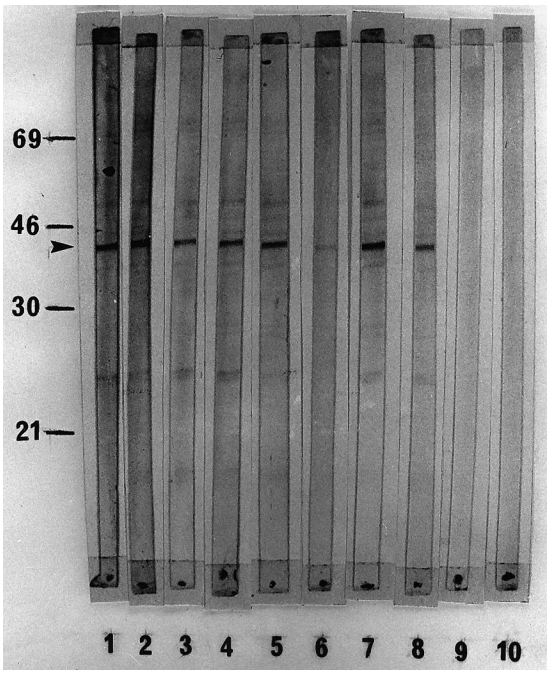

Fig. 2. Western blots of ripe tomato fruit stromal proteins probed with *Psy*1 monoclonal antibody lines. Blots of 12.5% SDS-PAGE were probed with different mab lines, as described in Experimental. Lane 1, A102; lane 2, A104; lane 3, A131; lane 4, A135; lane 5, A151; lane 6, A152; lane 7, A153; lane 8, A154; lane 9, control with mab to a brain protein; lane 10, control incubated with culture medium. The arrow indicated the position of the immuno-reactive phytoene synthase.

38 kDa was detected on western blots (Fig. 2). This mass corresponds to that predicted for the mature phytoene synthase from ripe tomato fruit, based upon the predicted amino acid sequence [9]. Those lines with positive responses to the 38 ± 3 kDa protein were further screened against stromal proteins from down-regulated *Psy*1 ripe tomato fruit which contain no *Psy*1 gene product (Bird *et al.*, 1991), in order to eliminate lines that gave responses that were not specific to phytoene synthase. The lines were also screened for their ability to inhibit phytoene synthase activity in ripe fruit stroma. The immunoreactive protein band at 38 kDa was the most predominant protein. However, the prolonged visualisation period used with lines having weaker titres (e.g. A152) did result in some non-specific detection, e.g. proteins at 27 kDa and 50 kDa. These bands were also found in extracts of *Psy*1 down-regulated mutants.

After several rounds of subculturing and repeated screening as described above, 11 stable, homogeneous monoclonal lines were obtained. All the mabs detected phytoene synthase from ripe tomato fruit on western blots, with some lines, such as A135 and A154, giving particularly strong responses. Other lines such as A113 and A114, gave poorer responses on western blots but were able to inhibit enzyme activity (Table 1). Isotyping of the mabs indicated that nine lines

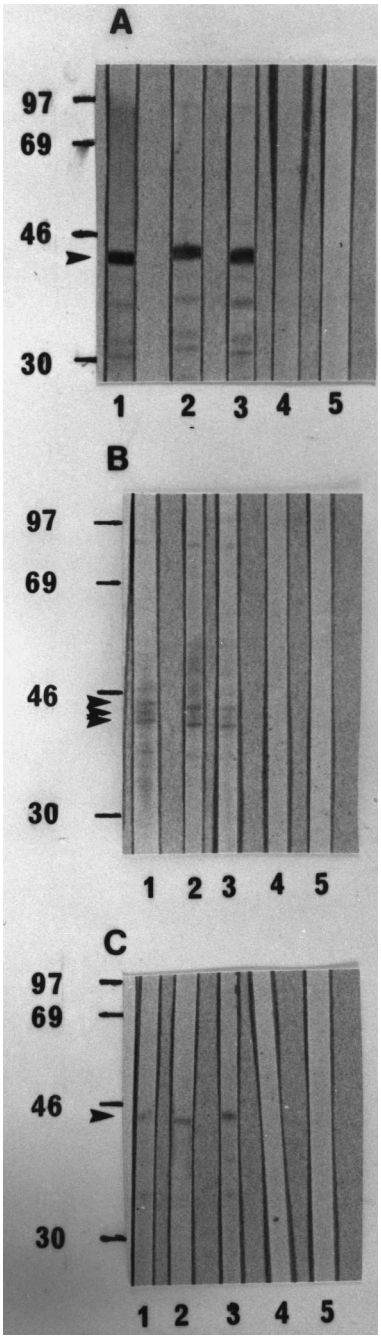

Fig. 3. Western blots of stromal proteins from pumpkin fruit (A), carrot root (B) and carrot shoots (C). The following mab lines were used: A135 (1), A102 (2), A154 (3), A114 (4). Lane 5 shows a control with a mab to a brain protein. Extracts were electrophoresed on curtain SDS gels and divided into strips (3×50 mm). Each lane contains 5–7 μg protein. Mabs were used without dilution. Arrows indicate positions of immuno-reactive phytoene synthases.

were of the IgM class and two (A113 and A114) were IgG/Ias.

The ability of the mabs to inhibit ripe fruit phytoene synthase activity was investigated in more detail by performing immunotitration curves with lines A114

Table 1. Characterisation of monoclonal antibodies prepared to phytoene synthase of ripe tomato fruit. Monoclonal culture supernatants contained 0.08–0.12 mg protein/ml. Each supernatant was mixed with ripe fruit stroma in a 1:10 (v/v) ratio. Following the colour reaction, blots were scanned by laser densitometry across each lane as described in Experimental. Phytoene synthase activity was assayed as described in Experimental. Additions of antibodies were standardised (20 μl) and controls were performed using an unrelated monoclonal line

Antibody line	Response on western blots (Absorbance units)	Phytoene synthase activity (% control)*
A102	0.86	50
A104	0.80	100
A113	0.20	50
A114	trace	15
A131	0.47	100
A135	1.10	100
A151	0.86	100
A152	0.55	100
A153	0.45	100
A154	2.40	80
A155	trace	100

*Control activity 25.68 MBq/mg protein from 66 MBq [1-^{14}C]IPP.

and A154. The amounts of mab added were standardised in each incubation. Increases in the amounts of A114 in the incubations reduced phytoene synthase activity (as measured by the incorporation of [1-^{14}C]IPP into phytoene) in an approximately proportional manner between 30 and 150 μg protein, with an apparent I_{50} value of 100 μg mab protein. In contrast, the A154 line did not inhibit phytoene synthase in a linear manner with respect to protein. It was only weakly inhibitory, with 40% inhibition at 150 μg mab protein.

In order to eliminate the possibility that inhibition of phytoene synthase activity was due to an effect on the prenyl phosphate synthases, reactions from IPP to GGPP, [^{3}H]FPP and [^{3}H]GGPP were used as alternative substrates. No such inhibition was detected (data not shown).

Intracellular location of phytoene synthase in ripe tomato fruit

Subcellular fractions of ripe tomato fruit were probed on western blots with lines A104, A135 and A154. Phytoene synthase (M_r 38,000) was found exclusively in the plastid fraction. Within the chromoplast, activity was located in the stroma, with only 1% of enzyme detectable in the chromoplast membranes. Determination of enzyme activity in the fractions using either [^{3}H]GGPP or [1-^{14}C]IPP as the substrate correlated with the cross reactions on western blots.

Cross-reactivity of monoclonal lines with phytoene synthase of other tomato tissues and carotenogenic organisms

Lines A102, 114, 135 and 154 were tested for their ability to cross react on western blots and to inhibit phytoene synthase activity of green tissues of tomato, other higher plant species and extracts of bacteria, algae and fungi.

No specific cross reactivity on western blots was found with any of the four lines to microbial phytoene synthases from *Erwinia uredovora*, *Phycomyces blakesleeanus*, *Scenedesmus obliquus* or *Synechococcus*. Likewise, phytoene synthase activities, determined on cell extracts of these organisms, was not inhibited by the mabs (data not shown). These data reflect the low homology between the phytoene synthase amino acid sequences of higher plants and microorganisms [5].

Chloroplast-containing tissues of tomato (leaf and mature green fruit) did not react with the mab lines on western blots (Table 2), and some inhibition (44%) of enzyme activity by A154 was found (Table 3), in contrast to the results with stroma from ripe tomato fruits which showed strong reactions on western blots and inhibition of enzyme activity (Table 1). Cell extracts of pepper, carrot, mango and pumpkin showed a variety of cross reactivities to the monoclonal lines on western blots (Table 2). Similarly, inhibition of phytoene synthase activity in these extracts by mabs was variable, with some incubations, e.g. carrot leaf, showing an apparent stimulation of activity. The extracts from both green and red pepper fruit exhibited non-specific interactions with the mabs, whereas carrot leaf yielded one immunoreactive protein (M_r 44,000). Carrot root tissue revealed three bands of M_r 39, 43 and 45,000, while pumpkin fruit stroma contained one cross-reacting protein, M_r 37,000.

Presumably the epitopes recognised by the mabs are not present in the phytoene synthases of all higher plant tissues. The detection of multiple immuno-reactive phytoene synthases in carrot chromoplasts could indicate the existence of PSY isoforms in carrot root tissue. The difference in PSY protein profiles in the carrot root and leaf tissue may reflect the different plastid types and carotenoid compositions in the two tissues, as with green and ripe fruit of tomato.

The molecular mass of the mature PSY-1 of tomato and other phytoene synthases has been an area of some disagreement. Predictions based on conserved consenses sequence alignments suggest that the most likely cleavage site of the plastid transit sequence yields a mature protein of 37 kDa [11]. From bacterial sequences, however, the mature protein is considerably smaller [5]. The melon *Psy* cDNA has two potential cleave sites; one at around amino acid residue 83, yielding a mature protein of molecular mass 38 kDa, and another at position 143, which would result in a protein of 31 kDa [30]. The *Capsicum* PSY

Table 2. Cross reactivity of mabs with higher plant extracts. PVDF membranes, containing cell extracts (5–7 μg protein per lane), were probed with culture supernatants from the four lines, as described in Experimental

	Cross reactivity on western blots			
Plant species	A102	A114	A135	A154
Ripe tomato fruit	+	t*	+	+
Green tomato fruit	−†	−	−	−
Tomato leaf	−	−	−	−
Red pepper fruit	nsr‡	nsr	nsr	nsr
Green pepper fruit	nsr	nsr	nsr	nsr
Carrot leaf	+	−	+	+
Carrot root	+	−	+	+
Ripe pumpkin	+	−	+	+
Ripe mango	−	−	−	−
Maize leaf	−	−	−	−

*trace, †no reaction, ‡non-specific reaction.

No cross-reactivity was found with partially purified phytoene synthase from maize (Holford. A. *et al*., unpublished data).

Table 3. Inhibition of phytoene synthase activity from higher plants by mabs raised to phytoene synthase from ripe tomato fruit. Stromal extracts (75–150 μg protein) were incubated with 0.5 μCi [1-^{14}C]IPP as described in Experimental. Incubations with each mab line contained the same amount of protein (50 μg)

	Control phytoene synthease activity	Phytoene synthase activity with mabs (% control)*			
Plant tissue/species	(MBq/mg protein)	A102	A114	A135	A154
Ripe tomato fruit	18.78	50	15	n.a.	80
Green tomato fruit	25.68	105	103	120	56
Green *Capsicum* fruit	2.12	105	103	120	56
Red *Capsicum* fruit	21.56	34	74	81	90
Carrot root	47.66	91	84	107	103
Carrot leaf	1.09	178	198	144	166
Maize leaf	41.08	100	97	102	93
Ripe pumpkin	49.42	125	129	88	120
Ripe mango	5.37	87	97	68	137

* Ripe tomato fruit stroma = 18.78 MBq/mg protein; n.a, not assayed.

is predicted to have a molecular mass of 38 kDa, but immunoblots with antiserum raised to the protein revealed a protein of 37 kDa. This antiserum detected a 45 kDa PSY protein in tomato. The molecular mass of the PSY-1 protein detected with the monoclonal antibodies in this study is 38 kDa. This agrees well with the predicted size derived from the putative cleave site (37 kDa) as well as the 41 kDa obtained using heterologous import assays [31]. The carrot and pumkin immunoreactive PSY proteins (Table 2) are all in the molecular mass range of PSY proteins reported to date. The multiple forms observed in the carrot root may reflect differences in mechanisms of plastid import and processing between chloroplasts and chromoplasts. The establishment of chromoplast import assays would provide a valuable insight into such processes.

The contrasting results with western blots and inhibition of phytoene synthase activity *in vitro* between chloroplast and chromoplast-containing tissues of tomato could indicate that different phytoene synthase proteins reside in the two tissues. If the enzyme in green tissues is premoninantly the *Psy*2 gene product, as claimed by Bartley and Scolnik [15], it would be expected that some of the mab lines would cross react with green tissue extracts, since the homology between *Psy*1 and *Psy*2 is high [15]. It is also feasible that the amounts of PSY-2 and -1 in green tissue is too low that to be detectable on western blots, correlating with studies that showed *Psy*1 and *2* transcripts in tomato leaf were detectable only by RT-PCR [15]. It is hoped that the monoclonal antibodies described in this article will aid in the evaluation of transgenic plants with altered phytoene synthase-1 and 2 levels and provide an insight into carotenoid formation in tomato fruit.

EXPERIMENTAL

Plant and microbial materials

Tomato plants (*Lycopersicon esculentum* Mill cv. Ailsa Craig) were grown in glasshouses with supplementary heating. Fruit were harvested at the mature green (approximately 7 weeks after fertilization) and firm red (7 days post-breaker) stages. Expanding leaf material was used. Other plants were purchased fresh locally. *Erwinia uredovora* and *Escherichia coli*, strain JM101, were obtained and maintained as described previously [17]. *E. coli* transformants harbouring pUR291 were selected from media containing 150 μg/ml ampicillin. *Phycomyces blakesleeanus* C5*carB*10 (−) (phytoene-accumulating mutant) was kindly provided by Prof. E. Cerdá-Olmedo (University of Sevilla, Spain), and grown as described in a previous publication [18]. *Synechococcus* PCC 7942 [19] and *Scenedesmus obliquus* were cultured as described previously [20]. Subcellular fractionation was performed on fresh tissue, whilst pigment analysis was typically performed on tissue frozen at −70° prior to analysis. The same sample set was used for western blotting and enzyme inhibition assays.

[1-^{14}C]IPP (56 mCi/mmol) and [^{3}H]FPP (56 mCi/mmol) were purchased from Amersham International. [^{3}H]GGPP (15 mCi/mmol) was obtained from American Radiolabeled Chemicals, St Louis, MO.

*Construction and expression of pUR*291-*Psy*1

Construction of the pUR291-*Psy*1 plasmid was achieved by digestion of pTOM5 with Bgl II/PstI to yield a 1.13 kb fragment with a 5′-deleted transit sequence of 97 bp, followed by an in frame fusion into pUR291 [21]. Transformation of *E. coli* JM101 with pUR291-*Psy*1 was performed by electroporation (BioRad Gene Pulser) following the manufacturer's instructions. Expression of the fusion protein in transformed cells was induced by IPTG (0.1 mM) using standard protocols [21].

PAGE and western blotting

Expression of the fusion protein was monitored by SDS-PAGE of *E. coli* proteins on a 7.5% resolving gel with a 5% stacking gel [22]. Induced cells (1 ml) were centrifuged at 10,000 *g* for 5 min, the pelleted cells resuspended in distilled water (100 μl) and an equal vol. of sample buffer (4% w/v SDS, 130 mM dithiothreitol, 20% v/v glycerol, buffered in 80 mM Tris-HCl, pH 6.8) added. The mixture was boiled for 3 min prior to electrophoresis. Samples (approximately 10 μl) were electrophoresed concurrently with protein standards at a constant current of 30 mA for 2 h. The gels were strained for protein with Coomassie Blue R250 and the molecular weight of the recombinant fusion protein estimated. The fusion protein was purified by prep. SDS-PAGE using identical condition to those described above except that maxi gels were used and electrophoresis allowed to proceed for a further 1 h after the disappearance of the dye front. Proteins from stromal fractions of plants and from carotenogenic bacteria, fungi and algae were separated on 12.5% SDS-PAGE. These protein samples were prepared by precipitation with MeOH and $CHCl_3$ [23], followed by resuspension of the dried pellets in sample buffer. On gels intended for western blotting, coloured molecular weight markers (Rainbow markers, Amersham International, Amersham, U.K.) were used to facilitate molecular weight determinations.

Proteins were electrophoretically transferred to PVDF membranes (Immobilon P, Millipore, Harrow, Middlesex) using a wet blotting apparatus (BioRad). The blotting buffer comprised 25 mM Tris, pH 8.3, 192 mM glycine, 0.05% SDS and 20% MeOH, maintained at 4°. Transfer proceeded for 1 h at 0.8 mA/cm. The blots were treated for 60 min with TBST, comprising 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% (v/v) Tween 20, containing 1% (w/v) BSA. The blocking soln was removed and the blot was incubated for 60 min with either the antiserum (1:1000 dilution) or monoclonal culture supernatant. Unbound antiserum was removed by washing in TBST (3 × 5 min). The blots were then incubated for 1 h with anti-mouse IgG-alkaline phosphatase (Promega), diluted 1:5000 in TBST (2 × 1 min). Following further washing in TBST (3 × 5 min) and TBS (2 × 1 min), visualization of the immunoreaction was performed by adding western blue-stabilized substrate for alkaline phosphatase. The reaction was stopped by washing in deionized water (2 min). Molecular mass values were obtained from comparison with protein markers. Control blots were performed with and without the primary and secondary antibodies, as well as with established monoclonal lines that produced unrelated mabs to brain proteins. Stained blots were scanned with a LKB Ultrascan XL laser densitometer (Table 1).

Preparation of monoclonal antibodies

Immunization protocol. Three female Balb/c mice, 6–8 weeks old, were immunized with the phytoene synthase/β-galactosidase fusion protein. The antigen was presented as a gel suspension in distilled water as adjuvant (10 μg fusion protein/mouse) and injected intraperitoneally. The mice were rested for two weeks between each boost. Serum was removed ten days after boosting to determine the antigenic response by western blotting.

Production of hybridomas. Monoclonal antibodies to the fusion protein were produced by polyethylene glycol-mediated fusion of Balb/c hyperimmune splenocytes to non-secreting PX3-Ag8.653 murine myeloma cells [27]. Antibody-secreting hybridomas were identified by positive reaction with the phytoene syn-

thase/β-galactosidase fusion protein on both ELISA and western blots. Selected cell lines were doubly subcloned by limiting dilution to ensure that they were clonal. Cell lines were frozen in culture medium containing 10% (v/v) DMSO and stored in liquid nitrogen. Antibodies were isotyped using an isotyping kit from Sigma, following the manufacturer's instructions.

ELISA. Standard ELISA protocols were used [25]. Microtitre plate wells were coated with extracts overnight at 4°. Plates were blocked with 1% (w/v) BSA, washed thoroughly and primary antibody applied. Plates were rewashed after 2 h and the secondary antibody (alkaline phosphatase-conjugated anti-mouse immunoglobulins) applied and left for 30 min before addition of the stop solution. Each plate was measured for absorbance at 550 nm.

Isolation of subcellular fractions. Plastids were prepared from plant tissues using the procedure described by [7]. The supernatant from the first centrifugation step was recentrifuged at 105,000 *g* to give microsomal (P_{105}) and cytosolic (S_{105}) fractions. Stromal and plastid membrane fractions were obtained from the isolated plastid fraction by resuspension in 0.4 M Tris-HCl buffer, pH 8.0, containing 5 mM dithiothreitol and 1 mM PMSF, followed by homogenisation with a hand-held Teflon homogenizer. Centrifugation at 105,000 *g* for 1 h separated the soluble stromal fraction from the pelleted membranes.

Separation and determination of carotenoids. Carotenoids and chlorophylls were extracted from tissues using 10% Et_2O in petrol, following the procedures detailed previously [7]. They were estimated by the method of Lichtenthaler and Wellburn [26]. Values are reported as the means of the three determinations. Individual carotenoids were separated by HPLC on a reversed-phase, C_{18} column (Spherisorb ODSl, 3 μm) using an eluting solvent of ACN–MeOH–*iso*-ProOH (17:2:1) at a flow rate of 1 ml/min [7] Where necessary, chlorophylls were removed by saponification of the lipid extracts with 6% (w/v) KOH.

Enzyme assays

Phytoene synthase was assayed by measuring the incorporation of [1-^{14}C]IPP, [^{3}H]FPP or [^{3}H]GGPP into phytoene. Incubations (500 μl) contained 0.5 μCi of either radiolabeled substrate, stroma (350 μl), $MgCl_2 \cdot 6H_2O$ (4 μmol), ATP (3 μmol), $MnCl_2 \cdot 4H_2O$, (6 μmol), 0.1% Tween 60, KF (0.5 μmol) in 0.4 M Tris-HCl buffer, pH 8.0, containing 5 mM dithiothreitol. Crude cell extracts of *Phycomyces*, *Scenedesmus* or *Erwinia* replaced the stromal fraction in the incubation where appropriate. Each incubation was at 25° for 6 h. Phytoene was extracted from the incubations by partition with hexane and purified by TLC and/or HPLC as described previously [7]. Prenyl phosphate synthases, converting IPP into GGPP, were assayed by isolating the prenyl pyrophosphates as prenyl alcohols. They were obtained by treating the aqueous phase remaining after hexane extraction with conc. HCl as described by [27]. The prenyl alcohols were separated and quantified by on-line radio-HPLC suing a previous protocol [7].

Protein determinations

Protein contents of cell extracts were determined in triplicate by the method of Lowry and co-workers [28].

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